MOLECULAR STUDIES OF AVIAN LEUKOSIS VIRUS

A Thesis

by

BLAYNE MYRON MOZISEK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	John El-Attrache
Committee Members,	Blanca Lupiani
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ABSTRACT

Molecular Studies of Avian Leukosis Virus. (December 2005)

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Chair of Advisory Committee: Dr. John El-Attrache

It was nearly a century ago that the viral etiology of sarcomas and leukemia (leukosis) in the domestic fowl was first described by Ellerman and Bang, working in Copenhagen, and Rous in New York. Through the following decades, in an attempt to control these oncogenic diseases which were becoming the main cause of mortality in commercial poultry, extensive investigation in many veterinary laboratories was undertaken. Throughout this period and continuing today, advancements in our understanding of the mechanisms of these viruses are ever expanding. This knowledge has exponentially expanded since the discovery and in turn the development of molecular, nucleic acid-based tools to analyze and interpret these viral genomes. This burgeoning field of research has shed light on countless topics including the elucidation of specific viral mechanisms of neoplastic induction. It has allowed for the creation of diagnostics which are vastly superior in sensitivity and specificity than to their non-molecular-based counterparts, and in the future will lead to the generation of disease resistance in the host. These topics, in specific regard to molecular studies of avian leukosis virus, will be further developed in this thesis.

DEDICATION

To all the sacrifices made

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. John El-Attrache, and my committee members, Dr. Blanca Lupiani, and Sanjay Reddy, for their guidance and support throughout the course of this research, and especially for the constant and continuing encouragement to pursue those goals which I may have thought to be unattainable.

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Finally, thanks to my mother and father. This accomplishment would not have been possible without your never-ending support and guidance. And to Kayla, for your love and support, for what could have only been…

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

LIST OF TABLES

Page

INTRODUCTION

Retrovirus classification

The family *Retroviridae* encompasses an extensive and diverse group of viruses found in all vertebrates (120). The earliest of these viruses studied were isolated from birds and mice. Examples of such retroviruses include avian leukosis virus (ALV), Rous sarcoma virus (RSV), mouse mammary tumor virus (MMTV), and murine leukemia virus (MLV). These viruses were the subject of intense study due to their propensity to form neoplastic disease in their host organisms. Investigations into the mechanisms by which these viruses were able to cause tumors eventually led to the discovery and development of the oncogene theory of tumorigenesis: the elucidation of oncogenes within their viral genomes or their interaction with host oncogenes following infection/integration in a direct or indirect way to contribute to tumor formation (49, 116).

As science progressed other viruses were isolated that shared common characteristics with the retroviruses described above, such as genome organization and replication strategies. Historically, these viruses were further classified into three subfamilies based on their patterns of pathogenicity: 1) the acutely oncogenic retroviruses, or oncoretroviruses; 2) the lentiviruses (associated with “slow” diseases or those with long latency periods); 3) and spumaviruses (“foamy” viruses, named because of the pathognomonic changes observed in infected cells) (19). Until recently these viruses were grouped as mammalian type B-D, avian type C, human T-cell leukemia

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This thesis follows the style of the Journal of Virological Methods.

virus / bovine leukemia virus (HTLV/BLV) type retroviruses, lentiviruses, and spumaviruses. The categories were based on electron microscopic (EM) appearance of their nucleocapsid structures, host range, and pathogenicity (19). Further study of these viruses enabled detailed comparison of their genomic structures and nucleic acid sequences, which resulted in further refinement of the retrovirus taxonomic classification system in use today and displayed in Table 1 (120).

Table 1. Retrovirus genera.

Retrovirus Class	Examples	Morphology
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)	C-type
Betaretrovirus	Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekte sheep retorvirus (JSRV)	B-, D-type
Gammaretrovirus	Murine leukemia virus (MLV) Feline leukemia virus (FeLV) Gabbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (RevT)	C-type
Deltaretrovirus	Human T-lymphotrpic virus (HTLV)-1, -2 Bovine leukemia virus (BLV) Simian T-lymphotropic virus (HTLV)-1, -2, -3	---
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1	---
Lentivirus	Human immunodeficiency virus type 1 (HIV-1) HIV-2 Simian immunodeficiency virus (SIV) Equine immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus Feline immunodeficiency virus (FIV)	Rod/cone core
Spumavirus	Human foamy virus (HFV)	Immature

Retrovirus genetics and proteins

The retrovirus genome is referred to as being "pseudodiploid" due to the two identical single-stranded positive sense RNA molecules which comprise its genetic material (19). The genetic material of the virus can be described in two different fashions, virion associated RNA or either proviral DNA (Figure 1).

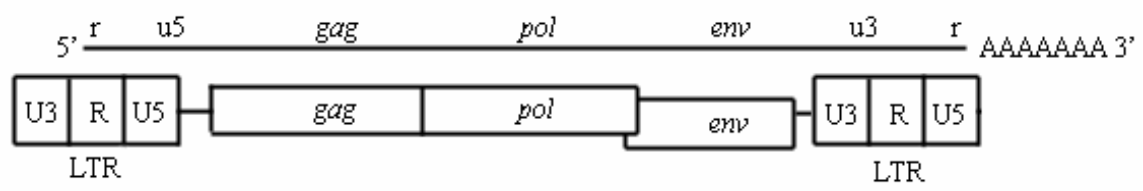

Figure 1. Both viral RNA and proviral DNA genome structures of a prototypical retrovirus. The genomic viral RNA, represented by a single black line, is shown at the top of the figure, with the structure of the resulting provirus after reverse transcription below. The locations of the open reading frames gag, pol, and env are shown. Reverse transcription of the RNA results in rearrangemnet of the termini of the genome, resulting in the structures of the LTRs (long terminal repeats) as indicated.

The 5' end of the genomic RNA begins with the "r" (for repeat) and "u5" (for unique 5' region) segments, followed by the viral genes *gag, pol,* and *env*. The 3' end of this sequence is terminated by the u3 (for unique 3' region) and r (identical to the 5' region) regions and a polyA tail. The virus then uses its prepackaged replication enzymes to convert this RNA molecule into a double-stranded DNA molecule via reverse transcriptase, from which it is then integrated into the host cell chromosomal DNA , where it is then referred to as a "provirus"(119).

Terminating the ends of the proviral DNA are the long terminal repeats (LTRs), which contain the regions U3, R, and U5. The mechanisms utilized for reading and translating the viral RNA during reverse transcription cause the u3 and u5 regions to be duplicated such that the 5' and 3' ends of the proviral DNA differ from that of the viral

RNA genome (54). This rearrangement and resulting formation of the LTRs at both ends of the genome enables appropriate expression of the viral genes (54). The U3 region of the 5' LTR contains the viral promoters and enhancers responsible for initiation of transcription of the viral genome at the 5' U3/R junction (69) . Yielding the viral *gag* and *pol* genes, expressed from an unspliced transcript, while the *env* gene is expressed from a spliced transcript (54). A termination signal is located at the 3' end of the genome along with the polyadenylation signal in the 3' LTR (54). The viral core structural proteins are encoded by the *gag* gene; these include the matrix (MA), capsid (CA), and nucleocapsid (NC). Within the reading frame of the *gag* gene some retroviruses also encode small regulatory proteins or peptides. The viral replication enzymes are encoded by the *pol* gene and these include both reverse transcriptase (RT) and integrase (IN). The *env* gene encodes the envelope (Env) glycoprotein which is post-translationally cleaved to form the transmembrane (TM) and surface (SU) subunits (78).

Virion structure

The mature retrovirus particle consists of a viral protein core, surrounded by a viral envelope made of cellular membrane-derived lipid bilayer and the viral encoded globular SU, 85 kD glycoprotein gp85, which mediates binding to the cellular receptor and a 37 kD TM glycoprotein anchor subunit that mediates fusion (69, 148). The cellular constituents of the envelope are acquired by the virus as it is assembled and buds from the infected cell's outer membrane (69). The structural core of the virion contains two copies of the positive sense RNA genome, found as a dimer and associated with the nucleocapsid protein, that is in turn shelled by the capsid protein (19). As described above, in addition to

the genome, retroviruses package within their core a unique set of enzymes, reverse transcriptase, protease, and integrase, enabling them to replicate through a double-stranded DNA intermediate. Outside the viral capsid is a matrix protein that appears to assimilate with the inner wall of the viral envelope (69). Newly budding immature viral particles contain the unprocessed Gag and Gag-Pol precursors of the proteins that will eventually make up the mature virus. The morphology of these immature particles is spherical, with a characteristic electron-lucent center (54). At maturation, viral proteins are processed and cleaved causing structure and morphology to change drastically (155). The mature particle retains its spherical shape to form a viral particle of approximately 100nm in diameter, but size in given preparations are not highly homogeneous (69). The glycoprotein surface projections are approximately eight nanometers in diameter (26). Virions exhibit a buoyant density in sucrose in a range of 1.16 to 1.18 g/mL and a sedimentation rate of approximately 600 S (54). The mature enveloped virions are sensitive to heat, detergents, and formaldehyde, while being somewhat resistant to UV light (54).

Endogenous retroviruses

Utilizing the proteins described above and the host cell replication scheme, viruses of the family *Retroviridae* have devised a unique method of perpetuating their genome. The incorporation of their genetic material into their hosts' chromosomal DNA, as described previously, has allowed for the continuance of a subset of these viruses to be transferred from host to host via Mendelian genes. Unlike the exogenous ALVs which are transmitted by conventional means through both vertical and horizontal transmission, a process that requires infectious virus; endogenous viral (*ev*) genes are inherited as host

genes and may or may not be expressed (30). Partial expression of *ev* genes can lead to one of the following phenotypes: 1) expression of viral group-specific (gs) antigen and subgroup E viral envelope antigen; 2) expression of subgroup E viral envelope antigen; and 3) spontaneous production of infectious subgroup E virus (41). Fully infectious endogenous viruses can be transmitted congenitally, horizontally, or genetically (41). These endogenous retroviruses have been reported in six classes of vertebrates and have become the subject of intense investigation (66). Of particular interest are the mammalian and avian endogenous retroviruses (AERs). This interest stems in large part from the recent finding that AERs were a contributing factor to the emergence of a novel, highly infectious subgroup of ALV, subgroup J (148). To date, three groups of AERs have been described: 1) the *ev* loci, 2) the endogenous avian retrovirus family (EAV), and 3) the avian retrotransposons (ART-CH) (11). The best characterized of these endogenous sequences are the *ev*s. Within the genome of the White Leghorn chicken 21 of these viruses have been described (3, 27, 67, 134). Some of these viruses encode for and express functional envelope glycoproteins, such as *ev*-3 and *ev*-6, while others such as *ev*-1, are classified as transcriptionally silent (8, 62, 63). Several non-defective *ev*s also exist which are able to produce infectious virus, such as *ev*-2 (11). Regardless of the *ev*, all which possess the *env* gene exhibit a common subgroup specificity designated E, as previously described. The sequence homology of this gene within all the subgroups of ALV expresses an identity of approximately 85-90% (11). While these endogenous viral sequences have been described in nearly all types of chickens, animals have been breed and selected to lack all known *ev* loci; these animals are referred to as *ev*-0 lines.

The ancient EAVs were the second class of endogenous viruses to be described (15, 16, 37). The identification of this group of endogenous virus was first made by low-stringency hybridization of *ev*-0 cell DNA with avian leukosis-sarcoma virus (ALSV) probes which revealed the presence of approximately 50 copies of EAV per genome in many avian species, including the White Leghorn chicken and the red jungle fowl, the progenitor of the domestic chicken (11). Of the described EAV loci, none appear to give rise to infectious virus and only one, E51, contains what appears to an intact *env* gene; all others include deletions of all or part of the SU. As a result, the description of a EAV functional envelope protein has not occurred and their subgroup specificity remains unknown (11).

Present at approximately the same rate as the EAVs within the chicken genome are the thirds class of endogenous viruses, the ART-CH (59, 94). These are comprised of small, ~3kbp, loci that include relatively short regions that show similarity to the ALSV genome. The ART-CH are transcriptionally active and have the potential to translate portions of the *gag* gene, although no protein product encoded by these elements has been described to date (12).

AVIAN LEUKOSIS VIRUS

Overview

Over the last century the body of literature accumulated and the wealth of knowledge discovered concerning the ALVs may perhaps make them the most well studied and mechanistically illuminated of the retroviruses. Henceforth, this group of retroviruses is the focus of the research performed under the direction of this thesis.

These viruses in chickens are grouped into the *Alpharetrovirus* genus and are classified into five pathogenic subgroups: A, B, C, D, and most recently J (106). Their distinctions arise from differences in envelope glycoproteins, virus-serum neutralization tests, virus interference, and host range (71). Of these exogenous subgroups, A and B are the most common ALVs, while subgroups C and D have been rarely reported in commercial poultry due in large part to the eradication efforts of the commercial poultry industry (21, 108). Chickens infected with exogenous ALV shed virus into albumen of eggs, vaginal and cloacal secretions, and congenitally transmit virus to the next generation. As described earlier, this group of avian retroviruses are best known and most studied due to their propensity to cause neoplastic disease, inducing leukosis affecting the erythroid, lymphoid, and myeloid series of hematopoietic cells, in addition to a number of solid tumors, including those affecting the mesenchyme, kidney, ovary, testis, liver, pancreas, and nervous system (10, 108, 117). These pluripotent neoplasms of ALVs cause severe economic losses due to condemnations at slaughter, loss of pedigree birds, and tumor mortality.

Neoplastic disease

The knowledge gained since the first isolation of ALVs has greatly contributed to the understanding of the molecular mechanisms responsible for many neoplastic conditions. Understanding these mechanisms has allowed the causative ALV to be placed into two categories, slowly or acutely transforming. The slowly transforming ALVs have a genetic structure like that of most typical retroviruses, that is their RNA genome is arranged in the 5'-*gag-pol-env*-3' format. The tumors they induce, as their name implies, are late onset, forming several months (~3) after initial infection. The mechanism by which these viruses are able to induce neoplastic disease, in particular lymphoid and erythroid leukosis (erythroblastosis) has been correlated to their proviral insertion upstream, downstream, or within host proto-oncogenes. Following this proviral integration the promoter/enhancer capacity of the viral LTR initiates the transcriptional up regulation of the host proto-oncogene, eventually leading to neoplasia (54). The determination of the cell type to be transformed is believed to be due to specific viral LTR sequences and the presence of specific LTR binding proteins on the cell (107).

The genetic composition of the acutely transforming ALVs is nearly identical to that of their slow transforming counterparts, with the exception of an oncogene or two within their genome. These oncogenes have been acquired by the virus through transduction of host oncogenes during the retrovirus replication process. These oncogenes can occur in variable locations within the viral genome and often lead to deletions within the structural genes of the virus such that the oncogene-carrying virion is replication defective and needs a replication competent helper virus to counter the genetic defect.

These ALVs are termed acutely transforming because they can induce neoplastic disease in their target cells, both in cell culture and *in vivo*, in a matter of a few days (39, 56). Fifteen of these avian viral oncogenes have been described (119). Their sequences are not completely homologous to their cellular counterparts and neither are the proteins they encode (107). These gene products, uncontrolled by normal regulatory processes, alter pathways of the cell concerned mostly with cell growth and differentiation. These 15 oncogenes can be placed into four main categories: growth factors, growth factor receptors, nuclear factors, and signal transducers.

The alteration of homeostatic expression of the Myc transcription factor is associated with a number of human and animal cancers (65). This transcription factor controls cell functions such as proliferation, differentiation, and apoptosis, through activation and repression of a number of target genes (58). These Myc regulated genes include those involved in angiogenesis, metabolism, and cell cycle and growth (28, 90). The function of Myc to influence cell cycle regulatory genes is thought to be important in its ability to induce of cell growth and proliferation (103). The over expression of Myc has also been shown to inhibit cellular differentiation, perhaps through its ability to block the cell cycle exit (65). In addition, Myc can also sensitize cells to apoptosis and it effectively induces blood vessel growth in a number of tumor models (17, 89, 93, 113). As explained earlier, ALV integration within the *c-myc* gene in immature B cells can result in metastatic bursal lymphomas (64, 91). Examination of the proviral integration within these neoplastic lymphoid cells show that nearly all proviruses have under gone deletion of the 5' LTR (82, 83), in turn allowing the 3' LTR to induce high levels of *c-myc* gene transcription (75).

These cells of the bursa, originating from approximately two stem cells that establish follicles during embryonic hemtaopoeisis, grow to number approximately 10,000 mature follicles (151). Upon infection at an early stage transformed or hyperproliferating follicles feature clonal proviral *c-myc* integration. These transformed follicles are filled with Myc-overexpressing lymphocytes which can be identified by their differential staining with methyl green pyronin (MGP), which indicates their increased ribosomal content (29). Of the 5 to 20 follicles which may be transformed, only one or a few of these hyperproliferating follicles progress to form a metastatic lymphoma after 3 months of age, presumably after additional mutations and/or proviral integration next to other proto-oncogenes(24, 144).

ALV has also been shown to induce erythroblastosis by mechanisms similar to those which cause ML, by integration of proviruses near another host oncogene, *c-erbB*. This cellular oncogene was first discovered due to its close amino acid homology with portions of the human epidermal growth factor receptor (EGF) which were strikingly similar to the oncogenes carried by the acutely transforming avian erythroblastosis virus (AEV) (147, 152). Data has shown that upon activation, *c-erbB* can assume an oncogenic role similar to that of its viral counterpart. Although, no alteration of *c-erbA* (the cellular homolog of the other oncogene of AEV) was found in leukemic samples analyzed (118). Furthermore, in the study of these leukemic samples, transcription of *c-erbB* but not *c-erbA* was highly elevated, suggesting that activation of the *c-erbB* gene alone is sufficient to cause erythroblast formation (118). The *c-erbB* oncogene encodes the EGF receptor, a protein kinase which is involved in growth signaling in many cell types, hemopoiesis, and

formation of transforming growth factor α (TGFα) (95, 100, 104). The EGF receptor contains three domains, an extracellular EGF binding domain, a short transmembrane domain, and a cytoplasmic domain (70). Interestingly, upon ALV infection most of the proviral integrations from tumors map within the 3' region of *c-erbB* intron 14, so that a truncated *gag-env-erbB* fusion protein is produced which is thought to have constitutive kinase activity (46, 86, 95, 118). These fusion products generally retain the complete provirus, and are generated by transcription read-through past the 3' LTR followed by alternative splicing (86, 95). From these fusion products and in turn read-through transcripts, recombinant viruses arise containing transduced *c-erbB,* forming acutely transforming viruses which can be observed in approximately 50% of the erythroblastosis tumors that arise (86, 95). Of those proviruses analyzed after initial infection, all were inserted in the same transcriptional orientation as *c-erbB*, and elevated expression of *c-erbB* related mRNA was consistently observed (51, 118). The *c-erbB* oncogene activated in this manner has lost the majority of its extracellular domain, but retains an intact intracellular domain (95). This molecule is strictly leukemogenic and can develop sacromatogenic potential only after accumulation of additional mutations in the kinase or C-terminal regulatory domain. Raines *et al.* observed that the truncated, structurally altered transcripts of *c-erbB* show a 100% correlation with the development of erythroblastosis, suggesting that disruption of the *c-erbB* locus is important for oncogenesis (114, 115, 118).

As described, examinations of ALV proviral integration sites within the *c-myc* or *c-erbB* genes of tumors has revealed a non-random pattern of proviral integration, giving insight to the oncogenic mechanisms of ALV. The integration of the ALV proviral DNA

into *c-myc* has been shown to cause bursal lymphomas, and predominantly this insertion occurs within the 3' region of *c-myc* intron 1 (122, 130). With the *c-myc* protein-coding sequence beginning in exon 2, the promoter/enhancer capabilities of the ALV LTR sequence have been shown to increase *c-myc* mRNA and protein roughly 50-fold (87). The integration of *c-erbB* observed in erythroblastosis are also non-random, so that the majority of tumors show integrations clustered within the 300 bp region of intron 14 (55).

Integration and replication

The replication of ALV is much like that of other retroviruses, with the exception of specific receptors used for attachment to the target cell by specific subgroups (69). The scenario described here is that of a replication competent virus, i.e. one that has not undergone transduction of cellular oncogenes and in turn, deletion of necessary structural genes. The first step in ALV infection, as with all other viruses, involves attachment to the host cell plasma membrane. This process is mediated by the interaction of the viral glycoproteins extending from its surface and specific receptor(s) on the host cell. Subsequent fusion of the viral envelope with the cellular plasma membrane occurs, either directly or following endocytosis, which results in the release of the viral core into the cytoplasm of the cell (26). In the case of ALV subgroup A (ALV-A), a small molecule with a single transmembrane domain distantly related to a cell receptor for low-density lipoprotein (LDL) has been identified, while that of ALV-B is related to the tumor necrosis factor α (TNFα) (36, *157*). The process of intracellular uncoating is not completely understood, however, subsequent early events are carried out in the context of a nucleoprotein complex derived from the capsid (69).

Replication begins with reverse transcription, by RT, of the virion RNA into complementary DNA (cDNA) using the 3'-end of a tRNA as a primer for the synthesis of a negative-sense cDNA transcript. The tRNA is a cellular molecule that is bound to the primer binding site (pbs) located just downstream of the 5' LTR. The initial short product (to the 5'-end of the genome) transfers and primes further cDNA synthesis from the nascent 3'-end of the genome by virtue of the identical r regions at the ends of the viral RNA genome (102). cDNA synthesis involves the concomitant digestion of the viral RNA via RNAse H activity of the RT protein. The products of this hydrolysis serve to prime virus-sense cDNA synthesis on the negative sense cDNA transcripts. In its final form, the linear dsDNA transcripts derived from the viral genome contain the LTRs composed of sequences from the 3' (U3) and 5' (U5)-ends of the viral RNA flanking sequence (R) found near both ends of the RNA. The process of reverse transcription is characterized by a high frequency of recombination due to the transfer of the RT from one template RNA to the other (69). The resulting dsDNA is then integrated, in an apparent random fashion, into a chromosome of the host cell where it resides as a provirus. This integration reaction is mediated by the viral integrase protein (57, 133), whereby the ends of the viral DNA are joined to the cellular DNA, involving the removal of two bases from each end of the linear viral DNA and the generation of a short duplication of cell sequences at the integration site (69). The viral DNA is inserted and cellular DNA repair enzymes complete the integration process (19). ALV lacks a nuclear transport function to move the proviral pre-integration complex into the intact nucleus, as a result the integration process is only able to occur in cells whose nuclear envelope has broken down as a result of the mitotic process, i.e. ALV

can only infect dividing cells (81). The integrated provirus in transcribed by cellular RNA polymerase II into virion RNA and mRNA species in response to the enhancer/promoter sequences of the proviral LTRs. An mRNA comprising the whole genome serves for the translation of the *gag* and *pol* genes (54). This results in the formation of a polyprotein which is later cleaved to yield the structural proteins, protease, RT, and IN, respectively (54). A smaller mRNA consisting of the 5'-end of the genome spliced to sequences from the 3'-end of the genome and including the *env* gene and the U3 and R regions, is translated into the precursor of the envelope proteins (54). Capsids assemble at the plasma membrane and are released from the cell by a budding process (69). Polyprotein processing of the internal proteins occurs concomitant with or just subsequent to the maturation of virions as described earlier (54).

AVIAN LEUKOSIS VIRUS SUBGROUP J

History of subgroup J

As part of a study examining the status of ALV infection among broiler breeder flocks in England in 1988, five isolates, designated HPRS-100 to HPRS-104, were obtained (105). Studies to elucidate the subtype of these new isolates using virus interference assays with RSV pseudotypes corresponding to known ALV subgroups revealed that they were unlike previously known ALVs (105). Further analysis employing antisera directed at the known ALVs, either singly or in combination, failed to neutralize these new isolates (105). Furthermore, host range studies were carried out utilizing chick embryos of varying ALV susceptibility phenotypes (105). The results of these assays also showed the new isolates to be unlike all previously known subgroups. Similar studies were also undertaken *in vitro* in cells derived from several avian species; interestingly isolate HPRS-103 was able to infect both chicken and jungle fowl cell lines, in addition to turkey embryo fibroblasts, a characteristic unknown to all current ALV subgroups (112). With this data, in comparison to all other known ALV subgroups, HPRS-103 was designated the prototype strain of the new subgroup J ALV (105). From the time of its original isolation in England to the point it was designated a novel ALV subgroup in 1991, the aggressive practices of acquiring superior genetics by the commercial poultry industry resulted in the global dissemination of ALV-J in primary breeding stocks, financially devastating the industry.

The ALV-J genome

Upon comparison to the other known and well studied subgroups of ALVs, the prototype ALV-J strain, HPRS-103, had an overall structure of typical slowly transforming replication-competent ALV viruses: LTR-leader-*gag/pol-env*-LTR (6). The sequences of the ALV-J LTR regions showed more than 90% homology to that of other ALVs (148), although the U3 region within HPRS-103 is lacking the enhancer protein (EFII) binding sites found in other ALVs. In addition to the conserved ATG start codon that initiates the transcription of the large open reading frame (ORF) encoding the Gag protein, the leader sequence has two unique ATG codons further upstream (148). The sequence of the HPRS-103 *gag* and *pol* genes are highly conserved (96 to 97 percent identity) with those of the other ALVs, with the exception of a 22 amino acid deletion in the 3' region of the protein due to the incorporation of a premature stop codon (148). This deletion however, is unlikely to affect its functions, as this region has been shown to be unnecessary for viral replication *in vitro* (72). Field strains of ALV-J isolated in the United States have been shown to have similar structure and very close sequence homology to the HPRS-103 prototype strain suggesting that the relationships among ALV-Js may be closer than other subgroups (12).

Unlike the similar LTR sequences shared between ALVs, the *env* gene of HPRS-103 is highly diverged from those of other ALV subgroups (7). The SU (gp85) domain of HPRS-103 has only about a 40% sequence homology with the corresponding sequences of subgroups A to E, as opposed to the 77 to 87% identity commonly found among other ALV subgroups (148). The TM (gp37) has about a 65% identity to that of subgroups A to

E, in comparison to the 92 to 95% identity among the other ALV subgroups (148). The insertion of a 219bp insertion within the TM domain of HPRS-103, termed the redundant TM (rTM), showed 97% homology to the corresponding region of other ALVs, indicating its ALV origin (6). Between the 3' end of the *env* gene and the LTR is a well conserved region among retroviruses that is essential for replication. Within this region are the direct repeat elements (DR1), which have been shown to have constitutive transport elements (CTEs) that facilitate cytoplasmic accumulation of unspliced RNA of unspliced RNA, necessary for proper processing of the viral transcripts(132).

The occurrence of a new subgroup of ALV led to speculation of its possible origins. Original database searches showed a closer relationship between the *env* gene of an endogenous family of avian retroviruses suggesting these endogenous sequences may have been the source of the recombination event (15). Later studies revealed the existence of a novel group of endogenous viral sequences within the chicken genome termed, EAV-HP, these sequences displayed over a 97% homology to that of HPRS-103 *env* gene (6). These EAV-HP elements, also referred to ev/J (11), were present in multiple copies within the genomes of all lines of chickens and in the ancestral jungle fowl, but were absent in most other avian species studied (137). To this point, all evidence suggests that the evolution of the new subgroup J ALV occurred as a result of recombination between an unknown exogenous ALV and the EAV-HP *env* sequences (7).

Unlike the sequences of the previously isolated ALV subgroups, HPRS-103 had a 150 bp region inserted in the 3' non-coding region downstream of the DR1 region referred to as the E element (also called the F2 or XSR) (7). Previously not known to be associated

with the ALV genome, the E element was only found in RSVs, either upstream (Schmidt-Ruppin SR-A strain) or downstream (Prague strain) of the *src* gene (14). Although the actual function of this region and its role, if any, in disease has yet to be elucidated, it does have the ability to bind the transcription factor c/EBP and may also act as an enhancer and its role in oncogenesis will be investigated and discussed later in the text (76, 125).

ALV-J's induction of neoplastic disease

The cells targeted during ALV infection all originate from multipotent progenitor stem cells which go on to differentiate into lymphoid, erythroid, myeloid, and other cell lineages (56). Before the evolution of ALV-J, subgroups A & B were the most predominant ALV subgroups, with the characteristic disease induced by these two subgroups being lymphoid leukosis (LL), primarily occurring in B cells of the lymphoid lineage (148). Based on descriptions of ALV-J-induced disease in experimental studies conducted with meat-type or layer-type chickens, subgroup J was found to induce late onset, occurring at a median age of 20 weeks, myeloid leukosis (ML) and renal tumors targeting mainly those cells of the myeloid lineage (109). In the majority of the cases described lesions were characterized by neoplastic enlargement of the liver and gross skeletal myelocytomas affecting the sternum, ribs, and vertebrae (148). The spleen, testes, kidney, and thymus also displayed accumulations of immature granulated myelocytes forming myeloid tumors (148). Other tumors identified include histiocytic sarcoma, haemangiosarcoma, mesothelioma, granulose cell tumors, pancreatic adenocarcinoma, fibroma, and an unclassified leukosis (107). In addition to the mortality resulting from tumor induction by ALV-J, field reports suggest that broilers generated from infected

breeding flocks may suffer from problems of uniformity, feathering, and inadequate performance, although these observations have not been proven experimentally (148). The ability of ALV-J to infect cells of the myeloid lineage was further substantiated by the demonstration of ALV-J to infect blood monocyte cultures from several different chicken lines (2). Furthermore, the ability of HPRS-103 to replicate in the medullary region of the lymphoid follicles of the bursa of Fabricius was reduced when compared to ALV-A that is able to induce LL (148). However, previous studies have shown that HPRS-103 and other ALV-J isolates do not transform chicken bone marrow cell cultures *in vitro* and that the tumors induced by these viruses occur after long latent periods (109). These observations and the demonstrations of the HPRS-103 genome to not carry any viral oncogenes suggested that ALV-J-induced oncogenesis was initiated by the activation of cellular oncogenes through the mechanism of insertional mutagenesis (7, 12, 74). To date however, the molecular mechanisms surrounding the myeloid tropism or the low bursal tropism have yet to be explained.

Interestingly, from about 60% of the cases of ML tumors induced by HPRS-103, acutely transforming viruses capable of rapid *in vitro* transformation of cultured bone marrow cells were isolated (110). The occurrence of these acutely transforming viruses was also noted in field cases, from which virus isolated from ML tumors was able to rapidly induce myeloid leukosis in experimentally infected chickens, suggesting that generation of acutely transforming ALVs is a common feature of ALV-J-induced neoplastic disease (22, 110). Recently the observation of lesions indicative of erythroblastosis have occurred in field samples submitted for diagnosis (149). From these

samples, just as with those of ML, acutely transforming viruses were isolated from these neoplastic tissues. As explained earlier, it was suggested that this rapid oncogenic potential was derived by the transduction of cellular oncogenes through recombination processes.

Oncogenesis initiated by ALV-J infection is predominantly found in the form myeloid leukosis (ML) (myleocytomatosis) (109), a characteristic thought to be associated with its ability to replicate well in blood monocyte cultures but less so in the lymphoid follicles of the bursa of Fabricius (2). Unlike acutely transforming leukemia viruses like MC29 or MH2 (56), HPRS-103 does not contain any oncogenes and the virus does not transform myeloid cells *in vitro*, as explained earlier (109). However, its ability to transform cells of the myeloid lineage are a direct consequence of its proviral integration next to the cellular proto-oncogene *c-myc*, subsequently enhancing and/or promoting the transcription of the oncogene via action of its LTR, resulting in the development of monoclonal tumors (55, 64). In experimentally infected embryos or 1-day-old chicks the development of bursal lymphomas will occur in approximately 50% to 100% of lymphoma-susceptible chicks by this method of cellular oncogene upregulation (20, 50). Studies by Fung indicate ALV-induced structural alterations of the *c-erbB* gene can induce the development of erythroblastosis (51). Unlike *c-myc*, whose enhanced or inappropriate expression of the unaltered protein may induce oncogenesis due to the actions of proviral LTR, *c-erbB* induced oncogenesis may be caused by disruptions in the coding sequence of the resulting EGF receptor gene (95). The fact that all proviral insertions observed in ALV induced erythroblastosis map to a small region in the middle

of the EGF receptor gene strongly suggests that specific truncation of the EGF receptor gene is required for oncogenesis (118).

Economic consequences of ALV-J

To place an exact total on the annual losses attributed to ALV-J at slaughter would be a nearly impossible task. However, estimations of these losses incurred by the commercial poultry industry as a whole and based on general neoplastic disease referred to as leukosis, can be made based on current statistics. These statistics published by the USDA (2005), predicted that in 2003 approximately 8.6 billion broilers were grown out in the United States. Of that total number, whole bird condemnations due to leukosis averaged 0.005% per company. So with efforts to control ALV-J by eradication in place at the breeder level (to be discussed in further detail later in the text), the industry still lost over $200K when the average price per pound of chicken meat was 30.5 cents (139). These are only losses incurred at slaughter; the losses due to poor growth and reduced feed conversion would significantly out number losses from condemned carcasses. Prior to the implementation of strict eradication policies for infected birds and the development of accurate diagnostics, the mortality in breeder females could have reached an overall high of 6% per month or higher at its peak, while mortality levels in males caused significant infertility issues (*156*). Tumors of various kinds would have been found in up to 60-70% of dead birds in severely infected flocks, with myelocytomas being the most common type of tumor (*156*). In the late 1990's it was not unusual for commercial broilers, hatched from breeder flocks with high virus shedding rates, to experience uniformity problems, paleness, abnormal feathering, inadequate performance and high late overall mortality (>5 wk of

age), usually associated with respiratory disease complications (*156*). Currently, the major economic impacts associated with ALV-J infection are distributed worldwide, but have been restricted to the primary breeder industry. A study conducted by Palya *et al.* in Hungary examined the effects of an ALV-J infection in a meat-type breeder flock; in total 24.3% of the animals died due to the direct or indirect consequence of the ALV infection and egg production fell behind the expected level by about 32% (101). As a consequence of the continuous high mortality rate and the suboptimal level of egg production, the flocks were slaughtered 2 months before the end of the normal production period (101). Worldwide, ALV-J associated loss rates, reported in breeders vary from 3-20% (5). Within the United States the few primary breeder companies who still remain have substantially increased their detection and eradication efforts and disease in this country is not as severe as in other parts of the world. But, efforts to control ALV-J by eradication have placed harsh selection pressures on valuable genetic stock resulting in the loss of pedigree birds. In addition to the eradication costs, the cost of continually monitoring breeder flocks for ALV-J infection can be extreme.

ALV-J diagnostics

Exogenous ALV and in particular ALV-J infections in a flock can be provisionally diagnosed by pathological identification of characteristic tumors and confirmed by various virological methods. The majority of ALV diagnostics in use today can be placed into two distinct categories: 1) those based on the detection of specific proteins or glycoproteins encoded by one or more of the three major genes of ALV, namely *gag, pol,* and *env*, and 2) the detection of specific sequences of proviral DNA or viral RNA by polymerase chain

reaction (PCR) and/or reverse transcriptase (RT)-PCR (41). The detection of both endogenous and exogenous ALVs can be further classified into biological, molecular, and serological assays (45, 108). The gold standard used today by the majority of the commercial poultry industry can be classified as a biological assay, whereby it employs cell culture to isolate and grow ALV before detection of p27 protein, the most abundant structural polypeptide produced by all ALVs. This time consuming step is necessary in a biological assay because most ALVs produce no cytopathic effects (CPE) in culture, making detection of p27 the basis of several diagnostic tests. Indirect biological assays such as complement fixation (CF) for avian leukosis (COFAL) (127), enzyme-linked immunosorbent assay (ELISA) (31, 45), phenotypic mixing (PM) (97), resistance inducing factor (RIF) (124), nonproducer cell activation (NP) (121), and flow cytometry (4) tests have been developed and used for detection of ALVs. Phenotypes of CEFs used for detection of ALV are listed in Table 2. Common diagnostic practices within the primary breeder industry aim to only detect all exogenous subgroups of ALV, making the C/E cell lines the most desirable. Although, other cell types such as those resistant to subgroup A (C/A) (32) and resistant to ALV-J (C/J) can also be used to confirm the subgroup of the isolated ALV (68). The differentiation of exogenous and endogenous ALV can be facilitated by culture in both cells resistant to endogenous (C/E) virus and cells susceptible to exogenous (C/O) virus. If the sample is positive for p27 in C/O cells but not in C/E cells, the sample is assumed to be positive for endogenous ALV, whereas positive results in both cell lines indicate an exogenous ALV. Most commonly, samples used for detection

of ALV include blood, plasma, buffy coat, meconium, cloacal/vaginal swabs, egg albumen, embryos, and tumors (33, 43, 45, 140).

Table 2. Phenotype of chicken embryo fibroblasts (CEFs) used for isolation and identification of most common ALVs from chickens. [A],[B](31), [C](32) , [D](68).

Designation of CEF phenotype	Susceptible to ALV of subgroup	Resistant to ALV of subgroup	Used for isolation of identification of ALV subgroup	Example of cell line and referece
C/O	A, B, C, D, E, J	None	All subgroups of ALV	15B1; Crittenden *et al.*, 1987
C/E	A, B, C, D, J	E	Exogenous ALVs only	Line 0; Crittenden *et al.*, 1987
C/AE	B, C, D, J	A, E	Rule out subgroups A and E ALV	Alv 6; Crittenden & Salter, 1992
C/EJ	A, B, C, D	E, J	Rule out subgroups E and J ALV	DF-1/J; Hunt *et al.*, 1999

Assays which test directly for ALV infection have largely relied on the detection of gs antigen (p27), a protein encoded by the *gag* gene of ALVs. Assays which rely on the detection of antibody can often be misleading due to the varying types and phases of ALV infection (148). For example, birds congenitally exposed or those birds infected by hatch mates may not develop an immune reaction, and therefore develop a persistent viremia and no antibodies to ALV (148). Using direct ELISA or the CF test, ALV p27 can be detected in samples of albumen, meconium, cloacal/vaginal swabs, or feather pulp (45, 108). Utilizing monoclonal antibodies against ALV p27 (35), the most common direct method of ALV detection is acELISA for p27, and commercially available kits have been developed. Monoclonal antibodies also exist for the detection of envelope glycoproteins (gp85) of AVL-J (40). Radioimmunoassays can also be used for the detection of p27 and were consistently found to be more sensitive than CF in detecting exogenous and endogenous

ALV (41). In sections of tumor of other tissue immunocytochemical staining procedures such as immunofluorescence, immunoperoxidase anti-peroxidase, or protein A-gold can also be used for detection of p27 associated with ALV virions (53).

Biological systems used to detect ALV, such as susceptible ALV-free chickens, chicken embryos, and CEFs are among the most reliable and most commonly used methods for isolation and identification of ALV. As described above, this ALV diagnostic technique allows the detection of infectious virus, antigen, and/or antibody and has been instrumental in control and eradication programs within the industry.

Today the most common method to isolate and identify ALV first involves samples to be cultured in CEFs. Since most ALVs produce no CPE, indirect biologic assays such as COFAL, acELISA, PM, RIF, and NP cell activation tests are used to detect the presence of ALVs. The acELISA has become the tool of choice for those who require a sensitive, practical, and economical assay for ALV detection. This method is carried out in two distinct parts; first the virus is propagated in CEFs (most often C/E) for approximately seven days, this is followed by the assaying the cell lysates, via acELISA, for ALV p27 gs antigen.

The latest and most rapidly advancing form of diagnosing ALV infection involves molecular technology. The complete ALV genome has been elucidated and this information has spawned the development of an array of primers and probes designed to detect many aspects of the ALVs (6, 7, 25). Unlike the methods relying on the biological amplification of ALV, this technology allows the rapid detection of ALV during early stages of infection (146). The use of molecular-based diagnostic techniques for the

detection ALV have been investigated (52, 73, 135, 136). PCR methods have been previously developed for the amplification of ALV-J from many different sample types including serum, whole blood, meconium, and feather tips (6, 52, 73, 135, 136). Primers developed for these assays have been targeted towards the sequences of the *env* gene or LTR regions of the ALV genome (135, 136). Additional regions of amplification for PCR include the 3' non-coding region, the E element, the H5-H7 located in the 3' region of the polymerase gene, and the 5' region of the gp85 portion of the *env* gene (135, 136). While the amplification of these regions of the ALV genome have proven sensitive and specific for the detection of ALV, the continual evolutionary pressure on these portions of the ALV genetic sequence create an ongoing trend of mutation (131, 148, 150).

The development of real-time monitoring systems for PCR amplification have been widely accepted and adapted for the detection and quantification of selected genes (98). This method of ALV detection gives superior sensitivity by the use of Taqman primer/probe chemistry, in addition, the length of the amplified transcripts are shorter and can be directed at smaller regions conserved within the ALV genome, affording greater specificity.

Control and eradication

The control of ALV-J presented a significantly challenging problem for the commercial poultry industry following its isolation in the late 1980s. In an effort to control this new subgroup of ALV, methods adopted by the industry have been primarily focused on it's eventual eradication (42, 96, 99, 111, 138). This goal however, has been an elusive one. The environment, population densities, and rearing practices of the modern-

day commercial poultry facility make isolating and eliminating infected birds in a timely manner very difficult. And unlike the other ALV subgroups, the extreme proficiency by which ALV-J is able to infect both horizontally and vertically, only compounds this problem, requiring eradication programs to be applied more rigorously (153, 154). Removal of ALV positive birds identified by the current industry 'gold standard,' cell culture coupled with acELISA, can at best occur approximately 8 days after the sample was collected. This period allows ample time for the horizontal transmission of virus to littermates and continued vertical transmission by breeders, inciting a chain of infection that is nearly impossible to break without detrimental consequences to breeding stock. The industry has taken steps to counter ALV-J's infectious proficiency; large whole-house breeder facilities have evolved into methods employing small pen rearing, limiting exposure to other birds within the same house (154).

Biosecurity is always a first defense against any exogenously spread disease and these measures should be applied to any scheme aiming at controlling and eradicating ALVs. The notion of conferring protection through vaccination has been investigated, but as with most, if not all retroviruses, their widespread antigenic diversity and lack of knowledge on immunogenic properties of different viral antigens, make development of an effective vaccine a challenging task (148). Currently, a shift in methods of surveying for and diagnosing exogenous ALV is taking place. The acELISA and anti-ALV antibody surveillance tests have been proven to not be completely reliable and less sensitive than diagnostic techniques currently available (1, 52, 149). However, molecular diagnostic techniques offer extreme sensitivity and specificity, provide rapid results, and can be

preformed in a high-throughput manner at a cost equivalent to or less than the current ‘gold standard’ method, as presented in the following manuscript.

DEVELOPMENT OF A HIGH-THROUGHPUT QUANTITATIVE REAL-TIME RT-PCR ASSAY FOR RAPID DETECTION OF EXOGENOUS AVIAN LEUKOSIS VIRUS

Overview

Avian leukosis viruses (ALVs) have caused significant economic losses worldwide in meat-type chickens by diminishing feed conversion, inciting neoplastic disease resulting in condemnations at slaughter, and increasing mortality. Based on differences in their envelope glycoproteins, virus-serum neutralization tests, virus interference, and host range these viruses in chickens are classified into six subgroups. The most recently discovered ALV, subgroup J, was isolated from meat-type chickens associated with myleocytomatosis in England in the late 1980s. The host range of ALV-J encompasses jungle fowl, turkeys, and meat-type birds at all breeding generations including commercial broiler flocks. Beginning in the early 1990s the commercial broiler industry was confronted with this new subgroup of ALV, when it began causing serious mortality and production problems worldwide. Eradication programs based upon virus isolation and identification were initiated by primary breeder companies in order to produce virus free breeding stock. A multitude of primary breeders have had meager success, in a large part due to the techniques employed for exogenous ALV detection. In order to increase sensitivity and provide a rapid analysis of samples, a qrRT-PCR assay was designed and developed by this laboratory to amplify a specific sequence conserved among the exogenous ALV subgroups A, B, C, D, and J while excluding amplification of endogenous viruses (ev) or ev sequences within the chicken genome. The sensitivity of the resulting one-tube

hydrolysis fluorogenic probe based reaction developed in this study was calculated to consistently amplify less than 20 copies of *in vitro* transcribed RNA. In addition, we analyzed 275 buffy coat samples drawn from pedigree broiler lines of one primary breeder company within the United States. The results of both virus isolation and qrRT-PCR from these samples were compared and the data correlated highly. The increased detection yielded by the qrRT-PCR assay was approximately 160% greater than VI. In addition, the lower range of detection of our qrRT-PCR assay was considerably greater than that detected following virus isolation. This contemporary method of exogenous ALV detection allows for the real-time quantification of ALV RNA copy numbers, is extremely sensitive, specific, cost effective, and easily performed in a high throughput manner, resulting in an assay able to provide a practical and efficient tool for commercial breeder operations to monitor and eventually eradicate ALV within their primary breeding stock.

Introduction

The avian leukosis viruses (ALVs) are grouped into the *Alpharetrovirus* genus of the family Retroviridae (120). These viruses in chickens are classified into six subgroups: A, B, C, D, E, and most recently J (106). Their distinctions arise from differences in envelope glycoproteins, virus-serum neutralization tests, virus interference, and host range (71). ALVs are known to cause a variety of neoplasms including erythroid, lymphoid, and myeloid leukoses (109, 110, 149). The ubiquitous endogenous ALVs of subgroup E have low pathogenicity and are transmitted vertically through inherited host genes, unlike the transmission of exogenous ALVs that occurs both vertically and horizontally (30). Of these exogenous subgroups, A and B are the most common ALVs, while subgroups C and D

have been rarely reported in commercial poultry due in large part to the eradication efforts of the commercial poultry industry (21, 108). The pluripotential neoplasms of ALVs cause severe economic losses due to condemnations at slaughter, loss of pedigree birds, and tumor mortality. The most recently discovered ALV, subgroup J, was isolated from meat-type chickens associated with myleocytomatosis in England in the late 1980s (105). ALV-J was predicted to be a recombinant of exogenous ALVs and the EAV family of endogenous avian retroviruses (6). The host range of ALV-J encompasses jungle fowl, turkeys, and meat-type birds at all breeding generations, including commercial broiler flocks (44, 109). The commercial broiler industry was first confronted with this new subgroup of ALV in the early 1990s, when its impact began causing serious production problems and mortality on a global scale (106, 141).

To date, the 'gold standard' assay employed by the commercial poultry industry to determine ALV infection involves the detection of the viral group specific antigen (gs) p27 by antigen-capture (ac) enzyme-linked immunosorbent assay (ELISA) (138). This technique however is not specific and when used exclusively it will detect p27 encoded by *ev* sequences, in turn applying undesirable, and costly selection pressure on pedigree stock (156). Since p27 is shared by both endogenous and exogenous ALVs, exclusive use of ELISAs cannot be used to identify and differentiate ALVs. To prevent the detection of *ev* p27 and increase specificity, samples must first be inoculated onto chicken embryo fibroblasts (CEFs) prior to acELISA (45). The most commonly used CEFs to isolate and detect AVLs by the commercial poultry industry include C/E, which are susceptible to infection only by exogenous ALV subgroups. Cells susceptible to infection from all

exogenous and endogenous ALV subgroups, C/O CEFs, can also be used for ALV isolation (31, 41, 45). This technique of virus isolation (VI) and antigen detection is time consuming, requiring 8-10 days to complete, it is also laborious, and expensive.

Methods to control ALV have been primarily focused on it's eventual eradication (42, 96, 99, 111, 138). This goal however has been an elusive one for the commercial poultry industry. The environment, population densities, and rearing practices of the modern-day commercial poultry facility make isolating and eliminating infected birds in a timely manner very difficult. The proficiency of ALV-J to infect both horizontally and vertically only compounds this problem (153, 154). Removal of ALV positive birds identified by cell culture coupled with acELISA can at best occur approximately 8 days after the sample was collected. This period allows ample time for the horizontal transmission of virus to littermates and continued vertical transmission by breeders, inciting a chain of infection that is nearly impossible to break without detrimental consequences to breeding stock.

The use of molecular-based diagnostic techniques for the detection ALV has been investigated (52, 73, 135, 136). Polymerase chain reaction (PCR) methods have been previously developed for the amplification of ALV-J from many different sample types including serum, whole blood, meconium, and feather tips (6, 52, 73, 135, 136). Primers developed for these assays have been targeted towards the sequences of the envelope (*env*) or long terminal repeat (LTR) regions of the ALV genome (135, 136).

Additional regions of amplification for PCR include the 3' non-coding region, the E element, the H5-H7 primer set spanning the 3' integrase region of the *pol* gene to the 5'

region of the gp85 portion of the *env* gene (135, 136). While the amplification of these regions of the ALV genome have proven sensitive and specific for the detection of ALV, the continual evolutionary pressure on these portions of the ALV genetic sequence create an ongoing trend of mutation (131, 148, 150).

The development of real-time monitoring systems for PCR amplification have been widely accepted and adapted for the detection and quantification of selected genes (98). The ability of this new quantitative real-time reverse transcriptase PCR (qrRT-PCR) technology to rapidly analyze and provide results is only limited by the time needed to extract the RNA/DNA template from individual samples. The high-throughput feasibility of this assay was retained by the use of magnetic bead technology, first described by Guesdon *et al*. (60). Adaptation of this magnetic bead technology to isolate both viral RNA and proviral DNA in a 96-well plate format was employed to acquire the template for this qrRT-PCR assay. The objective of this study was to develop a new high-throughput one-tube qrRT-PCR assay using a hydrolysis fluorogenic probe for the specific detection and quantification of only exogenous ALVs to rapidly identify and remove infected birds, limiting horizontal and vertical transmission. The application of this assay in the detection of exogenous ALV subgroups from clinical samples such as buffy coat (BC) and plasma is discussed; also a comparison of this assay to VI and acELISA is made.

Materials and methods

A. Diagnostic samples

BC and plasma samples were obtained from a U.S. primary breeder company. Diagnostic samples were shipped on dry-ice in 96-well microtiter plates, with each plate

corresponding to a random sample of 7 week-old pedigree birds within three different brooder houses (A,B, & C) on the same complex.

B. Nucleic acid extraction

Viral RNA was extracted from 50 µl plasma samples with the use of RNA-binding magnetic particles supplied by the MagMAX™ Viral RNA Isolation Kit developed by Ambion®, Inc. (Austin, Texas, USA). The manufacturer's protocol for cell free sample was followed. In an effort to simulate authentic high throughput evaluation of clinical samples in a 96-well plate format, the robotic extraction of viral RNA from plasma using the MagMAX™ components was carried out by Ambion®, Inc. (Austin, Texas).

Total nucleic acid from BC samples was also extracted with the use of the MagMAX™ kit (Ambion®, Inc. Austin, Texas). The manufacturers' protocol was optimized. Briefly, 10µl of BC samples were transferred into new 96-well plate, 60µl of nuclease free water was added and the plate was then shaken with a DPC MicroMix® 5, (form 5, amplitude 15) for 1 min. Fifty microliters of this suspension was then used for isolation of total nucleic acid following the normal MagMAX™ Viral RNA Isolation Kit protocol.

C. Development of a one-tube real-time quantitative RT-PCR

Sequence data and phylogenetic analysis by Garcia *et al.* (52) has demonstrated that the long terminal repeat (LTR) U3 region of the ALV genome is closely related among exogenous subgroups and more distant from U3 LTR regions of endogenous viruses. Using this preliminary data and additional exogenous ALV sequences obtained by this

laboratory, the DNASTAR group of programs was used to design a set of primers and probe within this region of the exogenous ALV genome (DNASTAR, Inc., USA).

Oligonucleotide primers used in the study were a forward primer (XALV-F) and a reverse primer (XALV-R) designed to amplify a conserved 112 bp region within the LTR of the ALV genome. This pair of primers was developed to specifically amplify all exogenous subgroups of ALV and did not amplify sequences of the endogenous subgroup E ALVs. The specificity of these primers was tested for all known strains of exogenous ALVs, subgroups A, B, C, D, and J, and on twelve samples derived from inbred and congenic chicken lines with known endogenous gene loci. Additionally, a BLAST search was carried out against the GeneBank database and no sequences of known endogenous virus identified. A fluorogenic probe labeled with 56-FAM and 3BHQ-1 was also developed that would bind within the location of the primers XALV-F and XALV-R (Integrated DNA Technologies, Inc., Coralville, Iowa).

Complementary DNA (cDNA) was amplified using the primers RUGZ-F and RUGZ-R from a recent ALV-J field isolate (*156*). Following RT-PCR, the amplicon was ligated to the pCR®-XL-TOPO® cloning vector (Invitrogen, Carlsbad, California, USA). The recombinant plasmid vector was transformed into the chemically competent One Shot® TOP10 strain of *Escherichia coli* (Invitrogen, Carlsbad, California, USA). Transformed colonies were selected following an 18 h incubation at 37^{o}C on Luria Broth (LB) plates containing 50 µg/ml Ampicillin. The plasmid was then purified from these cultures (QIAprep® Spin Miniprep Kit, Qiagen, Valencia, California, USA) an the desired insert and it's direction was visualized by the presence of a 649 bp product on 2% agarose

gel following PCR amplification using the primers XALV-F and M13 Forward Primer (Invitrogen, Carlsbad, California, USA). Purified plasmid DNA containing the desired insert in the proper orientation was linearized with Hind III (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 3 h at 37^{o}C an *in vitro* transcription was performed using a commercial kit (T7 RiboMAX^{TM} Express Large Scale RNA Production Systems, Promega, Madison, Wisconsin, USA). Following *in vitro* transcription, RNase-free DNase was added and incubated at 37^{o}C for 30 min. RNA transcripts were then precipitated via phenol/chloroform, washed and dried. Briefly, 750µl of Trizol LS was added to the *in vitro* transcribed RNA product, it was then vortexed and incubated at room temperature for 5 min. Two-hundred microliters of $CHCl_3$ was then added to a 1.5 ml Eppendorf tube, vortexed, and incubated for 10 min. This suspension was then centrifuged at 12,000 rpm at 4^{o}C for 10 min. Following the centrifugation the upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube, 500µl of isopropyl alcohol was added to the sample, vortexed, and incubated at room temperature for 10 min. This was followed by an additional spin at 4^{o}C at 12,000 rpm for 10 min, precipitating the RNA into a pellet. This pellet was finally washed once with approximately 1 ml 75% ethanol and repelleted by centrifugation. The resultant pellet was then redissolved in 50 µl of RNase free molecular grade H_2O and RNA concentration was determined using a biophotometer at A_{260} nm (Eppendorf, Hamburg, Germany). The RNA was divided into single use aliquots containing $1x10^6$ copied per microliter and stored at -80^{o}C.

qrRT-PCR was performed on control and viral RNA in ten-fold serial dilutions to determine amplification efficiencies. Standard curves for the respective RNAs were

calculated by plotting their threshold cycle (C_T) values versus their dilution factors. Control RNA was diluted from approximately 10^6 to 10^0 copies per microliter of sample input into the qrRT-PCR assay. A standard curve was then derived for extrapolation of viral RNA copy numbers from individual samples. The slopes of these standard curves were then compared to each other. Efficiencies were defined as equal if the difference of the slopes was smaller than 0.1 (61).

The total volume of the qrRT-PCR reaction was 25 µl and included 7 µl of template solution, the XALV-F and XALV-R primers at a concentration of 200 nmol and the hydrolysis fluorogenic probe at a concentration of 100 nmol. The remaining components used in this reaction were provided by the Quantitect™ Probe RT-PCR Kit (Qiagen, Valencia, California, USA) and formulated according to the manufacturer's instructions.

The protocol for the qrRT-PCR assay consisted of 50°C for 30 min, 95°C for 13.5 min, and 45 identical cycles of 95°C for 10 s, 52.5°C for 30 s, and 76°C for 30 s. The reverse transcription and cDNA amplification were carried out in a single tube in a Biorad iCycler™ thermocycler coupled with the MyiQ™ Single-Color Real-Time PCR Detection System (Biorad Laboratories, Hercules, California, USA). This system adds a 1.5 min cycle directly following the first cycle to reach 95°C in order to read initial fluorescence and calculate the needed adjustments to compensate for pipetting errors. The software that accompanies the detection system displays amplification data in real-time and derives individual C_T values according to a calculated base line value on completion of the reaction.

D. acELISA

In addition to qrRT-PCR, BC and plasma samples were inoculated onto cell culture to determine the presence of ALV-J by detection of p27 antigen. Briefly, 100µl of sample was simultaneously inoculated onto 48-well tissue culture plates containing $1.5x10^5$ DF-1 cells per well in 4% growth medium, containing 3.76 g Leibovitz L-15 Medium, McCoy's 5A Medium Mix, 1.1 g $NaHCO_3$, 20 ml (4%) fetal bovine serum (FBS),1 mg amphotericin B, 12.5 mg gentamycin, 1 mg DEAE dextran, 2000 units of heparin, and brought to a volume of 500 ml. Following 24 hr incubation the media was removed and replaced with maintenance media (1% FBS growth media without DEAE dextran and heparin) and incubated for an additional 7 days. Plates were then processed for p27 antigen by the addition of 50 µl 5% Tween 80 to each well followed by two freeze-thaw cycles. One hundred-microliter aliquots of cell lysates were collected from each well and transferred to 96-well plates for acELISA detection. ALV acELISA assay protocol and readings were performed according to the manufacture's recommendations (IDEXX Laboratories, Westbrook, ME) except a sample to positive ratio (S/P) cutoff value of 0.1, less stringent than the manufacture's recommendation of 0.2, was used to call positives. S/P ratios were calculated by subtracting the negative control mean absorbance value at 650 nm, A(650), from the sample mean and dividing this value by the value obtained from subtracting the negative control mean at A(650) from positive control mean at A(650). {[(sample mean – negative control mean)/(positive control mean – negative control mean)] = S/P}

E. Calculations

Simple κ coefficients were calculated using the formula: kappa = (observed agreement – chance agreement) / (total observed – chance agreement). κ values were interpreted as follows: <0 – no agreement, 1.0 to 0.19 – poor agreement, 0.20 to 0.39 – fair agreement, 0.40 to 0.59 – moderate agreement, 0.60 to 0.79 – substantial agreement, 0.80 to 1.00 – almost perfect agreement (77). Trend lines were calculated using the polynomial trend line feature of Microsoft® Excel with an order of two.

Results

A. qrRT-PCR

The specificity of the resulting primers and probe was tested against purified DNA from cells previously infected with ALVs A, B, C, D, and J, DNA from different chicken lines carrying endogenous loci and DNA from RAV-0 infected cells. Also a BLAST search was performed on the chicken genome, no results were found to correlate with *ev* sequences. A nearly 100% agreement was found when comparing sequence identity of the avian leukosis virus genome (Genebank accession number NC_001408) and the primers and probe set referred to earlier for exogenous ALV detection (13). The real-time PCR analysis of these samples only amplified the exogenous proviral DNA of ALVs A, B, C, D, and J. No amplification products were observed for *ev* RAV-0 or any of the genomic DNA from the chicken lines tested (data not shown).

B. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR of control RNA was used to generate amplification plots and standard curves for extrapolation of proviral DNA and viral RNA copy numbers

(Figure 2 & Figure 3). The sensitivity of the qrRT-PCR was determined by amplification of serial 10-fold dilutions of control RNA. Sensitvity yielded by the qrRT-PCR assay was determined to be approximately 20 copies of target sequence.

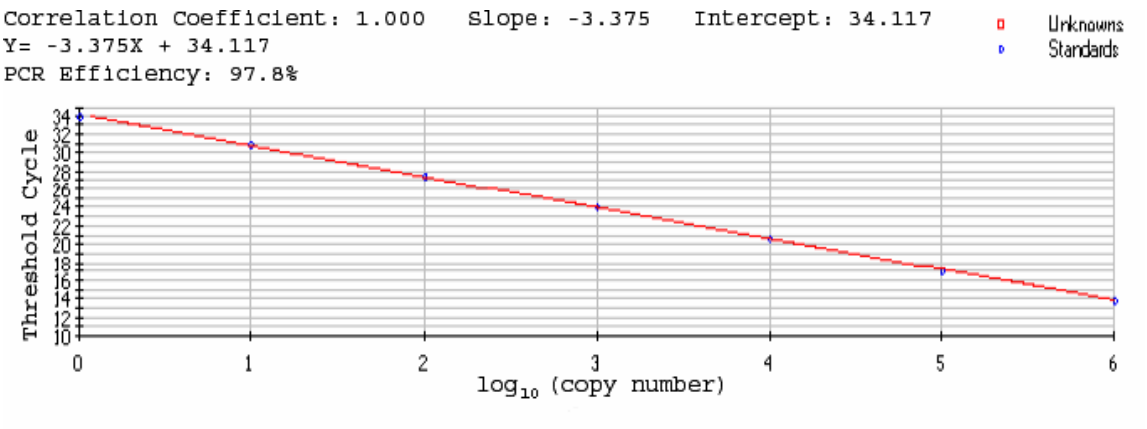

Figure 2. Standard curve depicting the threshold cycles (CT) values vs. the starting quantities of standard RNA dilutions of control RNA. The amounts of control RNA added were log dilutions from approximately 3.66 ng/µl to 3.66 ag/µl. The regression line is linear and CT values are highly correlated with starting RNA quantity. Exogenous ALV copy numbers were calculated based on the line equation derived by the standard curve.

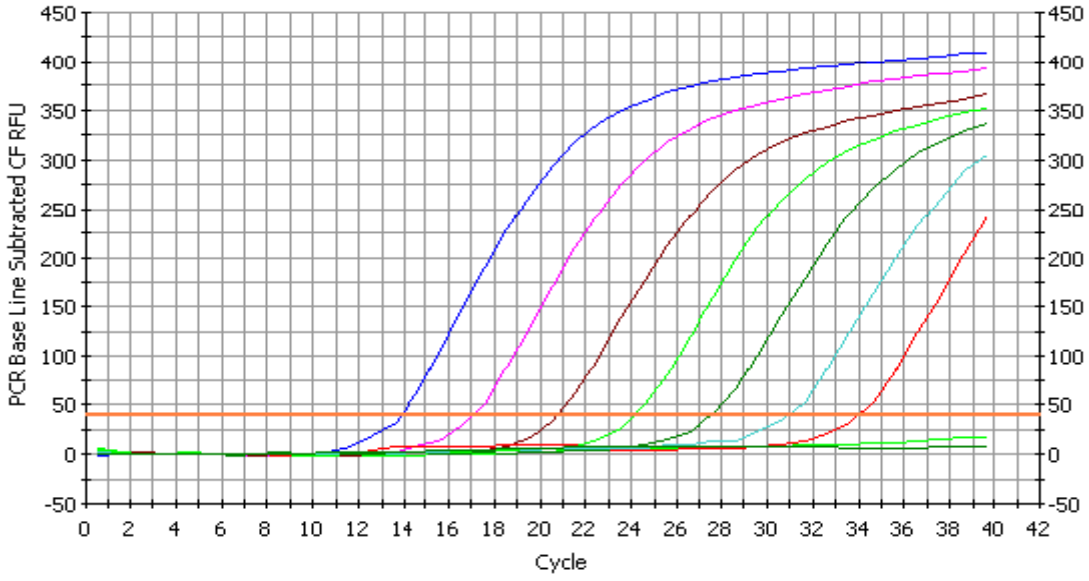

Figure 3. Amplification plot of control RNA dilutions containing (left to right) 7x108, 7x107, 7x106, 7x105, 7x104, 7x103, and 7x102 copy numbers, respectively for use in deriving a standard curve.

C. Detection of exogenous ALV from buffy coat samples

Virus isolation, qrRT-PCR of cell culture lysates (CC L), and qrRT-PCR of 275 BC samples from three grandparent flocks are shown in Table 3. A total of 24, 26, and 16 BC samples were positive for exogenous ALV via VI in DF-1 cells, whereas qrRT-PCR of the same lysates used in the acELISA, following VI, detected 29, 40, and 14 positive samples. qrRT-PCR of extracted nucleic acid from the BC samples of houses A, B, and C yielded 50, 65, and 57 positives, respectively (Table 3).

Table 3. Detection of exogenous ALV in buffy coat samples by VI and qrRT-PCR. [A]TP = true positive, number of samples VI+/qrRT-PCR+; TH = trure negative, number of samples VI-/qrRT-PCR-; FN = false negative, number of samples VI+/ qrRT-PCR-; FP = false positive, number of samples VI-/qrRT-PCR+; SE = % sensitivity, TP/(TP + FN) x 100; SP = % specificity, (TN/(FP + TN) x 100; κ = kappa coefficients, measure of agreement between virus isolation and qrRT-PCR.

		Positive Samples / Avg. value									
	No.		VI	qrRT-	Comparison of assays[A]						
Flock	samples	VI	CCS	PCR	TP	TN	FN	FP	SE	SP	κ
A	92	24	29	50	20	37	4	31	83.3	54.4	0.28
B	91	26	40	65	24	24	2	41	92.3	36.9	0.20
C	92	16	14	57	16	35	0	41	100.0	46.1	0.23

Virus isolation was compared to the results of qrRT-PCR (Table 3). True positive (TP) BC samples (VI+/qrRT-PCR+) detected in houses A,B, and C were 20, 24, 16, respectively. Twenty-four, 35, and 37 true negative (TN) samples (VI-/qrRT-PCR-) of BC were found in houses A, B, and C, respectively. False negative (FN) BC samples (VI+/ qrRT-PCR-) ranged from 0-5 per house. Notably, in the BC samples obtained from houses B and C there were 41 false positives (FP) samples (VI-/ qrRT-PCR+) (Table 3). The sensitivity (SE) of the qrRT-PCR assay developed detected copies of exogenous ALV nucleic acid ranging from 78.3% to 100% when compared to VI among the three houses, whereas the specificity (SP) ranged from 30.5% to 54.0% (Table 3).

Table 4 illustrates that increased S/P ratios, those samples found by VI to contain higher concentrations of p27 (higher virus titers), correlate to decreased C_T values in qrRT-PCR and in turn higher copy numbers of viral nucleic acid. In an S/P range of 0.000 to 0.099 the average value calculated among samples of all three houses was 0.037 in 208 VI negative samples, whereas qrRT-PCR amplified target sequence in 113 samples with an

average C_T of 38.52. In an S/P range of 0.100 to 0.199 the average value calculated was 0.133 in 11 VI positive samples and qrRT-PCR amplified target sequence in 9 samples with an average C_T of 36.58. Between the S/P values of 0.200 to 0.499 the average value calculated was 0.292 in 36 VI positive samples, while qrRT-PCR amplified target sequence in 32 of those samples with an average C_T of 34.92. Spanning the S/P range of 0.500 to 0.999, VI detected 9 positive samples with an average value of 0.718 and these samples were also found to be all positive by qrRT-PCR, with an average C_T of 34.42. There were 10 samples found to have an S/P ratio of greater the 1.000 following VI, those same samples were also found positive by qrRT-PCR and the average C_T was calculated as 31.37. The statistical analysis of this data was expressed by a simple κ coefficient. A simple κ coefficient of 1 indicates a perfect agreement between assays, whereas a κ coefficient of 0 indicates no agreement between assays.

Table 4. Sensitivity comparison of exogenous ALV detection by VI and qrRT-PCR. [A]Average value for VI stated as: S/P = ([Sample absorbance] - [average negative control absorbance])/positive control absorbance; VI CCS & qrRTPCR = CT (threshold cycle). [B]S/P cutoff. CqrRT-PCR value = CT (threshold cycle).

	Average combined values of houses A,B,&C	
	No. of samples / value	
S/P ranges[A]	VI	qrRT-PCR[C]
0.000-0.999	208 / 0.037	113 / 38.52
0.100[B]-0.199	11 / .133	9 / 36.508
0.200-0.499	36 / .292	32 / 34.92
0.500-0.999	9 / .718	9 / 34.42
≥ 1.000	10 / 1.38	10 / 31.37

Charted in Figure 4 are the BC samples found positive in VI and their corresponding S/P ratios in addition the C_T values obtained directly from the extracted nucleic acid of the same BC samples and from the CCL following VI. As depicted by the trend lines of the qrRT-PCR assays the C_T values, corresponding to viral RNA copy numbers, highly correlate with the increased S/P ratios. Accordingly the C_T values of the CCL contained an average of $10^{7.4}$ copies more than copies extracted directly from BC samples.

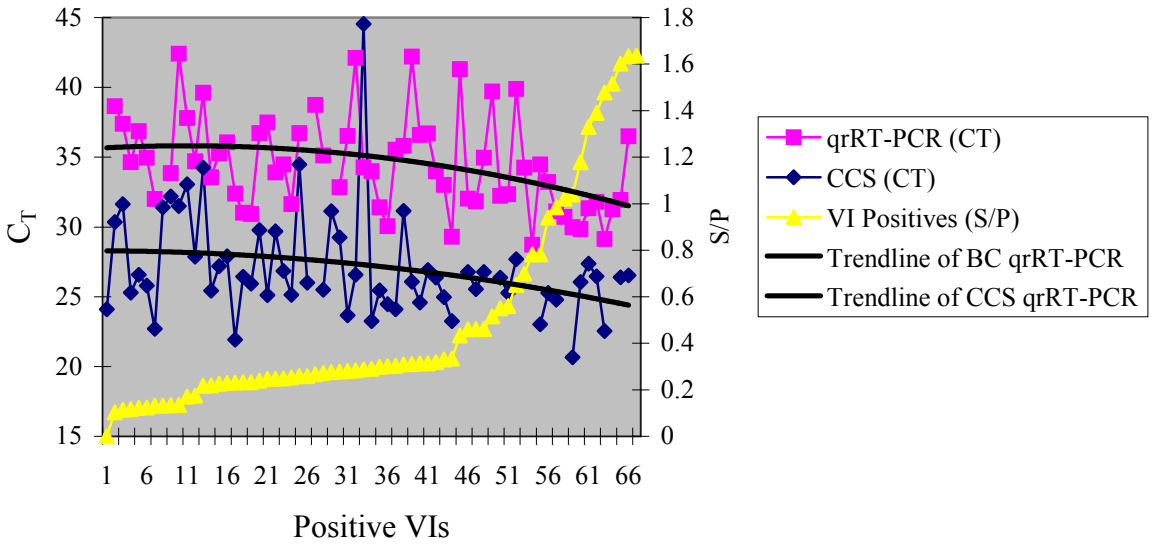

Figure 4. Trend lines of qrRT-PCR (quantitative real-time RT-PCR) CT (threshold cycles) values of BC (buffy coat) and CCL (cell culture lysate) samples versus the S/P (sample/positive) ratios following VI (virus isolation).

Discussion

The rapid identification and elimination of ALV positive birds is essential for the eradication of this virus from commercial primary breeding stock, and the continued financial success of the breeder company. The severe economic losses brought on by the rapid global dissemination of ALV-J within the primary breeder industry which occurred following its initial identification and isolation in the late 1980s clearly illustrate the need for the continual monitoring and eventual eradication of all exogenous ALV subgroups. Many obstacles will hinder if not prevent the elusive goal of eradication from ever being realized. The most important of these obstacles is the predominantly used diagnostic technique of VI, still considered the industry's 'gold standard.' This method requires within the sample infectious virus and in most instances the absence of inhibiting antibodies, two criteria which cannot be easily controlled. With these criteria met, and assuming no bacterial or fungal contamination of the cell culture has occurred, the elimination of positive birds can happen, at the earliest, seven days post acquirement of the sample. The dissemination of ALV-J infection in exposed birds is inevitable following this prolonged method of diagnosis, in turn inciting a course of infection that is near impossible to break. Secondly, the established dogma of ALV infection is based on the this method of diagnosis, which this study and others have shown to be less sensitive than PCR based technologies (52, 135). This dogma is founded on four classifications of ALV infection: the presence (+) or absence (-) of viremia (V), serum antibodies (A), and viral shedding (S) (148). These infection profiles can be further described or categorized as consistently positive, transiently positive, intermittently positive, and negative (153). Applying VI as a

diagnostic technique, Witter *et al*. (153) has provided detailed studies of bird responses to ALV-J infection. Consequently, VI responses of intermittently and transiently positive birds were characterized as inconsistent from weeks 12-62 post hatch, which may be explained by the presence of virus neutralizing antibodies present among these categories of birds between weeks 20 and 40 of age (153). The existence of the retrovirus infection classification described as V-A+S+ is evidence to the reduced sensitivity of VI when compared to PCR-based technologies.

As previously established by Garcia *et al*. (52) and further substantiated in this study, the 3' LTR region of the ALV genome is highly conserved among exogenous ALV strains, and is therefore considered a superior target of amplification for development of an ALV diagnostic for commercial applications. The primers and probe designed in this assay provide extreme sensitivity, detecting in most cases less than 10 virus particles (data not shown). In addition, the specificity of theses primers and probe was further shown by the absence of amplification when run against the DNA of ubiquitous *ev* loci. The amplification of all exogenous ALV subgroups, while excluding *ev* loci, is advantageous because it is the exogenous subgroups which cause detrimental effects within commercial poultry production, therefore making these subgroups targets of eradication.

The sensitivity obtained from the qrRT-PCR of BC samples was relatively high, except for the decrease that was observed in House A (Table 3). This can be explained by the extreme sensitivity of the assay and its ability to amplify the minutest quantities of target sequence, for this reason the Biorad MyiQ software calls positives based on amplification crossing the threshold and occurring before the negative control plus the

tolerance (Biorad, Hercules, CA U.S.A.). When compared to VI, more positive samples were in qrRT-PCR. Several postulations can be made to explain these occurrences. Firstly, the sensitivity of the VI must be questioned. Referring back to Figure 4, the trend lines of both qrRT-PCRs are nearly linear, suggesting that the lower range of detection of qrRT-PCR is greater than that of VI. If negative values of VI were included in this figure it would be expected that the C_T values will increase at the lower limits of the assay until there is a cessation of amplification. Therefore, what is considered false positive in this study may truly be false negative and the ALV present in the sample inoculated into tissue culture may have been inhibited by virus neutralizing antibodies. Retroviruses are notorious for their capacity to form replication defective particles, which may also contribute to this factor (128).

The extraction method utilized for the development of this assay has been compared to other established methods of extraction. The National Veterinary Services Laboratory (NVSL) has successfully implemented this extraction technique into their Exotic Newcastle Disease virus (ENDV) diagnostic; with over 100,000 samples analyzed not a single case of cross contamination or false positive/negative result has been observed (48). Data published by Ambion, Inc. further investigated the incidence of cross contamination when the extraction procedure was carried robotically using their MagMAX technology modified for whole blood samples. A checkerboard pattern was created which consisted of 24 positives surrounded by 72 negative samples no amplification was detected following total RNA isolation of negative blood samples (47).

The samples used for this assay were BC samples which contain the hemapoeitic progenitor cells the exogenous ALV subgroups typically infect (109). In addition to containing the proviral DNA, the extracellular fluid also has the potential to contain virus particles. This sample medium therefore presents two possible routes of target amplification, in turn increasing sensitivity. While providing a higher degree of sensitivity, this sample type is slightly more labor intensive compared to other cell-free sample types such as plasma. BC samples were compared to plasma and similar results were obtained (data not shown). When this assay was first undertaken a whole blood or BC extraction procedure had yet to be developed. Harsher lysis buffers and integration of proteases will make the extraction from these BC samples less labor intensive and therefore a more efficient route of exogenous ALV detection.

We have presented data that provides for a more sensitive, rapid, high-through-put, and cost effective means of exogenous ALV detection, providing a practical and efficient tool for commercial breeder operations to monitor and eventually eradicate ALV within their flocks. This new approach to exogenous ALV detection bridges a gap, allowing the full benefits of PCR based technologies to be used in high throughput diagnostic tests.

THE E ELEMENT OF AVIAN LEUKOSIS VIRUS SUBGROUP J: ITS EFFECT ON PATHENOGENESIS BY PROVIRAL INTEGRATION AND DEREGULATION OF C-ERBB AND C-MYC

Overview

The association of neoplastic disease and the deregulation of cellular oncogenes has been well characterized. In this study the deregulation of both c-myc and c-erbB mRNA was evaluated in commercial broiler chickens inoculated with wild-type and recombinant ALV-Js either expressing or lacking the E element within the ALV-J genetic sequence. In addition, the quantification of circulating ALV-J copy numbers and the integration of its proviral DNA within the chicken genome were investigated. These data were correlated and preliminary results suggest that the E element may have a greater efficiency at c-myc upregulation. At this point the neoplastic potential and pathogenicity of this region are difficult to predict. Further investigation and continuation of the experiment will allow for the development of neoplastic disease, greatly enhancing our ability to determine the effects of the E element during ALV-J infection.

Introduction

Viral upregulation and/or activation of cellular oncogenes has been associated with neoplastic formation in many animal models (21, 51, 65), and is commonly involved in many human diseases including Epstein-Bar virus, Burkitt's lymphoma, and prostate cancer (92). It was nearly a century ago that the viral etiology of sarcomas and leukemia (leukosis) in the domestic fowl was first described by Ellerman and Bang (1908), working

in Copenhagen, and Rous (1910) in New York (38, 123). Through the following decades, in an attempt to control these oncogenic diseases, which were becoming a significant cause of mortality in commercial poultry, extensive investigation in many veterinary laboratories was undertaken. Through this period the chicken has been an instrumental tool in discovering the mechanisms of pathogenesis of oncogenic viruses. Avian leukosis virus (ALV) and Rous sarcoma virus (RSV) have subsequently become the model systems of which to elucidate the mechanisms which lead to the formation of virally induced tumors.

Of particular interest are the ALVs, which are grouped into the *Alpharetrovirus* genus of the family Retroviridae (120). These viruses in chickens are classified into five pathogenic subgroups: A, B, C, D, and most recently J (106). Their distinctions arise from differences in envelope glycoproteins, virus-serum neutralization tests, virus interference, and host range (71). ALVs are known to cause a variety of neoplasms including erythroid, lymphoid, and myeloid leukoses (109, 110, 149). These pluripotential neoplasms of ALVs cause severe economic losses due to condemnations at slaughter, loss of pedigree birds, and tumor mortality. The most recently discovered ALV, subgroup J, was isolated from meat-type chickens associated with myleocytomatosis in England in the late 1980's (105). Beginning in the early 1990s the commercial broiler industry was confronted with this new subgroup of ALV, when it began causing serious production problems and mortality on a global scale (106, 141). The host range of ALV-J encompasses jungle fowl, turkeys, and egg and meat-type chickens at all breeding generations, including commercial broiler flocks (44, 109). ALV-J was predicted to be a recombinant of exogenous ALVs and the EAV family of endogenous avian retroviruses (6). In addition to a foreign envelope gene

acquired from this group of endogenous retroviruses, the genome of HPRS-103 the prototype of the envelope J subgroup of ALV (105), was found to contain an E element in the 3' non-coding region of it's genome. This enigmatic element, also know as F2 (14, 79) and XSR (145), had only been found in replication competent RSVs, but not in naturally occurring, replication-competent ALVs (13, 14). This novel addition to the ALV-J genome is of particular interest for several reasons. Firstly, it is predicted that this region is capable of forming a hairpin structure that might operate at either the level of DNA or RNA which may explain it's ability to bind the transcription factor c/EBP (125, 129). Secondly, the E element is found 5' of *v-src* in RSV SR, but 3' of *v-src* in RSV Pr, which presumably allows it's biological function to be exerted over distances of at least 1800 nucleotides (129). By analogy, it has been speculated (129) that this E region may be a transcriptional enhancer sequence, since the 72 bp murine leukemia virus enhancer sequence is capable of forming a strong hairpin structure and since enhancers can operate over long distances (9, 23, 80). This unique combination of characteristics has also been speculated to play a role in ALV-J oncogenesis, in particular, functioning as a promoter or enhancer within B cells (85). Upon studies to determine the pathogenicity of two recombinant ALVs Lupiani *et al.* (85) found there to be significant differences in oncogenic potential between two viruses, one of which was lacking the E element, leading to the speculation of a possible role this region may play in pathogenicity. Several ALV-J strains have been isolated globally with differences in the E element region, when compared to the prototype HPRS-103 strain; however comprehensive studies focused on determining the effects of these mutations within the ALV-J genome have yet to be performed (34, 142).

Oncogenesis initiated by ALV-J infection is predominantly found in the form myeloid leukosis (ML) (myleocytomatosis) (109), a characteristic thought to be associated with its ability to replicate well in blood monocyte cultures but less so in the lymphoid follicles of the bursa of Fabricius (2). Its ability to transform cells of the myeloid lineage are a direct consequence of its proviral integration next to the cellular proto-oncogene *c-myc*, subsequently enhancing and/or promoting the transcription of the oncogene via action of its long terminal repeat (LTR), resulting in the development of monoclonal tumors (55, 64). In experimentally infected embryos or 1-day-old chicks the development of bursal lymphomas will occur in approximately 50% to 100% of lymphoma-susceptible chicks by this method of cellular oncogene upregulation (20, 50). This transcription factor controls cell functions such as proliferation, differentiation, and apoptosis, through activation and repression of a number of target genes (58). These Myc regulated genes include those involved in angiogenesis, metabolism, and cell cycle and growth (28, 90). The over expression of Myc has also been shown to inhibit cellular differentiation, perhaps through its ability to block the cell cycle exit (65). In addition, Myc can also sensitize cells to apoptosis and it effectively induces blood vessel growth in a number of tumor models (17, 89, 93, 113). Examination of the proviral integration within these neoplastic lymphoid cells show that nearly all proviruses have under gone deletion of the 5' LTR (82, 83), in turn allowing the 3' LTR to more efficiently induce high levels of *c-myc* gene transcription (75).

ALV has also been shown to induce erythroblastosis by mechanisms similar to those which cause ML, by integration of proviruses near another host oncogene, *c-erbB*.

This cellular oncogene was first discovered due to its close amino acid homology with portions of the human epidermal growth factor receptor (EGF) which were strikingly similar to the oncogenes carried by the acutely transforming avian erythroblastosis virus (AEV) (147, 152). Data has shown that upon activation, *c-erbB* can assume an oncogenic role similar to that of its viral counterpart. The *c-erbB* oncogene encodes the EGF receptor, a protein kinase which is involved in growth signaling in many cell types, hemopoiesis, and formation of transforming growth factor α (TGFα) (95, 100, 104). The EGF receptor contains three domains, an extracellular EGF binding domain, a short transmembrane domain, and a cytoplasmic domain (70). Interestingly, upon ALV infection most of the proviral integrations from tumors map within the 3' region of *c-erbB* intron 14, so that a truncated *gag-env-erbB* fusion protein is produced which is thought to have constitutive kinase activity (46, 86, 95, 118). From these fusion products and in turn read-through transcripts, recombinant viruses arise containing transduced *c-erbB,* forming acutely transforming viruses which can be observed in approximately 50% of the erythroblastosis tumors that arise (86, 95). Of those proviruses analyzed after initial infection, all were inserted in the same transcriptional orientation as *c-erbB*, and elevated expression of *c-erbB* related mRNA was consistently observed (51, 118). Raines *et al.* observed that the truncated, structurally altered transcripts of *c-erbB* show a 100% correlation with the development of erythroblastosis, suggesting that disruption of the *c-erbB* locus is important for oncogenesis (114, 115, 118).

As described, examinations of ALV proviral integration sites within the *c-myc* or *c-erbB* genes of tumors has revealed a non-random pattern of proviral integration, giving

insight to the oncogenic mechanisms of ALV. The integration of the ALV proviral DNA into *c-myc* has been shown to cause bursal lymphomas, and predominantly this insertion occurs within the 3' region of *c-myc* intron 1 (122, 130). With the *c-myc* protein-coding sequence beginning in exon 2, the promoter/enhancer capabilities of the ALV LTR sequence have been shown to increase *c-myc* mRNA and protein roughly 50-fold (87). The integration of *c-erbB* observed in erythroblastosis are also non-random, so that the majority of tumors show integrations clustered within the 300 bp region of intron 14 (55).

There are many aspects of this ALV-J which remain to be elucidated. The research presented here will attempt to shed light on the role played by the E element during ALV-J infection. By quantifying viremia via quantitative real-time (qr)RT-PCR we will determine the effects of the E element on replication efficiency, a factor possibly altered by this region of the virus that has yet to be studied. In addition, we will also determine the levels of the oncogenes *c-myc* and *c-erbB* transcribed throughout the course of infection and correlate this data to viral load. ALV-J viremia and its outcome on virally induced oncogenesis both with and without the E element is an additional aspect which will be analyzed throughout this study. By combining this data and correlating the levels circulating ALV-J, *c-myc*, and *c-erbB* we aim to better demonstrate the role of the E element during ALV-J infection.

Materials and methods

A. Viruses

All viruses and molecular clones utilized in this study were kindly provided by Dr.

Blanca Lupiani (Texas A&M University / College of Veterinary Medicine). A detailed description of the origins and development of these viruses are discussed.

A field strain of ALV-J, R5-4, initially isolated by the Avian Disease and Oncology Laboratory (ADOL) served as the parent strain (ADOL R5-4) of ALV-J virus in this study (84). Using standard methods proviral DNA of ADOL R5-4 was molecularly cloned to form pALV-J. Briefly, DF-1 cells were inoculated with the ADOL R5-4 strain of ALV-J. Seven days post inoculation, DNA from infected cells was purified, digested with *Not*I and a library generated as described (126). Clones containing ALV-J sequences were identified by plaque hybridization using oligonucleotide probes corresponding to the viral LTR and *env* gene. Once the full length clone was identified, host DNA sequences flanking the proviral DNA were eliminated as follows. The clone was digested with *ApnL*I (a restriction enzyme that recognizes unique sites in both viral LTRs) re-ligated to itself to form circular molecules, digested with *Kpn*I (a unique restriction site located upstream of the env gene) and ligated to a pBS SK+ plasmid digested with *Kpn*I.

In order to generate a molecular clone with two LTRs, the 5' LTR and UTR were PCR amplified with a forward primer, containing a *Spe*I site, complementary to the 5'end of the LTR and a reverse primer complementary to the *BstE*II site of the 5' UTR. Similarly, the 3' UTR was PCR amplified with a primer complementary to the *Bsu*36I site, located in the 3' UTR, and a reverse primer, containing a unique *Not*I site, complementary to the 3' end of the LTR. These PCR products were ligated to the rest of the viral genome using the *BstE*II and *Bsu*36I restriction sites generating a molecular clone identical to the proviral DNA (pALV-J) (Figure 5). This process generated the molecular clone of the R5-

4 isolate (pALV-J/*kpn*I) with a single LTR (Figure 5). The pathogenicity of pALV-J was tested in meat-type chickens; day-old chicks were inoculated with 10^5 $TCID_{50}$ at hatch. Inoculated chickens tested positive for ALV-J viremia and developed tumors within 30 weeks of age, confirming pALV-J to be infectious (84).

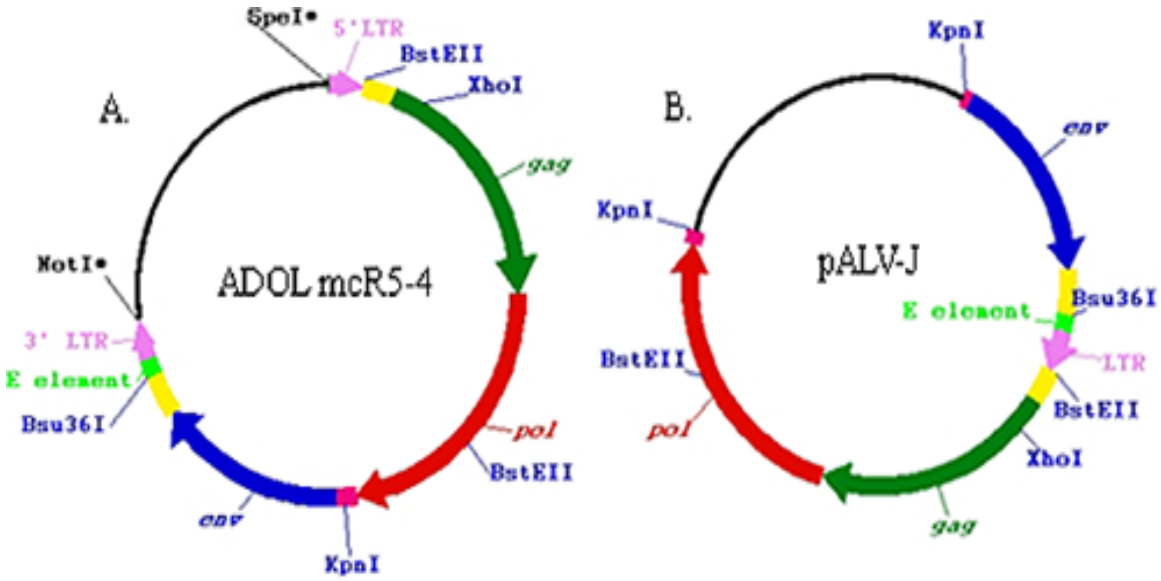

Figure 5. Schematic representations of two recombinant ALV-J viruses: pALV-J/KpnI and pALV-J. A. Schematic representation of the molecular clone, created by Katherin Conklyn from the original isolate R5-4; B. A easily manipulated plasmid vector pALV-J containing intact infectious ALV-J with multiple splice sites identified.

In order to generate a pALV-J virus without the E element (pALV-J /ΔE) the ALV-J 3'LTR was PCR amplified from pALV-J using a forward primer containing a *Bsu*36 I site overhang (underlined) (*Bsu*36-EI 5' CCC TCA GGA TAT AGT AGT TGC GCT TTT GCA TAG GGA GGG GGA 3') immediately downstream of the E element and a reverse primer with a *Not* I site overhang (underlined) (J-LTR.3 5' CAA GCG GCC GCT AAT GAA GCC ATC CGC 3') complementary to the 3'-end of U5. The PCR product obtained

was then subcloned into the pALV-J vector using *Bsu*36I and *Not*I restriction sites, yielding the virus pALV-J /ΔE (Figure 5).

B. Experimental design

One-day-old commercial broiler chicks were randomly selected and placed into 4 treatment groups: one serving as a negative control inoculated intra-abdominally with 0.1ml PBS, while the others were inoculated intra-abdominally with an equivalent volume buffered cell culture supernatant containing $10^{3.5}$ infectious units of the three described ALV-Js (ADOL R5-4, pAVL-J, and pALV-J /ΔE), receptively. Each group consisted of 22 chickens that were kept in isolation until the termination of the experiment. For individual identification purposes the birds were wing-banded according to their respective treatment and given number designations of 1-22. Those birds that lost wing bands during the trial were recorded as "no band" (NB) and data for these birds was not utilized. Liver, spleen, brain, kidney, marrow, and tumor tissue (if present) were collected and preserved in a 1:5 (vols/vols) ratio of RNAlater (Qiagen; Valencia, CA). For the purposes of this manuscript data will be interpreted from chickens that were sampled at 1, 5 and 11 weeks post inoculation (PI).

Samples were tested for viremia, *c-myc*, *c-erbB*, and glycerolaldehyde 3-phosphate (GAPDH) gene expression via qrRT-PCR. Chickens were also observed for tumors throughout the experiment, until termination. Chickens that were incapacitated and/or unresponsive were euthanized via CD. Samples of blood (when possible) and affected tissues from mortalities and euthanized chickens were collected for use in gene expression assays and preserved in RNAlater (1:5 dilution) (Qiagen; Valencia, CA). Additional tumor

tissues from necropsied chickens were fixed in 10% buffered formalin, stained with hematoxylin and eosin, and examined for ALV-J induced microscopic lesions.

C. Sample and tissue preparation from experimental chickens

At selected time points throughout the trial (at 0 and every odd numbered week) approximately 0.75 ml of blood was drawn from all birds of each treatment and dispensed into 2 ml Vacuette® tubes coated with EDTA K3 specific to each bird of each treatment. Subsequently, the samples were centrifuged (2000 rpm for 5min) and aliquoted out as follows: 2 rows (1-24 wells) of a 96 well plate were designated for each treatment, samples from each chicken (1-22) were placed into their respective well, while the 23rd and 24th wells severed as controls (extraction and qrRT-PCR). Dispensed into the wells of three respective plates were: 50 µl of plasma for viral (v)RNA extraction, 160 µl of plasma for future use, and 20 µl of BC preserved in 180 µl of RNAlater (Qiagen; Valencia, CA) for quantification of viremia, the housekeeping gene (GAPDH), and the oncogenes *c-myc* and *c-erbB*. To corroborate the results of qrRT-PCR a 48-well tissue culture plate was divided in two sections and 100 µl of plasma was aliquoted in duplicate from each bird for each treatment into its respective well, to be used for virus isolation (VI). Immediately following this processing, plates were sealed with adhesive covers and stored at -80^{o}C till extraction or VI procedures were carried out.

D. RNA isolation

vRNA was extracted from 50 µl plasma samples in a 96-well plate format with the use of nucleic acid-binding magnetic particles supplied by the MagMAX™ Viral RNA Isolation Kit developed by Ambion®, Inc. (Austin, Texas, USA). The manufacturer's

protocol for cell free sample was followed.

Total RNA (cellular and viral) was extracted from BC samples utilizing a modified protocol of the MagMAX™ kit (Ambion® Inc., Austin, Texas). The manufacturer's protocol was optimized. Briefly, the preprocessed 96-well plates containing 20 µl of BC and 180 µl of RNAlater (Qiagen; Valencia, CA) were centrifuged at 2500 rpm for seven minutes to pellet the cells. The residual RNAlater (Qiagen; Valencia, CA) was aspirated and 100 µl of the MagMAX™ Lysis/Binding Solution was added and the plate was then shaken on a DPC MicroMix® 5 (form 4, amplitude 18) for 5 min. The cellular debris was then pelleted by centrifugation at 2500 rpm for 5 min at 4°C. Following centrifugation approximately 80-90 µl of the Lysis/Binding Solution (avoiding the carryover of cellular debris) was transferred to a new 96-well plate. Twenty µl of the MagMAX™ Bead Suspension Solution was then added to each well and shaken as previously described. Following this step the magnetic particles were pelleted with 96-well Magnetic Ring-Stand, the residual Lysis/Binding Solution was aspirated, and 20 µl of DNAse I (1U to 1000 µl DNAse I buffer dilution) was added, shaken (form 5, amplitude 19) for 5 min and followed by a 15 min incubation at 37°C. Following the incubation, 100 µl of the Lysis/Binding Solution was added and shaken as described initially. This step was followed by a series of wash steps as described in the manufacture's protocol. Finally, total RNA was eluted in 30µl of the supplied elution buffer.

Tissue samples were extracted in much the same way. Residual RNAlater (Qiagen; Valencia, CA) was aspirated and approximately four volumes (0.1g tissue:0.4ml) of the MagMAX™ Lysis/Binding Solution was added the sample in a 1500 µl microcentrifudge

tube and pulse sonicated for ~5 sec. The tube was then vortexed, agitated for 5 min, and centrifuged at 8000 rpm for 2 min. Lysis/Binding Solution free of cellular debris was then transferred to clean tube, followed by the total RNA extraction method described above, for the exception of the tube format.

E. Primer design

The mRNA sequences of *c-myc*, *c-erbB*, and GAPDH were obtained through GeneBank (Table 5). The design of primers and probe sets was aided by the use of the PrimerSelect program within the DNASTAR software package (DNASTAR, Inc., USA) to comply with TaqMan criteria (18). Each primer pair was selected to have a maximal T_m difference of less than 2^oC, a GC content between 20% and 80%, no GC clamp, a length of between 9 and 40 nucleotides, fewer than 4 repeated G residues per primer, no hairpins with a stem size of less than or equal to 4, and an amplicon size of between 50 and 150 bp. When possible TaqMan probes were designed to have a T_m 10^oC greater than that of the flanking primer pairs, no G at the 5' end, and positioned over a splice site within the target sequence. Splice sites were determined by analysis of the chicken genome whole transcript products (Genebank, NCBI).

Table 5. Primers and probe sets and their location. [A]Chicken epideremal growth factor receptor mRNA (accession #NM_205497). [B]c-Myc chicken oncogene mRNA (accession # X68073). [C]Glycerolaldehyde-3-dehydrogenase chicken mRNA (142). [D]E element region, avian leukosis virus subgroup J genome HPRS-103 (accession #Z46390).

Primer/Probe Sequences	Terget gene	Nucleotide position	5' - Sequence - 3'
erbB-1269F	c-erbB[A]	1568-1589	TGCTACTGATCTCTATGCTTTT
erbB-1347R		1652-1673	TAACTTGAAAATACAGTCGTTG
erbB-1321P		1620-1639	AAGCAGCACGGCCAGTATTC
erbB-942conF T7		942-965	T7+CAGAACTGCCAGACTTTAACAAAA
erbB-1740conR		1721-1740	AGCAGAGGTTCTTGTTCTTC
cMyc-202F	c-Myc[B]	202-222	AACATCCACCAACACAACTAC
cMyc-283R		264-283	GAGGCTAAAGTTGGACAGTG
cMyc-240P		240-259	CAAGGTGGAATAGGGAGCGC
cMyc-45con T7		45-62	T7+CGACTCGGAAGAAGAACA
cMyc-408conR		389-408	CTTCAGCTCATTCCTTCGCT
GAPDH for	GAPDH[C]	422-447	TTGTTTCCTGGTATGACAATGAGTTT
GAPDH rev		482-502	ACATGGCATCCAAGGAGTGAG
GAPDH pro		450-479	ATACAGCAACCGTGTTGTGGACTTGATGGT
ALV-E for	E element[D]	7211-7230	AATGGGGCAATGTAAAGCAG
ALV-E rev		7591-7611	ACTTCCACCAATCGACGTGT
Exogenous ALV			proprietary information

F. Exogenous control RNA development

In an effort to reduce possible contamination resulting from the transfection and creation of plasmid vectors containing the dsDNA target sequence, control RNA sequences were developed employing PCR techniques. A forward primer was developed which contained an overhang of the T7 polymerase recognition sequence on its 5' end, resulting in a primer of 40 bp, 22 of which were the T7 recognition sequence. The reverse primer was then created to amplify a region which spanned multiple splice sites, allowing for the use of the same control sequence for the development of multiple primers and probe sets if they were required. Using the methods of total RNA extraction previously described a RT-PCR reaction was performed in a 50µl reaction volume, which included 25 µl Qiagen RT-PCR Probe MasterMix, 16.5 µl nuclease free water, 1.5 µl of both 10µM forward and reverse primers, 0.5 µl RT, and 5 µl eluted RNA product. The PCR reaction included a 30 min RT step @ 50^{o}C followed by a 15 min denaturation step at 95^{o}C. The amplification cycles included three steps: 1) 95^{o}C for 30 sec, 2) an annealing gradient spanning from 55^{o}C to 65^{o}C for 45 sec, 3) and a final extension step at 70^{o}C for one min. Agar gel electrophoresis (AGE) was carried out with 5 µl of this product to determine successful amplification and the annealing temperature which most efficiently amplified the control sequence. The two tubes containing the most target sequence, determined by band intensity of AGE, were PCR purified (Qiagen, Valencia, CA). This DNA product was then transcribed with the Promega T7 RiboMax kit according to the manufacture's protocol.

G. Virological and gene expression assays

Blood and tissue were processed as previously described from experimentally infected chickens as well as uninoculated controls and tested for viremia, *c-myc*, *c-erbB*, and GAPDH by quantitative real time RT-PCR (qrRT-PCR) as previously described (88). Breifly, seven microliters of eluted RNA sample was added to a mixture containing 12.5 µl of Qiagen QuantiTech® MasterMix, 3.5µl of water, 0.5µl of each primer (10µM), 0.5µl of probe (5µM), and 0.25µl RT, yielding a 25µl qrRT-PCR final reaction volume. ALV-J RNA copy numbers were extrapolated using data obtained from a standard curve of six log dilutions. Gene expression of *c-myc* and *c-erbB* was measured via qrRT-PCR with the same RNA and qrRT-PCR reagents used for quantifying viremia, with exception of the different primers and probe sets (Table 5).

H. Relative quantification of gene transcription

Current data has shown that GAPDH mRNA levels were found to show the least standard deviation when compared to chicken telomerase RNA and chicken telomerase reverse transcriptase (143). In concordance with this data all levels of gene expression and viremia were normalized to the housekeeping gene GAPDH by averaging the C_T values of this transcript for each sample of every plate. This value was then used to derive a standard deviation (SD), the difference of any GAPDH C_T value outside the range of the SD was applied to the respective well of each qrRT-PCR to normalize C_T values, correcting for any inconsistencies in the extraction process.

I. acELISA

In addition to qrRT-PCR, BC and plasma samples were inoculated onto cell culture to determine the presence of ALV-J by detection of p27 antigen. Briefly, 100µl of sample was simultaneously inoculated onto 48-well tissue culture plates containing $1.5x10^5$ DF-1 cells per well in 4% growth medium, containing 3.76 g Leibovitz L-15 Medium, McCoy's 5A Medium Mix, 1.1 g $NaHCO_3$, 20 ml (4%) fetal bovine serum (FBS), 1 mg amphotericin B, 12.5 mg gentamycin, 1 mg DEAE dextran, 2000 units of heparin, and brought to a volume of 500 ml. Following 24 hr incubation the media was removed and replaced with maintenance media (1% FBS growth media without DEAE dextran and heparin) and incubated for an additional 7 days. Plates were then processed for p27 antigen by the addition of 50µl 5% Tween 80 to each well followed by two freeze-thaw cycles. One hundred-microliter aliquots of cell lysates were collected from each well and transferred to 96-well plates for acELISA detection. ALV acELISA assay protocol and readings were performed according to the manufacture's recommendations (IDEXX Laboratories, Westbrook, ME) except a sample to positive ratio (S/P) cutoff value of 0.1, less stringent than the manufacture's recommendation of 0.2, was used to call positives. S/P ratios were calculated by subtracting the negative control mean absorbance value at 650 nm, A(650), from the sample mean and dividing this value by the value obtained from subtracting the negative control mean at A(650) from positive control mean at A(650). {[(sample mean – negative control mean) / (positive control mean – negative control mean)] = S/P}

Results

A. Propagation of viruses

Viruses pALV-J, ADOL R5-4, and pALV-J /ΔE were propagated in DF-1 cells. Confirmation of virus presence and titer was determined by qrRT-PCR and acELISA, repectively. Cell culture supernatants containing the respective viruses were diluted to contain $10^{3.5}$ infectious particles per 0.1 ml with buffered cell culture media. Presence or absence of the E element was verified by PCR followed by AGE of infected DF-1 cellular DNA using the forward primer 5'-ACT TCC ACC AAT CGA CGT GT-3' and reverse primer 5'-AAT GGG GCA ATG TAA AGC AG-3' (Table 5). These primers spanned a 400 bp region encompassing the E element. Positive conformation of the missing E element was observed via AGE by 150 bp reduction in the size of the pALV-J /ΔE amplicon, when compared to those of ADOL R5-4 and pALV-J. Outliers were corrected for each treatment and time point, retaining the central ~75% of the data points: all data analyzed for GAPDH at each time point was sorted by C_T value and the outer most data points were removed, this same method was carried out for *c-myc* expression levels, and finally ALV-J C_T values, leaving 16 data points for each treatment.

B. ALV-J-induced viremia and deregulation of cellular oncogenes

Levels of circulating ALV-J determined by qrRT-PCR beginning with week one for all treatments were relatively low to non-existent, with the exception of pALV-J, which had copy numbers greater than 25 times that of the nearest virus, pALV-J (**Figure 6**). The circulating levels of pALV-J remained elevated through the trial and averaged 135% greater viremia than did pALV-J/ΔE, its closest counterpart. Levels of virus measured in

the negative control treatment calculated to be less than one copy per microliter, out of the linear range of the qrRT-PCR assay. While the level of pALV-J was substantially higher than the other treatments during week one, these levels reduced as the experiment progressed (**Figure 6**).

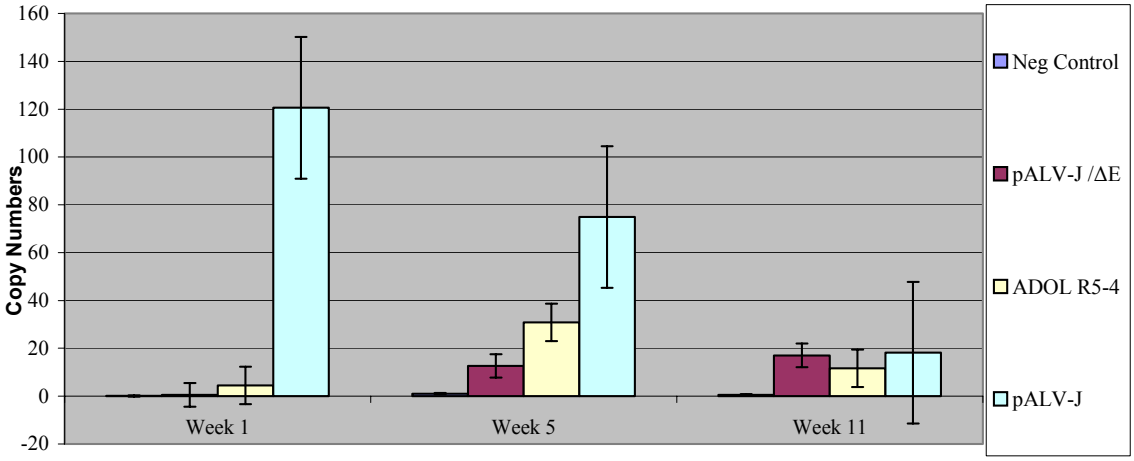

Figure 6. Normalized viremia levels in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4.

The expression of c-myc transcripts increased throughout the experiment, yet stayed relatively proportional to the negative control (**Figure 7**). The levels of *c-myc* gene expressed between the negative control and the pALV-J/ΔE treatment were nearly equivalent throughout the period of the trial, and averaged 28.4% greater than did *c-myc* expression in chickens infected with the pALV-J and ADOLmcPr5-4 viruses.

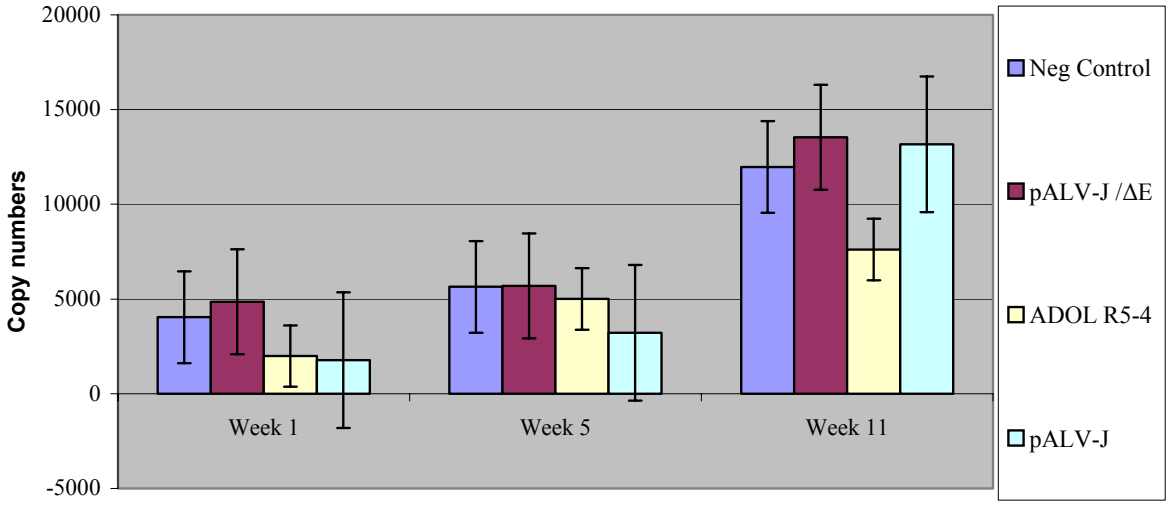

Figure 7. Normalized c-myc levels in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4.

As was previously shown, the levels of GAPDH expression were extremely constant over the course of infection (143). The range of variance was 1.1 C_T, averaging 2.6 SDs (**Figure 8**). A steady but slight increase was observed however, ranging 3.1 C_Ts over the data presented. The negative control and the pALV-J/ΔE infected treatments were consistently elevated over the values observed in the other two treatments (**Figure 8**).

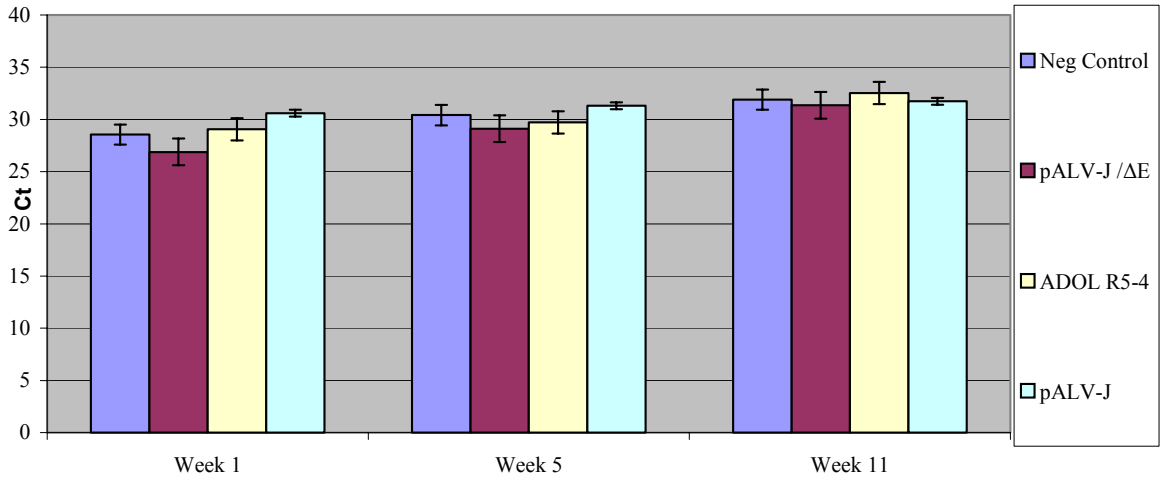

Figure 8. Normalized GAPDH expression in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4.

Configuration of the data to portray mRNA expression of the five chickens expressing the highest viremia showed steadily increasing levels of *c-myc* throughout the course of infection in those birds infected with experimental viruses, while expression levels in the negative control group remained relatively constant (**Figure 9**).

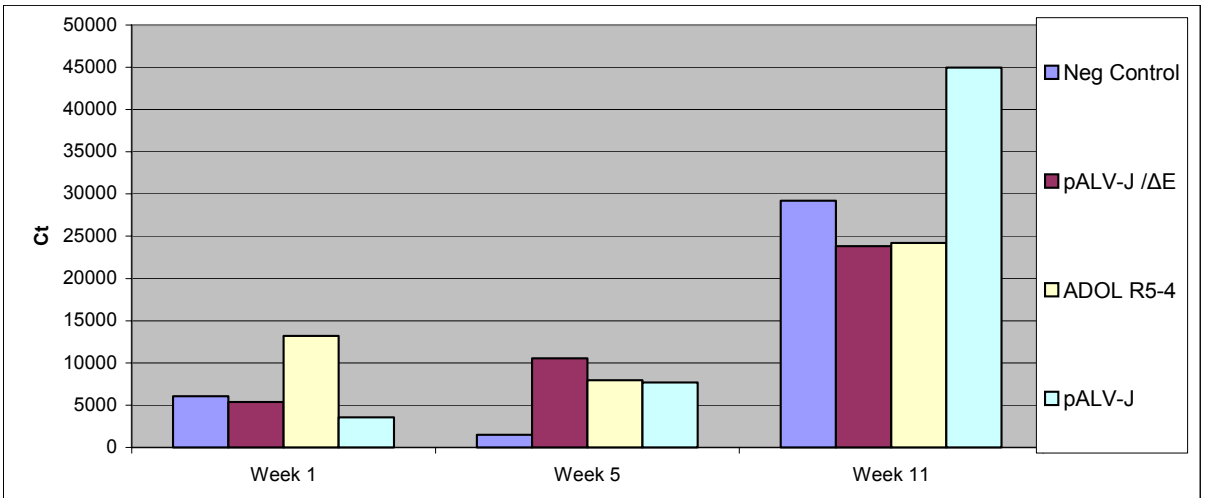

Figure 9. c-myc expression in 5 chickens expressing the highest viremia levels in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4.

Results presented in this format also showed GAPDH levels consistent with those observed in normalized values (**Figure 10**). The levels of viremia in those five birds with the highest circulating ALV-J also showed similar levels of circulating virus as those seen in the normalized data (**Figure 11**).

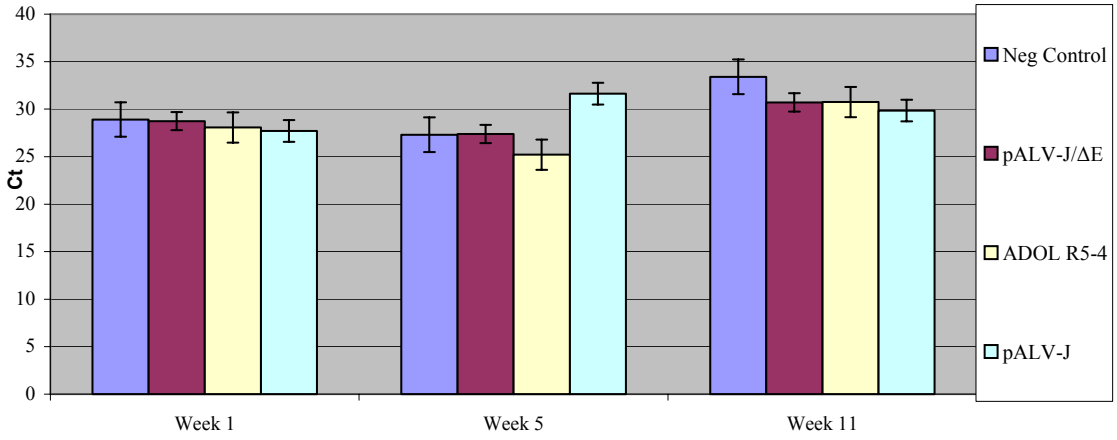

Figure 10. GAPDH expression in chickens expressing 5 highest levels of viremia levels in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4.

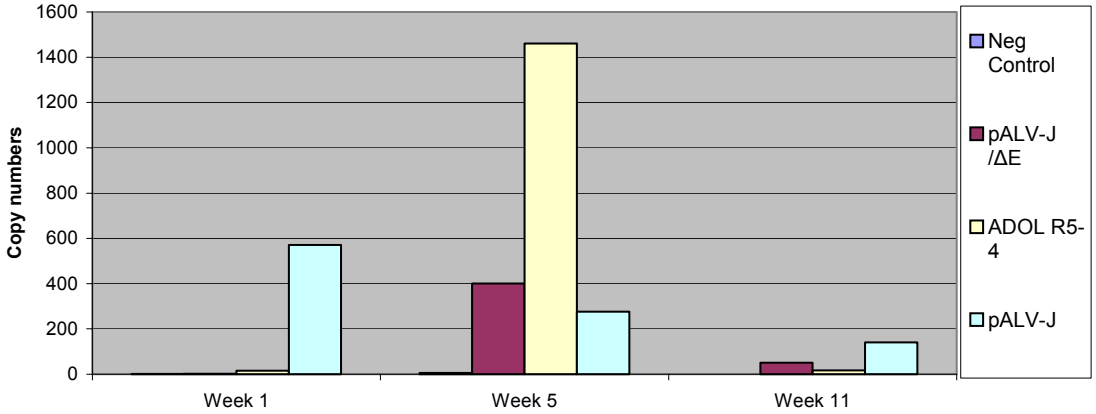

Figure 11. Viremia values in 5 chickens expressing the highest viremia levels in chickens challenged with pALV-J/ΔE, pALV-J and ADOL R5-4

C. Experiments in progress

This study remains ongoing and future work includes the incorporation of additional data points through a minimum of 30 weeks total, in addition to analyzing samples at time points within the span presented here. The expression of *c-erbB* data was not presented due to inefficient amplification of the target sequence. Prior to the production of control RNA, preliminary qrRT-PCRs utilizing extracted totRNA from chicken tracheal tissues resulted in satisfactory amplification. Further development of the control sequence revealed that lower limit of detection of the primers and probe set developed was approximately 10^4 copies. Two time points were analyzed by qrRT-PCR for *c-erbB* (Table 5), week one yielded three positive samples and week 5 yeilded no positives, at this point detection of *c-erbB* transcripts has been postponed until a more optimal set of primers and probe can be developed. In addition to the quantification of *c-erbB* expression, samples will also be isolated in cell culture and then analyzed via acELISA for ALV p27.

Discussion

Although the data presented do not completely support the hypothesis, certain trends can be observed and continuation of the experiment may lead to more definitive results. For instance, the high levels of pALV-J expressed early in infection are consistent with high numbers of replicating virus and in turn integrating virus. As the experiment progressed the circulating levels of this virus were cleared to levels similar to that of the other viruses. Yet, as copy numbers of this virus decreased, an almost proportional increase in *c-myc* mRNA expression was observed. This increased expression could be speculated to be caused by the integration of proviral DNA early during infection, resulting

in the increased *c-myc* transcription later. Further time points may reveal similar circumstances for pALV-J, as observed in week 5 increased replication lead to increased *c-myc* expression. However, the levels of *c-myc* transcript expression observed in the negative control counter the validity of the above arguments. Although, further investigation may reveal a plateau of *c-myc* expression that may be reached upon maturation of the chicken (18-20 weeks). ALV-J positive birds in this case may continue to show increasing amounts of *c-myc* as clonal replication within tumor tissue occurs.

The circulating levels of virus presented here differ from the conventional reasoning of ALV-J replication in chicks deficient of a mature immune system. In most scenarios infection at an early age leads to recognition of ALV-J as "self" and therefore the chick will not mount an immune response, producing no virus neutralizing antibodies allowing the virus to replicate unabated. The results here do not directly show this, however in some instances chicks may develop an immune response and clear or reduce circulating virus. This speculation can be tested with conventional, commercially available ELISA kits able to detect the anti-SU antibodies. Detection of these antibodies could explain the reduced viremia over the course of this experiment. If antibodies are present, this does not explain the substantial difference between the replication efficiencies of pALV-J and ADOL R5-4. It may be hypothesized that there are differences in the antigenic structure of their envelope glycoproteins. Even though ADOL R5-4 was the original isolate used to produce the molecular clone, pALV-J, sequencing should be performed to ensure have been sequenced their genetic similarity.

If the data is sorted and arranged based according to viremia levels and chickens in the top quartile of circulating ALV-J copy numbers are selected the data reveals *c-myc* levels more characteristic of the hypothesized scenario following ALV infection (**Figure 9**). Firstly, the negative control treatment's expression of c-myc transcripts is steady over the sample period and at week 1 data levels are relatively similar, with the exception of slightly increased pALV-J/ΔE copy numbers. This data configuration also shows the extreme level of amplification of ADOL R5-4 within this first week, when compared to the other treatments (**Figure 11**). The upregulation *c-myc* remains clearly distinguishable from the other viruses. Chickens infected with pALV-J/ΔE also show an upregulation of *c-myc* above the negative control. However, clear differences in the pathogenicity of this recombinant are difficult to distinguish. Further time points and analysis of tumor tissue will help further elucidate the role in pathogenicity this portion of the ALV genome.

The deregulation of *c*-erbB will be an important aspect of measurement to better understand and elucidate the functions of the E element. While the analysis of *c-erbB* upregulation was not successful in this phase of the experiment, the amplification of this region by qrRT-PCR was carefully plotted and significant time was spent isolating a specific amplicon. The initial unprocessed transcript, containing introns, is approximately 120kb, within this region are 12 exons coding for the epidermal growth factor receptor. In order to detect only processed functional c-erbB transcripts, all splice sites within the region were identified and these regions served as the basis for development of primer sets. Probes were first developed to span these splice sites, in order to eliminate or reduce the incidence of probe binding to unprocessed non-functional c-erbB. Upon the creation of

these probes, which was carried out both manually and with the aid of the PrimerSelect program supplied in the DNASTAR software package, selection of viable candidates was made based on T_m, ΔG values, and placement within the transcript. Past studies have revealed that upregulated c-erbB transcripts are structurally altered and often result in fusion products containing the 3' end of the c-erbB transcript. Based on these viable candidates, primer pairs were then developed to flank these regions. The use of currently available software provided little support in the development of these primers and their respective probe due to the extremely specific placement necessary to provide accurate quantification of the target gene. Alternative primer and probe sets have been developed and will be evaluated for their sensitivity.

The E element remains an enigmatic portion of the ALV genome. However, with further investigation, especially following the induction of tumors, our goal is to shed light on this elusive region, determining its role, if any, in pathogenicity. If found to cause increased formation of neoplastic disease, or increased replication efficiency as this experiment continues, or some other form of increased pathogenicity this information could potentially be used to determine other similar endogenous cytopathic factors produced by humans or animal species. In the case that this region is a factor in ALV-J pathogenicity, investigations into the mechanisms by which its able to cause the increased pathogenicity could potentially lead to novel therapeutics to treat other oncogenic diseases.

DISCUSSION AND SUMMARY

Overview

Retroviruses and their association with neoplastic disease have been described in the literature for nearly one century. Throughout this period a vast number of creative individuals have shed light on the elusive elements that form the mechanisms of their disease. Today the family *Retroviridae* encompasses a total of seven unique genera whose diseases are most often associated with the induction of cancer or immune deficiencies.

ALV, a member of the *Alpharetroviruses*, is an economically significant virus to the poultry industry. The disease incited by this virus within the chicken is known to cause a variety of neoplasms, which result in decreased production traits, condemnations at slaughter, and tumor mortality. The pathogenic forms of this ALV are classified into five subgroups, those being A, B, C, D, and J. Subgroup J ALV, the most recently of the ALVs to be discovered, became a significant economic threat to the commercial broiler industry soon after its identification (105). The efficiency at which ALV-J is able to spread facilitated its rapid global dissemination throughout meat-type poultry flocks. This virus had and remains to have its most profound effects on the primary breeder industry, which was impelled to implement eradication schemes to control this novel subgroup, leading to detrimental losses to pedigree stocks.

Diagnostic development

The pluripotent neoplasms induced by exogenous ALV infection have led to significant economic losses in the commercial broiler industry worldwide, primarily as a result of the introduction of ALV-J to pedigree stocks. The original isolation of the novel

subgroup was made in 1988 during a survey of ALV infection among breeding stock in England (105). By the early 1990s the commercial broiler industry was adopting eradication programs in an effort to control ALV-J. Optimization of current ALV diagnostic techniques of the time resulted in a 'gold standard' assay, still employed today by the majority of the industry. ALV-J arose by recombination of an unknown exogenous ALV and the envelope domain of an *ev* loci (11); as a result, the predominant gs antigen produced by ALV-J was p27, a protein also translated from *ev* derived transcripts. The ubiquitous *ev* loci, p27, circulates among nearly all lines chickens, requiring that the diagnostic for exogenous pathogenic ALV account for and/or eliminate the detection of endogenously produced p27. The resulting assay first employed isolation and growth of virus in cell culture free of endogenous viral transcripts for a minimum of seven days, followed by detection of the exogenously produced p27 protein via acELISA.

This method of exogenous ALV-J detection is time consuming, laborious, expensive, and is dependent on the presence of infectious virus. With methods to control ALV primarily focused on its eventual eradication the industry required a diagnostic that could provide a rapid, sensitive, and cost efficient method of detecting this virus. The existing gold standard allows ample time for the horizontal transmission of virus to littermates and continued vertical transmission by breeders, inciting a chain of infection that would be nearly impossible to break without detrimental consequences to breeding stock. However, with the advent of molecular based diagnostic techniques and real-time high throughput analysis tools, eradication became a more realistic goal. In an effort to provide, for the commercial poultry industry, an ALV diagnostic that could fulfill the

above mentioned criteria, a qrRT-PCR assay for the detection of exclusively all exogenous ALV subgroups was developed. An RNA isolation technique commercially available through Ambion Diagnostics® was incorporated into the assay to facilitate the high throughput capabilities required, in addition to RT-PCR technology able to provide rapid results in real-time. The resulting assay isolated totRNA from BC samples in a standard 96-well plate, producing the input for a qrRT-PCR reaction. This single-tube qrRT-PCR assay utilized a hydrolysis fluorogenic probe for the specific detection and quantification of only exogenous ALVs able to rapidly identifying positive samples, in turn expediting the removal of infected birds and limiting horizontal and vertical transmission and improving the likelihood of exogenous ALV eradication from commercial poultry.

The role of the E element of ALV-J throughout infection

In addition to the foreign envelope gene acquired by ALV-J during recombination, a little described region previously only known to exist in RSV and referred to as the E element was discovered in the 3' non-coding region of the ALV-J genome (105). The implications of this enigmatic region have yet to be directly studied during viral infection, but implications to its role in pathogenicity have been speculated (85). Giving merit to these speculations are several physical characteristics of this region, computer modeling predicts the capability of this region to form a hairpin structure that might operate at either the DNA or RNA level, possibly explaining it's ability to bind the transcription factor c/EBP (125, 129). Secondly, the E element is found 5' of *v-src* in RSV SR, but 3' of *v-src* in RSV Pr, which presumably allows it's biological function to be exerted over distances of at least 1800 nucleotides (129). By analogy, it has been speculated (129) that this E

region may be a transcriptional enhancer sequence, since the 72 bp murine leukemia virus enhancer sequence is capable of forming a strong hairpin structure and since enhancers can operate over long distances (9, 23, 80).

Results of this study are still in the preliminary stages, however the data obtained to this point show trends which, if continue to develop, will give a better understanding of the functions of the E element. Currently, trends are being observed that show high levels of ALV-J copy numbers, early during infection, lead to higher and more rapid up regulation of c-myc transcripts. Data presented to this point concerning the replication efficiency of each virus is difficult to interpret; two relatively identical viruses (pALV-J and ADOL R5-4) are producing very different results, both in the capability to deregulate *c-myc* and especially concerning their replication efficiency. The isolation of the initial R5-4 strain from the field, and the resulting quasispecies population, may be a factor in the observed differences. Following completion of the experiment representative virus from each treatment will be sequenced, once again, and mutations within their genomes analyzed to possibly explain the observed differences. Analysis of tumor tissue will yield important information concerning the region of ALV proviral integration; observation of proviral integration sites via PCR will provide valuable data as to the efficiency by which a recombinant ALV with or without the E element will site specifically integrate into or near host oncogenes.

Aspects of the E element presented here monitor the key points of ALV pathogenesis throughout the course of infection, specifically replication efficiency of the virus (viremia), its effect on the deregulation of the two oncogenes *c-myc* and *c-erbB* (in

future) correlated to the induction of neoplastic disease. In addition to the quantification of transcribed oncogene products and vRNA via qrRT-PCR, the integration of proviral DNA within those cells transformed by ALV-J will be investigated.

Future directions of ALV-J research

Techniques developed and data presented within the pages of this thesis add to the current, ever-expanding understanding of this economically important virus. The diagnostic technique developed here, if adopted and effectively implemented into commercial industry practices, stands to be a critical tool in the eradication of ALV from commercial poultry. The E element is an obscure portion of the ALV genome which has been speculated to be a factor in pathogenicity (85). The experiments presented here aim to decipher what roles the E element may play in ALV-J pathogenicity, with particular interest being paid to viremia, deregulation of the oncogenes *c-myc* and *c-erbB*, and determination of proviral DNA integration sites within the chicken genome.

Methods used to bring ALV subgroups A & B under control have been supported by the knowledge of the cellular receptor(s) used by the virus to gain entry into the host cell, by enabling the selection of chicken lines which lack the specific receptor(s). In the same manner, elucidation of the cellular receptor of ALV-J is an important and still elusive goal in its eventual eradication. This knowledge would allow scientist to select for and possibly genetically engineer chicken lines resistant to ALV-J.

Genetic engineering holds unlimited potentials for not only the poultry industry, in terms of ALV-J, but for all viruses and for aspects of life. The history of mankind has been marked by a series of revolutions beginning with agriculture and recently that of the

computer. In every case the introduction of these new technologies was met with some form of resistance, this period of skepticism was eventually followed by an acceptance within the populations. Today we are on the fringes of a genetic revolution; the genetic code to almost all forms of life can be, have been or are being described everyday. While this code may today be easy to extract from any organism, translating this code into useful information is extremely time consuming and difficult. As science progresses we are continually stepping closer to elucidating what all of the all the code means, forming the detailed pages of a blueprint that is life. Today much of this technology and its potential uses are not accepted by the populations of the world. Much like there was skepticism to the revolutions of the past, there will be, and currently is, reluctance to accept the idea of gene manipulation. But as science inches closer to solving this intricate puzzle and more information about the positive potentials of gene therapy surface, it is my opinion that the population will slowly begin to gain acceptance for this revolutionary means of treating infection and disease, therefore giving scientists an unlimited number of options to control this economically devastating virus.

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ABSTRACTS, PRESENTATIONS, PUBLICATIONS, & MEETINGS

B. Mozisek and J. El-Attrache. Development of a quantitative real-time RT-PCR for rapid detect o and quantification of exogenous avian leukosis virus.
To be submitted. Journal of Virological Methods. 2005.

B. Mozisek, J. El-Attrache, Q. Hoang, R.C. Willis, W. Xu, M.A. Bounpheng, and X. Fang.
High Throughput Viral RNA isolation and quantitative real-time RT-PCR assay for detection of exogenous Avian Leukosis Virus.

B. Mozisek and J. El-Attrache. Development of a high-throughput quantitative real-time RT-P R or rapid detection of exogenous avian leukosis viruses. International Poultry Scientific Forum: SCAD/SPSS Atlanta, Georgia January 24-28, 2005.

J. El-Attrache , B. Lupian , **B. Mozisek**, C. Cardona, J. Li, and S. Reddy. Molecular Character zat o of Recent Avian Influenza Viruses Isolated in the United States. Poster presented at the Working Together: *Research & Development Partnerships in Homeland Security, Conference;* Boston, Massachusetts; sponsored by the Department of Homeland Security, 2005.

Invited representative of Texas A&M University's National Center for Forgein Animal and Zoonotic Disease Defense at the DHS Scholars and Fellows Oreintation Meeting, Washington DC; Nov., 2005. Hosted by the Department of Homeland Security & Oak Ridge Institute for Science and Education.