JOHN EL-ATTRACHE
Classical and molecular studies enhancing the detection and control of avian leukosis virus
(Under the direction of PEDRO VILLEGAS)

The study is to enlighten the poultry industry in regards to diagnostics and possible therapeutic agents that can be adopted for avian leukosis virus (ALV) infected flocks. The ability to substitute primary cell culture with a continuous cell line is investigated. Secondly, the sensitivity and superiority of a developed nested reverse transcriptase-polymerase chain reaction (RT-PCR) in comparison to virus isolation coupled with antigen capture-enzyme linked immunosorbent assay (ac-ELISA) in the identification and detection of avian leukosis virus were evaluated and discussed. Lastly, the design and evaluation of a virtually un-degradable morpholino (MO) antisense oligo to potentially inhibit avian leukosis virus subgroup J (ALV-J) is assessed.

The permissibility of the DF-1 cell line and C/E secondary chicken embryo fibroblasts (CEF) to propagate and subsequently detect ALV-J by ac-ELISA was similar when expressed in terms of percent positive and although not significantly different (Chi-square test, $p>0.05$) virus propagation in the DF-1 cell line allowed for the identification of more positive plasma samples at both (sample to positive) S/P ratios of 0.2 and 0.1. A 20% - 25% loss of sensitivity and significant difference at a 0.2 S/P ratio ($p<0.05$) was observed for ALV-J detection for DF-1 viral propagation followed by ac-ELISA when compared to nested RT-PCR.

Seven virus isolation negative plasma samples which were positive for ALV-J viral RNA by nested RT-PCR were analyzed and several amino acid residues changes within the spacer region (SP), were identified. Some of these

same amino acid changes identified in the ALV-J genomic RNA from the VI-plasma samples have been shown to abrogate infectivity of ALV molecular infectious clones. In addition, ALV-J antibody positive samples were detected between 2-3 $\log_{10}$ dilutions lower in cell culture when compared to detection by nested RT-PCR, while antibody negative samples exhibited similar detection limits for both assays.

A significant reduction ($p < 0.05$) in the production of p27 was observed for only the MO targeted to the ADOL-7501 primer binding site (PBS). Real time quantitative LightCycler® RT-PCR and an endpoint dilution assays were confirmed a one log reduction in viral RNA copies and infectious units detected when compared to the groups treated with a non-specific MO.

INDEX WORDS: antisense oligos, avian leukosis virus, ALV-J, cell culture, DF-1, morpholinos, nested RT-PCR, real time LightCycler® RT-PCR, retrovirus, therapeutic agents

CLASSICAL AND MOLECULAR STUDIES ENHANCING THE DETECTION AND CONTROL OF AVIAN LEUKOSIS VIRUS

by

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CLASSICAL AND MOLECULAR STUDIES ENHANCING THE DETECTION AND CONTROL OF AVIAN LEUKOSIS VIRUS

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DEDICATION

To my family, I whole-heartedly believe that I would have nothing if it were not for the encouragement, love and devotion of my wife Stacie and the merriment I receive from my two daughters Morgan and Parker. Without them all I would be an insignificant soul.

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CHAPTER I

INTRODUCTION

PURPOSE OF THIS STUDY

There are ten subgroups (A-J) of avian leukosis viruses (ALV). Only exogenous subgroups A, B and J and endogenous subgroup E are prevalent in poultry. ALV subgroup J (ALV-J) seems to be a recombinant ALV, because of its genomic similarity to other viruses of the ALV group. The control and eradication of avian leukosis virus (ALV) infections in the commercial poultry industry depends on the readily availability of diagnostic tests. Several diagnostic assays have been available and established for over 30 years and for almost a decade now, have been applied for the identification and ultimate eradication of ALV-J from infected flocks. ALV-J, which was originally isolated in Europe and first reported as a new subgroup in 1992, quickly became the most widespread and economically important ALV to infect meat type birds. ALV-J has since been recognized to naturally infect virtually all commercial poultry meat type genetic lines worldwide and in turn has been associated as the cause of pluripotent tumor developments in infected birds, as well as mortality, reductions in growth and egg production and general non-uniformity among all infected generations.

ALV-J most often induces a late onset of myelocytic leukosis. Myeloid progenitor cells are precursors to all chicken blood cells except for B and T

lymphocytes. It is known that ALV-J has a propensity to infect these myeloid progenitor cells and therefore induce a late onset of myelocytic leukosis. ALV-J can be transmitted either vertically or horizontally. Vertical and early horizontal transmissions are often responsible for the immunotolerance phenomenon, which is the lack of the host immune system to recognize ALV-J as a foreign antigen and successfully mount an immune response. This results in an immunotolerant infection, which, in turn, leads to active shedding.

Several methodologies have been implemented to detect and eradicate ALV-J. The assays and techniques used for eradication followed methodologies used to eradicate other exogenous ALVs from layer type chickens. Antigen capture-enzyme linked immunosorbent assay (ac)-ELISA is the most common diagnostic assay used by the industry to detect the presence of both exogenous and endogenous ALV. This test can detect p27 antigen in meconium, cloacal swabs, albumen, serum, plasma, blood and cell culture. However, since this assay also detects endogenous ALV, undue selection pressure can be placed upon a population if there is a high prevalence of endogenous virus in the genetic line being tested. In addition, the test is dependent on the production of the p27 antigen and sensitivity can be sacrificed if the host is concurrently producing p27 antibodies thereby inhibiting the ac-ELISA reactions.

The antibody ELISA for ALV-J was developed and was made available commercially for the sole purpose of detecting ALV-J antibodies. The gp85 *env* antigen used in this ELISA test was genetically engineered in a baculovirus vector utilizing the *env* from the HPRS 103 ALV-J isolate. The sensitivity of this

ELISA test can be sacrificed in two distinct ways. First, the immunotolerance phenomenon occurs quite often in ALV-J, and without a complete immune response, no antibodies will be produced and detected by this assay. Secondly, the recombinant gp85 antigen used in the antibody ELISA test has been shown to be highly divergent from not only other European isolates, but also ALV-J isolates from the United States. Therefore, antibody production from highly divergent ALV-J isolates may go undetected.

Virus isolation and identification in cell culture has been the choice system used by the broiler breeder industry for the detection of ALV-J. It is often referred to as the 'gold standard' for ALV-J detection and identification. This test is used to differentiate endogenous and exogenous ALVs via the propagation of suspect virus samples in primary chicken embryo fibroblasts (CEF) derived from line O C/E, which excludes the propagation of endogenous viruses.

Even after more than 10 years of application of readily available assays for the detection of ALV-J and the use of effective methodologies described for the detection of previous ALV infections in chickens, several broiler breeder companies still have not been successful in the complete eradication of ALV-J. This slow rate of eradication has been associated with the propensity of viruses from this subgroup to spread horizontally rapidly in young meat type birds due to a viremic infection that produces virus shedders.

Numerous studies have been initiated to better understand ALV-J. They include studies on molecular pathogenesis of myeloid leukosis, immune responses, genetic resistance and effects on performance. Although quite a bit

of information has been learned about this avian retrovirus, investigations still need to be conducted in order to augment eradication in infected flocks. Ideally, the results of the studies herein can be applied to complement and enhance both ALV and ALV-J detection and assist in eradication by abrogating progressive infections in flocks.

OBJECTIVES AND ORIGINALITY

The evaluation and comparison of line 0 C/E primary CEF and DF-1 continuous cell culture for avian leukosis virus detection and isolation was conducted. The institution of stringent ALV-J screening procedures, which in the laboratory environment involves virus isolation on C/E cells coupled with ac-ELISA along with more extensive screening and testing, has reduced the prevalence of ALV-J in the US poultry industry. The cell culture screening procedures are still used by most of the industry worldwide (52, 117). The C/E chicken embryo fibroblasts (CEF) are currently provided to the industry via embryonated eggs by private industry (46). These embryos have extremely low livability and viability and in turn are costly. In addition, preparation of primary and secondary C/E CEF is quite labor intensive. A continuous fibroblastic cell line (DF-1) derived from the EV-O line of chickens was recently deposited into the American Type Culture Collection (ATCC) depository. This is the first non-transformed continuous chicken cell line which does not have any endogenous viruses and can propagate avian leukosis sarcoma viruses ALSV (43, 67, 73). Substitution of the C/E CEF virus isolation with DF-1 would considerably

decrease cost and labor. Since no report has been issued on the ability of the continuous cell line DF-1 to propagate plasma samples of naturally infected birds, the ability of the DF-1 cell line to propagate ALV-J was evaluated and compared to the C/E CEF standard.

Secondly, sequence analysis was performed on the p27 gag protein which included the major homology region (MHR) and spacer region (SP) of ALV-J isolates. Since the p27 gag protein is considerably conserved throughout the same genera of the retrovirus family, sequence analysis has been predominately reported on the hypervariable *env* and the unique E element of ALV-J. Most of the molecular analysis done in this study were performed on ALV-J isolates detected by nested RT-PCR but undetected by ac-ELISA. Antigen capture-ELISA is the most common diagnostic assay used by the industry to detect the presence of both exogenous and endogenous ALV. This test can detect p27 antigen in meconium, cloacal swabs, albumen, serum, plasma, blood and cell culture. The use of stringent ALV-J screening procedures, which in the laboratory environment involves virus isolation on C/E cells coupled with ac-ELISA along with more extensive screening and testing, has reduced the prevalence of ALV-J in the US poultry industry (51, 181). However, when individual sample comparisons of virus isolation versus nested RT-PCR are performed, a significant amount of ALV-J positive samples are detected by RT-PCR which are not readily detected by virus isolation coupled with ac-ELISA. Since ac-ELISA is the backbone of ALV detection for the commercial industry, it was pertinent to assay these undetected virus isolates and examine the p27 gag

protein sequence data for possible reasons of abrogating infectivity and resulting in the absence of detection. Classical viral analysis was also performed on these analyzed samples in order to rule-out or include the possibility of interference of detection and infection via neutralizing antibodies.

Lastly, the development and analysis of antisense oligomers was performed in order to determine possible therapeutic applications against ALV-J. There are no commercial vaccines that protect chickens and turkeys from infection with ALV or REV. Therefore, the only defense that the primary breeder industry and the broiler breeder industry have had is eradication. This is primarily based upon the elimination of dams that test positive for the ALV p27 antigen by ac-ELISA. Although several companies have used previously successful eradication programs installed for over a decade, the problems of ALV-J infections are still confronted today (50, 117). Possible use of viral vaccines to increase host resistance has been suggested. In a series of attempts to inactivate leukosis viruses by various means, researchers have demonstrated that the ability of these virus preparations to induce antibody was destroyed almost concurrently with inactivation. Attempts to produce attenuated strains of an avian leukosis virus have also failed and minimal success has been obtained with immunization using viral antigens. In addition, recombinant ALVs expressing subgroup envelope glycoproteins have been produced that could have potential as vaccines (113). However due to frequent mutation events in the avian leukosis genome, none of these immunization attempts have been successful.

Analysis of therapeutic antisense oligomers for avian leukosis sarcoma viruses (ALSV) has been reported in literature and as far back as 1979 (82, 113, 179). However, the uniqueness of this project is provided through the use of a novel morpholino oligomer that is adequately soluble, consistently stable, resistant to host nucleases and can be delivered effectively *in vitro* and *in vivo*. Morpholinos were first reported in 1997 and consist of a morpholino moiety (one strong nitrogen nucleophile), which was converted from a riboside moiety (two weak hydroxyl nucleophiles) and connected with a phosphorodiamidate intersubunit linkage (157). As far as we know, morpholino oligomers have not been examined as a therapeutic instrument for any genera of the retrovirus family. In this study, a total of 5 antisense morpholino oligomers were designed and evaluated. Three morpholinos were targeted towards open reading frames (ORFs) in order to inhibit translation of viral proteins and two morpholinos were targeted towards the ALV-J primer binding site (PBS) in order to inhibit proper binding of tRNA-Trp.

CHAPTER II

LITERATURE REVIEW

RETROVIRUSES

Retroviruses were discovered at the turn of the century in two investigations devoted to neoplastic diseases in chickens (169). In 1908, the Danish team of Vilhelm Ellermann and Oluf Bang showed that chicken leukosis, a form of leukemia and of lymphoma, was caused by a virus and shortly following in 1911 Peyton Rous at the Rockefeller Institute of New York reported the cell free transmission of a sarcoma in chickens (45, 128). The agents discovered by Ellerman and Bang are known as avian leukosis viruses and the virus isolated by Rous bears the discoverers name and collectively these viruses are known as avian leukosis sarcoma viruses (ALSV). Mammalian retroviruses were first described in 1936 by John Bittner (mammary carcinoma in mice) and later confirmed in 1957 by Ludwik Gross who later identified a potent leukemia virus in mice by a combination of inbreeding and inoculation at a early age (14, 60). During the next two decades, many such viruses that cause neoplastic disease in mice, cats, cattle and monkeys were identified (169). Although the first description of a retrovirus which later turned out to be the lentiviral disease equine infectious anemia (EIA) was first described in 1904 by Vallee and Carre (164), the recognition of the viral origin of this disease was not established until elaborate work was performed on the Visna virus, a neurological disease in

sheep caused by a lentivirus, which enabled further characterization of EIA (142). Of course, several lentiviruses which were associated with immunodeficiency were later described and characterized and often serve as models for human immunodeficiency virus (HIV).

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition and replicative properties (28). Retroviruses are enveloped RNA viruses (80-120 nm in diameter) that have a dimeric positive sense ssRNA genome, which codes for the unique enzyme reverse transcriptase. This enzyme allows transcription of the genomic RNA directly into DNA. This DNA is then able to integrate into a more or less random site of the host DNA. Proviral DNA inserted into the host genome is then transcribed and translated to synthesize budding viral particles (27).

Retroviruses are broadly divided into two categories (simple and complex), distinguishable by the organization of their genomes (28). All retroviruses contain three major coding domains with information for virion proteins and terminal non-coding sequences, which include two direct repeats (R), a unique 5' (U5) and a unique 3' (U3) sequence. The coding domains are; *gag*, which directs the synthesis of internal virion proteins that from the matrix, the capsid and the nucleoprotein structures: *pol*, which contains the information for the reverse transcriptase (RT) and integrase enzymes; and *env*, from which are derived the surface and transmembrane components of the viral envelope protein. An additional, smaller, coding domain present in all retroviruses is *pro*,

which encodes the virion protease (169). Simple retroviruses usually carry only this elementary information, whereas complex retroviruses code for additional regulatory non-virion proteins derived from multiple spliced messages (28). Some retroviruses carry oncogenes. Identified as transforming viruses, they are able to rapidly cause tumors in animals and oncogenically transform cells. With the exception of some strains of Rous sarcoma viruses (RSV), retroviruses that carry oncogenes are defective, having suffered variable deletions of one or more of the viral genes needed for replication during or after the acquisition event (170).

Retroviruses are further organized into seven groups based upon evolutionary relatedness of the *pol* gene. These genera are established by the International Committee on the Taxonomy of Viruses (ICTV) and are; Avian sarcoma and leukosis (ASLV), Mammalian type B, Mammalian type C retrovirus, Human T-cell leukemia-bovine leukemia, D type, Lentivirus and Spumavirus (170).

Replication. The retrovirus replication cycle can be readily distinguished as occurring in two phases. The first phase would include binding and entry of the virion core into the cytoplasm of the host cell, followed by reverse transcription and synthesis of the double stranded DNA from the RNA genome and integration into the host genome (28). This phase is noted by the necessity of proteins found within the virion and can proceed without viral gene expression. The second phase would include the synthesis and processing of genomic mRNAs to

produce associated virion proteins as well as assembly and budding of the finishing virions (28). A tremendous amount of research has focused on all facets of these two phases of retrovirus replication in order to understand normal and abnormal viral processing, natural and induced evolution as well as natural persistence.

Mutation rates of retroviruses often executed by reverse transcriptase is estimated to be 2×10^{-5} substitutions per site per round of copying of the genome and as a result often produce quasispecies as well as non-infectious or replicative defective virions (37, 38, 104, 105).

The initial step in the first phase of replication includes viral attachment to specific cell surface receptors. In the case of Avian Leukosis Virus Subgroup A, LDL has been identified as the cell receptor and a tumor necrosis factor receptor of different and distinct forms has been identified for subgroups B, D and E, while the CD 4 cellular receptor and co-receptors CXCR4, CCR3 and CCR5 have been identified for HIV (1, 2, 10, 62, 70, 85, 178). After adsorption of the virion to the cell surface receptor, the virus envelope and the cell membrane fuse to release the virion core into the cytoplasm (28). The virus is then uncoated either directly into the cytosol or endocytic vesicles. The retrovirus genome remains in close proximity of necessitated proteins (RT, integrase and nucleoprotein) for replication. Virus transcription is initiated instantaneously after virion uncoating and is carried out by RT in the core structure to copy the genome RNA into DNA. RT recognizes a tRNA molecule base paired to the primer binding site (PBS) located in the 5' end of the genome and extends this tRNA molecule to form a

DNA copy of the genomic 5' end (Figure 1). Of the numerous retroviruses studied, all use one of just a few classes of cellular tRNAs as primers for reverse transcription. For example, most mammalian retroviruses use $tRNA^{Pro}$, $tRNA^{Lys3}$ or $tRNA^{Lys1,2}$ while ASLV viruses have PBS complimentary to $tRNA^{Trp}$ (53, 172). RNase H activity removes the hybridized RNA and the cDNA jumps and hybridizes to the R sequence to the 3' end of the genome and extension of a full length cDNA is performed while most of the hybrid RNA is removed except for a polypurine tract that is just upstream of the U3 on the RNA genome. The tRNA primer is removed from the 5' end of the newly synthesized DNA while RT extends the plus strand DNA until the PBS is synthesized and RT makes its second jump to the PBS on the 3' end of the minus strand and hybridizes. Both DNA strands are then completed. Complete or incomplete DNA duplexes are then transported to the nucleus and are still associated with virion proteins. Integration of the viral DNA then occurs more frequently at random sites in the cellular DNA to form the provirus. Concerted integration of retrovirus DNA termini into the host chromosome *in vivo* requires specific interactions between the *cis*-acting attachment sites at the viral termini and the viral integrase in *trans* (26).

In the second main phase of retrovirus replication, viral RNA is then synthesized by cellular RNA polymerase II using the integrated provirus as a template (28). The retroviral LTR contains several replication and transcriptional regulatory elements that have been shown through mutational analysis to inhibit infection, integration, protein expression packaging, as well as transcription (6,

65, 68, 99, 132, 134). In addition, alterations within the first short open reading frame (ORF) and the main ORF located in the LTR of RSV resulted in accumulation of transformation defective viruses as well as a delayed onset of replication (96, 97).

Correct polyadenylation sites within the viral genome are necessary for the host cell to properly process retroviral transcripts (28). The lack of proper polyadenylation at the correct site results in read through transcripts which contain nonviral sequence at their 3' end and have been predicted to be in up to 32% of wild type ALSV particles and as a result, virus replication has been shown to be reduced up to six times less in polyadenylated mutated ALSV when compared to wild type viruses (28, 66). The transcripts are then processed to produce genomic and messenger RNA. Research groups have focused on the processing of the capsid proteins and the role that this proper processing plays in retrovirus replication and infectivity (23, 118, 173). Via mutational analysis, proper processing of ALSV capsid proteins via cleavage events that occur at identified amino acid residues within the spacer region (SP), which is located between the capsid (CA) and nucleocapsid (NC), have been shown to be required for infectivity of ALSV infectious molecular clones (17, 177). If virion proteins are synthesized and processed correctly, they assemble to form the virion, which completes assembly at the cell membrane and subsequently buds from the cell surface.

AVIAN LEUKOSIS SARCOMA VIRUSES

Avian leukosis sarcoma viruses (ALSVs) are type C retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis, erythroblastosis, myeloblastosis nephromas and / or nephroblastomas and other related connective tissue, epithelial endothelial tumors (113). These viruses were later associated with diseases described as 'lymphosarcomata' in 1868 by Roloff and fowl leukemia in 1896 by Caparini (22, 127). Since these original descriptions, reviews throughout the literature have described and comprehensively characterized similar diseases (19). Today, these viruses are prevalent in the poultry industry worldwide and cause severe economic losses from tumor mortality, condemnations and loss of pedigree birds (51, 107, 174).

The virion. Similar to other genera *Retroviridae* family, mature ALSVs are enveloped, have a spherical to conical core with several associated proteins important for replication, a dimeric RNA genome and is the virions are estimated to be 100 nm in diameter (28, 170).

The genome. For the most part, the organization of genome ALV RNA is simple (117, 170). Genomic organization for virtually all ALVs is 5' – R-U5-*gag*/*pro*-*pol*-*env*-U3-R – 3'. The *gag* gene encodes for five non-glycosylated proteins, which are p27, the major group specific capsid antigen; p19 the matrix protein; p12 the nucleocapsid, p15 a protease and p10. The virion envelope encodes two glycoproteins, which are gp85 (SU), the viral surface protein and gp37 (TM), the

transmembrane protein, which anchors the SU protein to the envelope. The *po*l gene encodes for RT, which is p68 and the integrase (IN) protein p32. For sarcoma viruses (RSVs), an *onc* gene usually replaces the *env* or *gag* gene and predominately renders the virus defective in replication (57, 159, 161). The direct repeats (R) along with the U5 and U3 region on each end of the viral genome are coined the long terminal repeat (LTR) regions of the genome. These LTR regions include promoter and enhancer sequences controlling transcription of viral DNA to RNA (113, 132). LTR regions often show large deletions of viral sequences, although the LTR is preserved to drive high level viral and cellular oncogene transcription (59, 132, 161). LTR alterations have been shown to change cell specificity, pathogenicity and increase RSV transcription (16, 41, 63, 64, 92, 141, 160, 182).

ALSV groups. ALVs infecting chickens can be classified into subgroups based on specific envelope surface glycoprotein responsible for specific viral interference patterns, virus neutralizing antibodies and host range (113, 160, 171). Historically, the classification of ALV subgroups was defined using host range in chickens that differ in susceptibility to infection, patterns of receptor interference, and virus neutralization (73, 74, 75). Six subgroups of ALVs have been identified to infect commercial poultry (A, B, C, D, E, and J) (51, 110, 115). Subgroups A and B occur as common exogenous viruses in the field, are spread vertically and horizontally, are oncogenic and cause principally lymphoid leukosis (LL) (20, 21, 113). Subgroups C and D are also exogenous viruses and have

been rarely reported in the field (113). Subgroup E viruses, include the ubiquitous endogenous leukosis viruses of low pathogenicity (145).

Endogenous leukosis viruses. Endogenous leukosis viruses, which are typified by Subgroup E ALV, contain complete viral genomes and partial endogenous virus (ev) sequences, which are stably inserted into the host cellular genome and are transmitted vertically to the progeny. The normal chicken genome contains several classes of avian retrovirus-like elements that are present as either complete or defective genomes in almost all normal chickens (32, 33, 36). There have been 4 repeatedly identified families of endogenous viruses and they include the chicken repeat 1 (CR-1), ALV-E (*ev*), avian retrotransposon from the chicken genome (ART-CH) and the endogenous avian virus family (EAV-0).

CR-1 elements are short interspersed DNA elements within the chicken genome (61, 135). They are believed to be contained within the chicken genome at rates of up to 2% (7,000 to 20,000 repeats per haploid genome) and have been identified in several avian and reptilian species (61, 165).

More than twenty-nine *ev* loci have been identified in the meat and layer type chicken genome and these gene loci usually do not cause a direct negative impact on bird performance, however, they can facilitate immunotolerance and interfere with detection of exogenous ALVs (36, 130, 145). Several ev loci have been identified and are well characterized. For example, the *ev*2 loci encodes for the prototype ALV-E RAV-0, and *ev*21 can also replicate and produce infectious

endogenous virus with a subgroup E envelope. Other examples of ev loci include ev3 and ev6 which produce env glycoproteins with similar sequence to functional glycoproteins (35, 89, 125, 146, 147). This family has been hypothesized to be the youngest group of endogenous viruses due to their distribution being restricted to the *Gallus* species (40).

ART-CH are usually small elements less than 3 kb and exist in the chicken genome with about 30-50 haploid copies (61). They are composed of a functional LTR with short but incomplete regions similar to ALSV *gag*, *pol* and *env* and do not code for functional proteins (98, 135).

EAV-0 is a group that has between 40 to 100 copies per haploid genome in the chicken (40). They usually have a typical proviral genome structure of 5'-LTR-*gag*-*pol*-*env*-LTR-3' and often show deletions within the *env* (81, 124, 135). They exist in species other than *Gallus* and therefore are hypothesized to be more ancient than *ev* loci (18). Characterized representatives of this family include E13, E33, E51, EAV-O and EAV-HP (11, 77, 151). There is evidence of expression, however, no evidence of functional proteins have been observed (11, 135, 151).

Exogenous leukosis virus. For the most part, all exogenous viruses display the same physical and biochemical characteristics (49). Exogenous ALVs are transmitted either vertically (congenital) from hen to progeny or horizontally from bird to bird by direct or indirect contact, while endogenous ALVs are usually transmitted genetically in the germ cells of both sexes (131). Of all three methods

of transmission early horizontal and congenital transmission of exogenous ALVs are more likely to induce oncogenesis (113, 114). Subgroups A and B were considered the most prevalent ALVs in the commercial chicken until the recent discovery of the subgroup J which most often causes myeloid leukosis in meat type birds (49, 174). Subgroups C and D are highly uncommon and are not considered economically important to the industry (47, 80, 106, 113, 117, 129). Due to exogenous and endogenous transmission of ALVs, four serological classes can occur in mature chickens in relation to a transmitted ALV infection: 1) no viremia, no antibody (V-, A-); 2) no viremia, with antibody (V-, A+); 3) with viremia, with antibody (V+, A+); 4) with viremia, no antibody (V+, A-) (113).

Viral transformation. Viruses within ALSV have the ability to transform host cells after integration (113). Two main types of transformation occur upon integration. Acute transformation usually occurs with viruses carrying differing viral oncogenes, which were derived from normal cellular sequences and are responsible for early onset neoplastic transformation (115). Several proto-oncogenes have been identified in the chicken genome and proviral insertion of several subgroups of exogenous viruses have been documented to activate the c-*myc*, c-*myb* and c-*erb* genes of chickens and cause oncogenesis (34, 54, 122).

Slow transformation viruses do not posses a viral oncogene and usually have two modes of inducing cellular transformation. Studies have shown that ALVs usually becomes integrated within the host c-*myc* locus, which is then expressed by the viral LTR promoter sequence (59, 80, 126). Outside of

displacement of host oncogene, viral insertion either upstream or downstream of host oncogene can induce transformation. Known as promoter insertion, this is believed to initiate the lymphomagenic process (7, 41, 54, 90, 113, 121, 136).

AVIAN LEUKOSIS VIRUS SUBGROUP J (ALV-J).

Avian leukosis subgroup J, a recently discovered member of the leukosis/sarcoma group, was first isolated from meat-type chickens (106, 109). ALV-J behaves as an exogenous virus causing mainly myeloid leukosis and nephromas (Figure 2) in meat type chickens (106, 110).

ALV-J induces a late onset of myelocytic leukosis. Myeloid progenitor cells are precursors to all chicken blood cells except for B and T lymphocytes. Studies on the cell tropism of strain HPRS-103, the prototype strain of ALV-J isolated in the United Kindgom, have shown that, in contrast to subgroup A ALV, it has a tropism for cells of the myelomonocytic series, but low tropism for bursal cells, consistent with its induction of myeloid, and not lymphoid leukosis (3). ALV-J *gs* antigen and viral transcripts have also been identified in several different cells and tissues outside of the bone marrow cells (3, 4, 5, 154). Chickens exposed experimentally at 11 days of embryo incubation *in ovo* had the greatest detectable nucleic acid in the adrenal gland, heart, kidney, and proventriculus (5). Congenitally infected chickens examined by ISH had similar staining patterns to those inoculated *in ovo* with minor differences in staining intensity within the organs (154).

ALV-J infection can affect meat-type birds at all breeding generations as well as commercial broiler flocks (48, 49, 52). Myeloid leukosis problems induced by ALV-J has emerged as a serious cause of mortality and other production problems in meat-type chickens throughout the world. The subgroup J virus is unique not only for its ability to induce myeloid leukosis but also for its broad genetic host range (48, 49, 52, 106, 108).

HPRS-103, which is the prototype strain of ALV-J, shares the same group antigen of other ALVs, however, differs from them in envelope properties and has been assigned a new subgroup because of these differences (9). The composition of the ALV-J genome is similar to that of other simple ALVs, however, differs when sequence comparisons are made. Analysis of the sequence of an infectious clone of the complete proviral genome indicates that HPRS-103 is a multiple recombinant of at least five avian leukosis and sarcoma virus (ALSV) sequences and one endogenous avian retrovirus (EAV) sequence (8, 9). The ALV-J proviral genome is approximately 7841 bp in length and ALV-J is thought to have evolved by recombination with *env*-like sequences of the EAV family of endogenous retroviruses (9). Identification and characterizations of ALV-J recombination events *in vitro* have been performed. After three passages in CEF, which contained a replication defective recombinant endogenous virus expressing a subgroup A envelope, the *env* gene of three different ALV-J isolates quickly recombined with the endogenous sequence and were able to infect DF-1/J cells which were resistant to ALVs expressing the J envelope (93). This recombination event follows models observed and described for retroviruses

(28). The *env* region is more closely related (75% similar) to the *env* gene of the defective EAV-E51 but divergent (35% dissimilar) from those of other ALSV subgroups (9). ALSV normally have a 92%-95% similarity between themselves (115, 116). The gp85 viral surface glycoprotein of the *env* gene has also been reported to be highly variable among different strains of ALV-J (144, 145). This variability (5%-10%) has resulted in several antigenic variants of ALV-J (144, 166). In addition to the highly variable *env* gp85 region, ALV-J genomes contain an E element (exogenous virus specific region) in its 3' non-coding region, previously found only in many transforming ALSVs.

Exogenous ALV-J is transmitted by conventional means through vertical and horizontal transmission. Field observations and preliminary experimental studies suggest that horizontal transmission of ALV-J in meat-type chickens is highly efficient and can lead to tolerant infection (persistent viremia and lack of antibody) and consequently shedding and congenital transmission to the next generation (51, 109).

Recently, existences of novel endogenous retrovirus elements in the chicken genome, with close sequence homology (95%) to the *env* gene of ALV-J and avian retrotransposon ART-CH, have been reported (11, 133, 135). RT-PCR experiments have demonstrated that at least some ev/J proviruses are expressed in chicken embryo fibroblasts (133). It remains to be elucidated if mRNA from these expressions produce *env*-related antigens which in turn might interfere or influence the ability of different ev/J strains to control infection by exogenous ALV-J.

In addition to the identification of endogenous virus sequence homologies, representative viruses of the ALV-J subgroup, which have the innate ability to rapidly transform host cells upon infection, have been characterized (25, 111, 168). Several ALV-J isolates have been characterized as acutely transforming viruses because of their ability to transform bone marrow cell cultures (168). Although the original isolate HPRS-103 of ALV-J and other isolates similar to HPRS-103 induce late occurring oncogenesis, several acutely transforming viruses that have induced a rapid onset of tumors have been isolated from late-onset tumors (25, 111, 168). This would suggest that the generation of acutely transforming ALVs is a common feature of ALV-J induced oncogenesis (25). Characterization of an acutely transforming virus isolated from a late-onset tumor has identified a 72 kDa *gag-myc* fusion protein encoded by its genome (25). This *v-myc* protein is observed in previously characterized acutely transforming viruses, however, *v-myc* proteins encoded by other characterized acutely transforming viruses have expressed gag-myc fusion protein of different and various sizes (12, 13, 24). This involvement of the *myc* gene in the induction of myeloid tumors by ALV-J confirms that irrespective of the cell targets for transformation, ALVs make use of the common *myc* mediated pathway for the induction of tumors (25).

AVIAN LEUKOSIS VIRUS DIAGNOSTIC TECHNIQUES

For almost a decade, it has been a major concern of the poultry industry to use applicable diagnostic assays to detect and identify ALV-J (62, 148, 153).

Typical control and eradication programs for primary breeders include the combined use of virus isolation, antigen capture (ac)-ELISA with additional analysis via molecular methods (52, 117, 153, 174, 175). The predominant method for detection of ALVs in chickens includes the detection of viral group-specific antigen (gs) by the ac-ELISA test (153, 175). Other assays that detect ALSVs have been well characterized and are proficient in their ability to detect viral presence. However, these assays such as phenotypic mixing, resistance-inducing factor, and the complement fixation test are often time consuming and labor intensive and are not suitable for routine sampling (31, 46, 52, 100, 137, 162, 163).

Virus isolation. The ac-ELISA test is not specific for exogenous virus and will detect gs antigens of endogenous virus in serum, plasma, albumen and meconium (112). In order to increase the specificity of the assay, propagation of the virus in different phenotypes of chicken embryo fibroblast is required prior to the ac-ELISA test. Virus isolation and identification in line 0 C/E cell culture is quite readily used by the broiler breeder industry and is often referred to as the ‘gold standard’ for ALV-J detection and identification (49). This cell line has the innate ability to eliminate the propagation of any present endogenous virus (46, 49). Although the combination of virus isolation followed by gs antigen ELISA has been very useful in the detection of ALV-J, this test is time consuming and requires a minimum of seven days to obtain results (46, 52, 112, 148, 150). In addition, due to intermittent patterns of both antigen shedding and virus

transmission to embryos, eradication programs based solely on dam testing are probably less effective than those where dam testing is combined with procedures to mitigate early horizontal transmission in progeny chicks (175). Therefore, additional assays have been utilized, developed and characterized for ALSV and ALV-J detection.

DF-1 continuous cell line. The institution of stringent ALV-J screening procedures, which in the laboratory environment involves virus isolation on C/E cells coupled with ac-ELISA along with more extensive screening and testing, has reduced the prevalence of ALV-J in the US poultry industry (52, 113, 115, 117). The cell culture screening procedures continue to be used by most of the international primary broiler breeder industry (180, 181). The line 0 C/E chicken embryo fibroblasts (CEF) are currently provided to the industry via embryonated eggs by private industry (Kestrel Inc. Waukee, Iowa). These embryos have extremely low livability and viability and in turn are costly. In addition, preparation of primary and secondary C/E CEF is quite labor intensive.

A continuous fibroblastic cell line (DF-1) derived from the EV-O line of chickens was recently deposited into the ATCC depository (ATCC CRL-12203). This is the first non-transformed continuous chicken cell line which does not have any endogenous viruses and can propagate ALSV (67, 86). This cell line has normal fibroblastic morphology and supports the replication of exogenous ALV subgroups A, B, C and J (67, 73). This cell line has also been shown to undergo the induction of cellular transformation by over 32 different viral and cellular

oncogenes present in the ALSVs (67, 86). In addition, several differences are observed when different ALV subgroups were propagated on DF-1 cells and not primary or secondary line 0 C/E CEF. These differences include the ability of subgroups B, C and D to induce plaque formation on DF-1 cell monolayers and clearly distinguish DF-1 from CEF (67). Properties of the DF-1 cell line have been beneficial for studies involving work with ALVs.

This cell line has recently been genetically engineered by Hunt *et al*. to be resistant to ALV-J infection (73). DF-1/J expresses the env protein from the ADOL-Hc1 ALV-J strain and serves to interfere with receptor binding of exogenous ALV-J viruses (73). Recombination events involved with ALV-J isolates have been characterized with DF-1/J (93). In addition, since DF-1/J has been shown to be resistant to a genetically diverse group of ALV-J isolates, this cell line could be readily used as a diagnostic tool. Samples containing only ALV-J will produce a positive p27 response using the DF-1 cells and a negative p27 response using the DF-1/J cells, and mixed exogenous samples would be positive on both cell types (73). The combinational use of the original DF-1 cell line along with the DF-1/J cell line would eliminate tedious characterization and/or virus neutralizations in order to determine the type of infectious subgroup and could be used to screen large numbers of samples (73). Economically, substitution of the C/E CEF virus isolation with DF-1 would considerably decrease cost and labor. The capabilities of the DF-1 cell line to propagate ALV-J have been evaluated and compared to the C/E CEF standard and although not significantly different, side by side analysis of over 400 plasma samples, revealed

that virus propagation in DF-1 cells allowed for the identification of more positive plasma samples by ac-ELISA at both S/P ratios (0.2 and 0.1) (43).

ALV-J antibody ELISA. The antibody ELISA was recently developed and is available commercially (Idexx International). The gp85 *env* antigen used in this ELISA test was genetically engineered from the HPRS 103 isolate (166). The sensitivity of this ELISA test can be sacrificed in two distinct ways. First, the immunotolerance phenomenon occurs quite often in ALV-J, and without a complete immune response, no antibodies will be produced and detected by this assay. Secondly, the recombinant gp85 antigen used in the antibody ELISA test has been shown to be highly divergent from not only other European isolates, but also ALV-J isolates from the United States (144, 167). Therefore, antibody production from highly divergent ALV-J isolates may go undetected.

Molecular methods. A more effective control of ALV infections mainly depends on the early detection and removal of infected birds to reduce contact with infected birds and the incidence of horizontal spread (117, 175). Polymerase Chain Reaction (PCR) based methods had been developed to provide a rapid tool for detection of ALVs proviral DNA and viral RNA (55, 117, 119, 120, 149, 150, 176). An ALV-A specific RT-PCR assay for detection of virus in albumen from unfertilized eggs has been utilized to assess the viral prevalence of infected layer flocks (119). ALV-J specific PCR methods have been developed to detect proviral DNA from buffy coats, from infected CEF and genomic RNA from serum

of naturally infected birds (145, 148). Although these assays have proven to be equally sensitive and in some cases superior to virus isolation (2% - 40% increase in sensitivity for ALV-J detection) (44, 55), a major concern in the application of PCR is both sensitivity and specificity due to both antigenic and genomic variants (143, 144, 145, 148, 167). Therefore, the majority of the primary breeder industry has a propensity to use molecular assays for the detection of ALV-J only as confirmatory analysis.

ANTISENSE OLIGONUCLEOTIDES

The strategy of employing nucleic acids with sequences complementary to specific target sequences to modulate and regulate gene expression is not a human innovation, and in fact is a technique used by bacterial cells to regulate gene expressions (94). Antisense technology has become a real alternative in the treatment of disease states arising from genetic abnormalities such as gene amplification or over-expression (72). Antisense oligonucleotides (ONs) are undergoing evaluation in clinical trials as treatments for a variety of diseases, including cancer (58, 71), viral infections such as Hepatitis C and Cytomegalovirus (78, 101), and inflammatory disorders such as Crohn's disease (72, 94). The first ON drug Fomivirsen, which was developed by Isis Pharmaceuticals (Carlsbad, CA, USA), has received approval for marketing in the USA and Europe for Cytomegalovirus retinitis infections in AIDS patients (58, 94, 101).

Design. Before addressing the delivery of antisense ONs or even ribozymes, which are catalytic RNA biomolecules discovered by Thomas Cech in 1981, it is first necessary to have a design that is active (72). Even with the sequence of mRNA being known, an active ON or ribozyme can be neutralized by the secondary structures mRNA present by rendering the target sequence inaccessible. Therefore, programs, which predict the secondary structures of RNA such as MFOLD, have been used to predict hybridization-accessible sites. However, this approach is often unsuccessful because of the inabilities of currently available computer algorithms to accurately predict the complex secondary and tertiary structures of RNA molecules (69, 72). As an alternative to secondary or tertiary structure predictions by computer algorithms, techniques such as RNaseH mapping and ONs scanning arrays have been employed to successfully design ONs and ribozymes.

Since RnaseH readily recognizes DNA-RNA heteroduplexes and selectively degrades RNA, hybridizing target mRNA with selected and designed libraries of ONs in the presence of RNaseH and analyzing existing cleavage fragments reveals accessible target sites. This strategy has been used successfully to select potent antisense molecules against the human multidrug resistance (MRP1) gene, the RNA of hepatitis C virus and active ribozyme constructs (72, 79, 139).

The use of ON scanning arrays involves the use of scanning combinatorial ON arrays for the determination of accessible sites (72). It is possible to combine RNaseH mapping and ON scanning arrays by using the former to select a region

with good accessibility, followed by the latter to precisely identify active antisense sequences (72).

Delivery. The aims for optimal delivery of ONs and ribozymes are enhanced cellular uptake, improved exit from subcellular compartments and correct targeting (spatial and temporal) to the desired site of action (72, 94). Optimal delivery includes successful applications for both *in vitro* and *in vivo* systems. Successful targeting will require effective target-matched delivery and pharmacodynamics, in which effective concentrations of ONs at the target site will need to be maintained for an appropriate length of time (72). This appropriate length of time has to match the half-life of the target protein and the desired level of effectiveness. It has been predicted that a delivery time period of four half-lives would be required to achieve greater than 90% inhibition (72).

Several delivery systems have been tested *in vitro* for the delivery of ONs and ribozymes. They include 1) lipid delivery systems, which include various liposomes and lipoplexes, can readily dissociate from ONs and ribozymes in a bioavailable form within the cell, 2) dendrimers which are comprised of highly branched three dimensional structures that facilitate nucleic acid binding and can be synthesized with various functional groups, 3) biodegradable polymers which provide sustained release of the bound nucleic acid, 4) receptor mediated endocytosis where a modified nucleic acid can be conjugated to a transferring receptor antibody and 5) carrier peptide mediated delivery which contain small proteins of approximately 10-16 amino acids (protein transduction domains) and

can be used to promote the delivery of active agents across biological membranes by a receptor and transporter independent mechanism (72). Delivery systems used can be varied depending on target site and most importantly target compartment. Ultimately, effective delivery will lead to a more widespread use of both antisense ONs and ribozymes as biological tools, drug-target validation agents and therapeutic agents (72).

Chemistries. Initially, the lack of biological stability was a major problem exhibited in traditional ONs with unmodified phosphodiester (PO) backbones because of their rapid degradation by host endo and exo nucleases. The end of this era was characterized by the recognition that nuclease resistance is generally a desirable trait in antisense oligomer chemistry, and this realization prompted an extensive exploration of various chemical modifications in oligomer structure (58). First generation antisense oligos comprised natural genetic material and often contained crosslinking agents for binding their targets irreversibly (157). As design challenges became more fully appreciated, a number of non-natural antisense structural types have been developed and are displayed in Figure 3. The most widely studied are phosphorothioate (PS) ONs in which one of the non-bridging oxygens of the phosphodiester backbone is replaced with sulfur (58, 83, 87, 94, 101, 157). Various chemical modifications such as PS ONs have been applied in an effort to increase ribozyme stability, however, due to some essential ribonucleotides for catalytic activity, ribozymes cannot be wholly modified (72). Additional structural ONs types, which have

been studied and reported in the literature, include dumbbell RNA/DNA chimeric oligonucleotides (102), peptide nucleic acids (PNAs) (15, 39, 152) and morpholinos (30, 56, 76, 78, 84, 95, 157).

Morpholinos. Morpholino (MO) based ONs are unique oligos that are designed to be between 18-25 genetic letters in length and were first reported in 1997 by Summerton (156, 157). MOs contain six-membered morpholine rings instead of the five-membered ribose or deoxyribose rings common to RNA and DNA respectively (94). The intersubunit linkage is a non-ionic phosphorodiamidate structure (157). These artificial nucleotide chemistries offer a number of advantages over natural and other artificial nucleotides.

Since PS oligos have previously dominated the antisense field, the development of MO based ONs was based on the improvement of PS antisense activity. These improvements have been reviewed and examined and are considered advantageous over PS oligos in that, 1) PS oligos have poor target specificity probably due in large part to RNaseH activity present with PS oligo delivery and not MO delivery, 2) MO have no non-antisese effects such as PS oligos, which have additional and multiple non-antisense effects such as binding to many proteins and extracellular surfaces and 3) MO oligos are virtually indestructible by host nucleases unlike PS oligos, 4) effective targets can be predicted by MOs more easily due to their higher affinity for complementary RNA than PS oligos and as a result are able to invade stable RNA secondary structures (95, 140, 155, 156, 157, 158).

In addition, MO oligos are delivered faster and simpler than PS oligos. Reliable *in vitro* delivery can be accomplished via scrape delivery and special delivery via a non-toxic MO/DNA/ethoxylated polyethyleneimine (EPEI) complex (56, 76, 95, 103, 123). Efficient and effective *in vivo* delivery of MO oligos, have also been reported.(29, 42, 88, 138).

Avian Leukosis Antisense Oligonucleotides. Antisense oligodeoxynucleotides (ODN) have been examined as potential inhibitors of ALSVs (82, 179). The first report was in 1978, by Zamecnik and Stephenson with a 13 ODN targeted towards the 35S RNA in the U5 LTR (179). Inhibition of RSV production via reduction of reverse transcriptase (RT) activity in CEF was observed. Several plausible explanations were given for inhibition of reverse transcription, however, no ODN controls were included in this study and therefore, non-antisense activity cannot be ruled out for (RT) inhibition.

A second report published in 1998 by Kim *et al*., included proper ODN controls and examined the possible inhibition of viral replication in CEF via analysis of p27 *gs* antigen production (82). Several ODNs were designed to specific targets throughout the ALV genome and one ODN out of the eight designed exhibited anti-viral activity. This ODN targeted the primer binding site (PBS) (Figure 1) of the ALV genome. However, when the corresponding region was specifically employed as a target for intracellular antisense RNA expression, there was no significant inhibition of ALV (82). Although an *in vivo* expression of antisense RNA is unlikely to be effective (probably due to degradation by host

endo and exo nucleases), specific and significant reduction of virus production via the PBS targeted ODN would be a promising application for modern modifications of antisense oligonucleotides such as morpholinos.

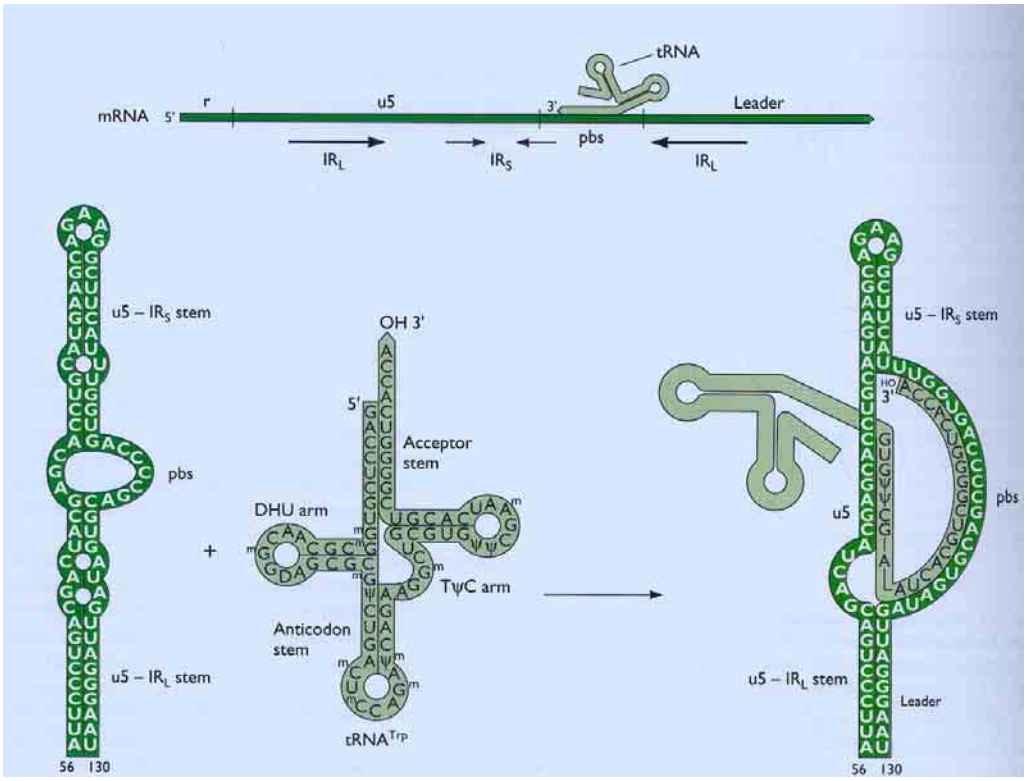
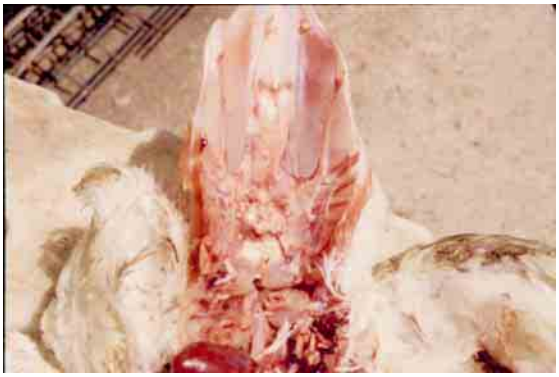


Figure 1. Primer tRNA binding to the retroviral RNA genome. (Top) Linear representation of the 5' terminus of retroviral RNA. The r, U5, and leader regions are indicated. A tRNA primer is shown schematically annealed to the PBS. Two inverted repeat sequences (IR) that flank the PBS are represented by arrows. (Bottom) Avian sarcoma/leucosis virus RNA can form an extended hairpin structure around the PBS in the absence of primer tRNA (left). (Middle) Primer $tRNA^{Trp}$ in the cloverleaf structure. Modified basesare indicated. (Right) Viral RNA annealed to $tRNA^{Trp}$. The U5-leader and U5-IR stem structures are indicated. An interaction with the TψC arm of the primer and U5 RNA is also shown (91).

A

B

C



D

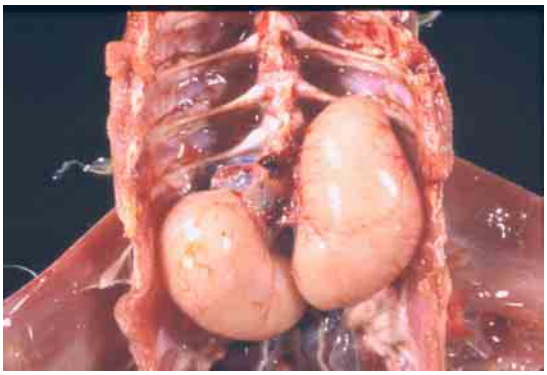


Figure 2. Various myelocytomas observed in chickens naturally infected with ALV-J. A) Cranial myelocytoma. B) Myelocytoma originating from wing. C) Myelocytoma originating form the keel bone. D) Gonad myelocytoma. (Photos provided courtesy of Pedro Villegas, DVM, Ph.D and Dr. Alberto.

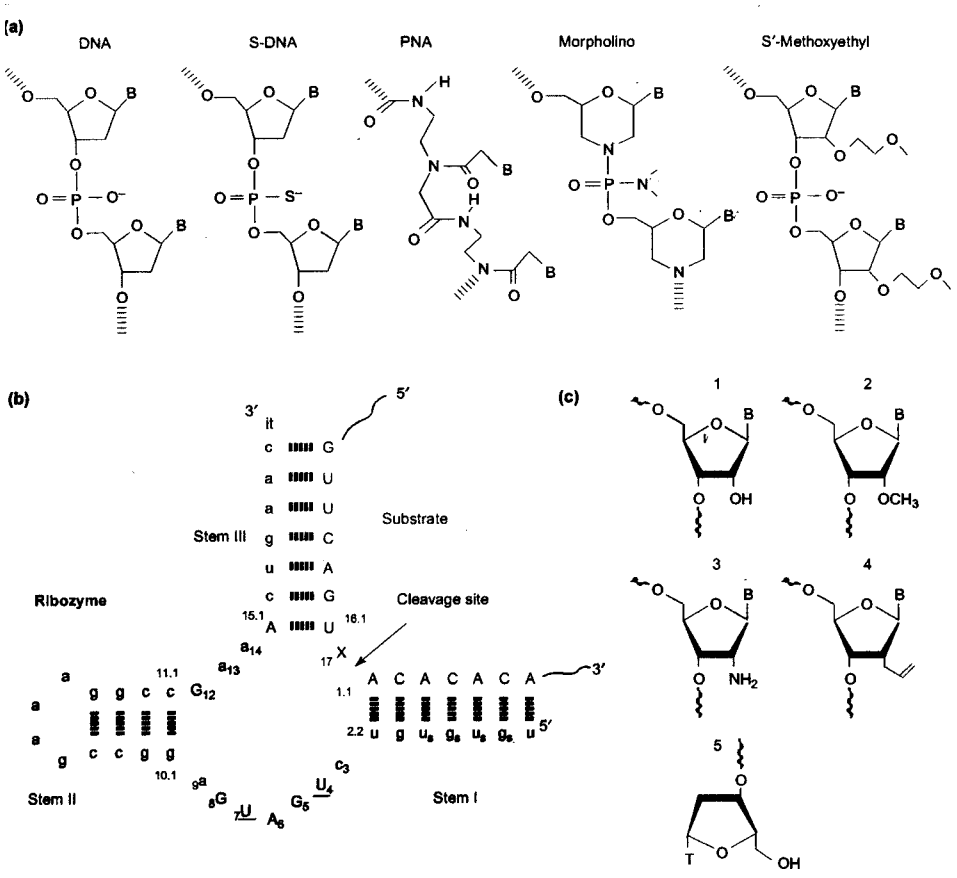


Figure 3. Commonly used nucleic acid chemistries. (a) Depicts phophodiester DNA chemistry and various standard chemical modifications aimed at enhancing the stability of ODNs. Abbreviations: S-DNA, phosphothiorate DNA; PNA peptide nucleic acid. (b) A hammerhead ribozyme: upper-case letters represent unmodified RNA; lower case letters represent 2'o-methyl ribonucleotides; underlinded letters represent either 2'-amino or 2'-C-allyl ribonucleotides. 3'-3'-inverted thymidine is represented by 'it'. (c) shows the structures of various forms of modified RNA; 1, unmodified RNA; 2, 2'O-methyl RNA; 3, 2'-amion RNA; 4, 2'-C-allyl RNA; 5, 3'-3'-inverted thymidine (72).

REFERENCES

1. Adkins, H. B., J. Brojatsch, J. Naughton, M. M. Rolls, J. M. Pesola, and J. A. Young. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc. Natl. Acad. Sc.i U S A. 94:11617-11622. 1997.

2. Adkins, H. B., J. Brojatsch, and J. A. Young. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. 74:3572-3578. 2000.

3. Arshad, S. S., K. Howes, G. S. Barron, L. M. Smith, P. H. Russell, and L. N. Payne. Tissue tropism of the HPRS-103 strain of J subgroup avian leukosis virus and of a derivative acutely transforming virus. Vet. Pathol. 34:127-137. 1997.

4. Arshad, S. S., L. M. Smith, K. Howes, P. H. Russel, K. Venugopal, and L. N. Payne. Detection of avian leukosis virus subgroup J (HPRS-103) using in situ hybridization. Avian Pathol. 27:s91. 1998.

5. Arshad, S. S., L. M. Smith, K. Howes, P. H. Russell, and K. Venugopal. Tropism of subgroup J avian leukosis virus as detected by *in situ* hybridization. Avian Pathol. 28:163-169. 1999.

6. Aschoff, J. M., D. Foster, and J. M. Coffin. Point mutations in the avian sarcoma leukosis virus 3' untranslated region result in a packaging defect. J. Virol. 73:7421-7429. 1999.

7. Baba, T. W., and E. H. Humphries. Avian leukosis virus infection: analysis of viremia and DNA integration in susceptible and resistant chicken lines. J. Virol. 51:123-130. 1984.

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

9. Bai, J., L. N. Payne, and M. A. Skinner. HPRS-103 (exogenous avian leukosis virus, subgroup J) has an env gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. J. Virol. 69:779-784. 1995.

10. Balliet, J. W., K. Gendron, and P. Bates. Mutational analysis of the subgroup A avian sarcoma and leukosis virus putative fusion peptide domain. J. Virol. 74:3731-3739. 2000.

11. Benson, S. J., B. L. Ruis, A. M. Fadly, and K. F. Conklin. The unique envelope gene of the subgroup J avian leukosis virus derives from ev/J proviruses, a novel family of avian endogenous viruses. J. Virol. 72:10157-10164. 1998.

12. Bister, K., C. Loliger, and P. H. Duesberg. Oligoribonucleotide map and protein of CMII: detection of conserved and nonconserved genetic elements in avian acute leukemia viruses CMII, MC29 and MH2. J. Virol. 32:208-219. 1979.

13. Bister, K., G. Ramsay, M. J. Hayman, and P. H. Duesberg. OK10, an avian acute leukemia virus of the MC 29 subgroup with a unique genetic structure. Proc. Natl. Acad. Sci. U S A. 77:7142-7146. 1980.

14. Bittner, J. J. Some possible effects of nursing on the mammary gland tumour incidence in mice. Science. 84:162. 1936.

15. Boerger, A. L., S. Snitkovsky, and J. A. Young. Retroviral vectors preloaded with a viral receptor-ligand bridge protein are targeted to specific cell types. Proc. Natl. Acad. Sci. U S A. 96:9867-9872. 1999.

16. Boulden, A., and L. Sealy. Identification of a third protein factor which binds to the Rous sarcoma virus LTR enhancer: possible homology with the serum response factor. Virology. 174:204-216. 1990.

17. Bowzard, J. B., J. W. Wills, and R. C. Craven. Second-site suppressors of Rous sarcoma virus Ca mutations: evidence for interdomain interactions. J. Virol. 75:6850-6856. 2001.

18. Boyce-Jacino, M. T., K. O'Donoghue, and A. J. Faras. Multiple complex families of endogenous retroviruses are highly conserved in the genus Gallus. J. Virol. 66:4919-4929. 1992.

19. Burmester, B. R., and H. G. Purchase. The history of avian medicine in the United States. V. Insights into avian tumor virus research. Avian Dis. 23:1-29. 1979.

20. Calnek, B. W. Lymphoid leukosis virus: a survey of commercial breeding flocks for genetic resistance and incidence of embryo infection. Avian Dis. 12:104-111. 1968.

21. Calnek, B. W. Lesions in young chickens infected by lymphoid leukosis virus. Avian Dis. 12:104-111. 1968.

22. Caparini, U. Fetati leucemici nei polli. Clin. Vet. (Milan). 19:433-435. 1896.

23. Castillia, C. S., and E. Hunter. Mutational analysis of the major homology region of mason-pfizer monkey virus by use of saturation mutagenesis. J. Virol. 66:7021-7032. 1992.

24. Chen, C., B. J. Biegalke, R. N. Eisenman, and M. L. Linial. FH3, a v-myc avian retrovirus with limited transforming ability. J. Virol. 63:5092-5100. 1989.

25. Chesters, P. M., K. Howes, J. C. McKay, L. N. Payne, and K. Venugopal. Acutely transforming avian leukosis virus subgroup J strain 966: defective genome encodes a 72-kilodalton gag-myc fusion protein. J. Virol. 75:4219-4225. 2001.

26. Chiu, R. Avian retrovirus DNA internal attachment site requirements for full site integration in vitor. J. Virol. 74:8292-8298. 2000.

27. Coffin, J. Retroviridae: The Viruses and Their Replication. In: Fundamental Virology, B. Fields, D. Knipe, and P. Howley, eds. Lippincott-Raven, Philadelphia. pp. 763-843. 1995.

28. Coffin, J. M. Retroviridae and their replication. In: Virology, 3rd ed. B. N. Fields, D. M. Knipe, and P. M. Howley, eds. Lippincott-Raven, Philadelphia. pp. 1767-1848. 1996.

29. Coonrod, S. A., L. C. Bolling, P. W. Wright, P. E. Visconti, and J. C. Herr. A morpholino phenocopy of the mouse mos mutation. Genesis. 30. 2001.

30. Corey, D. R., and J. M. Abrams. Morpholino antisense oligonucleotides: tools for investigating vertebrate development. Genome Biol. 2:REVIEWS1015. 2001.

31. Crittenden, L. B., D. A. Eagen, and F. A. Gulvas. Assays for endogenous and exogenous lymphoid leukosis viruses and chick helper factor with RSV(--) cell lines. Infect Immun. 24:379-386. 1979.

32. Crittenden, L. B. Exogenous and endogenous leukosis virus gene - a review. Avian Pathol. 10:101-112. 1981.

33. Crittenden, L. B. Genes, viruses and avian leukosis. Bioscience. 31. 1981.

34. Crittenden, L. B., and H. J. Kung. Mechanism of induction of lymphoid leukosis and related neoplasms by avian leukosis viruses. In: Mechnaisms of Viral Leukaemogenesis, J. M. Goldman, and O. Jarrett, eds. Churchill-Livingston, Edinburgh, Scotland. pp. 64-88. 1984.

35. Crittenden, L. B., E. J. Smith, and A. M. Fadly. Influence of endogenous viral (ev) gene expression and strain of exogenous avian leukosis virus (ALV) on mortality and ALV infection and shedding in chickens. Avian Dis. 28:1037-1056. 1984.

36. Crittenden, L. B. Retroviral elements in the genome of the chicken: Implicatons for poultry genetics and breeding. Crit. Rev. Poultry Biology. 3:73-109. 1991.

37. Domingo, E. Quasispecies and the implications for virus persistence and escape. Clin. Diagn. Virol. 10:97-101. 1998.

38. Duarte, E. A., I. S. Novella, S. C. Weaver, E. Domingo, S. Wain-Hobson, D. K. Clarke, A. Moya, S. F. Elena, J. C. de la Torre, and J. J. Holland. RNA virus quasispecies: significance for viral disease and epidemiology. Infect. Agents Dis. 3:201-214. 1994.

39. Dunn, K. J., B. O. Williams, Y. Li, and W. J. Pavan. Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. Proc. Natl. Acad. Sci. U S A. 97:10050-10055. 2000.

40. Dunwiddie, C. T., R. Resnick, M. Boyce-Jacino, J. N. Alegre, and A. J. Faras. Molecular cloning and characterization of gag-, pol-, and env-related gene sequences in the ev- chicken. J. Virol. 59:669-675. 1986.

41. Dutta, A., M. Y. Stoeckle, and H. Hanafusa. Serum and v-src increase the level of a CCAAT-binding factor required for transcription from a retroviral long terminal repeat. Genes Dev. 4:243-254. 1990.

42. Ekker, S. C., and J. D. Larson. Morphant technology in model development systems. Genesis. 30:89-93. 2001.

43. El-Attrache, J. Comparative analysis of the EV-O-derived cell line DF-1 and line-0, C/E chicken embryo fibroblast for the isolation and detection of subgroup J avian leukosis virus. 137th Annual Meeting of the American Veterinary Medical Association. AVMA, Salt Lake City, UT. pp. 716. 2000.

44. El-Attrache, J., P. Villegas, and M. Garcia. Molecular and classical analysis of nested RT-PCR ALV-J positive plasma samples undetected by virus isolation. 138th AVMA Annual Convention. Veterinary Software Publishing, Inc., Boston, MA2001.

45. Ellermann, V., and O. Bang. Experimentelle Leukamie bei Huhnern. Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. Orig. 46:595-609. 1908.

46. Fadly, A., and R. Witter. Oncornaviruses: Leukosis/ Sarcoma and Reticuloendotheliosis. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 185-196. 1998.

47. Fadly, A. M., W. Okazaki, and L. B. Crittenden. Avian leukosis virus infection and congenital transmission in lines of chickens resisting selection for reduced shedding. Avian Dis. 27:584-593. 1983.

48. Fadly, A. M. Leukosis and Sarcomas. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, H. G. Purchase, L. H. Harp, and C. H. Domermuth, eds., vol. 1. American Association of Avian Pathologists, Kennett Square. pp. 135-142. 1989.

49. Fadly, A. M. Avian Retroviruses. Vet Clin North Am Food Anim. Pract. 13:71-85. 1997.

50. Fadly, A. M., and E. J. Smith. An overview of subgroup J-like avian leukosis virus infection in broiler breeder flocks in the united states. In: Avian Tumor Viruses Symposium. American Association of Avian Pathologists, Reno, Nevada. pp. 54-57. 1997.

51. Fadly, A. M., and E. J. Smith. Isolation and some characteristics of a subgroup J-like avian leukosis virus associated with myeloid leukosis in meat-type chickens in the United States. Avian Dis. 43:391-400. 1999.

52. Fadly, A. M. Isolation and identification of avian leukosis viruses: a review. Avian Pathol. 29:529-535. 2000.

53. Flint, S. J., L. W. Enquist, R. M. Krug, V. R. Racaniello, and A. M. Skalka. Reverse transcription and intergration: Hallmarks of the Retroid Viruses. In: Virology, A. S. M. Microbiology, ed. ASM Press, Washington. pp. 198-233. 2000.

54. Fung, Y. K. T., W. G. Lewis, L. B. Crittenden, and H. J. Kung. Activation of the cellular oncogene c-erb by LTR insertion: Molecular basis of induction of erythroblastosis by avian leukosis virus. Cell. 33:357-368. 1983.

55. Garcia, M., J. El-Attrache, N. Ikuta, V. R. Lunge, A. S. K. Fonseca, S. M. Riblet, and P. Villegas. Current advances in the diagnosis of avian leukosis virus by the polymerase chain reaction. International Symposium on ALV-J and other Avian Retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 234-247. 2000.

56. Ghosh, C., and P. L. Iversen. Intracellular delivery strategies for antisense phosphorodiamidate morpholino oligomers. Anti. Nucleic Acid Drug Dev. 10:263-274. 2000.

57. Golde, A. Characteristics of infection by RSV. III. Duration of the latent period in unsynchronized chick embryo fibroblasts. Intervirol. 5:185-193. 1975.

58. Goodchild, J., and P. Iversen. Editorial overview: Oligonucleotides - from design to the clinic. Curr. Opin. Mole. Therap. 3:223. 2001.

59. Goodenow, M. W., and W. S. Hayward. Long terminal repeats of myc-associated proviruses appear structurally intact but are functionally impaired in tumors induced by avian leukosis virus. J. Virol. 61:2489-2498. 1987.

60. Gross, L. Spontaneous leukemia developing in C3H mice following inoculaion in infancy, with Ak-leukemic extracts, or AK-embryos. Proc. Soc. Exp. Biol. Med. 76. 1951.

61. Gudkov, A. V., E. A. Komarova, M. A. Nikiforov, and T. E. Zaitsevskaya. ART-CH, a new chicken retroviruslike element. J. Virol. 66:1726-1736. 1992.

62. Hafner, S., M. A. Goodwin, E. J. Smith, A. Fadly, and L. C. Kelley. Pulmonary sarcomas in a young chicken. Avian Dis. 42:824-828. 1998.

63. Hara, H., and A. Kaji. The U3 region of the long terminal repeat of a subgroup A transformation-defective rous sarcoma virus (tdPH2010) converts a noncytopathic virus to a cytopathic virus. Virus Genes. 15:171-180. 1997.

64. Hara, H., and A. Kaji. Two point mutations in the U3 region of the long terminal repeat convert a subgroup A transformation-defective rous sarcoma virus to a cytopathic virus. Folia Biol. 45:39-46. 1999.

65. Hejnar, J., J. Plachy, J. Geryk, O. Machon, K. Trejbalova, R. V. Guntaka, and J. Svoboda. Inhibition of the Rous sarcoma virus long terminal repeat driven transcription by in Vitor methylation: Different sensitivity in permissive chicken cells versus mammalian cells. Virology. 255. 1999.

66. Herman, S. A., and J. M. Coffin. Efficient packaging of readthrough RNA in ALV: implications for oncogene transduction. Science. 236:845-848. 1987.

67. Himly, M., D. N. Foster, I. Bottoli, J. S. Iacovoni, and P. K. Vogt. The DF-1 chicken fibroblast cell line: Transformation induced by diverse oncogenses and cell death resulting from infetion by avian leukosis viruses. Virology. 248:295-304. 1998.

68. Hindmarsh, P., M. Johnson, R.Reeves, and J. Leis. Base-pair substitutions in avian sarcoma virus U5 and U3 long terminal repeate sequences alter the process of DNA integration in vitro. J. Virol. 75:1132-1141. 2001.

69. Ho, S. P. Mapping of RNa accessible sites for antisense experiments with oligonuclotide libraries. National Biotech. 16:59-63. 1998.

70. Hu, Q. X., A. P. Barry, Z. X. Wang, S. M. Connolly, S. C. Peiper, and M. L. Greenberg. Evolution of the human immunodeficiency virus type 1 envelope during infection reveals molecualr corollaries of specificity fo coreceptor utilization and AIDS pathogenesis. J. Virol. 74:11858-11872. 2000.

71. Hudziak, R. M., J. Summerton, D. D. Weller, and P. L. Iversen. Antiproliferative effects of steric blocking phosphorodiamidate morpholino antisense agents directed against c-myc. Anti. Nucleic Acid Drug Dev. 10:163-176. 2000.

72. Hughes, M. D., M. Hussain, Q. Nawaz, P. Sayyed, and S. Akhtar. The cellular delivery of antisense oligonucleotides and ribozymes. Drug Disc. Today. 6:303-315. 2001.

73. Hunt, H. D., L. F. Lee, D. Foster, R. F. Silva, and A. M. Fadly. A genetically engineered cell line resistant to subgroup J avian leukosis virus infection (C/J). Virology. 264:205-210. 1999.

74. Hunt, L. A., and S. E. Wright. Comparison of the oligosaccharide moieties of the major envelope glycoproteins of the subgroup A and subgroup B avian myeloblastosis- associated viruses. J. Virol. 45:233-240. 1983.

75. Ishizaki, R., A. J. Langlois, and D. P. Bolognesi. Isolation of two subgroup-specific leukemogenic viruses from standard avian myeloblastosis virus. J. Virol. 15:906-912. 1975.

76. Iversen, P. L. Phosphorodiamidate morpholino oligomers: Favorable properties for sequence-specific gene inactivation. Curr. Opin. Mol. Therap. 3:235-238. 2001.

77. Jirong, B., and L. N. Payne. HPRS-103 (Exogenous avian leukosis virus, subgroup J) has an env gene related to those of endogenoous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. J. Virol. 69:779-784. 1994.

78. Jubin, R., N. E. Vantuno, J. S. Kieft, M. G. Murray, J. A. Doudna, J. Y. Lau, and B. M. Baroudy. Hepatitis C virus internal ribosome entry site (IRES) stem loop IIId contains a phylogenetically conserved GGG triplet essential for translation and IRES folding. J. Virol. 74:10430-10437. 2000.

79. Jubin, R. Hepatitis C IRES: Translating translation into a therapeutic target. Curr. Opin. Mole. Therap. 3:278-287. 2001.

80. Karakoz, I., J. Geryk, and J. Svoboda. In vivo effect of three transformation-defective mutants of subgroup C avian sarcoma viruses. Folia Biol. 26:62-69. 1980.

81. Kawai, S., M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki, and K. Toyoshima. Characterization of Y73, an avian sarcoma virus: a unique transforming gene and its product, a phosphopolyprotein with protein kinase activity. Proc. Natl. Acad. Sci. U S A. 77:6199-6203. 1980.

82. Kim, K. E., D. W. Salter, and J. B. Dodgson. Examination of antisense RNA and oligodeoxynucleotides as potential inhibitors of avian leukosis virus replication. Poult. Sci. 77:1400-1410. 1998.

83. Kipshidze, N., J. Moses, L. R. Shankar, and M. Leon. Perspectives on antisense therapy for the prevention of restenosis. Curr.Opin. Mol.Ther. 3:265-277. 2001.

84. Kipshidze, N., J. Moses, L. R. Shankar, and M. Leon. Perspectives on antisense therapy for the prevention of restenosis. Curr. Opin. Mol. Ther. 3:265-277. 2001.

85. Klatzman, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier. T-Lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature. 312:767-768. 1984.

86. Klein, J. S., I. Givol, E. V. Barsov, J. M. Whitcomb, M. VanBrocklin, D. N. Foster, M. J. Federspiel, and S. H. Hughes. The EV-0 derived cell line DF-1supports the efficient replication of avian leukosis sarcoma viruses and vectors. Virology. 248:305-311. 1998.

87. Kole, R., and P. Sazani. Antisense effects in the cell nucleus: Modification of splicing. Curr. Opin. Mol. Ther.. 3:229-234. 2001.

88. Kos, R., M. V. Reedy, R. L. Johnson, and C. A. Erickson. The winged helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development. 128:1467-1479. 2001.

89. Kuhnlein, U., R. W. Fairfull, R. Gowe, A. Kulenkamp, L. Mou, and D. Zadworny. Synergism between the endogenous viral loci ev6 and ev9 in inducing immunological tolerance to avian leukosis virus. Br. Poult. Sci. 34:93-104. 1993.

90. Kung, H. J., Y. K. Fung, L. C. Crittenden, A. Fadly, and S. K. Dube. The molecular basis of avian retrovirus-induced leukemogenesis. Hamatol Bluttransfus. 26:445-451. 1981.

91. Leis, J. Reverse Transcriptase. In: Reverse Transcriptase, A. M. Skalka, and S. P. Goff, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 33-47. 1993.

92. Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. 61:693-700. 1987.

93. Lupiani, B., H. Hunt, R. Silva, and A. Fadly. Identification and characterization of recombinant subgroup J avian leukosis viruses (ALV) expressing subgroup A ALV envelope [In Process Citation]. Virology. 276:37-43. 2000.

94. McGinty, J., and D. Jones. Oligonucleotides - from design to the clinic. Curr. Opin. Mol. Ther. 3:221-222. 2001.

95. Morcos, P. A. Achieving efficient delivery of morpholino oligos in cultured cells. Genesis. 30:94-102. 2001.

96. Moustakas, A., T. S. Sonstegard, and P. B. Hackett. Alterations of the three short open reading frames in the rous sarcoma virus leader RNA modulate viral replication and gene expression. J. Virol. 67:4337-4349. 1993.

97. Moustakas, A., T. S. Sonstegard, and P. B. Hackett. Effects of the open reading frames in the rous sarcoma virus leader RNA on translation. J. Virol. 67:4350-4357. 1993.

98. Nikiforov, M. A., and A. V. Gudkov. ART-CH: A VL30 in chickens? Journal of Virology. 68:846-853. 1994.

99. Ohi, Y., and J. L. Clever. Sequences in the 5' and 3' R elements of human immunodeficiency virus type 1 critical for efficient reverse transcription. J. Virol. 74:8324-8334. 2000.

100. Okazaki, W., H. G. Purchase, and B. R. Burmester. Phenotypic mixing test to detect and assay avian leukosis viruses. Avian Dis. 19:311-317. 1975.

101. Orr, R. M. Technology evaluation: Farmivirsen, Isis Pahrmaceuticals Inc/CIBA Vision. Curr. Opin. Mol.r Ther. 3:288-294. 2001.

102. Park, W. S., N. M. Kurosaki, T. Abe, K. Takai, N. Yamamoto, and H. Takaku. Inhibition of HIV-1 replication by a new type of circular dumbbell RNA/DNA chimeric oligonucleotides. Biochemical and Biophysical Research Communications. 270:953-960. 2000.

103. Partridge, M., A. Vincent, P. Matthews, J. Puma, D. Stein, and J. Summerton. A simple method for delivering morpholino antisense oligos into the cytoplasm of cells. Anti. Nucleic Acid Drug Dev. 6:169-175. 1996.

104. Pathak, V. K., and H. M. Temin. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc. Natl. Acad. Sci. U S A. 87:6019-6023. 1990.

105. Pathak, V. K., and H. M. Temin. 5-Azacytidine and RNA secondary structure increase the retrovirus mutation rate. J. Virol. 66:3093-3100. 1992.

106. Payne, L. N., S. R. Brown, N. Bumstead, K. Howes, J. A. Frazier, and M. E. Thouless. A novel subgroup of exogenous avian leukosis virus in chickens. J. Gen. Virol. 72:801-807. 1991.

107. Payne, L. N. Developments in avian leukosis research. Leukemia. 6:150S-152S. 1992.

108. Payne, L. N. Myeloid leukemogenicity and transmission of a new strain of avian leukosis virus. New and evolving virus disease of poultry, Belfast. pp. 311-325. 1992.

109. Payne, L. N., A. M. Gillespie, and K. Howes. Myeloid leukaemogenicity and transmission of the HPRS-103 strain of avian leukosis virus. Leukemia. 6:1167-1176. 1992.

110. Payne, L. N., K. Howes, A. M. Gillespie, and L. M. Smith. Host range of Rous sarcoma virus pseudotype RSV(HPRS-103) in 12 avian species: support for a new avian retrovirus envelope subgroup, designated J. J. Gen. Virol. 73:2995-2997. 1992.

111. Payne, L. N., A. M. Gillespie, and K. Howes. Recovery of acutely transforming viruses from myeloid leukosis induced by the HPRS-103 strain of avian leukosis virus. Avian Dis. 37:438-450. 1993.

112. Payne, L. N., A. M. Gillespie, and K. Howes. Unsuitability of chicken sera for detection of exogenous ALV by the group-specific antigen ELISA. Vet. Rec. 132:555-557. 1993.

113. Payne, L. N., and A. M. Fadly. Leukosis/Sarcoma Group. In: Diseases of Poultry, 10th ed. B. Calnek, J. Barnes, C. Beard, L. McDougald, and M. Saif, eds. Iowa State University Press, Ames, Iowa. pp. 414-466. 1997.

114. Payne, L. N., K. Howes, L. M. Smith, and K. Venugopal. Current status of diagnosis, epidemiology and control of ALV-J. In: Diagnosis and Control of Neoplastic Diseases of Poultry, A. M. Fadly, ed. American Association of Avian Patholgists, Kennet Square1997.

115. Payne, L. N. Retrovirus-induced disease in poultry. Poult. Sci. 77:1204-1212. 1998.

116. Payne, L. N. HPRS-103: a retrovirus strikes back. The emergence of subgroup J avian leukosis virus. Avian Pathol. 27:S36-S45. 1998.

117. Payne, L. N. History of ALV-J. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 3-12. 2000.

118. Pepinsky, R. B., I. A. Papayannopoulos, E. P. Chow, N. K. Krishna, R. C. Craven, and V. M. Vogt. Differential proteolytic processing leads to multiple forms of the CA protein in avian sarcoma and leukemia viruses. J. Virol. 69:6430-6438. 1995.

119. Pham, T. D., J. L. Spencer, and E. S. Johnson. Detection of avian leukosis virus in albumen of chicken eggs using reverse transcription polymerase chain reaction. J. Virol. Meth. 78:1-11. 1999.

120. Pham, T. D., J. L. Spencer, V. L. Traina-Dorge, D. A. Mullin, R. F. Garry, and E. S. Johnson. Detection of exogenous and endogenous avian leukosis virus

in commercial chicken eggs using reverse transcription and polymerase chain reaction assay. Avian Pathol. 28:385-391. 1999.

121. Pizer, E., and E. H. Humphries. RAV-1 insertional mutagenesis: disruption of the c-myb locus and development of avian B-cell lymphomas. J. Virol. 63:1630-1640. 1989.

122. Pizer, E. S., T. W. Baba, and E. H. Humphries. Activation of the c-myb locus is insufficient for the rapid induction of disseminated avian B-cell lymphoma. J. Virol. 66:512-523. 1992.

123. Qin, G., M. Taylor, Y. Y. Ning, P. Iversen, and L. Kobzik. In vivo evaluation of a morpholino antisense oligomer directed against tumor necrosis factor-alpha. Antisense Nucleic Acid Drug Dev. 10:11-16. 2000.

124. Resnick, R. M., M. T. Boyce-Jacino, Q. Fu, and A. J. Faras. Phylogenetic distribution of the novel avian endogenous provirus family EAV-0. J. Virol. 64:4640-4653. 1990.

125. Robinson, H. L., S. M. Astrin, A. M. Senior, and F. H. Salazar. Host Susceptibility to endogenous viruses: defective, glycoprotein- expressing proviruses interfere with infections. J. Virol. 40:745-751. 1981.

126. Robinson, H. L., and G. C. Gagnon. Patterns of proviral insertion and deletion in avian leukosis virus- induced lymphomas. J. Virol. 57:28-36. 1986.

127. Roloff, F. Mag Ges Thierheilkd.34:190. 1868.

128. Rous, P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. 13:397-411. 1911.

129. Rout, P. K., P. K. Pani, and S. Naithani. Genetic control of cellular infection by subgroups A and C RNA tumour viruses in guinea fowl. Vet. Res. Commun. 17:153-161. 1993.

130. Rovigatti, U. G., and S. M. Astrin. Avian endogenous viral genes. Curr Top Microbiol. Immunol. 103:1-21. 1983.

131. Rubin, H., A. Cornelius, L. Fanshier, A. Cornelius, and W. F. Hughes. Tolerance and immunity in chickens after congenital and contact infection with an avian leukosis virus. Virology. 17:143-156. 1962.

132. Ruddell, A. Transcription regulatory elements of the avian retroviral long terminal repeat. Virology. 206:1-7. 1995.

133. Ruis, B. L., S. J. Benson, and K. F. Conklin. Genome structure and expression of the ev/J family of avian endogenous viruses. J. Virol. 73:5345-5355. 1999.

134. Russell, R. A., Y. Zeng, O. Erlwein, B. R. Cullen, and M. O. McClure. The R region found in the human foamy virus long terminal repeat is critical for both Gag and Pol protein expression. Virology. 75:6817-6824. 2001.

135. Sacco, M. A., D. M. Flannery, K. Howes, and K. Venugopal. Avian endogenous retrovirus EAV-HP shares regions of identity with avian leukosis virus subgroup J and the avian retrotransposon ART-CH. J. Virol. 74:1296-1306. 2000.

136. Salter, D. W., E. J. Smith, S. H. Hughes, S. E. Wright, A. M. Fadly, R. L. Witter, and L. B. Crittenden. Gene insertion into the chicken germ line by retroviruses. Poult. Sci. 65:1445-1458. 1986.

137. Sarma, P. S., T. S. Log, R. J. Huebner, and H. C. Turner. Studies of avian leukosis group-specific complement-fixing serum antibodies in pigeons. Virology. 37:480-483. 1969.

138. Satou, Y., K. S. Imai, and N. Satoh. Action of morpholinos in Ciona embryos. Genesis. 30:103-106. 2001.

139. Scherr, M., and J. J. Rossi. Rapid determination and quantitation of the accessibility to native RNAs by antisense oligonucleotides in murine cell extracts. Nucleic Acids Res. 26:5079-5085. 1998.

140. Schmajuk, G., H. Sierakowska, and R. Kole. Antisense oligonucleotides with different backbones. Modification of splicing pathways and efficacy of uptake. J. Biol. Chem. 274:21783-21789. 1999.

141. Short, M. K., S. A. Okenquist, and J. Lenz. Correlation of leukemogenic potential of murine retroviruses with transcriptional tissue preference of the viral long terminal repeats. J. Virol. 61:1067-1072. 1987.

142. Sigurdsson, B. Observations on three slow infections of sheep. Maedi paratuberculosis, rida, a slow encephalitis of sheep with general remarks on infections which develop slowly and some of their special characteristics. Br. Vet. J. 110:255-270. 1954.

143. Silva, R. F., and E. J. Smith. PCR as a tool for the diagnosis of avian tumor viruses and tumors. Avian Tumor Virus Symposium. American Association of Avian Pathologists, Reno, Nevada. pp. 19-20. 1997.

144. Silva, R. F., A. M. Fadly, and H. D. Hunt. Hypervariability in the envelope genes of subgroup J avian leukosis viruses obtained from different farms in the United States. Virology. 272:106-111. 2000.

145. Smith, E. J., and D. W. Salteri. Selective shedding and congenital transmission of endogenous avian leukosis viruses. J. Virol. 1050-1054. 1986.

146. Smith, E. J., A. M. Fadly, and L. B. Crittenden. Interactions between endogenous virus loci ev6 and ev21. 2. Congenital transmission of EV21 viral product to female progency from slow- feathering dams. Poult. Sci. 69:1251-1256. 1990.

147. Smith, E. J., A. M. Fadly, and L. B. Crittenden. Interactions between endogenous virus loci ev6 and ev21. 1. Immune response to exogenous avian leukosis virus infection. Poult. Sci. 69:1244-1250. 1990.

148. Smith, E. J., S. M. Williams, and A. M. Fadly. Detection of avian leukosis virus subgroup J using the polymerase chain reaction. Avian Dis. 42:375-380. 1998.

149. Smith, L. M., S. R. Brown, K. Howes, S. McLeod, S. S. Arshad, G. S. Barron, K. Venugopal, J. C. McKay, and L. N. Payne. Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. Virus Res. 54:87-98. 1998.

150. Smith, L. M., K. Howes, S. S. Arshad, G. S. Barron, K. Venugopal, and L. N. Payne. Development and application of PCR tests for the detection of subgroup J avian leukosis virus. Avian Pathol. s90. 1998.

151. Smith, L. M., A. A. Toye, K. Howes, N. Bumstead, L. N. Payne, and K. Venugopal. Novel endogenous retroviral sequences in the chicken genome closely related to HPRS-103 (subgroup J) avian leukosis virus. J. Gen. Virol. 80:261-268. 1999.

152. Snitkovsky, S., and J. A. Young. Cell-specific viral targeting mediated by a soluble retroviral receptor- ligand fusion protein. Proc. Natl. Acad. Sci. U S A. 95:7063-7068. 1998.

153. Spencer, J. Progress towards eradication of lymphoid leukosis viruses - a review. Avian Pathol. 13:599-619. 1984.

154. Stedman, N., T. Brown, and C. Brown. Localization of avian leukosis virus subgroup J in naturally infected chickens by RNA *in situ* hybridization. Vet. Pathol. In press. 2001.

155. Stein, D., E. Foster, S. B. Huang, D. Weller, and J. Summerton. A specificity comparison of four antisense types: morpholino, 2'-O-methyl RNA, DNA, and phosphorothioate DNA. Anti. Nucleic Acid Drug Dev. 7:151-157. 1997.

156. Summerton, J., D. Stein, S. B. Huang, P. Matthews, D. Weller, and M. Partridge. Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. Anti.Nucleic Acid Drug Dev. 7:63-70. 1997.

157. Summerton, J., and D. Weller. Morpholino antisense oligomers: design, preparation, and properties. Anti. Nucleic Acid Drug Dev. 7:187-195. 1997.

158. Summerton, J. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta. 1489:141-158. 1999.

159. Taplitz, R. A., and J. M. Coffin. Selection of an avian retrovirus mutant with extended receptor usage. J. Virol. 71:7814-7819. 1997.

160. Temin, H. M., and V. K. Kassner. Avian leukosis viruses of different subgroups and types isolated after passage of Rous sarcoma virus-Rous-associated virus-0 in cells from different ring-necked pheasant embryos. J. Virol. 19:302-312. 1976.

161. Tsichlis, P. N., and J. M. Coffin. Recombinants between endogenous and exogenous avian tumor viruses: role of the C region and other portions of the genome in the control of replication and transformation. J. Virol. 33:238-249. 1980.

162. Tsukamoto, K., Y. Kono, K. Arai, H. Kitahara, and K. Takahashi. An enzyme-linked immunosorbent assay for detection of antibodies to exogenous avian leukosis virus. Avian Dis. 29:1118-1129. 1985.

163. Tsukamoto, K., H. Hihara, and Y. Kono. Detection of avian leukosis virus antigens by the ELISA and its use for detecting infectious virus after cultivation of samples and partial characterization of specific pathogen-free chicken lines maintained in this laboratory. J. Vet. Med. Sci. 53:399-408. 1991.

164. Vallee, H., and H. Carre. Sur la nature infectieuse de l anemie du cheval. C.R. Hebd. Seances. Acad. Sci. Ser. D Sci. Nat. 139:331-333. 1904.

165. Vandergon, T. L., and M. Reitman. Evolution of chicken repeat 1 (CR1) elements: evidence for ancient subfamiliesand multiple progenitors. Mol. Biol. Evol. 11:886-898. 1984.

166. Venugopal, K., K. Howes, G. S. Barron, and L. N. Payne. Recombinant env-gp85 of HPRS-103 (subgroup J) avian leukosis virus: antigenic characteristics and usefulness as a diagnostic reagent. Avian Dis. 41:283-288. 1997.

167. Venugopal, K., L. M. Smith, K. Howes, and L. N. Payne. Antigenic variants of J subgroup avian leukosis virus: sequence analysis reveals multiple changes in the env gene. J. Gen. Virol. 79:757-766. 1998.

168. Venugopal, K., K. Howes, D. M. Flannery, and L. N. Payne. Isolation of acutely transforming subgroup J avian leukosis viruses that induce erythroblastosis and myelocytomatosis. Avian Pathol. 29:327-332. 2000.

169. Vogt, P. K. Historical Introduction to the General Properties of Retorviruses. In: Retroviruses, J. M. Coffin, S. H. Hughes, and H. E. Varmus, eds. cold Spring Harbor Laboratory Press, Plainview, NY. pp. 1-26. 1997.

170. Vogt, V. M. Retroviral Virions and Genomes. In: Retroviruses, J. Coffin, S. Hughes, and H. Varmus, eds. Cold Spring Harbor Laboratory Press, New York. pp. 27-71. 1997.

171. Weiss, R. A., N. Teich, H. Varmus, and J. Coffin 1982. RNA Tumor Viruses, 2nd ed. Cold Spring Harbor, New York.

172. Whitcomb, J. M., B. A. Ortiz-Conde, and S. H. Hughes. Replication of avian leukosis viruses with mutations at the primer binding site: use of alternative tRNA as primers. J. Virol. 69:6228-6238. 1995.

173. Willems, L., P. Kerkhofs, L. Attenelle, A. Burny, D. Protetelle, and R. Kettmann. The major homology region of bovine leukemia virus p24 gag is required for virus infectivity in vivo. J. Gen. Virol. 78:637-640. 1997.

174. Witter, R. L. Avian tumor viruses: persistent and evolving pathogens. Acta. Vet. Hung. 45:251-266. 1997.

175. Witter, R. L., L. D. Bacon, H. D. Hunt, R. E. Silva, and A. M. Fadly. Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. Avian Dis. 44:913-931. 2000.

176. Woensel, V., and A. V. Blaaderen. Detection of proviral DNA and viral RNA in various tissues early after avian leukosis virus infection. Leukemia. 6:135S-137S. 1992.

177. Xiang, Y., R. Thorick, M. L. Vana, R. Craven, and J. Leis. Proper processing of avian sarcoma/leukosis virus capsid proteins is required for infectivity. J. Virol. 75:6016-6021. 2001.

178. Young, J. A., P. Bates, and H. E. Varmus. Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. 67:1811-1816. 1993.

179. Zamecnik, P. C., and M. L. Stephenson. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. U S A. 75:280-284. 1978.

180. Zavala, G. Update on myeloid leukosis in meat-type chickens. Committe on transmissible diseases of poultry and other avian species, Louisville, Kentucky1997.

181. Zavala, G. Thesis. Department of Medical Microbiology. University of Georgia. 2001.

182. Zheng, Y. H., H. Sentsui, M. Sugita, T. Nakaya, M. Kishi, K. Hagiwara, Y. Inoshima, C. Ishihara, Y. Kono, J. L. Lu, and K. Ikuta. Replication ability in vitro and in vivo of equine infectious anemia virus avirulent Japanese strain. Virology. 266:129-139. 2000.

CHAPTER III

COMPARATIVE ANALYSIS OF THE EV-O DERIVED CELL LINE DF-1 AND LINE-0, C/E CHICKEN EMBRYO FIBROBLASTS FOR THE ISOLATION AND DETECTION OF AVIAN LEUKOSIS VIRUS SUBGROUP J[1]

[1] El-Attrache, J., P. Villegas, M. Garcia, N. Ikuta and Alejandro Banda. To be submitted to Avian Diseases.

SUMMARY: Plasma samples were obtained from three different primary breeder pedigree flocks and one grandparent flock at various ages from flocks diagnosed with subgroup J avian leukosis virus (ALV-J). The plasma samples were uniformly divided and used as inoculum for paired viral propagation in the DF-1 cell line and in C/E chicken embryo fibroblasts (CEF). Antigen capture enzyme linked immunosorbent assay (ac-ELISA) and ALV-J specific nested reverse transcriptase-polymerase chain reaction (RT-PCR) were used to detect and confirm ALV-J isolation. The permissibility of the DF-1 cell line and C/E secondary CEF to propagate and subsequently detect ALV-J by ac-ELISA was similar when expressed in terms of percent positive and, although not significantly different (Chi-square test, $p > 0.05$) virus propagation in the DF-1 cell line allowed for the identification of more positive plasma samples at both S/P ratios of 0.2 and 0.1. However, when nested RT-PCR was used as a confirmation assay for the detection of ALV-J in one of the pure-line pedigree flocks, a 20% - 25% loss of sensitivity and significant difference at a 0.2 S/P ratio ($p < 0.05$) was observed for ALV-J detection for DF-1 viral propagation followed by ac-ELISA.

INTRODUCTION

Avian leukosis viruses (ALVs) are type C retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis, erythroblastosis, myeloblastosis nephromas and / or nephroblastomas and other related connective tissue, epithelial endothelial tumors (16). ALVs that infect chickens are readily classified into six groups (A, B, C, D, E and J) based upon the properties of their viral envelope (14, 16). Exogenous ALVs consist of all of the above subgroups with the exception of subgroup E. These exogenous viruses contain no oncogenes, are not defective in replication, and are prevalent in the poultry industry worldwide (5). Historically, subgroups A and B occur as the most common exogenous viruses in the field, are spread vertically and horizontally, are oncogenic and cause principally lymphoid leukosis (LL) (2, 16, 20). However, most recently ALV-J, which was first isolated from meat type chickens (13, 15), has become the most prevalent subgroup inducing myeloid leukosis (ML) tumors in meat-type chickens (5, 9).

Current methods for detection of ALVs in chickens include detection of viral group-specific antigen (gs) p27 by the antigen capture (ac)-ELISA test (20). Monoclonal antibodies against ALV have been developed and are also being used in testing samples for the presence of ALV p27 (1, 5). This test is not specific for exogenous virus and will detect gs antigens of endogenous virus in serum, plasma, albumen and meconium. Since p27 is shared by endogenous and exogenous ALV, direct assays cannot be used in identification of isolated ALV. In order to increase the specificity of the assay, propagation of the virus in

different phenotypes of chicken embryo fibroblast (CEF) is required prior to the ac-ELISA test. The phenotype of CEFs used most commonly to isolate and detect ALV-J include C/O which are susceptible to infection from all exogenous and endogenous ALV subgroups, and C/E which are only susceptible to infection from exogenous ALV subgroups (3, 4, 5). Virus isolation and identification in cell culture is quite readily used by the primary breeder industry and is often referred to as the 'gold standard' for ALV isolation and identification.

Using stringent ALV-J screening procedures, which in the laboratory environment often involves virus isolation on C/E cells coupled with ac-ELISA along with more extensive screening and testing, has reduced the prevalence of ALV-J in the primary breeder industry (12, 17). These cell culture screening procedures continue today for most of the industry worldwide. The C/E phenotype chicken embryo fibroblasts (CEF) are available to the industry via embryonated eggs. Preparation of primary and secondary C/E CEF requires a minimal two days of preparation, extensive laboratory materials and is quite labor intensive.

A continuous fibroblastic cell line (DF-1) derived from the EV-O (endogenous virus free) line of chickens has been deposited into the ATCC depository. This is the first non-transformed spontaneously immortalized CEF continuous chicken cell line which does not have any endogenous viruses and can propagate ALSV (11). The immortal DF-1 cells divided more rapidly than primary and other immortal CEF cells and are easily maintained (7, 10, 11). Recent studies suggest that the transcriptional activation of mitochondrial-

encoded genes and the elevated respiratory function should be one of the characteristics of these rapidly dividing immortal cells (10). The ability to substitution of the C/E CEF virus isolation with DF-1 would considerably decrease preparation time, cost and labor. However, the capabilities of the DF-1 cell line to propagate field samples of ALV-J must be evaluated and compared to the C/E CEF standard.

MATERIALS AND METHODS

Cell culture. Line 0 C/E phenotype embryos were supplied by Kestrel Inc. (Waukee, IA) for the production of C/E primary and secondary CEF from 9-day old embryos. The EV-0 derived DF-1 cell line was obtained from the American Type Culture Collection ATCC repository (Manassas, VA).

Plasma samples. A total of 400 plasma samples were collected from various pure-line pedigree and grandparent flocks (Table 1). Whole blood was individually collected in 5 ml draw EDTA Vacutainer® brand tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma and residual buffy-coat was collected from the whole blood samples via centrifugation of the Vacutainer® tubes at 4 C for 10 min at 500 g. Each plasma sample was divided in two separate tubes and frozen at –80 C until used for virus isolation and nested reverse transcriptase-polymerase chain reaction (RT PCR).

Virus isolation and detection. Virus isolation procedures for both cell culture systems were followed as described by Fadly and Witter (4). Briefly, for line O

C/E CEFs, secondary cells were suspended in F-10/M199 media (Sigma Chemical, St. Louis, MO) containing 2 μg/ml of DEAE and 4 units/ml of heparin at a cell concentration of 3.5 x 10^5 cells/ml. Fibroblasts were transfer to 24-well tissue culture plates. Plates were incubated until confluent monolayers were observed (approx 16 to 18 hrs.). Fifty μl of each plasma sample was added to individual wells. After inoculation, the plates were gently rocked to promote viral absorption and incubated. Medium was changed 24 hours after an initial incubation and cultures were maintained in the incubator for a period of 7 days. The above-mentioned procedure was followed for virus isolation in DF-1 cells except that DF-1 cells were propagated in high glucose DMEM media (Sigma Chemical, St. Louis, MO) and incubated at 39 C.

Following incubation, 40μl of 5% Tween 80 solution was added to each well, plates were frozen and thawed three times and 100 μl material was collected for antigen capture-enzyme linked immunosorbent assay (ac-ELISA). Avian leukosis virus ac-ELISA assay readings were performed following manufacturers recommendations (IDEXX Laboratories, Westbrook, Maine) with the exception of using two cutoff values of 0.2 and 0.1 S/P ratios.

Endpoint dilution assay. A limited dilution assay was performed on five plasma samples from group D (Table 1) with relatively high S/P ratios (> 2.0). Ten fold dilutions were inoculated as previously described (4), and consisted of 10^3 to 10^8 dilutions with a total of five treatments per sample. An S/P ratio greater than 0.1

were considered positive and the formula devised by Reed and Muench determined the 50% endpoint (18, 21).

Nested RT-PCR. Nested RT-PCR which amplifies a 213 bp product in the 3' non-coding region was utilized for the confirmation of ALV-J detection via virus isolation coupled with ac-ELISA for group D (Table 1) and followed as described by Garcia *et al.* (6). Briefly, RNA was extracted from the plasma for each individual sample in group D following the procedures described for the Trizol LS reagent (Life Technologies, Gaithersburg, MD) where 100 μl of plasma was added to 750 μl of Trizol LS reagent, vortexed and incubated at room temperature for 5 min. Two hundred μl of chloroform was added, vortexed and centrifuged at 12,000 g for 15 min at 4 C. Supernatant was removed and the RNA pellet was washed with ethanol. The RNA pellet was allowed to air dry and was re-suspended in 25 μl of DEPC water.

Reverse transcriptase reaction was performed with 10 units of M-MLV of RT (Life Technologies, Rockville, MD) in MMLV 1x buffer for 1 hour at 37 C followed by 2 min. at 94 C followed by nested amplifications utilizing primers Leu 3.2/ Leu 7 as external primers, and Leu 11/ Leu 12 as internal primers. Briefly, first amplification reaction contained 1 mM dNTP's, 5.0 μM Leu 3.2F and Leu 7R, 0.4 U of Taq polymerase (Life Technologies, Rockville, MD) in 1X Taq buffer, 1mM $MgCl_2$ and 2 μl of sample. The second amplification reaction was assembled as the first with the internal primers added at a concentration of 50 μM. The first amplification reaction was performed for 20 cycles of denaturing at

94 C for 20 sec, annealing at 60 C for 40 sec. and extension at 72 C for 60 sec. The second amplification was performed for 40 cycles of denaturing at 94 C for 20 sec and 68 C for 60 sec. Nested amplifications were carried in one tube by adding the reaction mixture of the first amplification into the second amplification reaction.

Statistical analysis. Statistical analysis was performed using JMP version 3.2.6 software (SAS Institute, Cary, NC). Data are reported as Chi-square approximation or Chi-square test and significance was accepted at $p < 0.05$.

RESULTS

Virus isolation and detection. Initial statistical analysis which included a nonparametric Kruskal-Wallis test (Chi-square approximation) was performed for all the data collected for virus isolations performed in C/E cells and the DF-1 cell line at both S/P ratios (0.2 and 0.1). No differences were observed among the four different groups with respect to the different ages and genetic lines (Table 2).

When a cutoff S/P ratio of 0.2 was used to identify positive ALV samples, no significant differences were observed between the two different assays. However, more ALV positive samples were identified by ac-ELISA after propagation in the DF-1 cell line for three of the four groups while identified ALV positives for group A were identical (Table 3).

No significant differences were observed between the two different assays at a cutoff S/P ratio of 0.1. Again for groups B, C and D more ALV positives were identified by ac-ELISA after propagation in DF-1 cells. However, in group A more ALVs were identified after propagation in C/E cells (Table 3).

Independent test analysis (Chi-square test) was performed for both 0.2 and 0.1 ratios and ensured validity of the data generated in the two different cell culture systems.

Endpoint dilution assay. Results from the limited dilution assays of five plasma samples with relatively high S/P ratios (> 2.0) are shown in Table 4. End point dilutions were identical for samples 3 and 4, while the DF-1 cells exhibited greater endpoint detection in two samples (samples 1 and 2) and a greater endpoint detection was exhibited in C/E cells for only sample 5.

Nested RT-PCR. Nested RT-PCR was able to detect more positive ALV-J plasma samples in group D when compared to virus isolation in DF-1 cells and was indifferent to the cutoff S/P ratio used via ac-ELISA (Figure 4). Only the differences observed between ALV detection via DF-1 propagation coupled with ac-ELISA when a cutoff S/P ratio of 0.2 was used and nested RT-PCR was statistically significant. Independent test analysis (Chi-square test) for both 0.2 and 0.1 ratios ensured the validity of the data generated from nested RT-PCR and DF-1 cell line.

DISCUSSION

The control of ALV infections mainly depends on the early detection and removal of virus shedding birds to reduce the spread of congenital and contact infection to other birds (19). Propagation in phenotype select CEFs and detection by p27 ac-ELISA is the most commonly used assay for the identification of exogenous ALVs (5). As a result of the abundant use of this assay for identification and detection of exogenous ALVs by the poultry industry and select research laboratories, line 0, C/E phenotype embryos are commercially available at a premium price. These embryos often have inconsistencies in viability and livability and must be incubated no less than 8-9 days in order to produce ample numbers of CEFs. In addition, two rounds of cell culture trypsinization must occur in order to eliminate the majority of epithelial cells and to have a predominately homogenous population of secondary CEFS. The use of a continuous cell line such as DF-1 would eliminate the difficulties associated with the production of secondary CEFs. It would therefore be advantageous for companies involved in ALV screening to adjust established cell culture protocols towards the utilization of the DF-1 continuous cell line.

The continuous, non-transformed DF-1 chicken fibroblastic cell line has been reported in the literature to have all the properties necessary for the replication of virtually all ASLV vectors and viruses (11). In addition, Hunt *et al.* have genetically engineered the DF-1 phenotype to resist infection to both ALV subgroups E and J (9). This phenotype of DF-1 would be beneficial in identifying or excluding the possibility of ALV-J in appropriate samples.

Several AVL-J isolates as well as reference exogenous ASLV strains have been shown to successfully propagate in DF-1 cells after several passages in culture (8, 11). The research presented here substantiates the use of the DF-1 cell line as an appropriate vehicle to propagate ALV-J field samples for the detection of group specific p27 gag protein via ac-ELISA. Plasma samples were purposely collected from different genetic lines, generations and ages in order to represent flock populations that would be designated for the screening of ALV-J infection. No statistical differences were observed among these different population in regards to genetics, generation and age and therefore allowed grouping of all submitted samples for further statistical analysis.

No statistical differences ($p<0.05$) were observed between the DF-1 cell line and C/E CEFs, virus propagation in DF-1 cells allowed for the identification of more positive plasma samples at both S/P ratios (0.2 and 0.1). A total of 16 more positive samples were identified by ac-ELISA with a positive S/P cutoff ratio of 0.2 and the number was reduced to 13 more positive samples identified with a positive S/P cutoff ratio of 0.1 after propagation in DF-1 cells. Virtually no distinct differences were observed when limited dilution assays were performed using ALV-J positive samples with relatively high S/P ratios for both the DF-1 and C/E cell culture.

In this study, ALV-J specific nested RT-PCR was initially used as a confirmation assay in order to verify the numerous positives for group D identified by both cell culture systems. However, it was found that nested RT-PCR performed directly on plasma samples without propagation in cell culture was

significantly ($p<0.05$) more sensitive than virus propagation in DF-1 cells coupled with detection by ac-ELISA with a positive S/P cutoff ratio of 0.2. However, this significance was lost when a positive S/P cutoff ratio of 0.1 was used after samples for group D were propagated in DF-1 cells. Nested RT-PCR was able to identify 14 more ALV-J positives for group D with a positive S/P cutoff ratio of 0.2 and 11 more ALV-J positives for group D with a positive S/P cutoff ratio of 0.1.

DF-1 cells share similar morphological, viability, and maintenance characteristics with C/E CEF cell culture when used for ALV diagnostic purposes. All together, the DF-1 cell line is easier to manage, upkeep, propagate and store. The utilization of the DF-1 cell line for ALV-J diagnostic screening would be much more cost effective when compared to the use of C/E cell culture. In addition, the combinational use of an established molecular assay such as the nested RT-PCR presented here after sample propagation in the DF-1 cell line, would assist in the confirmation of positive samples with suspect S/P ratios along with the identification of virus propagation/ac-ELISA false negatives.

Table 1. Identification of four hundred plasma samples (100/group) collected from various pure-line pedigree and grandparent flocks.

Group	Description	Age (weeks)
A	Pure-line pedigree	6-8
B	Pure-line pedigree	18
C	Grandparent	62
D	Pure-line pedigree	40

Table 2. Kruskal-Wallis (Chi-square approximation) analysis exhibiting no significance ($p < 0.05$) and further validating that plasma samples were drawn from identical populations with respect to knowledge about age and genetics.

Cell culture	Chi-square quotient	P value
DF-1 (0.2 S/P)	4.35	0.2262
DF-1 (0.1 S/P)	4.43	0.2182
C/E (0.2 S/P)	3.46	0.3258
C/E (0.1 S/P)	5.73	0.1258

Table 3. ALV percent positive samples identified by p27 ac-ELISA with a positive S/P cutoff ratio of 0.2 and 0.1 after propagation in either the DF-1 cell line or C/E cells. No significance was observed ($p < 0.5$).

	0.2 S/P ratio		0.1 S/P ratio	
Group	DF-1	C/E	DF-1	C/E
A	15	15	17	18
B	24	14	24	14
C	22	21	22	21
D	26	21	29	28

Table 4. Endpoint dilution assays of ALV-J positive plasma samples with relatively high S/P ratios performed in the DF-1 cell line and C/E cells using a positive S/P cutoff ratio of 0.1. 50% endpoints were determined using Reed and Muench calculations

	Sample number (S/P ratio)				
Cell culture	1 (2.133)	2 (2.170)	3 (2.165)	4 (2.031)	5 (2.108)
DF-1	10^{-6}	10^{-6}	10^{-4}	10^{-5}	$10^{-4.8}$
C/E	10^{-5}	$10^{-5.8}$	10^{-4}	10^{-5}	$10^{-5.0}$

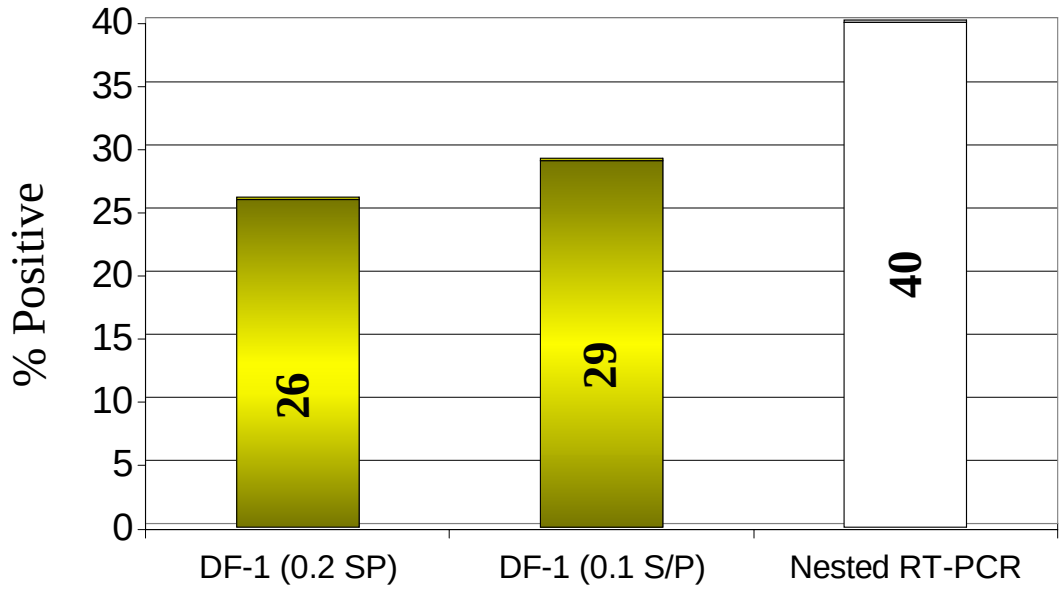


Figure 4. ALV positive samples identified by p27 ac-ELISA with a positive S/P cutoff ratio of 0.2 and 0.1 after propagation in the DF-1 cell line compared with nested RT-PCR. Total number of ALV positive samples per treatment is indicated in parenthesis. Significance was observed between nested RT-PCR and DF-1 cells at a positive cutoff S/P ratio of 0.2 ($p<0.5$).

REFERENCES

1. de Boer, G. F., and A. D. M. E. Osterhaus. Application of monoclonal antibodies in the avian leukosis virus gs-antigen ELISA. Avian Pathol. 14:39-55. 1985.

2. Calnek, B. W. Lesions in young chickens infected by lymphoid leukosis virus. Avian Dis. 12:104-111. 1968.

3. Crittenden, L. B., S. McMahon, M. S. Halpern, and A. M. Fadly. Embryonic infection with the endogenous avian leukosis virus Rous- associated virus-0 alters responses to exogenous avian leukosis virus infection. J. Virol. 61:722-725. 1987.

4. Fadly, A. M., and R. Witter. Oncornaviruses: Leukosis/Sarcoma and Reticuloendotheliosis. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 185-196. 1998.

5. Fadly, A. M. Isolation and identification of avian leukosis viruses: a review. Avian Pathol. 29:529-535. 2000.

6. Garcia, M., J. El-Attrache, N. Ikuta, V. R. Lunge, A. S. K. Fonseca, S. M. Riblet, and P. Villegas. Current advances in the diagnosis of avian leukosis virus by the polymerase chain reaction. International Symposium on ALV-J and other Avian Retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 234-247. 2000.

7. Himly, M., D. N. Foster, I. Bottoli, J. S. Iacovoni, and P. K. Vogt. The DF-1 chicken fibroblast cell line: Transformation induced by diverse oncogenesis and cell death resulting from infetion by avian leukosis viruses. Virology. 248:295-304. 1998.

8. Hunt, H., B. Lupiani, and A. Fadly. Recombination between ALV-J and endogenous subgroup E viruses. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 50-62. 2000.

9. Hunt, H. D., L. F. Lee, D. Foster, R. F. Silva, and A. M. Fadly. A genetically engineered cell line resistant to subgroup J avian leukosis virus infection (C/J). Virology. 264:205-210. 1999.

10. Kim, H., S. You, I. J. Kim, J. Farris, L. K. Foster, and D. N. Foster. Increased mitochondrial-encoded gene transcription in immortal DF-1 cells. Exp. Cell. Res. 265:339-347. 2001.

11. Klein, J. S., I. Givol, E. V. Barsov, J. M. Whitcomb, M. VanBrocklin, D. N. Foster, M. J. Federspiel, and S. H. Hughes. The EV-0 derived cell line DF-1supports the efficient replication of avian leukosis sarcoma viruses and vectors. Virology. 248:305-311. 1998.

12. McKay, J. C., and A. G. Rosales. Control and eradication of ALV-J. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 248-255. 2000.

13. Payne, L. N., S. R. Brown, N. Bumstead, K. Howes, J. A. Frazier, and M. E. Thouless. A novel subgroup of exogenous avian leukosis virus in chickens. J. Gen. Virol. 72:801-807. 1991.

14. Payne, L. N. Biology of Avian Retroviruses. In: The Retroviridae, 1st ed. J. A. Levy, ed., vol. 1. Plenum Press, New York. pp. 489. 1992.

15. Payne, L. N., A. M. Gillespie, and K. Howes. Myeloid leukaemogenicity and transmission of the HPRS-103 strain of avian leukosis virus. Leukemia. 6:1167-1176. 1992.

16. Payne, L. N., and A. M. Fadly. Leukosis/Sarcoma Group. In: Diseases of Poultry, 10th ed. B. Calnek, J. Barnes, C. Beard, L. McDougald, and M. Saif, eds. Iowa State University Press, Ames, Iowa. pp. 414-466. 1997.

17. Payne, L. N. History of ALV-J. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 3-12. 2000.

18. Reed, L. J., and H. Muench. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27:493-497. 1938.

19. Smith, L. M., S. R. Brown, K. Howes, S. McLeod, S. S. Arshad, G. S. Barron, K. Venugopal, J. C. McKay, and L. N. Payne. Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. Virus Res. 54:87-98. 1998.

20. Spencer, J. L. Progress towards eradication of lymphoid leukosis viruses - a review. Avian Pathol. 13:599-619. 1984.

21. Villegas, P. Titration of Biological Suspensions. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 248-254. 1998.

CHAPTER IV

IDENTIFICATION OF NESTED RT-PCR ALV-J POSITIVE PLASMA SAMPLES UNDETECTED BY VIRUS ISOLATION[1]

[1] El-Attrache, J., P. Villegas, M. Garcia and Nilo Ikuta. To be submitted to Avian Diseases.

SUMMARY: A total of 184 individual plasma samples from two avian leukosis virus subgroup J (ALV-J) infected flocks were aliquoted and divided equally. Each individual sample was tested for the presence of ALV-J by virus isolation in DF-1 cells coupled with p27 antigen capture-enzyme linked immunosorbent assay (ac-ELISA), and by testing of the plasma directly via a nested reverse transcriptase-polymerase chain reaction (RT-PCR) which amplifies a 213 bp product in the 3' non-coding region of ALV-J. Samples from one flock underwent two additional passages in DF-1 cells and one separate passage in C/O chicken embryo fibroblasts (CEFs) to ensure the lack of viral propagation and the absence of endogenous viruses respectively, seven samples that failed detection by virus isolation coupled with ac-ELISA but were detected by nested RT-PCR were analyzed. Molecular analysis was performed and included an additional nested RT-PCR amplification followed by restriction fragment length polymorphism (RFLP), which confirmed the presence of ALV-J RNA in these VI- plasma samples, an additional ALV-J specific RT-PCR utilizing previously published *env* specific H5 and H7 primers and capsid (CA) p27 amplification and analysis. Sequence analysis of the complete CA gene along with the major homology region (MHR) of the CA gene for these virus isolation (VI-) plasma samples revealed no noteworthy changes. However, several amino acid residues changes located between the CA and nucleocapsid (NC) gene, known as the spacer region (SP), were identified. Some of these same amino acid changes identified in the ALV-J genomic RNA from the VI- plasma samples have been shown to abrogate infectivity of ALV molecular infectious clones.

Phylogenetic analysis of the p27 CA gene did not elucidate any noted divergence among the VI- and VI+ ALV-J samples as well as other exogenous ALVs. Endpoint dilution assays were performed for VI+ samples in order to determine if the presence of ALV-J antibody interfered with propagation of ALV-J in DF-1 cells coupled with detection by ac-ELISA. On average, ALV-J antibody positive samples were detected between 2-3 $\log_{10}$ dilutions lower than by nested RT-PCR, while antibody negative samples exhibited similar detection limits for both assays.

INTRODUCTION

Avian leukosis viruses (ALVs) are type C retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis, erythroblastosis, myeloblastosis nephromas and / or nephroblastomas and other related connective tissue, epithelial endothelial tumors (21). In commercial poultry flocks worldwide, ALVs are prevalent in several breeding flocks causing serious economic losses from tumor mortality, carcass condemnation and loss of pedigree birds (26). ALVs that infect chickens are readily classified into six groups (A, B, C, D, E and J) based upon the properties of their viral envelope (19, 21). Exogenous ALVs consist of all of the above subgroups with the exception of subgroup E. These exogenous viruses contain no oncogenes, are not defective in replication, and are prevalent in the poultry industry worldwide (11). Historically, subgroups A and B occur as the most common exogenous viruses in the field, are spread vertically and horizontally, are oncogenic and cause principally lymphoid leukosis (LL) (5, 21, 27). However, most recently ALV-J, which was first isolated from meat type chickens (18, 20), has become the most prevalent subgroup inducing myeloid leukosis (ML) tumors in meat-type chickens (11, 15).

Current methods for detection of ALVs in chickens include detection of viral group-specific antigen (gs) p27 by the antigen capture (ac)-ELISA test (27). Monoclonal antibodies against ALV have been developed and are also being used in testing samples for the presence of ALV p27 (2, 11). This test is not specific for exogenous virus and will detect gs antigens of endogenous virus in

serum, plasma, albumen and meconium. Since p27 is shared by endogenous and exogenous ALV, direct assays cannot be used in identification of isolated ALV. In order to increase the specificity of the assay, propagation of the virus in different phenotypes of chicken embryo fibroblast is required prior to the ac-ELISA test. The phenotype of CEFs used most commonly to isolate and detect ALV-J include C/O which are susceptible to infection from all exogenous and endogenous ALV subgroups and C/E which are only susceptible to infection from exogenous ALV subgroups (8, 10, 11). Virus isolation and identification in cell culture is quite readily used by the primary breeder industry and is often referred to as the 'gold standard' for ALV isolation and identification.

A more effective control of ALV infections mainly depends on the early detection and removal of infected birds to reduce contact with infected birds and the incidence of horizontal spread (26, 30). For almost a decade, it has been a major concern of the primary breeder and associated industries to use applicable diagnostic assays to detect and identify ALV-J (18, 28). The use of multiple stringent ALV-J screening procedures, which in the laboratory environment often involves virus isolation on C/E cells coupled with ac-ELISA along with more extensive screening and testing, has reduced the prevalence of ALV-J in the primary breeder industry (17, 22). These screening procedures utilizing cell culture continue today for most of the industry worldwide (22). Preparation of primary and secondary C/E CEF for ALV-J detection and screening requires a minimal two days of preparation and at least seven additional days to obtain a

result (11). In addition, extensive laboratory materials are required and the procedures utilized are quite labor intensive.

Therefore the use of molecular techniques for better diagnostic assistance and ALV-J eradication from breeder flocks has been suggested (16, 17, 22, 25, 26). ALV-J specific PCR methods have been developed to detect proviral DNA from buffy coats, infected CEF and genomic RNA from the plasma of naturally infected birds (12, 26). Smith *et al.* found that PCR was in some cases superior to conventional assays for the detection of ALV-J in samples from experimentally infected birds (26). In addition, we have found that the utilization of a nested RT-PCR to identify the presence of ALV and ALV-J directly from plasma and applied to the screening of field samples has resulted in an increase of sensitivity ranging from 2%-26% when compared to virus isolation coupled with ac-ELISA detection (12). The authors were interested in elucidating some plausible explanations regarding the increased sensitivity of the nested RT-PCR assay. Therefore, analysis was performed and reviewed regarding the ability of antibody-mediated neutralization to reduce detection limits of virus isolation coupled with ac-ELISA. In addition, the examination of p27 genomic mutations that would render incomplete viral particles or replication incompetent non-infectious particles or even specific p27 protein mutants undetectable by ac-ELISA group was examined for seven virus isolation negative samples.

MATERIALS AND METHODS

Cell culture. SPF C/O phenotype embryos were supplied by Sunrise Farms. (Catskill, NY) for the production of C/O primary and secondary chicken embryo fibroblasts from 9-day old embryos. The EV-0 derived DF-1 cell line was obtained from ATCC repository (Manassas, VA).

Plasma samples. Two sets of plasma samples were collected from two different Pure-line pedigree flocks. Ninety-eight plasma samples from flock A (40 weeks of age) were analyzed in the molecular assays (nested RT-PCR, RT-PCR and sequence analysis) and 86 plasma samples from flock B (18 weeks of age) were analyzed in the end point dilution assays. Whole blood was individually collected in 5 ml draw EDTA Vacutainer® brand tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma and residual buffy-coat was collected from the whole blood samples via centrifugation of the Vacutainer® tubes at 4 C for 10 min at 500 g. Each plasma sample was divided in two separate tubes and frozen at –80 C until used for virus isolation and nested RT PCR.

Virus isolation and detection. Virus isolation procedures for both cell culture systems were followed as described by Fadly and Witter (10), and were performed on samples from flocks A and B. Briefly, for SPF C/O CEFs, secondary cells were suspended in F-10/M199 media (Sigma Chemical, St. Louis, MO) containing 2 μg/ml of DEAE and 4 units/ml of heparin at a cell concentration of 3.5×10^5 cells/ml. Fibroblasts were transfered to 24-well tissue culture plates. Plates were incubated until confluent monolayers were observed

(approx 16 to 18 hrs.). Fifty μl of each plasma sample was added to individual wells. After inoculation, the plate was gently rocked to promote viral absorption and incubated at 39 C. Medium was changed 24 hours after initial incubation afterwards cultures were maintained in the incubator for a period of 7 days. The above-mentioned procedure was followed for virus isolation in DF-1 cells with the following exceptions. DF-1 cells were propagated in high glucose DMEM media (Sigma Chemical, St. Louis, MO) and incubated at 39 C.

Following incubation, 40 μl of 5% Tween 80 solution was added to each well, plates were frozen and thawed three times and 100 μl material was collected for ac-ELISA assay. Avian leukosis virus ac-ELISA assay readings were performed following manufacturers recommendations (IDEXX Laboratories, Westbrook, Maine) with the exception of using a positive S/P cutoff ratio value of 0.1.

ALV antibody detection. The presence or absence of ALV and ALV-J antibody for flock B was determined by the avian leukosis antibody test kit – subgroup J and the avian leukosis antibody test kit following manufacturers recommendations (IDEXX Laboratories, Westbrook, Maine).

RNA extraction. RNA from individual plasma samples in flock A and B were extracted following the procedures described for the Trizol LS reagent (Life Technologies, Gaithersburg, MD). Briefly, 100 μl of plasma was added to 750 μl of Trizol LS reagent, vortexed and incubated at room temperature for 5 min. 200

μl of chloroform was added, vortexed and centrifuged at 12,000 g for 15 min at 4 C. Supernatant was removed and the RNA pellet was washed with ethanol. The RNA pellet was allowed to air dry and was re-suspended in 25 μl of DEPC water.

Nested RT-PCR. Nested RT-PCR was utilized for the confirmation of ALV-J detection via virus isolation coupled with ac-ELISA for flocks A and B and followed as described by Garcia *et al.* (12). Briefly, a reverse transcriptase reaction was performed with 10 units of M-MLV of RT (Life Technologies, Rockville, MD) in MMLV 1x buffer for 1 hour at 37 C followed by 2 min. at 94 C followed by nested amplifications utilizing primers Leu 3.2/ Leu 7 as external primers, and Leu 11/Leu 12 as internal primers (Table 5). Briefly, first amplification reaction contained 1 mM dnTP's, 5.0 μM Leu 3.2F and Leu 7R, 0.4 U of Taq polymerase (Life Technologies, Rockville, MD) in 1X taq buffer, 1mM MgCl2 and 2 μl of sample. The second amplification reaction was assembled as the first with the internal primers added at a concentration of 50 μM. The first amplification reaction was performed for 20 cycles of denaturing at 94 C for 20 sec, annealing at 60 C for 40 sec. and extension at 72 C for 60 sec. The second two-step amplification was performed for 40 cycles of denaturing at 94 C for 20 sec and 68 C for 60 sec. Nested amplifications were carried in one tube by adding the reaction mixture of the first amplification into the second amplification reaction. After amplification, samples were subjected to electrophoresis at 10V/cm for 30 min. on 1% agarose gels and stained with ethidium bromide.

Endpoint dilution assay. A limited dilution assay was performed on three antibody negative plasma samples, two ALV-J antibody positive plasma samples (S/P ratios greater than 1.0) and one ALV-J and ALV antibody positive plasma sample from flock B. Dilutions were inoculated onto DF-1 monolayers as previously described (10), and ranged from 10^0 to 10^7 ten-fold dilutions with a total of five treatments per sample. An ac-ELISA S/P ratio greater than 0.1 and the amplification of a 213 bp nested RT-PCR product directly from the ten-fold dilutions were considered positive. The formula devised by Reed and Muench determined the 50% endpoint (24, 29).

Classical and molecular verification of ALV-J identification. Fifteen of the virus isolation negative (VI-) and one virus isolation positive (VI+) plasma samples underwent additional classical and molecular analysis. Classical analysis included an additional passage in DF-1 cells to identify any possible infectious ALV-J in the plasma sample. In addition, one passage was performed in C/O CEFs to ensure the absence of any endogenous virus.

After classical analysis, seven VI- plasma samples of flock A were again amplified by nested RT-PCR and the 213 bp products were digested with the restriction endonuclease (RE) *Dde* I (New England Biolabs Inc., Beverly, MA) according to manufacturer's recommendation in order to confirm the unique RE site located within the amplicons of ALV-J RNA. Digested fragments were subjected to electrophoresis on a 12.5% polyacrylamide gel and visualized by rapid silver staining.

H5 and H7 RT-PCR. Extracted RNA of seven selected VI- and one VI+ individual plasma sample(s) from flock A were reverse transcribed to cDNA and amplified with commercially available Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ). All reagents with the exception of the H5 and H7 primers (Table 5) previously described by Smith *et al*. (26), were supplied with the RT-PCR beads. Briefly, 5 μl of extracted RNA and 1 μl of each primer (H5 and H7) was added along with 43 μl of DEPC treated water. Reverse transcription was performed at 50 C for 35 min followed by a single denaturing step at 94 C for 2 min and 35 cycles of amplification consisting of 30 sec at 94 C, 30 sec at 55 C and 1 min at 68 C. A final extension step was performed at 68 C for 7 min. After amplification, the 545 bp amplicons were subjected to electrophoresis at 10V/cm for 30 min. on 1% agarose gels and stained with ethidium bromide.

p27 capsid RT-PCR. The same procedure stated for H5 and H7 amplification of extracted plasma RNA from the seven selected VI- and one VI+ samples of flock A were performed with the Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) for p27 capsid amplification, with the following modifications. Ten μl of extracted RNA and 1 μl of each forward (EF133) and reverse (ER982) ALV p27 designed primers were added to 38 μl of DEFC water and applied at an annealing temperature of 61.5 C to amplify the complete p27 capsid gene (Table 5).

Phylogenetic, nucleotide and deduced amino acid sequence analysis. The 850 bp RT-PCR p27 capsid amplicons were purified by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). The DNA products were cloned via a TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Positive transformed colonies were selected and after growth overnight in LB broth, the pCR 2.1 DNA plasmid was purified with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) following the manufacture recommendations and examined for the proper 850 bp insert. Plasmid products were sequenced at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA) via the dideoxy-mediated chain-termination method with universal M13 forward and reverse primers. Two separate internal primers (IR595 and IF577) were designed to provide complete sequence of the cloned RT-PCR products (Table 5). Sequence data of the p27 CA and SP region was assembled with Seqman TM (Dnastar Inc., Madison, WI). Nucleotide sequence analysis and deduced amino acid sequence analysis was performed by the Clustal method with the package Megalign TM (Dnastar Inc.).

RESULTS

Plasma samples, virus isolation and detection, ALV antibody detection and nested RT-PCR. A summation of nested RT-PCR and virus isolation comparisons for flock A and virus isolation, antibody detection and nested RT-PCR for flock B are exhibited in Table 6. For both flocks, nested RT-PCR identified more positives than virus isolation coupled with ac-ELISA with flock A

and flock B having approximately 11.2% and 17.5% more ALV-J positives identified, respectively. In addition, a correlation above 86% was observed between the ALV-J positives identified by DF-1 propagation coupled with ac-ELISA and detected by nested RT-PCR (Table 7).

ALV-J antibody assessment for flock B revealed the presence of four serological classes assessed in Table 8. Viremia, with antibody (V+/A+) was more readily detected by nested RT-PCR. ALV antibody was detected in only nine samples (Table 6). Three of those nine samples were ALV-J antibody negative.

Endpoint dilution assay. Results of the limited dilution assays from the three antibody negative and three antibody positive plasma samples with S/P ratios greater than 1.0 are shown in Table 9. End point detections for the antibody negative samples were similar for both DF-1/ac-ELISA and nested RT-PCR assays. However, nested RT-PCR exhibited greater endpoint detection than DF-1/ac-ELISA for the antibody positive plasma samples.

Classical and molecular verification of ALV-J identification. After two passages in DF-1 cells and one passage in C/O CEFs, no virus was detected by p27 ac-ELISA for seven isolates. Seven of the 15 VI- that were identified by nested RT-PCR for flock B were further analyzed (Figure 5). Restriction enzyme analysis with *Dde* I was performed on all seven VI- nested RT-PCR positive samples (Figure 6). All seven amplicons, which were derived from the VI-

plasma samples, possessed the *Dde* I site characteristic of ALV-J but not present in other exogenous viruses or endogenous ALVs (Figure 6).

H5 and H7 RT-PCR. Additional confirmation of the amplification of ALV-J genomic RNA from VI- plasma samples of flock B was performed with ALV-J *env* specific primers (Figure 7). Six of the seven VI- plasma samples were amplified with this set of ALV-J specific primers giving an approximate 545 bp product. Plasma sample 6VI- was not amplified with the H5 and H7 set of primers.

p27 capsid RT-PCR. An approximate 850 bp product encompassing the complete CA gene was amplified from the seven VI- plasma samples of flock B (Figure 8). All seven VI- plasma samples were amplified by the EF133 and ER982 p27 designed primers.

Phylogenetic, nucleotide and deduced amino acid sequence analysis. The p27 capsid of three VI- samples (16VI-, 20VI-, 30VI-) and one VI+ (10VI+) was completely sequenced. Minimal nucleotide divergences (< 6%) were observed for the three VI- and one VI+ ALV-J samples when compared to other exogenous and endogenous subgroups. The nucleotide similarities were 100% among subgroup A and B as well as among 16VI- and 20VI-. The deduced amino acid sequence of these four ALV-J samples shared and extremely high identity with the European ALV-J strain HPRS-103 (99.6% to 100%) as well as the US ALV-J strain ADOL-7501 (96.7%-97.1%). Again, subgroups A and B shared 100%

amino acid similarities as well as 16VI- and 20VI-. Phylogenetic analysis of the 717 bp p27 CA is shown in Figure 9. No distinct branches were observed for this cladogram. The major homology region (MHR) within the CA was homologous among all exogenous subgroups and ALV-J analyzed samples (Figure 10). A noted threonine residue was substituted for a methionine for all analyzed exogenous viruses except for sample 30VI- and the reference Prague C strain (4).

The spacer region (SP) for sequenced VI- and VI+ ALV-J samples exhibited several amino acid substitutions when compared to the Prague C strain (Figure 11). Amino acids changes at residue 473 was repeated for 20VI- and 30VI- where an arginine was substituted for glutamine which was observed in the Prague C reference and the rest of the analyzed exogenous ALVs. Amino acids residue number 481 (serine) was changed for 17VI- (tyrosine) and 20VI- (phenylalanine). Sample 16VI- had to amino acid changes at residues 487 (isoleucine to valine) and 488 (methionine to tryptophan). ADOL 7501 also had to amino acid changes at residues 486 (leucine to valine) and 491 (valine to alanine).

DISCUSSION

The 3' LTR nested RT-PCR assay performed directly on plasma samples was a more sensitive assay than virus isolation in DF-1 cell coupled with ac-ELISA for the detection of ALV-J. Approximately 14.5% more ALV-J positives were identified by nested RT-PCR. A correlation was observed between

positives identified by DF-1 virus isolation and nested RT-PCR. Although few false negatives were identified for nested RT-PCR by DF-1 virus isolation, these numbers are probably attributed to non-mechanical RNA extraction methodologies, which can be easily resolved by commercial kits and applications. This assay allows for the rapid detection and identification of exogenous ALVs and can be readily applied in a diagnostic setting to enhance screening of ALV-J infected flocks.

The larger issue at hand that needs to be addressed when nested RT-PCR is compared to virus isolation coupled with ac-ELISA for the detection of ALV-J is the greater sensitivity that is consistently observed with molecular assays. The identification of VI- viral RNA in samples has been observed for both HIV and Dengue virus (1, 9). In both instances, the presence of neutralizing antibodies has played a role in minimizing detection by virus isolation and ac-ELISA. When nested RT-PCR and VI assessments were made in flock B, all four of the serological classes that have been noted to occur in ALV infected flocks were present. The majority of nested RT-PCR/VI- samples were obtained from the V+/A+ serological group. Our observations show that plausible neutralizing antibodies identified by ALV-J antibody ELISA minimized endpoint detection when compared to nested RT-PCR. Differences as great as 100-fold was observed between virus isolation and nested RT-PCR. Further substantiation for possible antibody interference of virus detection was recognized when antibody negative serum was analyzed via limited dilution assays, no reduction in end point detection was observed.

Methodologies to enhance virus infectivity assays have been well established and in animal virology physical particle:infectivity ratios as low as 100-1000:1 are considered respectable (14, 23). However, when this ratio falls below or even hovers around these respectable numbers, infectivity is not readily achieved. Assays that rely on HIV-1 protein production, reverse transcriptase activity or cell viability do not directly enumerate the number of infectious events in a cell culture and are limited if the level of virus replication is low (1, 14). The same ought to be assumed for ALSVs due to several events that can happen during replication that could render a large population of viral particles non-infectious. One example would be read through transcripts which contain nonviral sequence at their 3' end have been predicted to be in up to 32% of wild type ALSV particles and virus replication has been shown to be reduced up to six times less in poly-adenlyated mutated ALSV when compared to wild type viruses (13).

The role of proper processing of ALSV capsid proteins via cleavage events that occur at identified amino acid residues within the SP region between the CA and NC in the *gag* polyprotein have been shown to be required for infectivity of ALSV infectious molecular clones (3, 31, 32). In addition, viral DNA synthesis defects have been observed in assembly competent Rous sarcoma MHR CA mutants (4). Although no amino acid substitutions were observed in the MHR and the sequence for the entire CA gene was highly conserved among field isolates and other exogenous ALVs, several amino acid residue substitutions were identified for ALV-J nested RT-PCR positive samples within the SP region.

For two of the samples, (17VI- and 20VI-) amino acid substitutions occur at single residue sites that have been shown to abrogate infectivity of molecular infectious clones of Rous sarcoma induced by site directed mutagenesis (31). Amino acid substitutions observed for 16 VI- and 30 VI- have not been characterized by infectious molecular clone studies.

Nested RT-PCR would be continuously advantageous over virus isolation for the detection of ALV-J at the onset or latter stages of infection when viremia is low and replication and infectious factors mentioned above could possibly diminish ALV-J infectivity in cell culture. In addition, the presence of ALV neutralizing antibodies present in the sample would also diminish detection levels. This assay allows for the rapid detection and identification of exogenous ALVs undetected by virus isolation coupled with ac-ELISA and can be readily applied in a diagnostic setting to enhance screening of ALV-J infected flocks.

Table 5. Sequence, position, targets and expected PCR product sizes of oligonucleotide primers utilized in this study. Nucleotide position based on HPRS-103 (GenBank accession number Z46390).

Primers	Target	Nucleotide position	Sequence
Leu 3.2F	U3	7514-7543	GGAAATGTAGTGTTATRCRATACTCTTATG
Leu 7R	U5	7813-7834	ATCCGCTTCATGCAGGTGCTC
Leu 11F	U3	7594-7617	CGTCGATTGGTGGAAGTAAGGTGG
Leu 12R	U5	7785-7806	TCAGGGAATCGACGGTCCGGCC
H5	IN	5258-5277	ATAGAGCCAGAGGCACCT
H7	*env*	5783-5802	CATAGCTTCGTCTACGCCCATA
EF133	p10	1267-1288	TGGGCAAGGATCAGGGAGGAGC
ER982	p12	2095-2118	CACGACCACCCCGACCCAGTTTG
IR595[A]	p27	1707-1729	GAGCCGACGCCGTAATAGCAACC
IF577[A]	p27	1711-1734	GCTATTACGGCGTCGGCTCTCCAG

[A]Primers utilized for sequencing

Table 6. Summary of diagnostic assays performed on plasma samples from two separate Pure-line pedigree flocks.

Flock	Number	DF-1 Virus isolation positive	Nested RT-PCR positive	ALV-J antibody positive	ALV antibody positive
A	98	28	39	ND	ND
B	86	21	36	34	9

Table 7. Correlation of DF-1 virus isolation coupled with ac-ELISA positive samples detected by nested RT-PCR for two separate Pure-line pedrigree flocks.

Flock	Virus isolation positive	RT nested PCR positive	Percent correlation
A	28	24	86
B	21	19	90

Table 8. Assessment of the four ALV-J serological classes occurring in flock B when viremia is detected by either DF-1 virus isolation coupled with ac-ELISA or by nested RT-PCR.

Detection Method	V-A-	V-A+	V+A-	V+A+
VI	35	30	17	4
Nested RT-PCR	35	15	18	18

V = virus isolation or nested RT-PCR positive
A = antibody

Table 9. Endpoint dilution assays of ALV-J antibody positive (S/P ratios greater than 1.0) and antibody negative plasma samples from flock B performed in the DF-1 cell line and detected via ac-ELISA using a positive S/P cutoff ratio of 0.1 and nested RT-PCR. 50% endpoints for DF were determined using Reed and Muench calculations.

		Endpoints	
ALV-J Antibody	Sample Number	DF-1	Nested RT-PCR
Positive	1	$10^{-2.8}$	10^{-5}
	2	$10^{-4.8}$	$10^{-5.2}$
	3[A]	$10^{-1.5}$	10^{-3}
Negative	1	10^{-6}	10^{-4}
	2	10^{-6}	$10^{-5.8}$
	3	10^{-4}	10^{-4}

[A]ALV antibody detected by ELISA.

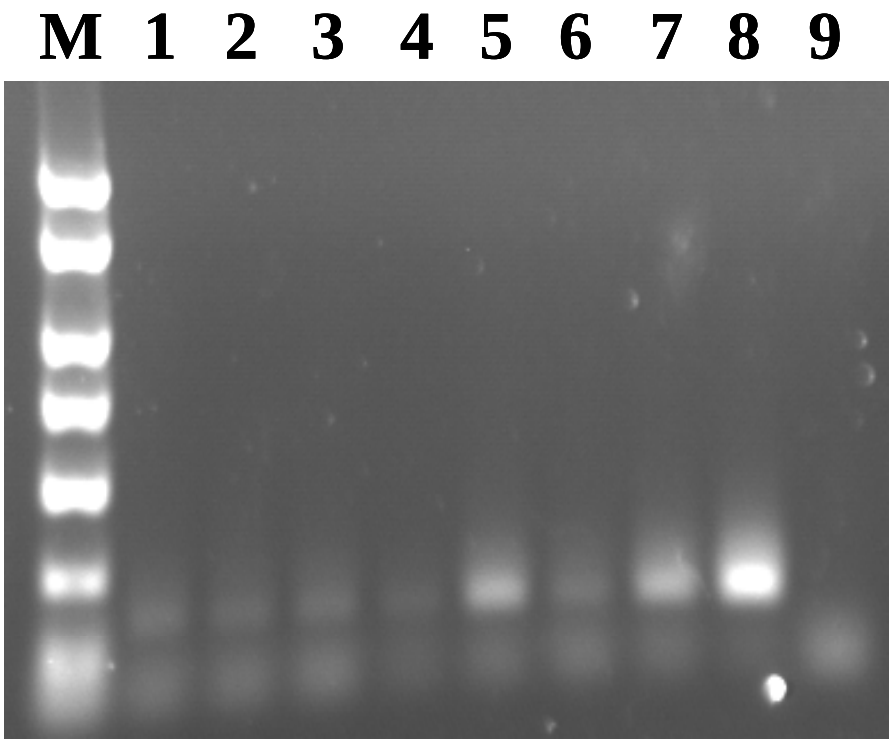


Figure 5. Nested RT- PCR amplification of an approximate 213 bp product from virus isolation negative (VI-) plasma samples of flock A utilizing the 3' LTR (Leu 3.2/7, Leu 11/12) nested primers. Lane 1, plasma sample 6VI-; lane 2, 17VI-; lane 3, 19VI-; lane 4, 20VI-; lane 5, 30VI-; lane 6, 35 VI-; lane 7, 60 VI-; lane 8, virus isolation positive plasma sample 10VI+ and lane 9, negative extraction control.

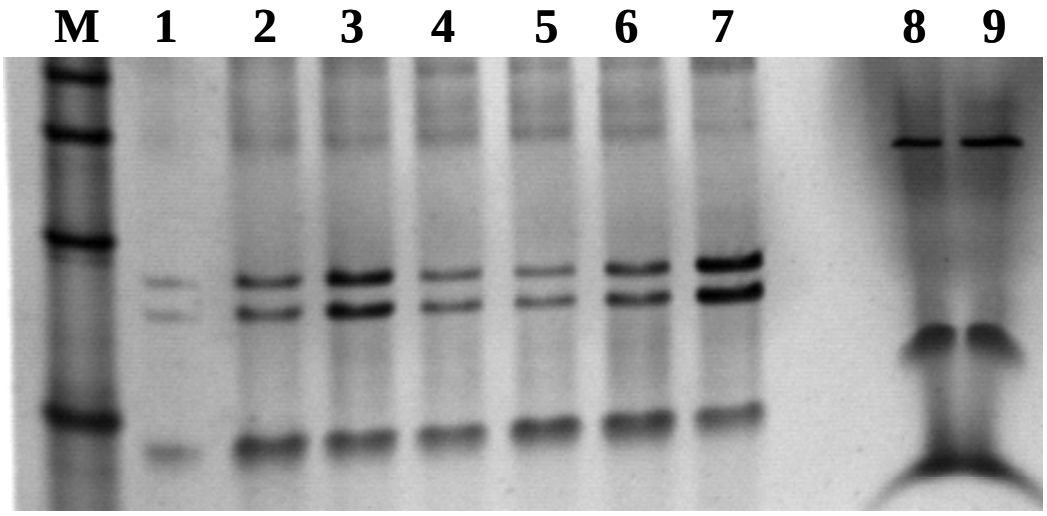


Figure 6. RFLP analysis for the confirmation of ALV-J subgroup of the seven VI-213 bp nested RT-PCR LTR products amplified from flock A plasma samples. Lane 1, plasma sample 6VI-; lane 2, 17VI-; lane 3, 19VI-; lane 4, 20VI-; lane 5, 30VI-; lane 6, 35 VI-; lane 7, 60 VI-; lane 8 and lane 9, uncut 213 bp product.

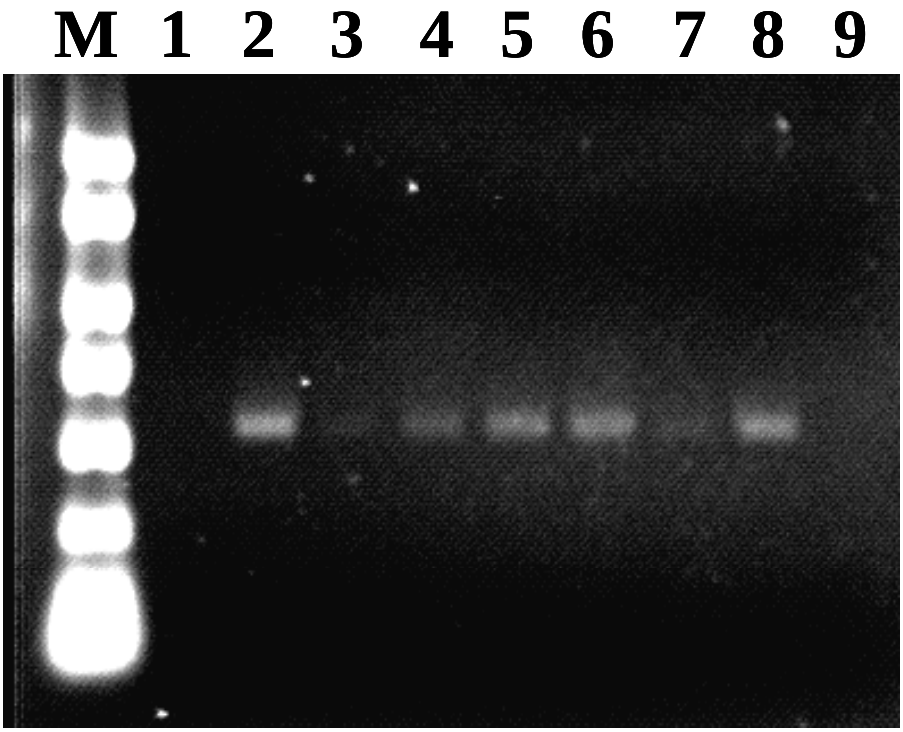


Figure 7. RT-PCR amplification of an approximate 545 bp product from virus isolation negative (VI-) plasma samples of flock A utilizing the ALV-J *env* specific primers (H5 and H7). Lane 1, plasma sample 6VI-; lane 2, 17VI-; lane 3, 19VI-; lane 4, 20VI-; lane 5, 30VI-; lane 6, 35 VI-; lane 7, 60 VI-; lane 8, virus isolation positive plasma sample 10VI+ and lane 9, negative extraction control.

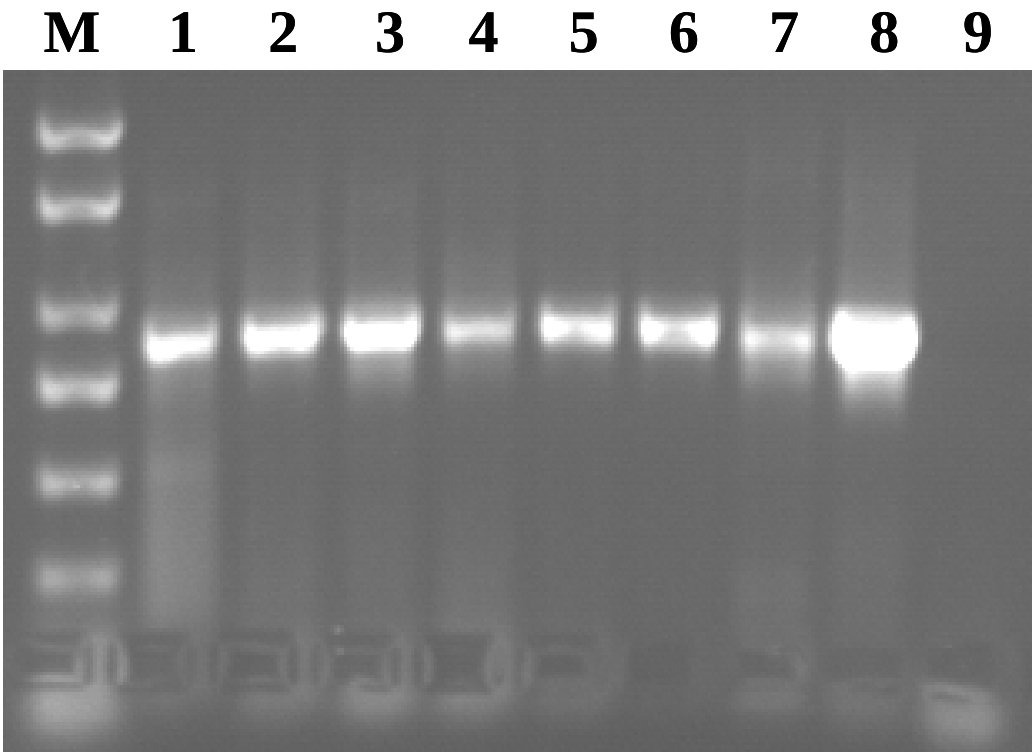


Figure 8. RT-PCR amplification of an approximate 850 bp product from the seven virus isolation negative (VI-) and one positive virus isolation (VI+) plasma sample(s) of flock A utilizing the p27 capsid primers (EF133 and ER982). Lane 1, plasma sample 6VI-; lane 2, 17VI-; lane 3, 19VI-; lane 4, 20VI-; lane 5, 30VI-; lane 6, 35 VI-; lane 7, 60 VI-; lane 8, virus isolation positive plasma sample 10VI+ and lane 9, negative extraction control.

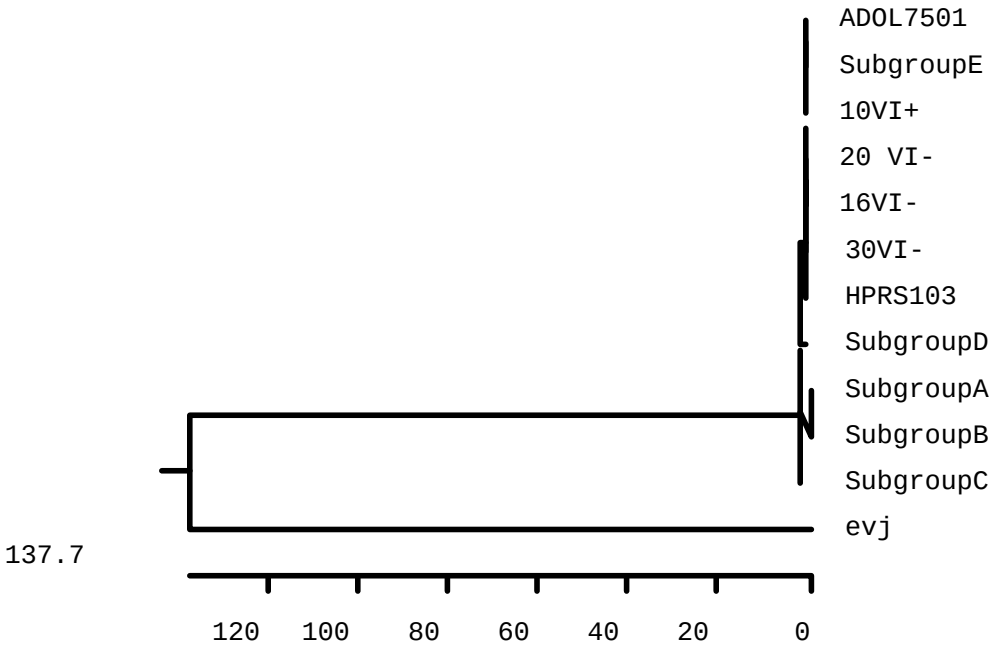


Figure 9. Phylogenetic analysis of the 717 bp p27 CA gene. The relationship among four ALV-J field samples and representatives from exogenous and endogenous subgroups of ALV is shown. This cladogram tree was generated using the Clustal method with PAM 250 residue weight table. This tree was rooted to an outgroup sequence of ev-j.

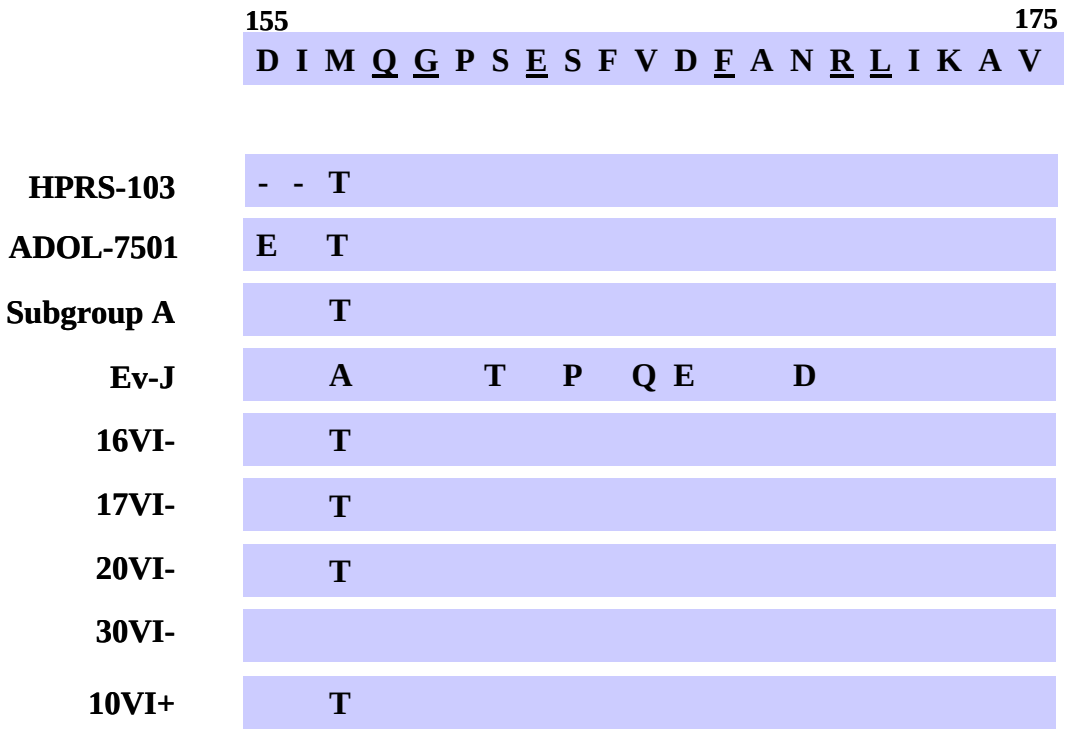


Figure 10. Deduced amino acid sequences of the major homology region (MHR) from residues 155-175 (numbering of the MHR of Cairns *et al*.(4)). Blank spaces are indicative of amino acid sequence identical to the Prague C strain *gag* gene. Underlined amino acids indicate areas of amino acid substitutions that abrogate replication of infectious molecular clones derived from the Prague C strain (4, 6).

Figure 11. Deduced amino acid sequences of the spacer region (SP) from residues 469-492 (numbering of the SP of Craven *et al.* (7)). Bold-faced residues are indicative of amino acid residues that differ from the Prague C strain. Underlined amino acids indicate areas of combinational amino acid substitutions and highlighted amino acid residues 475, 481 and 489 are single amino acid substitutions that abrogate infectivity of infectious molecular clones derived from the Prague C strain (31).

REFERENCES

1. Achkar, J. M., X. H. Wang, P. Nyambi, M. K. Gorny, S. Zolla-Pazner, and J. C. Bandres. Polymerase chain reaction-based assay for antibody-mediated neutralization of HIV-1 reveals a population of nonneutralized virus undetected by conventional p24 assay. J. Acquir. Immune Defic. Syndr. 24:203-210. 2000.

2. Boer, G. F. d., and A. D. M. E. Osterhaus. Application of monoclonal antibodies in the avian leukosis virus gs-antigen ELISA. Avian Pathol. 14:39-55. 1985.

3. Bowzard, J. B., J. W. Wills, and R. C. Craven. Second-site suppressors of Rous sarcoma virus Ca mutations: evidence for interdomain interactions. J. Virol. 75:6850-6856. 2001.

4. Cairns, T. M., and R. C. Craven. Viral DNA synthesis defects in assembly-competent rous sarcoma virus CA mutants. J. Virol. 75:242-250. 2001.

5. Calnek, B. W. Lesions in young chickens infected by lymphoid leukosis virus. Avian Dis. 12:104-111. 1968.

6. Craven, R. C., A. E. Leure-duPree, R. A. Weldon, and J. W. Wills. Genetic analysis of the major homology region of the Rous sarcoma virus Gag protein. J. Virol. 69:4213-4227. 1985.

7. Craven, R. C., A. E. Leure-duPree, C. R. Erdie, C. B. Wilson, and J. W. Wills. Necessity of the spacer peptide between CA and NC in the Rous sarcoma virus gag protein. J. Virol. 67:6246-6252. 1993.

8. Crittenden, L. B., S. McMahon, M. S. Halpern, and A. M. Fadly. Embryonic infection with the endogenous avian leukosis virus Rous- associated virus-0 alters responses to exogenous avian leukosis virus infection. J. Virol. 61:722-725. 1987.

9. De Paula, S. O., C. Nunes, R. Matos, Z. M. de Oliveira, D. M. Lima, and B. A. da Fonseca. Comparison of techniques for extracting viral RNA from isolation-negative serum for dengue diagnosis by the polymerase chain reaction. J. Virol. Methods. 98:119-125. 2001.

10. Fadly, A. M., and R. Witter. Oncornaviruses: Leukosis/Sarcoma and Reticuloendotheliosis. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 185-196. 1998.

11. Fadly, A. M. Isolation and identification of avian leukosis viruses: a review. Avian Pathol. 29:529-535. 2000.

12. Garcia, M., J. El-Attrache, N. Ikuta, V. R. Lunge, A. S. K. Fonseca, S. M. Riblet, and P. Villegas. Current advances in the diagnosis of avian leukosis virus by the polymerase chain reaction. International Symposium on ALV-J and other Avian Retroviruses. World Veterinary Poultry Asscociation, Rauischholzhausen, Germany. pp. 234-247. 2000.

13. Herman, S. A., and J. M. Coffin. Efficient packaging of readthrough RNA in ALV: implications for oncogene transduction. Science. 236:845-848. 1987.

14. Ho, W. Z., R. Cherukuri, S. D. Ge, J. R. Cutilli, L. Song, S. Whitko, and S. D. Douglas. Centrifugal enhancement of human immunodeficiency virus type 1 infection and human cytomegalovirus gene expression in human primary monocyte/macrophages in vitro. J. Leukoc. Biol. 53:208-212. 1993.

15. Hunt, H. D., L. F. Lee, D. Foster, R. F. Silva, and A. M. Fadly. A genetically engineered cell line resistant to subgroup J avian leukosis virus infection (C/J). Virology. 264:205-210. 1999.

16. McKay, J. C. A poultry breeders approach to avian neoplasia. Avian Pathology. 27:s74-77. 1998.

17. McKay, J. C., and A. G. Rosales. Control and eradication of ALV-J. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 248-255. 2000.

18. Payne, L. N., S. R. Brown, N. Bumstead, K. Howes, J. A. Frazier, and M. E. Thouless. A novel subgroup of exogenous avian leukosis virus in chickens. J Gen Virol. 72:801-807. 1991.

19. Payne, L. N. Biology of Avian Retroviruses. In: The Retroviridae, 1st ed. J. A. Levy, ed., vol. 1. Plenum Press, New York. pp. 489. 1992.

20. Payne, L. N., A. M. Gillespie, and K. Howes. Myeloid leukaemogenicity and transmission of the HPRS-103 strain of avian leukosis virus. Leukemia. 6:1167-1176. 1992.

21. Payne, L. N., and A. M. Fadly. Leukosis/Sarcoma Group. In: Diseases of Poultry, 10th ed. B. Calnek, J. Barnes, C. Beard, L. McDougald, and M. Saif, eds. Iowa State University Press, Ames, Iowa. pp. 414-466. 1997.

22. Payne, L. N. History of ALV-J. International Symposium on ALV-J and other avian retroviruses. World Veterinary Poultry Association, Rauischholzhausen, Germany. pp. 3-12. 2000.

23. Reading, S. A., M. J. Edwards, and N. J. Dimmock. Increasing the efficiency of virus infectivity assays: small inoculum volumes are as effective as centrifugal enhancement. J. Virol. Methods. 98:167-169. 2001.

24. Reed, L. J., and H. Muench. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27:493-497. 1938.

25. Smith, E. J., S. M. Williams, and A. M. Fadly. Detection of avian leukosis virus subgroup J using the polymerase chain reaction. Avian Dis. 42:375-380. 1998.

26. Smith, L. M., S. R. Brown, K. Howes, S. McLeod, S. S. Arshad, G. S. Barron, K. Venugopal, J. C. McKay, and L. N. Payne. Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. Virus Res. 54:87-98. 1998.

27. Spencer, J. L. Progress towards eradication of lymphoid leukosis viruses - a review. Avian Pathol. 13:599-619. 1984.

28. Venugopal, K., K. Howes, G. S. Barron, and L. N. Payne. Recombinant env-gp85 of HPRS-103 (subgroup J) avian leukosis virus: antigenic characteristics and usefulness as a diagnostic reagent. Avian Dis. 41:283-288. 1997.

29. Villegas, P. Titration of Biological Suspensions. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th ed. D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 248-254. 1998.

30. Witter, R. L., L. D. Bacon, H. D. Hunt, R. E. Silva, and A. M. Fadly. Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. Avian Dis. 44:913-931. 2000.

31. Xiang, Y., R. Thorick, M. L. Vana, R. Craven, and J. Leis. Proper processing of avian sarcoma/leukosis virus capsid proteins is required for infectivity. J. Virol. 75:6016-6021. 2001.

32. Yu, F., S. M. Joshi, Y. M. Ma, R. L. Kingston, M. N. Simon, and V. M. Vogt. Characterization of Rous sarcoma virus Gag particles assembled in vitro. J. Virol. 75:2753-2764. 200

CHAPTER V

THE DESIGN AND EVALUATION OF MORPHOLINOS AS POTENTIAL INHIBITORS OF AVIAN LEUKOSIS VIRUS REPLICATION[1]

[1] El-Attrache, J., P. Villegas, S. Callison and A. Banda. To be submitted to Antisense and Nucleic Acid Drug Development.

ABSTRACT: ALV-J, which was originally isolated in Europe and first reported as a new subgroup in 1992, quickly became the most widespread and economically important ALV to infect meat type birds internationally. Antisense phosphorodiamidate morpholino oligomers (MOs) have been analyzed as potential inhibitors of avian leukosis virus subgroup J (ALV-J) replication. A total of five morpholinos were designed and evaluated. Three MOs were targeted to different sites complementary to the 5' ALV-J genome and two additional MOs were designed complementary to the tRNA primer binding site (PBS). Inhibition of replication was analyzed *in vitro* by p27 antigen capture-enzyme linked immunosorbent assay (ac-ELISA) from cell-free supernatant of DF-1 cells infected with ALV-J pre- and post- treatment with the appropriate morpholinos. A significant reduction ($p < 0.05$) in the production of p27 was observed for only the MO targeted to the ADOL-7501 PBS when compared to the treatments with a non-specific MO control. Viral RNAs were extracted from the pooled cell free supernatant of the PBS targeted MO treatments and a one log reduction in viral RNAs was observed by Real time LightCycler® RT-PCR This one log reduction in viral RNAs correlated with a one log reduction of infectious units observed when an endpoint dilution assay was performed for the same treatments.

INTRODUCTION

Avian leukosis viruses (ALVs) are type C retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis, erythroblastosis, myeloblastosis nephromas and / or nephroblastomas and other related connective tissue, epithelial endothelial tumors (Payne et al., 1997). ALVs that infect chickens are readily classified into six groups (A, B, C, D, E and J) based upon the properties of their viral envelope (Payne et al., 1991; Payne et al., 1997). In commercial poultry flocks worldwide, ALV-J is prevalent in several breeding flocks causing serious economic losses from tumor mortality, carcass condemnation and loss of pedigree birds (Fadly, 2000; Smith et al., 1998; Witter et al., 2000).

There are no commercial vaccines that protect chickens from infection with ALV. Therefore, the only defense the poultry industry utilizes against ALV-J infection has been eradication. This is primarily based upon the elimination of dams and infected birds that test positive for ALV gs antigen by ac-ELISA through various programs (Fadly, 2000; Payne et al., 1997). Although several companies have had successful ALV-J eradication programs installed for over a decade, the problems of ALV-J infections are still confronted today (Payne, 2000).

Antisense oligos offer the prospect of safe and effective therapeutics for a broad range of intractable diseases. This includes the use of antisense oligos as an antiviral such as Vitravene®, an antisense phosphorothioate with sequence complimentary to the coding region of the major immediate-early gene of human

cytomegalovirus (Orr, 2001; Summerton et al., 1997). Extensive antiviral research has been performed analyzing the efficacies of retrovirus replication inhibition by antisense oligos targeted at several areas of the virus genome (Bordier et al., 1992; Chadwick et al., 2000; Wei et al., 2000). In particular, studies involving the inhibition of HIV-1 replication by targeting the primer binding site (PBS) and LTR regions with both traditional genetic oligos and non-traditional antisense oligomers have been successful in the inhibition of replication (Archambault et al., 1994; Chadwick et al., 2000; Hildinger et al., 2001; Ushijima et al., 2001). In contrast to the research that has been performed for inhibition of HIV-1 replication, only few published studies have reviewed the efficacies of antisense oligos inhibiting the replication avian leukosis sarcoma viruses (ALSVs). Zamecnik and Stephenson in two separate papers reported the inhibition of Rous sarcoma virus replication, viral RNA translation and cell transformation with a single phosphorothioate antisense oligo (Stephenson et al., 1978; Zamecnik et al., 1978), while *Kim et al.* analyzed eight traditional antisense oligos targeted at several regions within the genome along with the efficacies of intracellular antisense RNA expression (Kim et al., 1998).

Antisense phosphorodiamidate morpholino oligomers (MOs) are a relatively new class of antisense agents with high specificity and efficacy (Ghosh et al., 2000; Summerton et al., 1997). They are uncharged molecules in which the ribose moiety is replaced with a morpholino group (a six membered morpholino ring) and the phosphodiester linkage is replaced by a nonionic phosphorodiamidate linkage (Summerton et al., 1997). Similar to other oligo

chemistries, however, due to additional advantageous chemistries, MOs have several advantages over other designed oligos in that they exhibit minimal non-specific activity, high specificity and complete resistance to host nucleases (Summerton, 1999; Summerton et al., 1997). MOs have been highly successful in their use as antisense oligos (Iversen, 2001). The experiments reported here were designed to study the effects that the non-traditional MOs have on ALV-J replication in DF-1 cell culture. The 5' end of ALV-J genome was chosen as a target for 5 different MOs.

MATERIALS AND METHODS

Cell culture and virus preparation. The EV-0 derived DF-1 cell line was obtained from American Type Culture Collection repository (Manassas, VA). The ALV-J strain ADOL-7501 was obtained from U.S. Department of Agriculture, Avian Disease and Oncology Laboratory (ADOL, East Lansing, Michigan). ALV-J propagation procedures for the DF-1 cell line followed procedures described previously for ALV propagation and detection (Fadly et al., 1998). Briefly, DF-1 cells were suspended in supplemented DMEM media (Sigma Chemical, St. Louis, MO) containing 2 μg/ml of DEAE at a cell concentration of 3.5×10^5 cells/ml. Cells were transfered to 75-cm^2 flasks and incubated until confluent monolayers were observed (approx 16 to 18 hrs). ADOL-7501 was inoculated on confluent monolayers and cell free supernatant was collected for virus stock 5-7 days post-inoculation. The virus was titrated, aliquoted and frozen in liquid nitrogen before use in the MO pre-treatment assays. Infected monolayers were

sub-cultured a total of three passages before utilization in the MO post-treatment assays.

Morpholinos (MOs). A total of five MOs were designed and targeted towards consensus sequence of the ALV-J strains ADOL-7501 and HPRS-103 (GenBank accession numbers AY027920 and Z46390 respectively) and are summarized in Table 10. Primer Select (Dnastar Inc., Madison, WI) was used to analyze designed MOs in order to ensure the lack of MO excessive or unwanted dimers and/or hairpins. The twenty-five genetic letter length MOs were synthesized and provided by Gene Tools, LLC (Corvallis, OR). Each 300 nMole MO was re-suspended in 600 μl of RNase/DNase free water to give a stock solution of 0.5mMolar. The random 100 nMole MO 6 control was supplied by Gene Tools and was the was re-suspended in 200 μl of RNase/DNase free water to give a stock solution of 0.5 mMolar. MOs 1 and 2 were targeted against the two small open reading frames (ORFs) upstream from the main ORF to which MO 3 was targeted. MO 3 also targeted the only ALV splice site for the single polyadenylated mRNA. MO 4 was targeted to the tRNA primer binding site (PBS) immediately downstream of the U5 – IR_S stem. MO 5 was also targeted to the PBS and designed to overlap MO 4 approximately 11 bp downstream from the MO 4 5' end. MO 1, 2 , 3 and 6 were all tagged at the 3' end with carboxyfluorescein. Gene Tools LLC provided reagents and instructions for a 'Special Delivery' protocol for all MOs. This consisted of the MOs paired to a complementary DNA which when applied was bound electrostatically to a

weakly-basic ethoxylated polyethylenimine (EPEI) 20 minutes before application. This allowed for efficient endocytosis of the MO/DNA/EPEI complex.

***In vitro* delivery.** Intracellular delivery was assessed for the carboxyfluoroscein tagged MO 3. DF-1 cells were cultured on Lab-Tek® Chamber Slides (Nunc, Naperville, IL) until a confluent monolayer was observed. MO 3 was prepared at recommended concentration (1 μM) in three different mediums: 1) With 'Special Delivery' components 2) with 'Special Delivery' components in the presence of 20% fetal calf serum and 3) with MO 3 alone. Twenty-four hours post-delivery, coverslips were removed, rinsed three times with PBS and fixed in 100% methanol and observed under direct UV microcopy for fluorescence.

***In vivo* delivery and toxicity assessment.** Delivery and toxicity of the MOs alone and with 'Special Delivery' components were evaluated in day of age SPF chickens. 0.2 ml of the MO 3 alone, with special delivery components or special delivery components alone were administered at 10 μM and 1 μM MO concentrations via oral and intratracheal (IT) routes. 24 hrs post administration, the chicks were necropsied and esophagus, proventriculus, trachea and lungs were taken for histopathological analysis.

For delivery analysis the tracheas and esophagus were collected separately, minced and trypsinized in a 0.25% trypsin solution in phosphate buffer solution (PBS) and 0.02% EDTA for approximately 10 minutes. Trypsinized cells were centrifuged and re-suspended in 1 ml. Approximately 100

μl of the prepared solution was delivered to a cytofunnel slide chamber and placed on a Cytospin 3 Cytocentrifuge (Thermo Shandon, Pittsburgh, PA) for 5 min at 1000 rpm. Delivered cells on the cytofunnel slide were analyzed for fluorescence.

MO pre-treatment cell culture assay. Gene Tools, LLC, provided preparation and delivery protocols of the MOs and the following adaptations were performed. Briefly, for a 24-well plate preparation, each MO (5.6 μl of stock MO/DNA) was prepared with the appropriate 'Special Delivery' solution (5.6 μl of 200 μM stock EPEI) and RNase/DNase free sterile water, vortexed and incubated at room temperature for 20 min. After incubation, 1.8 ml of media was added to the MO mixture. One half ml of the final MO/Special Delivery/Media mixture was added in triplicates to a confluent monolayer of DF-1 cells. After 3 hours of incubation, the complete delivery solution was removed and replaced with DMEM maintenance media supplemented with 1% FCS. Sixteen to eighteen hours after administration, media was removed and ADOL-7501 was added at a high multiplicity of infection (HMOI) of 1 and a low MOI of 0.01. Twenty-four hours post-inoculation, media was removed and replaced with 2.0 ml/well of fresh DMEM maintenance media. One hundred and twenty hours post-inoculation, cell free supernatant was removed and initially screened for p27 CA reduction by ac-ELISA followed by LightCycler® viral RNA and endpoint dilution assessments.

MO post-treatment cell culture assay. ALV-J (ADOL-7501) infected DF-1 monolayers were sub-cultured a total of three passages before utilization in the MO post-treatment assays. Cell free supernatant after the second complete passage had a titer of $10^{6.5}$ IU/ml. DF-1 cells were transferred to 24-well plates. After a 80%-100% confluent monolayer was observed (16-18 hours), as described for the pre-treatment assay, the appropriate MOs were replicated in a total of five in treatments, incubated for 3 hours, removed and replaced with 2.0 ml/well of DMEM maintenance media supplemented with 1% FCS. Media was removed at 48, 96 and 144 hrs post-treatment and subsequently replenished then initially screened for p27 CA reduction by ac-ELISA followed by LightCycler® viral RNA and endpoint dilution assessments.

p27 ac-ELISA. Two hundred μl of the cell free supernatant sample was transferred to U-bottomed microtiter plates and 12.5 μl of 5% Tween 80 solution was added to each sample before the plates were frozen and thawed two times. One hundred μl of this prepared material was collected for ac-ELISA assay. Avian leukosis virus p27 ac-ELISA assay readings were performed and S/P ratios were calculated following manufacturers recommendations (IDEXX Laboratories, Westbrook, Maine). Viral growth as measured by p27 ac-ELISA was expressed in percentage and was obtained by dividing the averaged MO treatment S/P ratio by the average S/P ratio obtained for the MO control.

RNA extraction. All reagents and supplies were provided by the QIAamp® Viral RNA Kit (Qiagen, Valencia, CA). Triplicates from the pre-treatment assay and

the set of five samples from the post-treatment assay were pooled. One hundred and forty μl of pooled cell free infected and/or non-infected cell culture media from each group was used to extract ALV RNA. Protocols provided by the QIAamp viral RNA mini spin protocol were utilized as recommended. ALV RNA was eluted with 60 μl of RNase/DNase free water.

Real time LightCycler® RT-PCR. For the RT-PCR reaction, a commercial Roche Light Cycler-RNA Amplification Kit SYBR Green I (Roche Diagnostics Corp., Indianapolis, IN) was used. All reagents with the exception of the H5 and H7 ALV-J specific primers previously described (Smith et al., 1998), were provided with the commercial kit Two *u*l of DNase-RNase free water, 4 *μ*l of reaction mix (SYBR Green I, 5x), 1 *u*l of the forward primer H5 (ATAGAGCCAGAGGCACCT), 1*u*l of the reverse primer H7 (CATAGCTTCGTCTACGCCCATA), 1.6 *u*l of $MgCl_2$ (25mM) and 0.4 *u*l of enzyme mix were used per 10 *u*l of RNA sample. The final mixture was deposited in 20 *u*l capillaries (Boehringer Mannheim GmbH, Germany). RT was conducted for 10 min at 55 C. The PCR reaction included a denaturation period at 95 C for 30 seconds and 45 cycles of denaturation at 95 C for 0 seconds, annealing at 55 C for 10 seconds and polymerization at 72 C for 25 seconds. After the 45^{th} cycle, a melting curve analysis was obtained at 65 C for 10 seconds. Samples exhibiting fluorescence peaks at temperatures between 83 C and 85 C were considered positive for ALV-J (Figure 12). A RNA control which was developed by the in vitro transcription of a plasmid inserted with a 545 bp

H5/H7 amplicon of ADOL-7501 and quantified via spectrophotometer analysis was used to establish a viral RNA standard curve (Kim et al., 2000). Approximately 250 fg/μl of RNA control corresponds to 5 x 10^5 viral RNA copies/μl. All the products obtained by using the Light Cycler were run in a 1.5% agarose gel with ethidium bromide to confirm the presence of the 545 bp ALV-J specific products (data not shown).

End point dilution assay. An endpoint dilution assay was performed on pooled cell free supernatant collected from the post-treatment cell culture assays. Supernatant from a single triplicate sample for MOs 4, 5 and 6 was inoculated onto DF-1 monolayers. 10^0 to 10^8 ten-fold dilutions were performed with a total of five treatments per sample. The formula devised by Reed and Muench determined the 50% endpoint (Reed et al., 1938; Villegas, 1998).

Statistical analysis. Statistical analysis was performed using JMP version 3.2.6 software (SAS Institute, Cary, NC). Data was assessed with the non-parametric Kruskal-Wallis test and differences between test groups were analyzed by Dunn's multiple comparison tests. Significance was accepted at ($p < 0.05$) for the average S/P ratio of the MO treatment (1-5) groups compared against the average S/P ratio of the MO control group.

RESULTS

***In vitro* delivery.** Intracellular fluorescence was observed in DF-1 cell culture 24 hrs post-treatment for MO 3 (Figure 13A). A marked reduction in fluorescence was observed for the MO 3 which was incubated and delivered with 20% FCS (Figure 13B). Minimal to background fluorescence was observed for delivery of MO 3 without 'Special Delivery' solution (Figure 13C).

***In vivo* delivery and toxicity assessment.** Observation of the cytofunnel slides under direct UV microscopy (10X objective) revealed no cellular fluorescence. No distinct toxic effects were observed in the trachea, lungs, esophagus, and proventriculus for all experimental groups. The trachea epithelium was complete with normal cilia and no presence of inflammatory infiltrate and the lungs for all experimental and control groups had diffuse mild to moderate congestion. No pathological alterations of mucosal epithelium, no circulatory disturbances and no presence of any inflammatory process were observed for the esophagus. Proventricular glands were normal with no circulatory or inflammatory disturbances.

MO pre-treatment cell culture assay. Triplicate cell free supernatants from each of the MO (1-6) treatments for both HMOI and LMOI were assayed by p27 ac-ELISA and S/P ratios were averaged, compared and assessed for the statistical reduction ($p < 0.05$) of p27 production and are presented in Table 11. No significant differences were observed at HMOI and LMOI among the MO test

groups (1-5) when the average S/P ratios were compared to the MO 6 control. MOs 2 exhibited a non-significant 47% and 31% average reduction in growth for the HMOI and LMOI assays respectively. In addition MO 3 also exhibited a non-significant HMOI 42% and a LMOI 44% reduction in viral growth.

MO post-treatment cell culture assay. No significant differences were observed in the cell free supernatants collected at 48 hrs and 96 hrs post-treatment for all MOs (Table 12). All groups exhibited a marked increase in their S/P ratios from 48 hrs to the 96 hrs collection of cell free supernatant. At 144 hrs post-treatment MOs 4 and 5 exhibited a significant reduction of p27 S/P ratio when compared to the MO 6 control. This corresponded to a 35% and 43% reduction in viral growth for MOs 4 and 5. S/P ratios exhibited at 144 hrs for all treatment groups (MOs 1-5) and virus control were considerably lower than the S/P ratios recorded at 96 hrs.

Real time LightCycler® RT-PCR. A standard curve was established with 10^3 to 10^9 dilutions of quantified 545 bp control RNA (Figure 14). Amounts of ALV-J RNA were calculated based on the standard curve. Quantitative LightCycler® RT- Real time LightCycler® RT-PCR PCR analysis was performed on extracted viral RNA from cell free supernatant for MOs 4, 5 and 6 in both pre-treatment and post-treatment assays (Figures 15 and 16). For the HMOI pre-treatment assay, MO 5 had an approximate 2 log reduction in the amount of viral RNA amplified when compared to the MO 6 control. Both MOs 5 and 6 exhibited an

approximate one log difference when compared to the virus control viral RNA LightCycler® estimation (Figure 15). No differences were observed for the LMOI pre-treatment assay and as predicted by ac-ELISA, the virus control had a viral RNA estimation two logs lower than MO treatments.

LightCycler® RT-PCR PCR analysis for the post-treatment assays included all three collection times (48, 96 and 144 hrs). No log differences were observed between the MO treatments 4 and 5 and the control MO 6 for collection times of 48 hrs and 96 hrs. An approximate 1 log difference was in viral RNA concentration was observed between MO 4 and the control MO 6 for the collection time of 144 hrs (Figure 16).

End point dilution assay. A one log difference in the amount of infectious units was observed for the pooled MO 4 treatment group when compared to the virus control and the treatment and control groups MO 5 and 6, respectively (Table 13). The MO 5 treatment and MO6 control groups had infectious units detected by ac-ELISA of approximately 10^7 and equal (no greater than 0.5 log difference) to the virus control.

DISCUSSION

MOs designed for this experiment were based on previously published successful antisense oligo design and regions within the retrovirus genome that are known and/or have been shown to abrogate replication or infection. MO 1 and 2 were targeted towards the first and third small ALV ORF because of

alterations to one or both of these reading frames has been shown to inhibit viral propagation (Moustakas et al., 1993). In addition, these two small ORFs have been implicated in attenuating translation downstream as a possible internal ribosome entry site (Deffaud et al., 2000). If successful as an antisense oligomer, the effects of MO 3 which was targeted to the main ORF and in addition the downstream splice site for the single polyadenylated mRNA transcript would obvious avert translation and prevent the formation of the spliced mRNA necessary for the *env*. MOs 4 and 5 were targeted to the PBS, where other antisense oligos have been shown to successfully abrogate replication and infection in both lentiviruses and ALSVs (Bordier et al., 1992; Kim et al., 1998; Lee et al., 1998; Wei et al., 2000). Chadwick and Lever were able to demonstrate a 10^6 TCID50/ml reduction in replication with antisense RNA targeted at the HIV-1 PBS, while Kim et al., were able to demonstrate up to a 70% reduction in p27 ac-ELISA titer of ALV infected cells (Chadwick et al., 2000; Kim et al., 1998).

In this study MOs were successfully delivered intracellulary without visual toxic effects into DF-1 cells as determined by fluorescence via the 'Special Delivery' (MO/DNA/EPEI complex) solution provided by the manufacturers. In addition, *in vivo* delivery into day of age chicks at a 10 μM MO concentration resulted in no observations of toxic effects histopathologically. Although no delivery was noted by fluorescence in trachea or esophageal mucosa (probably due to lack of assay sensitivity), the lack of toxic effect would warrant additional

studies focusing on improving the efficacies and analysis via flow cytometry of *in vivo* delivery.

The MOs 2, 3, 4 and 5 were able to reduce the production of p27 in DF-1 cell culture ranging from 31% to 43%. However, only MOs 4 and 5 significantly reduced the production of p27 ($p < 0.05$) when measured by ac-ELISA. Kim et al. reported similar findings, where the only significant reduction of p27 as recorded by ac-ELISA was accomplished by the antisense oligo designed towards the PBS (Kim et al., 1998).

Real time quantitative LightCycler® RT-PCR assays which were performed on pooled samples from both the pre-treatment and post-treatment assays also confirmed a minimal reduction of viral RNA concentration (one to two logs) in the MO 4 and 5 treatment groups. Viral RNA concentration (viral RNA copies/μl) determined by real time quantitative LightCycler® RT-PCR correlated with the one log reduction in infectious units/μl observed via endpoint dilutions for MO 4. Similar results were reported when the the real time quantitative LightCycler® RT-PCR assay was utilized to correlate the ALV-J virus $TCID_{50}$ titer in cells (IU/μl) and viral RNA copies/μl (Kim et al., 2000)

All together, the inhibition of p27 production as recorded by ac-ELISA for the PBS MO 4 and 5 was not as substantial as what was reported by Kim et al., 1998. These findings were disappointing considering the superior antisense efficiencies of MOs. Due to efficient delivery of MOs via the 'Special delivery' solution, a delivery concentration of 1 μM is recommended and has successfully been utilized as an antisense oligo (Morcos, 2001). However, effective inhibition

of retroviral replication as reported by Chadwick and Lever and Kim et al., has been accomplished with concentration deliveries of 10 μM or greater by other antisense oligos (Chadwick et al., 2000; Kim et al., 1998). Therefore, due to the benefits of MO utilization, it would be advantageous to examine the potential and limitations of MO antisense applications in similar experiments at higher concentrations.

Table 10. Sequence, position and targets of test and control morpholinos (MOs). Nucleotide position based on HPRS-103 (GenBank accession number Z46390).

MOs	Target	Nucleotide position	Sequence
1	ORF-1	260-284	GGAATCGACGGTCCGGCCATCAACC
2	ORF-3	402-426	GGTCATCGTCCGCTCCCCGAATAAG
3	Main ORF	602-626	ATCACCTTTATGACGGCTTCCATAC
4	PBS	323-347	AACTATCACGTCGGGGTCACCAAAT
5	PBS	334-358	CACTATTCCCTAACTATCACGTCGG
6	Control	Random	CCTCTTACCTCAGTTACAATTTATA

Table 11. Assessment of ADOL-7501 viral growth in DF-1 cells pre-treated with the identified MOs (1-6) and inoculated at different MOIs. The average S/P ratios of the triplicate samples are shown. Statistical differences were extrapolated from comparisons of each MO (1-5) averaged S/P ration versus the control MO (6) S/P ratio and were analyzed by Dunn's multiple comparison test ($p < 0.05$).

	p27 ac-ELISA S/P ratios						
MOI	1	2	3	4	5	6	Virus control
1	.708	.397	.428	.838	.633	.736	.661
0.01	.654	.379	.304	NR[1]	NR[1]	.542	.347

[1]NR indicates that a S/P ratio cannot reliably be estimated due to little or no viral replication observed in the test.

Table 12. Assessment of viral growth in DF-1 cells pre-inoculated with $10^{6.5}$ IU/ml of ALV-J strain ADOL-7501 and sub-cultured three times before treatment with the identified MOs. The average S/P ratios of the triplicate samples are shown and statistical differences between test groups were analyzed by Dunn's multiple comparison test ($p < 0.05$) are extrapolated from comparisons of each MO (1-5) averaged S/P ratio versus the control MO (6) S/P ratio.

	p27 ac-ELISA S/P ratios at		
MOs	24-48 hr	48-96 hr	96-144 hr
1	1.74	2.13	1.95
2	1.77	1.99	1.76
3	1.82	2.14	1.79
4	1.49	2.23	1.50[A]
5	1.45	2.29	1.30[A]
6	1.60	2.05	2.28
Infected cells	1.87	2.10	1.62

[A]Significant p27 reduction when compared to the MO 6 control ($p < 0.05$).

Table 13. Endpoint dilution assays for post-treatment groups MO 4-6 and virus control. Cell free supernatant from the appropriate treatment was diluted, inoculated and incubated in the DF-1 cell line for 7 days. Cell free supernatant was collected and p27 was detected via ac-ELISA using a positive S/P cutoff ratio of 0.1. 50% endpoints for DF were determined using Reed and Muench calculations.

Treatment	DF-1
MO 4	$10^{-6.2}$
MO 5	$10^{-6.6}$
MO 6	$10^{-7.2}$
Virus control	$10^{-7.0}$

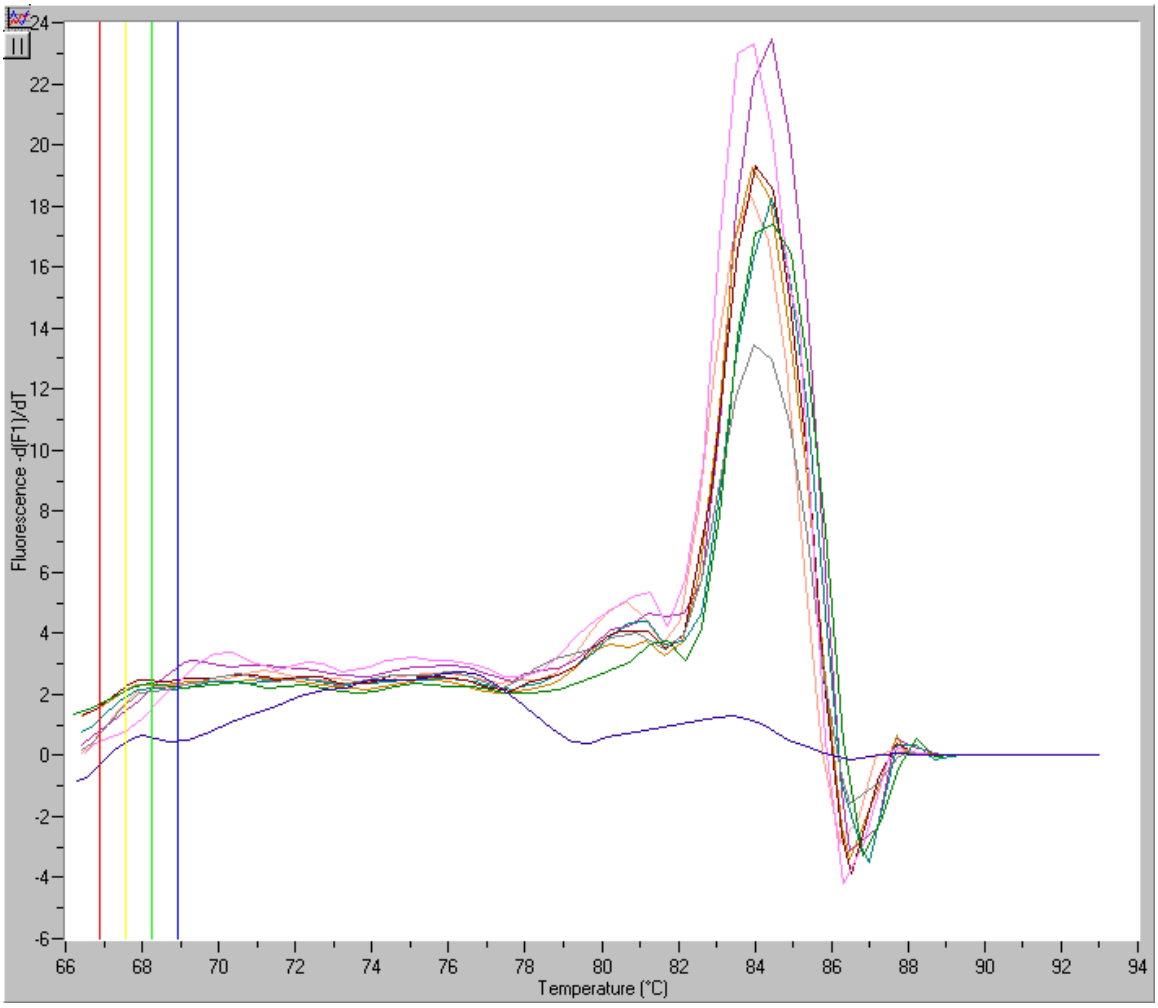
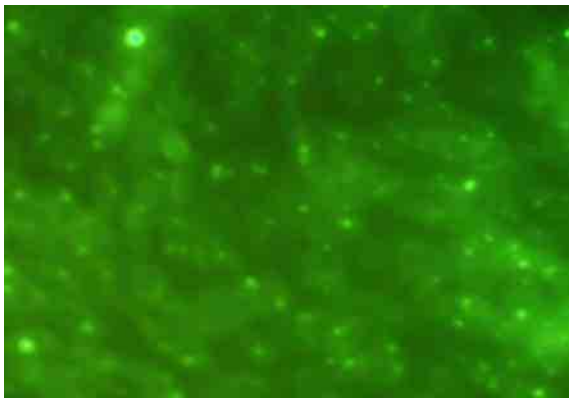


Figure 12. LightCycler® melting curve analysis of amplified ADOL 7501 viral RNA from eight different treatment groups. Cell control extraction is exhibited by the flat violet line at the base of the compact melting curve (-d(F1)/dt fluorescence versus temperature (°C)).

A)



B)

C)

Figure 13. Direct UV microscopic fluorescence (400X) detected in DF-1 cells 24 hrs post-treatment of MO 3 in different mediums. A) MO 3 with 'Special Delivery' components (B) MO 3with 'Special Delivery' components in the presence of 20% fetal calf serum and (C) MO 3 alone without any 'Special Delivery' components.

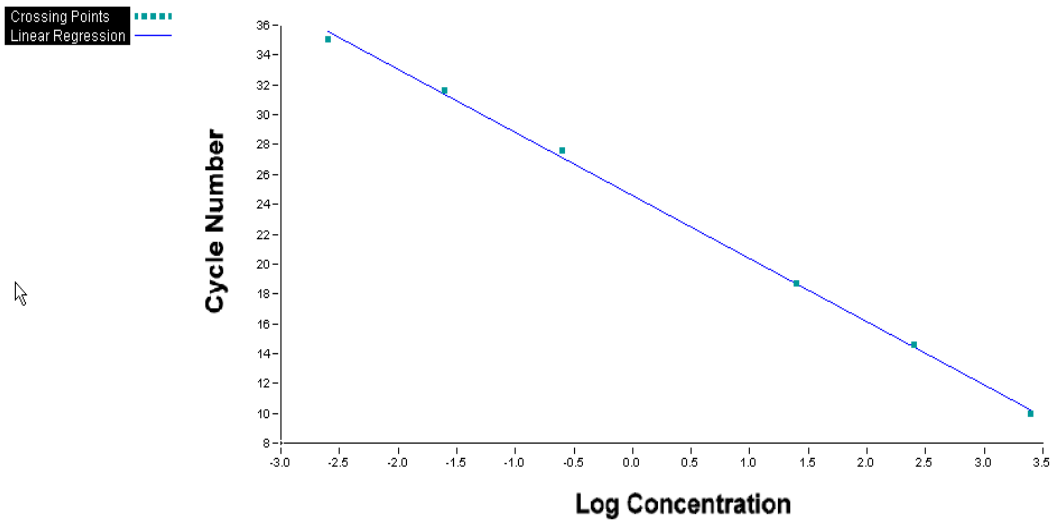


Figure 14. Second derivative maximum standard curve utilizing an arithmetic baseline adjustment and showing cycle number versus log concentration of standard RNA. The regression line is linear and cycle numbers are correlated with starting RNA quantity. Standard curve has a Slope = -4.228, Y-intercept = 24.61, an error = 0.688 and a correlation coefficient = -1.0.

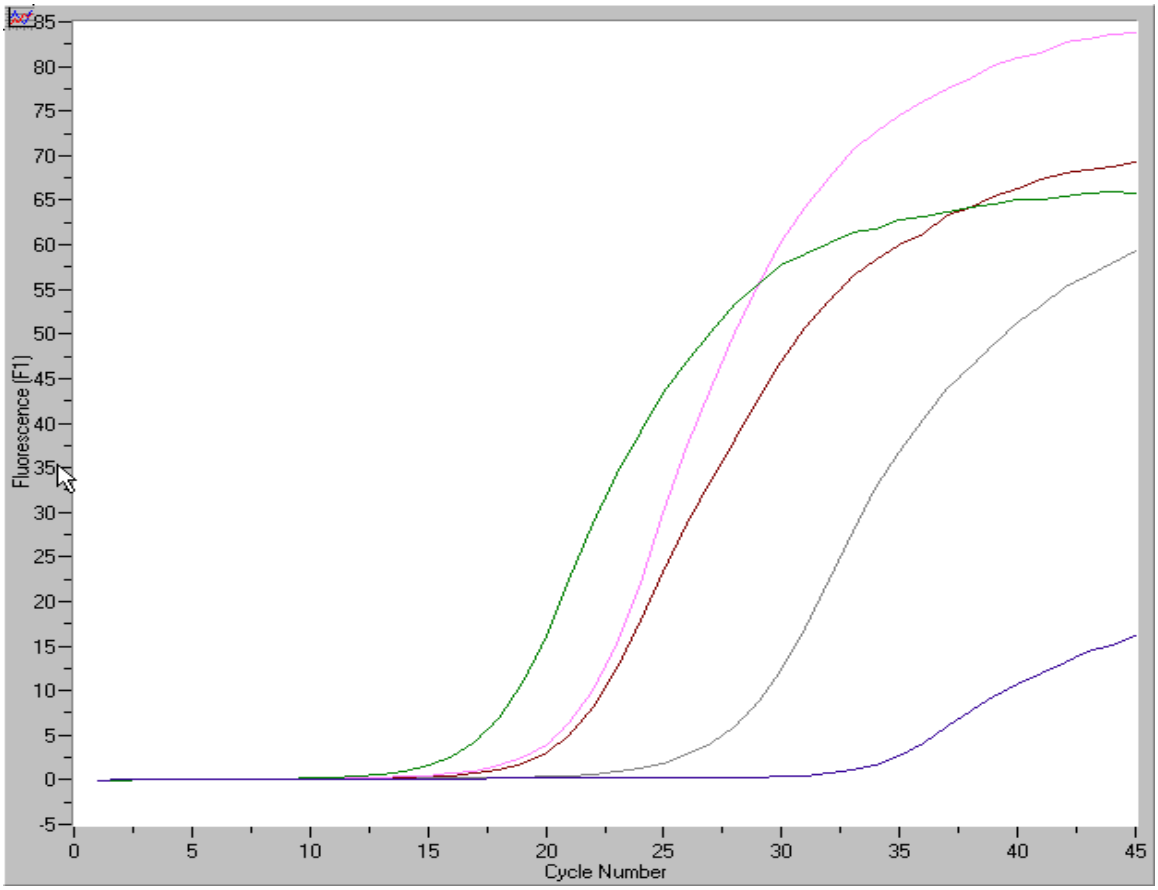


Figure 15. LightCycler® amplification plot of ADOL-7501 viral RNA (fluorescence versus cycle number). Assessment of ADOL-7501 viral RNA from cell free supernatant of DF-1 cells pre-treated with the MOs 4-6 and inoculated at a HMOI. MO 4 = brown line; MO 5 = gray line; MO 6 = pink; virus control = green line and cell control = blue line.

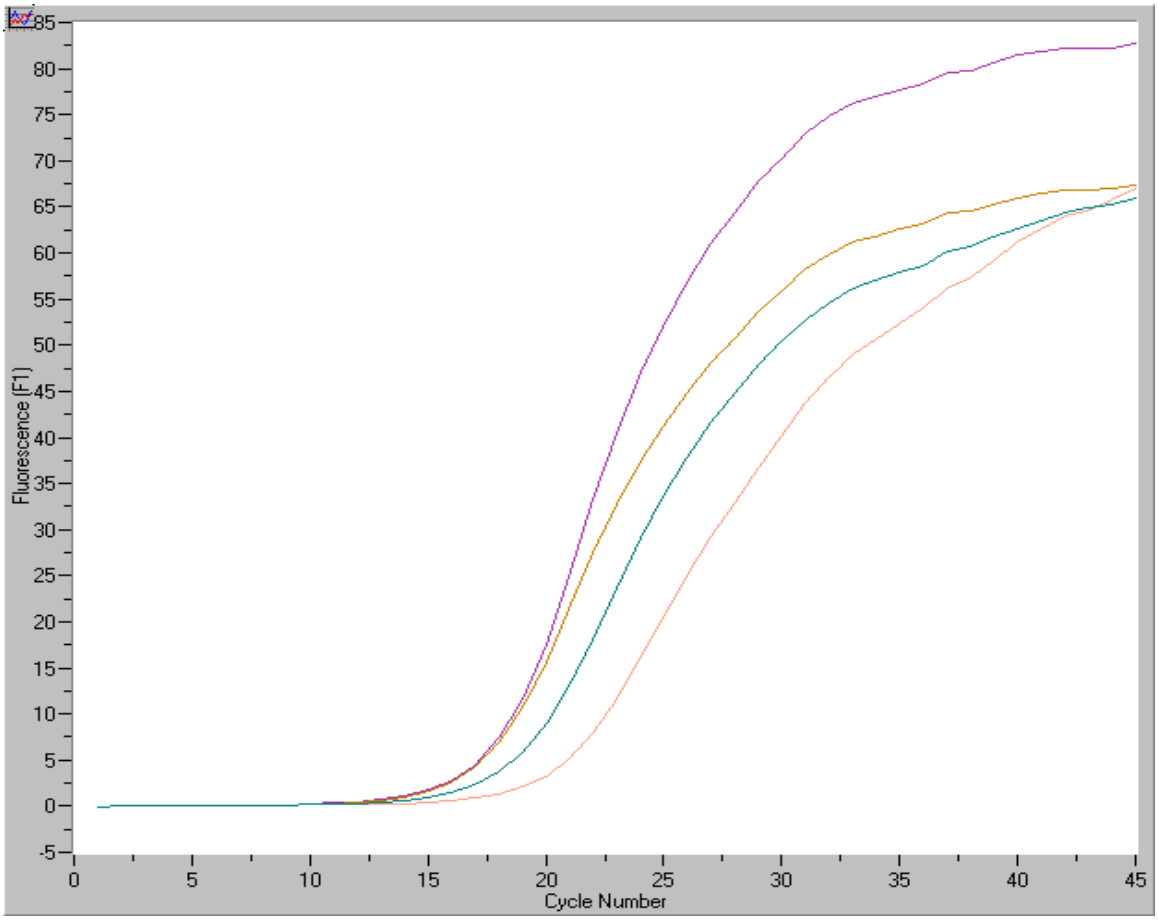


Figure 16. LightCycler® amplification plot of ADOL-7501 viral RNA (fluorescence versus cycle number). Assessment of ADOL-7501 viral RNA from cell free supernatant of DF-1 cells collected 144 hrs post-treatment. The DF-1 cells were infected with ALV-J and sub-cultured three times prior to treatment with the MOs 4-6. MO 4 = Georgia peach line; MO 5 = violet; MO 6 = Texas sunset orange; virus control = green line.

REFERENCES

ARCHAMBAULT, D., STEIN, C.A., and COHEN, J.S. (1994). Phosphorothioate oligonucleotides inhibit the replication of lentiviruses and type D retroviruses, but not that of type C retroviruses. Arch Virol **139**(1-2)**,** 97-109.

BORDIER, B., HELENE, C., BARR, P.J., LITVAK, S., and SARIH-COTTIN, L. (1992). In vitro effect of antisense oligonucleotides on human immunodeficiency virus type 1 reverse transcription. Nucleic Acids Res **20**(22)**,** 5999-6006.

CHADWICK, D.R., and LEVER, A.M. (2000). Antisense RNA sequences targeting the 5' leader packaging signal region of human immunodeficiency virus type-1 inhibits viral replication at post-transcriptional stages of the life cycle. Gene Ther **7**(16)**,** 1362-8.

DEFFAUD, C., and DARLIX, J.L. (2000). Rous sarcoma virus translation revisitied: Characterization of an internal ribosome entry segment in the 5' leader of the genomic RNA. J Virol **74**(24)**,** 11581-11588.

FADLY, A.M. (2000). Isolation and identification of avian leukosis viruses: a review. Avian Pathol **29**(6)**,** 529-535.

FADLY, A.M., and WITTER, R. (1998). Oncornaviruses: Leukosis/ Sarcoma and Reticuloendotheliosis. 4th ed. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, Eds.),

(American Association of Avian Pathologists, Kennett Square, PA), pp. 185-196.

GHOSH, C., and IVERSEN, P.L. (2000). Intracellular delivery strategies for antisense phosphorodiamidate morpholino oligomers. Antisense Nucleic Acid Drug Dev **10**(4)**,** 263-74.

HILDINGER, M., DITTMAR, M.T., SCHULT-DIETRICH, P., FEHSE, B., SCHNIERLE, B.S., THALER, S., STIEGLER, G., WELKER, R., and LAER, D.V. (2001). Membrane anchored peptide inhibits human immunodeficiency virus entry. J Virol **75**(6)**,** 3038-3042.

IVERSEN, P.L. (2001). Phosphorodiamidate morpholino oligomers: Favorable properties for sequence-specific gene inactivation. Current Opinion in Molecular Therapeutics **3**(3)**,** 235-238.

KIM, K.E., SALTER, D.W., and DODGSON, J.B. (1998). Examination of antisense RNA and oligodeoxynucleotides as potential inhibitors of avian leukosis virus replication. Poult Sci **77,** 1400-1410.

KIM, Y., BROWN, T.P., GHARAIBEH, S., STEDMAN, N.L., and PANTIN, M.J. (2000). *137th AVMA/AAAP Annual Convention, Salt Lake City*.

LEE, R., KAUSHIK, N., MODAK, M.J., VINAYAK, R., and PANDEY, V.N. (1998). Polyamide nucleic acid targeted to the primer binding site of the HIV-1 RNA genome blocks in vitro HIV-1 reverse transcription. Biochemistry **37**(3)**,** 900-10.

MORCOS, P.A. (2001). Achieving efficient delivery of morpholino oligos in cultured cells. Genesis **30**(3)**,** 94-102.

MOUSTAKAS, A., SONSTEGARD, T.S., and HACKETT, P.B. (1993). Alterations of the three short open reading frames in the rous sarcoma virus leader RNA modulate viral replication and gene expression. J Virol **67**(7)**,** 4337-4349.

ORR, R.M. (2001). Technology evaluation: Farmivirsen, Isis Pahrmaceuticals Inc/CIBA Vision. Current Opinion in Molecular Therapeutics **3**(3)**,** 288-294.

PAYNE, L.N. (2000). *International Symposium on ALV-J and other avian retroviruses, Rauischholzhausen, Germany*.

PAYNE, L.N., BROWN, S.R., BUMSTEAD, N., HOWES, K., FRAZIER, J.A., and THOULESS, M.E. (1991). A novel subgroup of exogenous avian leukosis virus in chickens. J Gen Virol **72**(Pt 4)**,** 801-7.

PAYNE, L.N., and FADLY, A.M. (1997). "Leukosis/Sarcoma Group." 10th ed. Diseases of Poultry (B. W. Calnek, J. Barnes, C. Beard, L. McDougald, and M. Saif, Eds.) Iowa State University Press, Ames.

REED, L.J., and MUENCH, H. (1938). A simple method for estimating fifty percent endpoints. Am J Hyg **27,** 493-497.

SMITH, L.M., BROWN, S.R., HOWES, K., MCLEOD, S., ARSHAD, S.S., BARRON, G.S., VENUGOPAL, K., MCKAY, J.C., and PAYNE, L.N. (1998). Development and application of polymerase chain reaction (PCR) tests for the detection of subgroup J avian leukosis virus. Virus Res **54**(1)**,** 87-98.

STEPHENSON, M.L., and ZAMECNIK, P.C. (1978). Inhibition of Rous sarcoma Viral RNA Translation by a Specific Oligodeoxyribonucleotide. Proc Natl Acad Sci U S A **75**(1)**,** 285-288.

SUMMERTON, J. (1999). Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim Biophys Acta **1489**(1)**,** 141-58.

SUMMERTON, J., and WELLER, D. (1997). Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev **7**(3)**,** 187-95.

USHIJIMA, K., SHIRAKAWA, M., KAGOSHIMA, K., PARK, W.S., KUROSAKI, N.M., and TAKAKU, H. (2001). Anit-HIV-1 activity of an Antisense phosphorothioate oligonucleotide bearing imidazole and primary amine groups. Biorganic and Medicinal Chemistry **9,** 2165-2169.

VILLEGAS, P. (1998). Titration of Biological Suspensions. 4TH ed. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (D. E. Swayne, J. R. Glisson, D. J. Jackwood, J. E. Pearson, and W. M. Reed, Eds.), (American Association of Avian Pathologists, Kennett Square, PA), pp. 248-254.

WEI, X., GOTTE, M., and WAINBERG, M.A. (2000). Human immunodeficiency virus type-1 reverse transcription can be inhibited in vitro by oligonucleotides that target both natural and synthetic tRNA primers. Nucleic Acids Res **28**(16)**,** 3065-74.

WITTER, R.L., BACON, L.D., HUNT, H.D., SILVA, R.E., and FADLY, A.M. (2000). Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. Avian Dis **44**(4)**,** 913-31.

ZAMECNIK, P.C., and STEPHENSON, M.L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A **75**(1)**,** 280-284.

CHAPTER VI

DISCUSSION and CONCLUSIONS

Virus isolation and nested RT-PCR sensitivity. No statistical differences ($p<0.05$) were observed between the DF-1 cell line and C/E CEFs, virus propagation in DF-1 cells allowed for the identification of more positive plasma samples at both S/P ratios (0.2 and 0.1). A total of 16 more positive samples were identified by ac-ELISA with a positive S/P cutoff ratio of 0.2 and the number was reduced to 13 more positive samples identified with a positive S/P cutoff ratio of 0.1 after propagation in DF-1 cells. Virtually no distinct differences were observed when limited dilution assays were performed using ALV-J positive samples with relatively high S/P ratios for both the DF-1 and C/E cell culture.

In this study, ALV-J specific nested RT-PCR was initially used as a confirmation assay in order to verify the numerous positives for group D identified by both cell culture systems. However, it was found that nested RT-PCR performed directly on plasma samples without propagation in cell culture was significantly ($p<0.05$) more sensitive than virus propagation in DF-1 cells coupled with detection by ac-ELISA with a positive S/P cutoff ratio of 0.2. However, this significance was lost when a positive S/P cutoff ratio of 0.1 was used after samples for group D were propagated in DF-1 cells. Nested RT-PCR was able to identify 14 more ALV-J positives for group D with a positive S/P cutoff ratio of

0.2 and 11 more ALV-J positives for group D with a positive S/P cutoff ratio of 0.1.

The control of ALV infections mainly depends on the early detection and removal of virus shedding birds to reduce the spread of congenital and contact infection to other birds. Propagation in phenotype selected CEFs and detection by p27 ac-ELISA is the most commonly used assay for the identification of exogenous ALVs. As a result of the abundant use of this assay for identification and detection of exogenous ALVs by the poultry industry and select research laboratories, line 0, C/E phenotype embryos are commercially available at a premium price. These embryos often have inconsistencies in viability and livability and must be incubated no less than 8-9 days in order to produce ample numbers of CEFs. In addition, two rounds of cell culture trypsinization must occur in order to eliminate the majority of epithelial cells from and end of with a predominately homogenous population of secondary CEFs. The use of a continuous cell line such as DF-1 would eliminate the difficulties associated with the production of secondary CEFs. It would therefore be advantageous for companies involved in ALV screening to adjust established cell culture protocols towards the utilization of the DF-1 continuous cell line.

DF-1 cells share similar morphological, viability and maintenance characteristic with C/E CEF cell culture when used for ALV diagnostic purposes. All together, the DF-1 cell line is easier to manage, upkeep, propagate and store. The utilization of the DF-1 cell line for ALV-J diagnostic screening would be highly cost effective when compared to the use of C/E cell culture. In addition,

the combinational use of an established molecular assay such as the nested RT-PCR presented here after sample propagation in the DF-1 cell line, would assist in the confirmation of positive samples with suspect S/P ratios along with the identification of virus propagation/ac-ELISA false negatives.

Nested RT-PCR ALV-J positive plasma samples undetected by virus isolation. The 3' LTR nested RT-PCR assay performed directly on plasma samples was a more sensitive assay than virus isolation in DF-1 cell coupled with ac-ELISA for the detection of ALV-J. Approximately 14.5% more ALV-J positives were identified by nested RT-PCR. A correlation was observed between positives identified by DF-1 virus isolation and nested RT-PCR. Although few false negatives were identified for nested RT-PCR by DF-1 virus isolation, these numbers are probably attributed to non-mechanical RNA extraction methodologies, which can be easily resolved by commercial kits and applications. This assay allows for the rapid detection and identification of exogenous ALVs and can be readily applied in a diagnostic setting to enhance screening of ALV-J infected flocks.

The larger issue at hand that needs to be addressed when nested RT-PCR is compared to virus isolation coupled with ac-ELISA for the detection of ALV-J is the greater sensitivity that is consistently observed with molecular assays. The identification of VI- viral RNA in samples has been observed for both HIV and dengue. In both instances, the presence of neutralizing antibodies has played a role in minimizing detection by virus isolation and ac-ELISA. Our

observations show that plausible neutralizing antibodies identified by ALV-J antibody ELISA minimized endpoint detection when compared to nested RT-PCR. Differences as great as 100-fold was observed between virus isolation and nested RT-PCR. Further substantiation for possible antibody interference of virus detection was recognized when antibody negative serum was analyzed via limited dilution assays, no reduction in end point detection was observed.

Methodologies to enhance virus infectivity assays have been well established and in animal virology physical particle:infectivity ratios as low as 100-1000:1 are considered respectable. However, when this ratio falls below or even hovers around these respectable numbers, infectivity is not readily achieved. Assays that rely on HIV-1 protein production, reverse transcriptase activity or cell viability do not directly enumerate the number of infectious events in a cell culture and are limited if the level of virus replication is low. The same ought to be assumed for ALSVs due to several events that can happen during replication that could render a large population of viral particles non-infectious. One example would be read through transcripts which contain nonviral sequence at their 3' end have been predicted to be in up to 32% of wild type ALSV particles and virus replication has been shown to be reduced up to six times less in poly-adenlyated mutated ALSV when compared to wild type viruses.

The role of proper processing of ALSV capsid proteins via cleavage events that occur at identified amino acid residues within the SP region between the CA and NC in the *gag* polyprotein have been shown to be required for infectivity of ALSV infectious molecular clones. In addition, viral DNA synthesis

defects have been observed in assembly competent Rous sarcoma MHR CA mutants. Although no amino acid substitutions were observed in the MHR and the sequence for the entire CA gene was highly conserved among field isolates and other exogenous ALVs, several amino acid residue substitutions were identified for ALV-J nested RT-PCR positive samples within the SP region. For two of the samples, (17VI- and 20VI-) amino acid substitutions occur at single residue sites that have been shown to abrogate infectivity of molecular infectious clones of Rous sarcoma induced by site directed mutagenesis. Amino acid substitutions observed for 16 VI- and 30 VI- have not been characterized by infectious molecular clone studies.

Nested RT-PCR would be continuously advantageous over virus isolation for the detection of ALV-J at the onset or latter stages of infection when viremia is low and replication and infectious factors mentioned above could possibly diminish ALV-J infectivity in cell culture. This assay allows for the rapid detection and identification of exogenous ALVs undetected by virus isolation coupled with ac-ELISA and can be readily applied in a diagnostic setting to enhance screening of ALV-J infected flocks.

Morpholinos. MOs designed for this experiment were based on previously published successful antisense oligo design and regions within the retrovirus genome that are known and/or have been shown to abrogate replication or infection. MO 1 and 2 were targeted towards the first and third small ALV ORF because of alterations to one or both of these reading frames has been shown to

inhibit viral propagation. In addition, these two small ORFs have been implicated in attenuating translation downstream as a possible internal ribosome entry site. If successful as an antisense oligomer, the effects of MO 3 which was targeted to the main ORF and in addition the downstream splice site for the single polyadenylated mRNA transcript would obvious avert translation and prevent the formation of the spliced mRNA necessary for the *env*. MOs 4 and 5 were targeted to the PBS, where other antisense oligos have been shown to successfully abrogate replication and infection in both lentiviruses and ALSVs.

In this study MOs were successfully delivered intracellulary without visual toxic effects into DF-1 cells as determined by fluorescence via the 'Special Delivery' (MO/DNA/EPEI complex) solution provided by the manufacturers. In addition, *in vivo* delivery into day of age chicks at a 10 μM MO concentration resulted in no observations of toxic effects histopathologically. Although no delivery was noted by fluorescence in trachea or esophageal mucosa (probably due to lack of assay sensitivity), the lack of toxic effect would warrant additional studies focusing on improving the efficacies and analysis via flow cytometry of *in vivo* delivery.

The MOs 2, 3, 4 and 5 were able to reduce the production of p27 in DF-1 cell culture ranging from 31% to 43%. However, only MOs 4 and 5 significantly reduced the production of p27 ($p < 0.05$) when measured by ac-ELISA.

Real time quantitative LightCycler® RT-PCR assays which were performed on pooled samples from both the pre-treatment and post-treatment assays also confirmed a minimal reduction of viral RNA concentration (one to two logs) in the

MO 4 and 5 treatment groups. Viral RNA concentration (viral RNA copies/μl) determine by real time quantitative LightCycler® RT-PCR correlated with the one log reduction in infectious units/μl observed via endpoint dilutions for MO 4.

All together though, the inhibition of p27 production as recorded by ac-ELISA for the PBS MO 4 and 5 was not as substantial as what was reported by Kim et al., 1998. These findings were disappointing considering the superior antisense efficiencies of MOs. Due to efficient delivery of MOs via the 'Special delivery' solution, a delivery concentration of 1 μM is recommended and has successfully been utilized as an antisense oligos.

However, effective inhibition of retroviral replication has been accomplished with concentration deliveries of 10 μM or greater by other antisense oligos. Therefore, it due to the benefits of MO utilization, it would be advantageous to examine the potential and limitations of MO antisense applications in similar experiments at higher concentrations.