PATHOGENICITY, TRANSMISSION AND MOLECULAR CHARACTERIZATION OF RETICULOENDOTHELIOSIS VIRUS STRAIN APC-566

by

TAYLOR MARCELO CORREA BARBOSA

(Under the Direction of Pedro Villegas)

ABSTRACT

Reticuloendotheliosis virus (REV) infection is associated with runting, mortality, immunosuppression, and chronic neoplasia with T and/or B cell lymphomas in a variety of avian species. The pathogenicity and transmission of a newly identified isolate from an Attwater's Prairie Chicken (APC-566) was studied using an experimental model in Japanese quail. REV infection was associated with higher mortality and decreased egg production, hatchability, and fertility in infected quail compared to uninfected quail. A deleterious effect on body weight gain was observed in infected breeder quail and their progeny. REV APC-566 was oncogenic in quail, chickens and turkeys and the majority of their REV-associated lymphosarcomas contained CD3+ cells. REV-infected turkeys expressed reduced serological responses against standard inactivated vaccine antigens. The complete proviral sequence of the REV APC-566 was determined. This isolate had higher similarity with a REV genome inserted into a Fowlpox virus. An immunosuppressive peptide region located within the envelope gene was 100% identical in all sixteen REV isolates sequenced, including viruses from wild and commercial birds.

INDEX WORDS: Reticuloendotheliosis virus, REV APC-566, Retrovirus, Japanese quail, pathogenicity, transmission, immunosuppression, performance data, sequencing, full genome.

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TAYLOR MARCELO CORREA BARBOSA

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TAYLOR MARCELO CORREA BARBOSA

Major Professor: Dr. Pedro Villegas

Committee: Dr. Guillermo Zavala
Dr. Maricarmen Garcia

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

Dedico este trabalho a pessoa mais importante na minha vida, meu Pai. Obrigado por ter me ensinado e aconselhado em todos os momentos da minha vida, ensinado-me que os valores e a dignidade de um homem são seu maior patrimonio. Hoje, ele com certeza guia meus passos junto com Deus. Descanse em paz meu pai. Saudades.

"*I dedicate this thesis to the most important person in my life, my Dad. Thank you for teaching and advising me in all moments of my life, and teaching me that the man's principles and dignity are his most valuable patrimony. Today, along with God, he surely guides my steps. Rest in peace. I miss you."*

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INTRODUCTION

Most neoplastic conditions in commercial poultry are associated with oncogenic viruses, primarily herperviruses and/or retroviruses. Marek's disease (MD) is caused by a cell-associated lymphotropic *Alphaherpesvirus* and induces a lymphoproliferative disease of chickens affecting the peripheral nervous system and other tissues and visceral organs. Retroviral induced tumors are caused by members of the avian leukosis/sarcoma virus group (ALSV) or by the reticuloendotheliosis virus group (REV). Reticuloendotheliosis (RE) is a neoplastic and immunosuppressive condition in several avian species. The REV group is immunologically, morphologically, and structurally distinct from the Avian Leukosis/Sarcoma group. The most common clinical diseases induced by REV include chronic lymphomas and runting disease associated with immunosuppression. REV has a widespread distribution and it has been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens. REV infection represents an economic concern mainly as a contaminant of biologic products produced in chicken embryo cells or tissues or as a barrier for exports of breeding stock to certain countries. Part of the objectives of this research was to examine the pathogenicity and transmission of an REV isolate from Attwater's prairie chickens (APC) using Japanese quail as a biological research model.

The Attwater's prairie chicken - APC (*Tympanuchus cupido attwateri*), is a wild species of grouse on the verge of extinction. APCs were previously known to be

susceptible to REV and presently they are endangered in part due to enzootic REV infection. The wild population of APCs was once estimated at more than one million birds inhabiting 2.4 million ha of prairie habitat on the coastal areas of Texas and Louisiana (USA) by 1941. Human-induced habitat loss and fragmentation has further reduced this subspecies to three small, isolated subpopulations totaling less than 70 birds in 1998. Great efforts are currently made in a preservation and reproduction program at the Fossil Rim Wildlife Center (Glen Rose, TX) to prevent extinction of the APC. However REV was detected in the 1990s in this captive population of APCs, which has represented a significant challenge for the preservation of this avian species and for the release of birds raised in captivity into their natural habitat. Therefore, a specific objective of studying this newly identified isolate (REV APC-566) was to determine its ability to induce disease and its transmissibility, both being critical aspects in populations of wild birds and commercial poultry.

The approach used for this research involved infecting Japanese quail embryos, hatching and raising the quail chicks in various groups with different rates of infection and reproducing infected quail to study the dynamics of REV infection in them and in their progeny. Japanese quail were kept up to 20 weeks of age and thus it was possible to evaluate egg production, fertility and hatchability, and to examine the effects of REV infection on such economic parameters. Progeny was produced from the quail infected with REV as embryos, with the purpose of evaluating their status of viremia at hatch or at 6 weeks of age. The REV effects on growth and viability were measured in the second generation. The ability of REV APC-566 to induce tumors was tested in Japanese quail

infected as embryos, and in SPF chickens and SPF turkeys infected at one day of age. REV APC-566 was oncogenic in all three species, as it has been in APCs.

Humoral and cellular immune responses are frequently depressed in chickens infected with REV. This virus has the ability to induce severe and rapid immunosuppression in chickens. Studies on immunosuppression appear to be necessary since diagnostic efforts have revealed that REV infection in prairie chickens not only results in mortality and lymphomas, but also coincides with other infectious diseases such as parasitic disease, septicemia and avian pox. Because APC is an endangered species the immunosuppressive effects of REV APC-566 were evaluated in SPF turkeys. Such evaluation involved studies on serological responses against inactivated viral antigens and protection against *Pasteurella multocida* in REV-infected and non-infected turkeys.

REV was first isolated in the USA in 1966. Since then, several reports of infections around the world have become available. To date, only one American and one Chinese isolates have been fully sequenced. Few sequences from other isolates have been published and they mainly target the LTR regions. Another objective of this research was to sequence in full the first proviral genome of an oncogenic REV isolated from wildlife. The proviral genome of REV APC-566 was compared with the proviral sequences of other REVs. Sequencing of the full proviral genome of the oncogenic and immunosuppressive REV APC-566 was accomplished by generating overlapping oligonucleotide PCR primers that spanned the entire genome of REV. The PCR amplicons were directly sequenced and assembled in a full length contig using computer software. The phylogenetic analysis of REV APC-566 was also done by using specialized computer software. The transmembrane (TM) protein of multiple REV

isolates from four different avian species was partially sequenced and their similarities and divergences were examined phylogenetically. The envelope gene was highly conserved, contrasting with the wide diversity observed in the envelope gene of most other retroviruses. As expected the *gag* and *pol* genes were the most conserved in all analyzed sequences. A short oligopeptide coding sequence within the TM region of the *env* gene was 100% similar among all REV sequences examined and highly similar to homologous sequences of *Murine leukemia virus* (MLV) in which such immunosuppressive oligopeptide was originally identified. The only region where differences were significant was the long terminal repeat (LTR), in which most of the REV isolates examined revealed a variety of deletions and insertions, primarily in the U3 region. The precise localization of regulatory elements in the REV LTR may contribute to the development and improvement of *in vitro* gene expression, which may provide novel information about the mechanisms of oncogenicity of REV and improved diagnostic tools for its detection in infected birds.

CHAPTER 2

LITERATURE REVIEW

Virus induced tumors in avian species are generally of mesodermal origin, transmissible and primarily associated with herpesvirus or retrovirus infection (38). The most commonly diagnosed neoplastic conditions of infectious origin in avian species include Marek's disease (MD), and a variety of leukoses and reticuloendotheliosis (REV). All three conditions cause economic losses from tumor-associated mortality as well as poor performance. Some of these neoplastic diseases have served as highly suitable models for studying various phenomena of neoplasia (38).

Marek's disease (MD) is caused by a cell-associated lymphotropic *alphaherpesvirus* and induces a lymphoproliferative disease of chickens affecting the peripheral nervous system and visceral organs. In the absence of control measures, MD can cause devastating losses in commercial layer and broiler flocks (70, 107)

Members of the avian leukosis sarcoma group (ALSV) are classified as *alpharetroviruses* (former *avian type C retrovirus*) and induce a variety of leukoses, sarcomas, tumors of hemopoietic origin and other related neoplasms (69). The term "leukosis" is used because a leukemic blood picture is not always present during the course of leukemia-like proliferative diseases of the hemopoietic system (70) Neoplastic changes induced by the ALSV group involve the lymphopoietic, erythropoietic, and myelopoietic systems. Lymphoid leukosis, a lymphoproliferative disease of chickens,

affecting primarily the bursa of Fabricius and visceral organs, is the most common form of leukosis arising from infection with a member of the ALSV group of viruses known as avian leukosis virus (ALV) (36). Other neoplasms of hemopoietic origin, which can also be seen in ALV-infected chickens include erythroblastosis, myeloblastosis, myelocytomatosis and related neoplasms such as nephroblastoma and osteopetrosis. Of these, myelocytomatosis is frequently caused by avian leukosis virus subgroup J (ALV-J) especially in meat type chickens (70).

Reticuloendotheliosis (RE) is a neoplastic and immunosuppressive condition induced by members of the Reticuloendotheliosis virus group (REV). These viruses are immunologically, morphologically, and structurally distinct from the Avian Leukosis/Sarcoma Virus group (61, 72). The most common clinical diseases induced by REV are chronic lymphomas and an immunosuppressive runting disease. REV has a widespread distribution, albeit clinical disease by REV occurs rather infrequently. REVs have been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens (2, 16, 34, 57, 78, 105, 114). The principal economic concerns regarding REV infection involve potential contamination of biological products manufactured using chicken embryo cells or embryos, or as a barrier to exports of breeding stock to certain countries (110).

General characteristics of the Retroviruses.

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition and replicative properties (22). The virions are 80 – 100 nm in diameter, and their outer lipid

envelope incorporates and displays the viral glycoproteins. The shape and location of the internal protein core is characteristic for various genera of the family. The virion has two copies of RNA which range from 7,000 to 12,000 nucleotides in size and it is linear, single stranded, non-segmented and of positive polarity. The main characteristic of the *retroviridae* family is its replicative strategy which includes as essential steps reverse transcription of the virion RNA into linear double stranded DNA and the subsequent integration of this DNA into the genome of the cell. The integrated viral DNA is known as proviral DNA. Such steps are done by two unique enzymes present in the virions. Reverse transcription is accomplished by an RNA-dependent DNA polymerase ordinarily known as **reverse transcriptase** and integration is achieved by an enzyme called **integrase** (22, 99).

Based on viral genome organization, retroviruses are divided into two main groups: simple and complex (100). Simple retroviruses usually carry only elementary genetic information, whereas complex retroviruses code for additional regulatory non-viral proteins derived from multiple spliced messages. All retroviruses contain three major coding domains with information for synthesis of virion proteins: *gag*, which directs the synthesis of internal viral proteins that form the matrix, the capsid, and the nucleoprotein structures; *pol*, which contains the information for the reverse transcriptase and integrase enzymes; and *env*, from which are derived the surface and transmembrane components of the viral envelope protein. An additional, smaller, coding domain within or adjacent to *gag* present in all retroviruses is *pro*, which encodes the viral protease (100).

The Retroviridae family is currently divided in three subfamilies: *Orthoretrovirinae*, *Spumaretrovirinae* and the unclassified members of the *Retroviridae* family (13). The most important is the Orthoretrovirinae, which is divided in 6 genera: *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus* and *Lentivirus* (Table 2.1). Within the classification of retroviruses, REVs are placed in the genus gammaretrovirus (formerly *mammalian C-type*) whereas the avian leukosis viruses are classified in a separate genus termed *Alpharetrovirus*. The phylogenetic relationship of REV with gammaretroviruses is supported on the basis of morphology, nucleic acid sequences, amino acid sequences of major polypeptides, and immunologic determinants and receptor interference patterns (31, 110).

Reticuloendotheliosis Virus Group

The Reticuloendotheliosis virus group of retroviruses (REV) comprises RNA-containing viruses with similar molecular and physical characteristics. REVs are immunologically, morphologically, and structurally distinct from the leukosis/sarcoma group of avian retroviruses. REVs belong the genus *gammaretrovirus*. No endogenous REV sequences in host DNA have been recognized, in contrast to ALV endogenous sequences that are commonly present in a variety of hosts (25, 26).

The REV group contains both *replication-defective* acutely oncogenic viruses and *non defective* viruses, which are usually slowly transforming. REV-T is defective for replication in chicken fibroblast tissue cultures and possesses a unique oncogene of cellular origin (v-*rel*), which is responsible for its acute oncogenicity (44, 45). Stocks of REV-T also contain a *non defective* helper REV that replicates in chicken embryo

fibroblast cultures but lacks acute oncogenic properties (44). The helper virus has been variously designated as REV-A or as *non defective strain T*. The REV group now includes strain T, REV-A, *chick syncytial virus* (CSV), *duck infectious anemia virus* (DIAV), and *spleen necrosis virus* (SNV) (110). Similar *non defective* strains have been (19) and continue to be isolated from turkeys, chickens, ducks, pheasants, geese and prairie chickens (110).

The REV morphology is typical of retroviruses with the virions being 100 nm in diameter (119) and with surface projections about 6 nm long and 10 nm in diameter (51). The viral density is around 1.16 – 1.18 g/ml in sucrose gradients (8). The morphology of the viral particles is shown in figure 2.1. As all retroviral particles, REV contains two copies of its genome linked near the 5' end. Thus, they are diploid and are the only viruses so equipped. A direct consequence of diploidy is the formation of heterozygote virions in cells that are infected with two or more genetically distinct but related retroviruses. Such heterozygote virions give rise in the next cycle of infection to stable genetic recombinants that are formed during the process of reverse transcription (91, 99).

The *non defective* REV has a genome of approximately 8.3 kb and the replication-defective strain T genome is only about 5.7 kb due principally to a large deletion in the *gag-pol* region and a smaller deletion in the *env* (18). The REV-T genome contains a substitution of about 1.5 kb in the *env* region that represents the transforming gene, identified as *v-rel* (116). The *v-rel* oncogene is not known to be present in *non defective* REVs or other avian or mammalian retroviruses (99, 106). Such oncogene is related to the *c-rel* gene of normal avian cells, including turkeys cells from which the oncogene was most likely transduced (116). This particular gene codes for a phosphoprotein product

identified as pp59$^{v\text{-}rel}$. The *v-rel* protein is usually complexed with cellular proteins and it is responsible for the acute oncogenicity of replication-defective REV-T (10, 84).

Like most of the retroviruses, the primary translational products are three polyproteins translated from polycistronic messages corresponding to Gag, Pol, and Env sequences (35). These are cleaved proteolytically to generate the functional virion components. The processed Gag proteins are referred to as MA (matrix), CA (capsid), and NC (nucleocapsid). The viral protease (PR) cleaves Gag and Pol precursors. It is encoded by the *pro* gene and maps between *gag* and *pol*. The Pol cleavage products are RT (reverse transcriptase) and IN (integrase). The Env precursor is translated from a spliced mRNA and it is processed by a the cellular machinery into SU (surface) and TM (transmembrane) proteins that are linked by disulfide bonds (96, 97, 99, 104). The RNA gene order is 5'-gag-pro/pol-env- 3' as shown in the figure 2.2 and discussed in chapter 5.

Virus Replication

The replication cycle is similar in all simple retroviruses. Virion entry depends on the envelope glycoprotein binding to a specific cell surface receptor that has not yet been identified (110). Entry of virions into cells probably is accomplished by direct membrane fusion. Reverse transcription begins when the viral particle enters the cytoplasm of the target cell. The process of reverse transcription occurs in the cytoplasm and generates a linear DNA duplex. This DNA is collinear with its RNA template, but it contains terminal duplications known as long terminal repeats (LTRs) that are not present in viral RNA (90), as shown on figure 2.2. At the completion of its synthesis, the viral DNA

molecule is a blunt-ended linear molecule whose termini correspond to the boundaries of the LTRs. Soon after completion of viral DNA synthesis, usually while still in the cytoplasm, the viral integrase cleaves the 3' termini of the viral DNA eliminating two bases from each 3'end strand. Oncoretroviruses generally gain access to the nucleus during mitosis, when the nuclear membrane is disassembled (12). The viral DNA with the integrase complex encounters the host DNA in the nucleus. It is known that specific host target sequences are not required for integration (7, 53), but the whole process is still not completely understood (12). Viral RNA transcription and translation are initiated through promoter and enhancer sequences present in the LTR. Three polyproteins are encoded, Gag, Gag-Pol, and Env. The gag precursor polyprotein is myristylated (97). The Gag polyprotein has the ability to direct the budding of virus-like particles from the cell (85). The Gag protein is also involved in packaging most of the other components of the virion, including the two copies of genomic RNA (85). The RNA is linked to the Gag protein by the packaging or encapsidation signal that is located upstream from the *gag* gene (27). The Env proteins are initially cotranslationally glycosylated by cellular enzymes in the lumen of the rough endoplasmic reticulum and further processed by proteolysis and transported to the plasma membrane, where the cleavage products are incorporated into budding viruses (96, 98).

Once all the polyproteins (Gag, Gag-Pro-Pol and Env) are synthesized, they migrate together (along with two copies of viral RNA) to a common site on the cell membrane to be assembled into viral particles. It is the Gag protein, in groups of several hundred molecules, that alone provides the driving force for the release of uniform particles from the plasma membrane (85). The budding of virions is the final stage of

replication and it has been observed both at the plasma membrane and at smooth-walled, intracytoplasmic vesicles (51). The first viral particles can be seen 24 hours post infection, and maximum production occurred 2-4 days after infection in chicken embryo fibroblasts (51). As all retroviruses, REV undergoes an obligatory maturation step in the formation of an infectious particle. Core particles assembled either in the cytoplasm or at the plasma membrane have an immature morphology. The viral Protease is directly involved in maturation of the viral particles by cleaving the Gag and Gag-Pro-Pol precursors (85). The defective strain REV-T requires a *non defective* RE helper virus to supply some of these functions during replication (44).

REV replication may be accompanied by subtle cytopathic changes. Syncytial cell formation has been reported in infected cultures (23), but degenerative changes are more commonly seen by microscopy starting at 3 days after infection and being more pronounced 4 days after infection (92). From 2 to 10 days after infection there is a decrease of the number of cells in culture. However, not all the cells in the infected cultures die, which allows for replication of the surviving cells and an increase of cells with concomitant disappearance of the cytopathic effect (92). A theory has been proposed for this phenomenon (93): infected cells synthesize unintegrated viral DNA, a part of which is integrated at multiple sites in the cellular genome (53), and thus the unintegrated LTR may be toxic for the infected cells in culture. Progeny virus then superinfects the already infected cells, leading to an accumulation of unintegrated viral DNA. Cells with large amounts of unintegrated DNA die, while those cells able to prevent early superinfection have few copies of unintegrated viral DNA and survive (93). In contrast, normal cellular structures may not be altered (51), which is more commonly

observed. Detection of REV is normally accomplished by indirect methods such as immunofluorescence (113), immunoperoxidase staining (14), enzyme immunoassay (82) or more recently by detection of proviral DNA by PCR (1, 62).

Origin and Evolution of REV

Several evolutionary studies have been done to search the origin of REV. It is well known that REV is more closely related to *Gammaretroviruses* or *Mammalian Type C* viruses than to other avian retroviruses (17, 51, 61, 119). Immunological relationships between the major structural proteins of the endogenous Owl monkey virus (OMC-1), the endogenous Deer kidney virus (DKV) and REV have been established, suggesting that OMC-1 and DKV are in the evolutionary lineage that led to the generation of a group of oncogenic viruses capable of crossing the interclass barrier between mammals and birds (5). Another independent study (73) showed significant nucleic acid homology between REV, the endogenous macaque viruses (MAC-1 and MMC-1) and the colobus monkey virus (CPC-1). Phylogenetic analyses showed a very close relationship between all four viruses, suggesting that the REV group of viruses and macaque-colobus isolates may be descendants from a common ancestral virus (59, 73). Interference assays have shown that spleen necrosis virus (SNV) shares the receptor with the type D simian retroviruses (SRV) and the Baboon endogenous virus (BAEV) (54). However, phylogenetic alignments had shown a distant relationship between REV and BAEV in another independent study (59).

Since RE viruses have no detectable genetic homologies with the DNAs of their avian hosts (50), several theories have been proposed: 1) an ancestor of the REV was

probably transmitted from a monkey to an avian host (59), with an interspecies transmission occurring probably recently and not directly from CPC-1 or MAC-1, but from their monkey virus ancestor (59, 73); 2) REV has a common evolutionary origin with the BAEV from a simian type D retrovirus, REV resulting from an adaptive radiation of a BAEV-like mammalian retrovirus in birds (54). This theory also suggests the existence of another type C virus, still unknown, related to BAEV, which would be the true REV progenitor; and 3) REV could have the OMC-1 and DKV in its evolutionary lineage based on their close antigenic relationship and probably an interaction of both somehow generated a group of oncogenic viruses capable of crossing the interclass barrier (5).

Although these studies tried to establish the evolutionary linkage of REV to *mammalian type C* viruses, their true origin still can not be established. Viruses in the REV group are very closely related to one another. Thus, REV may represent recent variants of a single virus whose ancestor may have been transmitted to birds from a mammalian endogenous form as an infectious virus a long time ago. Alternatively, the REV group may have arisen by a relatively recent infection of birds with an as yet undiscovered endogenous *mammalian type C* retrovirus (6).

REV Strain classification

The REV group includes as representative strains the *replication defective* REV-T turkey isolate and the *non defective* REV-A (44), the duck isolates spleen necrosis virus (SNV) (95) and duck infectious anemia virus (DIAV) (60), the chicken isolate chicken syncytial virus (CSV) (23) and several other *non defective* strains (24, 57, 103). The

REVs are remarkably uniform in antigenicity (115) and except for the defective strain T, have similar structural and chemical properties (51). The REV group encompasses a single serotype (19), but these viruses can be divided in 3 subtypes on the basis of neutralization assays and differential reactivity with monoclonal antibodies (19). REV isolates differ also in certain biologic properties including pathogenicity (115), albeit such differences have not been used for strain classification.

Phylogenetic analyses have indicated little variation among REV *pol* and *env* genes (9). The Env protein, which is normally the most divergent protein in most exogenous retroviruses, displayed at least 93% similarity amongst various REVs in a recent study (9). A molecular analysis of the full proviral genome showed various REV sequences being relatively conserved. The homology for all three genes and LTR fragments of 3 REV strains (SNV, Fowlpox REV insertion and China HA9901) was over 92%, regardless of the avian species of origin and chronology of isolation (103).

REV Hosts and Epizootiology

Non defective REV strains have been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens (2, 16, 34, 57, 78, 105, 114). The *defective strain* REV-T was isolated from turkeys (18). Chickens and turkeys have been used most often as experimental hosts. REV has a widespread distribution in flocks of turkeys, ducks and chickens. The prevalence of seropositive flocks may be high (2) and tends to increase with the age of the flock. However, reports of REV-associated clinical disease in commercial poultry are infrequent. Most outbreaks are associated with contaminated vaccines, thus involving a

high proportion of chickens affected (4, 39, 52, 89, 101, 118). However, REV has been demonstrated in flocks with a naturally occurring immunosuppressive disease in Korea (79) and cases of REV-associated lymphomas were reported in chickens (40, 109), turkeys (24, 67, 114), ducks (68), prairie chickens (34), quail (16, 78) and other avian species.

Responses to infection

After REV infection is established in a susceptible bird, it generally results in one of two possible scenarios. First is the *tolerant infection* with persistent viremia in the absence of antibody response against REV. Tolerance is induced readily in chickens by embryo inoculation (115) and by vertical transmission of virus from infected dams (114). This type of infection rarely occurs in chickens infected at hatch and it is unlikely to happen in later age infections (110, 115). Despite of the fact that some birds develop antibodies against REV in some cases (63), *tolerant infection* is usually associated with higher rates of vertical transmission and tumor development, and birds are typically stunted and immunosuppressed (110).

The second kind of response to infection is the *transient or non tolerant infection* and it normally happens when the first exposure occurs at hatching or later in life. The infection is followed by development of a robust antibody response with cessation of viremia (57, 115). Antibodies against REV have been detected as early as 1 week post infection in some, but not all ducks infected at 25 days of age. All ducks thus infected seroconverted by 4 weeks post infection (57). Chickens infected post hatch showed antibody responses as early as 16 days post infection (101).

Persistence of REV in the peripheral blood lymphocytes has been described in ducks after development of antibodies (57). Non infectious RE viral antigens also have been found in blood of chickens for several weeks following the disappearance of infectious virus (3). *Transient viremia* only rarely results in clinical disease (71, 111). Vertical transmission from hens infected in the adult life and prior to seroconversion has not been documented.

Transmission of REV

REV can be transmitted at low rates by close contact as observed in chickens, turkeys and ducks (56, 63, 67, 68). Horizontal transmission of REV occurs slowly and possibly very close contact might be necessary between infected and non-infected birds. Commercial flocks may become infected with REV at older ages (111). A natural reservoir has not been described for REV, and it is still a mystery how birds are infected in nature considering that REVs are quickly degraded outside the host at ambient temperatures (15). However, it is known that REV may be transmitted by insects. REV can be isolated from mosquitoes (*Culex annulirostris*) in contact with viremic chickens, which demonstrated an apparent mechanical transmission of the virus to recipient chickens exposed to carrier mosquitoes (66). REV has also been isolated from *Triatoma infestans* and *Ornithodoros moubata* shortly after feeding on infected chickens (94). In a recent report, positive insects were not found near REV antibody-positive flocks, suggesting that viremia had ceased or that the virus load or infection rates were too low to be transmitted mechanically (29). However, in the same study REV was detected in mosquitoes for up to 5 hours post blood feeding. In addition, REV was detected in the

feeding mixture for up to 96 hours (29). House flies also were reported to contain REV within their digestive tract for 4 days postexposure to sugar with added REV (29). In order to explain late seroconversion in commercial poultry flocks, mosquitoes would have to acquire the virus from a nearby population, which so far appears unlikely (110).

Another form of transmission has been described as accidental contamination of commercial vaccines with REV. Major economic consequences have been reported in REV-contaminated Fowlpox and Marek's Disease (MDV) vaccines. Partial (41, 62) or full length (43, 81) REV sequences have been found integrated the genome of vaccine Fowlpox viruses. Partial integration of REV also has been reported in Marek's Disease virus (28, 120). MDV vaccines contaminated with free REV particles have been found in several occasions (52, 55, 88, 89, 118).

Chickens inoculated with REV-contaminated MDV vaccines showed delayed growth, abnormal feather development, anemia, and leg paralysis as main symptoms. The culling rate may exceed 50% in some flocks (118). In another study with a contaminated MDV vaccine, chickens infected at 2 days of age showed feather abnormalities, undergrowth, anemia and leg paralysis (52). After experimental inoculation in day old chicks, the same contaminating virus induced persistent infection for at least 61 days and was proven to shed horizontally to non inoculated chickens, which rapidly seroconverted (52). Vaccine contamination may occur as a result of SPF flock contamination, and vaccine master seed or working seed virus contamination (110).

REV has the ability to integrate partially or entirely in large DNA viruses. Examples include REV proviral DNA integrated into Fowlpox field isolates, and the Australian S vaccine whose application results in seroconversion and presence of REV

particles in chickens (30, 43, 80). Although the mechanisms for acquiring such host genes and evolutionary alterations in the genome of pox viruses remain poorly understood, REV integration could play a role in DNA virus evolution and it may confer some benefit to Fowlpox virus for infection of poultry previously vaccinated against Fowlpox. Coinfection *in vivo* with MDV and REV may lead to LTR insertion into the MDV genome, but the consequences of LTR insertion have not been examined in inoculated chickens (28). Thus, insects carrying Fowlpox virus with REV inserts or MDV containing REV inserts may constitute a model for horizontal transmission in nature.

A very low rate of REV vertical transmission has been observed. CSV was detected in one out of 132 progenies of tolerant hens (115), and in three of 98 eggs produced by persistently infected dams (102). In a natural REV field outbreak, 59 turkey breeder hens produced 1.8% (5/278) positive poults (114). REV proviral DNA was found in three of 262 progeny chicks (1.1%) from 25 viremic female chickens and in 10 of 820 progeny chicks (1.2%) form 18 viremic male chickens (77). However, in another study (64) REV was isolated from 18 of 35 chicks (51%) derived from eggs of viremic hens that were set within 24 hours of lay. The low rates of congenital transmission may be explained by the presence of few tolerant birds and the relatively low titer of virus shed by dams into eggs (115). This REV behavior is different from avian leukosis virus, which may be shed into eggs at a relatively high titer (48, 75).

Pathology and Pathogenesis

REV causes various syndromes in multiple avian species, including runting, an acute non-neoplastic syndrome with high mortality, severe immune suppression and T and/or B cell lymphomas (108). REV infection can cause dramatic economic losses from a runting syndrome or chronic neoplasia with mortality. Significant losses can occur when REV-contaminated vaccines are administered to very young chickens. The body weights of infected chickens may be 20-50% lower than uninfected controls by 3-5 weeks after infection (65). Weight gain reduction has also been reported in infected ducks (72). Some chickens may have abnormal feather development, termed "*Nakanuke*" (55) where barbs of the primary and secondary wing feathers are abnormally stuck to the rachis providing a stick-like appearance to the feathers.

REV infection can result in chronic lymphomas in chickens, turkeys, ducks, Japanese quail, pheasants, prairie chickens and geese (24, 32-34, 40, 57, 78, 113, 115). Such lymphomas are characterized by a population of large stem cells with large nucleoli and few cytoplasmic organelles (20). Birds infected at an early stage with REV (*non defective* and REV-T) may develop B-cell and/or T-cell lymphomas (24, 109, 115). Macroscopically and microscopically the lesions are very similar to lymphoid leukosis and may be not be easily differentiated without a more specific test (110). The B-cell lymphomas have been proven to be induced by *non defective* REVs in a similar manner as ALV. That is, the provirus integrates into or in close proximity to the *c-myc* locus and uses a promoter insertion mechanism to activate *c-myc* expression (86). Activation of the c-myc protooncogene results in deregulated, over-expressed c-Myc protein leading to uncontrolled cell proliferation and/or programmed cell death (apoptosis) in

nontransformed cells (37). Conversely, Myc-induced apoptosis may result blockage of tumorigenic cells. Thus, cell transformation involves both the activation of the *c-myc* oncogene and transformation inhibition of the Myc-induced apoptosis pathway (37). REV can also induce T-cell lymphomas by integration of proviruses upstream of the c-myc coding exons, with most of the proviruses being oriented in the opposite transcriptional direction as the c-myc gene (49). This insertion pattern is in contrast to the pattern in B-lymphomas induced also by REV, in which integration occurs primarily upstream from the second *c-myc* exon (87). The *defective* REV-T strain is able to cause acute oncogenesis in a different pathway. REV-T pathogenicity is strongly linked to the *v-rel* gene inserted in its genome, which is a protooncogenic protein present in host genomic DNA of turkeys (58, 74, 116).

REV can causes a variety of neoplasms in infected birds, but the most common is lymphoma. Lymphomas were found in *tolerant* and *non tolerant* CSV-infected chickens and in *non tolerant* REV-A-infected birds with latent periods of 17 to 43 weeks. However, the frequency of lymphomas was higher in *non tolerant* chickens (115). In the same experiment CSV caused myxosarcomas at 35 weeks of age and fibrosarcomas at 65 weeks of age, whereas REV-A caused adenocarcinomas in 92 week-old chickens infected as embryos. REV-A also caused enlargement of peripheral nerves in *tolerant* chickens with a latent period of 50 to 82 weeks (115). The duck REV RU-1 strain caused lymphosarcomas as early as 58 days of age in ducks infected as embryos, but it also induced histiocytic sarcomas of various degrees of differentiation and spindle-cell sarcomas in ducks kept until 6 months of age (57)

Immunosuppression

Humoral and cellular immune responses are frequently depressed in chickens infected with *non defective* REV strains. Depressed antibody responses to Marek's disease virus (101), Newcastle disease virus (117), sheep erythrocytes and *Brucella abortus* (112) have been reported in chickens. Embryonic or neonatal infection in ducks caused inability to mount antibody responses against bovine serum albumin (BSA) and sheep red blood cells (SRBC) (57). REV infection has been reported to decrease immune responses against bacteria such as *Salmonella typhimurium* (64). Chickens infected with *S. typhimurium* died at higher frequencies when co-infected with REV. REV infection at early ages is also known to increase the severity of coccidiosis caused by *Eimeria tenella*, since body weights were significantly decreased and intestinal mucosal lesion scores and mortality were statistically greater in REV-infected chickens (65).

The induction of the immunosuppression caused by REV has been described (11, 76). *Non defective* REVs have the ability to cause severe and rapid immunosuppression in chickens (76). The PHA (phytohemagglutinin A) assay used to evaluate the mitogenic activity of splenic lymphocytes obtained from REV-A-infected birds revealed a severe compromise of mitogenic responses within 1 week post-infection and such reduced mitogenic responses remained suppressed for at least 2 to 4 weeks (11). However, in the same study, the REV-A infected-birds were no longer impaired in their ability to respond to PHA by 5 weeks after infection and were no longer viremic with REV. Thus, continued virus replication is thought to be necessary for maintenance of the suppressor cell activity in the lymphocyte population (11). Therefore, congenital or very early exposure to REV, which is more likely to induce long, lasting viremia is probably more

prone to result in significant suppressor cell activity. In addition, thepresence of the REV envelope glycoprotein on the surface of REV-transformed cells is known to be involved in the induction of the suppressor cell population during the course of the disease (76).

A 26 amino acid oligopeptide in the transmembrane protein (p15E), which is highly conserved among the envelope proteins of murine, feline, bovine, and simian retroviruses as well as human T cell leukemia virus (HTLV), and to a lesser extent, human immunodeficiency virus (HIV), may play an important role in the immunosuppressive properties of these viruses (42). Similar sequences were found in gp20E of Mason-Pfizer monkey virus (MPMV) and REV-A of turkeys (83). This immunosuppressive peptide (CKS-17) was first reported as a synthetic sequence corresponding to a portion of the consensus alignment of p15E from Murine leukemia virus (MLV), FeLV and HTLV (21). *In vitro* studies have shown that CKS-17 inhibits IL-2-dependent cell proliferation, alloantigen-stimulated lymphocyte proliferation, monocyte respiratory burst, natural killer cell activity, monocyte-mediated tumor cell killing, IL-1-mediated signal transduction, delayed type hypersensitivity reactions, LPS-induced mortality in mice, pokeweed mitogen (PWM)-induced IgG production, and activity of murine cytotoxic T lymphocytes (21, 42).

Immunosuppression is probably one of the most important consequences of vertical transmission or vaccine-derived REV infection in commercial poultry, but is less likely to result from contact infection after hatch (111) and it has not commonly been associated with seropositive breeder flocks (110).

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Table 2.1. Classification of retroviruses based on genetic arrangement and reverse transcribing elements[1].

Subfamily	Genus	Representative
Orthoretrovirinae	*Alpharetrovirus*	*Avian leukosis virus*
	Betaretrovirus	*Jaagsiekte sheep retrovirus*
		Mason-Pfizer monkey virus
	Deltaretrovirus	*Bovine leukemia virus*
	Epsilonretrovirus	*Snakehead retrovirus*
	Gammaretrovirus	*Reticuloendotheliosis virus group*
		Murine Leukemia virus
	Lentivirus	*Human immunodeficiency virus 1*
Spumaretrovirinae	*Spumavirus*	*Feline foamy virus*
unclassified *Retroviridae*	*Human endogenous retroviruses*	*Human endogenous retrovirus*
	Avian retroviruses	*Avian myeloblastosis-associated virus*

[1] Summarized and adapted from Hull (2001) (46) and the ICTVdb web site (47).

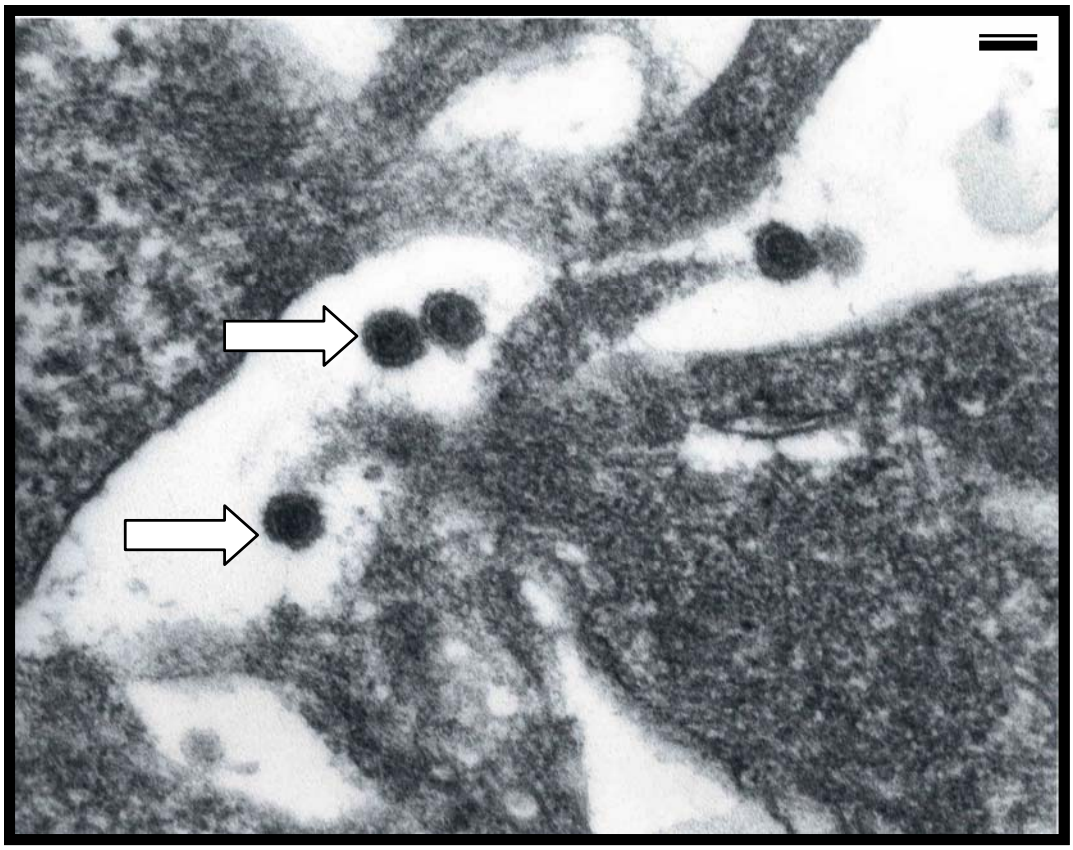

Figure 2.1. Transmission Electronic Microscopy of thin sections of DF-1 cells infected with REVAPC-566. Viral particles indicated by arrows. The bar at the top right corner of the figure represents 100 nm in length.

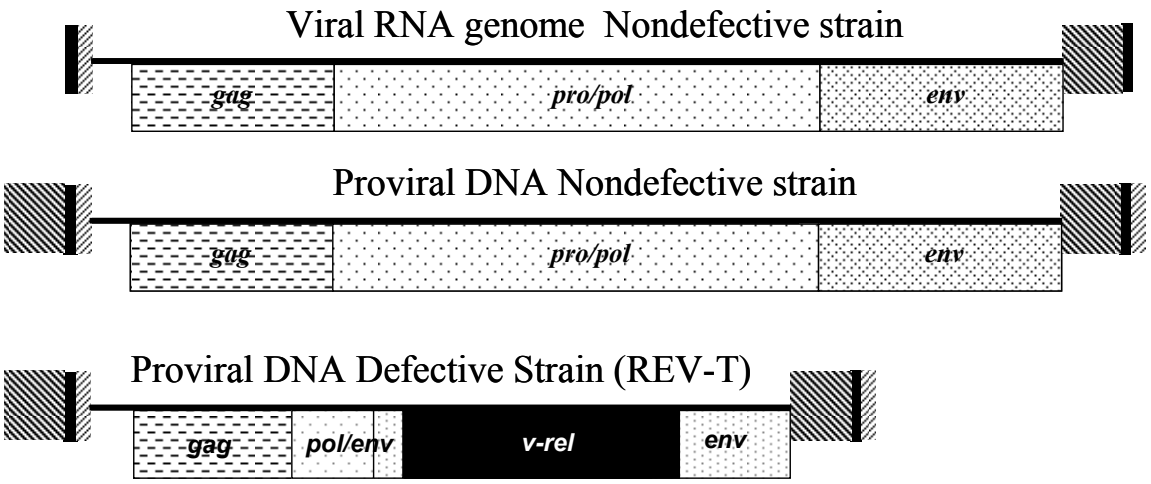

Figure 2.2. The major coding regions in the simple retroviral genome of Reticuloendotheliosis virus. Viral RNA and proviral DNA of a *non defective* REV strain are shown in the two top diagrams. The proviral DNA of the defective REV-T strain is illustrated in the bottom diagram (figure not to scale).

CHAPTER 3

PATHOGENICITY AND TRANSMISSION OF RETICULOENDOTHELIOSIS VIRUS STRAIN APC-566 IN JAPANESE QUAIL[1]

[1] Barbosa, TMC., Zavala, G., Cheng, S., Villegas, P. (2006). Submitted to *Avian Diseases.*

ABSTRACT

The pathogenicity and transmission of a field isolate of reticuloendotheliosis virus (REV) was studied using an experimental model in Japanese quail (JQ). The original REV (designated APC-566) was isolated from Attwater's Prairie Chickens (*Tympanuchus cupido attwateri*), an endangered wild avian species of the Southern United States. The pathogenicity of the REV isolate was examined after inoculations in JQ embryos. The transmissibility of the REV isolate was studied in young naive JQ placed in contact with experimentally infected quail. Vertical transmission was not detected by virus isolation and indirect immunofluorescence. Seroconversion readily occurred in contact quails demonstrating horizontal transmission. REV antibody titer increased between 6 and 20 weeks of age in all groups of quail infected as embryos. REV inoculations posthatch (3, 6, 14 and 23 weeks-old) resulted in strong antibody responses coinciding with interruption of viremia, as early as 3 weeks post inoculation. The APC-566 isolate induced tumors beginning at 6 weeks of age in quails infected as embryos. Most of the tumors detected in Japanese quail were lymphosarcomas and 81% of these neoplasias contained CD3+ cells by immunoperoxidase. REV APC-566 was also oncogenic in chickens and turkeys infected at one day of age, with tumors appearing as early as 58 days post infection in chickens, and at 13 weeks of age in turkeys. This study was conducted in part as an attempt to understand the potential for pathogenicity and transmission of REV isolated from endangered avian species. This in vivo research model in Japanese quail proved useful for REV pathogenicity studies.

INTRODUCTION

The reticuloendotheliosis viruses (REV) constitute a group of avian retroviruses with high similarity to the mammalian Type-C retroviruses classified as Gammaretroviruses. REVs are immunologically, morphologically, and structurally distinct from the Avian Leukosis/Sarcoma group of retroviruses (18, 25). The REV group includes the replication defective REV-T, the non defective REV-A, spleen necrosis virus (SNV), chicken syncytial virus (CSV), and duck infectious anemia virus (DIAV) as representative strains. REV-T was first isolated from turkeys and carries a transduced cellular oncogene known as v-rel (4). Non-defective REV strains have been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens (1, 3, 9, 17, 31, 38, 44). REV causes various syndromes in multiple avian species, including runting, an acute non-neoplastic syndrome with high mortality, severe immune suppression and T and/or B cell lymphomas (40). The virus is normally isolated and propagated in chicken or duck embryo fibroblasts or in DF-1 cells, a spontaneously immortalized fibroblastoid cell line derived from East Lansing chicken line zero embryos (ELL-0) (12). Japanese quail (Coturnix coturnix japonica) may be used as a model for studying vertically transmissible avian diseases because of their rapid development and because they are biologically related to domestic chickens (Gallus gallus) (26). The Attwater's prairie chicken - APC (Tympanuchus cupido attwateri), is a wild species of grouse on the verge of extinction. APCs were previously known to be susceptible to REV (9) and presently they are endangered in part due to enzootic REV infection. REV is transmissible vertically via the egg and horizontally by direct contact with infected birds (6, 20,

44), mechanically by insects (8, 22), by integration into large DNA viruses or accidentally by injection of contaminated vaccines (10, 11, 15, 19, 27, 32, 34, 35). Viremic chickens, turkeys and ducks may transmit infectious REV to their progeny but at a lower frequency than ALSV (42). The mechanisms for horizontal transmission among commercial flocks and the identity of biological reservoirs of infection are poorly understood. The objectives of the present studies included examination of the pathogenicity and transmission of a field isolate of REV (APC-566) using Japanese quail as a model.

MATERIALS AND METHODS

Quails. Commercially produced Japanese quail (*Coturnix coturnix japonica*) fertile eggs were obtained from a local hatchery after testing the breeders to insure absence of REV viremia and antibodies in the breeding population used to produce the eggs.

History of REV isolate. During 2004 and 2005 our laboratory received multiple clinical samples from captive APCs and greater prairie chickens (GPC) maintained at the Fossil Rim Wildlife Center (Glen Rose, TX) in a program intended to avoid the extinction of the APC. The samples consisted of refrigerated whole blood with anticoagulant, skin scabs and formalin-fixed tissues. One REV PCR-positive blood sample was randomly chosen for further evaluation. The virus isolated from such sample was designated APC-566 corresponding to the identification of the APC specimen. REV APC-566 was isolated from a male hatched in May of 2001. The APC-566 breeder specimen died in April of 2005 from an internal hemorrhage. None of the tissue samples from this bird revealed any significant lesions on microscopic examination,

although samples from several birds of the same captive reproduction colony were diagnosed with lymphosarcomas and REV was isolated from many of them. REV APC-566 was isolated and propagated from plasma in DF-1 cells, and frozen at -80C until used for these experiments. The REV APC-566 genome has been entirely sequenced in our laboratory and its genomic sequence is available at GenBank (accession No. DQ387450).

Viruses and virus titration. A virus stock was prepared by passaging three times DF-1 cells infected with REV APC-566. After the third passage the virus stock was titrated and frozen at -80 C until used. The virus titer in the stock was calculated by the Reed and Muench method using indirect immunofluorescence to identify REV positive virus cultures in DF-1 cells.

PCR. PCR was conducted to amplify part of the long terminal repeat of REV (LTR) using as template genomic DNA extracted and purified from whole blood or from infected DF-1 cells. The PCR reactions were performed according to pre-established procedures (19), in which amplifications are accomplished using the forward primer, REV-1 (5' CATACTGGAGCCAATGGTT 3') and the reverse primer, REV-2 (5' AATGTTGTACCGAAGTACT 3'). Denaturing was done at 95 C for 10 min and 35 cycles of 95 C for 1 min. Annealing was done at 55 C for 45 seconds and extension at 72 C for 30 sec.

Indirect fluorescent antibody (IFA) test. DF-1 cells were plated in either 24-well plates or 35mm plates at a concentration of approximately 150,000 cells/ml and inoculated with serial ten-fold dilutions of REV APC-566 and incubated at 39 C for 7 days. The inoculated cells and control cultures were then trypsinized, re-plated and incubated until they reached approximately 80% confluence, after which they were fixed with a cold acetone-alcohol solution (60:40 v/v). IFA was performed following a modified pre-established procedure (5). Skim milk (5%) was

used as a blocking agent during 1 hour to reduce non-specific fluorescence, followed by incubation of 1 hour with 1:100 diluted polyclonal anti-REV antibody (Charles River SPAFAS, Franklin, CT). The cells were then washed twice in PBS and incubated for 1 hour with 1:250 diluted fluorescein isothiocyanate (FITC)-labeled goat anti-chicken IgG (Zymed Laboratories, San Francisco, CA). All incubations were done at room temperature. A final double wash in PBS preceded examination with a fluorescence microscope.

Sample collection. Whole blood for plasma and serum collection from quail was obtained in heparinized tubes (Beckton Dickinson Vacutainer, Franklin Lakes, NJ) and in sterile microcentrifuge tubes, respectively. Any birds dying naturally during the experiments or euthanatized at the termination of the experiments were examined for gross lesions. Liver, spleen and any tissues with suspected neoplasia were collected for microscopic examination. After fixing the tissues in 10% buffered neutral formalin, all tissue sections were processed and stained with hematoxylin & eosin using standard histological techniques and examined by light microscopy.

Antibody assays. Antibodies to REV were detected using a commercially available enzyme-linked immunosorbent assay (REV ELISA), as per the manufacturer's recommendations (IDEXX Laboratories, Westbrook, ME). Selected serum samples rendering positive or negative REV ELISA results were used for confirmation by Western-blot assay as described below.

Western-blot assay. Total protein from APC-566 infected DF-1 cells was lysed with 2· SDS loading buffer (Laemmli sample buffer, Biorad Laboratories, Hercules, CA) with 5% 2-mercaptoethanol and subjected to heating at 95 C for 10 minutes prior to SDS–PAGE. Proteins were transferred to a nitrocellulose membrane (Biorad Laboratories, Hercules, CA) and blocked

overnight at 4 C in blocking buffer (5% skim milk powder in PBS buffer). The membrane was incubated with a 1:25 diluted quail primary anti-REV antibody in 5% blocking buffer for 2 h at 37 C. After washing three times with PBS, the membrane was incubated with a 1:500 dilution of alkaline phosphatase-conjugated anti-chicken IgG (whole molecule) (Sigma Aldrich, St. Louis, MO) and incubated for 10-30 minutes at room temperature. The membrane was thereafter washed with distilled water.

Immunohistochemistry. The characterization of neoplastic cells was done using a modified previously described procedure (7, 33). Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was done by microwaving (10 min at full power) in a Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA). The tissue sections were blocked (Universal Blocking Reagent; Biogenex, San Ramon, CA) and then incubated overnight at 4 C (or for 2 h at 37 C) with 1:1000 diluted rabbit anti-CD3 polyclonal antibody (Vector Laboratories, Burlingame, CA). The sections were then incubated with 1:250 diluted biotinylated goat anti-rabbit antibody, and then with avidin-biotin alkaline phosphatase (Vector Laboratories, Burlingame, CA). Substrate development was accomplished using the VECTASTAIN Elite ABC Kit (Vector Labs). All sections were counterstained lightly with haematoxylin and a slide cover slip was applied for a permanent record. Negative controls were prepared with the omission of the anti-CD3 antibody and with tissue slides made from normal thymus and bursa of Fabricius obtained from healthy Japanese quail.

Experimental Design.

Experiment 1. The objective of this experiment was to study the susceptibility of Japanese quail to REV APC-566 infection and evaluate the pathogenicity and transmission of REV APC-566 in Japanese quail infected as embryos.

First quail generation (F1). One hundred µl of the REV APC-566 virus stock was inoculated into Japanese quail embryos via the yolk-sac at 4 days of embryonic development. The titer of the inoculum was $10^{3.4}$ $TCID_{50}$ in 100 µl. Six experimental groups of quail chicks were produced to contain various proportions of infected individuals at hatch. The experimental groups were made to contain 0, 5, 10, 25, 50 and 100% chicks inoculated as embryos. The control group (group 1) contained 120 chicks from non-inoculated embryos. Groups 2, 3, 4, 5 and 6 contained 60 chicks each at hatch (Table 1). The experimental quail groups were raised in separate pens until 6 weeks of age. At this age 18 females and 12 males per group were placed in standard quail egg production cages at a 1:3 male:female ratio. Any extra birds not needed for the reproductive phase of the experiments were bled, humanely euthanatized and examined by necropsy. Daily egg production was recorded and all eggs produced were collected daily and stored at 18 C prior to incubation. Eggs were set for incubation to produce weekly hatches. All experimental breeder quail were kept until 20 weeks of age, at which time they were bled, humanely euthanatized and examined postmortem. Appropriate tissues were collected for PCR, immunocytochemistry and histopathology.

Second quail generation (F2). All eggs produced during the 12^{th} and 13^{th} weeks of age were incubated and hatched in two different dates (*F2a and F2b*). Thirty chicks produced per breeder group, or less if not enough chicks were produced, were raised in pens and kept until 6 weeks of age. At the termination of the experiment all second generation quail were bled,

humanely euthanatized and examined postmortem. Spleens, livers and suspect tissues were collected for microscopic examination.

Viremia at hatch was measured in day-old quail serum. All eggs produced between the 16^{th} and 18^{th} weeks of age were incubated in pools of eggs produced over the course of seven days and hatched in three different dates. Thirty chicks were bled at hatch per breeder group. Serum was used for virus isolation as described.

Experiment 2. The objective of this experiment was to study serological responses in growing and adult quail infected post hatch. One hundred µl of the REV APC-566 virus stock was inoculated via the intramuscular (i.m.) route. The titer of the inoculum was $10^{4.1}$ $TCID_{50}$ in 100 µl. Four experimental groups of quails with different ages were used. Each experimental group contained 20 birds, and at the time of REV inoculation the birds in each group were 3, 6, 14 and 23 weeks of age, respectively. Five additional quails per age group were kept as controls. Every 3 weeks after infection, 5 birds per group were bled, euthanatized and examined postmortem until the termination of the study (12 weeks post inoculation). All samples obtained were tested for virus isolation, ELISA and histopathology as appropriate.

Experiment 3. This experiment was performed to examine the susceptibility of chickens and turkeys to REV APC-566. SPF chicken fertile eggs were purchased from Sunrise Farms (Catskill, NY), incubated and hatched at the Poultry Diagnostic and Research Center (PDRC) facilities. After hatch the chicks were placed in groups of 8 chicks in Horsfall-type isolators including a group of negative controls, and inoculated with REV APC-566 virus stock i.m. using a dose of $10^{4.1}$ $TCID_{50}$ per bird. The SPF chickens were kept up to 9 weeks of age, soon after detection of neoplastic disease.

SPF turkey (*Meleagridis gallopavo*) fertile eggs were obtained from the Southeast Poultry Research Laboratory (USDA/ARS, Athens, GA). The eggs were incubated and hatched, and the poults housed and inoculated as described for the SPF chickens. The SPF turkeys were kept in isolators until 8 weeks of age, and then in pens until 14 weeks of age, soon after detection of neoplastic disease. Any bird that died during the experiment was examined postmortem and appropriate samples were obtained for histopathology. Soon after detection of neoplastic disease, all SPF chickens and SPF turkeys were bled, humanely euthanatized and examined postmortem. Liver, spleen, kidney, pancreas, bursa, thymus, small intestine, heart and nerve were collected for microscopic examination.

Statistical analysis. The statistical significance of differences in quail antibody response (geometric mean titer) was evaluated using the ANOVA Duncan test using the SAS software package (SAS institute Inc., Cary, ND). The χ^2 test was applied to analyze differences between mortalities. *P* values of ≤ 0.05 or ≤ 0.01 were used to define statistical significance.

RESULTS

Susceptibility to infection

Japanese quail inoculated as embryos with REV APC-566 exhibited a markedly delayed growth at 3 week post hatch (results not shown). REV was detected by PCR in nine growing quails that had been infected as embryos and died naturally, demonstrating that REV APC-566 successfully infected Japanese quail. REV was not detected in negative control quails of similar age. Several performance data indices were negatively affected in REV-infected Japanese quail and they will be described elsewhere (Barbosa et al., unpublished). Briefly, body weight was significantly reduced in breeder quail (F1) at 8 weeks of age, and in broiler quail (F2) at 3 and 6 weeks of age. Total mortality was higher at 6 and 20 weeks of age in the breeder quail that were infected as embryos. Egg production, hatchability and fertility were severely affected as well.

The mortality rate recorded is shown in Table 1. Japanese quail infected as embryos expressed 61.6% total mortality by 20 weeks of age in contrast with 10.8% in the negative controls, albeit not all the mortality was associated with tumor development. The mortality rate was significantly higher ($P<0.05$) at 6 weeks of age in any of the REV-infected groups in comparison with the controls. Mortality by 20 weeks of age was also significantly higher ($P<0.01$) in the groups containing 25%, 50% and 100% quails infected as embryos in comparison with the uninfected controls (Table 1).

Viremia

The results of virus isolation are shown in Table 2. The rates of viremia followed a pattern similar to the infection rate, where the groups with the highest proportion of inoculated

birds also had the highest number of viremic birds. At 6 weeks of age all birds that were positive by virus isolation were antibody-negative (tolerant) and those that were antibody-positive were not viremic at the time of sampling. Japanese quail from group 6 (100% infected as embryos) were not sampled at 6 weeks of age due the small number of survivors at that point. Generally, by 20 weeks of age the percent viremic birds had decreased when compared to an earlier age (6 weeks). However, the survivors in the group with 100% embryo-infected birds still had 64% REV-positive quail by 20 weeks of age.

All 335 samples from the quail broilers (F2) tested at 6 weeks of age were negative for REV on virus isolation. Likewise, all 540 blood samples tested from day-old quail (F2) were REV-negative on virus isolation.

Antibody responses

Serological responses to REV are summarized on Table 2. Anti-REV antibodies steadily increased in most of the REV-positive groups between 6 and 20 weeks of age. As mentioned above, all 6-week-old viremic breeders were REV antibody negative and vice-versa. The same pattern was observed at 20 weeks of age except in group 6 (100% infected) in which 8/14 quails (57.1%) were viremia and antibody positive; 5/14 (35.7%) were viremia negative and antibody positive; and only 1/14 (7.1%) was viremia positive and antibody negative. One contact quail from group 2 (5% infected quail) and another one from group 3 (10% infected quail) showed seroconversion at 6 weeks of age, indicating horizontal transmission had occurred.

Western blots were used to confirm ELISA results in select samples. Eight selected ELISA-positive samples also tested positive in Western blot assays. ELISA-negative samples were also negative on Western blots (results not shown). Anti-REV antibodies were not detected

in any sera from two hatches of F2 broiler quail produced by infected breeders and raised up to 6 weeks (results not shown).

Oncogenicity and pathology

REV APC-566 induced tumors in Japanese quail infected as embryos, as well as in SPF chickens and SPF turkeys infected at hatch. Adult Attwater's prairie chickens in the same captive colony from which REV APC-566 was isolated also developed tumors after natural infection with REV. Remarkably, the majority of Japanese quail breeders that expressed tumors were viremia negative and antibody-positive, except for one bird in group 6 (100% infected embryos), which was viremic and antibody negative (tolerant).

Macroscopic lesions in visceral organs of Japanese quail involved an enlarged liver and spleen with multiple white to grayish foci. Internally, the primary macroscopic lesions included gross enlargement of visceral organs and presence of multiple masses in the liver, spleen, pancreas, kidney, gonads and serosal membranes. In six-week-old Japanese quail, REV induced gross enlargement of the liver and spleen, which exhibited diffuse grayish areas suggestive of neoplasia. Such neoplastic organs occasionally filled almost completely the coelomic cavity.

In adult Attwater's prairie chickens, the lesions observed often included lymphomatous cutaneous involvement represented by gross thickening of the skin and scabbing. By 28 days of age, SPF chickens showed feather development abnormalities (Figure 1, panel d) and at 58 days of age, the same chickens developed gross enlargement of the spleen with multiple gray foci throughout the splenic parenchyma (Figure 1, panel e). A thirteen-week-old REV-infected SPF turkey died naturally with a grossly enlarged spleen and liver but no other obvious lesions.

Microscopically, most tumors consisted of solid sheets of infiltrating large uniformly-sized immature lymphoblasts with a clearly defined cytoplasmic membrane, an open and vesicular nucleus and a relatively prominent nucleolus (Figure 1). Mitotic figures were relatively rare in most cases. In all avian species examined (APC, Japanese quail, SPF chickens and SPF turkeys), the infiltrating lymphoblastoid cells were observed in a variety of visceral organs, including the liver, spleen, pancreas, gonads, heart, kidney and serosal membranes. The small intestine of Attwater's prairie chickens and turkeys was most severely infiltrated to the extent of neoplastic cells replacing completely all normal tissue in the lamina propria (Figure 1, panel b). One SPF turkey developed severe lymphocytic leukemia which was detected by 13 weeks of age (Figure 1, panels g-i). Cutaneous lymphosarcomas were found only in Attwater's prairie chickens. Intrafollicular bursal lymphomas were found exclusively in REV-infected SPF chicken (Figure 1, panel f). One tolerant (viremia-positive, antibody-negative) Japanese quail developed neoplastic lesions in the liver, where the cell type was not lymphomatous and could not be identified. In discrepancy with previous reports, peripheral nerve infiltrates were not detected in any of the avian species involved in this study.

All lymphosarcoma-positive tissues from Japanese quails were tested for T cells marker expression (CD3+). The transformed lymphocytes approximately 81% of quails with tumors (9/11) were identified as CD3+ cells. The extent of replacement of normal tissue architecture with CD3+ cells varied with the size of tumors. The remainder lymphosarcomas were not positive for CD3+ marker.

Responses to REV infection after hatch

Japanese quail first infected at 3, 6, 14 or 23 weeks of age mounted a strong antibody response and ceased to be viremic as assessed by virus isolation from whole blood as early as 3 weeks post inoculation (Table 3). Most of the birds were antibody positive in ELISA tests, except for two, one from the group of quail inoculated at 14 weeks of age (9 weeks post inoculation) and another one from the group inoculated at 23 weeks of age (12 weeks post inoculation). Over time REV ELISA titers tended to increase steadily in birds infected at younger ages, and to decrease in birds first infected at older ages ($P<0.05$) (Table 3).

DISCUSSION

Japanese quail proved to be a successful model for studying reticuloendotheliosis using a field REV isolated from wild birds. In this study the REV APC-566 obtained from Attwater's prairie chickens proved to be oncogenic in Japanese quail, SPF chickens and SPF turkeys. REV APC-566 also caused significantly decrease egg production, hatchability and body weight, and overall mortality reached 61% in quail breeders, in comparison with 10% in negative controls. In young Japanese quail REV APC-566 induced runting and decreased body weight gain (Barbosa et al., unpublished).

Not all quails infected as embryos became tolerant and most of them developed a detectable antibody response even after being infected with a relatively high dose of REV during embryonic development. However, as many as 64% of quail infected as embryos were still viremic by 20 weeks of age. Witter *et al.* (45) reported persistent viremia with absence of antibody in 36 of 38 chicks inoculated at 6 days of embryo incubation. In our study, three

categories of Japanese quail were observed, namely: a) tolerant (virus-positive, antibody-negative); b) partially tolerant (virus-positive, antibody-positive); and c) non-tolerant (virus-negative, antibody-positive). The rate of tolerance in quail was smaller than in chickens, as compared with results in previous reports (13, 16, 20, 45).

Working with chickens Witter, *et al.* (45) found that birds that are antibody-positive by 10 weeks of age tended to lose antibody titer with age. von Bülow (36) also working with chickens found maximum antibody levels by 3 weeks after infection, differing from our findings in quail. In our study, the geometric mean antibody titer (GMT) peak varied depending on the age at first infection, as shown on Table 3. GMTs increased progressively with age when Japanese quail were infected at earlier ages and decreased steadily after a peak response 3 weeks post-infection in birds first inoculated at older ages.

The evidence of anti-REV antibodies being neutralizing *in vivo* in quail is based on our observation in experiment 2 where Japanese quail were not viremic in the presence of either high or low antibody titers, regardless of the time elapsed after infection. Moreover, in experiment 1, all lymphosarcomas were found in anti-REV antibody positive quails, which all had the viremia neutralized at time of sampling. It is possible that anti-REV antibodies combined with cellular immunity may have neutralized REV to the extent of limiting vertical transmission. REV was not detected in quail chicks from 5 separate hatches (with virus isolation attempts performed in 3 hatches at day of age, and in 2 hatches at 6 weeks of age) from breeder quail infected as embryos. These results resemble those of a similar study in turkeys (43) where there was no evidence of REV vertical transmission. Vertical transmission of REV has been observed at a very low rate when it happens. CSV was detected in one out of 132 progenies of tolerant hens

(45), and in three of 98 eggs produced by persistently infected dams (37). In a natural REV field outbreak, 59 turkey breeder hens produced 1.8% (5/278) positive poults (44). Salter *et al.* (30) found REV proviral DNA in 3 of 262 progeny chicks (1.1%) from 25 viremic female chickens and in 10 of 820 progeny chicks (1.2%) form 18 viremic male chickens. However, Motha and Egerton (21) isolated virus from 18 of 35 chicks (51%) from eggs of viremic hens that were set within 24 hours of lay. In our study, eggs were set for incubation on a weekly basis, which may have reduced the viability of REV, possibly accounting for the failure of REV detection in the progeny.

The absence of congenital transmission may also be explained by the presence of few tolerant quail and the relatively low titer of virus shed by dams into eggs, as described by Witter *et al* (45). Although embryos may have been infected congenitally, the virus dose to which the embryos were exposed may have been insufficient to induce detectable infection. However, the progeny of infected dams was clearly at a disadvantage in terms of their delayed growth (results not shown). This REV behavior is different from avian leukosis virus, which may be shed into eggs at a relatively high titer (14, 28). In comparison, congenital transmission of REV in quail appears to be relatively inefficient. Although eggs were stored at 18 C with approximately 75% relative humidity, it is possible that egg storage for up to 7 days prior to incubation may have decreased the viability of REV, but this possibility was not tested. In addition, REV detection in the progeny was attempted from serum, which may be a less sensitive method than REV detection in plasma and/or plasma-rich white blood cells.

Horizontal transmission was detected in two birds (from groups 2 and 3, respectively), both of which developed antibodies and were not viremic at the time of sampling (6 weeks of

age), resembling the results of experiment 2 where infection post hatch lead to a rapid serologic response and a short period of viremia. This finding regarding horizontal transmission is similar to observations in chickens, turkeys and ducks (16, 20, 23, 24), but the rate of transmission was reduced in Japanese quail. Motha (20) did not find viremia in contact birds and the seroconversion occurred in 3/8 contact chickens. Larose and Sevoian (16) found 6/49 contact birds with antibodies against REV. Interestingly, they did not detect transmission from birds inoculated at hatch. This suggested that horizontal transmission of REV occurs slowly and possibly that very close contact is necessary between infected and non-infected birds. REV, like other retroviruses, may be quickly degraded outside the host at ambient temperature (2). Japanese quail must be reared at a relatively high environmental temperature during the first 3 weeks of age (35C), which may also contribute to REV inactivation at a critical age of susceptibility to REV.

Almost all tumors were classified as lymphosarcomas. Non-lymphomatous spindle-shaped cell coalescing tumors were found in one REV-infected quail but the cell type could not be characterized. This quail was the only one to be antibody-negative, viremia-positive and tumor-positive. Witter *et al.* (45) showed that REV caused non-lymphomatous tumors in tolerant birds (viremia-positive, antibody-negative). CSV caused myxosarcomas and fibrosarcomas at 35 weeks of age, and REV-A caused adenocarcinomas in 65-week-old chickens infected as embryos. The unclassified tumor found in a virus-positive, antibody negative quail was likely caused by REV since PCR reactions from affected tissues were positive to REV with different sets of PCR primers and negative to MDV (results not shown).

Early tumors induced by REV have been described in ducks infected with the RU-1 strain, which produced neoplasia as early as 58 days post-infection (17). In the present study, RE tumors induced by APC-566 were observed in Japanese quail as early as 42 days of age and up to 20 weeks of age (at the termination of the study). REV APC-566 also induced lymphosarcomas in chickens and turkeys inoculated at day-old (Figure 1). In a similar study by Witter *et al.* (45), chickens classified as non-tolerant (virus-negative, antibody-positive) were not viremic through 93 weeks and had a high rate of neoplastic response (13/14 birds). In the present study, infected Japanese quail produced high antibody titers and some developed tumors, albeit at a lower rate (6.66% compared to 92.8% in Witter's study). One possible explanation is the fact that our breeder study was terminated at 20 weeks of age, which quite possibly was insufficient to allow tumors to develop in more individuals.

Our immunohistochemical studies showed that the majority of lymphosarcomas in quails were CD3+. The cell type involved in lymphosarcomas induced by REV in quails had not been characterized prior to the present study. T-cell lymphomas caused by nondefective REV have been described in experimental inoculated chickens (39) and naturally infected turkeys (7). Birds infected with REV frequently develop humoral and cellular immunodepression (29). Some of the lymphosarcomas were not confirmed as CD3+, which could represent either transformed B cells or T cells not expressing detectable CD3. B-cell lymphomas have been described in chickens inoculated with CSV and REV-T (41, 45). Further studies may be necessary to determine whether REV is capable of inducing B-cell lymphomas in Japanese quail. REV APC-566 caused bursal lymphomas in SPF chickens inoculated at day of age,

demonstrating that the same REV strain may induce different types of lymphomas, as described earlier (39).

Evaluation of new REV isolates is important to determine if they represent new challenges to the poultry industry. In this case, the REV isolate was obtained from a captive colony of endangered wildlife. Part of the objectives of this study was to evaluate the pathogenicity and transmission of REV isolated from endangered wild avian species, and to demonstrate its oncogenic potential in other commercial poultry such as turkeys and chickens. Japanese quail proved to be a good model to study reticuloendotheliosis when compared to chickens. One obvious disadvantage is the very small size of quail chicks at hatch, which makes it extremely difficult to obtain adequate blood samples at critical young ages.

We have observed relatively few genetic changes in multiple field REV isolates (Barbosa *et al.*, unpublished). Clinically and pathologically, REV infection remains consistent regardless of the strain of REV. REV APC-566 is an isolate obtained from an endangered species infected enzootically and thus deserves consideration. Moreover, REV APC-566 was oncogenic in Japanese quail, chickens and turkeys. The natural reservoir of REV, if there is one, is not known. Thus, understanding the ecology, pathogenesis and transmission of field isolates of REV is of relevance to wild bird preservation programs and also to the poultry industry.

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Table 3.1. Experimental design and mortality of Japanese breeder quail (first generation).

	No. of birds			Percent mortality		
Group	Inoculated	Uninoculated	Infected (%)	0 to 6 weeks	6 to 20 weeks	Total
Neg Control	0	60	0	2.5	16.6	10.8
5%	3	57	5	16.6**	3.3	18.3
10%	6	54	10	11.6*	16.6	20.0
25%	15	45	25	16.6**	20.0	26.6**
50%	30	30	50	18.3**	30.0	33.3**
100%	60	0	100	38.3**	51.8**	61.6**

* Statistical significance at $P < 0.05$

** Statistical significance at $P < 0.01$

Table 3.2. Viremia, antibody response and oncogenesis induced by REV APC-566 in F1 breeder quail at 6 and 20 weeks of age.

	Virus isolation + IFA[1]				**REV ELISA serology**				**Neoplasia**		**Total[2]**	
	6 weeks		**20 weeks**		**6 weeks**		**20 weeks**					
Group	**Pos/Tot[2]**	**%**	**Pos/Tot**	**%**	**Pos/Tot**	**%**	**Pos/Tot**	**%**	**Pos**	**%**	**Pos/Tot**	**%**
Neg Control	0/30	0	0/30	0	0/30	0	0/30	0	0/60	0	0/60	**0**
5%	3/20	15.00	0/29	0	1/20	5.0	0/29	0	0/60	0	4/49	**8.16**
10%	2/25	8.00	0/25	0	1/25	4.0	4/25	16.0	4/60	6.6	7/50	**14.0**
25%	4/21	19.00	3/24	12.50	2/20	10.0	4/24	16.6	4/60	6.6	14/45	**31.11**
50%	2/19	10.50	1/18	5.50	3/20	15.0	10/18	55.5	1/60	1.6	16/37	**43.24**
100%	ND[4]	ND	9/14	64.30	ND	ND	13/14	92.8	4/60	6.6	14/14	**100.00**

[1] Virus isolations detected in DF-1 cells and immunofluorescence.

[2] No. positive in at least one test (Virus isolation, Serology, histopathology)/total tested.

[3] No. positive/No. tested.

[4] Not done.

Table 3.3. ELISA antibodies in Japanese quail infected during the rearing period or as adults.

Age at infection (weeks)	Weeks Postinfection							
	3 weeks		6 weeks		9 weeks		12 weeks	
	Pos/Total[1]	GMT[2]	Pos/Total	GMT	Pos/Total	GMT	Pos/Total	GMT
3	5/5	2950[b3]	5/5	8682	5/5	8345	5/5	10754[a]
6	5/5	7903[a]	5/5	8898	5/5	8950	5/5	8050[a]
14	5/5	9714[a]	5/5	9339	4/5	5484	5/5	3938[b]
23	5/5	8414[a]	5/5	5764	5/5	6566	4/5	2987[b]

[1] Number of positive sera over total sera tested.

[2] Geometric Mean Titer.

[3] Different superscripts in the same column indicate significant differences at $P < 0.05$ (ANOVA – DUNCAN)

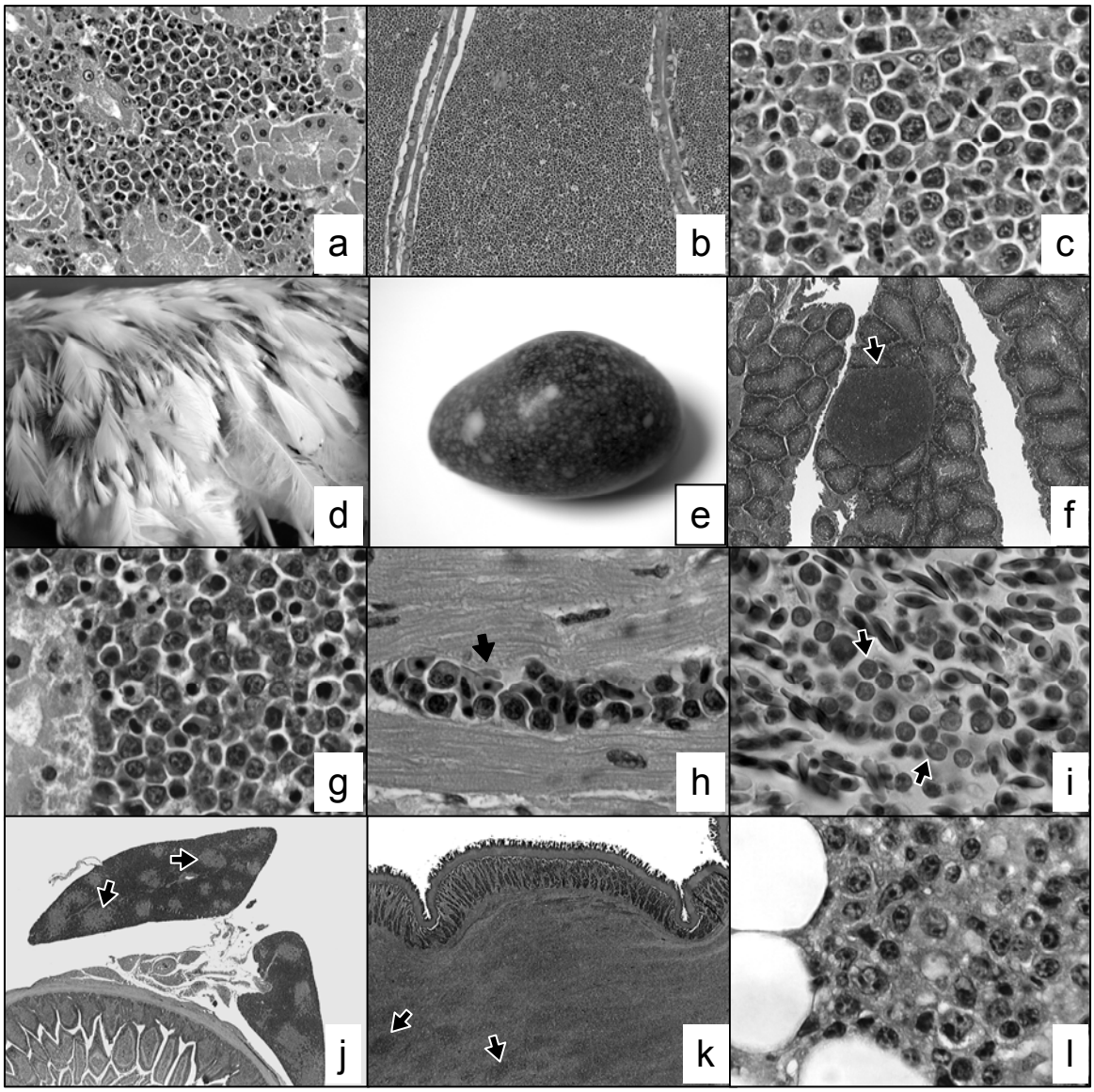

Figure 3.1. Gross and microscopic pathology induced by REV. Panels a-c correspond to Attwater's prairie chickens in the same captive colony from which REV APC-566 was isolated; panels d-f correspond to SPF chickens infected with REV APC-566 at hatch; panels g-i correspond to SPF turkeys infected with REV APC-566 at hatch; panels j-l correspond to Japanese quail infected with REV APC-566 as embryos. a) renal lymphosarcoma (H&E, 400X); b) solid sheets of infiltrating mononuclear cells in the lamina propria of the small intestine (H&E, 100X); c) renal lymphosarcoma containing

large immature uniformly sized lymphoblasts (H&E, 1000X); d) feather abnormalities (28-day-old SPF chicken); e) neoplastic foci in the spleen (58-day-old SPF chicken); f) follicular lymphoma (arrow) in the bursa of Fabricius (58-day-old SPF chicken) (H&E, 20X); g) renal lymphosarcoma (H&E, 1000X); h) myocardium with abundant intravascular large immature neoplastic lymphoblasts (arrow) (H&E, 1000X); i) a renal blood vessel exhibiting an overwhelming number of immature lymphoblasts (arrows) (Giemsa, 1000X); j) pancreas exhibiting multiple neoplastic foci (arrows) of immature mononuclear cells (H&E, 20X); k) neoplastic ventriculus displaying diffuse and coalescing neoplastic infiltrates of poorly differentiated mononuclear cells (arrows) in the muscularis (H&E, 20X); l) an aggregate of poorly differentiated neoplastic mononuclear cells in the adipose tissue of the heart (H&E, 1000X).

CHAPTER 4

EFFECTS OF RETICULOENDOTHELIOSIS VIRUS ON THE VIABILITY AND REPRODUCTIVE PERFORMANCE OF JAPANESE QUAIL[1]

[1] Barbosa, TMC., Zavala, G., Cheng, S., Lourenco, T., Villegas, P. (2006). Submitted to *Journal of Applied Poultry Research.*

ABSTRACT

The effects of Reticuloendotheliosis virus (REV) infection were studied using an experimental model in Japanese quail during two consecutive generations. The REV used in this study (APC-566) was isolated from Attwater's Prairie Chickens (*Tympanuchus cupido attwateri*). APC-566 induced tumors as early as 6 weeks of age. Mortality was significantly higher in groups of quail with a higher frequency of REV infection. Egg production, hatchability, and fertility rates decreased in infected quail as compared to uninfected control quail. The body weights of infected quail were significantly reduced at 8 weeks of age in the first generation of infected quail (breeders) and at 3 and 6 weeks of age in the second generation (quail broilers) compared to uninfected quail.

DESCRIPTION OF PROBLEM

Reticuloendotheliosis virus (REV) is an avian Gammaretrovirus, closely related to mammalian Type-C retroviruses. REV is immunologically, morphologically and structurally distinct from the avian leukosis/sarcoma viruses (ALSV). REV causes various syndromes in multiple avian species, including severe runting, an acute non-neoplastic syndrome with high mortality, severe immune suppression and T and/or B cell lymphomas. Non-defective REV strains have been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens [1]. REV infection can cause dramatic economic losses from a runting

syndrome or chronic neoplasia with mortality. Although these manifestations are not common, exposure to REV appears to be widespread in the field [2]. Significant losses can occur when REV-contaminated vaccines are administered to very young chickens. Weights of infected chickens may be 20-50% lower than uninfected controls by 3-5 weeks after infection [3]. Weight gain reduction has also been reported in infected ducks [4] .

The Attwater's prairie chicken - APC (*Tympanuchus cupido attwateri*), is a wild species of grouse on the verge of extinction. APCs were previously known to be susceptible to REV [5] and presently they are endangered partly due to enzootic REV infection. We have isolated REV from several APC specimens. One of such viruses was named APC-566 and randomly selected for these studies.

Japanese quail (*Coturnix coturnix japonica*) may be used as a model for studying vertically transmissible avian diseases [6] because of their rapid development and short generation interval, and because they are biologically related to domestic chickens (*Gallus gallus*) [7]. Japanese quail become sexually mature at approximately 6 to 7 weeks of age as compared to 24 to 28 weeks for chickens, and require much less space, labor, and maintenance cost. In addition, Japanese quail are prolific layers for most of the year [7]. The commercial quail industry is present in several regions of the world and involves egg and meat production.

To date, the effects of REV infection on the performance of commercial Japanese quail have not been examined and reported. This study was performed using Japanese quail as a model to better understand the effects of REV on the viability and performance of quail breeders and broilers.

MATERIALS AND METHODS

Quails. Commercially produced fertile eggs of Japanese quail (*Coturnix coturnix japonica*) were obtained from a local commercial hatchery after testing the breeders to insure absence of infectious REV and antibodies in the breeding population used to produce the eggs.

Viruses, virus isolation and virus titration. A field strain of REV named APC-566 REV was isolated in our laboratory from whole blood of an adult specimen of Attwater's prairie chicken (*Tympanuchus cupido attwateri*) held at the Fossil Rim Wildlife Center (Glen Rose, TX). REV was isolated in DF-1 cells and confirmed to be REV by indirect immunofluorescence, PCR and sequencing of the entire virus genome (GenBank accession No. DQ387450). A virus stock was prepared by passaging three times REV-infected DF-1 cells (ATCC UMNSAH/DF-1, CRL-12203). After the third passage in DF-1 cells the virus stock was titrated and frozen at -80ºC until used for this research. The virus titer in the stock was calculated by the Reed and Muench method using indirect immunofluorescence to identify REV positive virus cultures. Briefly, DF-1 cells were plated at a concentration of approximately 150,000 cells/ml and inoculated with serial ten-fold dilutions of APC-566 REV and incubated at 39ºC for 7 days. The inoculated cells and control cultures were then trypsinized, re-plated and incubated for approximately 2-4 days until they reached approximately 80% confluence and then fixed with a cold acetone-alcohol solution (60:40 v/v). IFA was performed following a modified pre-established procedure [8]. Skim milk in PBS (5%) was used as a blocking agent to reduce non-specific fluorescence, followed by incubation with polyclonal REV

antibody (Charles River SPAFAS, Franklin, CT). The cells were washed in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-chicken IgG (Zymed Laboratories, San Francisco, CA). A final wash in PBS preceded examination with a fluorescence microscope. REV-positive and REV-negative control cell cultures were included in all virological assays.

Experimental Design

First quail generation (F1). One hundred µl of APC-566 REV stock was inoculated into Japanese quail embryos via the yolk-sac at 4 days of embryonation. The titer of the inoculum was $10^{3.4}$ $TCID_{50}$ in 0.1 ml. Six experimental groups of quail chicks were produced to contain various proportions of infected individuals at hatch. The experimental groups were made to contain 0, 5, 10, 25, 50 and 100% chicks from inoculated embryos. The control group (G1) contained 120 chicks from non-inoculated embryos. Groups 2, 3, 4, 5 and 6 contained 60 chicks each at hatch. The experimental groups were raised in separate floor pens until 6 weeks of age. At this age, cloacal and esophageal swab samples were collected from 18 females and 12 males per group and all birds were placed in standard quail egg production cages at a 1:3 male:female ratio. Any extra birds not needed for the reproductive phase of the experiments were bled, euthanized and examined by necropsy. Daily egg production was recorded and all eggs produced were collected daily and either stored at 18ºC for weekly incubation or used for albumen sample collection. All experimental breeder quail were kept until 20 weeks of age, at which time they were bled, swabbed and examined postmortem. Any quail exhibiting clinical signs was immediately euthanized to avoid unnecessary distress. Tissues collected for PCR and/or histopathology included spleen, liver, kidney, heart,

brain, nerve, esophagus, gizzard, proventriculus, small intestine, pancreas, gonads, oviduct, skeletal muscle and thymus.

Second quail generation (F2). All eggs produced during the 12th and 13th weeks of age were pooled in two groups to produce two hatches, one hatch for eggs from week 12 (F2a) and another hatch for eggs from week 13 (F2b). Thirty chicks produced per breeder group (or less if 30 chicks were not available) were raised in floor pens and kept until 6 weeks of age. All second generation quail (F2) were weighed at 3 and 6 weeks of age and their weights were statistically compared. At the termination of the experiment (6 weeks of age) all quail were bled and examined on necropsy. Spleens, livers and suspect tissues were collected for microscopic examination.

Egg production, hatchability and embryodiagnosis. All eggs produced between the 6th and 19th weeks of age were collected and the hen-housed egg production calculated per group. All eggs produced during the 9th and 18th weeks of age were incubated for chick production. Embryodiagnosis was done in all the eggs not hatched and the causes of lack of viability were divided into five categories: a) infertile; b) early mortality (1-4 days of incubation); c) late mortality (14-17_days of incubation, i.e. after transfer) and/or cull chicks; d) pipped but not hatched; and e) broken egg shell.

Statistical analysis. The statistical significance of differences in quail body weights and embryodiagnosis was evaluated using the ANOVA Duncan and Scheffe using the SAS software package (SAS institute Inc., Cary, ND). The χ^2 test was applied to analyze differences between mortalities. A P value of ≤ 0.05 was used to define statistical significance.

RESULTS AND DISCUSSION

Uninoculated birds were negative for REV at all ages tested. REV was re-isolated from infected birds at various ages. REV APC-566 proved to be oncogenic in Japanese quail, producing lymphosarcomas and other tumors in up to 4.6% of birds from infected groups in F1 (results not shown). The mortality rate on the F1 generation was significantly higher in groups of quail with a higher frequency of REV infection (50% and 100% inoculated embryos). Most of the mortality in the remaining groups resulted from aggression and possibly overall unthriftiness (Table 1). Mortality caused by REV has been rarely reported in chickens [9, 10]

Affected birds in commercial flocks have been commonly culled prior to natural death and a culling loss of more than 50% between five and eight weeks has been described [11]. The present experiment showed that REV can cause high mortality rates in Japanese quail. Such mortality can be high, especially when the quail are kept for prolonged periods of time, as is the case in quail breeders.

The group with 100% infection as embryos showed the lowest average body weight at 8 weeks, being significantly different from all remaining groups. The uninfected control quail did not attain the highest body weights by 8 weeks of age. However, such birds were raised at a higher density, which may have contributed to their slower growth in the first generation of quail (F1). Early body weights in the second generation (3-6 weeks of age) were significantly reduced in the group where 100% of quail were infected, suggesting that REV infection may reduce growth significantly in the progeny of infected quail breeders (Table 2). Motha [12] showed that chickens infected

as embryos had significantly reduced body weights at 6, 25 and 51 days of age, when compared to uninfected birds. Weights of infected chickens were reduced 20-50% compared to uninfected controls [13-15].

Hen-housed egg production was significantly reduced in the groups where 50% and 100% of quail were infected. Embryodiagnosis of unhatched eggs revealed that hatchability and fertility may be affected by REV, since the group with the highest rate of infection as embryos (100%) showed significantly reduced fertility and hatchability rates (Table 3). Prior to our study, information about the possible effects of REV on the growth and egg production of Japanese quail was not available in the literature. The natural reservoir of REV is still unknown but REV is known to be capable of infecting a variety of avian species including commercial poultry. Thus, REV monitoring and control in the poultry industry must be exercised to avoid losses related to the ones herein described.

CONCLUSIONS AND APPLICATIONS

In this experiment we demonstrated that Japanese quail may be used as a model for studying detrimental effects of field strains of REV on viability and performance of commercial poultry in a relatively short period of time. Japanese quail embryos infected with REV had higher mortality caused by stunting and/or tumor development. REV infection was detrimental for body weights in two consecutive generations, and was also associated with decreased egg production, hatchability and fertility in Japanese quail.

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Table 4.1. Experimental design and mortality of Japanese quail breeders (first generation).

	No. of birds			Percent mortality		
Group	Inoculated	Uninoculated	Infected (%)	0 to 6 weeks	6 to 20 weeks	Total[3]
G1	0	60	0	2.5	16.6	10.8
G2	3	57	5	16.6**	3.3	18.3
G3	6	54	10	11.6*	16.6	20.0
G4	15	45	25	16.6**	20.0	26.6**
G5	30	30	50	18.3**	30.0	33.3**
G6	60	0	100	38.3**	51.8**	61.6**

* Statistical significance at $P < 0.05$

** Statistical significance at $P < 0.01$

Table 4.2. Mean body weight of growing quail breeders and quail broilers (g).

	F1 Breeder Quail		F2 Broiler Quail		
Group	N	8 weeks	N	3 weeks	6 weeks
G1	60	234.57 [a1]	60	120.97 [a]	227.21[a]
G2	30	242.34 [a]	60	111.53 [b,c]	226.92[a]
G3	30	238.10 [a]	60	112.35 [b]	217.19[a,b]
G4	30	236.08 [a]	60	104.26 [c,d]	218.02[a,b]
G5	30	225.38 [a,b]	52	99.04 [d]	216.06[a,b]
G6	18	214.29 [b]	29	98.73 [d]	208.89[b]

[1] Different superscripts in the same column indicate significant differences at $P < 0.05$ (ANOVA-DUNCAN).

Table 4.3. Mean hen-housed egg production and embryodiagnosis of incubated eggs from REV-inoculated Japanese quail.

			Embryodiagnosis[2]						
	Hens	Egg production[1]	Incubated eggs	Hatchability	Infertile	Early dead	Late dead/cull	Pipped	Broken egg
Group	(n)	%	(n)	%	%	%	%	%	%
G1	36	64.91 [a3]	1955	60.97 [a,b]	29.46 [a,b]	0.91	1.87	4.78	2.26
G2	18	80.22 [a]	1167	82.69 [a]	8.45 [a]	0.92	2.72	2.37	1.50
G3	18	68.54 [a]	973	73.47 [a,b]	19.99 [a,b]	0.86	2.34	1.92	0.86
G4	18	72.68 [a]	1064	59.47 [a,b]	30.81 [a,b]	0.87	3.15	2.87	1.67
G5	18	40.76 [b]	572	59.44 [a,b]	29.22 [a,b]	1.51	4.01	2.94	1.70
G6	13	42.70 [b]	483	49.43 [b]	36.92 [b]	1.71	4.17	3.50	2.57

[1]Average hen housed egg production for 14 weeks of egg production (weeks 6 through 19);

[2]Embryodiagnosis for 10 weeks of egg production and incubation (weeks 9 through 18).

[3] Different superscripts in the same column indicate significant differences at $P < 0.05$ (ANOVA – SCHEFFE).

CHAPTER 5

FULL GENOME SEQUENCE OF RETICULOENDOTHELIOSIS VIRUS STRAIN APC-566[1]

[1] Barbosa, TMC., Zavala, G., Cheng, S., Villegas, P. (2006). Submitted to *Virus Research.*

ABSTRACT

Reticuloendotheliosis virus (REV) infection has been associated with runting, high mortality, severe immunosuppression, and chronic neoplasia in adult birds with T and/or B cell lymphomas. REV has been described in Attwater's Prairie Chickens (APC) (*Tympanuchus cupido attwateri*). The complete proviral sequence of the REV strain APC-566 was determined. This virus was isolated from a blood sample obtained from an APC maintained in captivity in a reproduction program intended to avoid its extinction. The APC-566 strain of REV was inoculated in Japanese quail in which it produced tumors as early as 6 weeks post-infection. The proviral genome is 8286 nucleotides in length and exhibits a genetic organization characteristic of replication-competent gammaretroviruses. APC-566 contains two identical long terminal repeats (LTR) and a complete set of genes including *gag, gag-pol* and *env*. Alignments with other REV sequences showed high similarity with sequences found in *gag* and *pol* genes from other REVs. The APC-566 *env* gene showed high nucleotide homology with REV sequences inserted in fowl poxvirus (99.8%), and with spleen necrosis virus (SNV) (95.1%). Sequences coding for a previously reported immunosuppressive peptide contained in the TM region of the *env* gene are well conserved among all REV sequences analyzed. The LTR was the most divergent region analyzed, exhibiting various deletions and insertions. APC-566 has a unique insertion of 23 bp in U3 and shares deletions of 19 and 5 bp with chicken syncytial virus and REV inserts in fowl poxvirus. These results may explain the origin of REV in the APC population where the fowl poxvirus is endemic.

INTRODUCTION

Reticuloendotheliosis is an oncogenic and immunosuppressive disease of multiple avian species and is caused by reticuloendotheliosis virus (REV). REVs are a group of pathogenic avian retroviruses that are antigenically distinct from, and genetically unrelated to the avian leukosis sarcoma virus group of retroviruses (ALSV) (Witter and Fadly, 2003). REV shares some morphologic, structural and antigenic similarities to mammalian Type C retroviruses, and is currently classified as a *Gammaretrovirus*. REV causes runting, an acute non-neoplastic syndrome in young birds characterized by high mortality and severe immunosuppression, and chronic neoplasia in adult birds with T and/or B cell lymphomas. REV was isolated originally from a turkey with lymphoid tumors (Zeigel et al., 1966). Representative strains of REV include REV-T, a defective virus of turkeys and REV-A, a non-defective virus also from turkeys. Spleen necrosis virus (SNV) and duck infectious anemia virus are representative REVs from ducks, and chick syncytial virus (CSV) was originally isolated from a chicken. In addition, REV has been isolated from other avian species including geese, Japanese quail, pheasants and Attwater's prairie chickens (APC) (*Tympanuchus cupido attwateri*) (Drew et al., 1998; Schat et al., 1976).

The wild population of APCs was once estimated at more than one million birds inhabiting 2.4 million ha of prairie habitat on the coastal areas of Texas and Louisiana (USA) by 1941. Human-induced habitat loss and fragmentation has further reduced this subspecies to three small, isolated populations totaling less than 70 birds (Drew et al., 1998).

We report the sequencing and analysis of the complete proviral genome of the oncogenic REV strain APC-566. This data is a vital step in the study of this virus and opens the way to further studies to elucidate the pathogenesis and oncogenic mechanisms of this virus.

MATERIALS AND METHODS

Virus and provirus source. During 2004 and 2005 our laboratory received multiple clinical samples from captive APCs and greater prairie chickens (GPC) kept at the Fossil Rim Wildlife Center (Glen Rose, TX) in a preservation and reproduction program intended to avoid the extinction of the APC. Briefly, the samples consisted of refrigerated whole blood with anticoagulant, skin scabs and formalin-fixed tissues. One REV PCR-positive blood sample was randomly chosen for further evaluation. The identity of such sample was APC-566 corresponding to the identification of the APC specimen. APC-566 was a male hatched in May of 2001 and it was first diagnosed with REV infection in February, 2004. Thereafter this bird was treated on various occasions for several minor infections. APC-566 died in April of 2005 from an internal hemorrhage. None of the tissue samples from this bird examined on histopathology revealed any significant lesions.

REV APC-566 was isolated and propagated in DF-1 cells (ATCC UMNSAH/DF-1, CRL-12203), an immortalized fibroblastoid cell line derived from line 0 chicken embryos (Himly et al., 1998).

After 3 consecutive passages the virus was inoculated in the yolk sac of 4 day-old Japanese quail embryos. Quails were hatched and placed in pens until sexual maturity (6 weeks of age), at which time they were transferred to standard breeding and egg production cages. The birds were kept up to 20 weeks-old when all were humanely euthanized and examined postmortem. Suspect tumors were collected for microscopic examination and PCR. Lymphosarcomas were diagnosed in several tissue samples and one of these samples was used as source of proviral DNA.

DNA extraction, amplification and sequencing. Total genomic DNA was extracted from a lymphosarcoma using a commercial DNA extraction and purification kit (High Pure PCR Template Preparation Kit, ROCHE Biochemicals, Manheim, Germany). The DNA was kept at 4ºC and used as necessary for proviral DNA amplification.

Oligonucleotide primers were designed in our laboratory in overlapping regions for PCR amplification and direct sequencing from PCR products (Table 1). The oligonucleotide primers were designed using the OligoPerfect™ Designer web-based program (Invitrogen, Carlsbad, CA), based on published proviral DNA sequences of the REV-China strain HA9901 (GenBank NC006934). All oligonucleotides were synthesized at IDT – Integrated DNA Technologies, Inc. (Coralville, IA).

For PCR amplification, the initial cycle included denaturation for 8 min at 94 ºC, annealing for 2 min at 54 ºC and extension for 5 min at 72 ºC. This first step was followed by 35 cycles of denaturation for 30 sec at 94 ºC, annealing for 60 sec at 54 ºC and extension for 2-4 min at 72ºC (depending on the product size). One final cycle was performed with denaturation for 60 sec at 94 ºC, annealing for 3 min at 54ºC and extension for 5-10 min at 72 ºC in a DNA Engine DYAD^{TM} Peltier Thermal Cycler (MJ

Research, Waltham, MA). PCR products with expected sizes were gel-purified using a commercial DNA purification kit (QIAquick DNA Gel Purification Kit, QIAGEN, Inc., Valencia, CA). Sequencing of both strands was done using the dideoxy chain termination method at the Molecular Genetics Instrumentation Facility (MGIF), University of Georgia, Athens, GA.

Proviral DNA and predicted amino acid sequence analysis. The individual overlapping sequences from PCR products were aligned for preparation of a contiguous sequence of REV-APC 566 proviral genome using the Seqman 6.1 function in the DNASTAR sequence analysis software (DNASTAR, Inc., Madison, WI). The sequences resolved were aligned with REV China strain (GenBank NC006934), REV-A (GenBank S70398 and X01455), SNV (GenBank DQ003591 and M87666), CSV (GenBank M22223), REV proviral insertion in fowl poxvirus (GenBank AF246698 and AY255632) and MDV (GenBank S82226) using the MegAlign 6.1 sequence analysis function in DNASTAR (DNASTAR, Inc., Madison, WI, Madison, WI) and Mega 3.01: Molecular Evolutionary Genetics Analysis (Kumar et al., 2004), using the Clustal W alignment with weighted residues method.

Nucleotide sequence accession numbers. The sequence data obtained in this study have been submitted to the GenBank Nucleotide Sequence Database and are listed under Accession No. DQ387450.

RESULTS

Nucleotide sequences of the provirus. The complete proviral nucleotide sequence of reticuloendotheliosis virus strain APC-566 was fully determined. As shown in Fig. 1, the APC-566 REV genome is 8,286 nucleotides long. The base composition of the proviral DNA can be deduced to be 25.3% A, 22.3% T, 25.8% C, and 26.4% G. The APC-566 genome has a genetic organization typical of replication-competent Gammaretroviruses. The *pol* gene in gammaretroviruses is situated in the same continuous ORF as *gag* and expression of *pol* apparently occurs via termination suppression of an amber stop codon (Felsenstein and Goff, 1992). The *env* gene is located in a different ORF and its expression is driven by a spliced mRNA, and the splice donor (SD) and splice acceptor (SA) sequences were determined and are shown in Figure 1. The splice sequence has been described (Watanabe and Temin, 1983) and is similar in REV APC-566.

Analysis of the LTR and its regulatory elements. The REV APC-566 proviral sequence contains two identical long terminal repeats (LTR), each with 545bp pairs, with typical retroviral U3-R-U5 organization (Figure 2). Comparative analysis of LTR sequences was performed in an initial effort to identify sequences that might determine unique pathogenic properties. Multiple sequence analysis alignments were generated using the APC-566 LTR and several other REV LTR's previously reported. The REV APC-566 LTR showed 89.5% identity to the equivalent region in REV-A (Filardo et al., 1994). Such LTR region in APC-566 exhibited an identity of 98.3% to an MDV REV provirus insert (Jones et al., 1996); 97.9% to fowl poxvirus REV proviral inserts (Jones et

al., 1996; Singh et al., 2003); and 97.7% to CSV (Swift et al., 1987). On the other hand, the APC-566 only shares 73.5% identity with SNV LTR (Kewalramani et al., 1992). Within the LTR, the U3 region was the most divergent region, while the combined R and U5 regions exhibited a similarity of 94.5% with SNV and 99.4% with fowl poxvirus REV proviral inserts.

The U3 region in APC-566 contains insertions and deletions shared with CSV and REV proviral inserts in fowl poxvirus and MDV (Fig 2). A significant insertion, only present in APC-566, is located at the U3 5' end and consists of a repeat sequence of the first 23 bp. We also sequenced equivalent regions in two additional REV isolates from APCs in the same captive bird colony (APC-731 and APC-982), in which an identical pattern was observed (data not shown). Two short sequences of 19 and 5 bp respectively found only in the REV-A (Filardo et al., 1994) and REV strain HA9901 from China, are absent in the APC-566 strain of REV, CSV and REV proviral inserts of fowl poxvirus. The 19 bp insert forms part of a 22 bp direct repeat element, and contains an additional CAAT-like box unique to REV-A and the REV strain HA9901 from China (Fig 2). The regulatory elements for viral RNA synthesis (e.g. the CAAT box, the TATA box, and the polyadenylation signal) are indicated in Fig. 2 and tend to be conserved among our alignments and in previously described sequences (Ridgway et al., 1989).

The 5' LTR of REV APC-566 is followed by a primer binding site (PBS) complementary to the 3' end of $tRNA^{pro}$, which is common in type C viruses. An additional regulatory element is the packaging or encapsidation signal, which is located downstream from the PBS. Biochemical studies have indicated that the sequence

between the PBS and the splice donor site preceding the start codon of *gag* is important for dimeric RNA generation (Darlix et al., 1992).

Analysis of the *gag* and *gag-pol* genes. The gag protein of the APC-566 strain of REV is probably translated from the second available ATG codon at position 571. The gag precursor protein is 499 amino acids long, a similar protein size in analogous Type-C retroviruses. Alignment of APC-566 with available REV gag sequences revealed high similarity at the amino acid level, as shown by 99.8% identity with fowl poxvirus REV proviral insertion; 98.1% with the REV strain HA9901; and 97.4% with SNV. The *gag* cleavage sequences were localized as previously described (Tsai et al., 1985), p12 extending from amino acid 2 to 113; p18 from amino acid 114 to 199; p30 from amino acid 200 to 443; and p10 from amino acid 444 to 494 (Figure 1). The precursor Gag-Pro-Pol polyprotein is probably expressed by a translational read-through mechanism of a UAG termination codon at the 3' end of the *gag* gene (Felsenstein and Goff, 1992). As expected, the *pol* gene was the most conserved region among all sequences analyzed. The APC-566 *pol* gene is 100% identical to that of fowl poxvirus REV proviral inserts, and 98.3% and 96.8% identical to REV strain HA9901 and SNV, respectively.

Analysis of *env.* Envelope gene variability occurs in many retroviruses either because of immune pressure or recombination. Being a gammaretrovirus, the APC-566 *env* gene is probably translated from a spliced mRNA and it codes for two proteins, namely the surface (SU) and transmembrane (TM) proteins, in addition to a Leader sequence of 36 amino acids located in the NH2 terminal region. The precursor polyprotein is probably cleaved by enzymes in the Golgi apparatus (Bedgood and

Stallcup, 1992). The putative site of cleavage has been identified (Kewalramani et al., 1992) and we located this position on amino acid 398 (Figure 3).

The predicted *env* polyprotein sequence of APC-566 was aligned against other REV *env* genes. The REV *env* sequences analyzed did not contain any obvious insertions, deletions, or blocks of sequence with significant divergence, indicating that *env* variability among REV isolates is minimal. The *env* gene of APC-566 showed an amino acid identity of 95.1% to the SNV *env*. An identity of 95.6% was observed when compared to REV strain HA9901. The highest amino acid identity noted was with fowl poxvirus REV proviral inserts (99.8% of the predicted amino acid sequence of *env*). The previously described immunosuppressive peptide described in type D retroviruses and SNV (Kewalramani et al., 1992) was highly conserved in all sequences examined (Figure 3).

DISCUSSION

The complete sequence of a reticuloendotheliosis virus from a natural outbreak in wild birds is presented. Such REV was isolated from an endangered avian specimen (Attwater's prairie chicken), and is the first wild life REV to be fully sequenced. The genome organization of APC-566 was consistent with the genetic organization of gammaretroviruses, with two different ORFs and the *env* gene likely being translated from the only spliced mRNA as in all simple retroviruses. Prior to sequencing, we determined that the REV APC-566 isolate is oncogenic in quail, chickens and turkeys.

REV APC-566 also caused runting in young Japanese quail and decreased egg production and hatchability in quail breeders (data not shown).

Alignments and phylogenetic analysis proved that all available REV proviral sequences are closely related and show little variation. Several reports have shown differences in pathogenicity (Purchase et al., 1973; Witter, 1997). Such findings may be more related to regulatory sequences present in the LTR than to specific differences in the protein coding genes. As the LTR was the most divergent region on the genome, its unique deletions and insertions may play a role in the pathogenicity of REVs. Filardo, *et al.* (1984) indicated that an interaction of the LTR with the structural genes of the virus may be responsible for variation in virulence between CSV and REV-A. Our results did not show any significant differences of predicted amino acid sequences in the protein coding genes. It may be that the interaction of specific regions in the LTR with structural genes may play a determinant role in the differences of oncogenicity and pathogenicity among viruses belonging to the REV group. Such specific interactions have not been characterized, and their study may be required for better understanding of REV pathogenesis. REV can be differentiated genetically based on LTR sequences as indicated on Figure 2. We also suggest that diagnostic PCR targeting the *pol* gene may be more adequate than targeting the LTR due to higher genetic variation in the LTR than in the *pol* gene.

APC-566 sequences are more closely related to sequences resolved in CSV and fowl poxvirus (Singh et al., 2003; Swift et al., 1987), which suggests a possible origin of APC-566 and how it may have been introduced in the APC captive population (Drew et al., 1998). Full or partial REV integrated sequences have been reported in fowl poxvirus

and MDV (Calvert et al., 1993; Kim and Tripathy, 2001; Moore et al., 2000; Tadese and Reed, 2003; Zhang and Cui, 2005). The ability of REV to easily integrate into dsDNA viruses of high molecular weight is not well understood, but it certainly could be one strategy for virus spread (Kim and Tripathy, 2001), since the rate of congenital transmission is not as high as observed with other avian retroviruses such as avian leukosis virus (Witter and Crittenden, 1979; Witter et al., 1981). During 2004 and 2005 there was a confirmed fowlpox outbreak in the captive population of APCs, concomitant to enzootic REV. It is possible that REV may have been introduced simultaneously but independently from fowl poxvirus, or integrated into the fowl poxvirus genome. REV can also be mechanically transmitted by arthropods as well fowl poxvirus does (Motha et al., 1984), which could also explain why REV may be endemic in the APC captive population despite all efforts to control reticuloendotheliosis.

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Table 5.1. Primers used for PCR amplification and sequencing.

Location	**Forward 5':3'**	**Reverse 5':3'**
1 – 800	AATGTGGGAGGGAGCTCC	GTAACAAACAAACACACAAACCAC
500 - 1300	GGGTTGGGATCCGGACT	ACTTTTTGGCTAGCTAACAGAACTTT
1000 - 1800	TCTGACTTTAAGAAGAGGGCG	TCCTGACCTCCCGCCTA
1500 - 2300	TGGATTAGAGTTCGGAGCACA	TCCTCTCTCCGCTCTACTCTCC
2000 – 2800	AAAGCCCTACAGCTTACCTGG	TACCAATAGTTTCCCTTCTGTCC
2500 – 3075	CAAATAATAGATTTTCTAGTAGATACGGGA	AGTGGACGGGTCTCAGGA
2775 – 3575	TACGGACAGAAGGGAAACTATTG	CTTCTTCCCTGAAACCCG
2775 – 4075	TACGGACAGAAGGGAAACTATTG	GAGGCTTCTCTTGTGAGAAGAGC
2775 – 4575	TACGGACAGAAGGGAAACTATTG	TGGCGAATGCATAACGG
2775 – 5075	TACGGACAGAAGGGAAACTATTG	ATCTCTAGCTGCTCGATAAATCCC
4777 – 5575	CGAGAAGTAGCTATACGTCCTTTG	ACATCGTGCCCGGAGC
5277 – 6295	AGCAAAGAGAGAAACCTCCCA	AGGGCCTTGGCTGCTC
6052 – 6795	TTGGGACCACTGCCGA	ATTTCCATCGAGGGTGACTTC
6552 – 7295	GTGCGTGAAAGACTAGAGGAAATC	ATGGTCCCTGAAAGAGCCT
7052 – 7795	GTGCATACTGGCATCAATCG	GCAGTTAGCGAGCCAGC
7052 – 8295	GTGCATACTGGCATCAATCG	CCCCCAAATGTTGTACAGAAAT
8052 – 8295	AAAATGTCATGCAACATCCTGT	CCCCCAAATGTTGTACAGAAAT

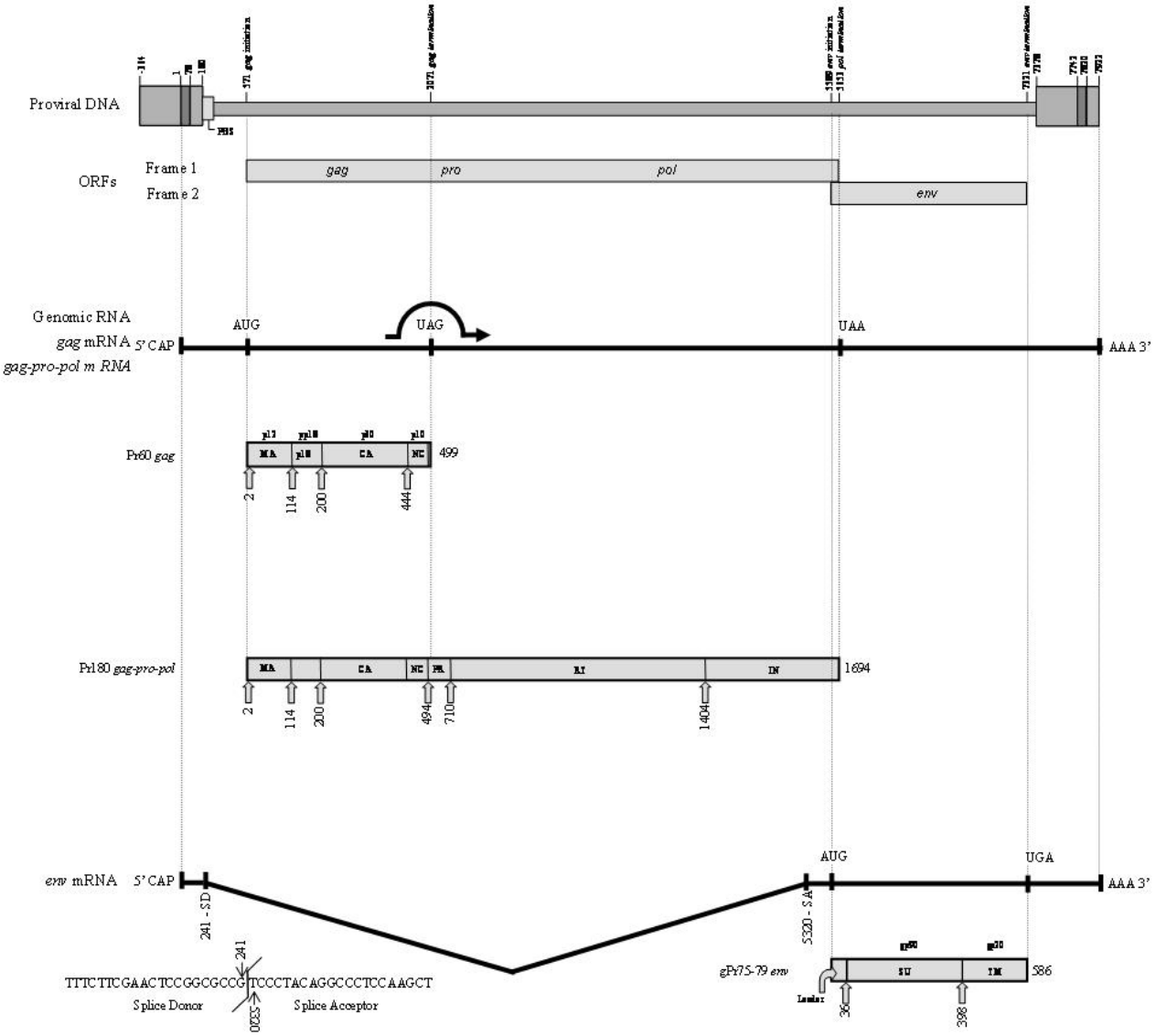

Figure 5.1. Organization of the APC-566 proviral genome. The organization of the REV APC-566 proviral genome is shown according to conventional criteria. Figure not to scale. The long terminal repeats (LTRs) are 544 bp each and the total length of the proviral genome is 8286 bp. The *gag* and *pol* genes are in the same open reading frame and the *env* gene is in the -1 frame. The *gag* gene extends from nucleotides 571 to 2070; the *pol* gene extends from nucleotides 22071 to 5653; and *env* gene extends from residues 5589 to 7321. The splice donor and acceptor sequences are shown with respective locations.

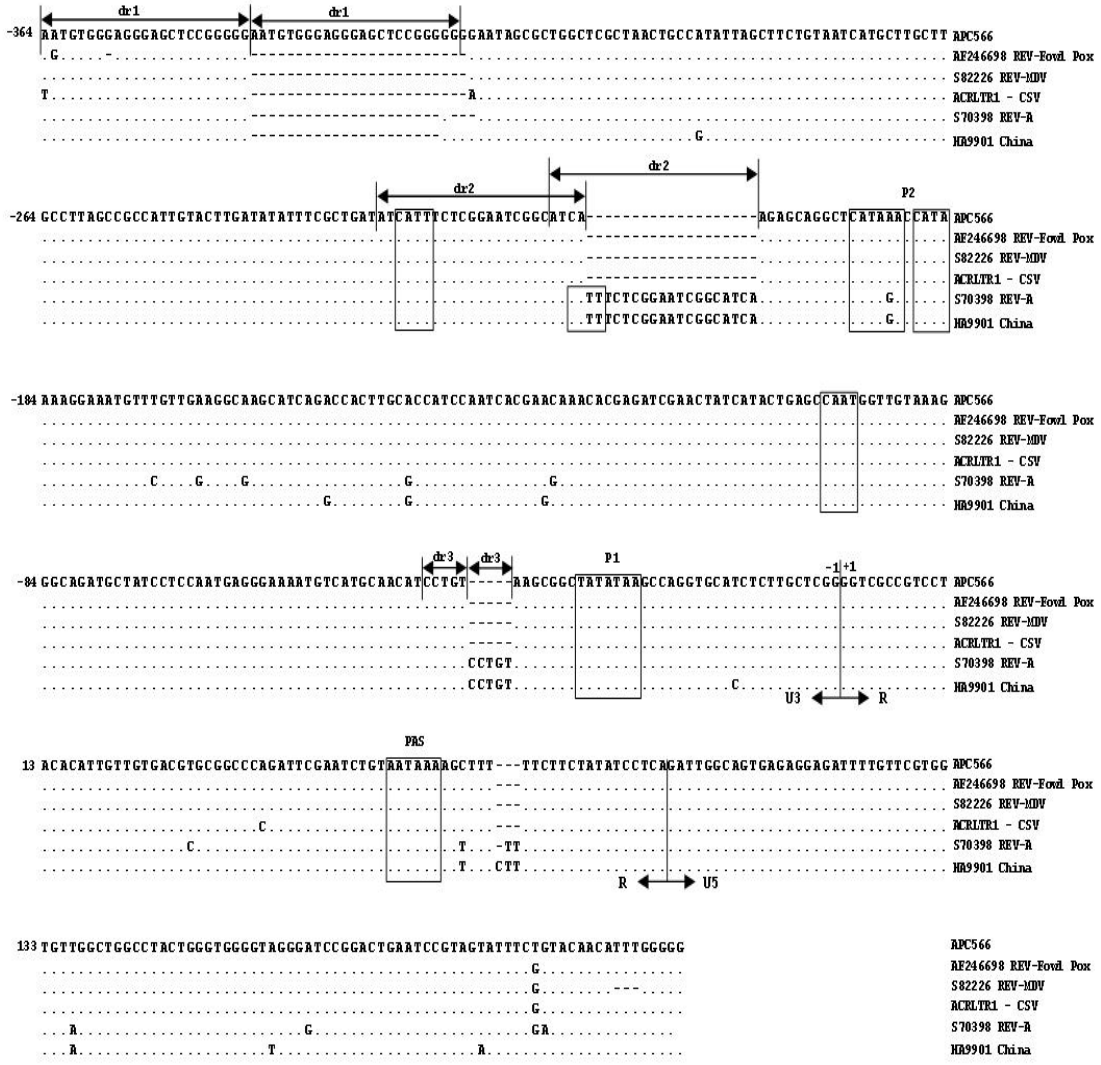

Figure 5.2. DNA sequence analysis of the LTR region. Positions of identity are indicated by dots while nucleotide differences are appropriately marked. The locations of U3, R and U5 are indicated. Regions important in establishing the initiation of transcription, including TATA-like boxes P1 and P2 (Ridgway et al., 1985), are shown. CAAT-like boxes that precede P1 and P2 are outlined as is the polyadenylation signal (PAS) (Filardo et al., 1994). Three different direct repeat (dr) regions are indicated. The first base in R is designated as position +1. Numbers shown at the left mark the positions relative to the start of transcription of REV APC-566.

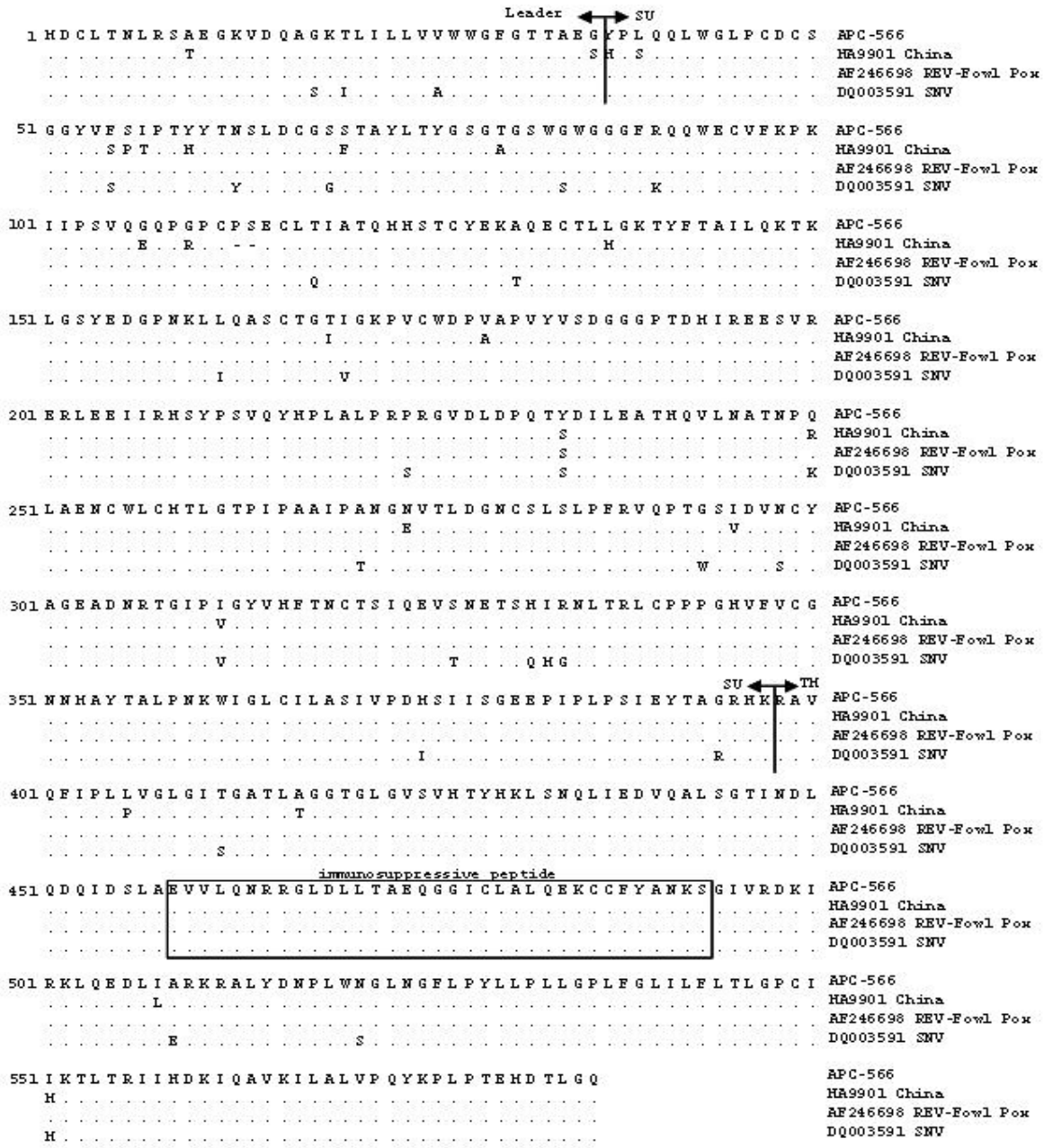

Figure 5.3. Alignment of the predicted amino acids of the envelope proteins. Only residue changes are indicated. Dots represent identical residues. The cleavage sites between the Leader, SU and the TM proteins are indicated. The immunosuppressive peptide sequence is indicated. Numbers shown at the left mark the positions relative to the start of translation.

CHAPTER 6

IMMUNE RESPONSES TO *PASTEURELLA MULTOCIDA*, PARAMYXOVIRUS AND BIRNAVIRUS IN TURKEYS INFECTED WITH A RECENT FIELD ISOLATE OF RETICULOENDOTHELIOSIS VIRUS[1]

[1] Barbosa, TMC., Zavala, G., Cheng, S., Villegas, P. (2006). To be submitted to *Avian Diseases.*

ABSTRACT

The effects of Reticuloendotheliosis virus (REV) on turkey immune responses against inactivated viruses (Newcastle disease virus and birnavirus) and *Pasteurella multocida* vaccines were evaluated. Compared with the non infected controls, two weeks post-vaccination there was a significant reduction of serological responses to IBDV and NDV as measured by enzyme-linked immunosorbent assays (ELISA) and to NDV as evaluated by ELISA and hemagglutination inhibiton (HI) in REV-infected turkeys. However, by 3 weeks post vaccination the serological responses were only numerically different. Turkeys infected with REV and challenged with *Pasteurella multocida* died sooner than their REV-free hatchmates. An immunosuppressive peptide (ISP) coding region, previously described in mammalian retroviruses was identified in the transmembrane coding region of the envelope gene from eleven REV isolates from endangered Attwater's Prairie Chicken (APC) isolates, one Greater prairie chicken (GPC) isolate, one turkey isolate, two broiler breeder isolates and the non-defective strain REV-A. All REV ISP sequences from APCs, GPC, chickens and turkeys share identical predicted amino acid sequences. The 5' end of the peptide is the most conserved region and few substitutions are seen at the 3' end when compared with the Murine leukemia virus (MLV) where this peptide was first identified. A look at the potential for immunosuppression by the new REV isolate APC-566, with serological responses to inactivated antigens in REV-infected birds is shown. Further researches on the functional influences of this peptide on avian cell mediators and immune responses and challenge

studies with live virulent viruses are needed to confirm the putative deleterious effects of such immunosuppressive peptide in REV isolates from birds.

INTRODUCTION

Reticuloendotheliosis (RE) is a neoplastic and immunosuppressive condition induced by members of the Reticuloendotheliosis virus (REV) group. They are immunologically, morphologically, and structurally distinct from the Avian Leukosis/Sarcoma group of retroviruses (17, 21). The REV group includes replication defective (REV-T) and non defective (REV-A) turkey isolates, spleen necrosis virus (SNV) from chickens, chicken syncytial virus (CSV), and duck infectious anemia virus (DIAV) as representative strains. REV-T was originally isolated from turkeys and carries a transduced cellular oncogene known as *v-rel* (5). Non-defective REV strains have been isolated from various avian species including chickens, turkeys, ducks, pheasants, geese, Japanese quail, peafowl and prairie chickens (2, 4, 9, 16, 23, 28, 30).

Humoral and cellular immune responses are frequently depressed in chickens infected with non-defective REV strains. Depressed antibody responses to Marek's disease virus (27), Newcastle disease virus (31), sheep erythrocytes and *Brucella abortus* (29) have been reported. Mammalian oncogenic and immunosuppressive retroviruses like murine leukemia virus (MLV), human T cell leukemia virus (HTLV) and Mason Pfizer monkey virus (MPMV) are know to carry highly conserved immunosuppressive sequences in their envelope gene, particularly in the transmembrane region (7, 11). Haraguchi *et al.* (11), identified a highly conserved short oligopeptide coding sequence

within p15 in the envelope transmembrane protein (TM) gene of murine (MLV), feline (FLV), simian (BaEV), human (HTLV), and turkey (REV-A) retroviruses.

REV APC-566 was isolated in our laboratory from an Attwater's prairie chicken kept in an inbreeding program in Texas and may be considered one of the most recent field isolates of REV. We have isolated and characterized REV APC-566 in terms of its oncogenicity, transmissibility and detrimental effects on economic aspects of breeding and growing birds. In Japanese quail, REV APC-566 negatively affected body weight of breeder quail at 8 weeks of age, and its progeny at 3 and 6 weeks of age. Total mortality was higher at 6 and 20 weeks of age in the breeder quail that were infected as embryos compared with non infected controls. Egg production, hatchability and fertility were also severely affected in quails, and REV APC-566 was also oncogenic in Japanese quail, SPF chickens and turkeys (Barbosa, *et al.* unpublished). Immunosuppressive studies seems to be necessary because in our diagnostics, REV infection in prairie chickens not only induces mortality and lymphomas, but also coincides with other infectious diseases such as parasitic disease, septicemia and avian pox (Zavala, *et al*. unpublished). Being APCs endangered of extinction (9), possible immunosuppression can not be studied in such avian species and therefore turkeys were chosen to assess the potential immunosuppression of this new isolate.

We report some aspects of potential immunosuppressive properties of immune responses of turkeys infected with REV APC-566, an isolate from wild endangered prairie chickens, which is a recent and still circulating REV isolate. Furthermore, we show the similarities of part the TM protein of multiple REV isolates from different wild

and domestic avian species, which share the same highly conserved immunosuppressive peptide found in other mammalian Type-C and Type-D retroviruses.

MATERIAL AND METHODS

Viruses. REV APC-566 was isolated from a blood sample of an Attwater's Prairie Chicken (GenBank accession number DQ387450). The isolate was propagated in DF-1 cells (ATCC #CRL-12203), a spontaneously immortalized fibroblastoid cell line derived from East Lansing chicken line zero embryos (ELL-0) (12). After 3 consecutive passages of infected cells in culture, the virus stock was frozen at -80 C until used. The virus titer ($10^{5.1}$ $TCID_{50}$/ml) in the stock was calculated by the Reed and Muench method using indirect immunofluorescence to identify REV positive virus cultures in DF-1 cells.

The viruses isolated in our laboratory and used for sequencing are listed in Figure 2. Attwater's Prairie Chicken (APC) and Greater Prairie Chicken (GPC) whole blood samples were collected from specimens kept at the Fossil Rim Wildlife Center, Glen Rose, TX, USA. Plasma samples were inoculated in DF-1 cells for REV isolation. After the second or third consecutive passage of infected cells, the virus stocks were frozen at -80 C. The commercial turkey isolate (REV 397-A) was isolated from tumors previously characterized as T-cell lymphomas (8). Frozen tumors from the original clinical case of RE were kindly provided by Dr. Rocío Crespo (California Animal Health and Food Safety Laboratory System, Fresno, CA). REV was isolated in DF-1 cells in our laboratory as described above. Commercial broiler breeders from Mexico (PMX) with an outbreak of neoplastic disease were diagnosed by PCR and histopathology in our laboratory with a

dual oncogenic infection with MDV and REV. For all viruses from Mexico, total genomic DNA was extracted locally using a commercial DNA extraction and purification kit (High Pure PCR Template Preparation Kit, ROCHE Biochemicals, Manheim, Germany). The DNA was kept at 4 C and used as necessary for proviral DNA amplification.

Turkeys. Specified pathogen free (SPF) turkey (*Meleagridis gallopavo*) fertile eggs were obtained from the Southeast Poultry Research Laboratory (USDA/ARS, Athens, GA). The eggs were incubated and hatched at the Poultry Diagnostic and Research Center (PDRC of the University of Georgia, Athens, GA) facilities, as described below at Experimental design.

Pasteurella multocida. A highly virulent field isolate of *Pasteurella multocida* serotype A: 3,4 obtained from a clinical case of fowl cholera (86-1913) was inoculated into the yolk sack of 6-day-old SPF embryonating chicken eggs. The allantoic fluid from the infected embryonating eggs was harvested upon the death of the embryos, which generally occurred within 24-48 hours post-inoculation. The allantoic fluid was streaked on blood agar plates and incubated at 37 C overnight. One overnight culture colony was inoculated into 100 ml of brain heart infusion (BHI) and incubated until the titer reached approximately 10^9 colony forming units (CFU) /ml.

Antibody assays. Antibodies to REV, NDV and IBD were detected using a commercially available enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's recommendations (IDEXX Laboratories, Westbrook, ME). Hemagglutination Inhibition (HI) was performed to measure NDV antibody titers. Briefly, individual serum samples were serially diluted two-fold in 96 well plates and

incubated with 8 units of antigen (NDV) for 30 minutes, and then 0.8% chicken red blood cells were added in all wells and incubated for 30 to 45 minutes, when the plates were read. The HI serum titers were determined by observing the highest serum dilution where hemagglutination inhibition occurred.

PCR and immunosuppressive peptide coding region sequencing. The REV *pol* PCR assay was conducted with proviral DNA extracted and purified from spleens and feather pulp. The PCR reactions were performed to partially amplify the polymerase gene (*pol*) using the forward primer, REV-7F (5' TACGGACAGAAGGGAAACTATTG 3') and the reverse primer, REV-8R (5' CTTCTTCCCTGAAACCCG 3'). A different set of primers designed in our laboratory, REV-16F (5' GTGCATACTGGCATCAATCG 3') and REV-17R (5' GCAGTTAGCGAGCCAGC 3') was used to amplify the TM region of envelope gene (*env*), where the immunosuppressive peptide is located. Denaturing was done at 95 C for 10 min and 35 cycles of 95 C for 20 sec, annealing was done at 57 C for 30 seconds and extension at 72 C for 1 min and 20 sec. Products with the expected size were sequenced by using the dideoxy chain termination method at the Molecular Genetics Instrumentation Facility (MGIF), University of Georgia, Athens, GA.

Predicted amino acid sequence analysis. Sequences from PCR products were aligned using the MegAlign 6.1 sequence analysis function in DNASTAR (DNASTAR, Inc., Madison, WI, Madison, WI) and Mega 3.01: Molecular Evolutionary Genetics Analysis (15), using the Clustal W alignment with weighted residues method. Additional REV sequences available from GenBank were incorporated in the alignments. Such sequences included REV China strain (GenBank NC006934), REV-A (GenBank S70398), SNV (GenBank DQ003591), REV proviral insertion in Fowl Poxvirus

(GenBank AF246698) and the retroviral sequences reported by Haraguchi, *et al.* (11), such as Mason-Pfizer monkey virus (MPMV), a murine leukemia virus endogenous to various strains of mice (AKV-M), mink-cell focus-forming virus of Moloney origin (MMCF), mink-cell focus-forming virus of AKR orign (AMCF), Friend leukemia virus (FriendLV), Gross leukemia virus (GrossLV), feline leukemia virus (FeLV), Moloney leukemia virus (MoLV), human T cell leukemia virus (HTLV), and the immunosuppressive domain of the retroviral p15E/p20E oligopeptide CKS-17.

Experimental design. One day-old SPF turkey poults were divided in 8 groups as shown in Table 1 and they were kept in Horsfall-type isolation units for 6 weeks at the PDRC. The infected groups were inoculated intra-abdominally with $10^{4.1}$ $TCID_{50}$/ml of the REV APC-566 virus stock. The titer of the inoculum was $10^{5.1}$ $TCID_{50}$/ml. At 3 weeks of age 4 groups (Table 1) were vaccinated intramuscularly with one dose of a *Pasteurella multocida* bacterin (Poulvac Pabac IV®, Fort Dodge Animal Health, Mendota Heights, MN) containing serotypes 1, 3, 4 and 3x4; and one dose of an inactivated bursal disease (standard & variant IBDV strains), ND, IB (Mass), and reovirus vaccine (BreederVac-IV-Plus®, Intervet, Millsboro, DE). Two weeks post-vaccination all turkeys were bled and 4 groups to include vaccinated and non-vaccinated turkeys (Table 1) were challenged with 1 ml of 10^6 CFU of *Pasteurella multocida* 86-1913 via the intra-muscular (IM) route. One week post-challenge the survivors were bled again, humanely euthanized and examined postmortem. Mortality was recorded twice daily. Two groups (REV-infected non-vaccinated and negative controls) were kept up to 14 weeks of age to observe any possible tumor development. At the termination of the experiment all remaining SPF turkeys were bled, humanely euthanatized and examined

postmortem. Spleen and feather pulp were collected and frozen at -80 C until used as proviral DNA sources for PCR. Liver, spleen, kidney, pancreas, bursa, thymus, small intestine, heart and nerve were collected for microscopic evaluation.

Statistical analysis. The statistical significance of differences in turkey antibody responses (geometric mean titer) was evaluated with the ANOVA Duncan test using the SAS software package (SAS institute Inc., Cary, ND). The χ^2 test was applied to analyze differences between mortalities. *P* values of ≤ 0.05 were used to define statistical significance.

RESULTS

Clinical signs and serological responses of SPF turkeys infected with REV APC-566. SPF turkeys inoculated with REV APC-566 at one day of age exhibited clinical signs of infection as judged by delayed growth when compared with non-inoculated birds during the first 3 weeks of life. REV infection was evident in all inoculated turkeys as determined by seroconversion by 5 weeks of age (Table 2). REV-induced neoplasia in turkeys was first detected at 13 weeks of age and was characterized by a grossly enlarged spleen and liver but no other obvious gross lesions. Histologically, infiltrates of lymphoblastoid cells were observed in a variety of visceral organs, including the liver, spleen, pancreas and kidney, and severe lymphocytic leukemia was also observed in histological preparations.

Evaluation of antibody responses to a specific antigen was done in turkeys after being vaccinated with killed Newcastle disease virus which can infect turkeys (1) and

chicken birnavirus (IBDV) which was used as antigen to evaluate the humoral immune response. Antibody responses were measured by ELISA (IBDV and NDV) and HI (NDV) at five and six weeks of age (two and three weeks post vaccination, respectively). Geometric mean titers (GMT) are shown in Table 2. Two weeks post vaccination the serological responses to all antigens involved (measured with ELISA for IBD and NDV, and with HI for NDV) were significantly reduced in the REV-infected groups of turkeys (Table 2). However, by 3 weeks post vaccination (6 weeks of age) the serological responses in the REV-infected turkeys were numerically lower but not statistically different from the responses of the REV-free vaccinated turkeys.

Development of active protection was tested with vaccination with *P. multocida,* since turkeys are know to be susceptible to this bacterial infection (10). Turkeys were challenged with *P. multocida* two weeks post vaccination with an inactivated vaccine. REV-infected turkeys challenged with *P. multocida* died sooner than REV-free turkeys after challenge (Figure 1). Despite earlier deaths in REV-infected turkeys after *P. multocida* infection, there were no statistical differences in the overall mortality patterns of REV-infected vs. REV-free turkeys.

Detection of proviral DNA in feather pulp. PCR amplification of REV proviral DNA was attempted targeting the polymerase gene from REV proviral DNA in feather pulp and spleen samples from 13 to 14-week-old turkeys that had been infected at one day of age. An REV PCR-positive reaction was detected in 4/4 (100%) spleen samples and in 3/4 (75%) feather pulp samples tested at 13 weeks of age. REV proviral DNA was not detected in turkeys kept as negative controls.

Detection and sequencing of an immunosuppressive peptide in the envelope gene of REV. An "immunosuppressive peptide" coding sequence located within the gp20E transmembrane protein (TM) coding region of REV-A has also been described in a wide variety of lymphotropic retroviruses (6, 11). In the present study, this immunosuppressive peptide coding region (ISP) was identified in eleven different APC isolates (APC-566, APC-793, APC-793, APC-782, APC-853, APC-854, APC-947, APC-951, APC-976, APC-1011 and APC-1006), one Greater prairie chicken isolate (GPC-G99), one turkey origin REV isolate (REV 397 A), and two broiler breeder isolates (PMX-16 and PMX-19), all isolated in our laboratory. The alignment of REV amino acid sequences and of other retroviruses is shown in Figure 2. All REV sequences regardless of their species of origin (APC, GPC, chicken and turkey) share identical predicted amino acid sequences, which also correspond to the same sequence found in the mammalian Mason-Pfizer monkey virus (MPMV), a known immunosuppressive Type D retrovirus (24). The 5' end of ISP is the most conserved region and only few substitutions were observed at the 3' end when compared with the original murin virus where this peptide was first described (6).

DISCUSSION

Reticuloendotheliosis virus infection caused impairment of serological immune responses in turkeys inoculated at day of age. The treatment groups were designed to have 10 poults each, but as SPF turkeys are very delicate birds and several died in the first 24 hours of life. These dead birds were responsible for the different number of birds

per treatment. Turkeys that died in this period were not counted as effects of treatment. The serological responses against NDV and IBDV were significantly decreased in turkeys infected with REV APC-566 at hatch and vaccinated vs. NDV and IBDV at 3 weeks of age. Decreased immunity against NDV in REV-infected chickens had been previously described (31), where SPF chickens were inoculated with REV isolated from a contaminated Marek's disease vaccine and subsequently challenged with NDV. In such study, the antibody response was suppressed and the duration of the NDV re-isolation was prolonged, regardless of the time of inoculation with NDV after REV infection. Death by NDV preceded by severe respiratory or neurological signs occurred more frequently in REV-infected chicks. In another study (27), REV was inoculated in chickens in association with herpesvirus of turkeys (HVT). The immune response against HVT was severely affected as a result of REV infection. Serological responses do not necessarily mean reduced protection against the pathogens included in this study, although it is well known that antibodies are important in protection against NDV and IBDV (20, 26). Serological responses are a partial indicator of an impaired immune system. However, actual challenge with live organisms would be necessary to reach more reliable conclusions.

In the present study, turkeys inoculated with REV APC-566 died sooner than REV-free control turkeys when challenged with virulent *P. multocida*. However, there were no statistical differences in the overall mortality. One possible explanation could be the high titer and virulence of the *P. multocida* challenge isolate used in this experiment, which is highly adapted to turkeys and known to be highly virulent for them. REV infection has been reported to decrease immune responses against other bacteria such as

Salmonella typhimurium (18). Chickens infected with *S. typhimurium* died at higher frequencies when co-infected with REV. REV infection at early ages is also known to increase the severity of coccidiosis caused by *Eimeria tenella*, since intestinal mucosal lesion scores, body weights and mortality were statistically greater in REV-infected chickens (19).

REV proviral sequences were detectable in feather pulp of SPF turkeys at 14 weeks of age. Despite the limited number of feather pulp samples tested (4 surviving turkeys) the rate of detection 14 weeks post-inoculation was high enough (75%) to suggest that, as it has been described for avian leukosis virus (ALV) (25, 32), feather pulp can be used successfully for REV detection using PCR. This non-invasive method eliminates the need of using sterile syringes, needles and blood collection tubes for each individual bird. In addition, the risk of cross contamination during sampling is low, and the samples may be stored refrigerated transiently or indefinitely frozen without any further processing (32).

The induction of the immunosuppression caused by REV has been described (3, 22). Rup, *et al.* (22) reported non-defective REV as having the ability to cause severe and rapid immunosuppression in chickens. The PHA (phytohemagglutinin A) assay used to evaluate the mitogenic activity of splenic lymphocytes obtained from REV-A-infected birds revealed a severe compromise of mitogenic responses within 1 week post-infection and such reduced mitogenic responses remained suppressed for at least 2 to 4 weeks. However, in the same study, the REV-A infected-birds were no longer impaired in their ability to respond to PHA by 5 weeks after infection and were no longer viremic with REV. Our results are consistent with such findings in that the turkeys were significantly

affected in their ability to respond to inactivated antigens (NDV and IBDV) at 5 weeks post-infection but not anymore at 6 weeks post-infection. However, the antibody responses against REV in the present study were quite robust, which can explain the cessation of viremia. In separate studies, the REV APC-566 strain from wild APCs used in this experiment also induced robust antibody responses and cessation of viremia by approximately 3 weeks post-inoculation in Japanese quail, chickens and turkeys, provided the birds were infected post-hatch (results not shown). Continued virus replication is known to be necessary for maintenance of the suppressor cell population (3). Thus, congenital or very early exposure to REV, which is more likely to induce long lasting viremia, is likely more prone to result in significant suppressor cell activity. In addition, presence of the REV envelope glycoprotein on the surface of REV-transformed cells has been reported to be involved in induction of the suppressor cell population during to course of the disease (22).

Lymphotropic retroviral infections often cause severe immunosuppression in various mammalian and avian species. Early studies suggested the transmembrane protein (p15E) of feline leukemia virus (FeLV) as the main factor of suppression of several immunological functions in vitro (14). Haraguchi, *et al.* (11) described a 26 amino acid oligopeptide in p15E, which is highly conserved among the transmembrane envelope proteins of murine, feline, bovine, and simian retroviruses as well as the human retroviruses human T cell leukemia virus (HTLV), and to a lesser extent, human immunodeficieny virus (HIV). Similar sequences were found in gp20E of Mason-Pfizer monkey virus (MPMV) and REV-A of turkeys (24). This immunosupressive peptide (CKS-17) was first reported by Cianciolo, *et al.* (6) as a synthetic sequence to correspond

to a portion of the consensus alignment of p15E from Murine leukemia virus (MLV), FeLV and HTLV. CKS-17 inhibits IL-2-dependent cell proliferation, alloantigen-stimulated lymphocyte proliferation, monocyte respiratory burst, natural killer cell activity, monocyte-mediated tumor cell killing, IL-1-mediated signal transduction, delayed type hypersensitivity reactions, LPS-induced mortality in mice, PWM-induced IgG production, and activity of murine cytotoxic T lymphocytes (6, 11).

REV-A may be considered an old isolate since it was first reported in 1979 (13). Intending to know more about the ability of newer REV isolates causing immunosuppression, we sequenced part of the TM protein of several RE viruses that were isolated or reported in the recent years. Our findings show that all REV isolates, independent of their origin, share the same oligopeptide sequence within the transmembrane protein (gp20E), suggesting that regardless of the REV strain, the immunosuppression potential is the same. Nevertheless, it should be mentioned that despite of those effects of this oligopeptide, it is still unknown if this short amino acid sequence is the sole factor used by retrovirus to cause immunosuppression.

Here we report an initial look at the potential for immunosuppression by REV APC-566, with serological responses to inactivated antigens and earlier mortality induced by *P. multocida* in REV-infected birds. However, certainly further researches on the functional influences of this peptide on avian cell mediators and immune responses, as well as challenge studies with live virulent viruses are needed to confirm the putative deleterious effects of such immunosuppressive peptide in REV isolates from birds.

REV APC-566 is a new and still circulating virus that can infect a variety of domestic and wild birds and it has been proven to infect, cause tumors and deleterious

effects on performance of commercial and wild poultry, and therefore it can also result potentially in immunosuppression once it infect commercial birds.

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Table 6.1. Groups and treatments of SPF turkeys, infected or not with REV APC-566.

Groups	Treatment			
	REV APC-566 1st day	**Vaccination at 3 weeks of age**	***P.* multocida Challenge at 5 weeks of age**	**Number of birds**
Uninfected control	-	-	-	7
PAST	-	-	+	10
REV	+	-	-	7
REV+PAST	+	-	+	6
REV+Vaccine	+	+	-	7
REV+Vaccine+PAST	+	+	+	9
Vaccine	-	+	-	7
Vaccine+PAST	-	+	+	9

Table 6.2. NDV, IBV and REV ELISA titers and NDV HI antibody titers in SPF turkeys infected with REV APC-566.

Treatment	Geometric Means Titers							
	5 weeks of age[1]				6 weeks of age[2]			
	ELISA			HI	ELISA			HI
	IBD	NDV	REV	NDV	IBD	NDV	REV	NDV
Uninfected control	5[c3]	1 [b]	1 [b]	5 [c]	1 [b]	5 [b]	1 [b]	0 [b]
PAST	3 [c]	2 [b]	1 [b]	5 [c]	ND[4]	ND	ND	ND
REV	5 [c]	2 [b]	6034 [a]	5 [c]	3 [b]	2 [b]	12090 [a]	0 [b]
REV+PAST	9 [c]	1 [b]	1926 [a]	5 [c]	ND	ND	ND	ND
REV+Vaccine	175 [b]	112 [b]	3949 [a]	54 [b]	287 [a]	1408 [a]	7688 [a]	54 [a]
REV+Vaccine+PAST	24 [c]	71 [b]	1606 [a]	32 [b]	ND	ND	ND	ND
Vaccine	683 [b]	867 [a]	1 [b]	149 [a]	864 [a]	2062 [a]	1 [b]	86 [a]
Vaccine+PAST	1154 [a]	605 [a]	1 [b]	123 [a]	ND	ND	ND	ND

[1] 2 weeks post vaccination

[2] 3 weeks post vaccination

[3] Different superscripts in the same column indicate significant differences at $P < 0.05$ (ANOVA – DUNCAN)

[4] Not Done – birds did not survive the *Pasteurella multocida* challenge.

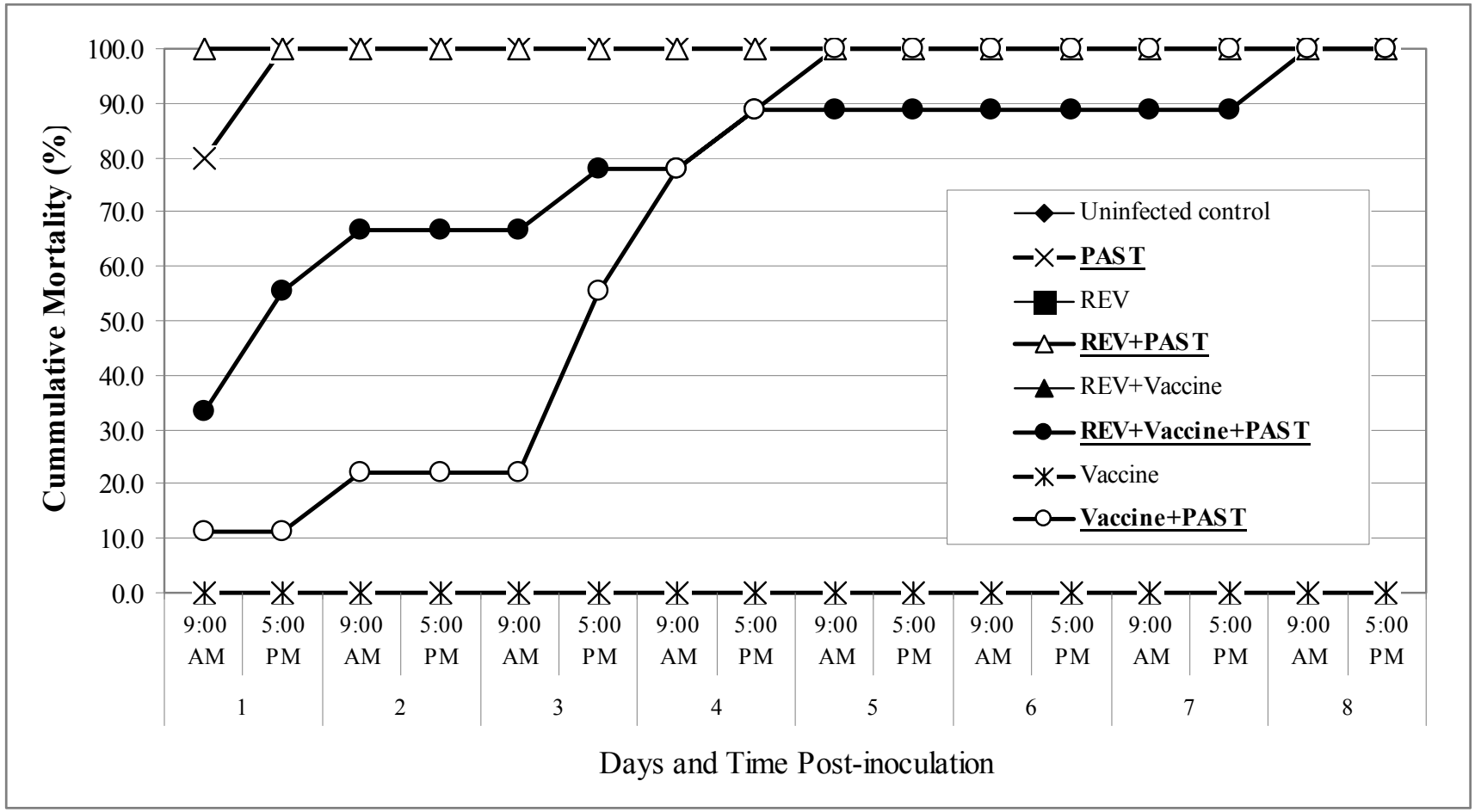

Figure 6.1. Cumulative mortality of SPF turkeys after *Pasteurella multocida* challenge. Mortality was recorded beginning 12 hours post-challenge.

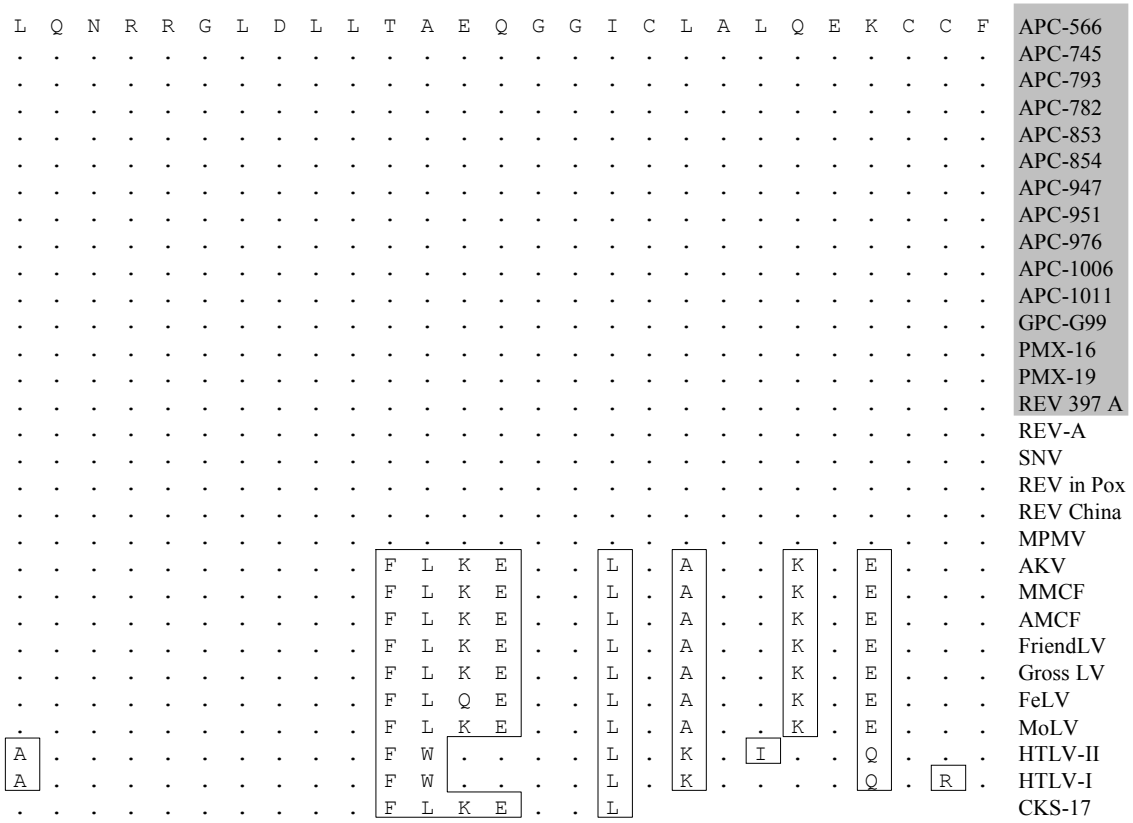

Figure 6.2. Alignment of amino acid sequences (single letter amino acid code) for the conserved region of retrovirus transmembrane envelope proteins of retroviruses. Only residue changes are indicated. Dots represent identical residues. REVs isolated, propagated and sequenced in our laboratory are shaded. Abbreviations: REV = Reticuloendotheliosis virus; SNV = Spleen necrosis virus; MPMV = Mason-Pfizer monkey virus; AKV-M = murine leukemia virus endogenous to various strains of mice; MMCF = mink-cell focus-forming virus of Moloney origin; AMCF = mink-cell focus-forming virus of AKR origin; FriendLV = Friend Leukemia virus; GrossLV = Gross Leukemia virus; FeLV = Feline Leukemia virus; MoLV = Moloney leukemia virus; HTLV = human T cell leukemia virus; CKS-17 = immunosuppressive domain of p15E/p20E of retrovirus

CHAPTER 7

DISCUSSION

Reticuloendotheliosis virus (REV) is a retrovirus of birds closely related with mammalian retroviruses. It has a worldwide distribution and despite of few reports of natural infection resulting in clinical disease in commercial poultry, REV antibodies can often be detected. REV is of considerable concern in specific-pathogen-free and poultry breeding companies since seropositive flocks are prohibited from being used for exports to various countries and vaccines must be produced with cells and embryos produced from REV-free SPF flocks. Significant costs are also incurred by vaccine companies and producers of SPF flocks with products that must be routinely monitored for possible REV contamination. REV is able to infect a variety of avian species. Since 1998, it is a serious problem in Attwater's prairie chickens (APC) and greater prairie chickens (GPC) in captive bird breeding facilities at the Fossil Rim Wildlife Center (Glen Rose, TX), as part of a preservation and reproduction program intended to prevent the extinction of the APC.

This research concentrated in the study of a newly identified REV strain (APC-566), which was isolated from one specimen of APCs. The virus was used for several experiments intended to enrich existing knowledge regarding the pathogenesis, transmission and molecular characteristics of REV. Japanese quail were used as a model for pathogenesis studies, because quail develop rapidly and they have a short generation

interval, in addition to being biologically related to domestic chickens. Oncogenicity by REV APC-566 was tested in turkeys and chickens. Turkeys were also used for examining the potential of REV APC-566 to induce immunosuppression. The molecular studies of REV concentrated in proviral genome sequencing, viral phylogenetics and viral genome sequence analysis.

Japanese quail proved to be a successful model for studying reticuloendotheliosis using a field strain of REV isolated from wild birds. REV infection was detrimental for body weight gain of quail breeders and their progeny, it was also associated with decreased egg production, hatchability and fertility in Japanese quail. Overall mortality reached 61% in infected quail breeders, in comparison with 10% in negative controls.

Not all quails infected as embryos became immunologically tolerant and most of them developed a detectable antibody response. However, as many as 64% of quail infected as embryos were still viremic by 20 weeks of age. The geometric mean antibody titer (GMT) increased progressively after post hatch infection in Japanese quail and decreased steadily after a peak response at approximately 3 weeks post-infection in adult quails. However, GMT REV antibodies increase continuously during the 12 weeks of the trial in young Japanese quail. Detectable seroconversion to REV coincided with complete cessation of viremia in quail infected after hatch, suggesting that anti-REV antibodies are neutralizing. However, the role of cellular immune responses was not evaluated in these experiments.

REV could not be detected in quail chicks of 5 separate hatches produced by breeder quail that had been infected as embryos, suggesting poor or undetectable vertical transmission in Japanese quail. A low rate of horizontal transmission was detected

indirectly through seroconversion of contact quail. We did not detect viremia in contact quails, which may resemble the results of REV infection in Japanese quail post hatch, when birds had a rapid serologic response and viremia was not detected either.

RE tumors induced by APC-566 were observed in Japanese quail as early as 42 days of age and up to 20 weeks of age. Almost all tumors were classified as lymphosarcomas and few were unclassified tumors. The rate of tumor-positive quail was low (6.6%) when compared with similar studies in chickens. However, in previous studies, the chickens infected experimentally were kept until 92 weeks, while the quail in the present experiment were kept only 20 weeks. It is possible that a higher number of quail would have developed neoplasia in a longer study. In addition to inducing tumors in quail, REV APC-566 also induced lymphosarcomas in chickens and turkeys inoculated at day-old. The majority of lymphosarcomas in quails contained a high number of CD3+ lymphocytes. Further studies may be necessary to determine whether REV is capable of inducing B-cell lymphomas in Japanese quail. REV APC-566 induced bursal lymphomas in SPF chickens inoculated at day of age and splenic lymphosarcomas in young turkeys.

REV APC-566 infection caused impairment of serological immune responses in turkeys inoculated at day of age. The serological responses against killed vaccine antigens were significantly decreased in turkeys infected with REV APC-566 at hatch and vaccinated at 3 weeks of age. In the present study, turkeys inoculated with REV APC-566 died sooner than REV-free control turkeys when challenged with virulent *P. multocida*, indicating that REV infection placed turkeys at a disadvantage regarding immune responses.

The complete sequence of a reticuloendotheliosis virus from a natural outbreak in wild birds is presented, REV APC-566 being the first wildlife REV to be fully resolved. The genome organization of APC-566 was consistent with the genetic organization of other gammaretroviruses. A phylogenetic analysis demonstrated that all available REV sequences are closely related and show little variation. The LTR was the most divergent region on the REV genome, with unique deletions and insertions. Whether such mutations in the LTR play a role in the pathogenicity of REV remains to be determined. Alignments did not show any significant differences of predicted amino acid sequences in the protein coding genes.

REV APC-566 sequences were more closely related to sequences resolved in CSV and Fowlpox virus REV inserts, which suggests the possibility of Fowl poxvirus being a vector for REV. It is also possible that REV may have been introduced simultaneously but independently from Fowlpox virus. Further studies seem necessary to explore the possible origin of REV circulating in APC and GPC captive populations.

Sequence data derived from the present molecular studies showed that all REV isolates examined share an identical 26-amino acid-long immunosuppressive oligopeptide (ISP) in the transmembrane region of the envelope protein, regardless of the virus origin. The ISP is highly conserved among the transmembrane envelope proteins of murine, feline, bovine, and simian retroviruses, and it has been reported to have deleterious effects on the immune system of several of these species. ISP was localized within the transmembrane protein coding region of REVs isolated from turkeys, commercial meat type chickens, APC and GPC, suggesting that regardless of the REV strain, the immunosuppression potential is the same. It is important to mention that despite the

consistent presence of this ISP, it is still unknown if this short amino acid sequence is the sole or main factor used by REV to cause immunosuppression in avian species.

In summary, evaluation of new REV isolates is important to determine if they represent new challenges to the poultry industry. In this case, the REV isolate was obtained from a captive colony of endangered wildlife. The objectives of this study included the evaluation of the pathogenicity and transmission of REV APC-566 using an *in vivo* model, and its molecular characterization. Japanese quail proved to be a suitable model to study reticuloendotheliosis. REV APC-566 is a newly identified REV that appears to continue circulate in captive wild birds. The resolved sequences of REV APC-566 proved to be closely related to CSV and Fowlpox virus REV insertion. REV APC-566 proved to be oncogenic in Japanese quail, chickens and turkeys, and exerted deleterious effects on the performance of Japanese breeder and broiler quail. REV APC-566 appeared to impair immune responses in SPF turkeys.