The Koala and its Retroviruses:
Implications for Sustainability and Survival

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A Novel Exogenous Retrovirus Isolated from Koalas *(Phascolarctos cinereus)* with Malignant Neoplasias in a United States Zoo

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Abstract. Koalas in US zoos were screened for koala retroviruses in an effort to determine the viral mechanism for koala retrovirus induced malignant neoplasias. Although the previously characterized koala retrovirus (KoRV-A) was present in all US koalas, some koalas were also infected by a novel koala retrovirus, termed KoRV-B. The genome of KoRV-B is highly related to KoRV-A; however, certain regions within the viral genome, including the envelope gene, displayed diversity. These differences are sufficient to allow KoRV-B to employ a receptor (a thiamine transporter) that differs from that used by KoRV-A (a phosphate transporter). Of great interest was the strong correlation between the presence of KoRV-B and malignant disease (lymphomas) in koalas. All koalas that died from lymphoma were KoRV-B positive as were the dead joeys ejected from the pouch of KoRV-B positive dams. We found no evidence of KoRV-B transmission from sires to offspring but did from dam to offspring through de novo infection, rather than via genetic inheritance like KoRV-A. Detection of KoRV-B in native Australian koalas should provide a history, and a mode for remediation, of leukemia/lymphoma currently endemic in this population.

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Endogenous retroviruses (ERVs) have played an integral role in mammalian evolution. Elements derived from these genetically inherited ERVs comprise as much as 8% of the human genome (Bromham, 2002) and are known to regulate the expression of highly conserved gene clusters (van de Lagemaat *et al*., 2003). The majority of ERVs are defective remnants of exogenously transmitted retroviruses that likely integrated into the germline of mammalian progenitors millions of years ago. The discovery of koala retrovirus (KoRV) (Hanger *et al*., 2000) described the first endogenous retrovirus that is still actively producing infectious particles capable of transspecies transmission while being retained as an inherited part of the host genome. KoRV isolates described to date in Australia, Germany, and Japan have shown very limited genetic diversity (>99% sequence identity), characteristic of an endogenous virus. However, considering the likelihood that koala genomes also contain newly integrated forms of KoRV, we screened cohorts of 13 koalas from the Los Angeles Zoo (LAZ) and 28 koalas from the San Diego Zoo (SDZ) to detect more diverse KoRV isolates (Xu *et al*., 2013).

PCR amplification of viral sequences from koala specimens obtained from the LAZ was performed using genomic DNA prepared from blood or tissue and from viral RNA present in plasma, with primers specific to KoRV. Additionally, a viral marker rescue assay was developed using human cells containing an integrated replication incompetent retroviral genome that expresses GFP (green fluorescent protein). The GFP genome can be rescued and assembled into virus if KoRV is present in the koala peripheral blood mononuclear cells (PBMCs) co-cultured with the human-GFP cells. If KoRV rescues the GFP genome then supernatant containing KoRV-GFP vectors can infect naïve target cells that will

subsequently express GFP.

PCR of infected target cells using primers for the KoRV *env* gene and the long terminal repeats (LTR) confirmed the existence in all assessed koalas from both SDZ and LAZ of a KoRV envelope gene almost identical to the endogenous KoRV previously described. Notably, a heretofore-uncharacterized KoRV envelope gene sequence was also identified in blood or tissue samples from six of 13 koalas from the LAZ, including three koalas that died of lymphoid leukemias and a joey ejected from the pouch of an infected dam at approximately one month of age. We refer to this new KoRV isolate as KoRV subgroup B or KoRV-B, and the original isolate as KoRV-A in keeping with the nomenclature previously established for other gammaretroviruses. Detection of KoRV-B envelope sequences was independently confirmed at the Centers for Disease Control (CDC) lab using freshly collected blood taken at multiple time points from the same koala.

We obtained the complete genome of KoRV-B from PBMC-derived genomic DNA using primers specific for the novel KoRV-B envelope gene sequences and primers derived from viral sequences flanking and within the LTR. KoRV-B differs from KoRV-A in the U3 region of the LTR (the region containing the viral promoter, and transcription regulatory sequences) and in its envelope gene. The U3 regions are represented at both ends of the integrated retroviral genome and can also direct expression of host genes flanking the viral integration site. If the adjacent gene is an oncogene, viral promoter activation of that gene can promote cancer. The envelope of KoRV-B differs significantly from KoRV-A in the receptor-binding domain (RBD). KoRV-B also contains the amino acid residue motif CETTG in its RBD. This motif is present in the RBD of all envelope proteins of infectious gammaretroviruses except for KoRV-A isolates and non-inducible ERVs (Oliveira *et al*., 2007).

KoRV-A and KoRV-B viruses exhibit different host ranges in cell culture, which indicates that they may use different receptors to infect cells. Murine MDTF cells are resistant to KoRV-A and KoRV-B, however expressing the human ortholog of the KoRV-A receptor confers susceptibility to infection by KoRV-A. The normal cell function of the KoRV-A receptor is that of a phosphate transporter (SLC20A1, formerly reported in the literature as PiT1). PiT1 has been reported to function as the viral receptor for gibbon ape leukemia virus (GALV) and feline leukemia virus subgroup B (FeLV-B) (Overbaugh *et al*., 2001). MDTF/PiT1 cells are susceptible to KoRV-A but resistant to KoRV-B, a finding consistent with KoRV-B using a receptor different from that used by KoRV-A to infect susceptible cells. Because gammaretroviruses tend to employ transporters as receptors, we individually expressed a panel of transporters in MDTF cells to determine whether any of these tested transporters conferred susceptibility to KoRV-B. Using this approach we discovered KoRV-B infects via the thiamine transporter (formerly referred to as THTR1 and now recognized as SLC19A2). The thiamine transporter was previously shown to be the receptor for feline leukemia virus subgroup A (FeLV-A) (Mendoza *et al*., 2006).

KoRV-B does not appear to be vertically transferred in the germline. KoRV-B positive sires do not transmit KoRV-B to their offspring if the dam is KoRV-B negative. KoRV-B positive dams can transmit KoRV-B to their offspring when the sire is KoRV-B negative. Necropsy tissue from a KoRV-B positive six-week old joey that died in pouch and was ejected from its KoRV-B positive dam is consistent with KoRV-B being transmitted in utero or in milk ingested in the pouch.

Most KoRV-A isolates from the 38 koalas analyzed from SDZ and LAZ contain envelope sequences closely related to or in many cases identical to the previously reported KoRV-A envelope sequences. However, genetic and phenotypic diversity in KoRV is well represented by KoRV-B, which utilizes thiamine transporter THTR1 (SLC19A2) as a receptor. It is possible that KoRV-B is a recombinant between the endogenizing KoRV-A and existent KoRV sequences in the koala genome, much like the origin of FeLV-B, a recombinant of exogenous FeLV-A and endogenous FeLV-B envelope sequences (Overbaugh *et al*., 2001). Whether KoRV-A serves as a founder virus in a manner analogous to FeLV-A giving rise to different KoRV subgroups/variants in addition to KoRV-B will need further investigation. Sequencing the koala genome will help resolve the composition of endogenous retroviral fragments that may have contributed to the generation of KoRV-B and other KoRV variants.

The correlation between the presence of KoRV-B infectious virus and malignant disease in koalas is strong even though the assessed sample size is small and we cannot exclude participation of KoRV-A in the observed pathology. Nonetheless, the ability to assess KoRV-B status, and therefore the likelihood of susceptibility to neoplastic malignancy could be of tremendous importance in sustaining and managing the koala population in captivity and better understanding the epidemiology of KoRV infection. Preventing KoRV-B-positive dams from breeding, sequestering KoRV-B-positive koalas from the rest of the koala population, and developing a KoRV vaccine may all be sensible approaches to reducing the impact of KoRV-B infection on the koala population.

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