Characterization of koala retrovirus impact on koala health in Japanese Zoos

（日本の動物園コアラにおけるレトロウイルス感染の影響に関する研究）

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Dedication

This thesis is dedicated to my beloved parents Late Hafiz Uddin and Late Jalemon Nesa and to my sweet family members with all my love.

Summary

Koala retrovirus (KoRV) remains as a significant threat to wild and captive koala populations. It exists in both endogenous (KoRV-A) and exogenous (KoRV-B to -J and KoRV-K) forms, which are considered to be linked to lymphoma/neoplasia and immunosuppression in koalas. The main objectives of this study are to characterize KoRV infection and their impact in captive koalas' health in Japanese zoo populations.

Towards this end, firstly, I investigated the co-prevalence of KoRV-A and KoRV-B, detected by type-specific PCR and sequencing. I also investigated KoRV proviral loads and found varying amounts of proviral loads in genomic DNA (gDNA) in peripheral blood mononuclear cells (PBMCs). I found 100% of the koalas examined were infected with KoRV-A and 60% (12/20) were coinfected with KoRV-B. The KoRV-A sequence was highly conserved, whereas the KoRV-B sequence varied among individuals. Interestingly, I observed possible vertical transmission of KoRV-B in one offspring in which the KoRV-B sequence was similar to that of the father but not the mother. Moreover, I characterized the KoRV growth patterns in concanavalin-A-stimulated PBMCs isolated from KoRV-B-coinfected or KoRV-B-uninfected koalas. I quantified the KoRV provirus in gDNA and RNA copy numbers in cells and culture supernatants by real-time PCR at days 4, 7, and 14 post-seeding. As the study population is housed in captivity, a longitudinal study of these koalas may provide an opportunity to study the transmission mode of KoRV-B. In addition, I characterized KoRV isolates by infecting tupaia cells, suggesting that tupaia may be used as an infection model for KoRV.

Secondly, to explores the impact of multiple KoRV subtypes infection on captive koala I surveyed a representative sample from a Japanese zoo population and investigated the proviral

and RNA load profiles in koala PBMCs and plasma. Six koalas were evaluated in the study and all koalas were infected with KoRV-A, and two koalas were coinfected with non-A subtypes (KoRV-B and/or KoRV-C). The highest viral RNA loads in PBMCs and plasma were found in a koala infected with multiple subtypes (KoRV-A, -B and -C). The other koala infected with multiple subtypes (KoRV-A and B) showed the highest proviral loads in PBMCs but the lowest RNA copy number in PBMCs and plasma. PBMCs from this animal were cultured for further investigation, and KoRV RNA was detected in the cells and culture supernatant after 7 and/or 14 days. The koalas harboring multiple subtypes had a higher white blood cell count than those harboring only KoRV-A and were judged to be leukemic, and they subsequently died due to lymphoma. Accordingly, I conclude that coinfection with multiple KoRV subtypes may be linked to more-severe disease. In a sequence alignment, KoRV-A *env* gene showed 100% sequence identity to the reference gene, whereas the KoRV-B and -C *env* genes varied from their reference gene sequences.

Thirdly, to further explores the subtype distribution and risk factors analysis of KoRV infection in koala whole blood samples were collected from seven zoos and investigated the proviral copies and RNA expression in KoRV-infected koalas. To ascertain any variation in viral load by institution, age, sex, or body condition score, I quantified KoRV proviral DNA and RNA loads in captive koalas (n=37) reared in Japanese zoos. All koalas were positive for KoRV genes (*pol, LTRs*, and *env* of KoRV-A) in gDNA, and 91.89% were positive for the *pol* gene in RNA. In contrast, the distribution rates of KoRV-B, KoRV-C, KoRV-D, and KoRV-F *env* genes in gDNA were 94.59%, 27.03%, 67.57%, and 54.05%, respectively. A wide inter-individual variation and/or a significant inter-institutional difference in proviral DNA ($p<0.0001$) and RNA ($p<0.001$) amounts (copies/10^3 koala β-actin copies) were observed in Awaji Farm England Hill Zoo koalas, which were obtained from southern koala populations,

suggesting exogenous incorporation of KoRV in these koalas. Significant ($p < 0.05$) age differences were found in KoRV RNA load ($p < 0.05$) and median total RNA load ($p < 0.001$), with loads higher in younger koalas (joeys and juveniles).

Finally, to know the KoRV transmission mode and pathogenesis KoRV isolates were obtained from a deceased male joey and its parents. I sequenced the KoRV long terminal repeat (*LTR*) and envelope genes isolated from the joey and its parents and found KoRV-A and KoRV-C in genomic DNA from both the parents and the joey. Notably, both parents were also positive for KoRV-B, whereas the joey was KoRV-B negative, further confirming that KoRV-B is an exogenous strain. The KoRV *LTR* sequence of the joey was considerably closer to that of its sire than its dam. For further characterization, total KoRV, KoRV-A, KoRV-B, and KoRV-C proviral loads were quantified in PBMCs from the parents and in blood samples from the joey. Total KoRV, KoRV-A, and KoRV-C proviral loads were also quantified for different tissues (bone, liver, kidney, lung, spleen, heart, and muscle) from the joey, revealing differences suggestive of a distinct tissue tropism (highest total KoRV proviral load in the spleen and lowest in bone). The amount of KoRV-C in the parents was less than that in the joey.

Over all this study contributes to an improved understanding of KoRV-related infection, pathogenesis and transmission mode in captive koala reared in Japanese zoos and highlight useful areas for future research.

Key words: Koala; Koala retrovirus; Endogenous; Exogenous; Prevalence; Conventional PCR; Real-time PCR; Zoo

Summary in Japanese (要旨)

コアラレトロウイルス(Koala retrovirus;KoRV)は、野生並びに飼育下のコアラにとって脅威となっている。KoRVは、内在性(KoRV-A型)もしくは外来性(KoRV-B~J, K型)の形式で感染し、コアラのリンパ腫や癌、免疫抑制と関連すると考えられている。本研究では、KoRV感染の性状を解析し、日本の動物園コアラの健康に及ぼす影響を明らかにする事を主な目的としている。

始めに、KoRVの主なサブタイプとしてKoRV-A型とB型があるが、このうちKoRV-B型は、病気との関連があり、コアラの健康に脅威となっている。そこで私は、KoRV-A型とB型の共感染の可能性をサブタイプ特異的なPCRとシークエンスで解析し、日本の3動物園のコアラにおける状況を検討した。また、コアラの末梢血リンパ球のゲノム中に組み込まれているプロウイルスの量も測定し、その組み込み量が一定ではないことを明らかにした。さらに、全ての動物園コアラがKoRV-A型に感染しており、B型には6割程度が共感染している事も明らかにした。KoRV-A型の遺伝子配列は高度に保存しているが、B型は個体によって変異がより多く入っていた。興味ふかい事に、子供のコアラの1例に父親のKoRV-B型と良く似た遺伝子配列のKoRV-Bが見つかった。一方、コアラの末梢血リンパ球(PBMC)をコンカナバリンAと培養し、細胞ゲノムや上清中ウイルス量の経時的な変化も解析した。KoRVはツパイ細胞にも感染したことから、ツパイを用いたKoRV感染動物モデルの作成が可能であると考えられた。

次に、日本の動物園コアラにおいてKoRVの単数もしくは複数のサブタイプが共感染した場合にどの様な影響を及ぼすかを検討した。解析した6頭のコアラは全てKoRV-A型に感染しており、このうち、KoRV-B, C型と共感染していた1例は末梢血リンパ球におけるKoRV-RNA量とウイルス量が最も高かった。KoRV-AとB型に感染した個体では、ゲノム中のプロウイルス量は最も高かったが、PBMCや血漿中のKoRV-RNA量は最も低かった。これらのPBMCを培養して調べたところ、培養後7日～14日目に細胞や培養上清にKoRV-RNAが検出された。複数のKoRVサブタイプに感染したコアラはKoRVA型のみに感染したコアラよりも白血球数が多く、その後リンパ腫で死亡した。このことから、複数のKoRVサブタイプに感染するとより病気になりやすいと考えられた。シークエンス解析より、KoRV-A型は保存しているが、KoRV-BやCのエンベロープ遺伝子はより変異が多くみられる事も明らかとなった。

さらに、日本全国の動物園（合計7動物園）におけるコアラでのKoRV感染の状況について、血液検査をして解析を行った。日本の動物園で飼育されているコアラのうち、37頭について解析を行った。また、KoRVの検出は、pol, LTR, env遺伝子領域で実施した。

その結果全てのコアラがKoRVA型を持っており、このうち91.89%がpol遺伝子RNAを持っていた。一方で、ゲノムDNA中のenv遺伝子でサブタイプを決定したところ、KoRV-B, C, D, F型はそれぞれ94.59, 27.03, 67.57, 54,05%であった。7つの動物園のうち、

淡路イングランドの丘動物園のコアラだけ、プロウイルスDNA量($p<0.0001$)やRNA量($p<0.001$)が他の動物園に比べて有意に少なく、KoRVが外来性に感染している可能性を示唆していた。これは、おそらく他の動物園のコアラがオーストラリア北部のクイーンズランド州から由来しているのに対し、淡路のコアラはオーストラリア南部から導入された為と考えられた。また、KoRV RNA量($p < 0.05$)やtotal RNA量($p < 0.001$)はより若い個体（幼獣）

で有意に多い事も明らかとなった。

最後に、KoRVの感染様式を調べた。死亡した子供のコアラ（生後半年、♂）を調べたところ、子供はKoRV-AとC型が陽性でB型は陰性であったが、両親はA, B,C型共に陽性であった。また、子供のKoRVの配列は父親のものにより相同性が見られた。さらに、KoRV-AやC型が子供の臓器でどの様に分布しているかも解析した。子供コアラにおけるKoRV-C型のウイルス量は両親よりも多かった。

以上の解析から、本研究により日本で飼育されるコアラにおけるKoRVと病原性との関連への理解が進み、将来の研究につながる事が期待される。

Declaration of originality of thesis by author

I, Md Abul Hashem, declare that this thesis is composed of my original work, and contains. I have clearly stated that any part of my thesis has not been submitted for another degree or diploma at any university or institute. I certify that, all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. The additional information and materials those are used from other published articles has been clearly cited and acknowledged in the text with a list of references at the end of thesis.

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List of Publications

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Parent Koalas to a Joey in a Japanese Zoo. Journal of virology, 94(11), e00019-20. https://doi.org/10.1128/JVI.00019-20. Chapter 6 of this thesis.

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Park, Kagoshima, Japan, with reference sequences. Reference sequences of KoRV-A (GenBank accession no. AF151794), KoRV-B (GenBank accession no. KC779547), and KoRV-C (GenBank accession no. AB828005.1) are also shown.

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List of Abbreviation

ERVs	Endogenous retroviruses
HIV-1	Human immunodeficiency virus-1
HIV-2	Human immunodeficiency virus-2
KoRV	Koala retrovirus
PBMC	Peripheral blood mononuclear cell
RT	Reverse transcriptase
mRNA	Messenger RNA
dsDNA	Double-stranded DNA
AIDS	Acquired immunodeficiency syndrome
SIVs	Simian immunodeficiency viruses
GALV	Gibbon ape leukemia virus
MbRV	Melomys burtoni retrovirus
FeLV	Feline leukemia virus
FPPR	Fusion peptide-proximal region
MPER	Membrane-proximal external region
SU	Surface glycoprotein
TM	Transmembrane protein
gDNA	Genomic DNA
LTRs	Long terminal repeats
GAG	Group-specific antigen
POL	Polymerase
ENV	Envelope
PCR	polymerase chain reaction

RT-qPCR	Reverse transcriptase quantitively polymerase chain reaction
WBCs	White blood cells
RBCs	Red blood cells
PCV	Packed cell volume
HGB	Hemoglobin
MCV	Mean corpuscular volume
MCHC	Mean corpuscular hemoglobin concentration
PCR	Polymerase chain reaction
MOI	Multiplicity of infection
QLD	Queensland
PRR	Proline rich regions
RBD	Receptor binding domain
BCS	Body condition score

Chapter 1

1.1. General Introduction

Retroviruses are unique among vertebrate viruses in that they possess—reverse transcription of viral RNA into DNA, and integration of the newly synthesized DNA into the genome of the infected cell. Integration may occasionally occur in the germ line, which can result in vertical inheritance and fixation in the host population [Gifford & Tristem, 2003; Blikstad et al., 2008; Jern & Coffin, 2008]. Endogenous retroviruses (ERVs) make up a significant proportion of vertebrate genomes although some of them remain inactive through mutation and deletion [Stoye, 2001]. In some species however, ERVs are replication competent and can produce disease through reactivation [Akiyoshi et al., 1998] such as in AKR mice which are germ free but carry murine leukemia viral oncogenes which are activated at their later stages of life and cause death [Ceccarelli and Rozengurt, 2002; Bong et al., 2013]. ERVs are associated with multiple sclerosis [Perron et al., 2001] leukemia, lymphoma [Patzke et al., 2002], and breast cancer [Wang-Johanning et al., 2003].

Retroviruses can infect new host species through transspecies transmission such as human immunodeficiency virus-1 (HIV-1) [Denner, 2016]. A unique example of ongoing active endogenization in genome is koala retrovirus (KoRV) [Tarlinton et al., 2006]. The full length of its genome is replication competent and once actively transcribed, type C retroviral particles are found in blood, tissues and peripheral blood mononuclear cell (PBMC) cultures [Hanger et al., 2000]. KoRV was firstly isolated from leukemic koala [Canfield et al., 1988] and later confirmed by genome sequencing in 2000 [Hanger et al., 2000].

KoRV is believed to be spreading through the Australian koala population, from northern to southern region. Notably, northern koala populations exhibit 100% prevalence of KoRV while southern koala populations exhibit less than 50% prevalence of KoRV [Simmons et al., 2012]. Northern koalas carry both the endogenous and exogenous variants of KoRV [Simmons et al., 2012]. However, still southern Australian koala populations carry only exogenous KoRV [Quigley et al., 2021]. The existence of KoRV in both endogenous and exogenous states means that it is transmitted both vertically (parent to offspring through the germ line) and horizontally (between infected animals). KoRV possesses several threats to koala health and survival. KoRV-related lymphomas have been reported in several captive populations [Hashem et al., 2021; Zheng et al., 2020; Xu et al., 2013; Tarlinton et al., 2005] and while not as prevalent, also occur in wild koala populations [Fabijan et al., 2017; Connolly et al., 1998]. KoRV has also been shown to be associated with chlamydial disease in wild koalas [Zheng et al., 2020; Fabijan et al., 2017; Waugh et al., 2017].

Captive (zoo-dwelling) koalas are available as target populations for investigations of KoRV. Once established as broadly representative of wild populations, captive koalas offer better documented medical histories and availability for sampling and examination procedures. Therefore, the purpose of this project was to know the characterization of KoRV and their impact in captive koalas' health in Japanese zoo populations.

1.2. Retroviruses

Retroviruses are RNA enveloped viruses which are belong to the family *Retroviridae.* A retrovirus is an encapsulated 100 nm in diameter of positive-sense single-stranded RNA virus with an internal spherical or conical core shaped (Figure 1.1) having genomes 7-10 kilobases

(kb) in length [Coffin, 1992]. Retroviruses are categorized into two subfamilies (*Spumaretrovirinae* and *Orthoretrovirinae*) within the family *Retroviridae*. This classification is based on the pattern of gene expression, processing of viral proteins and morphology [Dahlberg, 1988]. Spumaretroviruses, also known as foamy viruses, are complex retroviruses containing the characteristic feature of a large amount of reverse transcribed DNA [Colomer-Lluch et al., 2018]. Spumaviruses typically acquire their envelope while budding through the endoplasmic reticulum and not the cytoplasmic membrane. *Orthoretrovirinae* has seven genera Alpha, Beta, Delta, Epsilon, Gamma, Lenti, and Spuma-like retrovirus, all of which contain oncogenic members except for Lentiviruses [Rosenberg, 2011; Santillana-Hayat et al., 1996; Khan et al., 2018].

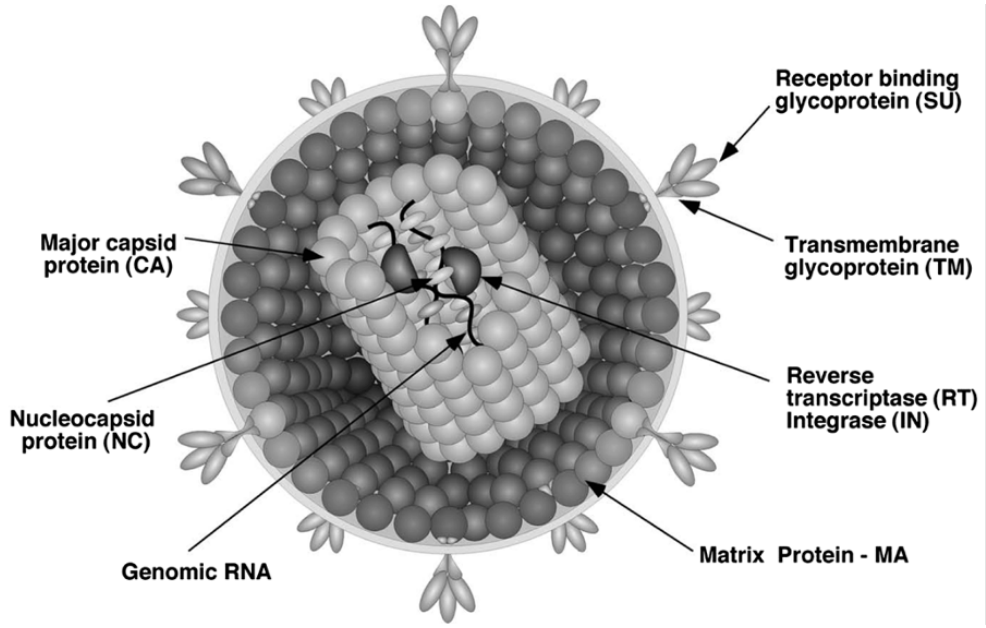


Figure 1.1. Retrovirus particle (not to scale) illustrating general structure. (Adopted from *Retroviridae* figures, ICTV 9th report, 2011).

1.2.1. Replication process of retroviruses

Retroviruses replicate through a unique enzyme reverse transcriptase (RT), which transcribed RNA into double-stranded DNA after entry into the host cell. Then newly synthesized viral DNA is integrated into the genome of the host cell resulting in the provirus. The provirus is then transcribed to form the RNA genome and messenger RNA (mRNA). The mRNA directs translation of viral proteins and processing of viral particles, resulting in budding of new virions from host cell surface [Jern and Coffin 2008]. There are seven steps in the replication cycle of the retrovirus are shown in figure 1.2 adopted from Saxena & Chitti, 2016.

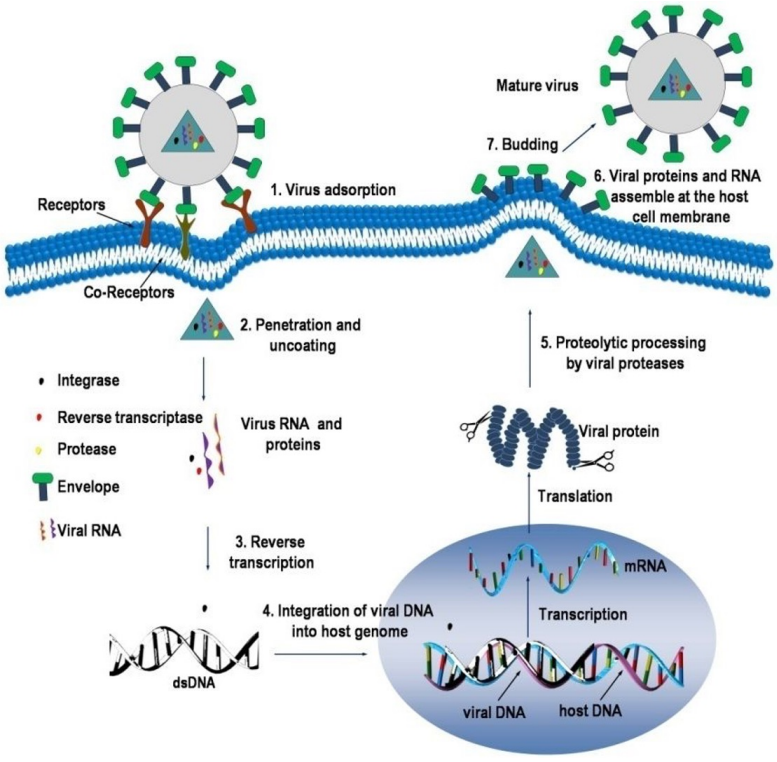


Figure 1.2. Replication cycle of retroviruses: Firstly, retroviruses are attached to one or more particular cell-surface receptors on the host cell by utilizing one of its glycoproteins. Secondly, infiltrate the host cell by direct fusion of the virion envelope with the plasma membrane of the host. Thirdly, the retrovirus undergoes partial uncoating thereby releasing its genome and three essential enzymes. At this stage, the RNA genome is converted by reverse transcriptase into double-stranded DNA (dsDNA). Fourthly, retroviral dsDNA integrates into the host genome followed by transcription and translation of the viral proteins occurs. Fifthly includes proteolytic processing of viral proteins. Sixthly, assembly of viral proteins and RNA. Finally, the immature viral particle acquires the host plasma membrane by budding and then the *gag*

and *pol* proteins of the retrovirus are cleaved by the retroviral protease, thus forming the mature and infectious form of the virus [Saxena & Chitti, 2016].

1.2.2. Mode of transmission of retroviruses

On the basis of transmission pattern, retroviruses may be classified as endogenous or exogenous viruses. Endogenous retroviruses are transmitted through cell to cell, mother to fetus transmission, and through biological fluids. Cell-to-cell transmission of retroviruses are much more efficient as compared with cell-free conditions, and as retroviruses reach through the tight cell-cell interface, they are out of reach of the immune system [Mayer et al., 1999]. Retrovirus employs various mechanisms of immune evasion, and it can destroy the immune system or subvert it to enable successful transmission [Jin et al., 2009].

Exogenous retrovirus are infectious viruses and transmitted horizontally from one host to another. Exogenous retroviruses affect a wide range of vertebrate hosts and causes a vast of diseases in humans and animals such as bovine leukemia, feline leukemia, chicken leukemia, mouse leukemia, equine infectious anemia, caprine arthritis-encephalitis, human adult T-cell leukemia, and acquired immunodeficiency syndrome (AIDS) [Gifford and Tristem, 2003].

1.3. Koala retrovirus (KoRV)

KoRV, a gamma retrovirus belongs to *Retroviridae* family that was first identified in 1988 in a leukemic koala [Canfield et al., 1988]. KoRV is a single-stranded positive-sense RNA virus, with a genome of 8.4 kilobases [Hanger et al., 2000]. KoRV constitutes a threat to koala (*Phascolarctos cinereus*) health both in the wild populations in Australia and in zoo populations around the world [Kayesh et al., 2020a].

1.3.1. Origin of KoRV

Retroviruses are capable to infect new host species through transspecies transmission, and prototypic such as human immunodeficiency viruses' type-1 (HIV- 1) and type-2's (HIV-2) are transmitted to humans from non-human primates. The HIV-1 transmitted in humans from chimpanzees and gorillas by multiple cross-species transmissions of simian immunodeficiency viruses (SIVs) and HIV-2 is transmitted in human from sooty mangabeys. Interestingly, SIVs are non- pathogenic for their natural host but after transmission to humans, HIV-1 and HIV-2 is threatening for human life by causing AIDS [Gao et al., 1994; Gao et al., 1999]. The most recent example of transspecies transmission is KoRV. Genome sequence revealed that KoRV is 78% similar to GALV [Hanger et al., 2000; Oliveira et al., 2006], an exogenous, oncogenic retrovirus isolated from captive gibbons at the SEATO medical research facility in Bangkok, Thailand [Hanger et al., 2000]. Phylogenetic analysis also revealed KoRV and GALV clustered in same group, separate from other gammaretroviruses [Tarlinton et al., 2008]. The genome of KoRV is similar to the GALV genome, suggesting a common ancestor [Simmons et l., 2014a].

Although the genetic similarities could suggest that GALV is the source of KoRV, but these two-host species are geographically separated by several thousand kilometers. Following the screening of DNA from a number of potential vertebrate hosts, four partial proviral sequences from a novel retrovirus were obtained from a native Australian rodent, the grassland melomys (*Melomys burtoni*). These sequences comprise a total of 2880 nucleotides and share remarkable identity with both KoRV and GALV. This virus has been named *Melomys burtoni* Retrovirus (MbRV) [Simmons, 2014b]. It shares such close identity with GALV that it could be considered another strain of GALV. The nucleotide sequence of MbRV shows 93% identity with

the sequence from GALV-SEATO and 83% identity with KoRV. The geographic ranges of the grassland melomys and of the koala partially overlap. Thus, a species jump by MbRV from melomys to koalas is comprehensible [Simmons, 2014b].

A recent study, GALV like retroviral sequence was reported in another subspecies of *Melomys burtoni* in Indonesia. This study explored the known distribution of GALVs in wild rodents. Phylogenetic relationship of KoRV with other retroviruses are shown in figure 1.3, adopted from McKee et al., 2017.

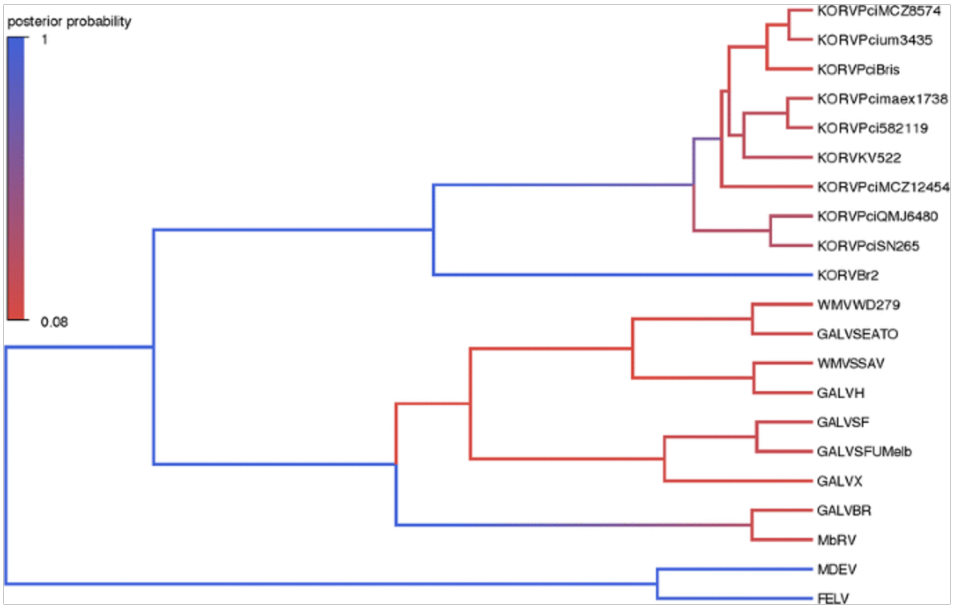


Figure 1.3. Multi-locus phylogenetic tree exposes evolutionary relationships of KoRV, GALV, MbRV, Mus dunni endogenous virus (MDEV), Woolly monkey virus (WMV) and Feline leukemia virus (FeLV). Colors are represented posterior probabilities of node placement, with warmer reds showing relatively low support and cooler blues showing high support [McKee et al., 2017].

1.3.2. Biological properties of KoRV

As a KoRV is assumed to have a simple genome that encodes envelope proteins, reverse transcriptase and structural proteins, and as is classified as a retrovirus (Figure 1.1). It has three core internal proteins (nucleocapsid, capsid and matrix), three enzymes (integrase, protease and reverse transcriptase) and an envelope protein with two subunits (surface glycoprotein (*SU* or *gp70*) and transmembrane protein (*TM* or *p15E*)) [Fiebig et al., 2006]. The full nucleotide sequence of this novel gamma retrovirus revealed the presence of all important components required for replication such as intact *gag, pol, env, LTR* regions in both 5′ and 3′ sites, a poly A tail and CAAT and TATA boxes [Hanger et al., 2000]. The genome ordered are shown in figure 1.4. *Pol* proteins are responsible for the synthesis of viral DNA and its subsequent integration into the host genome upon infection, while the protease is responsible for proteolytic cleavage of the gag and pol proteins during virion maturation. The *TM*, the smaller of the two envelope protein subunits, is embedded into the viral lipid bilayer which is derived from the plasma membrane of the host cell, during the process of budding. Within the *TM* protein, there are several epitopes for neutralizing antibody responses (the fusion peptide-proximal region (FPPR) and membrane-proximal external region (MPER)) [Fiebig et al. 2003], as well as a major immunosuppressive domain (IS or CKS-17 region) [Blinov et al., 2013]. KoRV has both exogenous and endogenous properties, is widely present in both wild and captive koala populations and exogenous properties are pathogenic for both wild and captive animals [Hobbs et al., 2017a; Zheng et al., 2020].

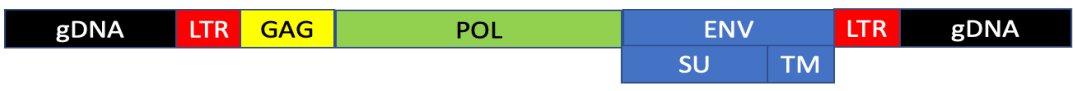


Figure 1.4. Structure of a KoRV. Genomic DNA (gDNA) (black) is flanking the integrated KoRV. Adjacent to gDNAare the duplicated long terminal repeats (*LTRs*) in red color. Three protein-coding domains are present: the group-specific antigen (GAG) in yellow color, the polymerase (*POL*) in green color and the envelope (*ENV*) domain in blue color with surface glycoprotein (*SU or gp70*) and transmembrane protein (*TM or p15E*).

1.3.3. Mode of transmission of KoRV in koalas

The transmission mode of KoRV shows some resemblance to other retroviruses. For example, HIV-1 and HIV-2 are known to jump species, and transmitted from non-human primates to humans [Gao et al., 1994; Gao et al., 1999]. Similarly, KoRV is a potentially fatal gammaretrovirus that crossed the species barrier into koalas. However, the species from which the virus jumped into koalas is unknown. The grassland melomys (*Melomys burtoni*) [Simmons et al., 2014b; Alfano et al., 2016] an Australian rodent, and bats have been suggested [Hayward et al., 2020] as a jumped-species for the source of KoRV transmission in koala population in Australia.

The endogenous KoRV has infected germ cells of host and integrates into the host (koala) genome at many locations, which suggests that integration is still recent and not uniform and fixed in the koala population [Tsangaras et al., 2014; Ishida et al., 2015; Cui et al., 2016; Löber et al., 2018]. Therefore, transmission is understood to be vertical (dam-to-offspring) for the endogenous form [Tarlinton et al., 2006; Quigley et al., 2018], but the mechanisms of horizontal transmission are not fully clear. Entry receptors have been reported for KoRV-A and KoRV-B [Xu et al., 2013; Oliveira et al., 2006], but not for the other subtypes (KoRV-C to KoRV-I and KoRV-K.

KoRV-B—an exogenous subtype—has different transmission modes and rates. An annual transmission of KoRV-B has been reported 3% for adult-to-adult individual contact, while there has been a 100% rate of KoRV-B transmitted from mothers to their joeys [Quigley, et al., 2018]. I previously reported on a case of sire-to-offspring transmission, which was differing from other reports where the transmission was from dam to offspring [Xu, et al., 2013; Quigley, et al., 2018]. Interestingly, I recently reported a KoRV-B-negative offspring born of

KoRV-B-positive parents [Hashem, et al., 2020]. Taken together, these results support the existence of a horizontal transmission mode for KoRV-B; however, little is known about the transmission modes for other subtypes. A recent study has reported the evidence for transmission of exogenous KoRV that primarily occurs between dam and joey [Joyce, et al., 2021]. A summary of the present status of KoRV transmission modes for different subtypes is shown in Table 1.1.

Table 1.1. A summary of the present status of KoRV transmission modes for different subtypes

Subtypes	Endogenous or exogenous	Mode of transmission	References
KoRV-A	Endogenous	Vertical	Tarlinton et al., 2005; Quigley et al., 2018; Simmons et al., 2012; Quigley et al., 2019; Hashem et al., 2019
KoRV-B	Exogenous	Both horizontal and vertical	Quigley et al., 2018; Quigley et al., 2019; Xu et al., 2013; Shojima et al., 2013; Hashem et al., 2019
KoRV-C	Exogenous	Both horizontal and vertical	Abts et al., 2015; Hashem et al., 2020
KoRV-D	Exogenous	Horizontal	Hobbs et al., 2017a; Quigley et al., 2019
KoRV-E	Exogenous	Horizontal	Hobbs et al., 2017a; Xu et al., 2015
KoRV-F	Exogenous	Horizontal	Chappell et al., 2017; Quigley et al., 2019
KoRV-G	Exogenous	Horizontal	Chappell et al., 2017
KoRV-H	Exogenous	Horizontal	Chappell et al., 2017
KoRV-I	Exogenous	Horizontal	Chappell et al., 2017
KoRV-K	Exogenous	Vertical	Joyce et al., 2021

1.3.4. Epidemiology of KoRV in koala populations

KoRV is a major threat to koala heath and conservation, and it shows an endemic in both wild and captive koala populations [Miyazawa et al., 2011; Xu et al., 2013; Fiebig et al., 2016; Quigley et al., 2018; Kayesh et al., 2019; Fabijan et al., 2019; Kayesh et al., 2020a]. However, the prevalence of KoRV varies depending on the geographical location of the koala population and its subtype [Simmons et al., 2012; Legione et al., 2017; Fabijan et al., 2019; Sarker et al., 2019; 2020].

Based on the geographical location of Australian koala populations are divided broadly into two populations: a northern koala population and southern koala population. In northern population, the prevalence of KoRV has been reported 100% regarding the presence of provirus copy number [Tarlinton et al., 2005; Quigley et al., 2018; Simmons et al., 2012; Fabijan et al., 2019; Quigley & Timms, 2020]. Whereas, the prevalence of KoRV in southern population previously showed much lower figures, ranges from 14.8 to 25% [Simmons et al., 2012; Legione et al., 2017; Waugh et al., 2017]; however, recent studies reported markedly higher figures, ranging from 42.4 to 99.0% [Fabijan et al., 2019; Sarker et al., 2019; 2020].

In the case of endogenous subtype (KoRV-A), the prevalence rate has been reported 100% in the northern koala populations [Tarlinton et al., 2005; Simmons et al., 2012; Quigley et al., 2018], while, the prevalence of KoRV-A has been reported 42.4% and 65.3% in the two largest South Australian koala populations: Kangaroo Island (KI) and Mount Lofty Ranges (MLR) respectively [Fabijan et al., 2019]. In northern koala population, additional subtypes have been reported such as KoRV-B (25%-100%), KoRV-D (88%-100%) [Chappell et al. 2017; Quigley et al. 2018a; Quigley et al., 2019; Sarker et al. 2019], KoRV-F (25%-44%), KoRV-G (11%–33%), KoRV-H (6%) and KoRV-I (6%–97%) [Quigley & Timms, 2020]. In the south, most studies have focused on total KoRV or KoRV-A detection alone; however, a recently study performed deep amplicon sequencing of southern koala, and found that all koalas were 100% positive for KoRV-A, KoRV-B and KoRV-D, 43% for KoRV-G and 64% for KoRV-I [Sarker et al. 2019].

Finally, there have also been reports of KoRV prevalence in captive zoo koala populations worldwide. The prevalence of KoRV in zoo populations mostly reflect the prevalence of their source of origin. The prevalence of KoRV in zoos consistent with northern

koalas, with 100% KoRV-A and 40%-68% KoRV-B (where there were at least five koalas) [Shojima et al. 2013; Xu et al. 2013; Fiebig et al., 2016; Hashem et al. 2019; Kayesh et al. 2019]. To date, there are ten identified KoRV subtypes (KoRV-A to I, KoRV-K) [Joyce et al., 2021], but most of the studies were did on KoRV-A and/or KoRV-B. A recent study reported the prevalence of KoRV-A (100%), KoRV-B (10.3%-59.3%), KoRV-E (7.7%-37.0%) and KoRV-F (5.1%-40.7%) in koala populations from a US zoo and two Australian zoos [Zheng et al., 2020]. Similarly, I investigated the prevalence KoRV subtypes in Japanese zoos kaola populations, and the respective prevalence's of KoRV-A, KoRV-B, KoRV-C, KoRV-D and KoRV-F were 100%, 94.59%, 27.03%, 67.57%, and 54.05% respectively [Hashem et al., 2022].

1.3.5. Genetic diversity of KoRV

Viruses are fastest-evolving organisms as they can evolve at rates as high as 10^{-3} substitutions per site per year, which represents an extraordinary one million times the rate of evolution of a typical cellular host organism [Jenkin et al., 2002; Hanada, et al., 2004; Aiewsakun and Sanjuán, 2012; Katzourakis, 2015]. The first KoRV full nucleotide sequence has been published in 2000 [Hanger et al., 2000] from wild and captive koalas, which is still referred to as the original KoRV sequence and now known as KoRV-A. Subsequently, this sequence had been independently identified in some zoo koala populations in Germany, Japan and the United States [Fiebig et al., 2006; Miyazawa et al., 2011; Xu et al., 2013]. The nucleotides sequence of KoRV-A has been 78% similarity with GALV [Hanger et al., 2000]. This original KoRV (KoRV-A) isolate was shown to use the same receptor for cell entry as GALV – the sodium-dependent phosphate transporter membrane protein PiT1 [Oliveira, Farrell and Eiden, 2006].

Until 2012, KoRV had been considered as a single virus. In 2013, an American research group reported a new KoRV strain from captive koala at Los Angeles Zoo suffering from lymphoma and this KoRV strain was designated as a KoRV-B [Xu et al. 2013]. In the same year, another research group in Japan, they isolated a new KoRV strain from koalas came from QLD koalas reared at the Kobe Municipal Oji Zoo in Hyogo, Japan and this strain was designated as a KoRV-J [Shojima et al. 2013]. Both strains used a different cell receptor, the thiamine transporter 1 (THTR1), to enter cells [Xu et al. 2013; Shojima et al. 2013]. Subsequently, through phylogenetic analysis Shimode, et al. revealed that KoRV-B and KoRV-J remained in the same subgroup with 91.8% identical of amino acid sequences, and commonly designated as a KoRV-B [Shimode et al., 2014], and additionally identified two novel subtypes KoRV-C and KoRV-D which contained enough differences in their putative receptor binding domains of their envelope genes within the variable region A (VRA) [Shimode et al., 2014].

Later, American research group isolated additional two unique KoRV variants that also used different cell receptors from PiT1 and THTR1 from koalas at the San Diego and Los Angeles Zoos and these KoRV subtypes were designated KoRV-E and KoRV-F (Xu et al. 2015). In 2017, Chappell et al. studied on wild koalas from southeast QLD targeting the VRA region of the KoRV envelope gene and found additional four new receptor binding domain diversity and established the additional subtypes KoRV-G, KoRV-H and KoRV-I [Chappell et al. 2017]. Currently KoRV also divided into three major clades based on the phylogenetic diversity of KoRV envelope VRA [Sarker et al. 2019].

More recently deep sequencing was utilized on an∼500 nucleotide (nt) region of the KoRV envelope(env) gene, encompassing the hypervariable RBD, for 109 captive koalas housed in two separate Southeast Queensland colonies. A total of 421 unique KoRV sequences

were generated from 109 koalas. On the basis of phylogenic analysis nine known (KoRV-A to KoRV-I) subtypes were identified with 60.4% sequence similarity, and one divergent group of sequences was found with 75.7% similar to those of the known subtypes within the RBD. Hence, designated as a novel subtype KoRV-K [Joyce et al., 2021].

1.3.6. Association of KoRV with clinical diseases

Human retroviruses have been shown association several diseases including leukemia, lymphoma, breast cancer and multiple sclerosis [Perron et al., 2001; Patzke et al., 2002; Depil, et al., Wang-Johanning et al., 2003]. Such causative associations have been postulated for KoRV, although a definite link between retroviruses and these diseases is still lacking.

The first comprehensive study of disease and death in Australian koalas was reported in 1961 and found that lymphoid leukemia and lymphosarcoma were found as one of the causes of death of koala [Backhouse and Bolliger, 1961; Heuschele and Hayes, 1961]. Subsequent studies have also reported cases of lymphoid neoplasia in the koala [McKenzie, 1981; Canfield et al., 1987a; Spencer and Canfield, 1996]. From that period to date, mortality surveys indicate that lymphoma and leukemia cause 3 to 5% of mortality in free-living koalas in QLD and New South Wales and up to 80% in captive koala populations in southeast QLD [Hanger, et al., 2000; Gonzalez-Astudillo, 2017]. Although lymphoid neoplasia is the most prevalent form, other types of neoplasia have been also reported in koalas such as ovarian neoplasms [Finckh and Bolliger, 1963], craniofacial tumors of bone and mixed cartilages [Sutton, 1986; Canfield et al., 1987b; Canfield et al., 1990b], mesothelioma [Canfield et al., 1990a; Canfield et al., 1990b], biliary adenocarcinoma, testicular teratoma [Canfield et al., 1990b], ductal

adenocarcinoma [Higgins and Canfield, 2009], and serosal myxosarcomas [Astudillo et al., 2015].

Chlamydia is well known as a major disease of both captive and free-living koalas [Brown et al., 1987]. It has been reported that the KoRV viral load positively correlates with leukemia, lymphoma, and chlamydial disease in koalas [Tarlinton et al., 2005; Tarlinton et al., 2008; Quigley et al., 2018; Fabijan et al., 2020]. The associations between chlamydial disease and KoRV infection in koala's links with immunosuppression has been reported [Legione et al. 2017; Waugh et al. 2017; Quigley et al. 2018].

Recently it has been reported that among ten variants of KoRV, KoRV-B is more pathogenic, and associated with neoplasia and chlamydial disease in koalas [Xu et al. 2013; Waugh et al. 2017; Quigley et al. 2018]. Lymphoma also has been reported in a KoRV-A positive female koala in South Australian, which was also affected with severe reproductive chlamydiosis [Fabijan et al., 2017]. Finally, the effect of KoRV expression over time on koala health has been investigated. While it has been previously reported that increases in KoRV RNA levels in plasma were associated with the development of neoplasia [Tarlinton et al., 2005]. A high level of viremia is correlated with an increased risk of neoplasia and immunosuppression in koalas [Tarlinton et al., 2008].

Besides of the neoplasia and chlamydial diseases, pneumonia has also been reported to affect koalas of all ages with nasal discharge, sneezing, coughing and sudden death due to per-acute pneumonia. A diverse range of pathogens have been associated with per-acute, acute and chronic pneumonia in koalas such as *Bordetella bronchiseptica* [Letcher et al., 1993; McKenzie et al., 1979], *Pseudomonas aeruginosa* [Oxenford et al., 1986], *Chlamydia.*

pneumoniae [Bodetti and Timms, 2000).], *Chlamydia. pecorum* [Mackie et al., 2016], *Nocardia asteroids* [Wigney et al., 1989], *Staphylococcus epidermidis* [Wigney et al., 1989], and *Streptobacillus moniliformis* [Russell and Straube, 1979]

1.3.7. Immune response of KoRV in koalas

The innate and adaptive immune system play a significant rule in host defense against infectious pathogens, while innate immune system is considered to be of particular importance [Chaplin 2010]. Usually, koalas have been regarded as immunologically "lazy" due to their slow responses in antibody production against antigen. Koala peripheral blood leukocytes are non-functional against B cell mitogens such as lipopolysaccharide, jacalin and protein-A but respond well against T cell mitogens such as phytohaemagglutinin, concanavalin A and pokeweed. These circumstances suggest poor humoral and cell mediated immune capabilities [Wilkinson et al., 1992a; Wilkinson et al., 1992b; Wilkinson et al., 1994]. The transcriptomic and proteomic study of koala milk revealed young koalas receive immunological protection during their lactation period as their milk comprises of novel proteins [Johnson, et al., 2018] and antimicrobial peptides [Morris et al., 2016]. Two subclasses of IgM and IgG immunoglobulin proteins are also found in koala serum [Wilkinson et al., 1991].

Generally, host-viral interactions are initiated through toll like receptors (TLRs), RIG-I like receptors (RLRs) and NOD like receptors. The recognition events induce cytokines, chemokines and type I interferons (IFNs) to protect the host through viral eradication (Kolli et al., 2013). The next generation study of koala immunomics fully characterized TLRs 2-10, RLR genes and the entire MHC repertoire [Abts et al., 2015]. Researchers have studied levels of expression of different cytokines in response to chlamydial infection and KoRV viral load

to identify genes with a role in local and systemic immunity and to characterize the regulation of immunity against infection titer. Some studies have been reported the cytokines expression of Th1 (*IFNγ, TNFα*), Th2 (*IL-10, IL4* and IL-6) and Th17 (*IL-17A*) pathways of the immune system [Maher et al., 2014; Kayesh et al., 2020b]. It has been reported that the higher expression levels of *IL-17* are associated with chlamydial disease severity in koalas [Maher et al., 2014; Mathew et al., 2014; Maher and Higgins, 2016]. The *IL-6* and *IL-10* has been upregulated in human PBMCs infected with KoRV which is suggestive of inflammation and immunosuppression [Fiebig et al., 2006]. A recent study has been reported a significant upregulation of *IL-6* in koala PBMCs infected with two exogenous KoRV subtypes (KoRV-B and KoRV-C) as compared with only the endogenous (KoRV-A) infection; however, no significant change in expression was observed for *IL-10*, and *IL-17A* [Kayesh et al., 2020b].

The cell surface receptors CD4:CD8 gene expression ratio is a potential biomarker for immunological evaluation [Lu, et al., 2015: Serrano-Villar et al., 2014]. Some cell surface receptors like CD4, CD8β, CLEC1B and CLEC4E have been identified in koala. A recent study reported a significantly decreased ratio of CD4:CD8 gene expression in KoRV-negative koalas [Maher et al., 2019].

Chapter 2

Aims of this study and structure of thesis

KoRV is one of the major threats to koala for long-term survival in wild and captive koala populations. The chapter 1 has presented an overview of the published literature and has explored areas requiring further study. Most of the studies have focused on wild koala population and a limited number of studies focused on captive koala in different countries. Therefore, this study was set up on captive koalas reared in different Japanese zoos to investigate the possible reasons for the different outcomes of KoRV infection and its subtype distribution.

This thesis contains seven chapters, structured as follows:

Chapter 1: Literature review of retroviruses and KoRV.

Chapter 2: Aims of this study and structure of thesis.

Chapter 3: Coinfection with koala retrovirus subtypes A and B and its impact on captive koalas in Japanese zoos. This chapter describes the prevalence of two major subtypes of KoRV in three Japanese zoos.

Chapter 4: Koala retrovirus (KoRV) subtypes and their impact on captive koala (*Phascolarctos cinereus*) health. This chapter describes the infection status for three KoRV

subtypes (KoRVA, B, and C), and proviral and RNA load profiles in animals with single- and multiple-subtype infections, using peripheral blood mononuclear cells (PBMCs) and plasma.

Chapter 5: Subtype distribution and expression of the koala retrovirus in the Japanese zoo koala population. This chapter describes the KoRV infection status focusing on proviral copies and RNA expression to ascertain any variation in viral load by zoo, age, sex, or body condition score, through quantifying KoRV proviral DNA and RNA loads in captive koala reared in seven Japanese zoos.

Chapter 6: Transmission of koala retrovirus from parent koalas to a joey in a Japanese zoo. This chapter describe the KoRV isolates obtained from a deceased male joey and its parents to investigate KoRV transmission mode and pathogenesis.

Chapter 7: General discussion. This chapter describes the major findings, limitations and conclusion.

Chapter 3

Coinfection with koala retrovirus subtypes A and B and its impact on captive koalas in Japanese zoos

3.1. Abstract

Koala retrovirus (KoRV) is unique among endogenous retroviruses because its endogenization is still active. Two major KoRV subtypes, KoRV-A and B, have been described, and KoRV-B is associated with disease and poses a health threat to koalas. Here, I investigated the co-prevalence of KoRV-A and KoRV-B, detected by type-specific PCR and sequencing, and their impact on the health of koalas in three Japanese zoos. I also investigated KoRV proviral loads and found varying amounts of genomic DNA (gDNA) in peripheral blood mononuclear cells (PBMCs). I found that 100% of the koalas examined were infected with KoRV-A and 60% (12/20) were coinfected with KoRV-B. The KoRV-A sequence was highly conserved, whereas the KoRV-B sequence varied among individuals. Interestingly, I observed possible vertical transmis- sion of KoRV-B in one offspring in which the KoRV-B sequence was similar to that of the father but not the mother. Moreover, I characterized the KoRV growth patterns in concanavalin-A-stimulated PBMCs isolated from KoRV-B-coinfected or KoRV-B-uninfected koalas. I quantified the KoRV provirus in gDNA and the KoRV RNA copy numbers in cells and culture supernatants by real-time PCR at days 4, 7, and 14 post-seeding. As the study population is housed in captivity, a longitudinal study of these koalas may provide an opportunity to study the transmission mode of KoRV-B. In addition, I characterized KoRV isolates by infecting tupaia cells. The results suggested that tupaia may be used as an infection

model for KoRV. Thus, this study may enhance our understanding of KoRV-B coinfection and transmission in the captive koalas.

3.2. Introduction

Koala retrovirus (KoRV) is a recently discovered gamma-retrovirus that is associated with leukemia, lymphoma, and immunodeficiency-like diseases in koalas (*Phascolarctos cinereus*) [Hanger et al., 2000]. It has provided new opportunities for retroviral research but also poses a new health threat for koalas. Recent studies have reported the isolation of KoRV from both wild and captive koala populations in Australia, as well as its isolation from koalas reared in zoos in other countries [Fiebig et al., 2006; Miyazawa et al., 2011; Shojima et al., 2013; Xu, et al., 2013; Fiebig et al., 2016; Kayesh et al., 2019]. KoRV is very closely related to gibbon ape leukemia virus (GaLV), a pathogenic exogenous retrovirus that is similar to murine leukemia virus (MuLV), feline leukemia virus (FeLV), and porcine endogenous retrovirus (PERV) [Hanger et al., 2000; Cui et al., 2012; Denner and Young, 2013a]. Mammals have been infected with retroviruses for millions of years, and when an exogenous retrovirus integrates into the host germ line, it is transmitted vertically from parent to offspring [Gifford and Tristem, 2003]. Although all vertebrate species contain endogenous retroviruses in their genomes [Bock and Stoye, 2000], most of these viruses are inactive due to the accumulation of substitution mutations and deletions [Boeke and Stoye, 1997]. An unusual feature of endogenous KoRV is that it is present as a full-length, replication-competent genome. It exists in koalas as both an endogenous and exogenous virus and provides an interesting model to study retrovirus endogenization into the host genome. To date, nine variants of KoRV have been characterized [Quigley et al., 2018]. KoRV-A is still endogenizing into the koala genome [Tarlinton et al., 2006; Stoye, 2006]. It has stably established itself in northern Australian koala populations and

is progressing southward [Quigley et al., 2018]. KoRV-B appears to be exogenous. It is associated with disease progression, and significantly effects koala health in the wild and in captivity [Xu et al., 2013; Fiebig et al., 2016; Denner & Young, 2013b; Quigley et al., 2018; Maher et al., 2016; Waugh et al., 2017]. Information on the impact of KoRV on koala health and the role of KoRV subtypes on disease induction is limited. The coinfection status of KoRV-B in the koala population of Japanese zoos has not been evaluated. I investigated the coinfection status of KoRV-B and KoRV-A in koalas reared in three different Japanese zoos and its effects on koala health. I also evaluated different health parameters by examining the blood of these koalas. I studied the prevalence of KoRV, determined proviral DNA copy numbers, and examined the effects of the KoRV genotype on the koalas' health. To expand our understanding of the growth of KoRV, with or without KoRV-B, I also investigated the growth kinetics of KoRV in koala peripheral blood mononuclear cells (PBMCs) stimulated with concanavalin A by measuring the KoRV RNA copy numbers in culture supernatants and cellular RNA fractions and KoRV proviral DNA copy numbers in the genomic DNA (gDNA) of cultured PBMCs. In addition, I evaluated the transspecies transmission of KoRV, as a previous report [Fiebig et al., 2006] showed a wide host range of KoRV infection.

3.3. Materials and Methods

In this study, 20 captive koalas reared in three different Japanese zoos, including Kobe Oji Zoo (5 koalas), Saitama Children's Zoo (4 koalas), and Hirakawa Zoological Park (11 koalas), were evaluated for coinfection with KoRV-A and KoRV-B and the effects of coinfection on koala health. Whole blood samples were collected using heparin or EDTA by venipuncture from koalas of different ages and genders (Table 3.1) following the Institutional Animal Care and Use Committee protocols.

Table 3.1. Koalas investigated in this study

Japanese Zoos	Koala	Age	Sex	KoRV-A	KoRV-B	Plasma viral RNA	WBC/µl blood	Health status
Kobe Oji Zoo	K1	4 y 5 m	M	Positive	Positive	Negative	**100,750**	**Lymphoma**
	K2	4 y 5 m	F	Positive	Positive	Negative	7,700	Healthy
	K3	2 y 2 m	F	Positive	ND	Negative	8,500	Healthy
	K4	8 y 3 m	F	Positive	Negative	Negative	4,900	Healthy
	K5	1 y 6 m	F	Positive	Positive	Negative	6,900	Healthy
Saitama Children's Zoo	S1	6 y 2 m	F	Positive	Negative	Negative	2,200	Healthy
	S2	5 y 9 m	F	Positive	Positive	Negative	4,300	Healthy
	S3	4 y 5 m	F	Positive	Positive	Negative	3,400	Healthy
	S4	3 y 9 m	F	Positive	Negative	Negative	4,100	Healthy
Hirakawa Zooligical Park	H1	15 y 7m	M	Positive	Positive	Negative	6500	Healthy
	H2	8 y 4m	M	Positive	Positive	Negative	7100	Healthy
	H3	5 y 10m	M	Positive	Positive	Negative	5300	Healthy
	H4	1 y 5 m	M	Positive	ND	Negative	8800*	Healthy
	H5	7 y 1 m	F	Positive	Positive	Negative	6600*	Healthy
	H6	3 y 9 m	F	Positive	Negative	Negative	7000*	Healthy
	H7	3y 1 m	F	Positive	Positive	Negative	9900	Healthy
	H8	5 y 5m	F	Positive	Negative	Negative	8200	Healthy
	H9	10y 3 m	F	Positive	Positive	Negative	5200	Healthy
	H10	3y 11 m	F	Positive	ND	Negative	9700*	Healthy
	H11	1 y 9m	F	Positive	Positive	Negative	8300	Healthy

ND-not detected; y-year; m-month

3.3.1. Plasma preparation

EDTA-treated whole blood samples were centrifuged at 3000 rpm for 5 min at 20 °C, and the extracted plasma was used for isolation of viral RNA using an ISOGEN-LS Kit (Nippon Gene, Japan) according to the manufacturer's instructions to assess the viral plasma load. Extracted RNA was treated with RQ1 RNase-Free DNase (Promega) to remove any contaminating DNA according to the manufacturer's instructions.

3.3.2. Hematological examination

The health of the koalas was examined, and general hematological data, such as the number of white blood cells (WBCs) and red blood cells (RBCs), hemoglobin concentration, packed cell volume (PCV), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) (Table 3.2), were examined as described previously [Kayesh et al., 2019].

Table 3.2. Hematological data assessed in koalas

Japanese Zoos	Koala	WBC (×102/µL)	RBC (×104/µL)	HGB (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Kobe Oji Zoo	K1	1007.5	149	6	17	114.1	40.3	35.3
	K2	77	256	7.9	38	148.4	30.9	20.8
	K3	85	263	9.4	30	114.1	35.7	31.3
	K4	49	248	9.9	34	137.1	39.9	29.1
	K5	69	304	12	41	134.9	39.5	29.3
Saitama Children's Zoo	S1	22	303	12.2	37.4	123.4	40.3	32.6
	S2	43	297	11.2	36	121.2	37.7	31.1
	S3	34	281	10.4	34.3	122.1	37	30.3
	S4	41	299	11.7	37.9	126.8	39.1	30.9
Hirakawa Zoological Park	H1	65	364	13.2	38.2	104.9	36.3	34.6
	H2	71	451	16.9	49.3	109.3	37.5	34.3
	H3	53	359	14	41.4	115.3	39	33.8
	H7	99	349	13.7	39.1	112	39.3	35
	H8	82	342	13	38	111.1	38	34.2
	H9	52	319	11	33.8	106	34.5	32.5
	H11	83	344	13.4	39.7	115.4	39	33.8

HGB- Hemoglobin; PCV- packed cell volume; MCV-Mean corpuscular volume; MCH-mean corpuscular hemoglobin; MCHC- mean corpuscular hemoglobin concentration

3.3.3. Koala PBMC cultures

Koala PBMCs were isolated from whole blood samples collected from koalas at the Kobe Oji Zoo, as described previously [Miyazawa et al., 2011] with some modifications [[Kayesh et al., 2019]. 1 × 10^6 cells/well were plated in three wells of a 6-well plate in 1 mL of RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate (Sigma), 50 μM β-mercaptoethanol, 1% MEM non-essential amino acids (Invitrogen), penicillin (100 units/mL), and streptomycin (100 μg/ml), stimulated with 20 μg of concanavalin A (Con- A) per mL, and incubated at 37°C in a 5% CO2 incubator. One day later, 1 ml of RPMI 1640 complete growth medium was added to each well. After 7 days, 1 ml of medium was replaced with fresh medium. The culture supernatants and cells were harvested at days 4, 7, and 14 post-culture, and total RNA and viral RNA were extracted from the cells and culture supernatants using a RNeasy Mini Kit (QIAGEN) and an ISOGEN-LS Kit (Nippon Gene, Japan), respectively, following the manufacturers' instructions. To remove any contaminating DNA present in the viral RNA, the supernatant was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. Then, the RNA was subjected to reverse transcription real-time PCR to determine the KoRV copy number as described below. The concentration and purity of each extracted RNA sample was determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., USA). RNA samples were stored at -80°C until further use.

3.3.4. Isolation of genomic DNA from PBMCs

Genomic DNA (gDNA) was isolated from PBMCs from EDTA-treated whole blood samples and from cultured PBMCs using a Wizard Genomic DNA Purification Kit (Promega)

according to the manufacturer's instructions. Extracted gDNA was used as a template for real-time PCR analysis for determination of the KoRV proviral copy number as described below.

3.3.5. Polymerase chain reaction and genotyping of KoRV

Phusion polymerase chain reaction (PCR) was performed using genomic DNA as a template for the detection of KoRV-A and KoRV-B variants, using primer sets that specifically amplify the env genes of KoRV-A and KoRV-B as described previously [Waugh et al., 2017]. The primer sequences were as follows: KoRV Universal Forward (for both KoRV-A and B), TCCTGGGAACTGGAAAAGAC; KoRV-A Reverse, GGGTTCCCCAAGTGATCTG; KoRV-B Reverse, GGCGCAGACTGTTGAGATTC. PCR conditions were as follows: denaturation at 98 °C for 2 min, 40 cycles at 98 °C for 30 s, annealing at 63.4 °C for 30 s, extension at 72 °C for 1 min, and extension at 72 °C for 5 min. To confirm the specificity of the KoRV-A-specific and KoRV-B-specific PCR assays, partial env gene PCR fragments were subcloned into pCR-Blunt II TOPO (Invitrogen) and sequenced. The resulting sequences were submitted to the GenBank database (GenBank accession nos. MK605475–MK605506).

3.3.6. Phylogenetic analysis

For phylogenetic analysis of KoRV, I used partial KoRV env gene sequences obtained from genotype-specific PCR products of KoRV-A and KoRV-B. Reference sequence for KoRV-A (GenBank accession no. AF151794) and KoRV-B (GenBank accession no. KC779547) were also included in this study. The gibbon ape leukemia virus (GALV) *env* gene sequence (GenBank accession no. M26927) was included as an outgroup. Phylogenetic trees were constructed by the neighbor joining method [Saitou and Nei, 1987], and evolutionary distances

were computed using the p distance method [Nei and Kumar, 2000]. Evolutionary analysis was conducted in MEGA7 [Kumar et al., 2016]. The robustness of the trees was assessed using bootstrap analysis with 1000 replicates.

3.3.7. KoRV copy number determination by real-time PCR

gDNA isolated from koala PBMCs was tested before and after culture to determine the total KoRV proviral DNA copy number by real-time PCR as described previously [Kayesh, et al., 2019]. The total KoRV copy numbers in the RNA isolated from the cultured cells and culture supernatants or plasma were also determined by real-time PCR as described previously [Kayesh, et al., 2019]. The primers used to detect the KoRV pol gene (GeneBank accession no. AF151794) by real-time PCR were as follows: forward primer, 5′-TTGGAGGAGGAATACCGATTACAC-3′; reverse primer, 5′-GCCAGTCCCATACCT GCCTT-3′ [Tarlinton, et al., 2005]. KoRV-A and KoRV-B proviral DNA copies isolated from the koala gDNA, PBMCs, or PBMC culture supernatant were quantified separately by real-time PCR using the primer sets described above, targeting the *env* genes of KoRV-A and KoRV-B, using reaction conditions described previously [Kayesh et al., 2019]. PCR specificity was confirmed by melt curve analysis. Standards were prepared from pre- quantified plasmids containing the target gene sequence. The koala β-actin gene was used as an endogenous control for normalization of the KoRV proviral DNA and KoRV RNA copy numbers. To detect koala β-actin (GenBank accession no. XM_021008442), I used the forward primer 5′-AGATCATTGCCCCACCT-3′ and the reverse primer 5′-TGGAAGGCCCAGATTC-3′ [Tarlinton, et al., 2006].

3.3.8. Infection of tupaia cells with KoRV isolates

The tupaia fibroblast cell line T-23 (clone 8 [T-238]) was established previously from the lung of a new-born male tupaia [Taketomi et al., 1986] and was provided by the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). T-238 cells were cultured as described previously [Kayesh et al., 2017]. To investigate the expanded trans-species transmission of the KoRV isolates, I infected T-238 cells at a multiplicity of infection (MOI) of 1 with KoRV-containing culture supernatants collected on day 4 from the PBMC cultures of koalas K1 and K4. To avoid including any cells from the inoculum, culture supernatants were centrifuged at 10,000 rpm for 3 min. I used 8μg of polybrene/mL (hexadimethrine bromide) (Sigma) during the infection of the T-238 cells and incubated them for 1 h at 37°C for viral adsorption. Cells were maintained for 4 and 6 days, and the cells and culture supernatants were then harvested to extract gDNA and viral RNA as described above. Finally, KoRV infection was confirmed by detecting the KoRV provirus in the gDNA isolated from the T-238 cells and KoRV RNA in culture supernatants by real-time PCR as described above. For provirus detection, I ran 50 real-time PCR cycles.

3.4. Results

3.4.1. Coinfection by KoRV-A and B in captive koalas

In this study, 20 captive koalas from three different Japanese zoos (Kobe Oji Zoo, Saitama Children's Zoo, and Hirakawa Zoological Park) were used to detect coinfection with KoRV-A and KoRV-B. All of the koalas tested were positive for KoRV-A (Table 3.1 and Figure 3.1A, B, and C). The *env* gene fragment was > 99% identical to the reference sequence of the KoRV-

A variant (GenBank accession no. AF151794), as determined using Genetyx software version 8 (Genetyx Corporation, Japan). In contrast, the prevalence of KoRV- B was 60% (12/20) (Table 3.1 and Fig. 3.1A, B, and C), and sequencing the env gene fragment showed more variability, with 91.9 to 99.3% identity to the reference sequence of the KoRV-B variant (GenBank accession no. KC779547).

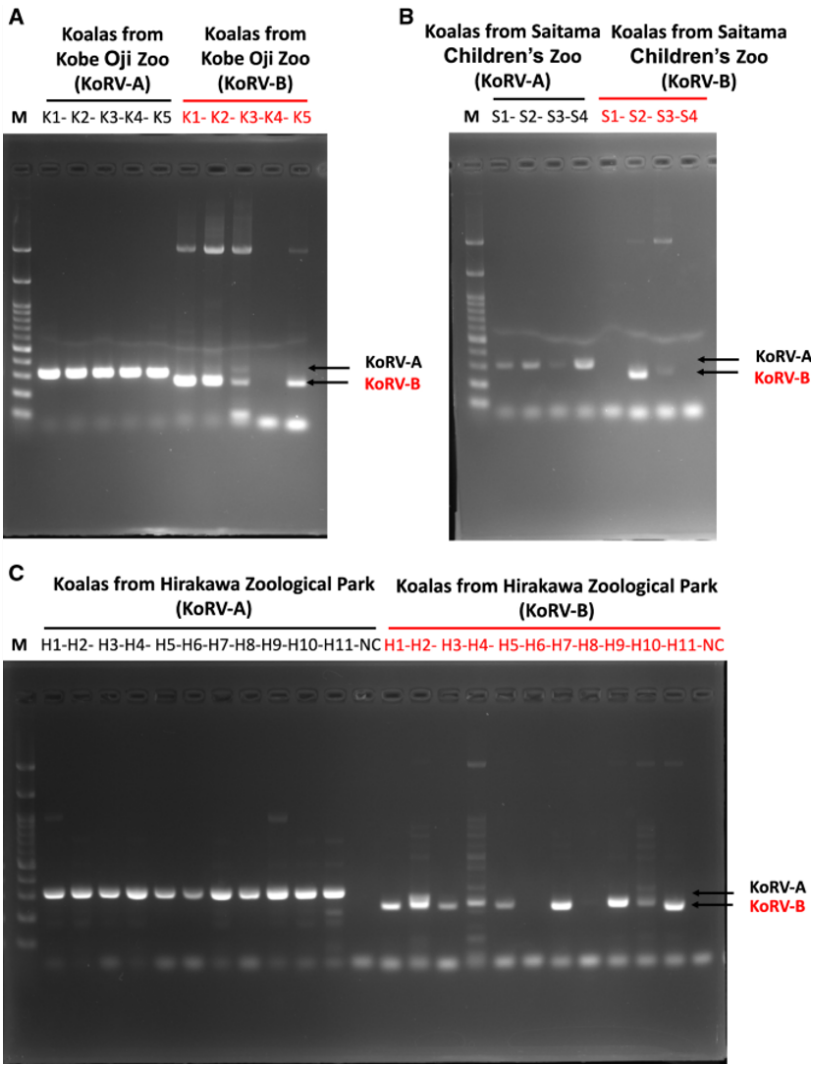


Figure 3.1. Genotyping PCR of KoRV in captive koalas of Japanese zoos. Results of the PCR analysis using primers specific for KoRV-A and KoRV-B in koalas from the Kobe Oji Zoo (a), Saitama Children's Zoo (b), and Hirakawa Zoological Park (c) are shown. In the gel image, K1–K5 indicate koalas from the Kobe Oji Zoo, S1–S4 indicate koalas from the Saitama Children's Zoo, and H1–H11 indicate koalas from the Hirakawa Zoological Park. NC indicates

no-template control, and 'M' indicates the 100-bp marker lane. The band specific for KoRV-A and KoRV-B has been indicated in the image for each koala.

3.4.2 Phylogenetic analysis of KoRV

Phylogenetic trees based on partial nucleotide sequences of the KoRV-A *env* (Figure 3.2A) and KoRV-B *env* genes (Figure 3.2B) revealed that KoRV-A was highly conserved (Figure 2A), whereas KoRV-B was clustered in subgroups, indicating strong evolutionary changes in the KoRV-B env gene (Figure 3.2B). The koalas H2 and H7, the offspring of H2 (♂) and H5 (♀), were in the same cluster; however, H7 and H2 lived in separate compartments, and H2 was separated from H5 and H7. H5 and H7 also lived separately, suggesting possible vertical transmission of KoRV-B from the father.

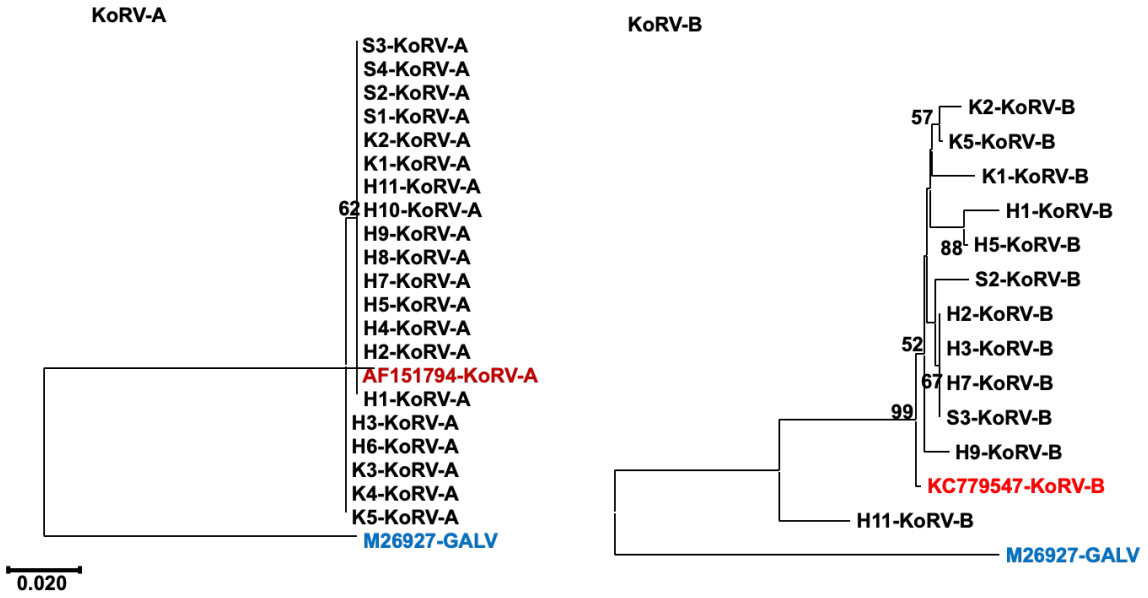


Figure 3.2. Phylogenetic trees con- structed based on the nucleotide sequences of partial env genes of KoRV-A (a) and KoRV-B (b) using the neighbor-joining method in MEGA7. Numbers at nodes indicate the percent bootstrap values for 1000 replicates, and values ≥ 50 are shown. Horizontal distances are proportional to genetic distances. Vertical distances are arbitrary. The scale bar at the bottom indicates the nucleotide distance.

3.4.3. Determination of KoRV proviral DNA copy number

The KoRV proviral DNA copy number was determined by real-time PCR. The copy number was normalized to koala β-actin. Among the koalas at Kobe Oji Zoo, the highest proviral DNA copy numbers were observed in koala K4 (Figure 3.3A), an 8-year, 3-month-old female that appeared healthy. The lowest proviral DNA copy numbers were in koala K1 (Figure 3.3A), a 4-year, 5-month-old male. For koalas reared in the Saitama Children's Zoo, the highest proviral DNA copy numbers were observed in koala S4 (Figure 3.3B), a 3-year, 9 months old female that appeared healthy. The lowest proviral DNA copy numbers were observed in koala S2 (Figure 3.3B), a 5-year, 10 months old female. I also quantified the proviral DNA copy numbers of KoRV- A and KoRV-B in koala PBMCs. In all koalas evaluated, the KoRV-A proviral DNA copy numbers were higher than the KoRV-B copy numbers (Figure 3.3C).

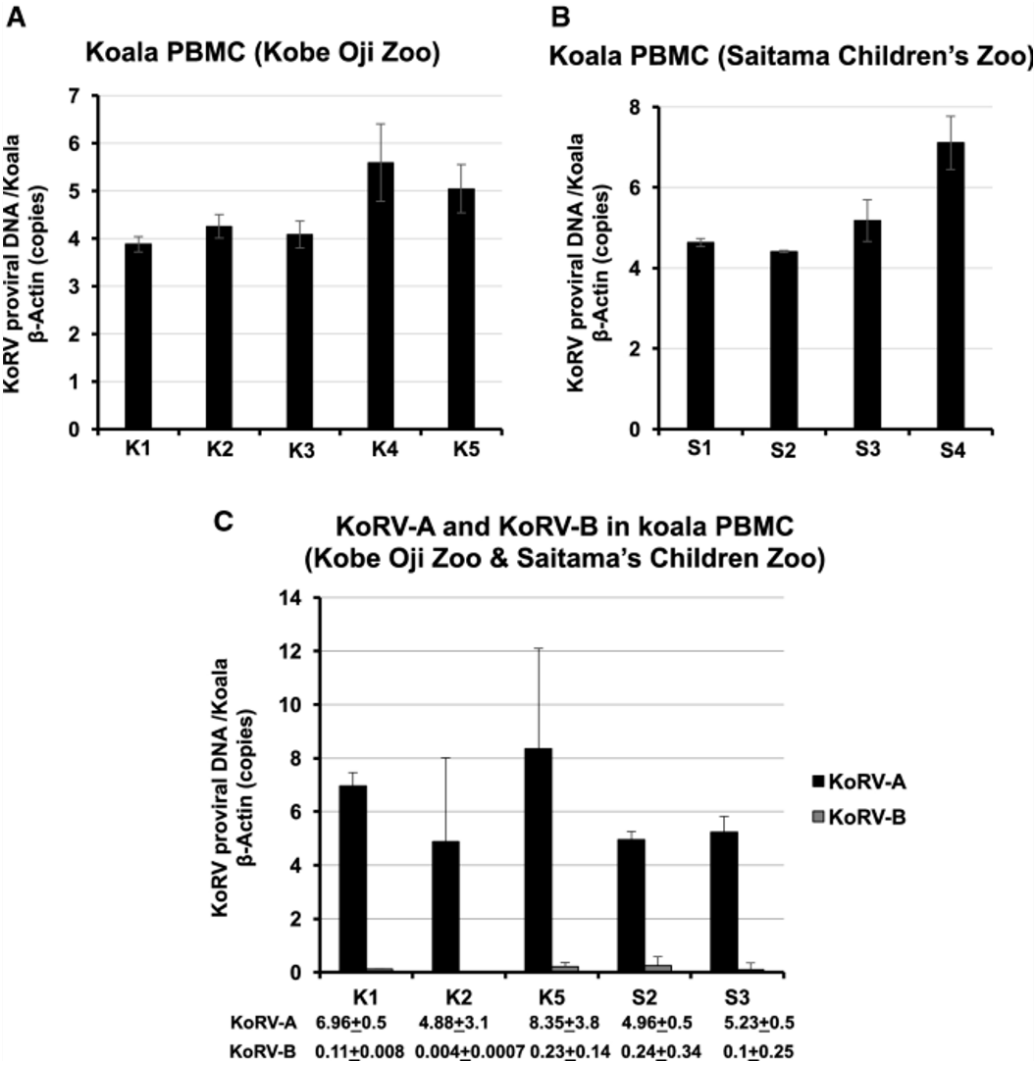


Figure 3.3. Normalized KoRV proviral DNA copy numbers in gDNA isolated from PBMCs of koalas of the Kobe Oji Zoo (a) and koalas of the Saitama Children's Zoo (b). c Normalized KoRV-A and KoRV-B proviral DNA copies in gDNA isolated from PBMCs of koalas of the Kobe Oji Zoo and the Saitama Children's Zoo. Provirus copy numbers of KoRV-A and B provirus was quantified by real- time PCR and normalized to koala β-actin in PBMCs.

3.4.4. Hematological analysis

The results of hematological analysis of koala whole blood samples are shown in Table 3.2. White blood cell (WBC) count is still a classical analytical tool for the detection of hematological abnormalities. Among koalas from different zoos, the normal WBC count ranged from 4100 to 9900 cells/µl of blood. Koala K1, however, had 100,750 WBCs/ µl (Table 2). In addition, the red blood cell (RBC) count, hemoglobin, and packed cell volume (PCV)

were lower in koala K1 (Table 3.2) than in other apparently healthy koalas. Koalas S1 and S3 from the Saitama Zoo had low WBC counts of 2200 cells/μl and 3400 cells/μl, respectively (Table 3.2).

3.4.5. KoRV plasma viral load

The KoRV plasma viral load was determined by real-time PCR. No KoRV RNA was detected in the plasma of any of the koalas (Table 3.1).

3.4.6. KoRV copy number in koala PBMC culture

Next, I evaluated the properties of KoRV in the PBMCs isolated from the Kobe Oji Zoo koalas. I determined the KoRV load in gDNA and total RNA and the viral RNA load in the culture supernatant, with or without coinfection with KoRV-B. I cultured PBMCs isolated from the Kobe Oji Zoo koalas, stimulated them with Con-A, and harvested them 4-, 7-, and 14-days post-culture. In most koala PBMC cultures, levels of KoRV in the gDNA decreased (K2, K3, K4, K5) from day 4 to day 14, but transcription increased (K4 and K5) (Figure 3.4A and B). Compared to day 4, the levels of KoRV RNA in the culture supernatant decreased for K1 at day 14, but increased for K2, K3, K4, and K 5 (Figure 3.4C). Differences in koala KoRV profiles may be due to host factors or the KoRV integration site, but the specific reasons were not determined. To identify which proviral genomic DNA was transcriptionally activated, I measured the levels of KoRV-A and KoRV-B in the culture supernatant at day 14 post-culture. I found that both KoRV-A and B were activated in koalas K1 and K5. In koalas K2, K3, and K4, only KoRV-A was transcription- ally activated (Figure 3.4D).

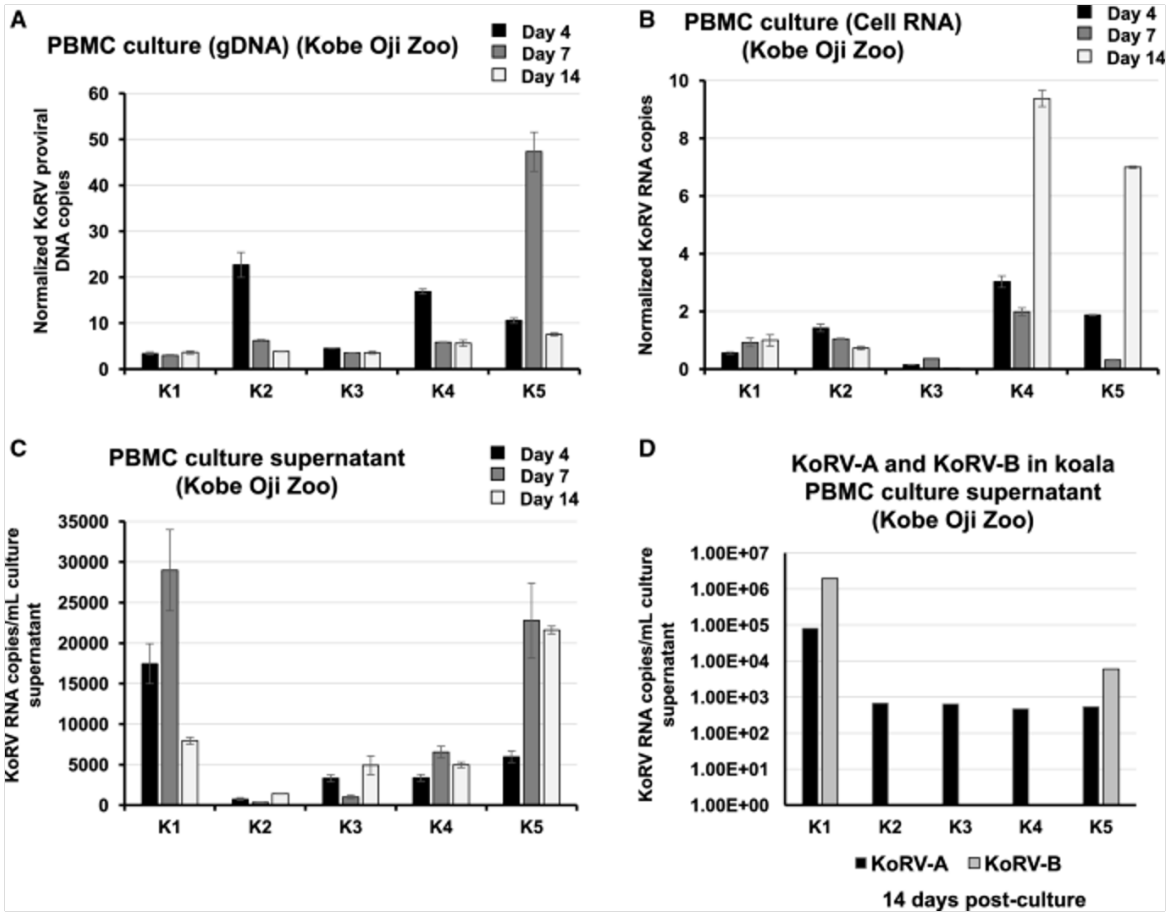


Figure 3.4. Level of KoRV in PBMC DNA (a), PBMC RNA (b), and culture supernatant (c) at the indicated time points. The KoRV genome copy number in cell DNA or RNA was normalized against koala β-actin in PBMCs. The number of KoRV RNA copies per ml of culture supernatant was calculated. d KoRV-A and KoRV-B RNA viral load/mL supernatant of koala PBMC at 14 days post-culture (d).

3.4.7. Susceptibility of tupaia cells to KoRV infection

To assess the susceptibility of T-238 tupaia cells to KoRV infection, T-238 s were cultured with KoRV-containing supernatant collected on day 4 from the PBMC of K1 (both KoRV-A and KoRV-B positive) and K4 (KoRV-A positive and KoRV-B negative). There was a significant decrease in the number of cells in the KoRV-infected wells compared to control wells at 4 days post-infection (dpi) (Figure 3.5A). At 6 dpi, cell depletion increased in the KoRV-infected wells (Figure 5B). The wells infected with culture supernatant containing both KoRV-A and KoRV-B showed more prominent cell loss than wells infected with KoRV-A

alone (Figure 3.5B). To confirm KoRV infection in the tupaia cells, I used real- time PCR targeting the KoRV *pol* gene to measure KoRV provirus copy numbers in the gDNA of control and KoRV- infected T-238 cells at 4 and 6 dpi (Figure 3.5C). I also quantified viral genomic RNA in the T-238 culture supernatant at 4 and 6 dpi (Figure 3.5D). I detected KoRV in both the gDNA and the culture supernatant of KoRV-infected tupaia cells, whereas no virus was detected in the control wells (Figure 3.5C and D).

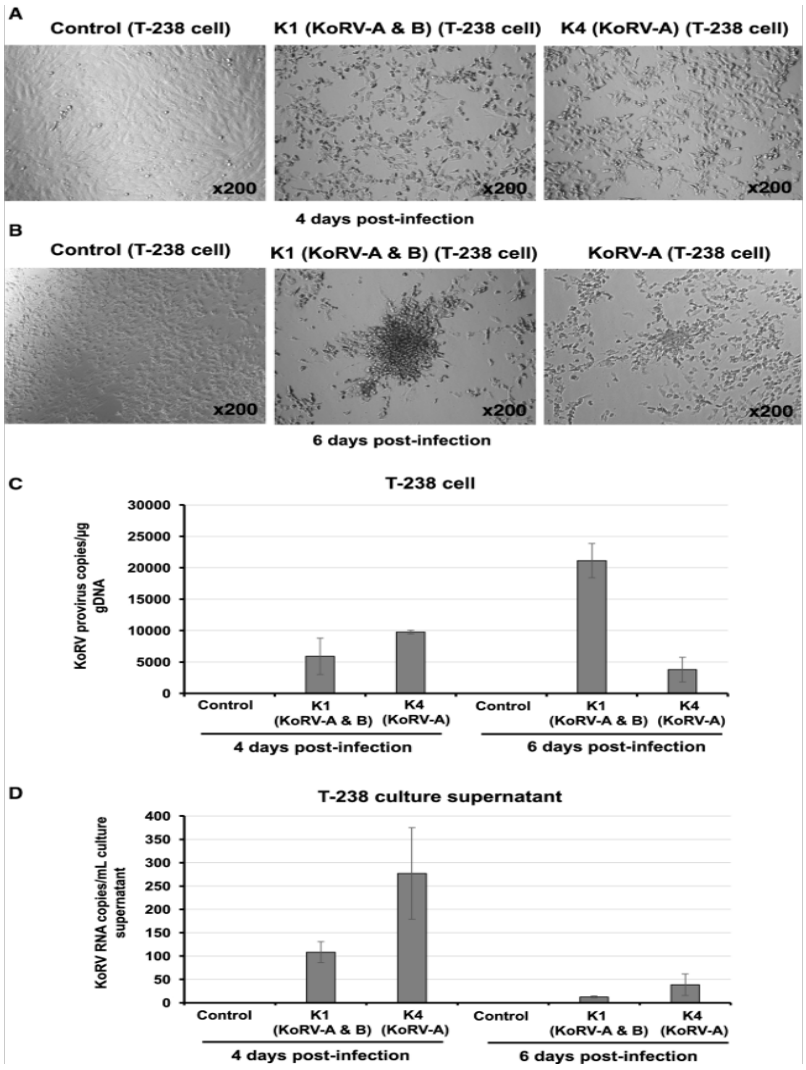


Figure 3.5. Infection of tupaia cells (T-238) with KoRV isolates. T-238 cells were infected with KoRV-containing culture supernatants collected on day 4 from the PBMC culture of K1 (KoRV-A and KoRV-B) and K4 (KoRV-A). a, b Bright-field microscopy images of T-238 cells upon infection with KoRV isolates at 4 days post- infection (dpi) (a) and 6 dpi (b). The

images shown here were taken using a BZ-X700 fluorescence microscope (Keyence Co., Japan) at 200×magnification. c, d Levels of KoRV provirus in gDNA isolated from KoRV-infected T-238 cells at 4 dpi and at 6 dpi (c) and the viral RNA load in T-238 culture supernatant at 4 and 6 dpi (d).

3.5. Discussion

In this study, I found a 100% prevalence of KoRV-A and a 60% prevalence of KoRV-B in koalas housed in three different Japanese zoos. In the Kobe Oji Zoo, 60% (3/5) of the koalas were positive for KoRV-B, whereas in the Saitama Children's Zoo, 50% (2/4) of the koalas were positive for KoRV-B. In the Hirakawa Zoological Park, 63.6% (7/11) of the koalas were positive for KoRV-B, indicating a high KoRV-B infection rate in Japanese zoos. From the phylogenetic analysis of KoRV-B (Figure 2B), I hypothesize that KoRV-B may be transmitted vertically. I found that some koalas were not infected with KoRV-B. These animals may be critical for follow-up studies to investigate the mode of KoRV-B transmission among these individuals. In a recent publication, Quigley et al. demonstrated a 100% infection rate of KoRV-A and a 24% infection rate of KoRV-B in the wild Queensland koala population [Quigley et al., 2018]. In another study, Simmons et al. showed that the prevalence of KoRV provirus in the koalas of Queensland and New South Wales was 100%, whereas in mainland Victoria, the Victorian Islands, and Kangaroo Island, it was 72.2%, 26.6%, and 14.8%, respectively [Simmons, et al., 2012]. Although the number of koalas used in this study was relatively small, the KoRV proviral DNA levels in the gDNA varied among individuals, which is consistent with previous findings [Kayesh et al., 2019; Tarlinton et al., 2005]. The exact cause of proviral DNA copy number variation has not yet been determined, but it might reflect the ongoing endogenization of KoRV within the koala genome. I found the highest proviral DNA copy number in koala S4 from the Saitama Children's Zoo, but this animal appeared healthy. In contrast, K1 from the Kobe Oji Zoo had the lowest proviral DNA copy number but

was diagnosed with lymphoma. This suggests a lack of association between proviral DNA copy number and disease occurrence, which is consistent with the findings of a previous study [Tarlinton et al., 2005]. In this study, koalas infected with both KoRV-A and KoRV-B had more KoRV- A proviral DNA copies than KoRV-B copies (Figure 3.3C). In addition, I did not detect KoRV RNA in the plasma of any apparently healthy koalas. It is important to note that KoRV RNA was not detected in the plasma of koala K1, which was diagnosed with lymphoma and infected with both KoRV-A and KoRV-B.

I examined the hematological parameters of these koalas and found that koala K1 had a high WBC count, a low RBC count, and low hemoglobin and PCV compared to other apparently healthy koalas. Koalas S1 (KoRV-B negative) and S3 (KoRV-B positive) from the Saitama Zoo had low WBC counts of 2200 cells/μl and 3400 cells/μl, respectively. In this study, I also observed that KoRV could replicate in mitogen-stimulated koala PBMCs, in which there was no significant difference in the KoRV proviral copy numbers in the gDNA of PBMCs from KoRV-B-positive and KoRV-B- negative koalas. I found that the declining proviral DNA levels in koalas K4 and K5 increased again at 14 days post- culture. The level of KoRV RNA in culture supernatants of PBMC cultures from K2, K3, K4, and K5 increased by day 14. Individual KoRV profiles differed. This may be due to host factors or the KoRV integration site, and this warrants further investigation. Furthermore, I detected KoRV-A and KoRV-B in the PBMC culture supernatants at 14 days post-culture, indicating that both KoRV-A and KoRV-B were transcriptionally activated in koalas K1 and K5 (Figure 3.4D).

In addition, I found that KoRV isolates can infect tupaia cells. This is the first study demonstrating the susceptibility of tupaia cells to KoRV infection, and it supports a previous study showing that KoRV has a wide host range [Fiebig et al., 2006].

I found that more than 50% of koalas in Japanese zoos are infected with KoRV-B and that 100% are infected with KoRV-A, supporting the idea that KoRV-A is endogenized. My findings also indicate that although KoRV-B infection could be an important factor for immune suppression, its direct association with disease occurrence needs further investigation in a larger-scale study. As several studies have shown an association of KoRV-B with chlamydial diseases, I will look for this interaction in future studies. In addition, this study indicates that if koalas are negative for plasma KoRV, I cannot rule out the possibility of impaired health status until whole blood analysis is performed. This study provides insights into the coinfection status of KoRV-B in captive koalas and its putative role in the health of the koala study population.

Chapter 4

Koala retrovirus (KoRV) subtypes and their impact on captive koala (*Phascolarctos cinereus*) health.

4.1. Abstract

Koala retrovirus (KoRV), a major pathogen of koalas, exists in both endogenous (KoRV-A) and exogenous forms (KoRV-B to J). However, the impact of infection with multiple subtypes is not well understood. Accordingly, in this study, I surveyed a representative sample from a Japanese zoo population to determine the infection status for three KoRV subtypes (KoRV-A, B, and C) and to investigate the proviral and RNA load profiles in animals with single- and multiple-subtype infections, using peripheral blood mononuclear cells (PBMCs) and plasma. Six koalas were evaluated in the study; all were infected with KoRV-A, and two koalas were coinfected with non-A subtypes (KoRV-B and/or KoRV-C). The highest KoRV total RNA and viral loads in PBMCs and plasma were found in a koala infected with multiple subtypes (KoRV-A, -B and -C). The other koala infected with multiple subtypes (KoRV-A and B) showed the highest proviral PBMC load but the lowest RNA copy number in PBMC and plasma. PBMCs from this animal were cultured for further investigation, and KoRV RNA was detected in the cells and culture supernatant after 7 and/or 14 days. The koalas harboring multiple subtypes had a higher white blood cell count than those harboring only KoRV-A and were judged to be leukemic, and they subsequently died due to lymphoma. Accordingly, I conclude that coinfection with multiple KoRV subtypes may be linked to more-severe disease. In a sequence alignment, the detected KoRV-A *env* gene showed 100% sequence identity to

the reference gene, whereas the KoRV-B and -C *env* genes varied from their reference sequences.

4.2. Introduction

Koala retrovirus has captured the interest of many virologists and, interestingly, exists in both endogenous and exogenous forms. Its evolutionary history has been well described elsewhere [Kayesh et al., 2020a; Quigley and Timms, 2020; Zheng et al., 2020]. Briefly, koalas may have first contracted this retrovirus – a relative of gibbon ape leukemia virus [Hanger et al., 2000], feline leukemia virus (FeLV) [Denner and Young, 2013a], *Melomys burtoni* retrovirus (MbRV) [Simmons et al., 2014], and porcine endogenous retrovirus – through transspecies transmission, with the process of endogenization beginning about 49,000 years ago, which is relatively recent in evolutionary terms [Ishida et al., 2015]. Endogenization appears to be complete in wild populations in northern Australia, where a prevalence of 100% has been established, but is still ongoing in some wild populations in southern Australia [Kayesh et al., 2020; Quigley and Timms, 2020; Hashem et al., 2019]. Health issues for koalas associated with KoRV have been reported elsewhere [Denner and Young, 2013a; Tarlinton et al., 2005; Xu et al., 2013; Waugh et al., 2017; Fabijan et al., 2017] and include neoplasia and chlamydial diseases [Zneng et al., 2020], which have been seen in both wild and zoo animals. Understanding KoRV is thus more than a subject of academic interest for virologists; such knowledge will have very real practical applications in the field of conservation.

The current knowledge about KoRV includes the fact that it exists in endogenous and exogenous forms, but further extensive investigation about the variant subtypes is required. KoRV-A, the endogenous form, is the most widely researched subtype, and it accounts for the

100% prevalence in north Australian populations. However, in addition to the endogenous subtype, another nine exogenous subtypes are known to exist (KoRV-B to J). Of these, KoRV-B is the best documented, and it is also believed to be the most pathogenic [Denner and Young, 2013a; Xu et al., 2013; Waugh et al., 2017]. I have previously found cases where KoRV-A represented less than 100% of the total KoRV (sometimes the combined KoRV-A and -B load was also below 100% of the total load), indicating that some koalas are infected with multiple subtypes. In another finding in our study, *IL-6* expression was significantly greater in PBMCs from koalas infected with multiple subtypes than in those from koalas infected with KoRV-A alone [Kayesh et al., 2020b]. There may be epidemiological implications with regard to these subtypes. Wild koalas in or from northern Australia and their zoo-reared descendants will all harbor KoRV-A from birth, but exposure to other variants may put them at risk of multiple-subtype infection and subsequent poor health outcomes. The zoo population targeted in our previous studies (at Hirakawa Zoological Park, Kagoshima, Japan) comprises koalas from a north Australian population and may thus represent a group at risk of infection with multiple subtypes. KoRV has been fully sequenced recently; it has a positive-sense, single-stranded RNA genome of 8.4 kb with gag, pol, and env genes, and long terminal repeats (*LTR*s) at the 5' and 3' ends [Hanger et al., 2000; Tarlinton et al., 2005; Tarlinton et al., 2006].

Accordingly, in this study, I set out to determine the infection status for the endogenous subtype (KoRV-A) and the two most common exogenous subtypes (KoRV-B and KoRV-C) in a representative sample of captive koalas in a Japanese zoo. I aimed to establish profiles based on proviral and RNA loads in plasma and peripheral blood mononuclear cells (PBMCs) and to make a preliminary assessment of health status for the different profiles based on white blood cell (WBC) count and mortality. Furthermore, I set out to align *env* gene sequences for any subtype identified in my study with reference sequences. The ultimate aim of this study is to

further understanding of KoRV and its subtypes in ways that will benefit both virologists and conservationists.

4.3. Materials and methods

4.3.1. Study animals and sample collection

Adult koalas housed at Hirakawa Zoological Park, Kagoshima, Japan, were chosen for blood collection. EDTA-treated whole blood samples were collected from koalas by venipuncture. The white blood cell (WBC) count was determined at the time of blood collection, and all procedures were in accordance with the regulations and bylaws of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University.

4.3.2. Extraction of DNA and RNA

Genomic DNA (gDNA) was extracted from EDTA-treated whole blood samples using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

Viral RNA and total RNA were extracted using an ISOGEN-LS Kit (Nippon Gene, Japan) and a RNeasy Plus Mini Kit (QIAGEN, Germany), respectively, according to the manufacturer's instructions. To remove any contaminating DNA, extracted RNA was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. The concentration and purity of each extracted DNA and RNA sample were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). RNA samples were stored at -80°C until analysis.

4.3.3. Peripheral blood mononuclear cells (PBMC) culture

Koala PBMCs were cultured from a whole blood sample from one animal, using a previously described method [Miyazawa et al., 2011] with some modifications [Kayesh et al., 2019]. PBMCs were added to two wells of a 6-well plate at 1×10^5 cells/well. The wells contained 1 ml of RPMI 1640 supplemented with 20% heat-inactivated FCS, 1 mM sodium pyruvate (SIGMA), 50 μM β-mercaptoethanol, 1% MEM non-essential amino acids (Invitrogen), penicillin (100 units/mL), and streptomycin (100 μg/ml). The plate was then incubated at 37°C in a 5% CO2 incubator overnight, and 1 ml of RPMI 1640 complete growth medium was added to each well the following day. At seven days after the start of incubation, the 1 ml of medium was replaced with fresh medium. Culture supernatants and cells were harvested at days 7 and 14 of culturing. DNA was extracted from cultured cells using a Wizard Genomic DNA Purification Kit (Promega). Total RNA and viral RNA were also extracted from the cells and culture supernatants using a RNeasy Plus Mini Kit (QIAGEN, Germany) and an ISOGEN-LS Kit (Nippon Gene, Japan), respectively, following the manufacturer's instructions.

4.3.4. Subtyping of KoRV

Using gDNA as a template, a polymerase chain reaction (PCR) was performed to detect KoRV subtypes (KoRV-A, -B or -C), using subtype-specific primers targeting the *env* gene (Table 4.1) under reaction conditions described previously [Hashem et al., 2019; 2020]. To confirm the KoRV subtypes, PCR fragments were subcloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

Table 4.1. Primers used in this study for identification of KoRV and KoRV subtypes

Target gene	Forward	Reverse	Amplicon size (bp)	References
Pol (KoRV)	TTGGAGGAGGAATACC GATTACAC	GCCAGTCCCATACC TGCCTT	523	[Tarlinton et al., 2006]
Env KoRV-A	TCCTGGGAACTGGAAAA GAC	GGGTTCCCCAAGT GATCTG	321	[Waugh et al., 2017]
Env KoRV-B	TCCTGGGAACTGGAAAA GAC	GGCGCAGACTGTT GAGATTC	271	[Xu et al., 2013]
Env KoRV-C	TCCTGGGAACTGGAAAA GAC	AAGGCTGGTCCCG CGAAGT	290	[Hashem et al., 2020]
β-actin	AGATCATTGCCCCACCT	TGGAAGGCCCAGA TTC	123	[Tarlinton et al., 2006]

4.3.5. Measurement of KoRV provirus and RNA copy numbers

The gDNA isolated from koala PBMCs was used as a template for the measurement of total KoRV proviral, KoRV-A, KoRV-B, and KoRV-C DNA copy numbers by real-time PCR as described previously [Hashem et al., 2020], using subtype-specific primers (Table 4.1) with Brilliant-III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Amplification and detection were carried out using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The specificity of the PCR was confirmed by melt curve analysis. Standards were generated from pre-quantified plasmids containing the sequence of the target gene. Koala β-actin was used as an endogenous control for the normalization of the KoRV proviral DNA copy numbers. The primers used for detection of koala β-actin are shown in Table 4.1.

The RNA copy numbers in the RNA isolated from koala PBMCs or plasma were also quantified by real-time PCR as described previously [Hashem et al., 2020], using gene-specific primers (Table 4.1) as described above. Koala β-actin was used as an endogenous control for the normalization of the KoRV total RNA copy numbers.

4.3.6. Multiple sequence alignment

KoRV-A, -B and -C *env* genes were isolated from koalas and sequenced. The sequence data were submitted to the GenBank database (GenBank accession nos. MT951447-MT951453). A multiple sequence alignment of KoRV *env* gene nucleotides was performed using MEGA7 [Kumar et al., 2016].

4.4. Results

4.4.1. Study population, KoRV status, WBC count, and mortality

Six adult captive koalas were included in this study (animal nos. KH1 to KH5 and H10): one male and five females; age range, 1 to 12 years). All six koalas were found to be positive for KoRV (Table 4.2).

Table 4.2. Study of population, subtyping and koala health information

Koala ID	Sex	Age	Detection of KoRV subtypes by PCR		Identified Subtypes	WBC (10^2/μl)	Clinical determinant
			In gDNA of PBMCs	In plasma viral RNA			
KH1	Female	2 Y	Positive	Positive	KoRV-A	102	Healthy
KH2	Male	2 Y	Positive	Positive	KoRV-A, -B and -C	**4000**	**Leukemic**
KH3	Female	12 Y	Positive	Negative	KoRV-A and -B	**2275**	**Leukemic**
KH4	Female	1 Y	Positive	Positive	KoRV-A	52	Healthy
KH5	Female	1 Y 10 M	Positive	Positive	KoRV-A	58	Healthy
H10	Female	6 Y 6 M	Positive	Positive	KoRV-A	125	Healthy

Y-year; M-month

Two koalas (KH2 and KH3) showed a high WBC count and were accordingly regarded as leukemic (Table 2). These two leukemic koalas were reported to have died before the end of the experiment, but their deaths occurred independently of any procedure or observation in

this study. Zoo veterinarians regarded lymphoma as the cause of death for these two animals. The other four koalas (KH1, KH4, KH5, and H10) survived and were regarded as clinically healthy.

4.4.2. Subtyping of koala retrovirus

KoRV subtypes were determined by PCR with gDNA as a template using subtype-specific primers (Table 4.1). Four koalas were found positive for KoRV-A only (KH1, KH4, KH5, and H10). One koala (KH2) was positive for KoRV-A, -B, and -C, and one koala (KH3) was positive for KoRV-A and -B (Figure 4.1A-C).

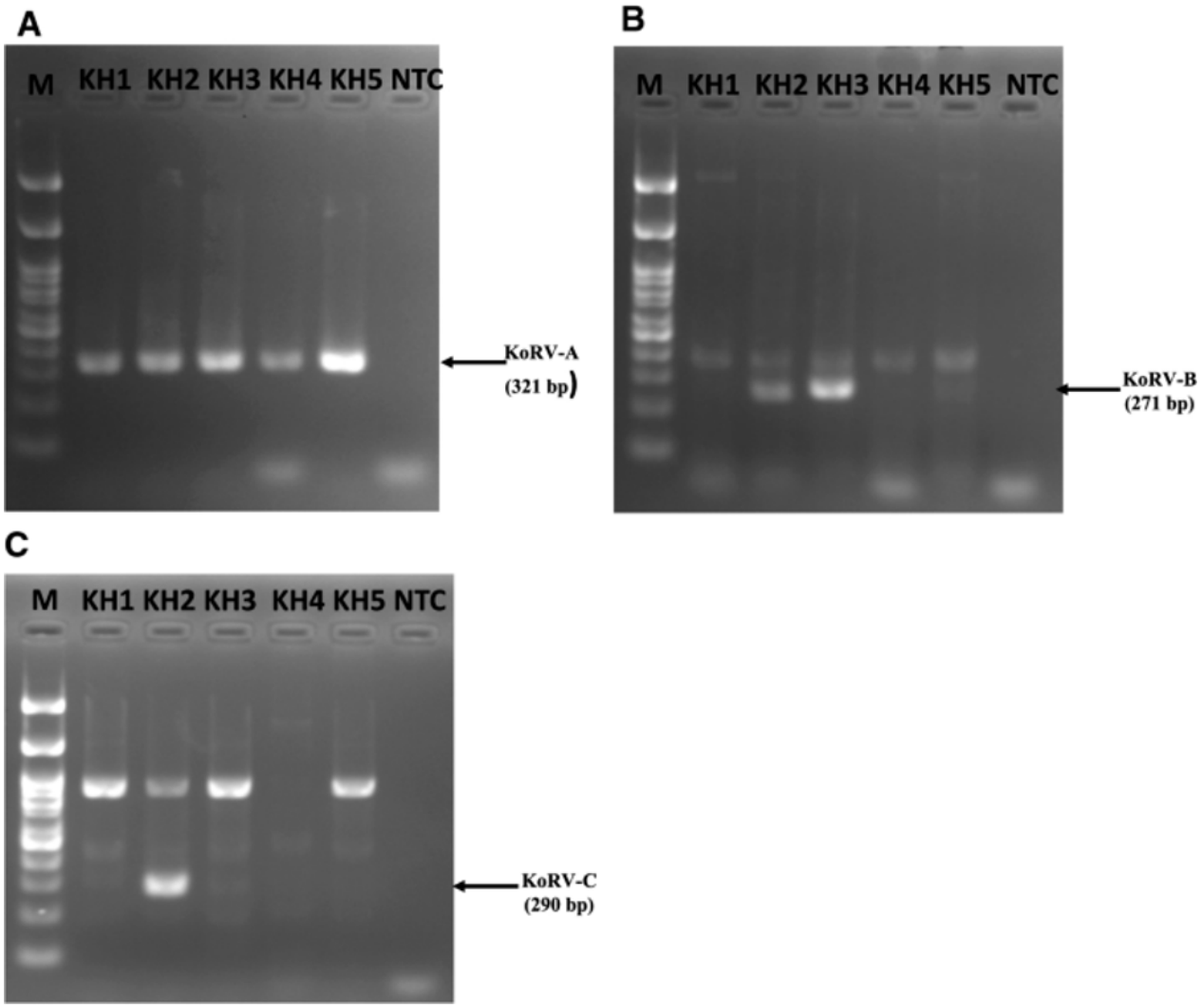


Figure 4.1. Subtyping of KoRV by PCR. Results of PCR analysis using primers specific for KoRV-A (A), KoRV-B (B), and KoRV-C (C) in koalas are shown. In the gel run image, KH1 to KH5 are the designations of the individual koalas, NTC is the no-template control, and 'M' indicates the 100-bp marker (TAKARA). The bands specific for KoRV-A, -B, or -C are indicated by an arrow.

4.4.3. Determination of KoRV proviral DNA and RNA copy numbers

KoRV proviral DNA and total RNA copy numbers were measured using real-time PCR and normalized against koala β-actin. I found variations in the amount of KoRV proviral DNA in the koala genome (Figure 4.2). I measured total KoRV proviral loads as well as KoRV-A, -B, and -C subtype-specific proviral loads. The highest total KoRV proviral load was found in koala KH3, and the lowest KoRV proviral load was found in koala H10 (Figure 4.2). All koalas

tested positive for KoRV-A, with koala KH2 additionally testing positive for KoRV-B and -C and koala KH3 additionally testing positive for KoRV-B.

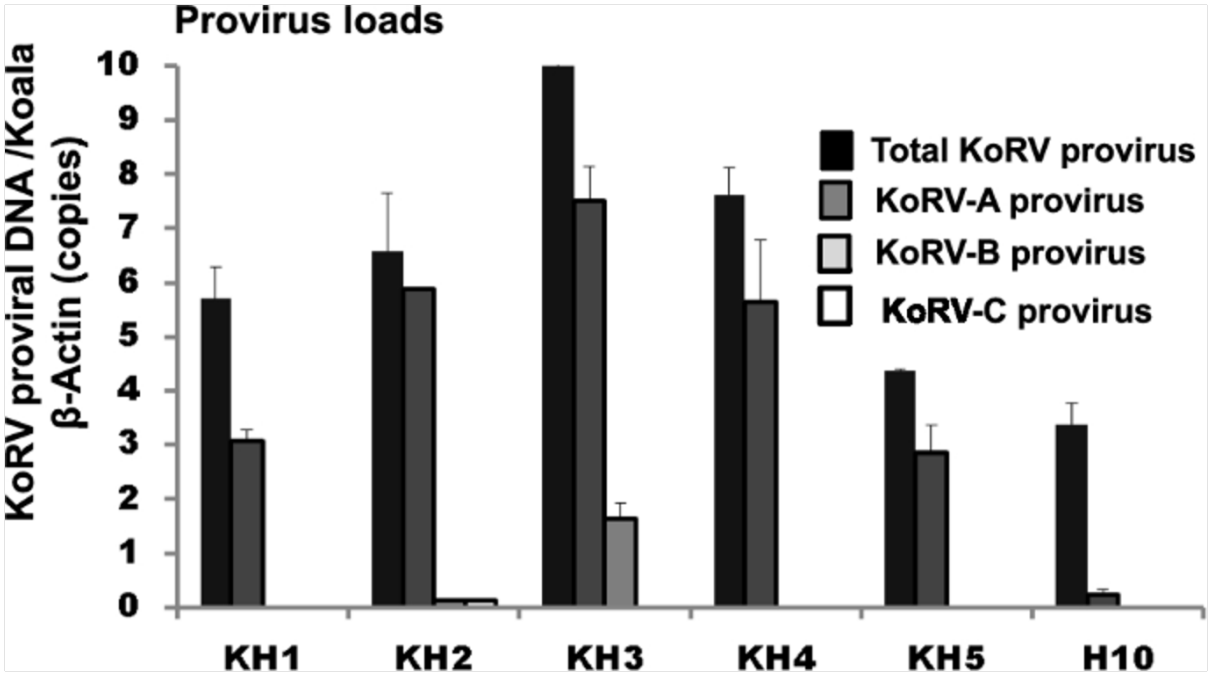


Figure 4.2. Quantitation of KoRV provirus in koala PBMCs. The total KoRV, KoRV-A, -B, and -C provirus DNA copy numbers in gDNA isolated from koalas PBMCs are indicated. The provirus copy numbers were measured by real-time qPCR and normalized with the corresponding koala β-actin in PBMCs. Vertical bars indicate SD.

I also determined the total KoRV RNA copy number targeting the KoRV *pol* gene and the KoRV-A, -B, and -C subtype-specific RNA copy numbers targeting KoRV *env* gene in RNAs isolated from koala PBMCs (Figure 4.3A) and plasma (Figure 4.3B). Both total and subtype-specific KoRV RNA were higher in one of the kolas with a multiple-subtype infection (KH2: KoRV-A, -B, and -C positive). However, koala KH3 (KoRV-A and -B positive) showed lower KoRV RNA copy numbers than other koalas (Figure 4.3A).

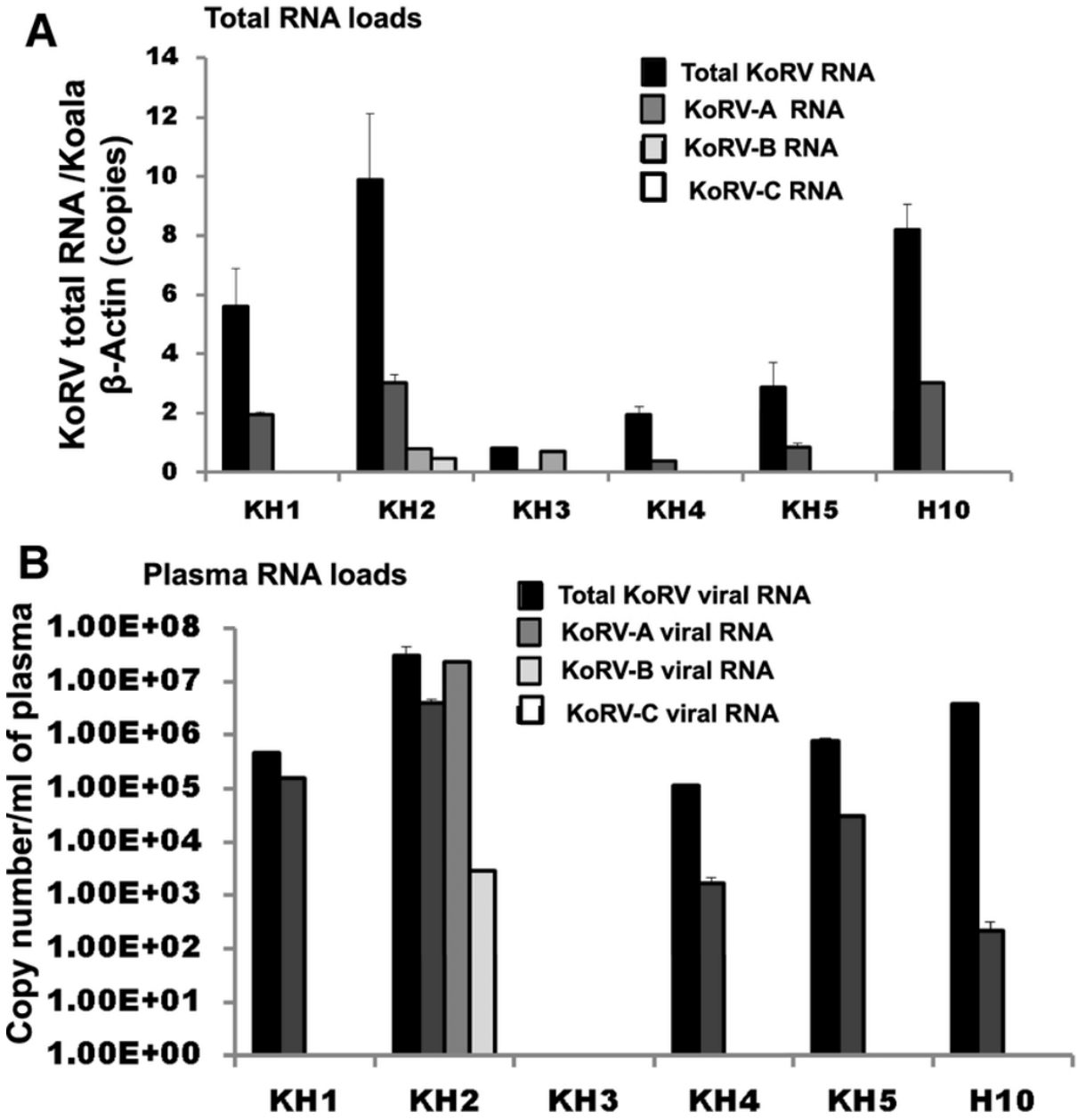


Figure 4.3. Quantitation of KoRV RNA in koala PBMCs and plasma. (A) Total RNA copy numbers in koala PBMCs. RNA copies were quantified by real-time RT-qPCR, and PBMC RNA copies were normalized to koala β-actin. (B) Total KoRV, KoRV-A, -B, and -C RNA copies per mL plasma. Vertical bars indicate SD.

4.4.4. Determination of the KoRV copy number in koala PBMC culture

The koala with undetectable plasma KoRV RNA (KH3) was targeted for further investigation with cell cultures of its isolated PBMCs. I measured the KoRV proviral load in the koala gDNA, as well as the total RNA and viral RNA load in the cultured cells and supernatant, respectively

(Figure 4.4A, B, and C). I detected KoRV proviral DNA in the genome and total KoRV RNA in cultured PBMCs at days 7 and 14 days of culturing. The culture supernatant contained detectable total KoRV RNA, KoRV-A, and -B RNA levels at day 7 of culturing, but not at day 14 of culturing (Figure 4.4C). I then measured RNA loads for total KoRV, KoRV-A, KoRV-B, and KoRV-C in plasma from each animal (Figure 4.4B). The highest plasma KoRV RNA load was in koala KH2 (KoRV-A, -B, and -C positive), but no KoRV was detected in the other koala with a multiple-subtype infection (KH3: KoRV-A and -B positive). The other four koalas had detectable levels of total KoRV and KoRV-A RNA in their plasma, albeit at lower levels than KH2 (Figure 4.4B).

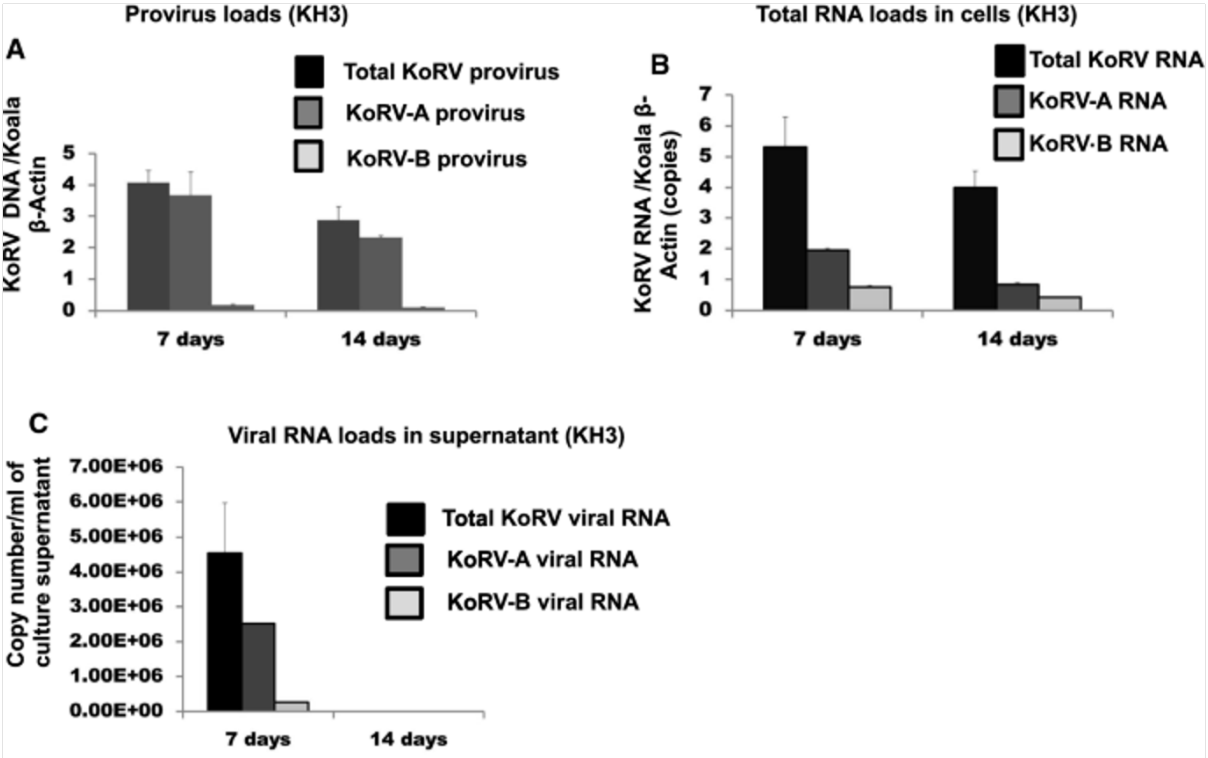
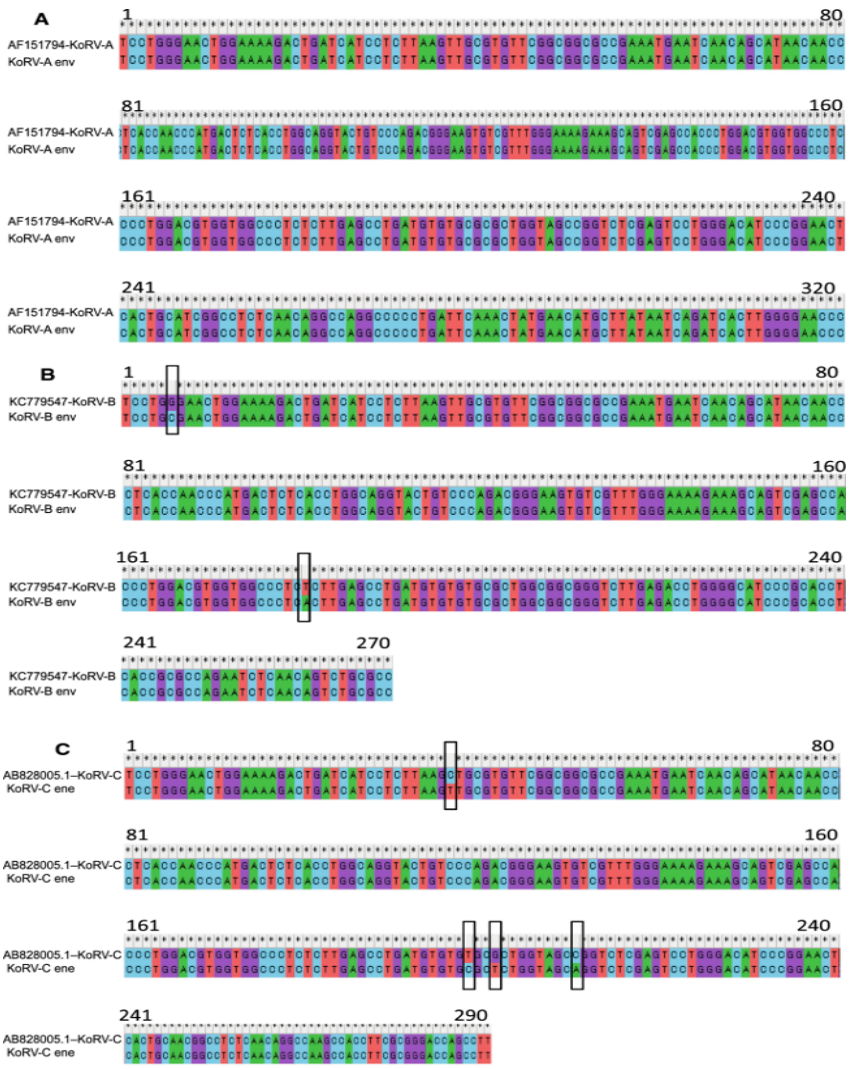


Figure 4.4. Distribution and quantity of KoRV subtypes in cultured koala PBMCs isolated from koala KH3. The koala PBMCs were cultured, and gDNA and RNA were purified from the cells and supernatant. (A) Total KoRV, KoRV-A, and KoRV-B provirus copy number in gDNA at days 7 and 14 post culture. (B) Total KoRV, KoRV-A, and KoRV-B RNA copy numbers in koala PBMCs at days 7 and days 14 post culture. Provirus copies were measured by real-time PCR and normalized to the corresponding koala β-actin. (C) RNA copy number per mL of koala PBMC culture supernatant at days 7 and days 14. Vertical bars indicate SD.

4.4.5. Sequence alignment

I sequenced the KoRV-A, -B, and -C partial env genes (GenBank accession nos. MT951447-MT951453) from the koalas in this study and performed sequence alignment with KoRV-A, -B, and -C *env* gene reference sequences (GenBank accession nos.: KoRV-A, AF151794; KoRV-B, KC779547; KoRV-C, AB828005.1). The KoRV-A partial *env* gene sequence in the study animals was 100% identical to the reference sequence (Figure 4.5A). The KoRV-B and KoRV-C partial *env* gene sequences in the study animal showed differences from the corresponding reference sequence in two and four nucleotides, respectively (enclosed by black boxes, Figure 4.5B and C).



Figures 4.5 Sequence alignment partial env genes of KoRV-A (from koala KH1-5), -B (from koalas KH2 and KH 3), and -C (from koala KH2). Boxes indicate the nucleotide difference of isolated KoRV-A, -B, and -C partial env genes from Hirakawa Zoological Park, Kagoshima, Japan, with reference sequences. Reference sequences of KoRV-A (GenBank accession no. AF151794), KoRV-B (GenBank accession no. KC779547), and KoRV-C (GenBank accession no. AB828005.1) are also shown.

4.5. Discussion

In this study, I determined KoRV profiles (for total KoRV and subtypes KoRV-A, -B, and -C) in six captive koalas in a Japanese zoo and made a preliminary assessment of the effects of multiple-subtype infection on koala health, based on WBC counts.

In my study population, two koalas had multiple-subtype infection (KoRV-B and/or -C in addition to KoRV-A), and four koalas had a single-subtype infection, carrying only the fully endogenized form (KoRV-A). Notably, the koalas with multiple subtypes had poorer health outcomes, showing elevated WBC counts indicative of leukemic status at the time of blood sampling. On the other hand, the four koalas with a single subtype (KoRV-A) appeared clinically healthy at the time of blood sampling.

My findings are consistent with a number of previous reports on KoRV and koala health. A recent study suggested that KoRV-A does not superinfect koalas harboring endogenized KoRV-A [Ishida et al., 2015], which was substantiated by the apparently clinically healthy status of the four koalas in our study with KoRV-A as a single-subtype infection. Associations between KoRV-B and lymphoma have been reported in both captive and wild populations [Zheng et al., 2020; Xu et al., 2013], findings which are reflected in the deaths due to lymphoma of the two KoRV-B-positive koalas in our study. Increased *IL-6* expression has also been reported previously in koalas with multiple-KoRV-subtype infection [Kayesh et al., 2020b],

which may be linked to associations of KoRV with immune suppression and neoplasia [Xu et al., 2013; Kayesh et al., 2020b].

I found that all of the koalas had detectable KoRV provirus, but with inter-individual variations in the proviral load. My findings on proviral load are consistent with those of a previous study [Tarlinton, et al., 2005] in which such variations were speculated to result from the ongoing endogenization of KoRV into the koala genome. The plasma viral RNA load has been advanced as an indicator of the stage and progression of other retroviral diseases in other species, with previous studies covering HIV1-infected humans [Piatak et al., 1993] and feline-immunodeficiency-virus-infected cats [Diehl et al., 1996]. Tarlinton et al. reported that a higher plasma viral load may be associated with neoplasia [Tarlinton et al., 2005]. The koala with the highest plasma load in our study died from lymphoma, but interestingly, KoRV could not be detected in the case of the other lymphoma-related death.

The two koalas with multi-subtype infection showed an apparently worse health status that the single-subtype-infected koalas, and they died due to lymphoma. Although one or the other of these koalas showed the highest value for each viral load parameter I examined, there were cases where koalas with single subtype (KoRV-A only) infection showed a higher proviral, total RNA, or plasma viral load than one of the koalas with a multi-subtype infection. Furthermore, the total load exceeded that of KoRV-A only for each koala designated as having single-subtype infection, suggesting that they may have harbored one of the subtypes (KoRV-D to J) not evaluated in this study.

During genotyping of the KoRV-C subtype with subtype-specific primers, I observed a larger amplicon for all of the koalas except KH4. This result is indicative of an extraneous

origin for the sequence. Accordingly, I sequenced larger fragments and found one DNA fragment from KH3 containing an N-terminal sequence (length: 181 nt) that was similar to that of the KoRV-C subtype envelope gene (GenBank accession no. MT951447.1), but the downstream sequence did not show any significant similarity to sequences in the GenBank database. This finding suggests that proviral recombination with KoRV-C might have occurred.

The koala with undetectable plasma KoRV RNA was chosen for further investigation with cell cultures of its isolated PBMCs. The culture supernatant contained detectable RNA levels at day 7 of culturing, but not at day 14. This finding may reflect a decrease in available cells releasing KoRV RNA into the culture supernatants at the later time point, as well as the fragile nature of the RNA.

Sequence alignment revealed 100% identity between the KoRV-A partial env gene in the study animals and the KoRV-A reference env gene, suggesting that KoRV-A has been fully endogenized in this population, whereas KoRV-B and -C are continuing to evolve and showed mutations as expected. Alignment of KoRV in our study population with reference sequences suggested that the state of KoRV in this Japanese zoo epidemiologically reflects that in wild koala populations in northern Australia, in which KoRV-A is fully endogenized but other subtypes, while actively replicated, are much less prevalent. I found 100% sequence identity to the reference sequence for KoRV-A but multiple differences in nucleotides for both KoRV-B and KoRV-C. I suggest that KoRV-B and KoRV-C are mutating with circulation in captive population, as would be expected for virus subtypes that are continuing to evolve. On the other hand, I used only the *env* genes for sequence alignment, and secure confirmation of a variant's ability to replicate requires identification of its full genome sequence. Further investigations are thus required to test our hypothesis on subtype replication in this captive koala population.

My findings are also consistent with previous reports of greater genetic diversity in the KoRV *env* gene for subtypes other than KoRV-A [Zheng et al., 2020; Xu et al., 2015; Quigley et al., 2018; 2019; Sarker et al., 2019].

In conclusion, coinfection with multiple KoRV subtypes may have a negative impact on koala health. I also suggest that single-subtype infection with KoRV-B may be implicated in the development of lymphoma and increased virus loads in plasma. Furthermore, infection with multiple KoRV subtypes may lead to increased KoRV loads in plasma, which could thus be a useful prognostic marker for koala health. For further understanding of disease association in multiple-subtype infection, a large-scale study targeting the full range of KoRV subtypes is warranted for further understanding of this marsupial retrovirus.

Chapter 5

Subtype distribution and expression of the koala retrovirus in the Japanese zoo koala population

5.1. Abstract

Koala retrovirus (KoRV) remains as a significant threat to wild and captive koala populations. It exists in both endogenous (KoRV-A) and exogenous (KoRV-B to -J and KoRV-K) forms, which are considered to be linked to lymphoma/neoplasia and immunosuppression in koalas. Therefore, in this study I investigated the proviral copies and RNA expression in koala retrovirus (KoRV)-infected koalas. To ascertain any variation in viral load by institution, age, sex, or body condition score, i quantified KoRV proviral DNA and RNA loads in captive koalas (n=37) reared in Japanese zoos. All koalas were positive for KoRV genes (*pol, LTRs*, and *env* of KoRV-A) in genomic DNA (gDNA), and 91.89% were positive for the *pol* gene in RNA. In contrast, the distribution rates of KoRV-B, KoRV-C, KoRV-D, and KoRV-F *env* genes in gDNA were 94.59%, 27.03%, 67.57%, and 54.05%, respectively. A wide inter-individual variation and/or a significant inter-institutional difference in proviral DNA ($p<0.0001$) and RNA ($p<0.001$) amounts (copies/10^3 koala β-actin copies) were observed in Awaji Farm England Hill Zoo koalas, which were obtained from southern koala populations, suggesting exogenous incorporation of KoRV in these koalas. Significant ($p < 0.05$) age differences were noted in KoRV RNA load ($p < 0.05$) and median total RNA load ($p < 0.001$), with loads higher in younger koalas (joeys and juveniles). Thus, the current study provides the distribution of KoRV subtypes in Japanese zoo koala populations and identifies several additional risk factors (sex, age, and body condition) associated with KoRV expression.

5.2. Introduction

Koala retrovirus (KoRV) can exist within its host in both endogenous and exogenous forms. The virus reportedly represents a pathogenic threat to these hosts— *Phascolarctos cinereus*, Australia's iconic koala—at a time their survival is already imperiled by a range of environmental, interspecific, and anthropogenic factors, as well as infectious diseases [Gonzalez-Astudillo et al., 2017; McAlpine et al., 2015]. Koalas are listed as vulnerable on the International Union for Conservation of Nature's Red List of threatened species [Woinarski and Burbidge, 2016], and the authorities in Australia are currently reviewing their survival status with a view to re-classifying the species as endangered [Foley, 2021]. Much has been learned about KoRV through virological and molecular epidemiological investigations in wild [Quigley et al., 2018; Sarker et al., 2020], and more latterly captive populations [Kayesh et al., 2019; Hashem et al., 2019; 2021]. However, much remains to be learned, and further investigations would be of benefit to koala conservationists, as well as being of interest to researchers studying the biology of retroviruses.

With its endogenous and exogenous subtypes, KoRV is a relatively conserved gamma retrovirus. It is closely related to gibbon-ape leukemia virus (GaLV) [Hanger et al., 2000; Tarlinton et al., 2005; Denner and Young 2013a], feline leukemia virus (FeLV) [Denner and Young 2013], and porcine endogenous retrovirus (PERV) [Denner and Young, 2013]. Its genome has been sequenced and is approximately 8.4 kb, and contains pol, gag, env genes as well as long terminal repeats at the 5' and 3' ends [Hanger et al., 2000; Tarlinton et al., 2005; Tarlinton et al., 2006]. A number of assays are available for detection of the virus targeting genomic DNA and viral RNA, and molecular characterizations can be performed for koalas to determine their KoRV infection status. For example, one report suggested that high viral RNA

loads in plasma may be associated with poorer health outcomes [Tarlinton et al., 2005]; thus, full molecular surveys are useful for elucidating aspects of KoRV infection. KoRV-A is the endogenous subtype, and has a near-universal prevalence of 100%, with only some small, highly localized populations where this subtype has yet to be fully endogenized into the genome [Quigley et al., 2018; Hashem et al., 2019; Kayesh et al., 2020; Zheng et al., 2020]. However, southern Australian koalas carry all exogenous KoRV subtypes (Quigley et al., 2021a). Since KoRV was first detected in 1988, in a leukemic koala [Canfield et al., 1988], a number of putatively exogenous subtypes have been identified in addition to the endogenous form. Up to the time of writing, a total of ten exogenous subtypes (KoRV-B to KoRV-I, and KoRV-K) have been identified [Joyce et al., 2021], and the first of these, KoRV-B, has so far received the most attention from researchers [Hashem et al., 2019; Kayesh et al., 2020; Zheng et al., 2020].

As a pathogen, KoRV has been linked to immunosuppression and tumors, and may also be implicated in chlamydiosis [Quigley et al., 2020; Kayesh et al., 2020; Kayesh et al., 2021]. Other reports have identified a specific role for KoRV-B in leukemia and chlamydial disease [Xu et al., 2013; Waugh et al., 2017]. I previously reported the possibility that co-infection with multiple subtypes may be linked to greater disease severity [Hashem et al., 2021]. KoRV integration may directly promote oncogenesis by altering oncogene expression [McEwen et al., 2021]. However, evidence on the relative subtype pathogenicity and transmissibility is still lacking [Zheng et al., 2020], and there is a need for further investigations harnessing molecular analyses with clinical and biometric evaluations.

Captive (zoo-dwelling) koalas are available as target populations for investigations of KoRV. Once established as broadly representative of wild populations, captive koalas offer

better documented medical histories and availability for sampling and examination procedures. This allows researchers to study prevalence and trends in infection by subtype, and to search for associations with clinical and demographic characteristics. Population level research has recently been published on koalas in American and Australian zoos [Zheng et al., 2020]. The results of that study showed universal infection with subtype A, and prevalences ranging between approximately 10% and 60% for the exogenous subtypes, which appeared to be significantly associated with leukemia and other cancers [Zheng et al., 2020]. Previously, I have reported on KoRV in Japanese zoo populations, but these investigations have been limited to comparisons of only two subtypes (KoRV-B and KoRV-A), small numbers of animals, and only three zoos [Hashem et al., 2019]. Thus, further investigations are needed in the wider populations of Japanese zoo-dwelling koalas, to establish prevalence by type and characterize associations with infection status. Furthermore, Japanese zoo-dwelling koalas represent a very useful population for KoRV evaluations, because the zoos participate in a sharing program with koalas occasionally being transferred for breeding and/or exhibition, and such transfers are important for modeling transmissibility.

Proviral copies may be considered a crucial indicator of the endogenous or exogenous KoRV status. For KoRV provirus quantification, several earlier studies targeted the KoRV pol gene in gDNA, which was normalized to the koala β-actin gene [Simmons et al., 2012; Sarker et al., 2020; Wedrowicz et al., 2016], as well as direct counting of proviral sequences in the koala genome [Hobbs et al., 2017]. Previous studies have reported that 54.7–165.0 proviral copies per cell are present in Queensland (QLD) koalas [Sarker et al., 2020; Simmons et al., 2012]. In contrast, 9.0×10^{-2}–1.29×10^{-4} proviral copies are present in Victoria Island koalas [Simmons et al., 2012; Wedrowicz et al., 2016], possibly suggesting that KoRV provirus is not yet endogenous in Victoria Island koalas.

Against this background, I set out to perform a cross-sectional molecular epidemiology study of KoRV infection for the captive koala population dwelling in Japanese zoos. The investigations were threefold: a molecular characterization targeting genomic DNA and plasma and cellular RNA, using a comprehensive suite of PCR analyses (conventional PCR, qPCR and RT-qPCR); KoRV subtyping for each individual; and an evaluation of associations of KoRV proviral and viral loads with a range of demographic and clinical characteristics (institution, sex, age, and body condition score) in this koala population. Overall, this study provides a clear understanding of the distribution of KoRV subtypes in the Japanese zoo koala populations. Additionally, several risk factors (sex, age, and body condition) that may influence KoRV expression can be identified and could be vital in maintaining health in zoo-dwelling koalas.

5.3. Materials and methods

5.3.1. Study population

I targeted captive koalas dwelling in seven Japanese zoos and available for blood sampling one time in the study period (June to October 2021) for inclusion in this study. Specifically, the zoos involved were Kanazawa Zoo, Tama Zoological Park, Kobe Oji Zoo, Awaji Farm England Hill Zoo, Hirakawa Zoological Park, Saitama Children`s Zoo, and Nagoya Higashiyama Zoo, and these represent all seven zoos in Japan which house koalas. At each zoo, the koalas were housed in an environment maintained within a constant temperature range (23~25°C) using an air conditioning system. Each habitat is designed, as far as possible, to simulate the koalas' natural habitat, and they were provided with an ad libitum supply of eucalyptus leaves. The koalas in the Awaji Farm England Hill Zoo originate from Victoria,

whereas the other koalas are mainly from Queensland. They are independently maintained, and no cross-breeding was performed, thus, maintaining genetic information in wildlife.

5.3.2. Sample collection and preparation

Between June and October 2021, a blood sample (1.5 to 2 ml) was collected from each koala with EDTA. All guidelines of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University, Japan (Approval Number: 19K001) were followed.

5.3.3. Extraction of genomic DNA

Genomic DNA (gDNA) was extracted from 300 µl of whole blood using a Wizard Genomic DNA Purification Kit (Promega) in accordance with the manufacturer's instructions, and finally eluted in 100 µl of the elution buffer provided in the kit. The concentration and purity of the extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Waltham, MA, USA).

5.3.4. Extraction of RNA

EDTA-treated whole blood samples of 1 mL volume were centrifuged at 3,000 rpm for 5 min at 20°C to obtain plasma samples, which were then stored at −80°C. To measure plasma viral load, viral RNA was isolated from plasma using RNeasy plus Mini Kit (QIAGEN, Germany), in accordance with the manufacturer's instructions.

Total RNA was also extracted using RNeasy plus Mini Kit (QIAGEN, Germany), in accordance with the manufacturer's instructions. The RNA concentration and purity of the extracted RNA were measured using a NanoDrop ND-1000 spectrophotometer.

5.3.5. Demographic and clinical assessments

The demographic and clinical assessments were performed at the time of blood sample collection, and records at the relevant zoos were consulted to determine the sex and age of each koala (Table 5.1). A qualified zoo veterinarian evaluated the condition of each koala by scoring the animal's body condition on a scale of 1 to 5. This score was determined by palpating muscle in the scapular region, with 'poor' corresponding to a score of ≤2, 'good' corresponding to a score of 3, and 'very good" corresponding to a score of 4-5 [Sarker et al., 2020].

Table 5.1. Details of koalas used in this study

Koala ID	Sex	Age	Group	BCS	Clinical Status	Clinical sign	WBC (10^2/ul)	Health status
Kanazawa Zoo								
KU_YZ_01	F	1Y 3M	Juvenile	5	very good	None	115	Satisfactory
KU_YZ_02	M	6Y 8M	Adult	3	Good	None	75.5	Satisfactory
KU_KAZ_3	F	8 Y 1 M	Adult	3	Good	None	66.5	Satisfactory
KU_KAZ_4	F	4 Y 3 M	Adult	3	Good	None	95.5	Satisfactory
Tama Zoological Park								
KU_TZ_05	M	6Y	Adult	3	Good	None		Satisfactory
KU_TZ_06	M	5Y	Adult	3	Good	None		Satisfactory
KU_KZ_07	F	11Y	Old	4	vert good	None	45	Satisfactory
KU_KZ_08	F	7Y 2M	Adult	5	very good	None	54	Satisfactory
KU_KZ_09	M	5Y 5M	Adult	5	very good	None	68	Satisfactory
KU_KZ_10	F	4Y 11M	Adult	5	very good	None	68.5	Satisfactory
KU_KZ_11	M	4Y	Young adult	5	very good	None	30	Satisfactory

KU_KZ_12	F	2Y 8M	Young adult	5	very good	None	147	Satisfactory
KU_KZ_13	F	2Y 3M	Young adult	4	very good	None	56	Satisfactory
KU_KZ_14	F	2Y 3M	Young adult	5	very good	None	95.5	Satisfactory
Awaji Farm England Hill Zoo								
KU_AZ_15	F	24 Y	Old	3	Good	Loose stool	50	Satisfactory
KU_AZ_16	F	13 Y	Old	3	Good	None	58	Satisfactory
KU_AZ_17	M	12 Y	Old	3	Good	None	97	Satisfactory
KU_AZ_18	M	7 Y	Old	3	Good	Loose stool	42	Satisfactory
Hirakawa Zoological Park								
KU_HZ_19	M	1Y	Juvenile	4	Very good	None	62	Satisfactory
KU_HZ_20	M	1Y	Juvenile	3	Good	Abdomenal bloating	50	Satisfactory
KU_HZ_21	F	1Y	Juvenile	4	Very good	None	82	Satisfactory
KU_HZ_22	F	1Y	Juvenile	4	Very good	None	88	Satisfactory
Saitama Children`s Zoo								
KU_SZ_23	M	3 Y	Young adult	3	Good	None		Satisfactory
KU_SZ_24	F	7 Y	Adult	3	Good	None		Satisfactory
KU_SZ_25	F	3 Y	Young adult	3	Good	None		Satisfactory
KU_SZ_26	F	2 Y	Juvenile	3	Good	None		Satisfactory
KU_SZ_27	F	2 Y	Juvenile	3	Good	None		Satisfactory
Nagoya Higashiyama Zoo								
KU_NZ_28	M	9 Y	Old	3	Good	None	76	Satisfactory
KU_NZ_29	M	4 Y	Young adult	3	Good	None	85	Satisfactory
KU_NZ_30	F	11 Y	Old	4	Good	None	23	Satisfactory
KU_NZ_31	F	11 Y	Old	2	Good	None	51	Satisfactory
KU_NZ_32	F	7 Y	Adult	3	Good	None	47	Satisfactory
KU_NZ_33	F	4 Y	Young adult	4	Good	None	57	Satisfactory
KU_NZ_34	F	10 M	Joey	3	Good	None	43	Satisfactory
KU_NZ_35	F	1 Y	Juvenile	4	Good	None	69	Satisfactory
KU_NZ_36	M	11 M	Joey	3	Good	None	69	Satisfactory
KU_NZ_37	F	3 Y	Young adult	3	Good	None	66	Satisfactory

M-male; F-female; Y-year; M-month; WBC-white blood cell

Each koala was observed for clinical signs and subjected to blood sampling for determination of white blood cell (WBC) count. The overall health status for each koala was determined by the veterinarian based on body condition score, clinical signs, and WBC data.

5.3.6. Determination of KoRV in genomic DNA

Conventional PCR was performed to detect the presence of KoRV LTR and proviral *env* genes (for KoRV-A, -B, -C, -D, -E, or -F) using multiple primers (Table 5.2).

Table 5.2. PCR primers used in this study for the detection of KoRV genes

Target gene	Primer sequences	Annealing temperature	Amplicon size (bp)	References
LTR	U3F>AATGAAGGAGGCAGAAATCATGAGGC Gag 1A>TTCCAGTGATCTAGTGTAAG	57 °C	505	Hashem et al., 2020
	U3F>AATGAAGGAGGCAGAAATCATGAGGC U5R>ATGAAAGACCCCAATGTTCG	61°C		
pol	F>CCTTGGACCACCAAGAGACTTTTGA R> TCAAATCTTGGACTGGCCGA	64 °C	523	Tarlinton et al., 2006
β-actin	F>AGATCATTGCCCCACCT R>TGGAAGGCCCAGATTC	59 °C	123	Tarlinton et al., 2006
***Env* KoRV-A**	F>TCCTGGGAACTGGAAAAGAC R>GGGTTCCCCAAGTGATCTG	63.4 °C	321	Waugh et al., 2017
***Env* KoRV-B**	F>TCCTGGGAACTGGAAAAGAC R>GGCGCAGACTGTTGAGATTC	63.4 °C	271	Xu et al., 2013
***Env* KoRV-C**	F>TCCTGGGAACTGGAAAAGAC R>AAGGCTGGTCCCGCGAAGT	62 °C	290	Hashem et al., 2020
***Env* KoRV-D**	F>TCCTGGGAACTGGGAAAAGAC R>TTGTATGACCGACCTTGGGG	58°C	286	This study
***Env* KoRV-E**	F>GGTGACCGAGTGCTGGTTAG R>CAGTGGCTGTAAAGCCAGGCC	-	-	Xu et al., 2015
***Env* KoRV-F**	F>GGTGACCGAGTGCTGGTTAG R>GGGATTCCCCAAGGCCCGGTC	65°C	469	This study

A nested PCR was performed using primer sets (Table 5.2) to determine the nucleotide sequence of the *LTR* for KoRV provirus, as described previously [Hashem et al., 2020]. For KoRV subtyping, a PCR was performed to detect KoRV subtypes, using subtype-specific primers (Table 5.2) targeting the env gene in gDNA. The reaction conditions were as largely as previously described [Xu et al., 2015; Hashem et al., 2019; 2020]. However, the conditions were slightly modified for the detection of subtypes KoRV-D and -F. The conditions for KoRV-D involved initial denaturation at 98°C for 2 minutes, denaturation at 98ºC for 30s, annealing at 58ºC for 30s followed by 40 cycles, extension at 72ºC for 1 min, and final

extension at 72ºC for 5 min. The same cycle conditions were applied for KoRV-F, except for the annealing phase, for which the condition was set at 65ºC for 30s.

The amplified PCR products were electrophoresed in a 1.5% agarose gel with 10% TAE buffer and ethidium bromide dye (ThermoFisher Scientific), and then visualized in Fusion Solo S imaging system. PCR reactions were directly purified with Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions. Next, the resulting target PCR fragments were subcloned into pCR-Blunt II TOPO (Invitrogen), and sequenced. The resulting sequences were submitted to the DDBJ database (accession numbers: KoRV-A LC667835-LC667871, KoRV-B LC667872-LC667905, KoRV-C LC667906-LC667915, KoRV-D LC667916-LC667940, KoRV-F LC667941-LC667960, and KoRV LTRs LC667961-LC667997).

5.3.7. Quantification of KoRV *pol* gene

The gDNA isolated from koala PBMCs was used as a template for the measurement of KoRV proviral DNA copy numbers. This measurement was conducted by real-time PCR, as described previously [Hashem et al., 2020; Kayesh et al., 2019], using the relevant primers (Table 5.2) with Brilliant-III Ultra-Fast SYBR Green Q-PCR and RT-qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), in accordance with the manufacturer's instructions. Amplification and detection were carried out using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The specificity of PCR was confirmed by melt curve analysis. Standards were generated from pre-quantified plasmids containing target gene sequences. Koala β-actin was used to normalize KoRV proviral DNA copy numbers. Primers used for the detection of koala β-actin are listed in Table 5.2.

The RNA copy numbers for koala PBMC or plasma isolates were also quantified by real-time PCR as described previously [Hashem et al., 2020], using gene-specific primers (Table 5.2) as described above. Koala β-actin was used as an endogenous control for the normalization of the KoRV total RNA copy numbers. Only the pol gene was evaluated with this methodology due to the conserved nature of this gene and its widespread use for KoRV diagnosis

5.3.8. Statistical analysis

A one-way ANOVA was performed using GraphPad Prism (Version 9) to compare KoRV proviral and RNA loads between the zoos. Using GraphPad Prism, a Mann-Whitney test was performed to reveal any statistically significant differences in KoRV loads within this population, based on sex, age, and body condition score. For analyses by age, the koalas were divided into the following cohorts: joeys (age =<1 year), juvenile (age = 1-2 years), young adult (age = >2-4 years), mature adult (age = >4 years) [Sarker et al., 2020]. Statistical significance was set at $P < 0.05$.

5.4. Results

5.4.1. Demographic parameters and clinical status (clinical signs, WBC count, body condition score, and overall health determination)

The study population comprised a total of 37 captive koalas across seven Japanese zoos. In this population, 13 koalas were males (age range: 11 moths to 12 years) and 24 were females (age

range: 10 months to 24 years). The demographic and clinical characteristics are summarized by zoo in Table 5.3, and presented in full for each individual koala in the Table 5.1.

Table 5.3. Study area and population characteristics

Variable	Name of the zoos						
	Kanazawa Zoo (n=4)	Tama Zoological Park (n=2)	Kobe Oji Zoo (n=8)	Awaji Farm England Hill Zoo (n=4)	Hirakawa Zoological Park (n=4)	Saitama Children` s Zoo (n=5)	Nagoya Higashiyam a Zoo (n=10)
Sex							
Male	1 (25%)	2 (100%)	2 (25%)	2 (50%)	2 (50%)	1 (20%)	3 (30%)
Female	3 (75%)	0 (0%)	6 (75%)	2 (50%)	2 (50%)	4 (80%)	7 (70%)
Age cohort							
Joey	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)
Juvenile	1 (25%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)	2 (40%)	1 (10%)
Young adult	0 (0%)	0 (0%)	4 (50%)	0 (0%)	0 (0%)	2 (40%)	3 (30%)
Adult & aged	3 (75%)	2 (100%)	4 (50%)	4 (100%)	0 (0%)	1 (20%)	4 (40%)
Clinical status							
Poor	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Good	3 (75%)	2 (100%)	0 (0%)	4 (100%)	1 (25%)	5 (100%)	10 (100%)
Very good	1 (25%)	0 (0%)	8 (100%)	0 (0%)	3 (75%)	0 (0%)	0 (0%)

Clinical signs and WBC count data are presented in the supplementary table (Table 5.1). Of the 37 koalas, 34 showed no abnormal clinical signs (the exceptions were two koalas showing loose stool, and one koala showing abdominal bloating). All koalas showed WBC counts within the normal range.

Body condition scores ranged from 3 to 5, and all scores corresponded to the ‘good’ or ‘very good’ category.

Based on the results for all examinations, the relevant veterinarian made an overall health determination of ‘satisfactory’ for the health of each koala in this study (Table 5.1).

5.4.2. Gene positivity rates for target DNA and RNA molecules in genomic DNA and plasma and PBMCs

To establish the molecular profiles for KoRV infection status in all koalas, I applied a range of conventional PCR and qPCR assays, targeting various loci in genomic DNA, koala plasma, or PBMCs. To detect proviral DNA, two conventional PCR assays (one for the env gene and the other for *LTR*) and one qPCR assay (targeting *pol* gene only) were performed using PCR primers (Table 5.2). To detect KoRV RNA in plasma and PBMCs, two RT-qPCR assays (targeting the *pol* gene only) were used. All 37 koalas (100%) tested positive for the presence of proviral DNA (in the conventional PCR assay targeting the *LTR* region and the qPCR assay targeting the *Pol* gene). Thirty-four of the 37 koalas (91.89%) tested positive for the KoRV *pol* gene using RT-qPCR assays for viral RNA and/or total RNA detection (Table 5.4).

Table 5.4. Rate of PCR positivity by conventional PCR, qPCR reactions using gDNA for KoRV provirus and RT-qPCR reactions using viral RNA & mRNA for KoRV in different zoos.

PCR reactions	Gene target	Name of the zoos							
		Kanazawa Zoo (n=4)	Tama Zoological Park (n=2)	Kobe Oji Zoo (n=8)	Awaji Farm England Hill Zoo (n=4)	Hirakawa Zoological Park (n=4)	Saitama Children`s Zoo (n=5)	Nagoya Higashiyama Zoo (n=10)	Total N=37
Conventional PCR	*LTR*	100%	100%	100%	100%	100%	100%	100%	100%
qPCR (gDNA)	*Pol*	100%	100%	100%	100%	100%	100%	100%	100%
RT-qPCR (viral RNA)	*Pol*	50%	100%	100%	75%	100%	100%	100%	91.9%
RT-qPCR (mRNA)	*Pol*	100%	100%	100%	25%	100%	100%	100%	91.9%

5.4.3. Prevalence of KoRV subtypes

Koalas were further screened for subtypes KoRV-A, -B, -C, -D, -E, and -F, targeting proviral env genes. The overall prevalence of KoRV-A was 100%, and the overall prevalences of KoRV-B, -C, -D, and -F subtypes were 94.59, 27.03, 67.57, and 54.05 %, respectively, but KoRV-E was not detected (Table 5.5). However, prevalence reached 100% for KoRV-B in five zoos (Kanazawa Zoo, Tama Zoological Park, Hirakawa Zoological Park, Saitama Children`s Zoo and Nagoya Higashiyama Zoo) and for KoRV-D in three zoos (Tama Zoological Park, Hirakawa Zoological Park, Saitama Children`s Zoo) [Table 5.5]. KoRV-C reached its highest prevalence in Saitama Children's Zoo (80.00%), followed by Tama Zoological Park (50.00%), Kanazawa Zoo (25.00%) and Hirakawa Zoological Park (25.00%) [Table 5.5]. KoRV-F reached its highest prevalence in Saitama Children's Zoo (80.00%), followed by Kanazawa Zoo (75.00%), Kobe Oji Zoo (62.50%), Tama Zoological Park (50.00%) and Nagoya Higashiyama Zoo (50.00%) [Table 5.5].

Table 5.5. Prevalence (%) of KoRV subtypes in different Japanese zoos

Name of zoos	Prevalence (%) of KoRV subtypes					
	KoRV-A	KoRV-B	KoRV-C	KoRV-D	KoRV-E	KoRV-F
Kanazawa Zoo (n=4)	100	100	25	50	ND	75
Tama Zoological Park (n=2)	100	100	50	100	ND	50
Kobe Oji Zoo (n=8)	100	87.5	12.5	62.5	ND	62.5
Awaji Farm England Hill Zoo (n=4)	100	75	0	50	ND	25
Hirakawa Zoological Park (n-=4)	100	100%	25	100	ND	25
Saitama Children`s Zoo (n=5)	100	100	80	100	ND	80
Nagoya Higashiyama Zoo (n=10)	100	100	20	50	ND	50
Overall; n=37	100	94.59	27.03	67.57	ND	54.05

5.4.4. Proviral and viral loads in koalas

KoRV proviral DNA and KoRV RNA copy numbers in cells were measured using real-time PCR targeting *pol* gene and normalized against koala β-actin.

KoRV proviral DNA copy numbers did not significantly differ between six of the seven zoos, with the highest median proviral load at Saitama Children's Zoo (4508.2 copies/10^3 koala β-actin copies), followed by Nagoya Higashiyama Zoo (4261.4) and Kanazawa Zoo (4049.4). The only zoo showing a significant difference was the Awaji Farm England Hill Zoo (6.65 copies/10^3 koala β-actin copies, low; $p<0.0001$) [Figure 5.1A].

In PBMC analyses, KoRV RNA copy numbers did not significantly differ between six of the seven zoos, with the highest median total RNA load at Hirakawa Zoological Park (1089.5 copies/10^3 koala β-actin copies), followed by Kanazawa Zoo (59.8 copies/10^3 koala β-actin copies) and Saitama Children's Zoo (55.7 copies/10^3 koala β-actin copies). The only zoo showing a significant difference was the Awaji Farm England Hill Zoo (0, low; $p<0.001$) [Figure 5.1B].

In plasma analyses, KoRV RNA copy numbers did not vary significantly between zoos. The highest median viral load was found at Nagoya Higashiyama Zoo (36646.5 copies/ml plasma), followed by Saitama Children`s Zoo (7911.4 copies/ml plasma) and Hirakawa Zoological Park (3152.61 copies/ ml plasma), and the lowest median viral load was found in Awaji Farm England Hill Zoo (94.76 copies/ ml plasma) [Figure 5.1C].

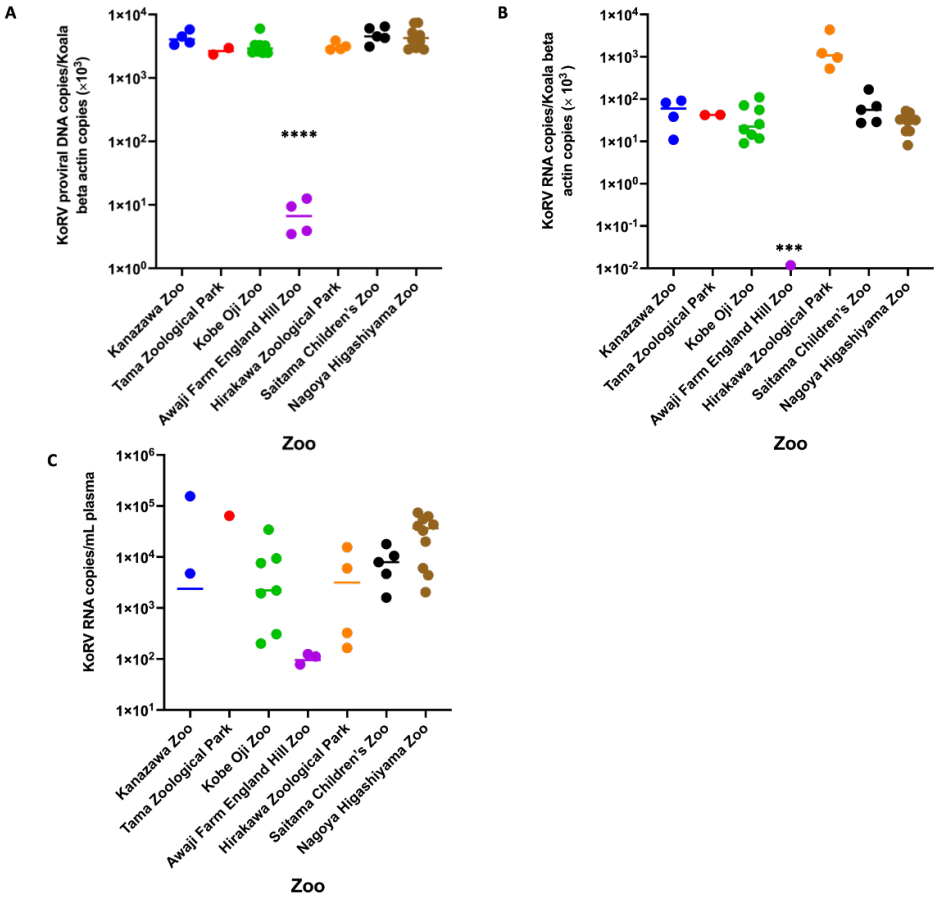


Figure 5.1. Quantitation of KoRV (A) provirus, (B) KoRV RNA in koala PBMCs and (C) viral RNA in plasma from different zoos. The KoRV provirus and RNA copy numbers were measured by real-time qPCR and RT-qPCR, respectively and normalized with the corresponding koala β-actin copies. All resultant copy numbers have been log10 transformed. Each dot denotes an individual's KoRV load with the median line displayed. The Y-axis denotes individual koala values (log transformed) for (A) KoRV proviral DNA copies/10^3 β-actin copies (B) KoRV RNA copies/10^3 β-actin copies and (C) KoRV RNA copies/ml plasma. The X-axis denotes the holding institution (zoo) of the koala population. Statistical significance was evaluated using one-way ANOVA test and is indicated by asterisks (***$p < 0.001$ and ****$p < 0.0001$).

5.4.5. Viral expression

To determine viral expression in whole blood and plasma, I analyzed cell and plasma RNA expression. No significant relationship was observed between whole blood and plasma values (Figure 5.2).

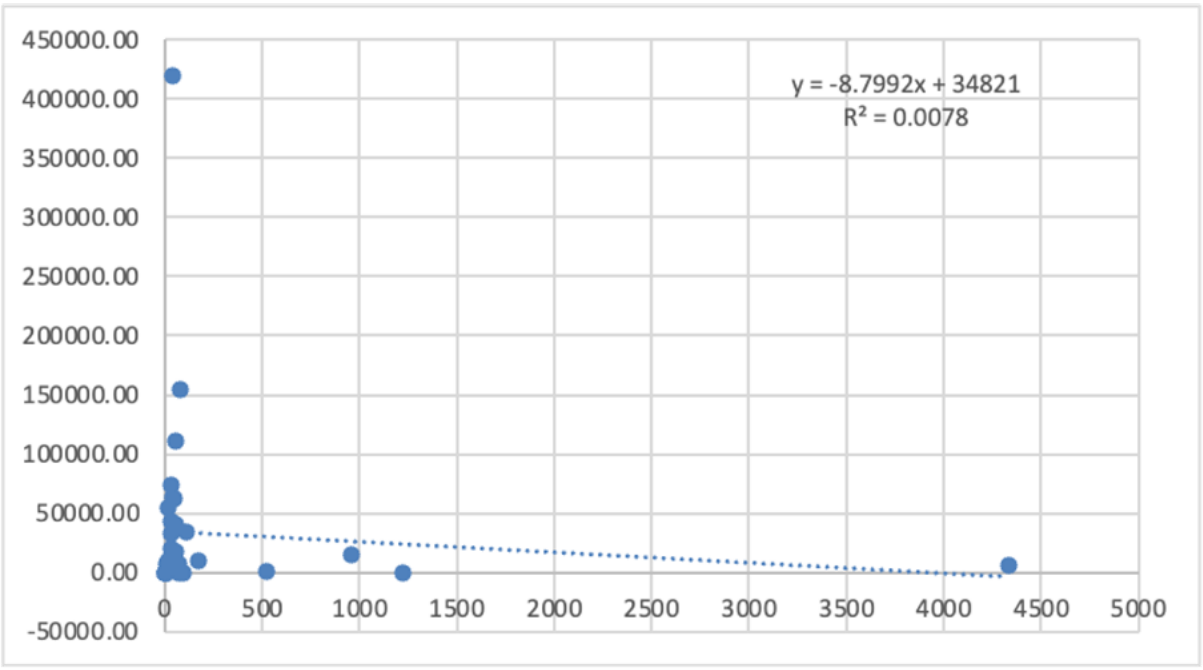


Figure 5.2. Correlation between pol gene whole blood and plasma. X-axis shows individual koala values (log transformed) of cell RNA copies/10^3 β-actin copies; Y-axis plasma RNA copies/ml plasma of those koalas.

5.4.6. Associations with demographic and clinical characteristics

To analyze possible factors in KoRV infection status, I investigated associations between demographic and clinical characteristics (sex, age, and body condition score) and KoRV proviral DNA and viral RNA loads (in plasma and PBMCs).

No significant sex difference was noted in proviral DNA load (Figure 5.3A), or KoRV RNA loads in PBMCs (Figure 5.3B) or plasma (Figure 5.3C).

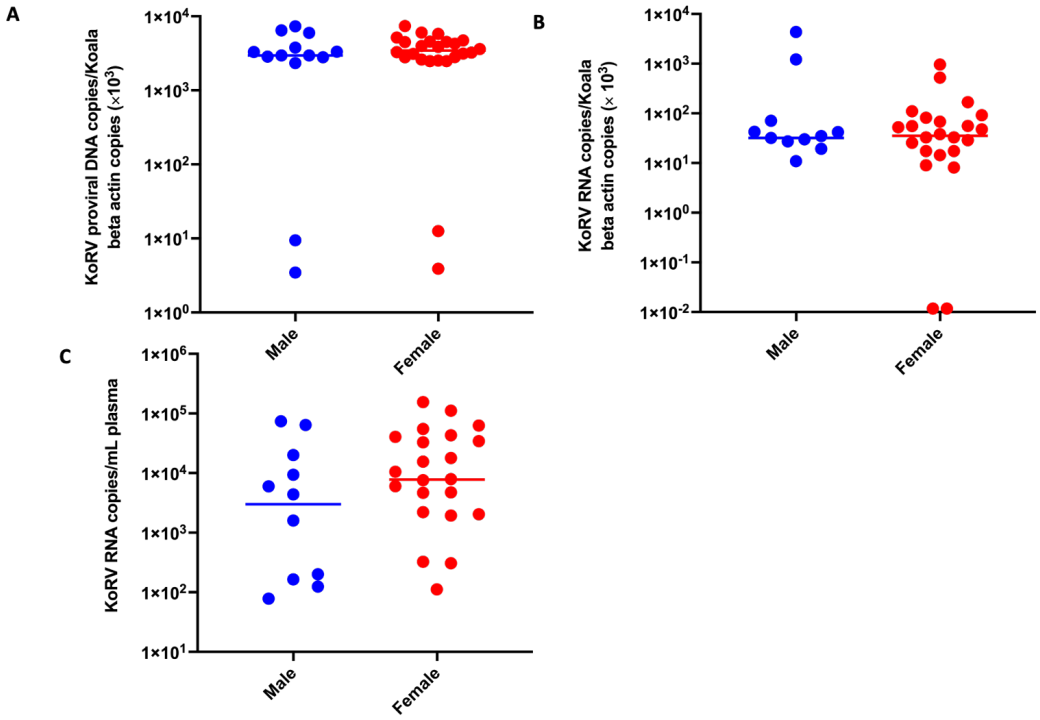


Figure 5.3. Comparison of (A) KoRV proviral DNA, (B) KoRV RNA and (C) viral RNA load between male and female koalas, evaluated using a non-parametric Mann-Whitney U test. All resultant copy numbers have been log10 transformed. Each dot denotes an individual's KoRV load with the median line displayed. The Y-axis denotes individual koala values (log transformed) for (A) KoRV proviral DNA copies/10^3 β-actin copies, (B) KoRV RNA copies/10^3 β-actin copies and (C) KoRV RNA copies/ml plasma. The X-axis denotes sex.

No significant age difference was noted in proviral DNA load (Figure 5.4A), but the youngest age cohorts (joeys and juveniles) showed a significantly higher KoRV RNA viral load ($P < 0.05$) in PBMCs (Figure 5.4B). No significant age difference was observed in plasma viral RNA (Figure 5.4C).

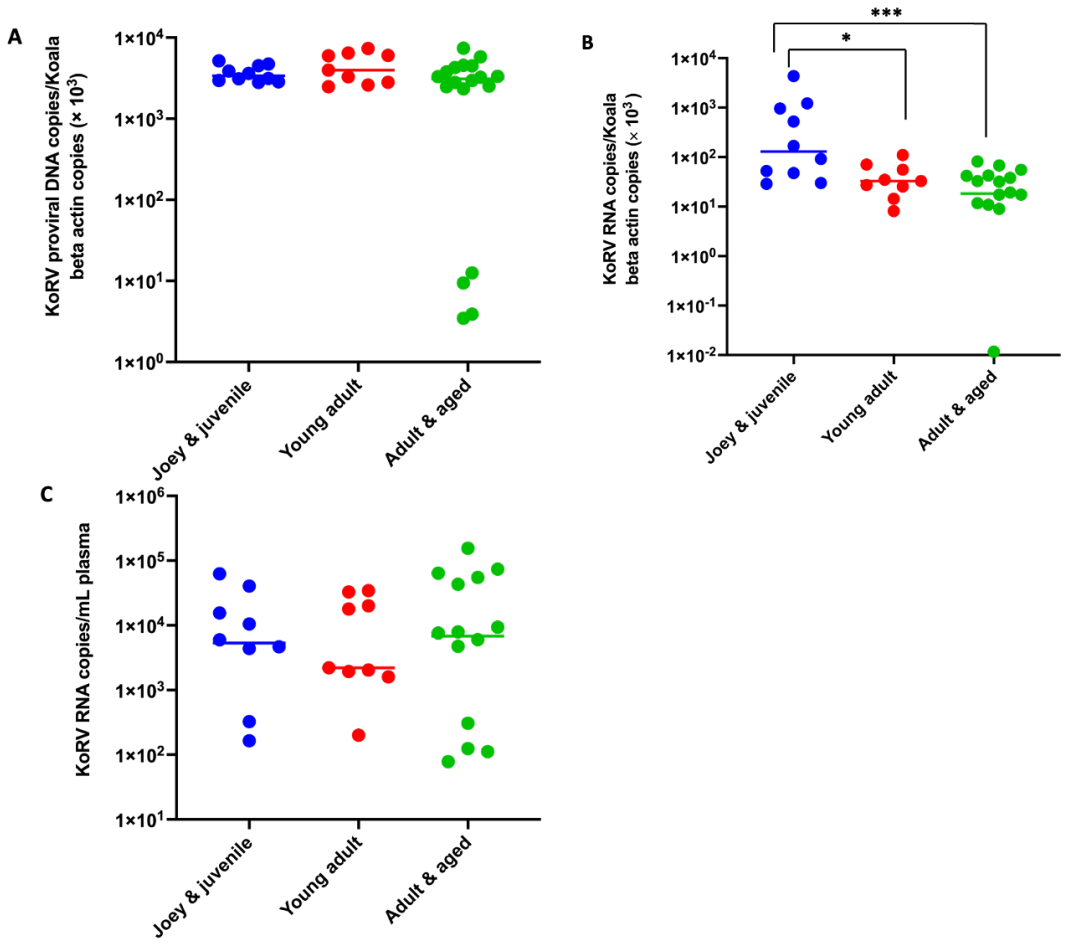


Figure 5.4. Comparison of (A) KoRV proviral DNA, (B) KoRV RNA and (C) viral RNA load between koala age cohorts. All resultant copy numbers have been log10 transformed. Each dot denotes an individual's KoRV load with the median line displayed. The Y-axis denotes individual koala values (log transformed) for (A) KoRV DNA copies/10^3 β-actin copies, (B) KoRV RNA copies/10^3 β-actin copies and (C) KoRV RNA copies/ml plasma. The X-axis denotes age. Statistical significance was evaluated using one way ANOVA and a non-parametric Mann-Whitney U test, and indicated by asterisks (*$p < 0.05$ and ***$p < 0.001$).

In analyses targeting body condition score, no significant difference was noted in proviral DNA load (Figure 5.5A) or KoRV RNA loads in PBMCs (Figure 5.5B) or plasma (Figure 5.5C).

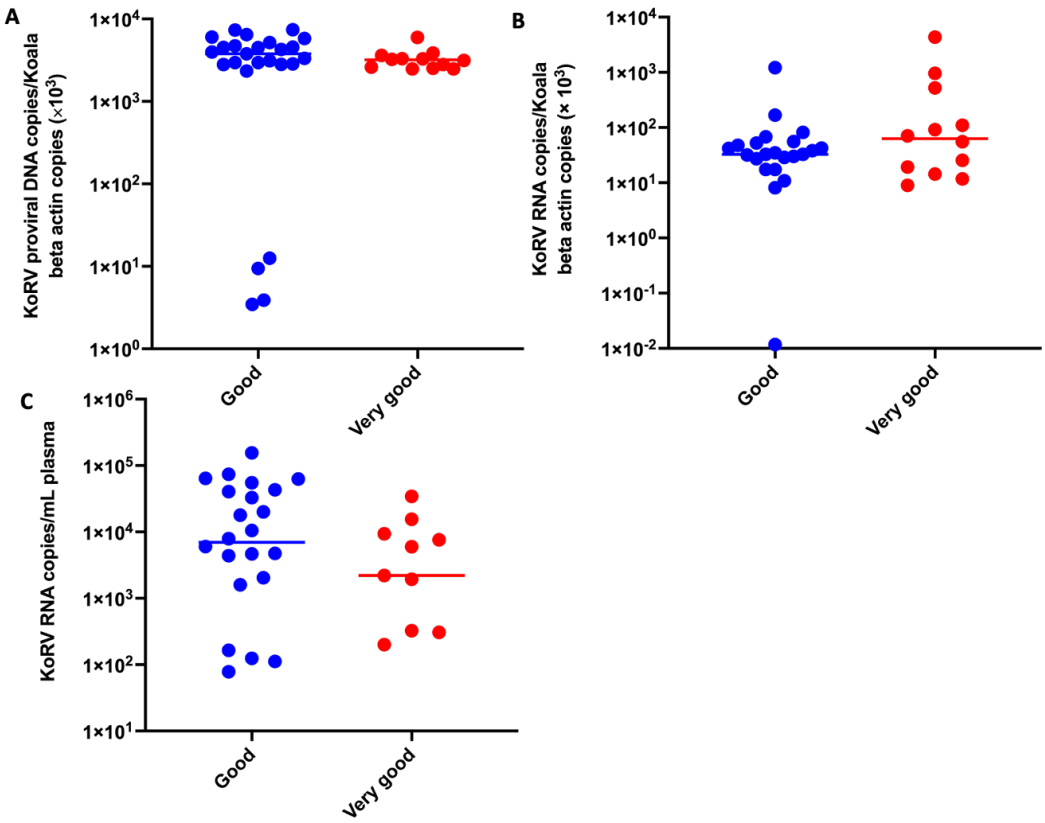


Figure 5.5. Comparison of (A) KoRV proviral DNA, (B) KoRV RNA and (C) viral RNA load by body condition score for koalas. All resultant copy numbers have been log10 transformed. Each dot denotes an individual's KoRV load with the median line displayed. The Y-axis denotes individual koala values (log transformed) for (A) KoRV proviral DNA copies/10^3 β-actin copies, (B) KoRV RNA copies/10^3 β-actin copies and (C) KoRV RNA copies/ml plasma. The X-axis denotes body condition score (all were "good" or "very good").

5.5. Discussion

To the best of my knowledge, this cross-sectional molecular epidemiology study is the first report on KoRV infection status and subtype prevalence across the captive koala population in Japanese zoos. I used a large suite of PCR assays to target gDNA and viral RNA in plasma and PBMCs for detection, performed KoRV subtyping, and investigated the associations between proviral DNA and viral or cell RNA loads and demographic and clinical parameters.

My study has established zoo-dwelling koalas in Japan as a well-profiled population for KoRV status. All seven zoos exhibiting koalas in Japan were covered by this study, and I collected data on 80.43% of the known national koala population (37/46) during the study period. This profiling of the Japanese captive population follows similar data collection for comparing American and Australian captive populations [Zheng et al., 2020] the latter study involved fewer zoos, with two zoos in Australia and one in the United States, but a larger number of animals, owing to the smaller total number of zoo-dwelling koalas in Japan. Zheng et al. showed a significant association between KoRV infection and plasma viral loads in multiple subtypes of KoRV in koalas with leukemia/lymphoma and other cancers [Zheng et al., 2020]. This report thus represents a comprehensive epidemiological snapshot of the captive koala population in Japanese zoos. This snapshot is particularly useful as koalas can be transferred between Japanese zoos for exhibition or breeding, through a program under the auspices of the Japanese Association of Zoos and Aquariums, in which all seven zoos participate.

I investigated differences in KoRV proviral DNA, and cellular and plasma viral RNA in koalas dwelling at different Japanese zoos. Similar to several previous studies [Tarlinton et al., 2005; Simmons et al., 2012; Kayesh et al., 2019; Sarker et al., 2020], all koalas (100%) were positive for KoRV proviral DNA. However, proviral loads showed wide inter-individual variation and a significant ($p<0.0001$) inter-institutional difference in the Awaji Farm England Hill Zoo. This study showed varied amounts of normalized proviral copies in different zoo populations. The number of proviral copies in six (except Awaji Farm England Hill Zoo) of seven zoos were 2337.91 to 7400.43 copies/10^3 koala β-actin copies. However, the Awaji Farm England Hill Zoo showed significantly fewer proviral copies (6.65 copies/10^3 koala β-actin copies, low; $p<0.0001$) [Figure 5.1A]. This decrease in proviral copies could be partly

explained by the history of the origin of these koalas, as they were obtained from Victorian koala populations. KoRV prevalence in southern koala populations is comparatively lower than that in northern koala populations [Tarlinton et al., 2006; Sarker et al., 2020; Michaela et al., 2021; Stephenson et al., 2021]. My findings on proviral loads are also similar to those of previous studies [Tarlinton et al., 2005; Sarker et al., 2020; Hashem et al., 2021]. Notably, I observed a significant ($p<0.001$) variation in viral RNA loads in PBMCs, which might be linked to spontaneous mutation [Combe and Sanjuán, 2014], genetic divergence, or factors involved in the isolation of RNA viruses [Jenkins et al., 2002]. I targeted measurements of viral RNA in this study because such viral loads have been suggested to indicate stage and progression of retroviral diseases in other species, specifically HIV in humans [Piatak et al., 1993] and FIV in cats [Diehl et al., 1996]. In my study, there were no significant variations of viral RNA copy numbers in plasma, suggesting a lower viral replication.

In terms of exogenous subtypes, my study presents an interesting degree of contrast to previous reports. I found high subtype prevalence across our study population. Of particular note was the prevalence of 94.59% for KoRV-B (100% at five of seven zoos evaluated). This is markedly higher than previously reported figures of 10.3% for a US zoo and 59.3% for two Australian zoos [Zheng et al., 2020], 84.4% for two Southeast Queensland colonies (Joyce et al., 2021), and 60% for three Japanese zoos (Hashem et al., 2019), and even the range of figures (24% to 48.55%) in wild Northern Australian populations [Quigley et al., 2018; Sarker et al., 2019]. The Japanese population is largely of North Australian origin (Queensland), where KoRV exogenous subtypes are reportedly more widespread [Kayesh et al., 2020], although some southern lineages are present to a smaller extent. The Japanese koala population is relatively recently established, with the older koalas in this study corresponding to an F0 generation captured from the wild in Australia, and the adult and juvenile koalas corresponding

to respective F1 and F2 generations born in captivity. Aside from KoRV-B, the respective prevalence of KoRV-C, KoRV-D and KoRV-F were 27.03%, 67.57%, and 54.05%. KoRV-C has been previously reported in Japanese zoos [Hashem et al., 2020; 2021], and recently at 0.9% in two Southeast Queensland colonies [Joyce et al., 2021] but subtypes D and F have never previously been reported in Japanese captive koalas [Xu et al., 2015]. A recent study reported the respective prevalences of KoRV-D and KoRV-F were 95.4% and 7.3% in two Southeast Queensland colonies [Joyce et al., 2021]. Interestingly, localized patterns of infection have been reported within Queensland for the exogenous subtypes. For example, KoRV-D and KoRV-F appear to show higher prevalences in different parts of northern and southern Queensland [Quigley et al., 2021a], yet they each showed a prevalence exceeding 50% in this study population (for which southern Queensland origins are the most common).

The higher subtype prevalences in Japanese koalas suggest that this population is ideal for further research into KoRV with a focus on the various subtypes and co-infection. Such research is crucial for conservationists, as exogenous infections spread rapidly through wild populations.

Exogenous subtypes have been suggested to be specifically linked to immunological conditions and heightened susceptibility to infectious diseases [Kayesh et al., 2021]. Therefore, future studies should assess the possibility of co-infection with *Chlamydia pecorum* in all healthy koalas characterized in this study [Hashem et al., in preparation]. These determinations were made by zoo veterinarians based on body condition assessments that showed no signs of emaciation, WBC counts within the normal range [Canfield et al., 1989], and the general absence of abnormal clinical signs. It has previously been suggested that KoRV infection in itself may not trigger clinical disease manifestations [Waugh et al., 2017], and that other stress

factors such as habitat loss and attacks by dogs may be implicated in aggravating roles [McCallum et al., 2018]. Our captive population has a secure habitat without intraspecific threats, and further studies on KoRV in this population may throw light on the role of environmental stress in clinical manifestations of disease. With no comparable data previously collected across Japanese zoos and no signs of clinical disease, my RNA results can only represent benchmarking data. However, these data will be useful and I suggest that future longitudinal studies in this population will be highly beneficial.

Japanese zoo-dwelling koalas appear to be a relatively homogenous population in terms of KoRV infection profiles. Age was the only demographic factor that showed any significant difference in key parameters; with juvenile kolas (defined roughly as joeys to adolescent koalas) showing significantly higher viral RNA loads in PBMCs (but not plasma) than older age cohorts (young adults or mature adults). This is in line with a previously reported association between age and viral load [Tarlinton et al., 2005]. However, most of koalas in this study were mature adults who had produced offspring. Therefore, I consider this finding is suggestive of widespread vertical (mother-to-offspring) KoRV transmission in the study population. I found no other significant differences in KoRV profile by clinical or demographic characteristics, and I found no significant differences in proviral or viral RNA loads between sex or the seven zoos; the institutional outlier was Awaji Farm England Hill Zoo. That zoo houses only a small number of koalas (four), all of which are mature adults, and the absence of juveniles may explain the low viral loads at this zoo. Notably, the koalas of the Awaji Farm England Hill Zoo were obtained from the southern region of Australia, and as mentioned earlier in the text, the prevalence was lower than that in the northern koala populations. Overall, given the homologous nature of KoRV in captive koalas of Japanese zoos, I regard this population as highly useful for future research, including longitudinal epidemiological studies.

This study has a couple of limitations. The issue of sire/dam-to-joey transmission was not addressed, although some koalas were offspring of others in this study population. My health assessment was limited to body condition score and WBC count. Accordingly, I propose further investigations into the molecular dynamics and transmission of KoRV in this population.

This study provides the overall prevalence of KoRV, viral expression, and several associated factors in captive koalas in Japanese zoos. In addition, the prevalence of multiple KoRV subtypes and their distribution in different Japanese zoos have been determined. The exogenous subtype of KoRV, notably KoRV-B, which is a more pathogenic subtype, was widespread in Japanese zoo koalas. In conclusion, this study highlights the potential risk factors for KoRV infection in captive koala populations. It enhances my current understanding of KoRV prevalence and its subtypes in Japanese zoo koalas and the risk factors associated with koala health, which could be valuable to improving the health and maintenance of zoo-dwelling koalas.

Chapter 6

Transmission of koala retrovirus from parent koalas to a joey in a Japanese zoo

6.1. Abstract

Koala retrovirus (KoRV) is of an interest to virologists due to its currently active endogenization into the koala (*Phascolarctos cinereus*) genome. Although KoRV has frequently been isolated in wild and captive koala populations, its pathogenesis and transmission remain to be fully characterized, and most previous research has concentrated on adult koalas rather than on joeys. Here, I characterized KoRV isolates obtained from a deceased male joey and its parents (animals reared in a Japanese zoo) to investigate KoRV transmission mode and pathogenesis. I sequenced the KoRV long terminal repeat (*LTR*) and envelope genes isolated from the joey and its parents and found KoRV-A and KoRV-C in genomic DNA from both the parents and the joey. Notably, both parents were also positive for KoRV-B, whereas the joey was KoRV-B negative, further confirming that KoRV-B is an exogenous strain. The KoRV *LTR* sequence of the joey was considerably closer to that of its sire than its dam. For further characterization, total KoRV, KoRV-A, KoRV-B, and KoRV-C proviral loads were quantified in peripheral blood mononuclear cells from the parents and in blood samples from the joey. Total KoRV, KoRV-A, and KoRV-C proviral loads were also quantified for different tissues (bone, liver, kidney, lung, spleen, heart, and muscle) from the joey, revealing differences suggestive of a distinct tissue tropism (highest total KoRV proviral load in the spleen and lowest in bone). The amount of KoRV-C in the parents was less than that in the joey. My findings contribute to an improved understanding of KoRV pathogenesis and transmission mode and highlight useful areas for future research.

KoRV is unique among retroviruses in that one strain (KoRV-A) is undergoing endogenization, whereas the other main subtype (KoRV-B) and another subtype (KoRV-C) are reportedly exogenous strains. Its transmission and pathogenesis are of interest in the study of retroviruses and are crucial for any conservation strategy geared toward koala health. This study provides new evidence on the modes of KoRV transmission from parent koalas to their joey. I found vertical transmission of KoRV-A, confirming its endogenization, but with closer conservation between the joey and its sire than its dam (previous reports on joeys are rare but have postulated dam-to-joey vertical transmission). This is also the first report of a KoRV-B-negative joey from KoRV-B-positive parents, contrasting with the few previous reports of 100% transmission of KoRV-B from dams to joeys. Thus, the results in this study give some novel insights for the transmission mode of KoRV.

6.2 Introduction

Koala retrovirus (KoRV) is from the genus Gammaretrovirus in the family *Retroviridae*. KoRV is endemic in both wild and captive populations, and it represents a major threat to koala health [Quigley et al., 2018; Miyazawa et al., 2011; Shojima et al., 2013; Xu et al., 2013; Fiebig et al., 2016; Kayesh et al., 2019]. KoRV is of particular interest to virologists because it exists in both endogenous and exogenous forms. Furthermore, compared with other retroviruses that have been endogenized into mammalian genomes for millions of years, the endogenization of KoRV started relatively recently (around 22,000 to 49,000 years ago) and is apparently still progressing, considering that some regional wild populations in Australia reportedly still do not show 100% prevalence [Ishida et al., 2015; Simmons et al., 2012].

A total of nine KoRV subtypes (KoRV-A to -I) have been identified up to the present [Quigley et al., 2018], among which KoRV-A and KoRV-B are regarded as the major subtypes. KoRV-A is the endogenous subtype; it is found in wild populations, with prevalences ranging from 27% to 100% [Simmons et al., 2012], and it is also endemic in zoo populations. Conversely, KoRV-B is an exogenous strain; it is widespread in both wild and captive populations and is regarded as a major threat to koala health through its pathogenic association with disease progression [Quigley et al., 2018; Xu et al., 2013; Fiebig et al., 2016; Waugh et al., 2017]. Furthermore, KoRV-B appears to be undergoing mutation and is spreading rapidly [Quigley et al., 2018].

The mode of transmission has been investigated for both subtypes. As with other endogenous retroviruses, KoRV-A is integrated into the germ line of the host and transmitted vertically to offspring. Reportedly, this vertical transmission apparently occurs between dams and their offspring [Quigley et al., 2018; Tarlinton et al., 2006]. In contrast, KoRV-B is transmitted horizontally; an annual transmission rate of 3% has been reported for adult-to-adult koala contact per year, as opposed to a 100% transmission rate for dams to joeys [Quigley et al., 2018; Tarlinton et al., 2006]. However, much work remains to be done to fully characterize KoRV and its transmission. In particular, reports on KoRV status in joeys are scarce.

KoRV infection dynamics has been reported in a previous study [Kayesh et al., 2019], and in another study, I found 100% prevalence for KoRV-A and 60% for KoRV-B at three Japanese zoos [Hashem et al., 2019]. An interesting finding in the latter study [Hashem et al., 2019] suggested that sire-to-offspring KoRV-B transmission had occurred in one case, which is not consistent with the findings of Quigley et al. [Quigley et al., 2018] and Xu et al. [Xu et

al., 2013]; this suggests the need for further research on transmission to offspring and the association between health impact and KoRV subtype and KoRV pathogenesis in joeys.

The koala population at Hirakawa Zoo in Kagoshima, Japan, was among those targeted in our previous KoRV research. Staff at the zoo recently found a 6-month-old joey which had died and been ejected from its dam's pouch. The KoRV statuses of the deceased joey's sire and dam (both KoRV-A and KoRV-B positive) were known from our previous studies. Further examination of the parents and the deceased joey represented an opportunity to expand my knowledge on the disease and its transmission between parents and offspring. Accordingly, in this study, I aimed to improve the understanding of KoRV transmission mode and pathogenesis in joeys by investigating the KoRV status of the parents and their joey and by characterizing KoRV in blood and tissues from the joey.

6.3. Materials and Methods

6.3.1. Animals

The joey was a 6-month-old male found dead and ejected from its dam's pouch by staff at the Hirakawa Zoological Park in Japan (Fig. 1A); its cadaver weighed about 450 g. This was the first joey born to this dam. Its sire and dam, identified as KoRV-A and KoRV-B positive in our previous study, were 5 and 3 years old, respectively, at the time of the joey's death. The dam and sire were born in zoos in Australia and Japan, respectively, and the dam had been reared at the Hirakawa Zoo for 1 year prior to the death of the joey.

6.3.2. Sample collection

EDTA-treated whole-blood samples were collected by venipuncture from two the adult koalas (sire and dam) housed at the Hirakawa Zoological Park in Kagoshima, Japan, in accordance with the protocols of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University. Tissue samples (bone, liver, kidney, lungs, heart, spleen, and muscle) were collected at necropsy from the dead joey at Hirakawa Zoological Park and stored at −80°C for further characterization. I collected whole-blood samples with heparin or EDTA by venipuncture from 2 koalas (parents) and tissue samples (clotted blood, bone, liver, spleen, lung, kidney, heart, and muscle) from their dead joey at Hirakawa Zoological Park, in accordance with the Institutional Animal Care and Use Committee protocols.

6.3.3. Hematological examination

To determine the health status of the parent koalas, a hematological panel was obtained with standard protocols, and this panel included white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

6.3.4. Preparation of plasma

Whole-blood samples (EDTA treated) were centrifuged at 3,000 rpm and 20°C for 5 min to obtain plasma samples, which were then stored at −80°C. To measure viral plasma load, viral

RNA was isolated from plasma using an Isogen-LS kit (Nippon Gene, Japan), according to the manufacturer's instructions. To remove any contaminating DNA, extracted RNA was treated with RQ1 RNase-free DNase (Promega), according to the manufacturer's instructions.

Isolation of genomic DNA and viral RNA.

Genomic DNA (gDNA) was isolated from EDTA-treated whole-blood samples using a Wizard genomic DNA purification kit (Promega), according to the manufacturer's instructions. gDNA was isolated from frozen tissue samples using a phenol-chloroform extraction method. The extracted gDNA was used for further characterization.

PCR, cloning, and sequencing.

PCR was performed using gDNA as a template to amplify the envelope genes of KoRV-A, -B, and -C with genotype-specific primer sets (Table 6.1), as described previously [Xu et al., 2013; Waugh et al., 2017]. After the end of the PCR run, the products were further incubated at 72°C for 10 min with LA-Taq polymerase. The resulting target PCR fragments were subcloned into the pCR2.1 TOPO vector (Invitrogen) and sequenced.

Table 6.1. Primers used for detection of corresponding KoRV

Target gene	Sequences	References
Pol (KoRV)	Forward: 5′-TTGGAGGAGGAATACCGATTACAC -3′ Reverse: 5′--GCCAGTCCCATACCT GCCTT-3′	Kayesh et al., 2019
β-actin	Forward: 5′-AGATCATTGCCCCACCT-3′ Reverse: 5′-TGGAAGGCCCAGATTC-3′	Kayesh et al., 2019
***Env* (KoRV-A)**	Universal forward: 5′-TCCTGGGAACTGGAAAAGAC-3′ Reverse: 5′-GGG TTC CCCAAG TGATCT G-3′	Hashem et al., 2019
***Env* (KoRV-B)**	Universal forward: 5′-TCCTGGGAACTGGAAAAGAC-3′ Reverse: 5′-GGCGCAGACTGTTGAGATTC-3′	Xu et al., 2013; Hashem et al., 2019

	OR Forward: 5′-CGGTGAAGGTTGACGGTATT-3′ Reverse: 5′-ACCCCAAGGTTCCATAGCTC-3′	
***Env* (KoRV-C)**	Universal forward: 5′-TCCTGGGAACTGGAAAAGAC-3′ Reverse: 5′-AAGGCTGGTCCCGCGAAGGT-3′	Genbank KP792564.1
LTR	U3Forward:5′-AATGAAGGAGGCAGAAATCATGAGGC-3′ Gag 1A Reverse: 5′-TTCCAGTGATCTAGTGTAAG-3′ U5Reverse: 5′-ATGAAAGACCCCAATGTTCG-3′	GenBank NC 039228.1

A nested PCR was performed to determine the nucleotide sequence of the long terminal repeat (*LTR*) for KoRV-A and the env region of KoRV-C, using gDNA as the template. The sequence data of KoRV-C env were submitted to GenBank (accession no. MT134110). Tissue was isolated from koala blood or tissue samples using the primer sets in Table 6.1, as described previously [Miyazawa et al., 2011; Hanger et al., 2000]. The cyclic conditions for the first PCR were as follows: initial denaturation at 98°C for 2 min, denaturation at 98°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 1 min, followed by 39 cycles, and final extension at 72°C for 5 min. The second PCR was performed using the product of the first PCR as the template, with annealing at 66°C. The resulting PCR fragments were then subcloned into the pCR2.1 TOPO vector and sequenced.

Multiple-sequence alignment.

The KoRV-A envelope gene and KoRV LTRs were isolated from the parent koalas and the joey koala and sequenced. The multiple-sequence alignment of the KoRV *env* gene nucleotides and *LTR* nucleotides was performed using the MEGA7 software [Kumar et al., 2016]**.**

6.3.5. Phylogenetic analysis

For phylogenetic analysis of KoRV *LTRs*, I used KoRV *LTR* sequences obtained from my study. The phylogenetic tree was constructed on the basis of the neighbor-joining method [Saitou and Nei, 1987], and evolutionary distances were computed using the p-distance method [Takahashi and Nei, 2000]. Evolutionary analyses were conducted using MEGA7 software [Kumar et al., 2016]

6.3.6. Real-time PCR

Isolated gDNA was used as the template to determine the KoRV proviral DNA copy number by real-time PCR, as described by Kayesh et al. [Kayesh et al., 2019], using primer sets (Table 6.1) with Brilliant-III Ultra-Fast SYBR green qPCR master mix (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Amplification and detection were carried out using a CFX Connect real-time PCR detection system (Bio-Rad, USA). The specificity of the PCR was confirmed by melt curve analysis. Standards were generated from prequantified plasmids containing the sequence of the target gene. Koala β-actin was used as an endogenous control for normalization of the KoRV proviral DNA copy numbers. The primer sets used for the detection of koala β-actin are shown in Table 6.1.

Isolated gDNA was also used for the determination of KoRV-A, KoRV-B, and KoRV-C proviral DNA copy numbers with real-time PCR using genotype-specific primer sets (Table 6.1) targeting the env genes of KoRV-A, KoRV-B, and KoRV-C, using reaction conditions as described previously [Hashem et al., 2019]. However, in the case of KoRV-B copy number determination, the reaction conditions were slightly modified, as follows: the initial

denaturation at 95°C was for 3 min, and this was followed by 40 cycles at 95°C for 5 s and 55°C for 10 s. PCR specificity was confirmed by melt curve analysis. Standards were prepared from pre-quantified plasmids containing the target gene sequence. The koala β-actin gene was used as an endogenous control for normalization of the KoRV proviral DNA copy numbers.

6.3.7. Plasma viral load determination by real-time PCR

The total KoRV copy numbers in the plasma were determined by real-time PCR, as described previously [Kayesh et al., 2019].

6.4. Results

6.4.1. Hematological examination of parents

The hematology results for the parents are shown in Table 6.2. All blood parameters were within normal ranges, indicating that the parent koalas were generally healthy.

Table 6.2. Hematological data from parent koala

Parameters	Sire	Dam
WBC ($\times 10^2/\mu L$)	48	113
RBC ($\times 10^4/\mu L$)	353	327
HGB (g/dL)	13.9	11.9
PCV (%)	39.4	35.2
MCV(fL)	111.6	107.6
MCH (pg)	39.4	36.4
MCHC (g/dL)	35.3	33.8
PLT ($\times 10^4/\mu L$)	2.9	22.2

WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet.

6.4.2. Postmortem examinations of the joey

The joey was found dead at 6 months of age, and the appearance of its cadaver is shown in Figure 6.1A (height, 29.5 cm; body weight, 483 g). For comparison, a healthy joey (7 months of age) is shown with its mother in Figure 6.1B. Necropsy revealed fluid in the thoracic (12.5 ml; relative density, 1.029 kg/m^3; total protein [TP], 3.4 g/dl; fibrin deposit, +) and peritoneal (10.2 ml; relative density, 1.035 kg/m^3; TP, 4.2 g/dl; fibrin deposit, +) cavities (Figure 6.1C); such findings would not be observed in healthy joeys. Due to the age of the joey and time elapsed since death, the cause of death could not be determined, and tissue specimens of sufficient quality for histopathology could not be obtained.

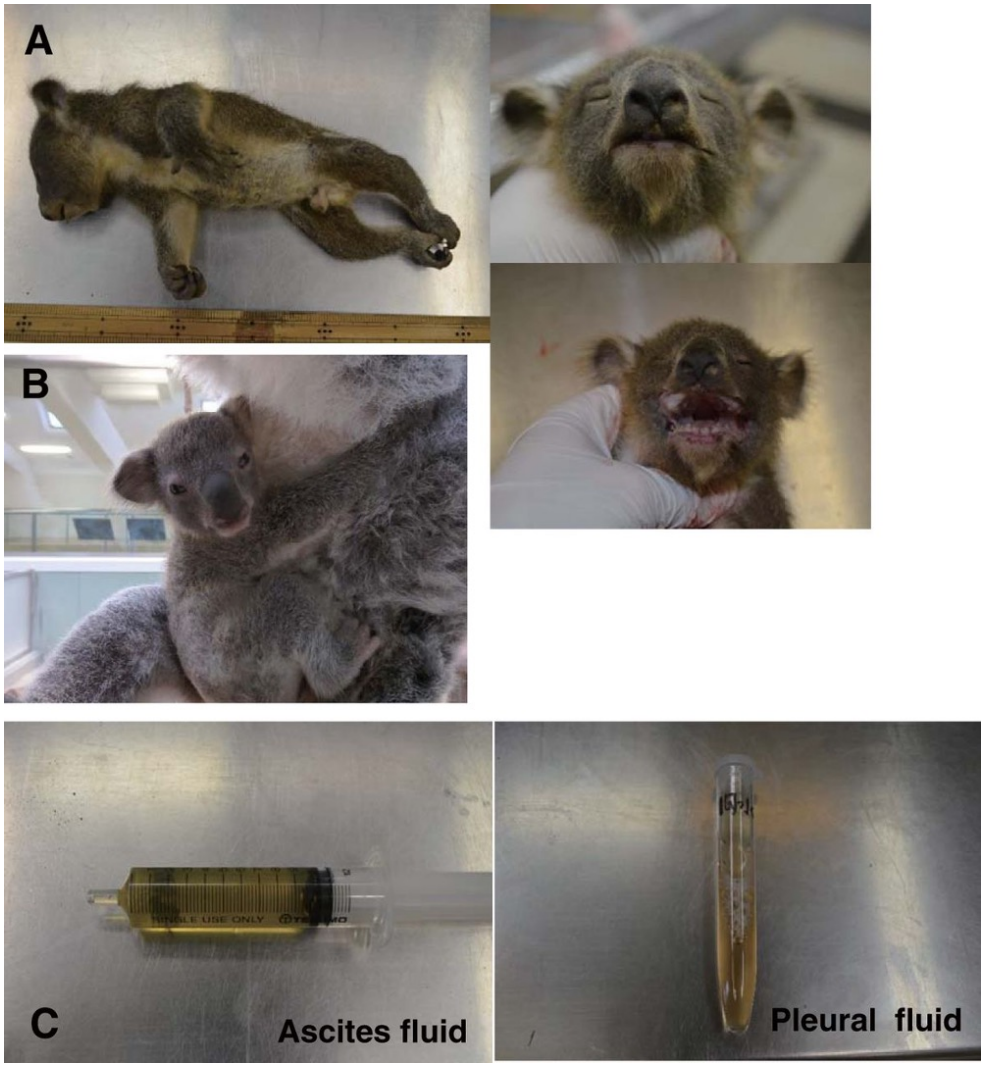
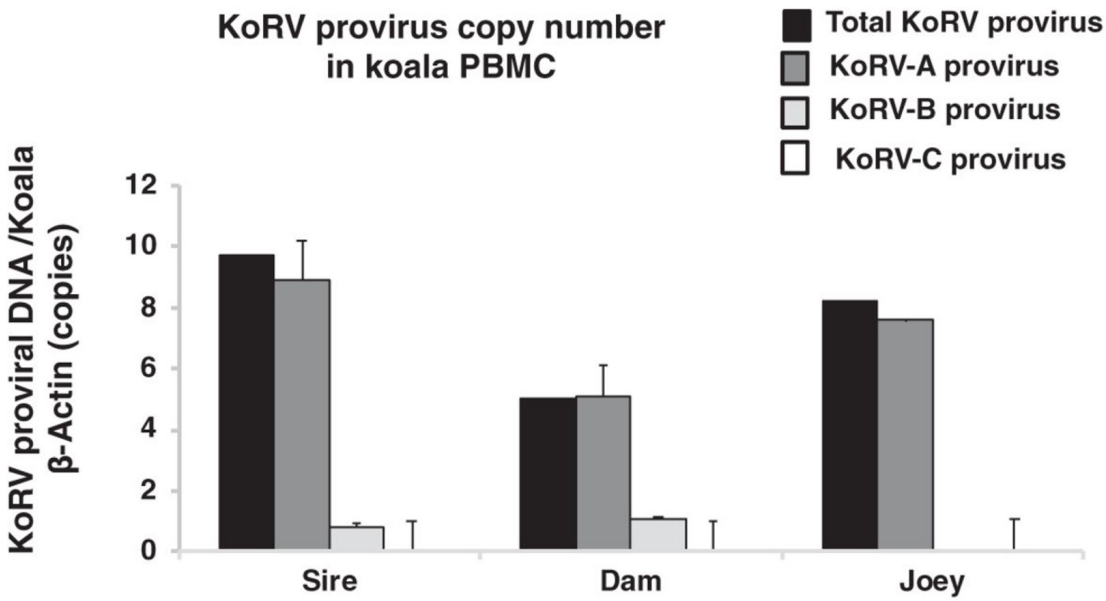


Figure 6.1. Sample (Joey) from Hirakawa Zoological Park. (A) The deceased joey (age, 6 months) and its appearance. (B) A healthy joey (age, 7 months) with its mother. (C) Left, fluid (about 10.2 ml) collected from the peritoneal cavity of the joey at necropsy. Right, pleural fluid collected from the joey's thoracic cavity at necropsy.

6.4.3. Determination of proviral copy numbers in parents and joey

Real-time PCR was performed using genomic DNA (gDNA) isolated from peripheral blood mononuclear cells (PBMCs) for the determination of KoRV proviral copy numbers in the parents and joey, with normalization against koala β-actin. Various proviral copy numbers were found in these koalas (Figure 6.2). I measured total KoRV proviral load as well as KoRV-A, KoRV-B, and KoRV-C proviral loads. The total proviral and KoRV-A copy numbers were

highest in the sire, followed by the joey, and lowest in the dam (Figure 6.2). KoRV-B was detected in both the dam and the sire but not in the joey. Furthermore, relatively small amounts of KoRV-C were detected in the joey and its parents, and the amounts in the sire and the dam were less than that in the joey (Figure 6.2).



Total KoRV provirus	9.7±1.28	5.02±0.95	8.21±0.02
KoRV-A provirus	8.9±0.1	5.12±0.11	7.61±0.003
KoRV-B provirus	0.82±7.5E-05	1.05±6.4E-05	Negative
KoRV-C proviruus	0.00028±0.0001	0.00028±0.0001	0.08±0.0009

Figure 6.2. Normalized KoRV proviral load in genomic DNA isolated from koala PBMCs. Normalized total KoRV, KoRV-A, KoRV-B, and KoRV-C proviral loads in parents and total KoRV, KoRV-A, KoRV-B, and KoRV-C proviral loads in samples from the parents (sire and dam) and joey are shown. Provirus copy numbers were quantified by real-time PCR and normalized against corresponding koala β-actin in PBMCs.

To investigate the tissue tropism of KoRV in the joey, the following organs/tissues were collected at necropsy (Figure 6.3A): heart (Figure 6.3B), lung (Figure 6.3C), spleen (Figure 6.3D), liver (Figure 6.3E), kidney (Figure 6.3F), and intestines (Figure 6.3G). Using real-time PCR, KoRV proviral copy numbers were determined in gDNA isolated from these samples.

The highest total proviral copy numbers were observed in the spleen and the lowest in bone (Figure 6.4). Contrastingly, the highest KoRV-A proviral DNA copy number was found in the liver and was the lowest in the heart. KoRV-C proviral load showed a pattern similar to that of KoRV-A, with the highest load in the liver and the lowest load in the muscle (Figure 6.4).

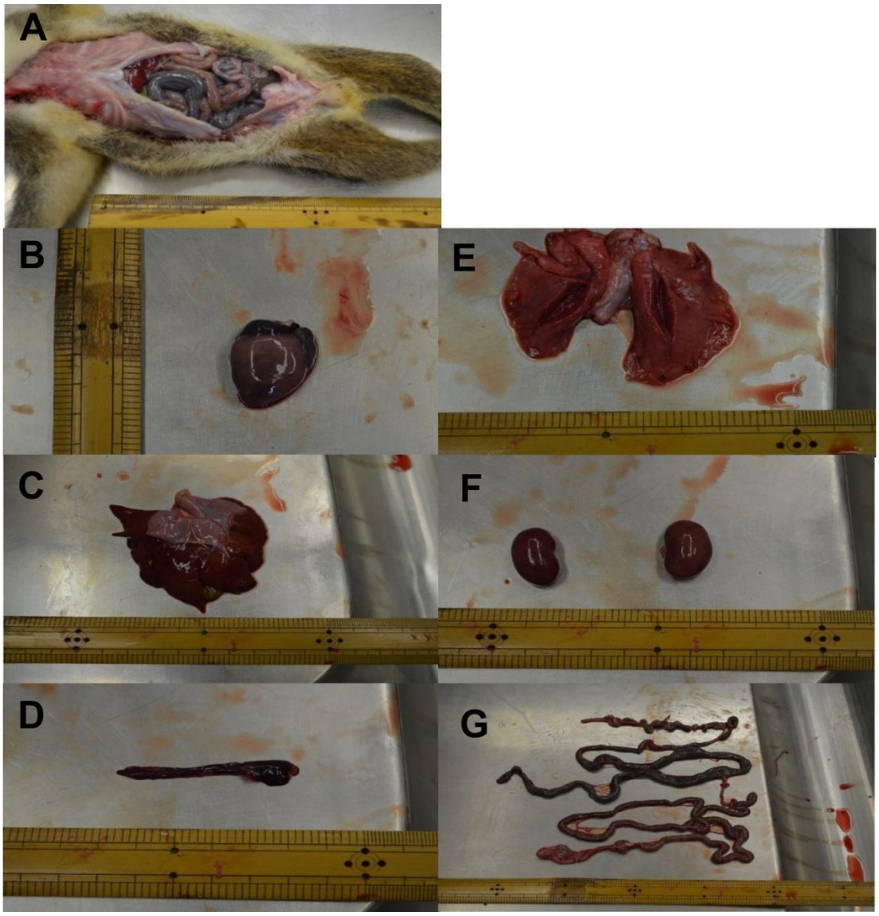
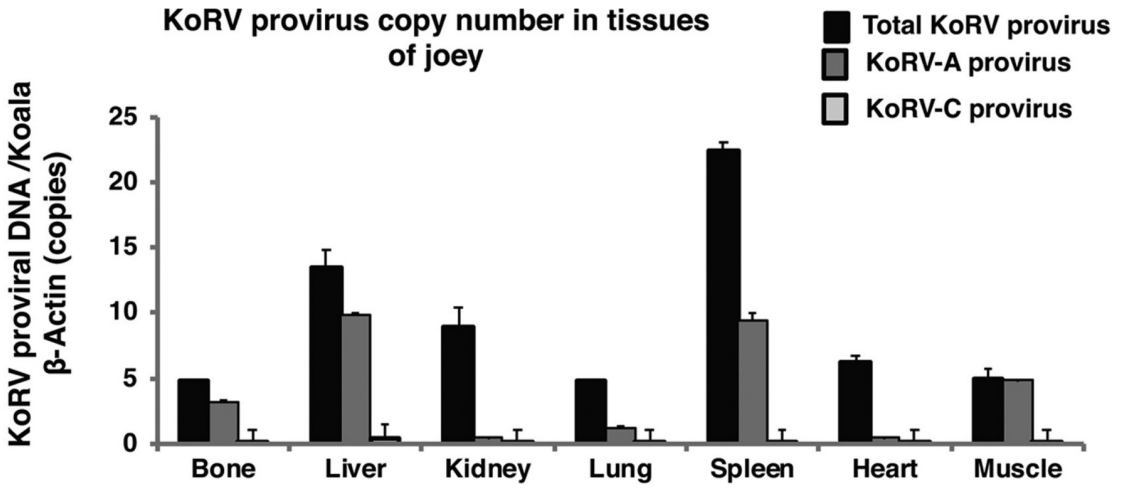


Figure 6.3 Postmortem examination of the male joey of 6 months old. (A to G) Opening of the abdominal cavity and investigation of the viscera (A), heart (B), liver (C), spleen (D), lung (E), kidney (F), and intestine (G) collected from a baby koala at necropsy.



	Bone	Liver	Kidney	Lung	Spleen	Heart	Muscle
Total KoRV provirus	4.8±0.06	13.53±1.35	8.997±1.41	4.8±0.06	22.4±0.62	6.3±0.47	5.01±0.81
KoRV-A provirus	3.2±0.07	9.920±0.01	0.498±0.02	1.263±0.05	9.380±0.7	0.48±0.03	4.83±0.02
KoRV-C proviruus	0.14±0.03	0.49±0.010	0.085±0.01	0.04±0.01	0.09±0.003	0.03±0.008	0.005±0.0006

Figure 6.4. Normalized KoRV proviral load in gDNA isolated from different tissues of a deceased baby koala. Normalized total KoRV, KoRV-A, and KoRV-C proviral loads in different tissues, including bone, liver, kidney, lung, spleen, heart, and muscle are shown. Provirus copy numbers were quantified by real-time PCR and normalized against corresponding koala β-actin in tissues.

6.4.4. Viral load in plasma

Reverse transcription-quantitative PCR (RT-qPCR) was performed to determine viral load in plasma. The viral copy numbers were 1,070,526/ml of plasma in the sire and 1,147,045/ml of plasma in the dam (Figure 6.5) and could not be determined in the joey due to postmortem blood coagulation.

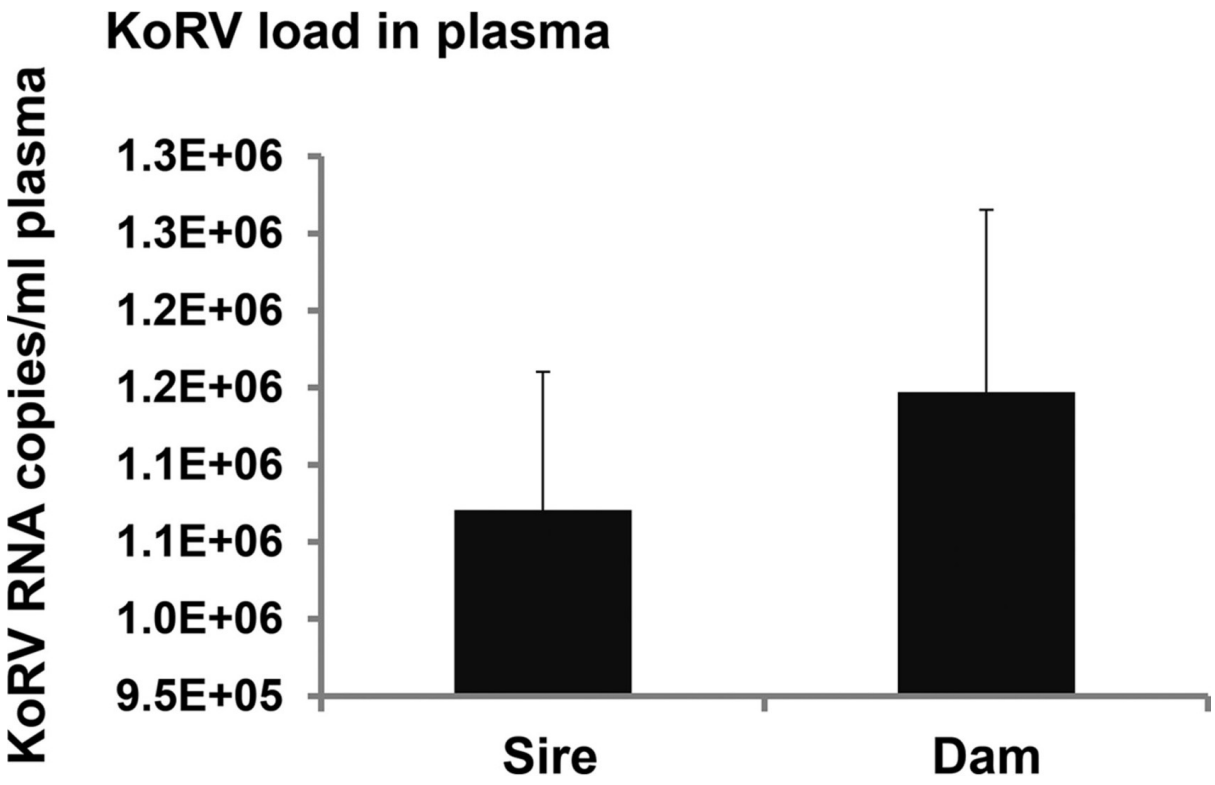


Figure 6.5. Amount of KoRV RNA copies in the plasma from the sire and dam. KoRV RNA copies per milliliter of plasma were calculated to show the plasma viral load.

6.4.5. Nucleotide sequence analysis of KoRV in parents and joey

PCR amplification of sequences with primers U3F and U5R (Table 6.1) resulted in expected bands of approximately 0.5 kb of the long terminal repeat (*LTR*) sequence (Figure 6.6A). Phylogenetic analysis revealed that the KoRV *LTR* sequence in the joey (505 nucleotides [nt]) was closer to that of the sire than that of the dam (Figure 6.6B). The KoRV *LTR* nucleotide sequences of the parents and joey were analyzed by multiple-sequence alignment (Figure 6.6C). The conserved sequences for the joey and its sire are enclosed in black boxes, and the joey-specific sequences are enclosed within red boxes. These results indicate that the *LTR* sequence of KoRV in the joey was closer to that of the sire.

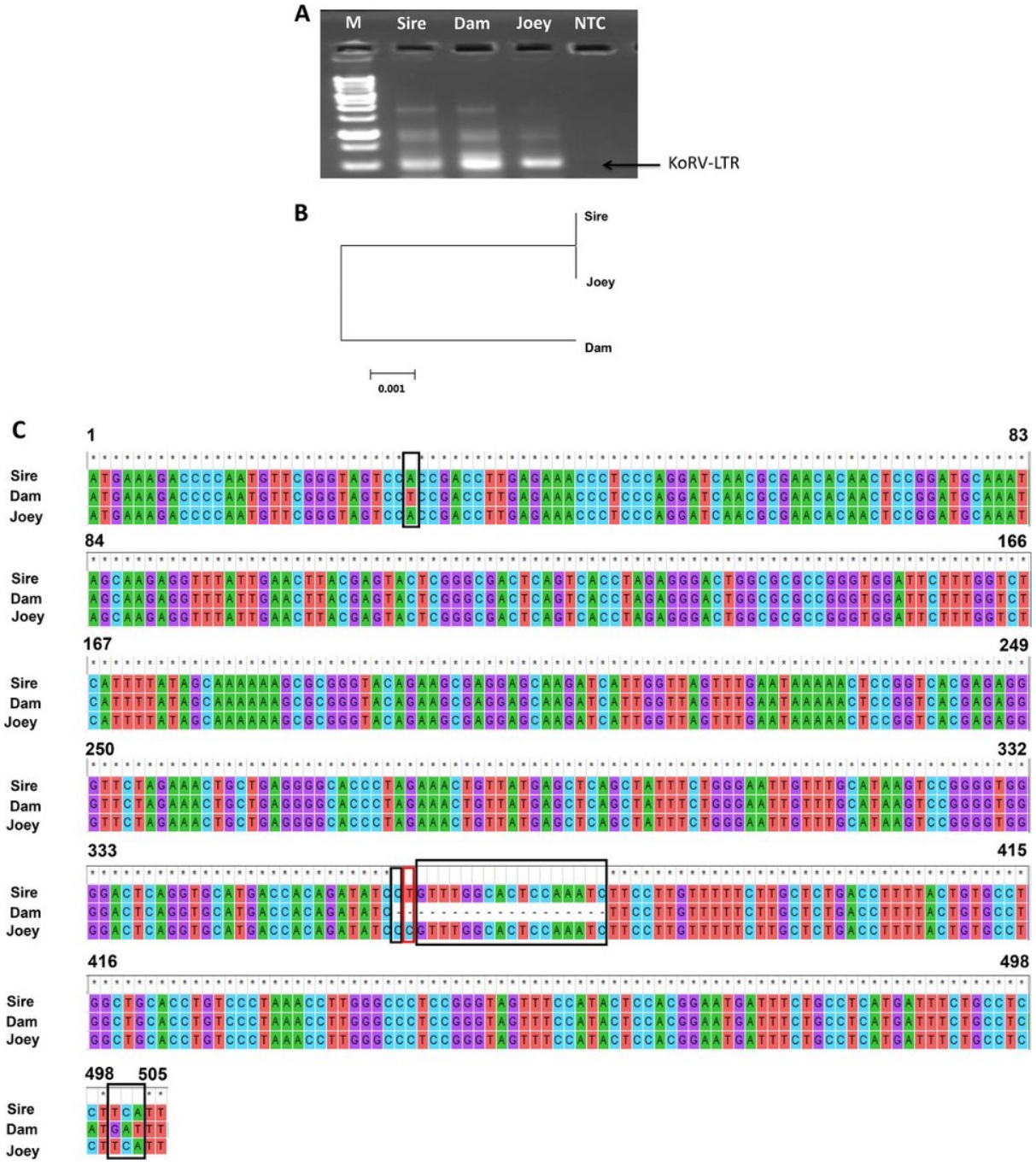


Figure 6.6. Characterization of long terminal repeats (*LTR*s) of KoRV in parents and joey. (A) Amplification of *LTR* gene using genomic DNA from the sire, dam, and joey. Lane NTC, no-template control; lane M, 1-kb marker (GeneDireX, Inc.). (B) A phylogenetic tree was constructed based on the nucleotide sequences of KoRV *LTR* using the MEGA7 software neighbor-joining method. The scale bar at the bottom indicates the nucleotide distance. (C) *LTR* multiple-sequence alignment. Black boxes indicate nucleotide differences between the joey with dam only, and the red box indicates nucleotide differences of baby from both the sire and dam. Asterisks indicate conserved sequences.

In addition, I sequenced the KoRV-A partial envelope gene (320 nt) from the joey and performed multiple-sequence alignment with the parents' KoRV-A *env* gene sequences (GenBank accession numbers MK605477 and MK605481) (Figure 6.7). Multiple-sequence alignment revealed differences in only two nucleotides, indicated by black boxes (Figure 6.7). One point was conserved between both parents and the joey, and one point was conserved between only the dam and the joey. Notably, using two sets of primers [Xu, et al., 2013; Hashem, et al., 2019], I also performed genotype-specific PCR for the detection of KoRV-B in the joey, but no PCR bands of the expected size were observed in any samples from the joey, whereas both the sire and dam were found to be KoRV-B positive (data not shown) with both of the primer sets [Xu, et al., 2013; Hashem, et al., 2019].

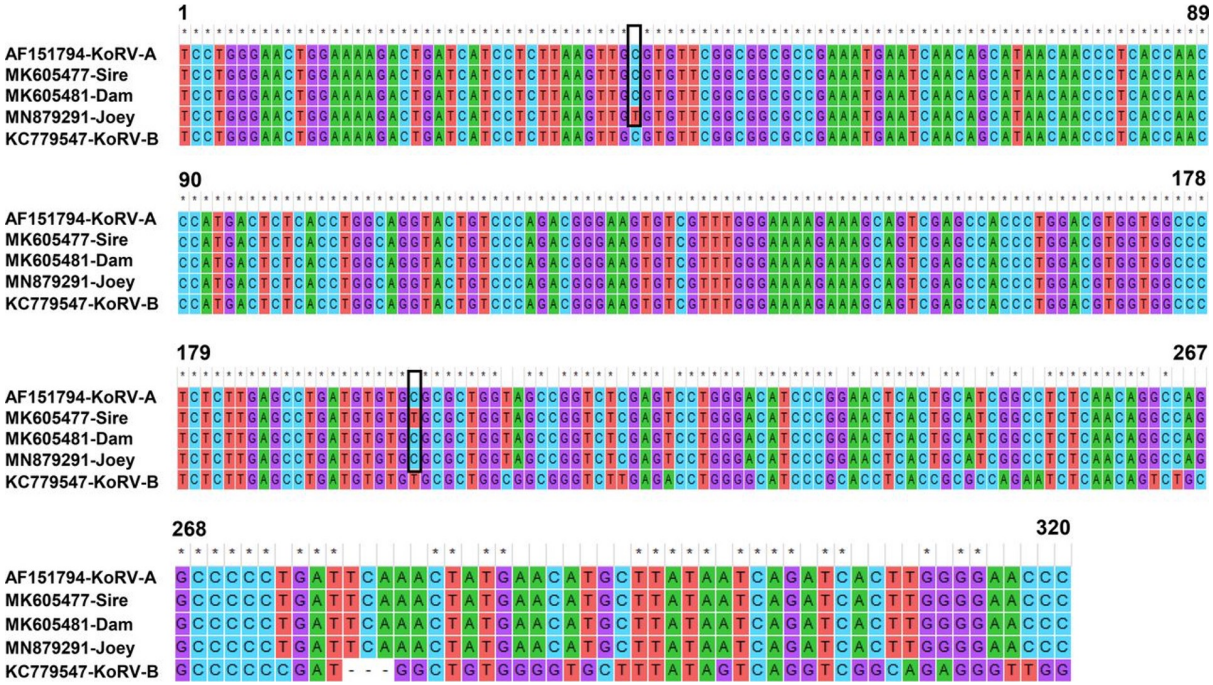


Figure 6.7. Multiple-sequence alignment of KoRV-A envelope gene. Boxes indicate nucleotide differences between the joey and the sire or dam. Asterisks indicate conserved sequences, and dashes indicate deleted sequences. Published sequences of KoRV-A (GenBank accession no. AF151794) and KoRV-B (GenBank accession no. KC779547) are also shown.

6.5. Discussion

In the present study, I investigated the subtype status, tissue tropism, and transmission mode of KoRV in a deceased joey koala from parents previously established to be KoRV-A and KoRV-B positive in our previous studies [Kayesh et al., 2019; Hashem et al., 2019].

For KoRV-A, I speculated that vertical parent-to-offspring transmission had occurred in this case, which is in line with previous findings of 100% vertical KoRV-A transmission in regional koala populations in Australia and the inheritance of KoRV after its endogenization into the genome [Quigley et al., 2018; Hashem et al., 2019]. Notably, the parents were born in captivity, one in a Japanese zoo and one in an Australian zoo. Furthermore, in contrast to reports that offspring inherit KoRV-A from their dams, I found that the joey had a KoRV *LTR* sequence closer to that of its sire than its dam. Quigley et al. [Quigley et al., 2018] reported on four joeys (age, 0.9 to 2 years) who had all apparently been subject to horizontal infection by their dams. Xu et al. [Xu et al., 2013) reported KoRV-B statuses for four of five joeys (age range, 1 month to 1 year) in a study population at a Los Angeles Zoo and concluded that KoRV-B had been transmitted from the dam to the joey. Differences in points of conservation in multiple-sequence alignment suggest KoRV-A that is continuing to evolve, and these differences are consistent with mutations that can be expected as the endogenized genome is passed from one generation to the next.

My findings for KoRV-B are of particular interest. The joey was found to KoRV-B negative, although both of its parents were found KoRV-B positive (in both this study and our previous study). Although adult-to-adult transmission of KoRV reportedly occurs at a low rate, the previous (limited) reports on parent-to-offspring transmission suggested that 100% of joeys

contract this subtype through horizontal (or de novo) infection from their dams. Such transmission could occur through uterine fluids, milk, or pap, which probably originates in the dam's cecum. KoRV-B-negative joeys with KoRV-B-positive sires have been featured in these reports; however, to my knowledge, this is the first report of a KoRV-B-negative joey from a KoRV-positive dam. As an understanding of transmission is key to any strategy for species conservation, further research on KoRV prevalence and transmission in joeys would appear to be necessary.

The cause of death, and more specifically, whether the cause of death was related to KoRV, could not be established in the limited postmortem examinations that were possible for this joey. The findings of fluids in the thoracic and peritoneal cavities could be consistent with the sort of immunodeficiency or neoplasia reportedly linked with KoRV-B. However, it has to be noted that that the joey in this case was negative for KoRV-B and positive for KoRV-A. According to a previous study, lymphoma and leukemia are found to be the most common forms of neoplasia in both captive and free-living koalas [Hanger et al., 2000]. Mortality surveys of wild koalas revealed that these two conditions account for around 3 to 5% of deaths of free-living koalas in the New South Wales and southern Queensland [Connolly et al., 1998; Heuschele and Hayes, 1961; McKenzie, 1981]. Accordingly, the necropsy findings may be consistent with lymphoma or leukemia as the cause of death for the joey in this study. Further research is needed on the possible pathological associations of KoRV-A in joeys.

The joey's parents were also suggested to be generally in good health, as determined from the hematology examinations in this study and recent veterinary observations, and they had not shown any signs of neoplasia or autoimmune disease, such as the "AIDS-like syndrome" described in KoRV-B-positive koalas, until recently.

I found various proviral loads in the joey organs and tissues examined in this study, indicating that the virus is still active and that replication and de novo integration in some organs are ongoing. This indication is consistent with previously reported differential proviral loads in a range of organs for porcine endogenous retroviruses (PERVs) [Mazurek et al., 2013; Denner, 2016; Fiebig et al., 2018]. One explanation for such differential proviral loads concerns the status of KoRV-A as a currently endogenizing retrovirus which is still actively infectious, thus causing new insertions in susceptible tissues. Moreover, the larger amount of KoRV-C in the joey than in the parents could reflect progressive integration into the joey's genome. This result may explain the inconsistency between total KoRV, KoRV-A, and KoRV-C provirus amounts, because they were quantitated using *pol* gene and *env* gene regions. The proviral load in the spleen exceeded the total amounts of KoRV-A and KoRV-C. This result suggests that a strain other than KoRV-A, -B, or -C could be present in the joey's genome.

Overall, my study confirms that KoRV-B is an exogenous variant of KoRV, and that KoRV-A is inherited by vertical transmission, as has been previously widely reported. However, I suggest that previous assumptions about the 100% dam-to-joey transmission rate for KoRV-B may need to be reexamined. A fuller understanding of transmission and pathogenesis is required to develop any conservation strategies, such as vaccination programs for wild animals, and will be also be of interest to virologists studying the exogenous and endogenous subtypes of this disease. In future research on the koala populations in Japanese zoos, I hope to further expand my knowledge of KoRV and provide answers to some of the questions raised by recent research.

Chapter 7

General discussion

As a pathogen, KoRV is a significant threat to the long-term survival of the koala. KoRV-related lymphomas and leukemia have been reported in several captive koala populations [Xu et al., 2013; Tarlinton et al., 2005]. It has been also associated with immunosuppression and tumors [Quigley et al., 2020; Kayesh et al., 2020; 2021. This association is yet to be definitively established and the pathogenesis of KoRV is incompletely understood. As KoRV appears to be rapidly spreading through the Australian wild koala population [Fabijan et al., 2017; Gonzalez-Astudillo et al., 2017; Legione et al., 2017] as well as captive koala population [Kayesh et al., 2019; 2020; Zheng et al., 2020; Joyce et al., 2021], it is important to determine the significance of infection and how KoRV causes disease to better plan management strategies. The prevalence and types of disease that generally occur in captive koalas are highly burdened by malignant neoplasia and lymphoma/leukemia [Tarlinton et al., 2005; Xu et al., 2013; Zheng et al., 2020]. This study provides some information about KoRV transmission, lymphoma/leukemia related diseases and several risk factors associated with demographic characteristics of koala reared in different Japanese zoos, which will improve our current understanding of KoRV pathogenesis and transmission.

7.1. Major findings

In this thesis, data was incorporated from different Japanese zoo-dwelling koalas to characterize the KoRV infection status and their subtype distribution. Based on data analysis I demonstrated following key information to zoo authority as well as researchers which could be helpful for proper management of koala health.

i) Firstly, I collected data from three zoos (Hirakawa zoological park, Kobe Oji Zoo and Saitama children's zoo). I found varying amounts of KoRV proviral loads in genomic DNA (gDNA) in peripheral blood mononuclear cells (PBMCs). I found that 100% of the koalas were infected with KoRV-A and 60% were coinfected with KoRV-B. I also investigated the trans-species transmission of KoRV in Tupaia cells.

ii) Secondly, I surveyed a representative sample from a Japanese zoo population to determine the single- and multiple-subtype infection status of KoRV. I found highest viral expression and WBC count in a koala infected with multiple subtypes than single subtype. Accordingly, I conclude that coinfection with multiple KoRV subtypes may be linked to disease severity.

iii) Thirdly, I collected a wide range of koala blood samples from seven zoos to ascertain any variation in viral load by institution, age, sex, or body condition score. I quantified KoRV proviral DNA and RNA loads and found a significant inter-institutional difference in proviral DNA ($p<0.0001$) and RNA ($p<0.001$) amounts (copies/10^3 koala β-actin copies) in Awaji Farm England Hill Zoo koalas. Significant ($p < 0.05$) age differences were noted in KoRV RNA load ($p < 0.05$) and median total RNA load ($p < 0.001$), with loads higher in younger koalas (joeys and juveniles).

iv) Finally, I reported new evidence on the modes of KoRV transmission from parent koalas to their joey. I found vertical transmission of KoRV-A, indicating its endogenization. I also reported a KoRV-B-negative joey from KoRV-B-positive

parents, contrasting with some previous reports of 100% transmission of KoRV-B from dams to joeys.

7.2. Limitations

Although this study generated some valuable information about KoRV in captive koalas, there were some limitations which couldn't be solved within the time of the Ph.D. candidature. At each zoo, the koalas were housed in an environment maintained within a constant temperature range (23~25°C) using an air conditioning system, therefore I could not evaluate environmental and seasonal impact of KoRV on captive koala population.

The issue of sire/dam-to-joey transmission was not addressed in whole population, although some koalas were offspring of others in this study population. My health assessment was limited to body condition score and WBC count. Accordingly, I propose further investigations into the molecular dynamics and transmission of KoRV in this population.

7.3. Conclusion

Overall, the work in this thesis significantly contributes to my current understanding of KoRV infection status in captive koalas reared in Japanese zoos. It has identified several viral and host parameters that are likely to contribute to development of KoRV-associated disease and has provided the basis for future, more specific work to further explore each of these parameters. Ultimately, this work will provide crucial information for development of KoRV prevention and management strategies.

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