Proceedings of the Second Koala Retrovirus Workshop

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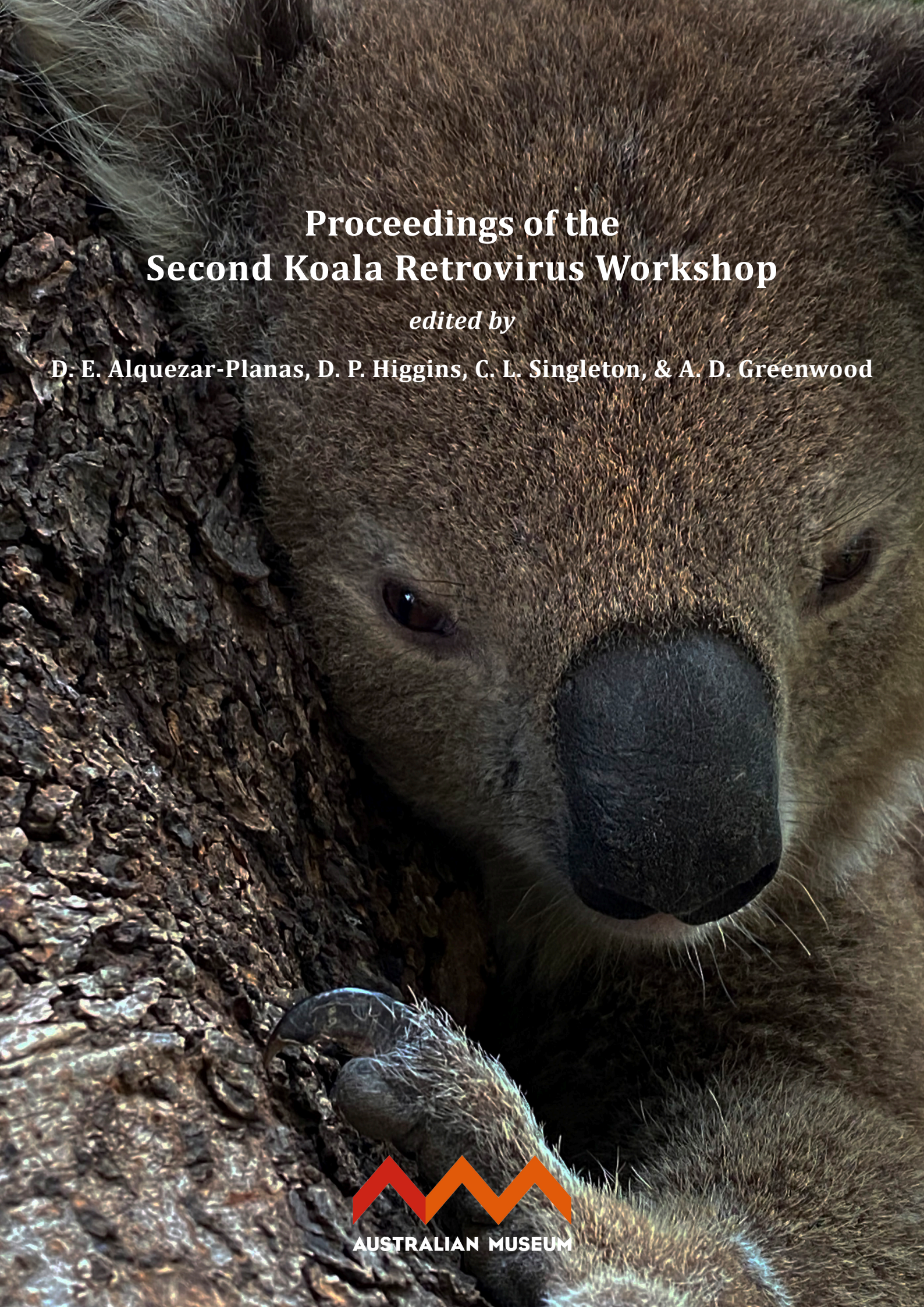
D. E. Alquezar-Planas, D. P. Higgins, C. L. Singleton, & A. D. Greenwood



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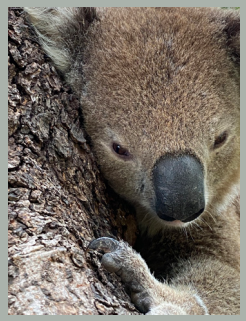

Cover photo
by Damien P. Higgins

A series of peer-reviewed papers, edited by David E. Alquezar-Planas, Damien P. Higgins, Cora L. Singleton, & Alex D. Greenwood, and a discussion summary, from the *Second Koala Retrovirus Workshop* held online, 25–27 May 2021. Published 21 June 2023, in *Technical Reports of the Australian Museum Online* number 38, ISSN 1835-4211 (online).

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Preface to the Second Koala Retrovirus Workshop Online 25–27 May 2021

David E. Alquezar-Planas[1], Damien P. Higgins[2], Cora L. Singleton[3], and Alex D. Greenwood[4]

[1] Australian Museum Research Institute, Australian Museum, Sydney NSW 2010, Australia

[2] Sydney School of Veterinary Science, Faculty of Science, The University of Sydney NSW 2006, Australia

[3] San Diego Zoo Wildlife Alliance, San Diego, California 92101, United States of America

[4] Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, 10315 Berlin, Germany; and School of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany

Abstract. In 2013, the first Koala Retrovirus Workshop was held in San Diego, bringing together biology and veterinary specialists to assess and discuss the state of knowledge on koala retrovirus (KoRV) and to form professional bridges and networks. Tremendous progress has been made in the years following the San Diego meeting, in large part due to ongoing international collaborations that were fostered to study KoRV. This volume presents peer-reviewed papers from most of the oral presentations and discussions held during the Second Koala Retrovirus Workshop in 2021. Unfortunately, the COVID-19 pandemic forced the workshop into an online only format. Despite this limitation, three days of discussions based on workshop presentations highlighted current knowledge and important information gaps, culminating in suggested ways forward, all summarized in this volume.

Since characterization of the koala retrovirus (KoRV) in 2000 (Hanger *et al*., 2000) and the discovery that it represents the only accessible model of the process of genome colonization (Tarlinton *et al*., 2006), molecular techniques have advanced to a state where full genomes of koalas and huge numbers of individual koalas in both healthy and diseased states can be examined (Greenwood *et al*., 2018). At the same time, a growing body of research supports association of KoRV with disease manifestations in koalas (Legione *et al*., 2017; Waugh *et al*., 2017; Fabijan *et al*., 2019; Quigley *et al*., 2019; Butcher *et al*., 2020; Saker *et al*., 2020; McEwen *et al*., 2021; Blyton *et al*., 2022). In the 10 years since the first Koala Retrovirus Workshop (2013), enormous strides have been made in understanding KoRV. However, the workshop clearly demonstrated that several knowledge gaps remained which precluded implementation of effective management strategies to support koala conservation efforts. This has become an increasingly urgent need. Koala population decimation following the major fires across much of the koala's Australian range in 2019–2020 highlighted the vulnerability of koala populations, brought about by decades of habitat reduction and fragmentation. As of February 2022, the Australian Government listed the combined koala populations across Queensland, New South Wales, and the Australian Capital Territory as endangered.

During this three-day workshop, invited speakers reviewed historical knowledge and presented recent discoveries in KoRV biology, with topics covering KoRV

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ORCID iD: Alquezar-Planas 0000-0001-5360-5263 Higgins 0000-0003-1215-6523 Singleton 0009-0007-1856-4073 Greenwood 0000-0002-8249-1565
Corresponding author: David E. Alquezar-Planas David.Alquezar@Australian.Museum
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genetic diversity and distribution, KoRV-associated diseases, anti-retroviral processes, the origins of KoRV, and new models for retroviral germline invasion. Scientists in many fields including: veterinarians, ecologists, and population managers, all contributed to discussions on KoRV status and the impacts this has on koala health, the challenges faced with managing koala populations (wild and captive) as well as maintaining fit for purpose zoological collections that will enable ongoing foundational research. Over three days following the presentations, discussion sessions focused on KoRV foundational biology and applied management of zoo and wild koala populations to consolidate knowledge, achieve consensus, and identify contrasting perspectives. Discussion summaries for the three-day workshop are also published in this series (Greenwood *et al*., 2023), outlining what we know, what we still do not know, and what we need to know about KoRV. We hope this will serve as a useful guide for current and future KoRV researchers to continue advancing our understanding KoRV and its impacts on koalas.

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An Overview of Koala Retrovirus Epidemiology in Australia

Rachael Tarlinton

School of Veterinary Medicine and Science, University of Nottingham,
United Kingdom

Abstract. Koala retrovirus (KoRV) epidemiology varies across koala (*Phascolarctos cinereus*) populations with distinct differences in viral prevalence, sequence diversity, and disease impact. Curiously the more genetically restricted southern populations are less impacted by KoRV with the virus not endogenized in its replication competent form in these animals. These southern animals do, however, have replication defective recKoRV variants in their genomes indicating historical exposure to KoRV and recKoRV. Whether southern animals are inherently resistant to KoRV infection and endogenization is not clear. It is also not clear whether the current regional epidemiological patterns will persist or whether exposure to animals with infectious KoRV or cross-breeding between different genetic populations will change the KoRV prevalence with time.

Introduction

Both koala (*Phascolarctos cinereus*) genetics and koala retrovirus (KoRV) prevalence vary regionally across Australia, with a stark demarcation between a more genetically diverse "northern" group (New South Wales and Queensland) and a genetically restricted "southern" group (Victoria and South Australia). These groups of animals also display markedly different disease profiles, with putatively KoRV-related disease syndromes at a much lower rate in the southern animals. All northern animals ever studied have endogenous KoRV-A alongside varying prevalence of other KoRV genotypes that do not appear to be endogenous. Endogenous KoRV loci are shared amongst closely related individuals but are not fixed across the species. Northern koalas also have multiple copies of defective KoRV variants in their genomes, where the central portion of the KoRV genome has been replaced by another koala retro-element termed Phascolarctid endogenous retroelement (PhER). These are known as recKoRVs and are also not fixed.

The southern animals were re-established from off-shore island colonies after localized extinction in the 1920's with a marked genetic bottleneck evident. Southern animals display varying KoRV prevalence without endogenous KoRV loci. Those animals that are KoRV positive tend to have lower viral loads than their northern counterparts. It was previously thought that many of these animals were KoRV free; however, recent work has demonstrated that many (perhaps all) animals that test negative for the KoRV *pol* gene PCR (the most used diagnostic for all KoRV variants) have recKoRV variants within their genomes. These are distinct from the recKoRV variants in the northern animals with an additional indel of unidentified DNA between the KoRV *gag* and PhER sequences. It is not clear at this stage what the significance of this is for potential to cause disease. It is possible that the presence of these variants inhibits infectious KoRV (as happens with defective endogenous retroviruses in other species). It is also possible that southern animals are simply not born tolerized to KoRV-A (or other variants) and are better able to control virus replication via their

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ORCID iD: Rachael E. Tarlinton https://orcid.org/0000-0003-3325-2311
Corresponding author: Rachael E. Tarlinton rachael.tarlinton@nottingham.ac.uk
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immune responses. Ancestors of the founder populations of the southern animals must have been infected with KoRV at some stage to have accumulated recKoRVs in their genomes but why KoRV-A is not also endogenous in these animals or what the implications are for cross-breeding of animals at border areas between populations is still unknown.

Discussion

Koala retrovirus is an unusual pathogen in that it is currently undergoing the transition between being an infectious transmissible virus and a retrotransposon carried by its host's genome (Tarlinton *et al*., 2006). Retroviruses are single stranded RNA viruses that make a DNA copy of themselves that is integrated in the host cell's DNA as part of their lifecycle. If this copy is integrated into a germ line cell (sperm, ova or progenitor of these in early stage zygotes) it becomes inherited. This process is surprisingly common with all vertebrates to date having multiple endogenous retroviruses integrated into their genomes. However, most have become attenuated with time, accumulating mutations that render them non-functional as a virus (Symer & Boeke, 2010). The process of re-integration into the genome can continue for some time after the original infectious virus becomes extinct, with the retroviral genomic copies forming a "fossil" record of a host's past viral exposure in evolutionary history.

KoRV is one of a small group of viruses that have both infectious forms currently circulating and accumulated host germline copies of the virus. This greatly complicates assigning attribution for disease pathogenesis in populations where animals are born with inherited germline copies of the virus. Though it is now apparent that both the accumulation of new somatic insertions of KoRV and the inheritance of viral insertions near or in oncogenes is the trigger for the very high rates of haematopoietic neoplasia seen in koalas (McEwen *et al*., 2021).

Koala populations have been through multiple bottlenecks with a marked population contraction approximately 30–40,000 years ago. Several distinct geographical barriers are evident in studies of koala population genetics with five distinct geographical clusters: North Queensland, South East Queensland, Mid-North Coast New South Wales, South Coast New South Wales and Victoria/South Australia (Johnson *et al*., 2018). The most recent and dramatic genetic segregation was the bottleneck in the southern population induced by hunting pressures upon European colonization. Most of the southern population was effectively extinct by approximately 1920, and this region was repopulated from a very small number of animals that had been removed to offshore island sanctuaries (Ruiz-Rodriguez *et al*., 2016). Consequently, the southern (Victoria/South Australia) population has a markedly lower genetic diversity than the other populations (Johnson *et al*., 2018; Ruiz-Rodriguez *et al*., 2016; Neaves *et al*., 2016).

This split in general koala conservation genetics is also evident in the distribution of their koala retrovirus complement, with marked differences evident between northern and southern koala populations (Sarker *et al*., 2019, 2020) as well as structuring of retroviral diversity at local population levels in the northern animals (Quigley *et al*., 2018). To date, all animals in the northern populations have the originally described variant of koala retrovirus known as KoRV-A, thought to be the endogenous variant with an attenuated CETAG envelope (*env*) protein motif (Quigley *et al*., 2018, 2021a) along with a defective variant of this strain with a frameshift mutation and stop codon in *env* (Quigley *et al*., 2021b). Diagnostic tests used for KoRV are usually PCR or qPCR based tests designed to detect KoRV polymerase (*pol*) or *env* genes (Tarlinton *et al*., 2005; Stephenson *et al*., 2021). Many animals in southern populations do not have KoRV based on these tests. KoRV-A is detected in some southern animals but at a rate and copy number per genome equivalent in individuals that implies it is solely exogenous (Speight *et al*., 2020; Legione *et al*., 2016). This is further supported by the increased prevalence (in animals that test positive for KoRV) of the presumed exogenous variant of KoRV-A with the more virulent CETAG motif (Quigley *et al*., 2021b).

Other strains of KoRV, based on sequencing of the hypervariable region of the surface unit of the *env* gene, have been described (Legione *et al*., 2016). These have never been detected without concurrent detection of KoRV-A, and it is not clear whether they circulate independently of KoRV-A or not (Sarker *et al*., 2019; Quigley *et al*., 2021b; Joyce *et al*., 2021). These also vary locally in different populations, with a general decrease in viral diversity and load evident further south in the koala population range (Sarker *et al*., 2019; McEwen *et al*., 2021). There has been much speculation as to whether these variants, particularly the B variant, are associated with increased virulence or differences in disease prevalence (Zheng *et al*., 2020; Xu *et al*., 2013; Waugh *et al*., 2017) but this has not been borne out in all studies (Quigley *et al*., 2019; Robbins *et al*., 2020).

The emerging picture from many groups' work on KoRV variants and prevalence is one of a distinct split between Victoria/South Australia animals and northern animals, with KoRV present in both endogenous and exogenous forms in the northern koalas but as an exogenous virus with reduced diversity in the southern animals. This coincides with different disease prevalence rates between these populations, with both neoplasia and clinical chlamydial disease at much lower rates in southern populations (Sarker *et al*., 2020; Quigley *et al*., 2021b; Fabijan *et al*., 2020).

The other set of KoRV variants present in the koala genome, known as recKoRVs, are recombinants between KoRV and an older retrotranspon, Phascolarctid endogenous retroelement (PhER) (Hobbs *et al*., 2017; Löber *et al*., 2018). This type of recombination between exogenous retroviruses and genomic transposons is well-described in other animal models such as cats and mice (Chiu & VandeWoude, 2021; Young *et al*., 2012) and can have considerable impact on the creation of viral variants with altered pathogenesis. These arise because of the way retroviruses replicate, involving jumps between two copies of viral RNA during the reverse transcription process, making them extremely prone to integrating other retroviral or even non-retroviral RNA into their genomes (Symer & Boeke, 2010). The recKoRVs were described as part of the koala reference genome analysis (Hobbs *et al*., 2017; Löber *et al*., 2018) and vary in copy number among animals, they are not functional as viruses and are unlikely to be able to retrotranspose themselves within the genome as they do not encode a complete reverse transcriptase reading frame. Our recent work has demonstrated that southern animals that test negative for KoRV with *pol* gene PCR or qPCR (and that would have previously been considered KoRV free) have recKoRV variants in their genomes (Tarlinton *et al*., 2021). These variants were found in all animals tested though do not appear to be fixed, with some loci (but not all) shared between animals from disparate genomic locations. They are not the same as those identified in northern animals, with the addition of an unidentified sequence between the KoRV *gag* and PhER reverse transcriptase sequence.

It is not clear what the significance of these recKoRV isolates are in koalas. They have most likely arisen and been transmitted alongside infectious KoRV variants as has been described for defective oncogene containing retroviruses in other species (Rubin, 2011). This implies that the ancestors of today's southern animals likely had infectious and or endogenous KoRV variants that were lost due to the extreme genetic bottleneck that the founder populations underwent during translocations. There is an additional possibility that the presence of these recKoRV variants may inhibit replication of infectious KoRV. Blockade of infectious virus has been described for endogenous retroviruses in other species including sheep and mice (Viginier *et al*., 2012; Nethe *et al*., 2005) and is thought to potentially be a driver of positive selection for endogenization of particular retroviral loci in genomes.

Elucidation of whether recKoRVs in southern animals have any effect on the lifecycle of infectious KoRV variants awaits further experimental work. This is not just an academic or evolutionary biology curiosity, in that these differences in KoRV prevalence and the linked prevalence of disease neatly distinguish the two largest genetic groups of animals in the range of the species. The recent bushfire events and translocations of animals associated with emergency responses and recovery have highlighted the fragility of the koala population in many areas. Consideration must be given to whether mixing of genetic populations should be avoided or whether this may have unintended consequences for either further population bottlenecks or infectious disease prevalence.

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Endogenous and Exogenous Koala Retrovirus Patterns in Wild Koalas across Australia

Bonnie L. Quigley and Peter Timms

Centre for Bioinnovation, University of the Sunshine Coast,
Sippy Downs QLD 4556, Australia

Abstract. Our understanding of koala retrovirus (KoRV) has advanced dramatically in recent years. Cross-sectional studies examining hundreds of wild koalas (*Phascolarctos cinereus*) from populations across their natural Australian range (Queensland–New South Wales–Victoria) have shed new light on KoRV abundance and diversity in the wild. A single strain of KoRV (the originally characterized Hanger strain from 2000) appears to be the dominant KoRV strain within koalas, endogenous in northern populations and the predominant exogenous strain in southern populations. Alongside this strain are potentially exogenous variants representing both intact and defective versions of some of the many recognized KoRV subtypes (KoRV-A to KoRV-M). The patterns of these may suggest a transition from endogenous KoRV in the north to exogenous KoRV in the south, occurring in southern New South Wales. They also highlight how actively the hypervariable region of the envelope gene of KoRV is diversifying, with fragmented koala populations across the country containing unique and distinctive KoRV proviral profiles. As more koala populations are examined with increasingly sensitive and specific genetic tools, our understanding of KoRV is poised to continue to evolve as quickly as the virus itself.

Introduction

Koala retrovirus (KoRV) is known to exist both endogenously and potentially exogenously in koalas (*Phascolarctos cinereus*) (Hanger *et al*., 2000; Quigley & Timms, 2020). At some point in the last 49,900 years, KoRV began endogenizing or permanently incorporating its provirus into koala germline genomes in the northern Australian koala population (Tarlinton *et al*., 2006; Ishida *et al*., 2015). In parallel, within almost all koala populations across Australia, potentially exogenous strains of KoRV have continued to diversify into 13 recognized subtypes (KoRV-A to -M, based on differences in the receptor binding domain region of the envelope gene (Shojima *et al*., 2013; Xu *et al*., 2013; Xu *et al*., 2015; Chappell *et al*., 2017; Blyton *et al*., 2021). Targeted studies of both endogenous and exogenous KoRV strains in recent years have led to impressive advances in our understanding of this virus across the natural koala range in Australia (Table 1).

Endogenous KoRV-A

KoRV-A is the original and most prevalent subtype of KoRV detected across Australia (Hanger *et al*., 2000; Chappell *et al*., 2017; Quigley & Timms, 2020). Genetic analysis identified KoRV-A provirus to be present in northern Australian koalas in a pattern consistent with it being endogenously incorporated into their genomes (Tarlinton *et al*., 2006). Additional studies have supported this endogenous status with quantified KoRV provirus within Queensland and northern New South Wales koala cells at levels at or above one copy per cell, with the majority of provirus being KoRV-A (Simmons *et al*., 2012; Hobbs *et al*., 2017; Sarker *et al*., 2020; Quigley, Wedrowicz, *et al*., 2021).

Recent examination of KoRV proviral strains across Australia has revealed that every KoRV positive koala examined, from anywhere in Australia, contained a single dominant KoRV proviral sequence, identical to the originally published Hanger *et al.* (2000) KoRV sequence (accession

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ORCID iD: Bonnie L. Quigley https://orcid.org/0000-0003-3787-0993 | Peter Timms https://orcid.org/0000-0002-1682-0025
Corresponding author: Peter Timms ptimms@usc.edu.au
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Table 1. Summary of endogenous and exogenous koala retrovirus (KoRV) across Australia.

	Endogenous KoRV-A	Exogenous KoRV-A	Exogenous other subtypes (B to M)
General	Hanger *et al*., 2000 strain AF151794.2, represents 8–96% of KoRV provirus detected in positive koalas	KoRV-A variants, containing both intact and defective envelope genes, also detected in KoRV positive koalas. Defective variants appear uniformly abundant across Australia.	Generally represent ≤ 2% of total provirus detected in positive koalas, with each subtype detected at << 0.1 proviral copies/cell
Queensland and northern NSW	All koalas tested KoRV-A positive, provirus detected at ≥ 1 copies/cell	Intact non-Hanger KoRV-A strains represent < 0.1% of KoRV-A	Greatest diversity of subtype strains detected within individual koalas
Southern New South Wales	All koalas tested KoRV-A positive, but provirus levels not suggestive of endogenization (KoRV-A provirus detected at ~0.2 copies/cell)	Non-Hanger KoRV-A strains becoming more abundant	
Victoria	Not all koalas KoRV-A positive, KoRV-A provirus detected at << 0.01 copies/cell, indicating lack of endogenization	Intact non-Hanger KoRV-A strains represent up to 20% of KoRV-A	Least diversity of subtype strains detected with individual koalas

number AF151794.2) (Quigley, Wedrowicz, *et al*., 2021). This strain, which contains an attenuated CETAG Env protein motif (Oliveira *et al*., 2007), was detected as 87–96% of all the KoRV provirus in Queensland and northern New South Wales koalas (Quigley, Melzer *et al*., 2021; Quigley, Wedrowicz, *et al*., 2021). This strongly suggested that the Hanger KoRV-A strain is the endogenous strain in Queensland and northern New South Wales koala populations.

Exogenous KoRV-A

In contrast to northern koalas, the evidence to date suggests that endogenization of KoRV-A is absent or at least very rare in southern koalas. Both presence/absence and quantification studies of KoRV in southern New South Wales and Victorian koala populations have detected koalas that appear to be KoRV negative, with KoRV proviral levels much less than one copy per cell when present (Simmons *et al*., 2012; Wedrowicz *et al*., 2016; Legione *et al*., 2017; Quigley, Wedrowicz, *et al*., 2021).

Detailed examination of these southern koala populations continues to find the Hanger *et al.,* 2000 KoRV-A strain to be the dominant KoRV strain present in KoRV positive koalas (Quigley, Wedrowicz, *et al*., 2021). However, the proportion of total provirus represented by this strain drops from an average of ≥ 87% in the north to only 67% of provirus per koala in the south (Quigley, Wedrowicz, *et al*., 2021). The KoRV proviral load within examined southern koalas notably contained a KoRV-A variant (A3003, accession number MN931401.1) with 15 single nucleotide polymorphisms (SNPs) when compared to the Hanger *et al.,* 2000 KoRV-A strain (Quigley, Wedrowicz, *et al*., 2021). This resulted in five non-synonymous amino acid changes in the Env protein, returning the KoRV-A A3003 variant to the more virulent CETTG motif (Oliveira *et al*., 2007)). While the KoRV-A A3003 variant was detectable in all KoRV positive koalas across Australia, A3003 abundance increased dramatically from an average of < 0.1% of provirus per koala in the north to ~21% of provirus per koala in the south (Quigley, Wedrowicz, *et al*., 2021). This data, coupled with proviral quantifications suggesting less than one in five cells per koala in southern New South Wales and less than one in a hundred koala cells per koala in Victoria contain KoRV provirus (Quigley, Wedrowicz, *et al*., 2021), is supportive though not definitive evidence that KoRV remains exogenous in these southern regions.

These detailed KoRV genetic analyses also revealed that defective KoRV variants are detectable in koalas across Australia and their abundance appears independent of endogenization status. A defective KoRV-A variant (A3002, accession number MN931400.1), which has a two base pair insertion when compared to the Hanger *et al.,* 2000 strain, creating a frameshift/stop codon in the envelope gene, was identified in every KoRV positive koala studied. Interestingly, this defective KoRV-A strain represented between 3–10% of all KoRV proviral reads detected in any koala from any part of Australia (Quigley, Wedrowicz, *et al*., 2021; Quigley, Melzer, *et al*., 2021).

Other potentially exogenous KoRV subtypes

Targeted proviral analysis continues to detect KoRV proviral variation falling under the identified subtypes KoRV-B to -M. Quantification of KoRV-B, KoRV-D, and KoRV-F proviral levels confirmed that these subtypes are present at much less than one copy per 10 koala cells, when detectable at all (Quigley, Wedrowicz, *et al*., 2021). Despite these variants composing only a small fraction (usually < 2%) of the total KoRV provirus present in any individual koala, they represent an impressive range of diversification among the koala populations studied (Quigley, Melzer, *et al*., 2021). Comparing koala populations separated by habitat fragmentation for as little as 90 years, distinct population shifts in their KoRV proviral diversity suggested that lineage diversification of KoRV is still an active process (Quigley, Melzer, *et al*., 2021).

Conclusions

As more koala populations across Australia are studied with increasingly sensitive and specific genetic tools, our understanding of KoRV will continue to evolve. Presently, it appears that most of the KoRV provirus load in koalas can be traced back to a single, dominant KoRV strain (the originally identified Hanger *et al.,* 2000 KoRV-A strain) that has endogenized into koala genomes in northern Australia and continues to circulate in southern Australia. However,

that individual strain itself may represent as many as seven distinct genome colonization events as determined by LTR variation identified (Ishida *et al*., 2015). Other defective and intact KoRV variants, encompassing all the recognized KoRV subtypes (A-M), vary in their distribution among koala populations across the country. Continued KoRV research will not only improve our understanding of this retrovirus for better koala conservation, but also expand our knowledge about the active process of diversification and endogenization of retroviruses in real time.

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Koala Retrovirus Genetic Diversity and Transmission: Advice for Breeders

Briony A. Joyce

The University of Queensland, St Lucia Queensland QLD 4072, Australia

Abstract. The rapid spread of koala retrovirus (KoRV) across Australia and international zoo populations has necessitated appropriate control measures. Along with pathogenicity, the genetic diversity of the virus and how it transmits between animals also needs to be considered when deciding the most suitable measures. Next generation sequencing has become the gold standard approach for KoRV diversity studies due to the high sensitivity, accuracy, and throughput. This approach has identified a large proportion of known KoRV diversity and has provided a broader understanding of KoRV prevalence and abundance within koala (*Phascolarctos cinereus*) populations, specifically identifying individuals with low diversity. Recent evidence has demonstrated that exogenous KoRV transmits from mother to joey, likely through the ingestion of milk and/or pap, and that koalas are not likely to acquire additional KoRV subtypes/sequences later in life. This finding strongly indicates that breeding with KoRV negative or endogenous KoRV-A positive only females is the best chance at alleviating exogenous KoRV from koala populations worldwide. Captive breeders are therefore urged to determine the KoRV profile of all animals included in their breeding program through deep sequencing methods (where feasible) and use this to inform their future breeding regimes.

Introduction

Koala retrovirus (KoRV) is a gammaretrovirus discovered in 2000, closely related to feline leukaemia virus (FeLV) and gibbon ape leukaemia virus (GaLV) (Hanger *et al*., 2000). Alike other retroviruses, KoRV is putatively associated with the onset of neoplasia and other associated cancers in koalas (*Phascolarctos cinereus*) (including leukaemia and lymphoma) and is suspected to cause immunodeficiency and opportunistic disease in this species (Tarlinton *et al*., 2005; Fabijan *et al*., 2020). Whilst habitat destruction and fragmentation, domestic dog attacks and vehicle collisions are among the greatest threats that wild koalas face, the putative KoRV-associated diseases are currently the major contributor towards captive koala mortality. Initially established from wild koala gene pools, captive koala breeding programs are now commonplace in zoos around Australia and internationally. These animals are often exchanged between institutions and, in some cases, exported overseas to increase genetic diversity within colonies. Occasionally, wild koalas are also incorporated into the captive setting and either used for display or as part of the breeding program. Animals approved for this integration are often hand raised and show no wild instincts or have sustained significant injuries, making them unfit to return to the wild. Understanding how to effectively manage these captive populations to reduce the impact from this virus is therefore crucial. The current advice based on recent publications will be addressed in this manuscript.

KoRV genetic diversity

KoRV was first discovered by Hanger *et al*. (2000) in koala genomic DNA through PCR with degenerate primers. This prototypic sequence was later classified as KoRV-A. Since its discovery, more than 10 additional subtypes (B-M) have been identified across multiple institutions around the world (Xu *et al*., 2013; Shojima *et al*., 2013; Xu *et al*., 2015; Chappell *et al*., 2017; Joyce *et al*., 2021; Blyton *et al*., 2021), each with a unique amino acid signature within the receptor binding

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ORCID iD: Briony A. Joyce https://orcid.org/0000-0001-7713-1648
Corresponding author: Briony A. Joyce b.joyce@uq.net.au
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domain of the KoRV envelope protein. It is hypothesized that this variation allows the subtypes to utilize different host cell receptors in attempt to overcome superinfection interference. However, this has only yet been explored for subtypes A and B, which use the sodium-dependent phosphate transporter (PiT1) and thiamine transporter 1 (THTR1) receptors, respectively (Oliveira *et al*., 2006; Xu *et al*., 2013; Shojima *et al*., 2013). Initial investigations into KoRV diversity focused primarily on PCR-based detection methods using subtype-specific primers. Whilst this approach led to the discovery of KoRV subtypes B-E (Xu *et al*., 2013; Shojima *et al*., 2013; Xu *et al*., 2015), it wasn't sensitive or high throughput enough to capture all the KoRV diversity within samples (Legione *et al*., 2017). This prompted the shift to next generation sequencing to allow greater detection of KoRV diversity. This method was first employed by Chappell *et al*. (2017) who detected 108 novel KoRV sequences and four new subtypes (F-I) in 18 wild koalas. This deep sequencing approach is now used as the gold standard for KoRV genetic diversity analyses and has helped detect well over 800 different KoRV sequences (Quigley *et al*., 2019; Sarker *et al*., 2019; Quigley *et al*., 2021; Joyce *et al*., 2021; Blyton *et al*., 2021). The magnitude of this is exemplified in the study recently conducted by our group which detected 421 unique KoRV sequences from 109 captive Australian koalas, the most diversity detected in a single study to date (Joyce *et al*., 2021). This dataset also revealed a novel KoRV subtype, KoRV-K.

Analysing KoRV subtype prevalence, abundance, and diversity is pivotal in understanding KoRV evolution within and among koala populations. KoRV-A is ubiquitous among the northern Australian populations of Queensland (QLD) and New South Wales (NSW), where it accounts for 94% of an animal's KoRV sequence reads on average (Joyce *et al*., 2021). However, the distribution and abundance of the remaining subtypes varies considerably among different populations. This is evident in our study where significant subtype differences were observed between two QLD koala colonies, despite the frequent sharing of animals and geographic proximity (Joyce *et al*., 2021). These differences in subtype prevalence and abundance are markedly greater among different regions (Joyce *et al*., 2022). Due to this high variability, the KoRV profile of all koalas housed in captive institutions should be established through deep sequencing methods. This information is pivotal for ensuring the appropriate management of these animals, especially when considering the transmission dynamics of this virus.

Potentially exogenous KoRV transmits from mother to joey

An aspect of KoRV biology is that it transmits via endogenous and potentially exogenous routes. At present, KoRV-A is the only subtype known to have endogenized into the koala genome, having been detected in koala sperm by Tarlinton *et al*. (2006) using fluorescence *in situ* hybridization. Similar work has not been conducted for the remaining KoRV subtypes so there has been no reported evidence of endogenization of these to date, and consequently, these variants are believed to only transmit via exogenous routes. However, many variants are defective, so an exogenous transmission mechanism is not clear. Based on recent studies, we know that if exogenous transmission occurs, it is primarily between mother and joey (Joyce *et al*., 2021).

Mother to joey transmission of KoRV-B has been noted in a few studies conducted worldwide since 2013 (Xu *et al*., 2013; Quigley *et al*., 2018; Zheng *et al*., 2020). However, the first substantial and statistically significant evidence of this transmission, observed for several KoRV subtypes, is from the recent study carried out by our team (Joyce *et al*., 2021). In this study, we conducted a large-scale sequence sharing analysis to track the transmission of KoRV sequences among captive koalas with known pedigree. Overall, we found very strong evidence of mother-joey transmission for all analysed subtypes (A, B, D, H–K), including non-endogenized KoRV-A. Interestingly, we found no evidence of father-joey or sexual transmission of this virus. Analysis of animals over time also revealed that KoRV infection occurs in the early stages of life and that koalas are less likely to acquire additional KoRV sequences or subtypes later in life. Notably, provirus re-integration can still occur within the animal, where substantial accumulation is associated with neoplasia (McEwen *et al*., 2021). Together, these findings highlight that KoRV transmission requires close contact—as seen between a mother and joey (Fig. 1)—and suggest that KoRV transmits through the ingestion of infected fluids. However, alternative scenarios remain possible such as excess integration on the X chromosome which would similarly skew integration site ratios to look like mother-joey transmission.

Whist the exact route of mother-joey transmission is yet to be investigated for KoRV, there are several postulations based on the various fluids shared between the two individuals. The most likely source of KoRV transmission is through the ingestion of infected milk and/or pap (semi-fluid faecal matter). Whilst no active virus has been recovered, KoRV sequences and peptides have been previously discovered in koala lactation milk (Morris *et al*., 2016). Exogenous viral transmission in both milk and faeces is seen to occur for other closely related retroviruses including FeLV, GaLV and mouse mammary tumour virus (Kawakami *et al*., 1977; Pacitti *et al*., 1986; Petropoulos, 1997; Gomes-Keller *et al*., 2008). Detection of KoRV-D in a neonate that failed to make it into the pouch due to consuming amniotic fluid also raises the possibility of viral transmission occurring *in utero* or during parturition (Joyce *et al*., 2022). This form of transmission has also been documented for GaLV (Kawakami *et al*., 1978). It should be noted that GaLV and FeLV contain the CETAG motif and KoRV contains CETTG, which drastically reduces KoRV infectivity, which may limit exogenous transmission. Investigation into whether and which koala excretions carry infectious virus is therefore required and crucial for our understanding of KoRV viral transmission.

Implications for koala breeding programs

The evidence collected thus far strongly indicates that the KoRV status of female koalas is important. Captive breeders are urged to preferentially breed with female koalas that are KoRV negative or positive for KoRV-A only. Where this is not possible/feasible, breeders should opt for females with the least KoRV genetic diversity. This has been shown to be effective in a southeast Queensland (SE QLD) zoo that actively removed KoRV-B positive individuals from their breeding program several years ago. This population is found to have drastically reduced KoRV diversity compared to all other populations analysed by our group (Joyce *et al*., 2022), in particular the two SE QLD populations from our recent publication that reside in the same geographic area (Joyce *et al*., 2021). Instigating this change across all captive institutions should therefore help alleviate the transmission of subtypes with unknown health risk within captive koala populations.

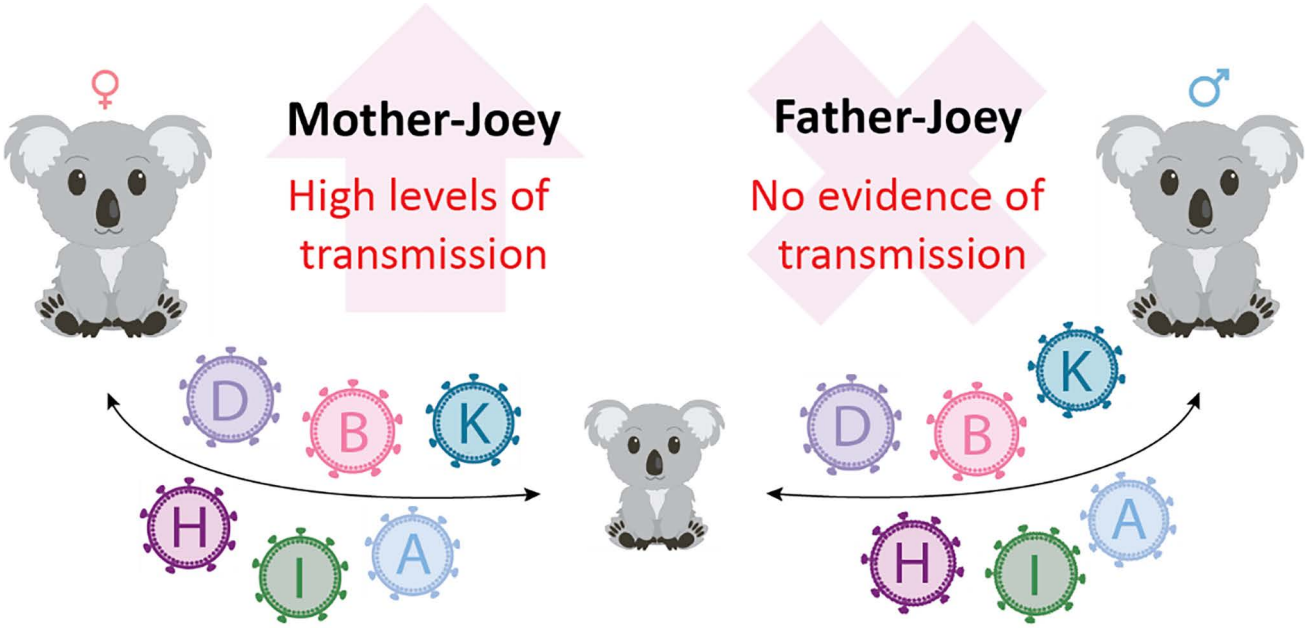

Figure 1. Schematic depicting key exogenous transmission dynamics of koala retrovirus. Letters refer to respective KoRV subtypes.

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Bats or Rodents, Who Started it? Short History of the Gibbon Ape Leukaemia Virus–Koala Retrovirus Clade

Saba Mottaghinia[1], Lee McMichael[2], and Joanne Meers[2]

[1] Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, 10315 Berlin, Germany

[1] CIRI, Centre International de Recherche en Infectiologie, Univ. Lyon, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, ENS de Lyon, F-69007, Lyon, France

[2] School of Veterinary Science, University of Queensland, Gatton QLD 4343, Australia

Abstract. The close genetic relationship between gibbon ape leukaemia virus (GALV) and koala retrovirus (KoRV) has puzzled scientists since its discovery. As the two hosts are separated geographically and taxonomically, it was hypothesized that cross-species transmission of an ancestor virus from another host into gibbons and koalas had occurred. The relatively recent introduction of KoRV into the koala genome and the apparent absence of GALV in wild gibbons suggest that this ancestor virus or a close relative may still be in circulation. Investigation into the nature of this ancestor virus may provide insights on the impact of KoRV on declining koala populations and will also broaden our understanding of host-virus coevolution. A variety of mammalian species have been identified to harbor GALV-like viruses, but the true host of the ancestral virus of KoRV and GALV remains uncertain. Here we provide a short history of the most prominent candidates: rodents and bats.

Introduction

The isolation of koala retrovirus (KoRV) in 2000 instigated one of the most intriguing mysteries in retrovirology (Hanger *et al.*, 2000). The virus had a very high sequence identity and phylogenetic relationship with gibbon ape leukaemia virus (GALV), which had been identified in captive white-handed gibbons (*Hylobates lar*) in the 1970s. The close relationship between these viruses indicated that cross-species transmission had likely occurred. However, the two species (koalas and gibbons) are evolutionarily and geographically distant (Fig. 1), thus the direct transmission of virus between the species seemed improbable. Researchers hypothesized that these viruses were introduced into each species via another host. The debates over identifying the precursor virus and the original reservoir host continue.

Based on published phylogenetic analysis of the GALV-KoRV clade, both bats and rodents are host to viruses in basal and crown positions (Greenwood *et al.*, 2018). However, what makes the rodent reservoir more prominent is the fact that 53% of all gammaretroviral-derived endogenous retroviruses (ERVs) are shown to have rodent origins (Hayward *et al.,* 2013). It has been proposed that while bats are highly capable recipients of cross-species retrovirus transmission events, rodents are more commonly the originator of these events (Cui *et al.,* 2015); for example, murine retrovirus transmission to porcine endogenous retrovirus (PERV) (Denner, 2007) and the likely tree shrew origin of *Rhinolophus ferrumequinum* retrovirus (RfRV) found in the greater horseshoe bat (Cui *et al.*, 2015).

Here we summarize the history of GALV and look into two prominent candidates for the "ancestor" of the GALV-KoRV clade: rodents and bats.

Keywords: endogenous retrovirus (ERV), cross-species transmission, koala retrovirus (KoRV), gibbon ape leukemia virus (GALV), woolly monkey virus (WMV), flying fox retrovirus (FFRV)
ORCID iD: Saba Mottaghinia 0000-0002-9409-3302 | Lee McMichael 0000-0002-7675-4859 | Joanne Meers 0000-0001-9305-0469
Corresponding author: Joanne Meers j.meers@uq.edu.au
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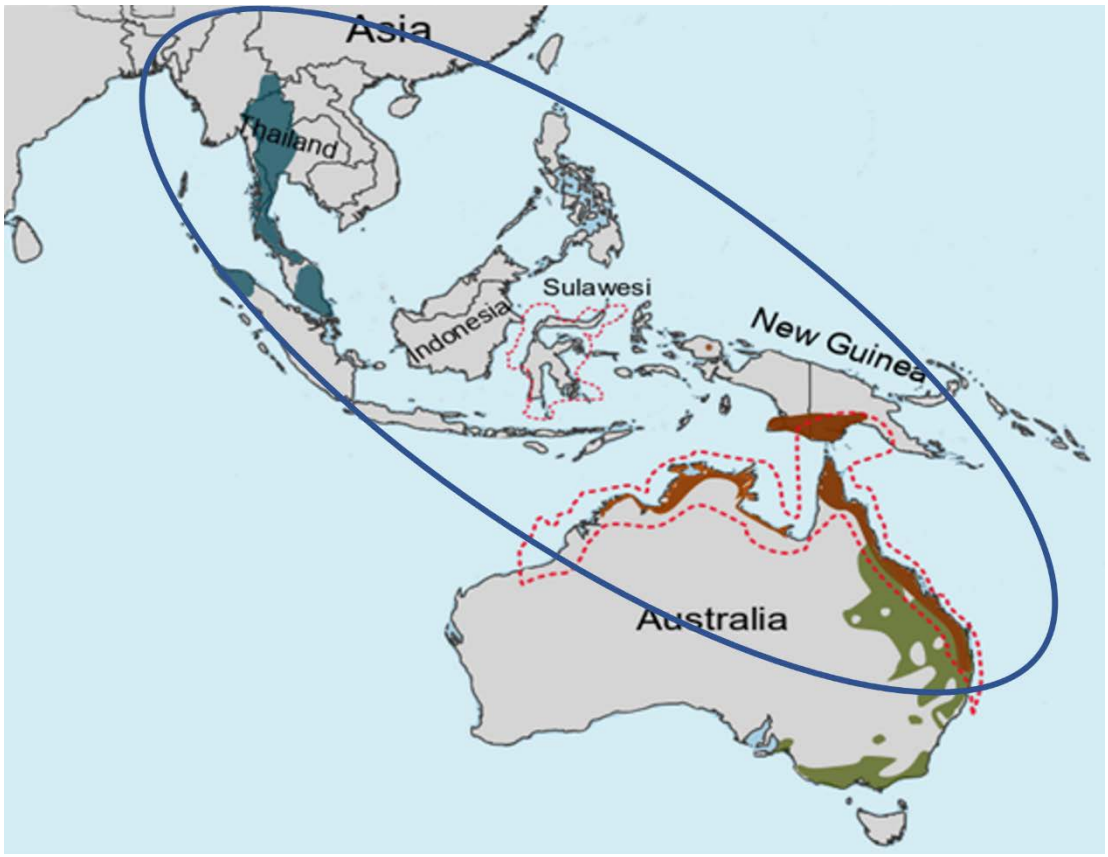

Figure 1. The approximate geographic distribution of white-handed gibbon (teal shade), *Melomys burtoni* (brown shade), koala (green shade), and *Pteropus alecto* (which includes *P. conspicillatus*) (red dotted line) that harbor GALV-SEATO, MbRV/MelWMV, KoRV and HPG/FFRV viruses, respectively. The distributions of other bat species harbouring KoRV/GALV-like viruses lie within the solid blue line, comprising *Synconycteris australis* (northern Australia, Papua New Guinea, Indonesia), *Macroglossus minimus* (northern Australia, PNG, Indonesia, SE Asia), *Hipposideros larvatus* (SE Asia, Indonesia). The distributions are based on the International Union for Conservation of Nature Red List of Threatened Species (https://www.iucnredlist.org/). The base image was generated using the free open source QGIS.

Gibbon ape leukaemia virus (GALV)

GALV is an exogenous retrovirus with oncogenic potential (Kawakami *et al.*, 1980). There are five recognized strains of GALV (Alfano *et al.*, 2016a) , including the initial isolates of GALV from cases of lymphoid neoplasia in captive gibbons at research facilities in Bangkok (GALV-SEATO) and in San Francisco (GALV-SF). The virus was subsequently detected in captive gibbons at other locations in the USA (GALV-Br) and in Bermuda (GALV-Hall's Island), and in cultured cells (GALV-X). Woolly monkey virus (WMV), which was isolated from a woolly monkey (*Legothrix lagothrica)* that had been housed with a GALV-infected gibbon, clusters phylogenetically with the five GALV strains (Alfano *et al.*, 2016a).

GALV infection (either virus or antibodies) has never been reported in wild gibbons. There has been no definitive evidence of GALV infection or GALV-induced disease in captive gibbons for almost 40 years (Brown & Tarlinton, 2017; McKee *et al.*, 2017), although a serological study in 2015 detected GALV antibodies in 21 out of 76 captive gibbons in North America (Siegal-Willott *et al.*, 2015). It has been suggested that the circulation of GALV in captive gibbons in the 1970s stemmed from an initial transmission event, mostly likely at SEATO in the mid to late 1960s, followed by transportation of gibbons from that region to research facilities in North America (Brown & Tarlinton, 2017; McKee *et al.*, 2017). The nature of the transmission event remains uncertain but was probably either iatrogenic inoculation of gibbons with material derived from humans and other species, or direct contact between gibbons and rodents, which were held in large collections at SEATO (Brown & Tarlinton, 2017).

Rodents as a plausible source or intermediate host to GALV-KoRV clade

Following the initial discovery of GALV, related retroviruses were detected in native Asian rodents, including *Mus caroli, Mus cervicolor*, *Vandeleuria oleacea* and *Mus dunni* (now *Mus terricolor*) (Lieber *et al.*, 1975; Callahan *et al.*, 1979). However, the techniques used at that time (serology and DNA hybridization) were of relatively low resolution. More recent work including sequencing, phylogenetic analysis and receptor usage of these rodent viruses has revealed that they cluster separately to the GALV-KoRV clade, and that although there is some relationship, they are not close enough to be considered the origin of GALV (Hayward *et al.*, 2013; Brown & Tarlinton, 2017).

In 2014, a novel virus sequence that clustered with GALV and KoRV was reported in a native Australian rodent, the grassland melomys (*Melomys burtoni*) (Simmons *et al.*, 2014). The *Melomys burtoni* retrovirus (MbRV) sequence was present in all 17 animals examined suggesting a likely endogenous virus. The unsuccessful attempts to isolate the virus in cell culture and the inability to detect expression of viral RNA in the animals provided further evidence of the endogenous nature of the virus.

After MbRV identification in Australia, Alfano *et al.* (2016b) screened 26 Southeast Asian rodent species. This

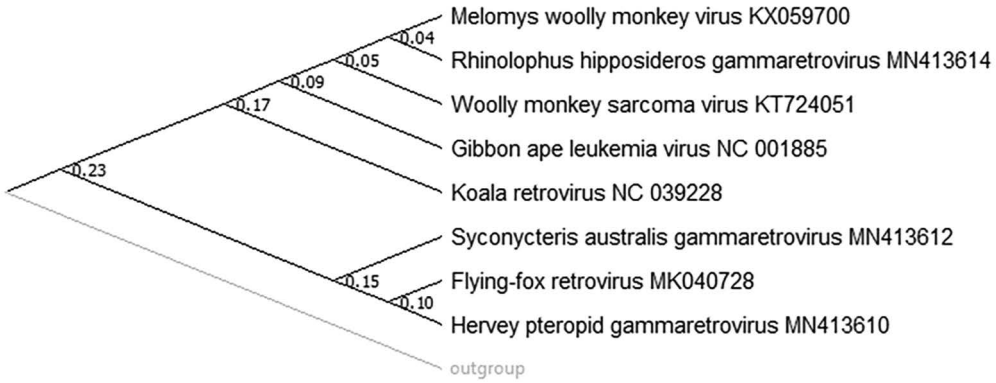

Figure 2. Time tree evolutionary analysis of representative KoRV and GALV-like retroviruses in the Australian and Asian regions. Generated using the RelTime method (Tamura *et al*., 2012). Divergence times for all branching points in the topology were calculated using the Maximum Likelihood method and JTT matrix-based model (Jones *et al*., 1992). The estimated log likelihood value of the topology shown is -7673.45. The tree is drawn to scale, with branch lengths measured in the relative number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

study resulted in identification and characterization of Melomys woolly monkey virus (MelWMV) in another *M. burtoni* subspecies in Maluku Island of Indonesia that with 98% nucleotide similarity nested within GALVs as a subspecies of WMV (Alfano *et al.*, 2016b). The single integration event of MelWMV into *M. burtoni* subspecies, is a defective ERV that has endured large deletions in the *pol* (corresponding to reverse transcriptase domain), *env* and *gag* genes. This suggests MelWMV is no longer capable of producing viral particles nor re-integrating into the host genome without a helper virus, a classic characterization of an ERV.

Although *Melomys* is currently confined to the Australo-Papuan region, this paraphyletic group along with 64 rodent species native to Australia, descends from a mixture of southeast Asian and Australo-Papuan "old endemic" rodents (Rowe *et al.*, 2008; Bryant *et al.*, 2011; Geffen *et al.*, 2011; Fabre *et al.*, 2018) (Fig. 1). Where there is no evidence of endogenization of GALV-KoRV-like sequences in bats, *Melomys* seem like a plausible source or intermediate host that shares a deep history with this viral group and their respective vertebrate hosts.

Bats and the evolution of GALV-KoRV like retroviruses

The recent characterization of novel gammaretroviruses with potential evolutionary relationships to GALV- and KoRV-like retroviruses in chiropteran species (bats) is not surprising, as bats are known reservoirs for many viruses, and the finding supports the suggestion that there may be several origins of retroviruses in bat species (Cui *et al.*, 2012a). Recent reports of gammaretroviruses in a variety of bat species inform investigations into the evolutionary origins of GALV and KoRV. These viruses include flying fox retrovirus (FFRV) variants in *Pteropus alecto* (McMichael *et al.*, 2019) and *P. conspicullatus* (McMichael *et al.*, unpublished data); Hervey Pteropid Gammaretrovirus (HPG), MmGRV and SaGRV from the Australian bat species *P. alecto*, *Macroglossus minimus* and *Syconycteris australis* respectively; and HlGRV and RhGRV, from the Asian bat species *Hipposideros larvatus* and *Rhinolophus hipposideros,* respectively (Hayward *et al.*, 2020).

The presence of intact open reading frames of FFRV (McMichael *et al.*, 2019) and the infectivity of HPG viral constructs (Hayward *et al.*, 2020) suggests that the flying fox retroviruses, FFRV and HPG, may be exogenous and infectious in nature. The hypothesis of "species jumping" of exogenous retroviruses closely related to GALV and KoRV (Simmons *et al.*, 2014; Greenwood *et al.*, 2018) suggests that the most likely candidate species of transmission are those species that transit between the Australian mainland and southeast Asia, with geographic ranges and feeding ecology that may result in close contact with both gibbons and koalas (Fig. 1). It has been suggested that bats that harboured distinct gammaretroviruses may have played an important role as reservoir hosts during the diversification of mammalian gammaretroviruses, and that bat retroviruses are not constrained by geographic barriers (Cui *et al.*, 2012a; Cui *et al.*, 2012b). Denner similarly suggests the hypothesis that retroviruses of bats are the origin of GALV and KoRV, which also deserve consideration (Denner, 2016).

Notwithstanding these hypotheses, molecular clock and phylogenetic analyses (Fig. 2), shows that the novel gammaretroviruses found in Australian megabat species from the genera *Pteropus*, *Macroglossus* and *Syconycteris*, are a divergent evolutionary lineage to that of GALV, KoRV and the KoRV-GALV-like Asian bat and rodent clades of gammaretroviruses. While the relationship between the KoRV and GALV-like gammaretroviruses is still unclear, it is likely that these retroviruses may have an unknown common ancestor. Thus, further investigation into the diversity of gammaretroviruses in Australian and Asian bat species may elucidate their evolutionary origins.

Conclusion and future aspects

Although retroviruses that are closely related to GALV and KoRV have been described in a variety of rodent and bat species, the definitive ancestral virus of both GALV and KoRV remains uncertain. Debate on the identity of the host of this ancestral virus continues, as does the question of whether this virus is still circulating in its host or has become a defective endogenous element.

The consensus for further retroviral screening of the rich

biodiversity within this region is clear, specifically *Melomys* and bat species across their biogeographical ranges. Different characteristics of these species, such as the short generation time of rodents and the unique intrinsic immunity of bats to viral infection, provide diverse opportunities to study the intriguing history of this group of viruses.

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The Koala Retrovirus: Lessons Learned from the Koala Genome

David E. Alquezar-Planas

Australian Museum Research Institute,
Australian Museum, Sydney NSW 2010, Australia

Abstract. The establishment of the Koala Genome Consortium in 2013 culminated in the publication of the first fully assembled koala genome. An international initiative involving 29 institutes across the globe, the publication has led to a much greater understanding of koala biology including knowledge on gene families putatively associated with detoxification of eucalypt leaves and the species' ability to taste and smell plant secondary metabolites. Similarly, the genomic resource has enabled comparative assessments facilitating immunogenomics, population genomic analysis, and, for the first time, genome-wide assessments of the koala retrovirus (KoRV). This summary outlines how the koala genome has increased our capacity to understand the genetics of KoRV—from a deeper understanding of KoRV viral subtypes and their recombinants to preferences for viral integration across the host genome.

Introduction

The koala (*Phascolarctos cinereus*) is an arboreal marsupial species that is endemic to the eastern Australian mainland and is the only living representative of the family Phascolarctidae. Having a unique biology, koalas are characterized by their evolutionarily unique physiological adaptations, such as their capacity to thrive almost exclusively on the consumption of eucalyptus leaves (Moore & Foley, 2000). In recent years, koala populations have experienced significant declines, which have been attributed to a range of factors including widespread habitat loss through land clearing and extreme climactic conditions such as those preceding and associated with the 2019–2020 summer bushfires (Phillips *et al*., 2021). Susceptibility to various infectious diseases such as chlamydiosis and potential pathogens such as the koala retrovirus (KoRV), has created additional selective pressures that collectively have impacted most koala populations to some degree. The multifactorial nature of these declines has underpinned the complexities of managing the species, particularly as populations across the range are threatened through a combination of these different factors.

Considering these widespread declines, the Koala Genome Consortium was established with the purpose of generating the first high-quality koala genome assembly to be used as a resource by researchers to enact measurable conservation outcomes (Johnson *et al*., 2014). The culmination of this work offers multiple insights into the species (Johnson *et al*., 2018), but additionally provides a unique resource for comparative genomic applications, including the study of KoRV, found across the koala genome.

KoRV is a gammaretrovirus that is in the process of endogenization across the koala genome. Endogenous retroviruses (ERVs) descend from exogenous retroviruses that infected a host germline and have since propagated through vertical transmission via parent to offspring. While most ERVs colonized their host genomes millions of years ago, KoRV is estimated to have entered the koala germline much more recently (Ishida *et al*., 2015) and may spread through either vertical or horizontal transmission. Belonging to the Retroviridae family of viruses, KoRV replication commences with the conversion of retroviral RNA via reverse transcription into double stranded DNA within the host cell. The viral DNA subsequently becomes integrated

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ORCID iD: David E. Alquezar-Planas https://orcid.org/0000-0001-5360-5263
Corresponding author: David E. Alquezar-Planas David.Alquezar@Australian.Museum
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into the host genomic DNA and inevitably forms a permanent alteration that may be studied through the koala genome.

Advancing our knowledge of KoRV through use of the koala genome or koala genome resources

A mere 10 years ago, KoRV sequence diversity was assumed to be comprised of a single genetic subtype, endogenous KoRV-A (Quigley & Timms, 2020). However, the years that followed outlined a much more complex evolutionary picture of KoRV diversity, including the identification of various other subtypes, such as exogenous KoRV-B, which utilizes a different receptor binding domain (THTR1) than does KoRV-A (Pit1) (Xu *et al*., 2013). Wider adoption of high-throughput sequencing applications has also aided in our understanding of KoRV sequence diversity. While much of the KoRV provirus has remained remarkably conserved, most sequence diversity has been characterized across the *env* hypervariable region within the receptor binding domain used for mediating cellular infection (Chappell *et al*., 2017; Sarker *et al*., 2021).

Despite these advances, the lack of a koala reference genome has complicated the ability to pair positional information within the host with KoRV sequence diversity. KoRV analysis is further compounded by the limited diversity across viral genes and the repetitive Long Terminal Repeat sequences that are characteristic of retroviral elements, which make sequence assembly using short-read applications methodologically challenging. Thus, while KoRV diversity could be characterized, genetic insights into KoRV have been done so in aggregate, where KoRV reads (potentially originating from various KoRV proviruses across the genome) are mapped to a full length assembled provirus (Löber *et al*., 2018). In this manner, assembling specific KoRV-like proviruses, pinpointing the genomic location of these integrants, and studying the effects that these integrations may have conferred to the host, was not possible.

In the past five years, through the analysis and utility of the koala genome, several studies have expanded our knowledge of KoRV that would otherwise not have been possible without access to this resource. Notably, a study by Hobbs *et al*., outlined the first comprehensive picture of full length endogenous KoRV proviruses within a single koala, achieved through the analysis of long PacBio sequence reads later used to assemble the first koala genome (Johnson *et al*., 2018). Analysis of the sequencing reads provided several additional insights including positional data on integration sites across the genome; the characterization of a newly identified endogenous recombinant retroelement termed recKoRV—the result of a recombination of an older ERV termed *Phascolarctos* endogenous element (PhER) and KoRV; and putative evidence of somatic cell integration by exogenous KoRV (Hobbs *et al*., 2017).

A key area of KoRV research and retrovirology that has flourished with access to koala genome resources is the study of viral integration sites. As a young retrovirus, integration site analysis of KoRV provides a unique opportunity to study retroviral endogenization within a mammalian host in real-time. As a North-South cline to viral infection appears the most likely explanation for KoRV infection and expansion across the koala genome; the resource has provided opportunities to study KoRV integration patterns across time through the analysis of historical and contemporary museum specimens (Cui *et al*., 2016).

Previous studies have shown that retroviral genera display differing integration site preferences (Kvaratskhelia *et al*., 2014). However, while integration into a specific genomic locus is random, retroviruses within the same family are statistically more likely to integrate within specific host genome features (Lafave *et al*., 2014). The recent development of a novel genetic assay termed sonication inverse PCR (SIP) has aided integration site analysis, particularly when coupled with long-read PacBio sequencing and comparative assessment to the koala genome (Alquezar-Planas *et al*., 2021). The tool was successfully applied to comprehensively compare KoRV and recKoRV integration sites of an unrelated koala to the reference genome. In doing so, the role that older ERVs play in the disruption and remobilization of active retroviruses like KoRV at the earliest stages of endogenization within the koala genome was able to be examined (Löber *et al*., 2018).

Another application of viral integration site analysis made possible through comparative assessment to the koala reference genome is the study of pathogenesis. Insertional mutagenesis mediated through viral integration is one of several known mechanisms by which a retrovirus may cause cancer in its host. These integrations may result in several deleterious effects, including the disruption of oncogenes and the up or down regulation of gene expression (Bushman, 2020). Like several other gammaretroviruses with known oncogenic capacity, KoRV has been long suspected of increasing cancer prevalence in koalas, particularly as lymphomas and leukaemias occur in high prevalence across the species. Through the analysis of paired healthy and neoplastic tissue from 10 koalas, a recent study by McEwen *et al*. (2021) provided the first supportive evidence of KoRV to underlie elevated cancer rates in koalas. The analysis of the paired tissue provided support for the identification of up to 172 integration sites uniquely found within the neoplastic tissue but absent in healthy tissue. Through the analysis, evidence for KoRV involvement in cancer development via different viral mechanisms are proposed (McEwen *et al*., 2021).

Conclusions and future perspectives

Through the analysis of koala genome reads, or the comparative assessments of proviral KoRV mapped back to the koala genome, the last five years has uncovered insights into KoRV biology, KoRV subtype diversity and the effects of viral integration on putative disease manifestations. With the decreased cost of sequencing technologies, the complete sequencing and annotation of hundreds of koala genomes across the species range is not far away. In fact, a large whole genome sequencing project lead by the University of Sydney with funding support from the NSW and the Australian Federal Government is presently underway and seeks to achieve this. Over the coming months, 450 koala genomes sequenced from across the species range will be uploaded into the public domain to support vital genomics research. The resource will enable key questions on koala biology, health and disease, and adaptations to climate change (among others) to be explored. The comparative assessment of this data is likely to provide further insights into KoRV that presently remain poorly understood. Retroviral infection processes, used by KoRV to propagate and spread, are one such area. Evidently, while some KoRVs are transcriptionally active, others are defective (as characterized by the disruption of open reading frames) but may still remobilise through

various mechanisms such as retrotransposition. Also, a virus in the process of endogenization is likely to propagate and spread using a broad range of mechanisms that differ to one that is exclusively exogenous or has seemingly reached equilibrium within its host. The complex evolutionary processes undergone by KoRV since its emergence and spread throughout the koala host is likely only to be fully uncovered through its analysis across multiple genomes, especially in related animals but also throughout its range. Another area of knowledge likely to continue to grow is our understanding of KoRV sequence diversity across the species. Recent technological developments have already enabled a much deeper understanding of viral subtypes that can be pinpointed to specific locations across the genome. Access to these same resources is likely to expand on how KoRV integrations may contribute to other diseases via immune modulation, either individually or in conjunction with other infectious agents. The years that follow are likely to provide fertile grounds for uncovering KoRV mysteries that would not otherwise be possible without access to the first koala genome.

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Defining Putative Koala Retrovirus-Associated Disease in Koalas

Amber K. Gillett

Australia Zoo Wildlife Hospital, Beerwah QLD 4519, Australia

Abstract. Koalas suffer from a wide range of diseases and illness, some of which are well understood, and others that are observed but have unclear aetiologies. A largely undescribed and poorly defined area in koala health is diseases presumed to be associated with koala retrovirus (KoRV) infection. Disease conditions putatively linked to KoRV infection are defined here as "putative KoRV-associated diseases" (PKAD). These include neoplasia, severe dermatological and oral conditions, life-threatening fungal and opportunistic infections, haematological disorders, chronic ill-thrift or poor body condition of undefined cause and other conditions suggestive of immune dysfunction. Multiple conditions are usually present at once and koalas invariably die despite treatment. The multifactorial nature of PKAD and the lack of clarity around KoRV's role in many conditions means that developing a standard case definition encompassing all presentations is difficult. As such, presenting conditions have been defined as dysplastic/neoplastic versus those associated with immune dysfunction (putative immune dysfunction disorders—PIDDS).

Introduction

Koala retrovirus (KoRV) is present in almost all koalas (*Phascolarctos cinereus*) throughout Australia as both endogenous (integrated into the germ line and heritable) and exogenous (replication-competent, transmissible) virus. KoRV subtypes A-M exist, with only the subtype A showing evidence of endogenization and being ubiquitous in koalas from Queensland (QLD) and New South Wales (NSW) (Quigley & Timms, 2020; Blyton, Young, *et al*., 2022). Koalas in South Australia and Victoria do not appear to have endogenous forms of KoRV; however, there is evidence of recombinant variants of KoRV (recKoRVs) across the koala's range which are thought to be largely defective, or non-replication competent (Löber *et al*., 2018; Tarlinton *et al*., 2022). Exogenous forms of KoRV have been detected across the koala's range and there is mounting evidence to suggest that viral load may be an important factor to consider when investigating links to disease in koalas (Maher *et al*., 2019; Fabijan *et al*., 2020; Quigley & Timms, 2020; Blyton, Pyne, *et al*., 2022).

Koalas suffer from a wide range of diseases and illness, some of which are well understood and others that have unclear aetiologies. Diseases presumed to be associated with KoRV infection are a largely undescribed and poorly defined area in koala health. This knowledge gap is partly due to our poor understanding of how KoRV might act as an aetiological agent, but also the need to clearly define what constitutes KoRV-associated disease. Diseases putatively linked to KoRV infection are defined here as "putative KoRV-associated diseases" (PKAD).

Putative KoRV-associated diseases comprise a suite of conditions that present in koalas similarly to those caused by other pathogenic gammaretroviruses which affect other species (e.g., feline leukaemia virus, murine leukaemia virus, gibbon ape leukaemia virus) (Hanger & Loader, 2014). Examples of such conditions include leukaemia, lymphoma, aplastic anaemia, tumours, and immunodeficiency disorders (Beatty, 2014; MacLachlan & Dubovi, 2017). Despite compelling similarities between disease presentations in koalas and other species affected with gammaretroviruses, further research is required to substantiate a causal link

Keywords: koala retrovirus, KoRV, leukaemia, myelodysplasia, ill-thrift, neoplasia, dermatitis, putative KoRV-associated disease (PKAD), immune dysfunction
ORCID iD: Amber K. Gillett https://orcid.org/0000-0002-7163-1761
Corresponding author: Amber K. Gillett amber@wildlifewarriors.org.au
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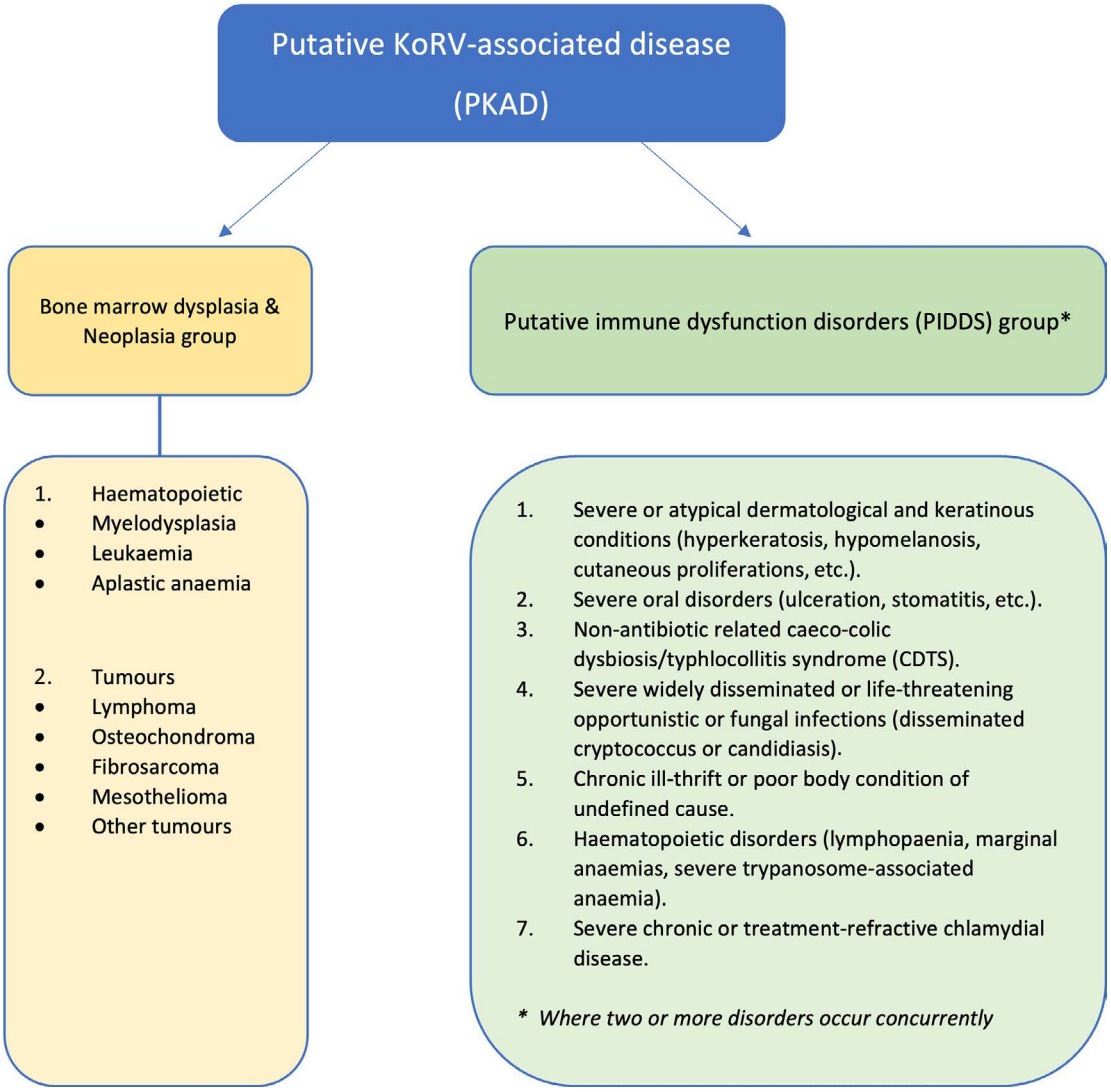

Figure 1. PKAD can be separated into two clearly defined groups: the Bone marrow dysplasia and neoplasia group and disorders associated with immune dysfunction, termed PIDDS. A presumptive classification of PIDDS should be considered in koalas concurrently afflicted by two or more conditions from the PIDDS group.

between KoRV infection and these conditions.

To aid in defining PKAD, these conditions can be separated into two groups (Fig 1): conditions clearly defined as bone marrow dysplasia and neoplasia, and conditions that reflect immune dysfunction, dysregulation, or disruption of normal cellular function, termed "putative immune dysfunction disorders" (PIDDS).

This manuscript aims to build on already published literature by Hanger & Loader (2014) outlining a suite of conditions that fall under the banner of PKAD.

Bone marrow dysplasia and Neoplasia group

Until recently, KoRV's role in the development of neoplasia was speculative, at best. However, in 2021 McEwen *et al*., identified KoRV-A genomic integrations near oncogenes and established that there was dysregulation of genes in koalas affected by leukaemia and a variety of tumours (McEwen *et al*., 2021). This provides the most compelling evidence to date that KoRV integration (at least for subtype A) probably leads to neoplasia in koalas.

Virtually all neoplastic presentations in koalas are fatal, either via expansion and compression of surrounding structures impeding function or through carcinogenesis. Hanger & Loader (2014) provide a comprehensive review of neoplastic conditions observed in koalas, describing in detail leukaemia, lymphoma, osteochondroma, fibrosarcoma and mesothelioma. Many other types of neoplasia have been reported in koalas (Gillett, 2014; Tong, 2019) but their relationship with KoRV infection still requires investigation.

Bone marrow dysplasia of humans is referred to as "myelodysplastic syndrome" or "myelodysplastic neoplasia" and is characterized by ineffective haematopoiesis or the failure of normal bone marrow stem cells to mature into normal functioning blood cells (Hasserjian, 2019; Sekeres & Taylor, 2022). Bone marrow dysplasia is often associated with peripheral cytopenias and morphologic dysplasia in haematopoietic elements and has an inherent tendency for leukaemic transformation (Gangat *et al*., 2016). Aetiological agents typically involved in bone marrow disorders of humans include: pharmaceutical drugs, manufactured toxins and chemicals, viruses, chemotherapy, radiation, congenital predisposition, idiopathic aplastic anaemia, and germline variants in haematopoietic stem cells (Shahidi, 1990; Sekeres & Taylor, 2022). Bone marrow dysplasia in koalas presents as a similar spectrum of haematopoietic disorders to those described in humans, with diagnoses in koalas usually assigned as aplastic anaemia, leukaemia or myelodysplasia.

Definitive causes of bone marrow dysplasia in koalas are unknown, though genetic inheritance, KoRV-induced germline variants in haematopoietic stem cells and exogenous viral infection remain plausible aetiologies. Antimicrobials such as chloramphenicol may induce short-term aplastic anaemia in some koalas but is extremely rare. A bone marrow assessment guide for koalas is available to assist with diagnoses of bone marrow dysplasia (Gillett & Hanger, 2019).

The putative immune dysfunction disorders (PIDDS) group

Koalas in this group often present with chronic ill thrift and poor body condition and are suffering from a variety of disorders consistent with immune incompetence, suppression, dysregulation or dysfunction. It is possible that gene dysregulation, immune dysfunction from KoRV integration, or a direct immunosuppressive effect of the KoRV transmembrane envelope protein p15E is responsible for manifestations of PIDDS, but further investigation is required. Individuals affected by PIDDS often die prematurely, though it is not uncommon for some koalas to suffer repeated episodes or combinations of disorders for months to years before their demise. Koalas colloquially referred to as "poor-doers" or suffering from "wasting" syndrome might also fall into this group.

A diagnosis of PIDDS may be complicated by coinfecting pathogens and, in some cases, these may exacerbate disease severity. It is not always clear if coinfections are primary or secondary in nature. For example, Phascolarctid gammaherpesvirus 1 & 2 (PhaHV 1&2) has been identified more commonly in koalas affected with chlamydial disease (Vaz *et al*., 2019), and trypanosome infection in koalas may cause severe anaemia (regenerative), dullness, lethargy, anorexia, peritoneal effusions, and nervous signs (tremors and seizures). As such, when faced with conditions included in this group, clinicians should be cautious of arriving at a diagnosis of PIDDS without first carrying out comprehensive clinical and diagnostic assessments to investigate other feasible causes of disease.

A presumptive classification of PIDDS should be considered in koalas concurrently afflicted by two or more conditions described in the PIDDS group. For example, concurrent planar/plantar hyperkeratosis, oral ulceration and severe candidiasis; or severe ulcerative tongue lesions, stomatitis, oral candidiasis, marginal anaemia and hypomelanosis of planar and plantar surfaces.

Conditions within the PIDDS group have been separated into 7 subgroups. These include:

1 **Severe or atypical dermatological or keratinous conditions.**

Severe or atypical dermatological or keratinous conditions include cutaneous growth disorders and proliferations, giant cell dermatosis (Fig. 2A), hyperkeratosis of the planar and plantar surfaces (Fig. 2B), hypomelanosis of the planar and plantar surfaces (Fig. 2C), autoimmune mediated dermatitis such as discoid lupus erythematosus, allergic dermatitis and severe generalized dermatitis which may or may not be associated with primary or secondary fungal or parasitic infections (Fig. 2D).

Histopathological findings may include parakeratotic hyperkeratosis, epidermal hyperplasia and dysplasia, lymphoplasmacytic, lymphohistiocytic and neutrophilic infiltrates and interstitial fibrosis.

2 **Oral disorders in the form of severe gingivitis, periodontal disease and ulceration of the tongue, buccal surfaces and lips.**

Ulcerative lesions may appear as deep dry lesions along or at the base of the tongue (Fig. 3A) or buccal surface. Ulcers may be obscured by a layer of firmly adhered masticated leaf. Cracking and deep fissures around the commissures of the mouth are often observed (Fig. 3B). Advanced periodontal disease includes extensive gingival recession, particularly around the incisors, inflamed gingiva and purulent exudate from affected sites (Fig. 3C). Oral candidiasis is usually present and may extend into the oesophagus and around the oesophageal sphincter. Affected koalas often display ptyalism, leaf drop, and pain associated with eating.

3 **Non-antibiotic related gastrointestinal caeco-colic dysbiosis/typhlocollitis syndrome (CDTS).**

CDTS results in altered caeco-colic homeostasis, disrupted motility, chemical and epithelial function, altered water content, and inflammatory changes in the caecum and proximal colon (Gillett & Hanger, 2019). The aetiology behind CDTS is unclear and likely multifactorial but is commonly associated with antimicrobial use (Gillett & Hanger, 2019). However, a subset of captive or wild koalas will develop this syndrome in the absence any predefined risk factors.

Affected koalas develop diarrhoea, seemingly spontaneously, and suffer rapid weight loss, dehydration and have normal or reduced appetites. Affected koalas may be found suffering from these symptoms in the wild, often sitting low or at the base of a tree, or they may develop this condition in captivity and often die despite attempts at treatment. There is no prior history of antibiotic or antifungal use in these animals.

At gross necropsy the caecum presents with varying degrees of pallor and content consistency. In some cases, the caecal content may be liquid and malodourous but the mucobacterial lining remains intact (Fig. 4A). Other cases may show complete separation of the caecal mucobacterial layer (resulting in a translucent caecal wall) and either soft, firm or dry caecal content (Fig. 4B). Histopathology usually reveals lymphoplasmacytic infiltrates in the lamina propria and submucosal layers of the gastrointestinal tract.

4 **Severe life-threatening or widely disseminated opportunistic or fungal infections, including multifocal cryptococcal and candidal infections.**

Multifocal cryptococcal lesions may be observed in the long bones, pelvis, mandible, maxilla, soft tissue structures, and dermis. Severe systemic candida infections with infiltration of the oesophageal and gastrointestinal mucosal layers and myocardial micro-abscessation have been observed (Hanger & Loader, 2014).

5 **Chronic ill-thrift or poor body condition of undefined cause.**

Both adult and joey koalas may present with ill-thrift and poor body condition, with some joeys also failing to thrive during their development to

Figure 2. Severe and atypical dermatological presentations in koalas. (*A*) Cutaneous proliferations caused by giant cell dermatosis. (*B*) Hyperkeratosis of the plantar surface. (*C*) Hypomelanosis of the plantar surface. (*D*) Severe crusting and ulceration of the face and limbs with secondary fungal infection.

adulthood. The processes behind why a joey might fail to thrive are currently unknown and are likely to be multifactorial, though investigations in this area are currently underway (D. Higgins, *pers. comm*.). Intense plasma cell and other mononuclear cell infiltrates within the gastrointestinal lining have been identified in some koalas with ill-thrift (Hanger & Loader, 2014) and suggest an underlying aetiology.

6 Haematopoietic disorders including marginal anaemias, lymphopaenias, and severe anaemia associated with trypanosome infection.

Anaemia and lymphopaenia of undefined cause have been observed in koalas. Affected individuals may be in moderate to poor body condition and may or may not be suffering from other disease manifestations suggestive of immune suppression. Bone marrow cytology appears normal in these cases.

Several koalas have been observed with clinical signs consistent with trypanosome infection in other species including severe regenerative anaemia, dullness, lethargy, anorexia, peritoneal effusions, nervous signs (tremors and seizures), and irregular parasitaemia. Affected animals include joeys and adults. Koalas affected by neurological symptoms invariably die within days, whilst those showing regenerative anaemias only may recover with blood transfusions and supportive care.

Histopathology of neurological trypanosome infected cases revealed an intense lymphocytic/plasmocytic choroiditis, extensive lymphocytic infiltration of the meninges, marked perivascular lymphocytic infiltration and macrophage reaction within the choroid plexus which extended into the surrounding meninges. Adjacent cerebral and cerebellar vessels were congested, and free trypanosomes were observed in blood vessels.

Figure 3. Oral disorders that may be found in koalas with a presumptive diagnosis of PIDDS. (*A*) Deep ulceration of the tongue, which may be obscured by or impacted with masticated leaf. (*B*) Cracking and deep fissures around the commissures of the mouth. (*C*) Severe stomatitis and advanced periodontal disease. Candida may also be present in the mouth and oesophagus.

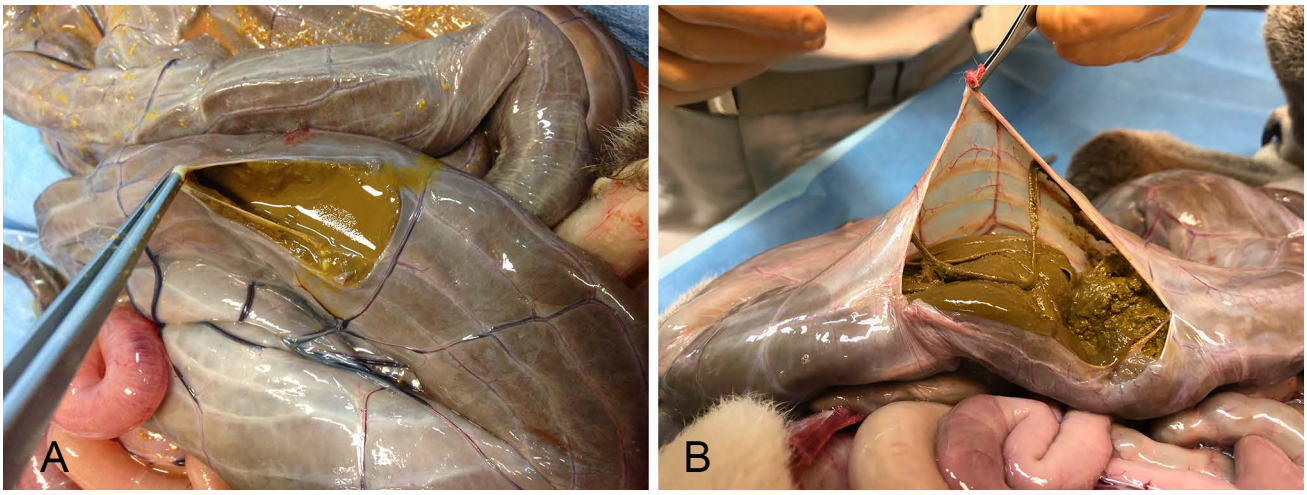

Figure 4. Pathological findings in koalas affected by caeco-colic dysbiosis/typhlocollitis syndrome (CDTS). (*A*) Liquid and malodorous caecal content with the mucobacterial lining grossly intact and an opaque caecal wall. (*B*) Extensive breakdown of the mucobacterial lining in a koala with chronic illness where the caecal content has fallen away from the mucosa entirely leaving a transparent caecal wall.

7 Severe chronic or treatment-refractive chlamydial disease.

Severe chlamydial disease is more common in northern koala populations with a positive association suggested between KoRV infection and chlamydial disease severity (Quigley & Timms, 2020). Other factors could contribute to chlamydial severity such as chlamydial virulence plasmids, environmental conditions affecting nutrition, and co-infection with other bacterial or viral pathogens.

Clinical and diagnostic evaluation

When aiming to establish a disease diagnosis in koalas, thorough physical examinations should be followed by comprehensive diagnostic tests (Gillett & Hanger, 2019). Diagnostic testing of any ill or compromised koala should include (at minimum) haematocrit, total plasma protein, blood film examination, bone marrow aspirate and cytological examination, abdominocentesis with cytological examination, urinalysis, abdominal ultrasonography, and full body radiographs. Additional tests could include a full haematology and biochemistry panel, blood gas analysis, and computed tomography or magnetic resonance imaging.

Most diagnostic techniques applied in koala medicine are similar to those in domestic species and can be easily performed by a skilled clinician. Details of techniques used in koalas have been described (Gillett & Hanger, 2019).

Discussion

Although the role of KoRV infection in the aforementioned conditions is still putative, there is growing evidence to suggest that it is highly likely that KoRV influences the immune system of koalas (Mathew *et al*., 2014; Higgins, 2019; Maher *et al*., 2019) and that viraemic load may be an important factor to consider.

Conditions within the PIDDS group may have multiple potential aetiologies, and at times affected koalas may appear to respond temporarily to targeted treatment. However, where multiple conditions are present in combination the likelihood of clinical resolution is extremely low, and koalas invariably die despite treatment. As access to molecular tests such as PCR for identifying KoRV subtypes become more widely available, there is the potential that clinicians may be tempted to infer a diagnosis or assign a prognosis based on a koala's subtype result. Given the complexity of KoRV's role in disease, the significance of a positive molecular KoRV test should always be viewed in light of the koala's clinical presentation, particularly until tangible evidence is found to link KoRV subtype or viral load to particular disease syndromes.

Thorough clinical and diagnostic evaluations are critical in disease diagnosis and where veterinarians are not intimately familiar with koala medicine and health, advice should be sought from those with experience in this area. It is recommended that fresh postmortem tissues (disease affected and unaffected) and whole blood in EDTA be collected and stored frozen (-80°C where possible) for future research into KoRV's relationship with clinical disease.

The multifactorial nature of PKAD and the lack of clarity around KoRV's role in many conditions means that developing a standard case definition encompassing all presentations is difficult. It is hoped that separating presentations into those defined as bone marrow dysplasia or neoplastic verses those associated with immune dysfunction will inform clinicians on a likely prognosis and assist with welfare and treatment choices. As research evolves and larger datasets are accumulated, it is anticipated that these two groups may be further defined.

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The Role of Koala Retrovirus Integrations in Promoting Neoplasia in Koalas (*Phascolarctos cinereus*)

Gayle McEwen[1] and Alex D. Greenwood[1,2]

[1] Department of Wildlife Diseases,
Leibniz Institute for Zoo and Wildlife Research, 10315, Berlin, Germany

[2] School of Veterinary Medicine,
Freie Universität Berlin, 14163, Berlin, Germany

Abstract. Koalas suffer unusually high rates of neoplasia. There has been a long-standing correlation between koalas with the koala retrovirus (KoRV) and development of neoplasia which has lacked a mechanistic explanation. We describe recent results that demonstrate that many KoRV integrations lead to neoplasia by (*I*) inserting into somatic cells preferentially near oncogenes, (*II*) inserting into germ cells near oncogenes predisposing koalas to cancer, and (*III*) transduction—replacing KoRV genes with oncogenes, resulting in drastic upregulation of the transduced gene. The high mortality associated with integration-driven promotion of neoplasia may explain the increased prevalence of dysfunctional recombinant KoRVs (recKoRVs), which, over time, could replace KoRV, thereby slowing the production of novel detrimental integration sites.

Introduction

Koalas develop neoplasia at rates at least an order of magnitude higher than humans and many other mammals (Gonzalez-Astudillo *et al.*, 2019). Approximately 3% of wild koalas from southeast Queensland brought into veterinary clinics had lymphoma and 7% had lymphoid neoplasms (Gonzalez-Astudillo *et al.*, 2019; Fabijan *et al.*, 2020). This is likely an underrepresentation as it is unlikely that wild koalas manifesting late stage leukaemia or lymphoma will be found when they die. In contrast, in zoos where observation of koalas is continuous, 25% developed lymphoma (Gillett, 2014). This tremendous cancer burden has been associated correlatively with koala retrovirus (KoRV) infection, either as a quantitative increase of KoRV expression in koalas suffering from neoplasms or as an increase in the number of variants of KoRV expressed (Tarlinton *et al.*, 2005; Quigley *et al.*, 2019). A causative relationship and mechanistic explanation for the elevated cancer risks faced by koalas with KoRV has not been provided until now.

In general, there are four major mechanisms by which KoRV as a gammaretrovirus could cause cancer in koalas: I. insertional mutagenesis in somatic cells, II. non-lethal insertional mutagenesis in the germline in or near genes that promote the development of cancer later in life, III. transduction of oncogenes whereby the retrovirus incorporates an oncogene sequence in its genome and expresses it above the normal host rate, and IV. immune suppression by expression of the immunosuppressive domain in the envelope protein preventing host immune cell recognition and clearing of cancerous cells. We have recently provided evidence supporting a role for I-III (McEwen *et al.*, 2021). Ten paired tumour and neoplastic tissues were obtained from koalas from Queensland, Australia. Seven of the animals died or were euthanized because of advanced lymphoma. Three animals, all from one population, died of osteosarcoma. The integration site (IS) profile for each tissue sample and assorted positive and negative controls was determined using a novel long inverse PCR approach followed by large fragment high throughput sequencing

Keywords: koala retrovirus, KoRV, koala lymphoma, koala leukaemia
ORCID iD: Gayle McEwen https://orcid.org/0000-0001-5134-1380 | Alex D. Greenwood https://orcid.org/0000-0002-8249-1565
Corresponding author: Alex D. Greenwood greenwood@izw-berlin.de
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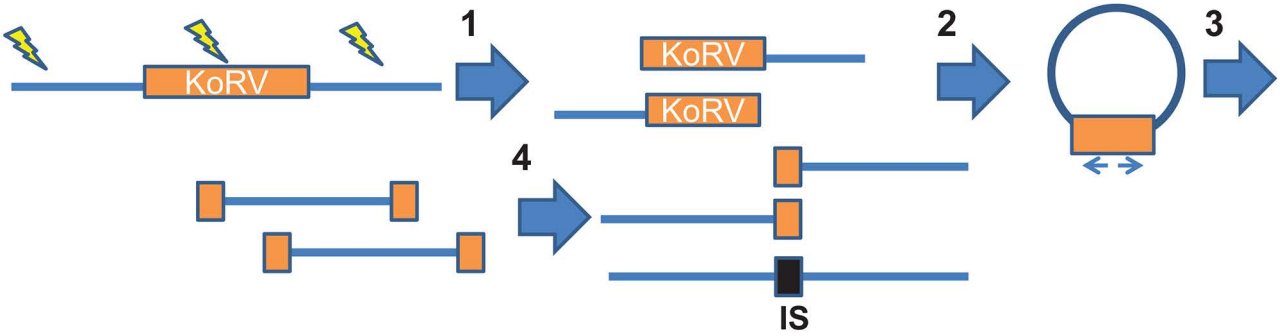

Figure 1. Summary strategy for comprehensive determination of integration sites by sonication inverse PCR (SIP). 1. DNA is randomly fragmented to an average size of 3 kb using high frequency sound waves. 2. The DNA molecules are turned from linear DNA into circular DNA. 3. Inverse PCR with primers based on the viral long terminal repeats (LTRs) are used to amplify fragments that extend partially into the virus and partially into the flanking sequence. 4. The resulting PCR products are sequenced on a long fragment high throughput sequencing platform and then mapped to the koala reference genome to identify the integration sites (Alquezar-Planas *et al.*, 2021).

(PacBio) called sonication inverse PCR (SIP) (Alquezar-Planas *et al.*, 2021). The end result of this sequencing is a comprehensive profiling of the endogenized KoRV IS in an average fragment size range of 2–3 Kb, yielding partial retroviral genome information at the 5' and 3' ends of the virus and several hundred to thousand base pairs of flanking sequence information. At higher sequencing depth, low coverage somatic cell integrations (which, unlike germline integrations, occur in only some cells of the body) can be detected (Fig. 1).

Mechanisms by which IS result in neoplasia

We detected 1002 unique IS among the 10 koalas. Of these 793 were endogenous retroviruses (ERVs), identified in both paired tissue types. There was an average of 100 KoRV ERVs per koala. The remaining IS were tumour specific (172) or healthy specific (37) representing somatic cell integrations. The vast majority of IS represented KoRV-A integrations and a smaller group of recKoRV integrations. KoRV-B and other variants were exceptionally rare among the individuals tested. In the neoplasms studied (also summarized in Fig. 2), KoRV IS were associated with the following mechanistic pathways:

I **Insertional mutagenesis**: The 172 tumour specific integrations identified were both associated with and enriched for genes known to be involved in the development of cancer (reaching statistical significance).

II **ERVs promoting cancer**: Of the ERVs identified, sharing of IS was directly correlated with spatial proximity to where the koala was sampled. Three koalas from one wild managed population (Lone Pine Sanctuary) all shared integrations in oncogenes associated with osteosarcomas, the type of cancer from which all three individuals died. Additional shared IS among the koalas in the data set were associated with oncogenes which may greatly increase the lifetime risk of developing cancer in a heritable manner. In both cases of somatic IS and ERV IS, multiple "hotspot" genes were identified where the same gene in several koalas had unique IS as either an ERV or a somatic integration. These were statistically significantly more likely to contain IS than other genes and the majority of these genes were known oncogenes such as c-myc and c-myb. In most cases, we observed that the IS near the various identified oncogenes promoted increased expression of these oncogenes, sometimes strongly increased above the level observed in koalas lacking such IS in the same tissues.

III **Transduction**: In one koala the KoRV LTRs and interrupted KoRV protein coding sequences flanked the *BCL2.1-xl* gene, a known promoter of invasion by various neoplasms (Trisciuoglio *et al.*, 2017). The oncogene interrupted most of the *env* gene of KoRV with the *gagpol* region experiencing deletions and interruptions by a small portion of the ZBTB18 gene. The result of this transduction was a greater than 500 fold increase in expression of *BCL2.1-xl*.

Somatic IS, ERVs and transduced oncogenes all resulted in increased expression of oncogenes in tumour tissues of affected koalas providing a molecular link between KoRV and cancer in koalas. This mutational excess likely puts enormous pressure on the koala host to purge deleterious KoRV IS from the population and on KoRV to attenuate. There is some evidence that this process is occurring and from a population genetic evolutionary perspective, quickly (Löber *et al.,* 2018).

KoRV recombinants (recKoRVs) and long term defence against novel IS

Most large-bodied species take several years to reach sexual maturity. In such species, neoplasms occurring before or during the optimal breeding years will likely be purged as individuals dying of cancer prior to having offspring, or having fewer offspring as a consequence, will be poorly or not represented in the subsequent generations. Thus, large-bodied long-lived species typically have ERVs which are highly degraded either by mutation, deletion or recombination preventing the expression of full-length viral transcripts or proteins (Katzourakis *et al.*, 2014). A class of recombinant KoRVs (recKoRVs) which replace much of the KoRV protein coding sequences with the highly disrupted genome of an ancient marsupial ERV (PhER) have been observed (Hobbs *et al.*, 2017; Löber *et al.*, 2018) (Fig. 3).

Some recombinant KoRV's, such as recKoRV1, have up to 10 copies, comprising approximately 10% of the total KoRVs in an individual. Seventeen distinct recKoRVs

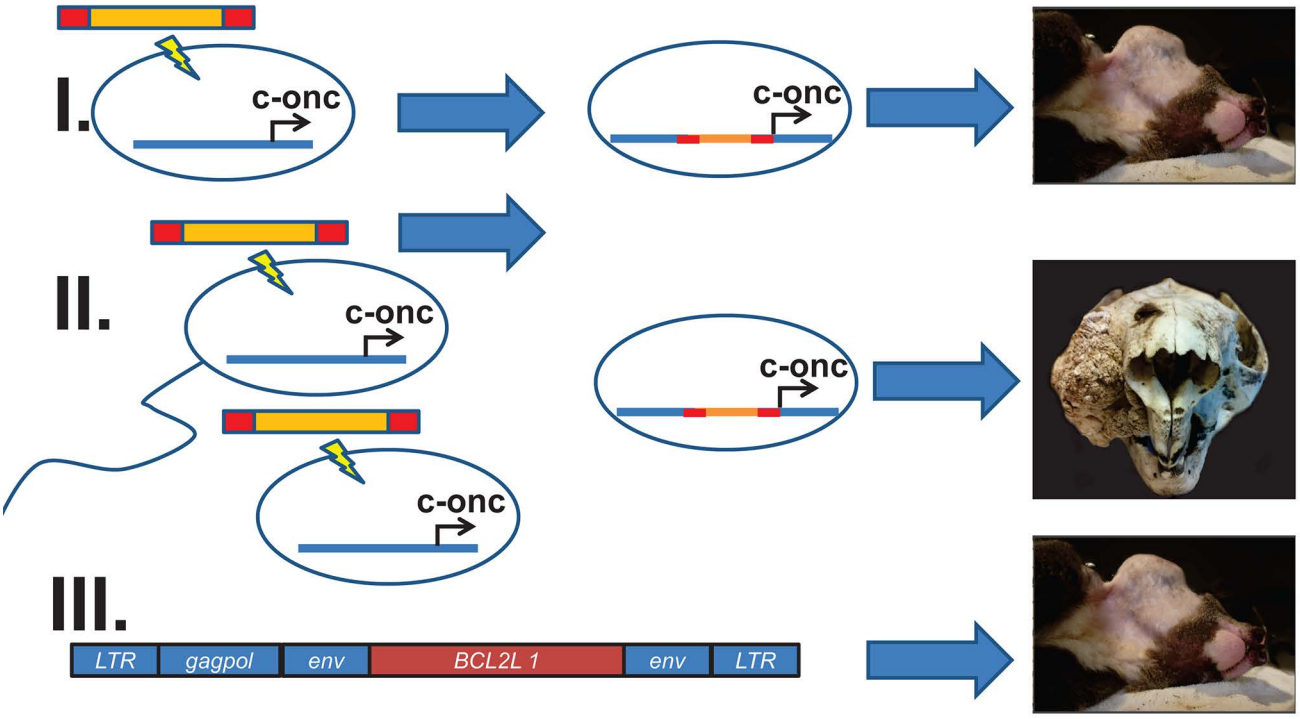

Figure 2. Three mechanisms identified whereby KoRV integrations or transduced KoRV lead to neoplasia (McEwen *et al.*, 2021). I. Somatic integrations lead to lymphoma II. Inherited KoRVs lead to development of osteosarcomas. III. Transduced KoRV results in lymphoma. Photographs kindly provided by Amber Gillett.

that had different spatial distributions, have been observed (Löber *et al.*, 2018). This suggests recKoRVs arise frequently and independently among koala populations. Over the long term, the more recKoRVs established in the koala genome, the fewer functional copies of KoRV will be available. As recKoRVs are likely dependent on KoRV for replication, it will become exceedingly rare for the recKoRV to mobilize autonomously. As recKoRVs are similar to disrupted endogenous retroviruses in other large-bodied species, it is likely that koalas are in a transition phase whereby intact retrovirus remains prevalent in the population, but the disrupted copies begin to increase in frequency, likely under strong selection. However, until competent KoRV becomes uncommon, both novel KoRV and recKoRV integrations will present considerable mutagenic and hence, cancer risks to koalas.

Practical aspects: selective breeding

While somatic mutations in captive populations cannot be controlled or predicted, selective breeding could help prevent or reduce the number of shared IS in oncogenes. This would require IS determination for the entire captive (or at least breeding) population of koalas, an undertaking that could be completed within a year. Selective breeding or introduction of wild koalas lacking specific IS could produce populations that have reduced numbers of ERVs in oncogenes that are heritable which may reduce, but not eliminate the elevated cancer risk. Somatic mutations will remain a problem. Monitoring of expression of specific genes with IS in existing koalas could potentially indicate if they are at risk of developing neoplasia with potential interventions that could be explored.

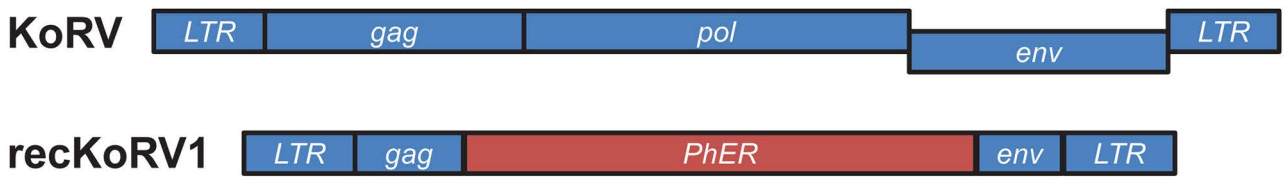

Figure 3. Structure of a common recombinant KoRV (recKoRV1). PhER is an ancient marsupial endogenous retrovirus that interrupts the KoRV genome removing much of the *gag* gene, all of the *pol* gene, and all but approximately 100 bp of the *env* gene. The LTRs and *gag* leader sequence remains intact (Löber *et al.*, 2018).

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Incidence, Trends, and Significance of Putative Koala Retrovirus-Associated Diseases in Monitored Wild Koala Populations in Southeast Queensland

Philippa A. McKay[1] and Brent D. Jones[2]

[1] Endeavour Veterinary Ecology Pty Limited, Toorbul QLD 4510, Australia

[2] Centre for Planetary Health and Food Security, Griffith University, Nathan QLD 4111, Australia

Abstract. Research indicates that northern koalas (*Phascolarctos cinereus*) are ubiquitously infected with koala retrovirus (KoRV). There is increasing evidence linking KoRV with neoplasia and a range of disorders associated with immunodeficiency, conditions observed at high rates in captive colonies, and sick koalas presenting to wildlife hospitals. However, less is known about the occurrence of these putative KoRV-associated diseases in wild populations. We analysed health data collected at the veterinary examinations of 691 koalas inhabiting three monitored wild koala populations in southeast Queensland between 2013 and 2020. At initial presentation, neoplasia and AIDS-like syndrome were detected at a prevalence of 1.16% (8/691; 95% CI 0.5–2.19%). Longitudinal data from koalas recruited into the monitoring programmes and receiving one or more subsequent examination revealed an incidence rate of 3.5 cases/100 koalas/year (95% CI 2.35–4.9). These findings indicate that a relatively small proportion of the populations studied were affected by these putative KoRV-associated diseases. However, the impact on individuals was severe, with high associated mortality in the diseased cohort. Furthermore, northern koala populations endure multiple threats, suffering severe declines in recent decades. We propose that the significance of putative KoRV-associated diseases on these populations should be considered within this context and that further research into the interactions between KoRV and other drivers of decline is warranted.

Introduction

Northern koala (*Phascolarctos cinereus*) populations in Queensland and New South Wales account for approximately two-thirds of the total range of this iconic native species and have suffered substantial declines in recent decades (McAlpine *et al*., 2015; Adams-Hosking *et al.,* 2016; Beyer *et al.,* 2018; Melzer *et al.,* 2000). Consequently, koalas in these regions were listed as "vulnerable" under the Australian Environment Protection and Biodiversity Act in 2012. Multiple threats have been implicated, including habitat loss and degradation, dog predation, vehicle strikes, bush fires, climate change and disease (Rhodes *et al.,* 2011; Beyer *et al.,* 2018; McAlpine *et al.,* 2015).

Koala retrovirus (KoRV) is highlighted as a major pathogen infecting koalas and receives ongoing attention for its suspected role in several diseases impacting populations (Quigley & Timms, 2020). KoRV-A is the endogenous form of this gammaretrovirus and is detected in 100% of northern koalas (Table 1). Other KoRV subtypes, designated KoRV-B through to KoRV-K, are believed to be exogenous, and have a much more variable prevalence geographically (Quigley & Timms, 2020; Joyce *et al.,* 2021). KoRV-B and KoRV-D are generally found to be the most predominant subtypes in southeast Queensland (SE QLD) koalas (Table 1).

Following infection, retroviruses insert into the host genome, with potentially mutagenic effects (Rabson & Graves, 1997). There is mounting evidence demonstrating

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ORCID iD: Philippa A. McKay https://orcid.org/0000-0003-2432-4811 | Brent D. Jones https://orcid.org/0000-0003-4256-3664
Corresponding author: Philippa A. McKay pip@endeavourvet.com.au
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Table 1. Prevalence of KoRV subtypes reported between 2012 and 2020 in wild koala populations in southeast Queensland (SE QLD). Dashes indicate that the relevant subtype was not tested for by that study. Koalas presenting to wildlife hospitals inhabited the Moreton Bay Region, Sunshine Coast, Gold Coast and Brisbane. *MBR* = Moreton Bay Region; *HV* = Old Hidden Vale Site located west of Ipswich.

Region in SE QLD	KoRV subtype % (number positive / number sampled)							References
	A	B	D	F	G	H	I	
Wildlife hospitals	100% (18/18)	78% (14/18)	94% (17/18)	44% (8/18)	11% (2/18)	6% (1/18)	6% (1/18)	Chappell *et al.,* 2017
MBR	100% (290/290)	29% (83/290)	—	—	—	—	—	Quigley *et al.,* 2018
MBR	100% (16/16)	25% (4/16)	88% (14/16)	25% (4/16)	0% (0/16)	0% (0/16)	0% (0/16)	Quigley *et al.,* 2019
Wildlife hospitals	100% (33/33)	100% (33/33)	100% (33/33)	0% (0/33)	33% (11/33)	0% (0/33)	97% (32/33)	Sarker *et al.,* 2019
MBR	100% (60/60)	40% (24/60)	97% (58/60)	77% (46/60)	2% (1/60)	0% (0/60)	0% (0/60)	Robbins *et al.,* 2020
HV	100% (20/20)	55% (11/20)	100% (20/20)	25% (5/20)	0% (0/0)	0% (0/0)	0% (0/0)	Robbins *et al*., 2020

a link between KoRV infection and neoplasia. Captive koala colonies suffer exceptionally high rates of neoplasia, particularly lymphoid neoplasms and leukaemia (Xu *et al.,* 2013; Gillett, 2014) and high rates are also reported in wild northern populations (Hanger & Loader, 2014; Gonzalez-Astudillo *et al.,* 2019; Fabijan *et al.,* 2020). Recently described KoRV proviral integration sites appear likely to influence the development of tumours (McEwen *et al.,* 2021). Plasma KoRV RNA levels are significantly higher in captive koalas with leukaemia or lymphoma (Tarlinton *et al.,* 2005) and a positive association has been demonstrated between neoplasia and both KoRV-B (Xu *et al.,* 2013; Quigley *et al.,* 2018) and KoRV proviral load (Sarker *et al.,* 2020). Associations have also been made between aspects of KoRV infection and certain chronic, severe diseases suggestive of dysfunction, dysregulation, or suppression of the immune system, including correlation between KoRV-B and overt chlamydial disease (Waugh *et al.,* 2017; Quigley *et al.,* 2018). An AIDS-like syndrome representing a suite of such conditions is recognized by clinicians and characterized in the literature (Gillett, 2014; Hanger & Loader, 2014; Quigley *et al*., 2018), although links to KoRV are currently largely putative.

Many of the studies investigating suspected clinical manifestations of KoRV infection have been conducted in captive koalas or sick individuals presenting to wildlife hospitals. However, there are far fewer data regarding the occurrence in longitudinally monitored, free-ranging koala populations. Our study seeks to quantify the potential impact of putative KoRV-associated diseases (PKAD) by examining veterinary records from 691 koalas inhabiting three monitored wild koala populations in SE QLD between 2013 and 2020. We estimate the initial prevalence detected when koalas enter the monitoring programmes. We then use longitudinal data to calculate incidence rates to establish the occurrence of KoRV-associated diseases over time. Finally, we examine variables of age, sex and familial trends and document outcomes for individuals in the diseased cohort. Our findings help to establish the significance of KoRV as one of many threats to the survival of koalas in SE QLD.

Materials and methods

Our study utilized three monitored wild koala populations in SE QLD. Moreton Bay sites 1 and 2 (MB1 and MB2) are located north of Brisbane approximately 20 km apart (27.2247°S 153.02°E and 27.3193°S 152.9571°E, respectively). Both are peri-urban/urban koala habitats composed of predominantly open eucalypt forest that has undergone varying levels of clearing and disturbance as part of infrastructure or extractive industry projects. The Old Hidden Vale site (HV) is geographically separated, located approximately 70 km away west of Ipswich (27.6594°S 152.4672°E). HV is rural koala habitat composed of grassland and open forest. All three are open populations, with new koalas recruited into monitoring programs over time as joeys or when moving into the area, and individuals are removed as they disperse or die.

Koalas were monitored using radiotelemetry and biotelemetry collars (K-Tracker, LX Group, Sydney) and tracked and sighted at least once every two weeks. Individuals underwent comprehensive veterinary examinations under anaesthetic approximately every six months, or more frequently for growing individuals or where health or welfare concerns were raised. Veterinary examinations were performed by veterinarians experienced with koalas and consisted of a full physical examination, including ultrasound of the urinary and reproductive tracts, and radiographs if indicated. Blood, urine, bone marrow aspirate, and peritoneal fluid were collected for cytological examination. All health data and clinical findings were recorded in a standardized database. Monitoring of activity data from biotelemetry collars and regular tracking of individuals enabled diseased koalas to be rapidly identified by field staff and recaptured for veterinary assessment. Similarly, deceased koalas were quickly located, and thorough necropsies performed.

We reviewed data for all koalas that had undergone at least one veterinary examination or necropsy. A case was considered positive for PKAD if consistent with one of the following categories: neoplasia, suspected but not confirmed neoplasia, and conditions suggestive of immune dysfunction or dysregulation (AIDS-like syndrome). Koalas were included in the AIDS-like syndrome cohort if they displayed two or more of the clinical signs outlined in Table 2. This is consistent with published inclusion criteria for this syndrome (Gillett, 2014; Hanger & Loader, 2014; Quigley *et al.,* 2018).

Initial prevalence was determined by the proportion of KoRV-associated disease cases identified at the initial veterinary examination upon entry into the monitoring program at each site. Incidence rate was then determined for the remaining susceptible koalas that underwent at least one subsequent veterinary examination, using the number of positive cases identified as the numerator and the total number of days all susceptible koalas were monitored for as the denominator. In the case of positive koalas, only the days monitored prior to being deemed positive were included in the calculation. The result was multiplied by 100 and 365 to give a rate of cases per 100 koalas per year. Sites were analysed as separate populations as well as the Moreton Bay region (MBR; MB1 and MB2) and SE QLD (MB1, MB2 and HV) combined.

Table 2. Clinical signs suggestive of immune dysfunction or dysregulation with examples. In our study, AIDS-like syndrome was diagnosed in koalas displaying two or more of these clinical signs.

Category of clinical signs suggestive of immune dysfunction / dysregulation	Examples of clinical sign
Dermatopathy	Generalized dermatitis, chronic otitis externa/media, paronychia
Oral lesions	Severe oral ulceration, severe periodontal disease
Chronic ill-thrift	Persistent or unexplained poor body condition
Fungal infections	Severe cryptococcosis
Severe, debilitating chlamydiosis	Severe chlamydiosis that fails to respond to treatment
Severe gastrointestinal disorders	Caeco-colic dysbiosis/typhlocolitis syndrome unrelated to antibiotic administration

The age and sex of koalas diagnosed with PKAD were analysed across all sites. Familial links were also explored in the MB1 population in a subset of koalas where the maternal lineage was known. The Winpepi software suite (Abramson, 2011) was used for all statistical analyses.

Results

Initial prevalence and incidence rate

The number of koalas subjected to an initial veterinary examination, those subjected to one or more subsequent examinations, and the duration of monitoring at each site are outlined in Table 3.

Six koalas at MB1 (n = 634) were diagnosed with PKAD at the first veterinary examination, giving an initial prevalence of 0.95% (95% CI 0.35–2.05%). Of these, one had lymphoma (16.7%), two had suspected myelodysplasia (33.3%) and three fell into the AIDS-like syndrome category (50.0%). Of the 541 koalas that went on to have subsequent examinations, PKAD was detected in 29 cases. These consisted of 11 cases of neoplasia (four lymphoma, one lymphoid leukaemia and lymphoma, three myelodysplasia, three osteochondroma), three cases of suspected neoplasia (two myelodysplasia, one lymphoma) and 15 cases of AIDS-like syndrome. The incidence rate was calculated as 3.4 cases/100 koalas/year (95% CI 2.29–4.89).

One koala at MB2 (n = 22) was diagnosed with neoplasia at initial veterinary examination, giving an initial prevalence of 4.55% (95% CI 0.12–22.84%). There were no cases of PKAD diagnosed in any of the koalas that went on to have subsequent examinations (n = 18).

One koala at HV (n = 35) was diagnosed with AIDS-like syndrome at the first examination, giving an initial prevalence of 2.86% (95% CI 0.07–14.92%). Of the 32 koalas that went on to have subsequent examinations, one was diagnosed with lymphoma and one with AIDS-like syndrome. The incidence rate was calculated as 5.3 cases/100 koalas/year (95% CI 0.73–18.98).

Excluding the geographically separate HV population, prevalence at initial examination (7/656) was 1.07% (95% CI 0.43–2.19%) and the incidence rate was 3.4 cases/100 koalas/year (95% CI 2.26–4.85) in the MBR (MB1 and MB2). Combining data from all three sites, prevalence at initial examination (8/691) was 1.16% (95% CI 0.5–2.19%) and the incidence rate was 3.5 cases/ 100 koalas/year (95% CI 2.35–4.9). The differences between the initial prevalence and incidence rates at each site were not statistically significant.

Age, sex, and familial variables

Across all three sites, there was no statistically significant difference between the number of male and female koalas presenting with PKAD (Upton's modified Chi-square test, $\chi = 0.004$; 1 degree of freedom; $p = 0.95$) (Campbell, 2007).

The mean age of diagnosis of neoplasia was 6.75 years across all sites. This was slightly lower for AIDS-like syndrome at 5.10 years. Koalas in this category presented over a wider range of ages (0.86–12.92 years) compared to koalas with neoplasia (3.83–11.00 years) (Fig. 1). The difference in means was not statistically significant.

Of the total 35 cases of PKAD identified in MB1, the dams of four individuals are known. The 21 female cases collectively had 29 joeys that were entered into the monitoring programs, and for which detailed health data are available. One potential familial link emerged from these data. An approximately five-year-old koala was diagnosed with abdominal lymphoma (Fig. 2) and her male offspring developed a pelvic osteochondroma at four years of age. As of the end of this study, her younger female offspring was alive, healthy, and continuing to be monitored. PKAD was not identified in any of the other 27 joeys, eight of which were still alive at the end of this study.

Outcomes

Of the 14 koalas diagnosed with neoplasia across all sites, 13 were humanely euthanized due to their disease presentation (92.9%). The mean survival time after diagnosis was 40

Table 3. The duration of monitoring and number of koalas sampled from each of the three sites (MB1, MB2, and HV).

Study site	Study duration	No. koalas given initial examination	No. koalas given one or more subsequent examinations	Mean no. of days monitored per koala
Moreton Bay site 1 (MB1)	03/2013–03/2021	634	541	574
Moreton Bay site 2 (MB2)	04/2019–05/2020	22	18	168
Hidden Vale site (HV)	05/2018–05/2020	35	32	433

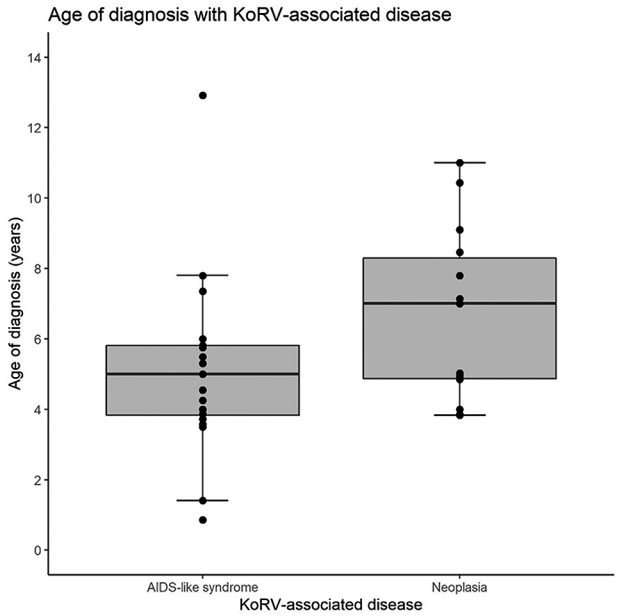

Figure 1. Graph showing the ages at which koalas from all three sites (MB1, MB2 and HV) were diagnosed with neoplasia and AIDS-like syndrome.

days, with 38.5% being euthanized within 14 days. The remaining koala was found dead. All five koalas with suspected neoplasia were also euthanized. Of the 25 koalas fulfilling the criteria for AIDS-like syndrome, nine were euthanized (45%), six were found dead, and outcomes for the remaining five are unknown as they were removed from the monitoring programs.

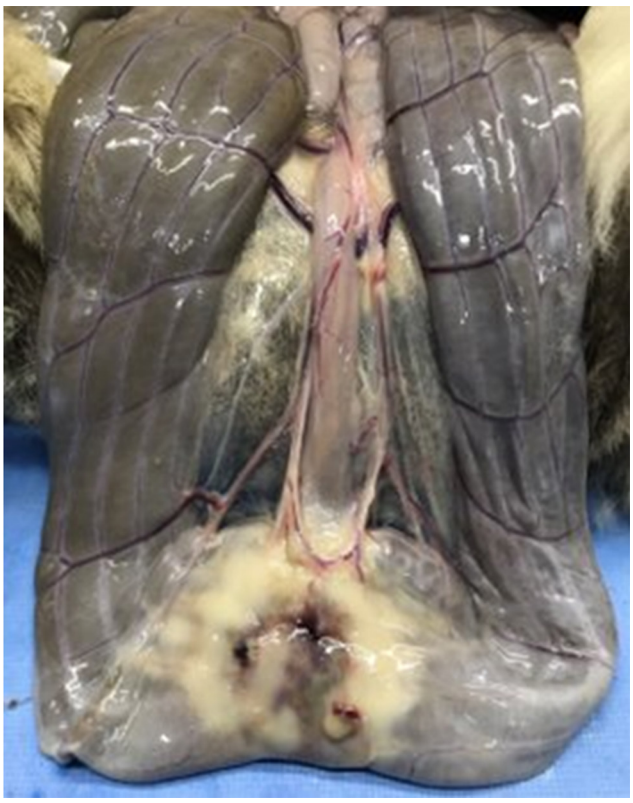

Figure 2. Photograph taken at necropsy of abdominal lymphoma diagnosed in an approximately five-year-old female koala from the Moreton Bay Site 1 (MB1). Photo credit to Endeavour Veterinary Ecology.

Discussion

Through an analysis of longitudinal health data, we sought to measure the occurrence of PKAD to determine their significance in wild koala populations in SE QLD. Previous studies have demonstrated high prevalence of endogenous and exogenous KoRV subtypes in this region, including koalas inhabiting our three study sites (Table 1). However, when surveying for potential clinical manifestations of KoRV infection, we found both prevalence and incidence rates to be relatively low. Combining data from all sites, we calculated prevalence of PKAD at initial presentation to be 1.16% (95% CI 0.5–2.19%), ranging from 0.95% (95% CI 0.35–2.05%) in MB1 to 4.55% (95% CI 0.12–22.84%) in MB2. The incidence rate for all sites combined was 3.5 cases/100 koalas/year, ranging from 3.4 to 5.4 cases/100 koalas/year between MB1 and HV, respectively. Differences in initial prevalence and incidence rate between sites were not statistically significant. This likely reflects the relatively small sample size from MB2 (n = 18) and HV (n = 32) when compared with MB1 (n = 541), and the variation in duration of monitoring at each site (7 years at MB1, 14 months at MB2, and 2 years at HV). This latter point is the most likely reason no further cases were identified at MB2 during the study.

As expected, our findings for the MB1 site were comparable to those reported by Quigley *et al.* (2018) in their study of 290 koalas from the same population. Between 2013 and 2017, 1.72% (5/290) of koalas presented with neoplasia and 2.41% (7/290) developed AIDS-like syndrome. Similarly, between 2013 and 2021, we reported neoplasia (including suspected cases) in 2.68% (17/634) and AIDS-like syndrome in 2.84% (18/634) of individuals. This is unsurprising given that in both studies, very similar inclusion criteria for diagnosing PKAD were employed (Quigley *et al.,* 2018) (Table 3).

In contrast, our calculations of PKAD occurrence in the MBR (MB1 and MB2) were lower than those determined by a previous analysis of a wild population in this area (Hanger & Loader, 2014). This study found prevalence at initial presentation to be 7.8% (23/296) between 2008 and 2013, compared to our detection of just 1.07% (95% CI 0.43–2.19%) between 2013 and 2021. In the previous study, longitudinal data were available for 126 koalas, in which the incidence risk was 12.5% per year. Again, this is notably higher than our incidence rate of 3.4 cases/100 koalas/year (95% CI 2.26–4.85), which converts to an incidence risk of 3.34% using the equation $CI = 1–e^{-I}$ where CI is incidence risk and I is incidence rate (Thrusfield *et al.,* 2018). These differences may reflect the use of more conservative criteria for diagnosing PKAD in our study. The aim of this was to avoid the inclusion of false positives in our analysis. This approach carries the risk of excluding cases that may be linked to KoRV and consequently, the true rate of disease in these populations is likely to be somewhere in between.

A single potential familial link was identified in those individuals in which dam and joey relationships were known. Given that this was limited to a very small subset of the population, further analysis was not pursued. Our study was conducted in a wild population and so information about the sires of individuals is unknown. However, this is unlikely to influence our analysis as endogenous KoRV-A is transmitted via germline DNA, and dam-to joey transmission is by far the most important mode of transmission for exogenous subtypes (Xu *et al.,* 2013; Quigley *et al.,* 2018; Joyce *et al.,* 2021).

Most of the data pertaining to PKAD in wild koala populations are collected from animals presenting to wildlife hospitals (Hanger & Loader, 2014; Fabijan *et al.,* 2020; Gonzalez-Astudillo *et al.,* 2020). In a retrospective survey of clinical records for the 10,082 koalas admitted to the Australia Zoo Wildlife Hospital, SE QLD, between 2004 and 2020, 10.9% were found to have presented with neoplasia or other PKAD (R. Booth, pers. comm.). While such datasets provide a valuable insight into the impact of disease on free-ranging populations, they represent a biased cohort constituting predominantly sick or injured individuals. This may explain the generally higher rates of neoplasia and other diseases falling under the umbrella of AIDS-like syndrome when compared to our study.

Individual animal welfare should also be considered when assessing the significance of PKAD on the health of koala populations. Neoplasia and AIDS-like syndrome commonly manifest as chronic, painful, and debilitating symptoms (Hanger & Loader, 2014; Fabijan *et al.,* 2020). In our populations, 72.26% of the diseased cohort were euthanized on welfare grounds upon veterinary intervention or found dead in the field. We found that individuals presented with symptoms over a very wide range of ages (Fig. 1). This was broadest for those with AIDS-like syndrome (0.86–12.92 years), likely reflective of the varied and often chronic nature of these conditions. These diseases lead to poor welfare outcomes for individuals, and may reduce reproductive success and life expectancy, particularly in koalas suffering from a young age.

Our findings suggest that neoplasia and other conditions suggestive of immune system dysfunction, dysregulation, or suppression impact a small percentage of the free-ranging populations that we studied in SE QLD. However, this should not diminish the potential impact of KoRV infection on wild northern koala populations. Rather, further research is needed to better understand the role of this virus within the framework of other threats and drivers of decline, such as high rates of chlamydial disease endured by these populations. Furthermore, studies that seek to contextualise the significance of KoRV infection will help to inform future management practices and ensure that the best health and welfare outcomes are achieved for our wild koalas.

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Koala Retrovirus Infection and Disease in South Australian Koala (*Phascolarctos cinereus*) Populations

Natasha Speight

School of Animal and Veterinary Sciences,
Faculty of Sciences, University of Adelaide, Roseworthy SA 5371, Australia

Abstract. Koala retrovirus (KoRV) infection, endogenous in all northern koalas (*Phascolarctos cinereus*), has been found to occur at lower, but increasing, prevalence in the Kangaroo Island and Mount Lofty Ranges koala populations in South Australia. Proviral and viral loads are also lower than in Queensland koalas, which may be due to exogenous spread of infection, or may be related to the variable presence of viral genes and fragmented expression that has been found in positive Mount Lofty Ranges koalas. However, high proviral loads and full expression across the KoRV genome in South Australian koalas has been found in individuals with neoplasia, particularly lymphoma, which can be as extensive and as severe as that observed in northern koalas. KoRV-A is the predominant subtype and no association with chlamydial status has been found except that high viral loads correlate with severity of chlamydiosis. Based on the complexity of KoRV infections in South Australian koalas, further research is needed to understand the differences in transmission and pathogenesis that occur.

Introduction

The understanding of koala retrovirus (KoRV) in koala (*Phascolarctos cinereus*) populations in South Australia (SA) has gradually increased over the past 15 years. Previous KoRV studies have focussed on Kangaroo Island, which holds one of the five geographically separated SA koala populations, the others being the Mount Lofty Ranges, Eyre Peninsula, the Riverland, and the lower southeast of the state. Kangaroo Island, at least prior to the 2019/2020 bushfires, and the Mount Lofty Ranges represent two of the largest SA populations and have generally been regarded as healthy, though genetically restricted. Recent KoRV research has been conducted with koalas from both of these populations, however the KoRV status of the other SA koala populations remains unknown.

Kangaroo Island

The Kangaroo Island koala population was founded from a small translocated group of koalas from French Island, Victoria, in the 1920s (Robinson, 1978), which subsequently expanded in numbers to the point of requiring population control measures (Duka & Masters, 2005). Their fecundity may be partly attributed to the recent finding that Kangaroo Island koalas are free of infection with *Chlamydia pecorum*, based on 170 koalas tested between 2014–2017 and analysis of over 13,000 veterinary records from a sterilization program (Fabijan *et al*., 2019). The total koala population, which was estimated at 50,000 in 2016, has now been reduced by approximately 80% in the recent bushfires to an estimated current population of 5,000–10,000 animals (DEW, 2020; Dunstan *et al*., 2021).

The earliest study of KoRV on Kangaroo Island in 2004 (n = 26) found no evidence of infection by end-point PCR (Tarlinton *et al*., 2006); however, a subsequent end point PCR based study conducted in 2007 found 15% prevalence within the animals sampled (n = 162) (Simmons *et al*., 2012). This low proportion of infected koalas, in conjunction with low proviral load, led researchers to conclude that transmission was exogenous, rather than endogenous, in this population (Simmons *et al*., 2012). However, a 2013 publication reported an updated prevalence of 30–35%

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ORCID iD: Natasha Speight https://orcid.org/0000-0001-5367-0241
Corresponding author: Natasha Speight natasha.speight@adelaide.edu.au
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within the animals sampled, based on unpublished data (Denner & Young, 2013), suggesting either a rapid increase in KoRV spread in Kangaroo Island koalas, a heterogenous distribution of KoRV in the island's population, or use of a more sensitive assay.

Our qPCR-based KoRV prevalence study in 2014–2017 targeted wild-caught Kangaroo Island koalas (n = 170) and detected KoRV in 42% of samples, with all positive animals positive for KoRV-A and negative for KoRV-B (Fabijan *et al*., 2019). The median proviral copy number was only 113 KoRV copies/10^3 β-actin copies; however, some koalas showed higher loads (maximum 12641 KoRV copies/10^3 β-actin copies), suggesting that in some individuals either exogenous KoRV infection was more extensive or that endogenous transmission was occurring. This mixed transmission pattern was further supported by the finding that of 19 mother-joey pairs, the infection status differed in five cases, with two pairs in which the mother was KoRV-positive and offspring negative, and three pairs where the offspring only was KoRV-positive (Fabijan *et al*., 2019). That several offspring were positive independent of their dam could represent dam infection below the detection limit, endogenous transmission from the sire, or exogenous transmission from other koalas.

Mount Lofty Ranges

The Mount Lofty Ranges koala population, near Adelaide, is principally derived from Kangaroo Island koalas translocated in the 1960s, with reported addition of individuals brought from New South Wales (Robinson, 1978) and Queensland (Lindsay, 1950). Little was known of the health status of this koala population, except that *Chlamydia pecorum* infection was common in the absence of clinical disease (Polkinghorne *et al*., 2013). Following this, high prevalence of the renal disease, oxalate nephrosis, was described at up to 55% in cohorts of necropsied individuals (Speight *et al*., 2013; Speight *et al*., 2018). In 2016, chlamydial infection was identified in 47% of wild-caught koalas (n = 75), associated in several cases with ocular and urogenital disease (Fabijan *et al*., 2019).

Our 2016 study of wild-caught Mount Lofty Ranges koalas (n = 75) identified a KoRV prevalence of 65% within the animals sampled, with a median proviral copy number of 35 copies/10^3 β-actin copies (maximum 574 KoRV copies/10^3 β-actin copies) (Fabijan *et al*., 2019). Only KoRV-A, not KoRV-B, was detected, and the likelihood of KoRV infection increased with age (Fabijan *et al*., 2019). KoRV was not found to be associated with chlamydial infection or disease, but periodontitis was more common in KoRV positive koalas (Butcher *et al*., 2020). Concurrent studies investigated putative KoRV-associated diseases, including the neoplastic conditions, leukaemia and lymphoma. The first documented case from 2014 was an older female koala initially presenting with hindlimb lameness, but found at clinical examination to have concurrent lymphosarcoma, reproductive chlamydiosis, and KoRV infection (Fabijan *et al*., 2017).

A large comparative study of KoRV in necropsied koalas from Queensland and SA found lymphoma in 4.3% (4/92) of the KoRV positive Mount Lofty Ranges koalas sampled (Fabijan *et al*., 2020). High proviral loads were found in both SA and Queensland koalas with neoplasia (Sarker *et al*., 2020); however overall, the SA koalas had lower proviral loads (median 2.71 × 10^3 KoRV DNA copies/10^3 β-actin copies) compared with Queensland koalas. Only 51% of SA koalas sampled had circulating virus detected, for which the load was also lower than koalas from Queensland (Sarker *et al*., 2020). However, high viral load in SA koalas was positively correlated with chlamydial disease severity, and in both populations, positively correlated with splenic lymphoid area, lymphocyte count, and metarubricyte count (Fabijan *et al*., 2020). KoRV-A was found to be the predominant subtype in the Mount Lofty Ranges cohort (Sarker *et al*., 2019).

As part of this large study, variable absence of KoRV DNA and RNA genes (Sarker *et al*., 2020) and defective expression (Tarlinton *et al*., 2017) was found in SA koalas in comparison with Queensland koalas, for which all genes were present (Sarker *et al*., 2020) with high expression (Tarlinton *et al*., 2017). A recent study comparing KoRV positive (all genes present on PCR) koalas, KoRV positive koalas with lymphoma, and KoRV negative (central KoRV genes absent on PCR) koalas in the Mount Lofty Ranges found only fragmented, or no expression, of central KoRV genes (*gag*, *pol* and *env*) in three positive and two negative SA koalas, respectively, despite expression of the terminal regions in all koalas (Stephenson *et al*., 2021). However, KoRV positive koalas with lymphoma showed high expression of all KoRV genes (Stephenson *et al*., 2021). These findings may explain the lower prevalence of KoRV in SA koala populations, the lower proviral and viral loads in positive animals, and the lower incidence of KoRV-associated neoplasia.

Conclusion

KoRV infection clearly shows a high level of complexity that differs among the koala populations across Australia. More research is needed to further understand the epidemiology and pathogenesis in both the Kangaroo Island and Mount Lofty Ranges populations of SA koalas. The infection profile of KoRV in southern koalas offers the ability for comparison with northern koala KoRV infections, and the hope that some koala populations in Australia can harbour koalas that are regarded as KoRV negative.

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Putative Koala Retrovirus-Associated Diseases in the Japanese Captive Koala (*Phascolarctos cinereus*) Population

Tetsuya Imanishi

JAZA Species Coordinator for Koala, Nagoya Higashiyama Zoo,
3-70 Higashiyama-motomachi Chikusa-ward Nagoya-city, 464-0847, Japan

Abstract. Japan began housing koalas (*Phascolarctos cinereus*) in 1984, increasing from six individuals in 1984 to a peak of 96 koalas in 1997. However, the number of koalas has almost halved since and as of 2020, 54 koalas remain in zoos in Japan. Although records of 330 koala deaths have been accumulated over 37 years, there have been no comprehensive reports on the relationship between the causes of death and koala retrovirus (KoRV) in the Japanese captive population. Based on the koala studbook updated by the Japanese Association of Zoos and Aquariums, we have investigated causes of death in the Japanese captive koala population. The most common cause of death was joeys falling. When combined with stunted joey growth, one-third of the koalas died within a year of birth. Deaths due to malignant neoplasms and opportunistic infections cannot be directly associated with KoRV infection because no test for KoRV had been performed before or during disease onset. It is suspected that KoRV may be associated with deaths due to the large number of cases of neoplasms, which accounted for 16.4% of all deaths.

Introduction

Captive koala (*Phascolarctos cinereus*) breeding began in Japan when three zoos introduced six koalas from Australian zoos in 1984. The number of koala individuals and institutions increased subsequently through further imports and reproduction. A total of 81 koalas have been imported so far, all but one from Australia. Eight koalas have been exported overseas to the United States, the United Kingdom, Australia and other countries. A total of 311 koalas have been born in Japan, and 330 koalas have died since 1984. After reaching a peak of 96 individuals in 1997 and 10 institutions in 1998, the number of koalas has halved in 15 years. As of the end of December 2020, 54 koalas were living at seven institutions in Japan.

The purpose of this study was to investigate the more than 300 cases of koala deaths that occurred in Japanese captive populations from the point of view of KoRV, which is thought likely to cause immunosuppression and malignant neoplasms (Tarlinton *et al*., 2005; Quigley *et al*., 2018; Zheng *et al*., 2020), and to search for a relationship between KoRV infection and mortality.

The Japanese koala studbook, started in 1984 and updated by the Japanese Association of Zoos and Aquariums in 2020, contains information on a total of 392 koalas, including 331 koalas that were born in Japan and 330 koalas that died in Japan. The results categorizing these koalas by cause of death are shown in Table 1. In cases where two or more causes of death were recorded together, malignant neoplasms were prioritized as the cause of death.

Deaths of 102 joeys less than 1 year old accounted for 30.9% of all deaths. Of these deaths, 66 cases were due to "joey falling," and 36 cases were due to "stunted growth of joey" including five cases of joey loss. About one third of the 311 koalas born in Japan died before the age of one year old. Although this is a very high mortality rate, the European

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ORCID iD: Tetsuya Imanishi https://orcid.org/0000-0003-0087-9214
Corresponding author: Tetsuya Imanishi t.imanishi.vm@city.nagoya.lg.jp
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Table 1. Causes and rates of death of koalas in the Japanese captive population 1984–2020.

causes of death	all koalas	northern koalas	southern koalas
	(n = 330)	(n = 299)	(n = 31)
Malignant neoplasms	54 (16.4%)	54 (18.1%)	0
lymphoma	29 (8.8%)	29 (9.7%)	0
leukaemia	14 (4.2%)	14 (4.7%)	0
other neoplasms	11 (3.3%)	11 (3.7%)	0
Opportunistic infections	13 (3.9%)	13 (4.3%)	0
cryptococcosis	12 (3.6%)	12 (4.0%)	0
pyocyanic disease	1 (0.3%)	1 (0.3%)	0
Immunodeficiency	2 (0.6%)	2 (0.7%)	0
Septicaemia	11 (3.3%)	11 (3.7%)	0
Joey falling	66 (20.0%)	60 (20.1%)	6 (19.4%)
Stunted growth of joey	36 (10.9%)	34 (11.4%)	2 (6.5%)
Old age	33 (10.0%)	24 (8.0%)	9 (29.0%)
Others	115 (34.9%)	101 (33.8%)	14 (45.2%)

captive population also shows a similar trend (Mulot, 2014).

In this studbook, 33 mortalities are recorded as "old age" for koalas showing no particular cause of death other than weakness of various body functions associated with old age. Mortality categorized as "others" includes 18 deaths of unknown cause (all older than 1 year), and diseases clearly unrelated to retroviruses such as intestinal torsion, traumatic shock, respiratory obstruction and spinal curvature, as well as various inflammations where it is not clear from the studbook whether the diseases were infectious or not.

A total of 80 mortalities (24.2%) were attributed to putative KoRV-associated diseases, which are divided broadly into two groups: 1) bone marrow dysplasia and neoplasia and 2) putative immune dysfunction disorders (Gillett, 2023). Mortalities resultant from "malignant neoplasms" accounted for 54 cases. Among those, 29 cases were lymphoma and 14 cases were leukaemia, both of which are thought to be associated with aspects of KoRV infection. Other neoplasms recorded in 11 cases include liver, uterine and ovarian tumours, colorectal cancer, squamous cell carcinoma, and peritoneal mesothelioma. Retroviruses are known to be directly associated with different types of cancers, sarcomas, and lymphomas in mammals including humans (Miyazawa, 2009). Therefore, the association of KoRV with neoplasms other than lymphoma and leukaemia cannot be excluded.

Some retroviruses cause immunosuppression in some animal species (Miyazawa, 2009). Hence, in addition to the two cases of suspected immunodeficiency, 12 cases of cryptococcosis and one case of pyocyanic disease, both of which are opportunistic infections, are suspected to be related to KoRV infection. Furthermore, 11 cases of mortality due to septicemia were recorded. Although the studbook has no record on the details of the causative organisms, it is possible that opportunistic bacteria, or bacteria that entered the bloodstream from the host's intrinsic flora, especially the intestinal flora, contributed to their development as a result of immunosuppression.

Of the 392 koalas listed in the studbook, 354 individuals are of northern lineage, 38 individuals are southern, and there are no hybrids. Koalas of northern and southern lineages have been separated and have not been kept in the same zoo except for exceptional cases. Therefore, although KoRV transmission could have occurred within the same koala lineage, it is unlikely that the infection has occurred between northern and southern koalas. When the causes of death of koalas from northern and southern lineages are compared, different trends are observed. Although the number of deaths among southern koalas is not large, accounting for only 31 cases or less than 10% of the total, none of the deaths were due to malignant neoplasms such as lymphoma or leukaemia. Northern koalas have a significantly higher rate of death from malignant neoplasms than the southern koalas (Fisher's exact test, $p = 0.0029$).

In a PCR-based study of 648 wild southern koalas, none were found to be infected with KoRV-B (Legione *et al*., 2017). Similarly, of 51 koalas in Japanese zoos in 2008, 27 of 40 northern koalas were found to be infected with KoRV-B, while all 11 southern koalas were found to be uninfected (Shojima *et al*., 2013). In light of reports finding a significant association of KoRV-B infection in wild koalas with other neoplasms (Quigley *et al*., 2018) and finding significantly higher proportions of leukaemia, lymphoma and other cancers in koalas infected with KoRV-B, -E and -F than those infected with only KoRV-A (Zheng *et al*., 2020), the fact that there are 54 deaths from malignant neoplasms only in the northern koalas of the Japanese captive population and none in the southern koalas further strengthens the suspicion of an association between these diseases and KoRV, especially KoRV-B. However, as KoRV-B negative koalas suffer high rates of neoplasia, it cannot be excluded that the koalas of northern Australian origin did not suffer from neoplasms caused by KoRV-A.

In conclusion, 80 of the 330 (24.2%) koala deaths in the Japanese captive population were due to putative KoRV-associated diseases (malignant neoplasms and possible opportunistic infections). However, since the viral expression of KoRV before and at the time of disease onset or even the presence of infection of KoRV has not been evaluated in all but a few cases, it is not possible to definitively link these causes of death to the virus.

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Koala Retrovirus Status and Putative Koala Retrovirus-Associated Diseases in Koalas (*Phascolarctos cinereus*) in North American Zoos

Cora L. Singleton and Chris Hamlin-Andrus

San Diego Zoo Wildlife Alliance, San Diego, CA 92101, United States of America

Abstract. The living koala population in North America is predominantly descended from koalas imported from a single Australian facility in 1976 and 1981, with several smaller imports from other facilities between 1985 and 2013. Koala retrovirus subtype B (KoRV-B) entered the North American population via imports in 2005, 2008, and 2013. The 2005 and 2008 KoRV-B positive lineages are deceased, but the 2013 KoRV-B positive lineage has seven surviving koalas, including one female of breeding age. Three koalas born to KoRV-B negative dams were documented as being KoRV-B positive at 15 months of age after nursing from a KoRV-B positive female. The prevalence of koala retrovirus subtype A (KoRV-A) detection in North America is 100% but the prevalence of KoRV-B detection is 17%. Lymphoid neoplasia is a common cause of mortality, dating back to founder koalas. Most cases of lymphoid neoplasia have occurred in presumptive KoRV-B negative koalas, and many cases have occurred between the ages of four and nine years. Familial clusters of lymphoid neoplasia are apparent. Additionally, myelodysplasia and fatal peripheral cytopenias are important putative KoRV-associated diseases that cause mortality in koalas in North America, with higher prevalence in koalas younger than two years of age. Since 2013, breeding of known KoRV-B positive koalas has been managed, to maintain separation from the remainder of the KoRV-B negative population.

Koalas in North America

The San Diego Zoo opened in 1916, after a small zoo exhibit was left behind at the end of the 1915–1916 Panama–California Exposition. Nine years later, in 1925, the zoo received international attention when Australia donated animals, including the first two koalas to arrive in the United States. In the 1950s, four koalas (two males and two females; 2.2) were imported but did not produce offspring. In the 1960s, two more importations occurred (three males and three females; 3.3). These koalas produced 11 offspring, three of which lived into the 1970s when this lineage ended. In 1976, six mature koalas (two males, four females, and one unknown sex pouch young; 2.4.1) were imported to San Diego Zoo. These koalas, plus an additional seven koalas (one male and six females; 1.6) imported in 1981 from the same facility in Australia, are the founders of the current North American koala population. Later, several other zoos imported smaller numbers of koalas from a variety of facilities in Australia. The last importation occurred in 2013.

The current North American koala population consists of 52 koalas living at 10 different zoos. San Diego Zoo Wildlife Alliance (SDZWA) cares for the largest colony of koalas outside of Australia (32 resident koalas) and manages the North American koala population through leadership of the Association of Zoos and Aquariums (AZA) Koala Species Survival Plan (SSP) program. All 10 zoos holding koalas participate in the Koala SSP program. In 1983, SDZWA established the Koala Education and Conservation Program (KECP) to support koala care, education, research, and

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ORCID iD: Cora L. Singleton https://orcid.org/0009-0007-1856-4073
Corresponding author: Cora L. Singleton csingleton@sdzwa.org
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Table 1. Koala retrovirus subtype B (KoRV-B) status of 52 koalas living in North America, March 2021.

	male		female		unknown		total	
	tested[a]	all[b]	tested[a]	all[b]	tested[a]	all[b]	tested[a]	all[b]
KoRV-B Positive	6	7	1	2	0	0	7	9
KoRV-B Negative	18	20	16	21	0	2	34	43
total	24	27	17	23	0	2	41	52
prevalence	25.0%	25.9%	5.9%	8.7%	0%	0%	17.1%	17.3%

[a] Laboratory tested using qPCR for KoRV-A and KoRV-B.

[b] Total number of koalas in each category, both laboratory-confirmed KoRV-B status and expected KoRV-B status based on KoRV-B status of the dam.

conservation. Five zoos in the United States and eight zoos in Europe are partners in the KECP.

History of koala retrovirus in North America

Based on the assumption of 100% transmission of koala retrovirus subtype B (KoRV-B) from dam to joey (Quigley *et al*., 2018), analysis of maternal pedigree of qPCR-confirmed KoRV-B negative and KoRV-B positive female koalas indicates that the founder koalas can be assumed to be KoRV-B negative. The first KoRV-B positive koalas (two full-sibling females) arrived in North America in 2005 and 2008. The female imported in 2005 and her four KoRV-B positive descendants over two generations died by 2016 from lymphoid neoplasia, age range 4.0 to 5.5 years (Xu *et al*., 2013). The female imported in 2008 and her single offspring died by 2014 at the ages of 7.5 years from mesothelioma and 2.5 years from pneumonia, respectively.

In 2013, two (1.1) of three (2.1) koalas imported from Australia were determined to be KoRV-B positive after arrival. The imported KoRV-B positive female died from lymphoid neoplasia at 11.5 years of age and six of her seven descendants are alive (4.2; age range 1.5–7.5 years). The imported KoRV-B positive male is still alive as of this writing.

Health surveillance and current koala retrovirus status

Health surveillance: Koalas receive regular comprehensive health examinations which include physical examination, computed tomography study, ultrasonographic exam, complete blood count, serum biochemistry panel, urinalysis, *Cryptococcus* spp. antigen test, cytological evaluation of a bone marrow aspirate (Dr Nicole Stacy, University of Florida), and koala retrovirus subtype A (KoRV-A) and KoRV-B qPCR (Molecular Diagnostics Laboratory, San Diego Zoo Wildlife Alliance) if status is undetermined. A complete post-mortem evaluation is conducted on every koala that dies, which includes gross examination, histopathological evaluation of a complete set of standardized tissue samples, and banking of selected biological samples. Gross and microscopic findings are recorded, and a cause of death is identified. For this discussion, lymphoid neoplasia includes lymphoma and lymphocytic leukaemia, and myelodysplasia refers to blood and bone marrow disorders ranging from subclinical nuclear dysplastic changes to fatal peripheral cytopenias.

KoRV testing: Testing for selected KoRV subtypes began in 2010 for research purposes, and this included testing of post-mortem samples dating back to 2008 in response to a familial cluster of mortalities due to malignant neoplasms (Xu *et al*., 2013). In 2014, diagnostic testing for detection of KoRV-A and KoRV-B was established at the SDZWA Molecular Diagnostics Laboratory. The KoRV assays (qPCR for KoRV-A and KoRV-B envelope genes) were developed based on protocols from Xu (Xu *et al*., 2013), William Switzer (Centers for Disease Control and Prevention, personal communication), and Maribeth Eiden (National Institute of Health, personal communication).

KoRV subtype prevalence: As of March 2021, 79% (41/52) of koalas living in North America had been tested for the presence of KoRV-A and KoRV-B using qPCR (Table 1). The 11 untested koalas were assigned expected KoRV-B status based on KoRV-B status of the dam. The KoRV-A subtype prevalence in North America was 100%. Overall, the KoRV-B subtype prevalence was about 17%, with a higher prevalence in males (25.0–25.9%) relative to females (5.9–8.7%).

Allonursing and KoRV-B transmission: Detailed breeding and parturition records are maintained for the North American koala population. One KoRV-B positive lactating female was living with three KoRV-B negative lactating females, all four with joeys of approximately the same age. Joeys were observed sharing dams often. Subsequently, all four joeys were confirmed to be KoRV-B positive by qPCR at 15 months of age. Since three of the joeys were born to KoRV-B negative dams and later tested KoRV-B positive, it is suspected that they acquired KoRV-B by nursing from the KoRV-B positive dam.

Sub-clinical myelodysplasia: Routine cytological evaluation of bone marrow aspirates began in 2017 to screen for sub-clinical myelodysplasia. Of 26 clinically healthy koalas evaluated to date, there was cytological evidence of cellular dysplasia in 77% (20/26) of koalas (Table 2). Dysplastic

Table 2. Koala retrovirus subtype B (KoRV-B) status and degree of myelodysplastic changes diagnosed by cytological evaluation of bone marrow aspirates from clinically healthy koalas living in North America.

	normal	minimal	mild	moderate	total
KoRV-B positive[a]	2	3	2	4	11
KoRV-B negative[a]	4	6	5	0	15
total	6	9	7	4	26

[a] Laboratory confirmed using qPCR.

Table 3. Crude and age-stratified mortality rates for lymphoid neoplasia (lymphoma and lymphocytic leukaemia) and myelodysplasia, two putative koala retrovirus-associated diseases, in koalas in North America 1959–2020.

	mortalities	necropsy reports	lymphoid neoplasia	myelodysplasia
1959–1975	21	12	8% (1/12)	0% (0/12)
1976–2020	247	195	21% (40/195)	7% (13/195)
pouch young (< 0.5 years)	63	24	0% (0/24)	0% (0/24)
immature (0.5–1.5 years)	29	28	0% (0/28)	21% (6/28)
mature (> 1.5 years)	155	143	28% (40/143)	5% (7/143)

changes ranged from minimal to moderate and were seen more frequently in the erythroid line than the myeloid line, and rarely in the megakaryocytic line. The presence and degree of dysplastic changes does not appear to be related to KoRV-B status. Assessment of cytological changes in individual koalas over time is in progress.

Mortality due to putative koala retrovirus-associated diseases

Two important putative koala retrovirus-associated diseases (PKAD) in the North American koala population are lymphoid neoplasia and myelodysplasia (Gillett, 2023). Between 1959 and 1975, 8% (1/12) of mortalities were attributed to lymphoid neoplasia (Table 3). Between 1976 and 2020, the cause of death of 21% (40/195) of koalas was attributed to lymphoid neoplasia and 7% (13/195) of koalas was attributed to myelodysplasia (Table 3). The mortality rate due to lymphoid neoplasia and myelodysplasia varies by age, KoRV status, and sometimes pedigree in the North American koala population.

Lymphoid neoplasia and KoRV status: Both KoRV-B positive and presumed KoRV-B negative koalas died from lymphoid neoplasia (Table 4). Of 171 immature (0.5–1.5 years of age) and mature (> 1.5 years of age) koalas that died between 1976 and 2020, 95% (162/171) were presumed KoRV-B negative and 5% (9/171) were confirmed KoRV-B positive. Of these 171 koalas, 23% (40/171) died from lymphoid neoplasia. Of the 40 koalas that died from lymphoid neoplasia, 82.5% (33/40) were presumed KoRV-B negative koalas, while 17.5% (7/40) were confirmed KoRV-B positive koalas. Mortality rate due to lymphoid neoplasia was 20% (33/162) in KoRV-B negative koalas and 78% (7/9) in KoRV-B positive koalas. Of the seven KoRV-B positive koalas that died from lymphoid neoplasia, five were from the same direct maternal lineage (Xu *et al*., 2013). It is not possible to determine if the mortality rate due to lymphoid neoplasia varies by KoRV status in the North American koala population based on this data, due to small sample size and confounding variables such as potential inbreeding effects and fixation of KoRV-A integration sites and dysregulation of oncogenes (McEwen *et al*., 2021).

Lymphoid neoplasia and age: The mortality rate due to lymphoid neoplasia varies by age in the North American koala population, with only mature koalas dying from lymphoid neoplasia (Table 3). Approximately 62% of deaths due to lymphoid neoplasia occurred in the age range 4–9 years. The highest mortality rate (82%) due to lymphoid neoplasia was age 4–5 years, which includes the familial cluster of five mortalities previously described (Xu *et al*., 2013). Mortality rate for lymphoid neoplasia ranged from 0% to 55% for other ages, with no cases of lymphoid neoplasia in koalas less than 2 years of age or greater than 18 years of age.

Lymphoid neoplasia and pedigree: Based on the assumption of 100% transmission of KoRV-B from dam to joey (Quigley *et al*., 2018), it appears that KoRV-B entered the North American koala population with importation of four positive koalas (full siblings 0.1 in 2005 and 0.1 in 2008; unrelated 1.1 in 2013). Therefore, there are two KoRV-B positive maternal lineages. Within one of these lineages, 15% (6/40) of the cases of lymphoid neoplasia occurred. In contrast, 45% (18/40) of lymphoid neoplasia can be traced back through two KoRV-B negative maternal pedigrees. Further analysis is required to evaluate the relationship of KoRV, lymphoid neoplasia, and pedigree in the North American koala population.

Myelodysplasia: The mortality rate due to myelodysplasia varied by age in the North American koala population (Table 3). Of the 13 koalas that died from myelodysplasia, 46% were immature koalas and 54% were mature koalas. From 1976–2020, myelodysplasia was a leading cause of death for immature koalas (6/28, 21%) but was an uncommon cause of death in mature koalas (7/143, 5%). In seven mature koalas that died from myelodysplasia, six were 5–9 years old and one was 12–13 years old. All 13 koalas that died from myelodysplasia were KoRV-B negative.

Husbandry and breeding management

Since 2013, breeding of confirmed KoRV-B positive koalas has been managed, to maintain separation from the remainder of the KoRV-B negative population. Between 2013 and 2018, most arranged breeding was between KoRV-B negative

Table 4. Koala retrovirus subtype B (KoRV-B) status and death due to lymphoid neoplasia (lymphoma and lymphocytic leukaemia) and myelodysplasia, two putative koala retrovirus-associated diseases, in koalas in North America 1976–2020.

	lymphoid neoplasia	myelodysplasia	other	total
KoRV-B positive[a]	7[c]	0	2	9
KoRV-B negative[b]	33	13	116	162
total	40	13	118	171

[a] Laboratory confirmed positive using qPCR.

[b] Both laboratory-confirmed negative using qPCR and presumed negative based on KoRV-B status of the dam.

[c] Five of seven cases from the same maternal lineage.

males and KoRV-B negative females. Prior to discovery of KoRV status, one KoRV-B positive female bred with two KoRV-B negative males. Starting in 2020, KoRV-B positive males were bred with the single remaining KoRV-B positive female.

Based on very low horizontal transmission of KoRV-B via casual contact (Quigley *et al*., 2018), the last remaining KoRV-B positive female koala was co-housed with several KoRV-B negative female koalas. After apparent KoRV-B transmission via allonursing, the KoRV-B positive breeding female was isolated during lactation.

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Synthesis of Discussions of the Second Koala Retrovirus Workshop, 2021

Alex D. Greenwood[1], David E. Alquezar-Planas[2], Philippa A. McKay[3], Baptiste Mulot[4], Geoffrey W. Pye[5], Amy Robbins[3], Cora L. Singleton[6], Rachael E. Tarlinton[7], and Damien P. Higgins[8]

[1] Department of Wildlife Diseases, Leibniz Institute for Zoo and Wildlife Research, 10315 Berlin, Germany; and School of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany

[2] Australian Museum Research Institute, Australian Museum, Sydney NSW 2010, Australia

[3] Endeavour Veterinary Ecology Pty Ltd, Toorbul QLD 4510, Australia

[4] ZooParc de Beauval & Beauval Nature, 41110 Saint-Aignan, France

[5] Disney's Animals, Science, and Environment, Bay Lake, FL 32830, United States of America

[6] San Diego Zoo Wildlife Alliance, San Diego, California 92101, United States of America

[7] School of Veterinary Medicine and Science University of Nottingham, United Kingdom

[8] Sydney School of Veterinary Science, Faculty of Science, The University of Sydney NSW 2006, Australia

Abstract. This document represents a synthesis of discussions held online at the Second Koala Retrovirus Workshop in 2021. The three days of discussions were based on workshop presentations and comprise: KoRV foundational science (Day 1); applied management of koalas in zoo populations (Day 2); and applied management of koalas in wild populations (Day 3). Each of these discussions gathers current knowledge, explores points of consensus and disagreement, and identifies important knowledge gaps. Recommendations arise regarding research strategy, interim measures for management, and support of research and management via initiation of working groups on KoRV diagnostics and biobanking.

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Table of Contents (continued)

DAY 1
Synthesis of Discussions KoRV 2021 Workshop Day 1: Foundational Science

Alex D. Greenwood, David E. Alquezar-Planas, and Rachael E. Tarlinton

Facilitators: Rachael E. Tarlinton, David E. Alquezar-Planas, and Alex D. Greenwood

Chat Managers: Larry Vogelnest, Gayle McEwen, and Laura Chao

Goal

To identify foundational knowledge gaps on KoRV subtypes, biology, and disease progression.

Day 1 talk titles

Section 1: Which koalas have KoRV infections

Tarlinton	Overview of KoRV epidemiology across Australia
McEwen	KoRV integration sites in wild and captive koalas and their effects on gene expression
Quigley	One virus two stories—endogenous vs exogenous spread of KoRV in koalas

Section 2: What do we know about the KoRV infection and the transmission process

Roca	Endogenous vs exogenous dynamics of KoRV
Joyce	KoRV genetic diversity and transmission dynamics in zoo populations
Vinette-Herron	KoRV transmission in a zoo population
Blyton	KoRV diversity across the geographic range and a correlative analysis of disease and KoRV
Stent	KoRV in the body: Identifying viral distribution and expression in tissues using in-situ hybridization

Section 3: Origins of KoRV

Meers	Overview of the origins of KoRV
McMichael	Flying fox retrovirus, part of the KoRV mystery or a threat to bats
Mottaghinia	Frequent Integration of Gibbon Ape Leukaemia Viruses in rodents within the Australian-Papua region

Section 4: The host – the koala

Alquezar-Panas	The koala genome from a KoRV perspective

Section 5: What do we know about the role of KoRV in disease? (KoRV and Disease)

Gillet	Overview of the clinical presentations of KoRV
McKay	Incidence trends and significance of KoRV-associated diseases in monitored wild koala populations in SE QLD
Greenwood	KoRV contributes to elevated cancer rates during germline invasion
Higgins	KoRV associations with neoplastic disease, including chlamydial disease

Section 6: What do we know about the role of KoRV in disease? (Regional perspectives)

Krockenberger	KoRV infection and disease in NSW koala populations
Booth	The incidence of KoRV related diseases in koalas in Queensland
Devlin	What can studies of free ranging Victorian koala populations tell us about KoRV
Speight	KoRV infection and disease in SA koala populations

Section 7: KoRV diagnostics and Therapeutics

Higgins	KoRV diagnostics
Etiene	KoRV defence by the host
Timms & Olagoke	The development of vaccines for KoRV
Chappel	RNA silencing
Lifson	Anti-retroviral drugs

Topics discussed

For each of the following, we present discussion points, unanswered questions and recommendations:

Updated overview of KoRV transmission dynamics.

Updated overview of KoRV infection and disease biology: Degree of certainty of causation for neoplasia, chlamydiosis, ill thrift and bone marrow disease, joey loss.

Updated KoRV and koala genomics.

Current state of anti-KoRV processes, natural and developed.

Overview of KoRV diagnostics.

Overview on therapeutic control.

Origins of KoRV.

The notation [U1], [U2], [U3] keys to unanswered question 1, 2, 3, etc. at the end of the discussion points.

The notation [R1], [R2], [R3] keys to recommendations 1, 2, 3, etc. at the end of the discussion points.

(A) Updated overview of KoRV transmission dynamics

Discussion points

There is considerable variation across different populations (with respect to both KoRV and recKoRV subtype) with structuring of subtypes regionally apparent [R1].

Viral diversity decreases on a north/south gradient with a major divide at the Victorian border between "Northern" and "Southern" animals [R1]).

Southern animals display a decreased viral load and diversity compared to northern animals and don't appear to have endogenous KoRV.

Southern koalas do, however, have recKoRV variants that are probably not replication competent. i.e., It is not clear if they can co-package or recombine with KoRV or are accumulating new integrations into cells or which tissues these are expressed in [R1]).

The recKoRVs are present in the northern animals with regional variations in recKoRV sequence apparent (again with a major north/south divide).

Many southern animals that were previously assessed as KoRV free have recKoRV variants [U1], [U2]).

A likely endogenous genotype of KoRV-A (based on the presence or absence of the CETAG motif in *env*) that is present in northern but not southern animals has been identified. The difference in disease status and virus load between the putative endogenous and exogenous KoRV-A variants are yet to be explored.

Variants other than KoRV-A and recKoRV do not appear to be endogenous [U3]).

With respect to *env* subtype association with disease, current evidence points towards virus load (diversity increases with load) as being more convincingly linked to clinical disease (neoplasia) in wild animals than particular *env* subtypes of KoRV.

Data presented from zoo populations does not clearly demonstrate increased disease prevalence in KoRV-B positive animals [R2]).

It is now clear that there are many envelope subtypes of KoRV with three major phyletic groups—KoRV-A, KoRV-B, and a large set of related "D like" quasispecies (A is the basal virus phylogenetically with other variants likely derived from it) [U4]).

KoRV-A and KoRV-B are clearly replication competent and transmissible in cell culture experiments.

Transmission of other variants has not been demonstrated in cell culture. Cell culture experiments have all required cell–cell transmission (rather than from viral supernatants) to establish infection in human (HEK293T) cell lines [R3]).

Variants other than KoRV-A and KoRV-B are likely non-functional and replicate by piggy backing off the KoRV-A replication and packaging mechanisms to replicate.

Breeding or selecting for animals that only carry KoRV-A (or endogenous KoRV-A) would be feasible (however few such animals have been robustly identified to date) and while transmission routes are unclear it is hard to develop management recommendations. The relative importance of inherited alleles vs re-integration in individuals vs infection between individuals (all three routes may be occurring) is also very unclear.

There has been a lot of focus on *env* subtypes, but other determinants of retroviral replication efficiency (such as LTR sequences) may also influence transmission and need to be explored further. There is

some data on variability in the LTR region ([U3] enhancer region) [R4]).

Studies of familial groups in wild animals demonstrate a higher similarity between maternal KoRV subtypes and offspring than paternal, indicating that the main route of KoRV transmission is likely maternal, though whether in-utero (early-stage embryos) or via milk and colostrum is unknown [R5]).

In mouse studies (MuLV) integration was primarily in embryo or new-born animals (rather than via sperm). Either way, selection for maternal lines with low viral loads/integrations is probably a good idea to minimize new retroviral integration.

It is also possible that genetic factors, inheritance of alleles on X chromosomes (X chromosomes tend to preferentially accumulate endogenous retrovirus insertions as they are larger than Y chromosomes) or epigenetic silencing of paternal chromosomes (it is not clear whether this occurs in all marsupials or just kangaroos) may also play a role in the apparent maternal transmission/inheritance pattern.

The relative immaturity of koalas at birth may also be relevant to how effectively their immune system is able to control viral infections (and ease of endogenization). Zoo studies indicate that maternal transmission is more likely but does not explain all transmission/inheritance patterns. There is data from murine retroviruses also indicating that endogenization of retroviruses primarily occurs in the female germ line. It is not clear at which stage, ova, zygote, foetus, pouch young this could be occurring in koalas, however, ethics approvals for funding would be difficult.

In-situ hybridization work indicates high viral loads exists in sexual (sperm) and respiratory tissue in southern animals [U5]).

Whether there is super-infection over the top of endogenous KoRV loads is likely but not clear how much is transmission and how much is within animal mutation. There are no documented cases of infection of variants other than KoRV-A without a concurrent KoRV-A infection (all animals with infectious KoRV have KoRV-A to date with a variable load of other variants [R6]).

Whether KoRV will/can endogenize in southern animals is not clear—it is present in semen but quite variable so may be a matter of chance for a locus to become inherited.

Variability of KoRV loads (and subtype) over time is not well studied—only a few have been followed with some quite stable and some quite variable. Viral loads tend to be higher in older animals [R7]).

Unanswered questions

U1—Presence and absence of recKoRV and their significance: In general, and where KoRV is absent (southern animals), what is the significance of recKoRV variants? Do they contribute to inhibition of infectious KoRV variants?

U2—Other recKoRV variants: It is not clear if there are additional recKoRV variants with other recombinations. For example, different segments of viral genes.

U3—Degree of endogenization: Whether non-endogenous subtypes are transmissible or arise within individuals is not known. It is not clear if the “endogenous” version of KoRV-A is as transmissible as the “exogenous” version (this is important for whether prevention of transmission needs to cover both). It seems likely from accumulated data from sequencing experiments that only KoRV-A endogenises, while other variants are only reported as somatic integrations. Only KoRV-A has been found in sperm.

U4—Recombination of variants: It is not clear if recombination occurs between different KoRV subtypes.

U5—Routes of infection: Routes of transmission and the subtypes that may be transmitted is not known. In sperm and in respiratory secretions routes of transmission appear likely for an exogenous virus; however, it is not known if these are all KoRV-A or whether other variants are found in sperm/semen.

Recommendations

Long read sequencing: Resolution of this question may be answered using long-read sequencing.

R1—Longitudinal studies: Longitudinal inheritance studies across related individuals (dam, sire and joey) in zoo populations may uncover patterns of KoRV integration sites and disease prevalence for different viral subtypes.

R2—Transmission of variants: (i) Resolution of infectiousness of variants other than KoRV-A & KoRV-B might require tools such as virus pseudotypes and basic virology (cell culture) work into the function (or not) of viral proteins and variants. (ii) Are these variants infectious or only arise within individuals. This affects whether control efforts need to be directed just against A/B or all variants).

R3—Determinates other than *env* subtypes: Env subtype characterization is being prioritized for KoRV classification. Other determinates, including looking at the whole virus, need to be investigated.

R4—Viral Isolation: Viral isolation followed by sequencing can determine transmissibility of virions and subtypes from dam to joey. Excretion of KoRV in milk is a topic that needs research as allo-nursing of young to minimize KoRV transmission may be a viable control option in zoo populations.

R5—Marsupial cell lines: A lack of koala (or marsupial) cell lines (particularly KoRV free cell lines) and cell culture systems is hindering answering these types of questions. Funded research for the development of continuous koala cell lines would be very advantageous to KoRV work.

R6—Variability of KoRV loads and subtype: A geographically wide study of KoRV and recKoRV across Australia is recommended to understand viral load and diversity.

(B) Updated overview of KoRV infection and disease biology, degree of certainty of causation for:

Neoplasia

Discussion points

Data for the association of KoRV with neoplasia is very convincing now. Insertional mutagenesis is a well described pathology for gammaretroviruses (like KoRV). There are clear associations with neoplasia type, KoRV-A integration location and familial patterns for endogenous KoRVs from genetic studies of tumours in related groups of animals. KoRV also clearly accumulates new somatic integrations in tumour tissues on top of a base line germline load of KoRV-A insertions (though at what stage of life these occur is not clear). There are in addition clear and consistent epidemiological links between KoRV load and neoplasia across multiple studies from different populations and research groups [U1]).

Heritability of neoplasia risk is also evident in zoo pedigrees. Breeding for low impact KoRV integrations may however be difficult due to the numbers and complexity of insertions and the very variable time lag to onset of neoplasia [U2]).

Joeys may also have endogenous integrations not present in parents (making selection difficult).

The prevalence rates of neoplasia are greater in zoo populations (which are longer lived and have other infectious diseases controlled for) than wild populations. The impacts of neoplasia on zoo populations are considerable (it is the major cause of death after juvenile mortalities) and there are still limited control options for disease [U3] [U4] [R1]).

Unanswered questions

U1—Links between titre and integrations: The association between specific integrations and higher titre in relation to cancer is not known.

U2—Screening of integrations: It is also not clear which integrations are the deleterious ones (to be selected against in breeding programmes).

U3—Mixing of different populations: It is unclear what the risks and impact of mixing populations with different KoRV status (e.g., across the NSW/Victorian border or in zoos) are for disease prevalence, particularly animals with/without endogenous KoRV.

U4—KoRV differences—North to South: It is not clear what is determining the differences in the northern and southern populations. For example, (i) Are there differences in immune tolerance in animals born with KoRV that are unable to control it? (ii) Is there a gradual spread south of infectious variants? (iii) Is there genetic resistance to infectious KoRV (either from existing KoRV or recKoRV loci or other immune or receptor variance)? Diagnostics for KoRV integations (and selection) are likely more effective at a population level for decreasing risk. Predicting risk for an individual animal will not be effective due to the number of variables involved, unless targeted approaches are used (e.g., looking at specific Integration site hotspots in or near known oncogenes).

Recommendations

R1—Biobanking: There is a need for bio-banking (with established protocols) to facilitate studies within and across different populations. This is not specific to neoplasia samples but broadly across any pathology specimen that could be used for diagnostic purposes and/or to research disease causality.

Chlamydiosis

Discussion points

Evidence is more equivocal for links between KoRV and Chlamydia spp. infection. While immunosuppression predisposing to other infectious diseases is a well-described consequence of retroviral infection in other species, it is harder to demonstrate than neoplasia causality, particularly with a lack of cell culture systems/protocols for marsupial immunology. Chlamydia spp. Infection is also largely absent from zoo populations (and hence not studied in a controlled environment in the same detail as neoplasia [R1]).

Evidence is stronger in southern populations (where chlamydial and infectious KoRV prevalence are both lower than in northern animals) for a statistical association between KoRV and clinical chlamydial disease.

While neoplasia rates (< 3%) are unlikely to impact on wild population viability, chlamydia does (rates are > 40% in some QLD populations). There are indications that there are differences in severity (or number of intractable chlamydial cases) between QLD and NSW animals (regional differences are marked and need to be compared).

Many studies have focussed on KoRV subtype and chlamydial infection whereas it appears likely from the data on viral loads and subtype diversity that viral load is a more appropriate measure of KoRV severity and studies of chlamydial association should include viral load (there is likely an increase in the risk of clinical chlamydial disease with increased viral load [R2]).

There are also other factors at play with chlamydial susceptibility (such as non-KoRV koala genetics, chlamydial genetics including virulence plasmids, environmental conditions affecting nutrition and other bacterial diseases).

Combined sequencing and epidemiology studies are still required in this area to explore interactions between Chlamydia spp. and KoRV. Chlamydia strains in the south are also likely less virulent than those present in the north (complicating studies) lacking virulence plasmids.

There is an additional need to describe the interactions between herpesviruses of koalas, KoRV and clinical disease as it seems (again based on how similar viruses behave in other species) that the gammaherpesviruses of koalas are likely to be immunomodulatory and play a role in immunosuppression and clinical chlamydial disease [R1]).

Unanswered question

What role if any does KoRV play in Chlamydiosis?

Recommendations

Disease associations between KoRV and other infectious diseases: Comparative studies of co-infected koalas across different populations with KoRV and other infectious diseases (*Chlamydia* spp. and herpesvirus) is required to understand epidemiology and disease (e.g., Does herpesvirus positivity correlate with and increase or decrease of KoRV titre?). Comparative studies with animals that don't have infectious KoRV (southern populations) would also assist in disentangling disease associations. It is recommended that specific populations are identified for study.

R1—Statistically significant studies: Statistically robust studies that demonstrate whether high viral loads in northern animals are definitively linked to clinical chlamydial disease are required. Additional studies researching how chlamydial infection may trigger changes in KoRV loads and immunosuppression are required. Statistically significant studies also need to take other variables into account (such as chlamydial stains and background koala genetics).

R2—Koala risk factors: Additional longitudinal studies of KoRV and chlamydiosis in wild animals are needed to follow individual animals risk factors for this disease and what specific triggers result in manifestation of clinical disease.

Ill thrift and joey loss, bone marrow disease, other diseases in southern populations

Discussion points

Ill-thrift and joey loss

Other disorders such as ill thrift and joey loss have been postulated as linked to KoRV (and this is possible based on retroviral disease in other animals). However, better case definitions and higher case numbers are needed to make definite links between KoRV and other disease syndromes [R1]).

Bone marrow disease

Histological data for this looks strong. Bone marrow dysfunction is also a very well described for other gammaretroviruses [R1]).

Other diseases

Southern populations display distinctly different disease profiles to northern ones with sarcoptic mange and oxalate nephrosis major diseases in southern animals [U1] [R1] [R2].

Unanswered questions

U1—Co-morbidity: The relationship between sarcoptic mange, oxalate nephrosis and other diseases such as KoRV or chlamydiosis is not well explored. Oxalate nephrosis is probably a genetic condition, but data, to date, do not indicate links with KoRV integrations or virus load.

Recommendations

R1—Establishing causal links: A study integrating veterinary pathology, KoRV titre and integration sites is recommended to establish possible links to joey loss, bone marrow disease and oxalate nephrosis. Timely biobanking of specimens would be required.

R2—Understanding mites: A study on mite populations my provide additional insight into sarcoptic mange.

(C) Updated KoRV and koala genomics

Discussion points

Currently there is one annotated QLD koala genome (with resequencing to achieve better genome quality underway).

A new project announced by the University of Sydney and the Office of the Chief Scientist will do Illumina short reads for 400 koala genomes at 30× coverage but there are no current plans for assembly or annotation. The 400-koala genome project will select a range of koalas from across the range (mostly focussed on NSW but with some Victoria and QLD animals). There is also an RNAseq (Illumina) dataset from QLD and SA animals (29 animals) [U1]. Update: sequence data now available but analysis plans not clear.

Also, a partial long read genome of a SA animal (University of Nottingham) is not complete [R1]. Update: now complete and available.

The current annotation status of the koala genome is not detailed enough to characterize anti-viral defence systems with confidence for many gene classes. Lack of retroviral control factors may be a factor in why koalas are so susceptible to endogenization [R1] [R2].

Unanswered question

U1—Methylation: It is not known what the methylation pattern for the koala genome is and whether the preferential silencing of the paternal chromosome evident in kangaroos is also the case in koalas.

Recommendations

Marsupial and koala genome sequencing and annotations: The sequencing and annotations of more marsupial and koala genomes is recommended. In general, antiviral defence systems are poorly characterized across marsupials. Long read sequencing of critical koala populations (both north and south) and computational resource to complete genome annotations will be necessary. Particularly for exploring KoRV insertion locations and sequence diversity, presence, or absence of defective or recombinant variants (and whether this changes with time or whether more are accumulating). Short read technology alone will not resolve repetitive element loci. Better quality genomes would also facilitate comparison of different populations for genetic differences that may affect disease prevalence.

R1—Other—omics studies: There is also a need for RNAseq or methylation studies to explore the interaction between KoRV load/replication and antiviral defence mechanisms.

(D) Current state of anti-KoRV processes, natural and developed

This area is underexplored with one paper on piRNA inactivation of KoRV. It is unclear if this mechanism (or others) differs among populations of koalas. It is also unclear how much this mechanism contributes to silencing of infectious KoRV. There is no data on methylation status (or other indicators of epigenetic control) for KoRV integrations and getting a handle on this would help with resolution of endogenous vs exogenous integration sites.

(E) Overview of KoRV diagnostics

Discussion points

KoRV diagnostics are PCR based. Cell culture and antibody detection methods are used in experimental studies, but clinical diagnostics is almost exclusively PCR based.

These are split into end point PCR for KoRV presence or absence or presence/absence of a particular subtype.

Usually these are *pol* gene (KoRV presence) or *env* gene (subtype).

qPCR methods are used for estimates of viral load. These are usually *pol* gene based [R1].

PCR and Illumina sequencing have been used experimentally for envelope subtyping but is still expensive and cumbersome (only large batches are done at present) for routine diagnostic work. Similarly, long read sequencing (Oxford Nanopore/Pacific BioSciences) is still largely an experimental technique [R2].

RNA and DNA viral loads and subtype assessment are correlated (either is ok, DNA is easier in terms of collection, preservation, and transport).

Diagnostics in southern animals is complicated by the presence of the recKoRV variants, testing for KoRV using *pol* and *env* gene PCRs/qPCRs may miss these. These animals probably don't harbour infectious KoRV, but caution should be taken when declaring animals KoRV free and multiple genes (including LTRs) used to assess the KoRV status of animals for translocation.

Use of RNA later may be resulting in reduced detection of viral loads. Different preservation methods should be compared head-to-head to select the most appropriate routine diagnostic sample [R2].

Recommendations

Standardizations of diagnostics: There is a need to standardize reference gene usage for KoRV. This should include PCR diagnostics that are established and universally applied for LTR, *gag*, *pol*, and *env*. Standardization should also occur for qPCR primers (as different studies use different methods of normalization for qPCR and beta-actin is not a single

copy gene).

R1—Next-generation sequencing (NGS) Diagnostics: It would be beneficial to develop a routine subtyping diagnostic on the KoRV envelope gene (or other) that could be used across diagnostic labs. This would include the development of bioinformatic pipeline(s) that assists identified testing labs with downstream analytical processes.

R2—Standardization of collected samples: Sample collection protocols need to be established and implemented universally.

(F) Overview on therapeutic control

Discussion points

There have been a number of small pilot trials of vaccination of QLD (animals with KoRV 30 animals in largest group) and SA (animals without KoRV A) with *E. coli* expressed KoRV-A envelope protein (linear epitope). These have not raised any safety concerns and have indicated that koalas can mount an antibody response to the vaccine.

There are no comparable situations in other virus/host systems where vaccination against an endogenous retrovirus is used (endogenous and exogenous FeLV are quite different).

Autoimmune reactions to the vaccine are possible (autoimmune reactions to ERVs can occur in people but causal relationships with disease are weak). Those with KoRV infections have a decreased viral load. However, the magnitude and whether this translates into later protection from clinical disease are still open points.

There is conflicting evidence from different studies (using different envelope protein preparations) over whether northern animals with endogenous KoRV have existing antibody responses to the virus or not.

The issue of virus tolerance and whether animals can mount an immune response when vaccinated (in animals that are born with it) is an important one for considering vaccine efficacy for disease prevention.

Vaccination for prevention of transmission/disease may be more relevant in southern populations (without endogenous KoRV).

Alternative formulations of vaccine (mRNA vaccines or conformational epitopes expressed in mammalian cells) may also be alternatives to be explored

Raltegralvir (integrase strand transfer inhibitor) and Tenofovir (reverse transcriptase inhibitor) have been trialed in one animal with a modest reduction in virus load.

Cell culture experiments (human cells) with integrase strand transfer inhibitors (Elvitigravir, Raltegralvir, Carbotegravir, Dolutegravir) show dose dependent inhibition of KoRV. These drugs will soon have long-acting slow-release injectable forms for use in humans (monthly dose) which will make animal treatment a lot more feasible than current daily oral dosing.

This is promising for the use of these drugs in KoRV infections. However, pharmacokinetics in koalas (whether these drugs survive transit through the specialized koala GIT) needs to be done and the effect on viral loads in animals measured [R1].

Drug treatment will not eliminate already integrated KoRVs—selection of drug classes (to be effective against suppressing virus expression rather than re-integration) should be carefully considered (data from human ERVs indicates non-nucleoside reverse transcriptase inhibitors are the most effective drug class at decreasing endogenous virus expression). Antiviral therapies would only be feasible for zoo koalas.

Recommendations

R1—Zoo studies: Controlled studies in zoos should be performed to explore promising drug candidates.

(G) Origins of KoRV

Discussion points

Indications to date are that there are closely related viruses in Melomys spp. rodents and a variety of bat species in SE Asia and Northern Australia (endogenous in Melomys spp., exogenous in bats [U1]).

One hypothesis postulated is that a third virus (now extinct) may have been the origin for recKoRV but this is speculative at this stage [U2] [R1].

Comparative genomics of marsupials/koalas for other genes that may affect retroviral control is also still necessary to try and explain why KoRV-like viruses have endogenized so readily in koalas (but remains exogenous in primates and bats)

Unanswered questions

U1—Pathway of viral transmission: The direction the virus travelled and the implications for infection in bat species are unresolved.

U2—KoRV endogenization: The timelines for KoRV endogenization/fixation are still unclear. Modelling of average time for loss of fixation for multiple alleles entering the genome in an initial infection would be helpful to resolve this. It is also still unclear whether KoRV genome diversity is due to a burst of viruses integrating on initial entry, or accumulation of new alleles over time (or a combination of both).

Recommendations

R1—Dating of KoRV Invasions: Dating of LTR divergence would also be helpful to resolve the issues of the time frame of KoRV integration; however, at this stage no LTR differences have been found. This question may be explored through the comparison of multiple complete koala genomes and long read analysis.

DAY 2
Synthesis of Discussions KoRV 2021 Worksop Day 2: Applied Management—Zoo Populations

Cora L. Singleton, Geoffrey W. Pye, and Baptiste Mulot

Facilitators: Geoffrey W. Pye, Baptiste Mulot, and Cora L. Singleton

Goals

Identify practical applications of the knowledge that we have whilst acknowledging that we are very far away from knowing everything about KoRV

Develop a consensus on what is known, what we should do, and level of certainty

Day 2 talk titles

Pyne—Zoo Populations Australia

Singleton & Hamlin-Andrus—North America Koala Population Update

Imanishi—Zoo Populations and Koala Retrovirus in Japan

Md Abul Hashem—Epidemiological study of KoRV Genotypes in Koala in Japanese Zoo

Volker Grün, Baptiste Mulot, & Kerstin Ternes—Koala EEP (European Zoo) Update

Topics discussed

For each of the following we present discussion points and recommendations or suggestions, with a focus on consensus and knowledge gaps to identify ways to progress management:

- Recap of Day 1 Foundational Science discussion
- Understanding of KoRV status for management
- Testing considerations
- Breeding decisions
- North-south hybridization
- International transfers
- Role of stress and movement in KoRV infection and disease expression
- Treatment: anti-retrovirals
- Co-infections: herpesviruses
- Biobanking

(A) Recap Day 1 foundational science discussion

Discussion points

- KoRV transmission
  - Endogenous KoRV-A
    - Vertically (Mendelian inheritance)
  - Non-KoRV-A
    - Horizontally or vertically primarily from dam to joey though not definitively proven and may differ among subtypes
      - Rare—sire to joey
      - Rare—between breeders
      - Rare—casual contact
- KoRV status /profile

- Management application
  - Subtype presence
  - Subtype prevalence & diversity
    - Higher proportion of non-KoRV-A, relative to KoRV-A, is associated with higher likelihood of disease within individuals
  - Viral load
    - Increased viral load is associated with clinical disease
  - Geographically distinct profiles
- Discovery/research
  - Integration sites
    - Can affect expression of nearby genes and can be linked with specific clinical diseases
    - Joey integration sites more reflective of dam than sire
    - Geographically distinct profiles
  - Defective or recKoRVs
    - Non-functional and possibly protective but insertions may still alter gene expression in neoplasia and possibly other diseases

- KoRV diagnostics (current state)
  - Clinical diagnostics—PCR based
    - PCR or qPCR for functional KoRV presence or absence = *pol* gene DNA
    - Reverse transcriptase qPCR to estimate viral load = *pol* gene RNA
    - Presence/absence of a particular subtype
      - *env* gene DNA PCR
      - Illumina sequencing (economically feasible on a batch basis only)
  - Experimental studies—cell culture and antibody detection methods
    - Long read sequencing (Oxford Nanopore/Pacific Biosciences) for KoRV typing and insertion site analysis

(B) Understanding of KoRV status for management: underlying principles

Key issues

Description of KoRV status varies across populations, which hampers ability to compare populations, make management decisions, and assess health outcomes

- It is unclear what we need to know
  - Individual animal health vs population management?
  - Disease expression?

Discussion points

- Not all KoRV-B is the same
  - At least two different lineages
  - Also KoRV-B intermediate sequences
- Is KoRV-B status related to disease manifestation?
  - Disease manifestation is not necessarily associated with presence of KoRV-B specifically
    - The presence and diversity of all non-KoRV-A subtypes is a more important than presence/absence of KoRV-B specifically
    - KoRV-related problems are not eliminated by restricting KoRV-B positive animals
    - Plenty of healthy KoRV-B positive animals
  - KoRV-B detection may just reflect more viral transcription
    - Viral diversity increases with viral load—more virus, more subtype diversity, more likely to detect KoRV-B
    - Higher viral load linked to clinical disease (neoplasia) in wild animals, more so than a particular KoRV subtype

PCR test for KoRV-B

Result indicates that the animal is above the threshold, not how far above the threshold

Cannot reverse the logic and say that KoRV-B animals are likely to have higher viral loads

KoRV-B commonly present (detected on amplicon deep sequencing) but not detected by qPCR as at low abundance or has polymorphisms at primer sites

What is the cutoff level for "high" viral load?

Need longitudinal monitoring of individual animals

Depends on copy numbers, location of integrations, and expression of those KoRVs

Low expression of KoRV in a bad place may be worse than high expression of a KoRV in a less bad place

Peter Timms group is following a large group of wild animals in QLD but it is not very clear that there are consistent patterns in viral diversity/load for an individual over time (except that animals with leukaemia have a massive spike in load and that load gradually increases with age)

Management decisions

Co-housing

Co-housing of koalas with different subtypes leads to very low transmission

Suggestions for keeping KoRV-B animals separated from KoRV-A only animals is not justified

Breeding

May be most important to have KoRV-A only (minimal to no other subtypes) breeding females, though this would generally restrict breeding to southern koalas

Use pedigree information

Who has bred a lot? Are there families where all offspring die young? Specific diseases running through specific lineages?

This pops out in pedigree analysis sometimes but is information that has not been systematically collected and must be followed up repeatedly

Disease association (what status is thought to have lowest disease expression and highest longevity)

Ideal appears to be low viral load, KoRV-A only, minimal deleterious integration sites

Prevalence (& diversity?) of non-KoRV-A subtypes is associated with disease manifestation

Subtype diversity is more important than presence/absence of KoRV-B specifically

Not all KoRV-B is the same

Plenty of KoRV-B healthy animals

There are many non-KoRV-B subtypes

Discussion summary

Three questions to ask of each koala

Which KoRV subtypes does it have?

Where are the KoRV integrations?

How much are these integrations being transcribed?

Test categories

qPCR—probably best diagnostic

Quick, inexpensive

Need to standardize

Test for viral load, look at % of KoRV-A

If KoRV-A is majority, then might be ok to stop

If not, then start looking for other variants

Subtype analysis—deep amplicon sequencing of *env* gene

Need bioinformatician and batching of samples but not as involved as would be for looking at IS

Maybe more useful to test breeding females

Whole genome studies for insertion sites and subtype diversity

Still in research arena due to expense, complexity and incomplete understanding

Cost per animal decreases as number of animals tested increases

Recommendations / Suggestions

Diagnostics working group

Standardized testing protocol, frequency of testing, which animals to test, testing tiers

Protocol

Need a global standardized test

or maybe indicate the test used

If can only perform one test—do viral load, select for koalas with low viral load

Define KoRV status/profile

Viral load

Subtype prevalence and diversity

Integration sites

recKoRVs

(C) Testing considerations

Key issues

Diagnostic testing for KoRV lacks uniformity and application, which hampers ability to compare populations, make management decisions, and assess health outcomes.

Transfer of biological samples for testing has challenges

Discussion points

Testing is not standardized

Agree upon methods

Primers and target are critical to agree upon (what you are amplifying)

Kits and enzymes can be changed based on local availability as tests validated in-lab

Set up a testing schedule

Based on test type and management need

Review and update on a regular basis

Amend with information about new variants

Viral load (qPCR)

Advantages

Easy and inexpensive

Informative and trackable

Easier to apply results to management decisions

Longitudinal testing

Changes in viral load may help to identify animals before clinical disease develops

Especially breeding animals and older animals

Important to monitor changes in viral load if treatment with antiretroviral drugs becomes feasible

Subtype diversity & prevalence (qPCR)

Application

Do a qPCR to distinguish variants

If you have a population that has never had KoRV-B, the population is unlikely to get KoRV-B over time unless you have an unlucky recombination event

False negatives possible as target region is hypervariable and polymorphisms can occur within primer sites; and some animals have extremely low target abundance, which may

- be below limit of detection.
- But there are many subtypes and relevance unknown—what to test for?

- KoRV D diversity is massive
  - Every wild population will turn up a new clade of different subtypes so if you design qPCR primers for specific subtypes, you'll quickly become outdated
  - Infer how much of the viral load is attributable to non-KoRV-A
  - qPCR for total viral load—qPCR for KoRV-A (original sequence) and all the rest (non-KoRV-A)
    - If low proportion of non-KoRV-A, then maybe not worried about it
    - If high proportion of non-KoRV-A, then consider more testing to sort out all of the subtypes
    - All the rest—could be B, D, non-functional but might not be important which
    - Lose information about combination of subtypes with this "fractional" method

- Integration sites
  - Probably important when looking at neoplasia in lines of individuals
    - Seeing families having a high rate of neoplasia—look at that line to see if there are particular IS that are in those oncogenes
      - May inform decision not to breed from that line.
      - However, the problem with this is how to avoid breeding in other "bad" IS?
  - In principle would be useful and not very expensive to get full IS profile of captive population and *env* variant diversity
    - Then all future testing would be on the few individuals bred into the population from the wild, and if those were from SA and Victoria, the problem would likely be quite minimal (low KoRV-A *pol*, less recKoRV)
  - Challenges
    - May be cost-prohibitive to screen all animals
    - Data is time consuming to analyse and understanding is still early

Potential Approach

- Tier 1 = PCR-based clinical diagnostics (quick, inexpensive, available)
  - Subtype presence or absence (*env* gene)
  - Viral load (qPCR, *pol* gene)
    - Select koalas with low viral load
    - Higher viral load, more likely to test positive for KoRV-B
    - *pol* gene PCRs are from Tarlinton original primers
  - Viral load + Subtype prevalence and diversity (endpoint PCR, *pol* gene)
    - Look at % of KoRV-A
      - if KoRV-A is majority, then might be ok to stop
      - if not, then start looking for other subtypes
  - In all cases need to remember
    - KoRV consists of multiple elements and detecting one of these does not necessarily indicate that all are present in functional form (i.e. PCR may be detecting retroviral elements in absence of complete virus). Context is important.
    - There is a need for a panel of qPCRs across multiple targets (e.g., *pol*, *env*, LTR etc)
  - Technical note for qPCR
    - TaqMan PCR is >10 more sensitive and reduces false positive signals, compared to standard qPCR.
    - TaqMan Probes are expensive to start with. But, once established, running costs are cheap. For example, most SARS-CoV-2 testing kits use TaqMan.
- Tier 2 = research studies (expensive, long time to results, need bioinformatician)
  - *env* gene amplicon deep sequencing
    - Only way to gain certainty of subtypes present

- Prioritize breeding females, or where *pol* high but KoRV-A low, or KoRV-B detected (indicating non-KoRV-A subtypes likely abundant)
- Easier than full genome sequencing and profiling
- Data analysis is time consuming and only feasible financially for large runs of samples
- Integration site and recKoRV analysis by long read sequencing
- Gold standard, if can afford cost and time for analysis
- Not diagnostic—can't necessarily say how a disease is going to progress
- For breeding selection
  - If two koalas are sharing an IS, it is important to know where that IS is to avoid driving an IS in an oncogene to homozygosity across your entire population, which could create a highly cancer prone koala populations
  - Select for the most harmless integrations that you can find
- recKoRV analysis also useful
  - Some of the novel integrations that land in bad places are recKoRVs
  - Functional KoRV can move the recKoRVs—so the recKoRVs can be harmful

Consider adding herpesviral load by qPCR

- May play an important role as immune modulators

Recommendations / Suggestions

Form KoRV Diagnostics Working Group

- Uniform testing protocols
- Regional testing centres
  - Europe—Nottingham or Berlin?
  - North America—San Diego Zoo Wildlife Alliance?
  - Australia—Koala Health Hub, Australia Museum
- Centralized data collection?
- Testing guidelines
  - Which suite of tests?
  - Which animals?
  - When to test?

Develop qPCR for herpesviruses, multiple KoRV elements, streamline amplicon deep sequencing

Define what is a "high" *pol* load or "high" level of non-KoRV A

(D) Breeding decisions

Key issues

How can information about KoRV status inform breeding decisions?

Discussion points

Transmission

- If occurs, appears to be primarily from dam to joey

Diversity of KoRV is associated with disease

- Strive to minimize the KoRV diversity
- KoRV-A only females are extremely valuable as likely to be more resilient, more healthy
  - Offspring will be KoRV-A only—No sequence sharing from sire to joey above unrelated background sharing
  - KoRV-A only joeys re-sequenced 18 months later, had no other subtypes present

Wild populations

- Random mating—low amounts of IS sharing, particularly in the oncogenes
- More IS sharing in geographically close koalas

Would be interesting to release KoRV-A only koalas to wild and see if they remain KoRV-A only

Managed populations

KoRV-A plays an important role in managed populations

Amount of IS sharing goes up dramatically, probably being driven into both chromosomes

If breeding individuals that have an abundance of IS shared in oncogenes, may end up with koalas that are extremely prone to developing cancers early

Goal—minimize fixing deleterious IS (make sure they don't go to high frequency in the population) over maximizing genetic variability in zoo populations

Maybe get genetic diversity from sire and minimize KoRV diversity through dam

If IS that showed up in a joey that neither parent had were heritable it would cause huge breeding problems—if they keep making new KoRVs independent of inheritance it would make it impossible to breed out undesirable lines.

KoRV testing for management

qPCR and subtypes using amplicon deep sequencing can give an indication of risk (more diversity and higher loads equals greater probability of deleterious IS)

But maybe full genome sequencing and IS analysis is important for the breeding animals

Recommendations / Suggestions

Breeding

Subtypes

Dam—prioritize KoRV-A only females

Joey KoRV status reflective of dam status

Sire—KoRV status less important

Joey KoRV status reflective of dam status

No sequence sharing from sire to joey above unrelated background sharing

Low rate of transmission between breeding partners

No sequence sharing between partners

Integration sites

Avoid pairings that fix deleterious integration sites

Maybe get genetic diversity from sire and minimize KoRV diversity through dam

Housing

Co-housing of koalas with different subtypes leads to very low transmission

Caution housing lactating females of different status—horizontal transmission through milk

Pedigree work

Learning if there are certain animals that are passing on disease

Can target animals that don't develop disease

(E) Northern-southern hybridization

Key issues

Could breeding northern males with southern females maintain genetic diversity (southern problem) while minimizing KoRV (northern problem)?

Discussion points

Southern koalas

KoRV exogenous

Southern koalas have exogenous KoRV-A (endogenous KoRV-A in the north)

Have minimized the problems with KoRV

But they have other genetic problems due to inbreeding

Neoplasia still associated with exogenous KoRV-A in South Australian koalas, but the dominance of KoRV-A in SA koalas makes it hard to find variants without deep sequencing

Genetic diversity reduced

Severe population bottleneck

Status of managed populations outside of Australia

North America

Phased out southern koalas a while ago

Not inclined to hybridize unless this is recommended by Australia

Europe

Only Longleit has southern koalas

Had decided to keep them separate

These would comprise a useful population for longitudinal studies comparing to captive northern koalas

Northern-southern hybrids

There does seem to be some northern blood in SA

Based on Blyton microbiome and KoRV work

Integration sites

Will still inherit about half of integrations from sire so might not get around the problems of fixation of integration sites

recKoRVs seem to have a hard VIC/NSW border

recKoRVs are present everywhere but different variants

There are at least 3 distinct variants of recKoRV1

recKoRV 1 seems to have spread the farthest but there is a hard boarder somewhere in western NSW...coastal NSW koalas have recKoRVs but they are not the same as the ones in Queensland

Disrupt co-evolution and local adaptation

Might be less of a problem for populations in zoos

Recommendations / Suggestions

No recommendations

(F) International transfers

Key issues

Some KoRV-B positive animals do well for long time, then die shortly after transfer

Australian export standards do not require KoRV testing

Discussion points

Europe

9 of 11 imported koalas died at 2–4 years of age

Would be interesting to determine time between transport and mortality

United States

Last import from Australia in 2013

Association of Zoos and Aquariums (AZA) Koala Species Survival Plan (SSP)

Determines koala movement and breeding needs for the North American population

Participating zoos cooperate to fulfill these recommendations

Australia

Stock animals for international zoos

Some facilities would not be able to test via the protocols that we are thinking of—need to discuss with Australian facilities

Southern-northern hybridization

Would facilities be interested in breeding southern-northern hybrids for export?

- Value to keeping southern and northern phenotypes separate
  - Reflection of what is happening in the wild, unaltered state
  - Phenotypes are very different
- Zoo and Aquarium Association (ZAA) has species as monitored and not managed
  - Group can make breeding and transfer recommendations but no guaranteed action
  - Recommendations are not adhered to
  - Are not asked to identify koalas for export
  - No idea of KoRV status
  - Australian zoo populations are not managed separately
    - Individual zoos do what they want
    - Northern and southern studbooks are managed separately
    - Some hybrids
- Export of koalas is much lower priority to a zoo than the larger commercial opportunities
  - Unlikely to be pulling out specific koalas for export
  - Commercial interests dictate number of koalas, how they are bred, where they live
  - Institutions vary in husbandry practices
  - Koalas have special jobs in zoos
  - Great resistance to regulating certain animals for export
- South Australia
  - Some consider future of koala in Australia a national hybrid and that southern koalas should be translocated to the north
    - Not enough koalas in wild in QLD and NSW—lots of populations are below self-sustainable level
    - 1960s northern koalas released into Mt Lofty ranges around Adelaide where they bred
    - Recent genetic work suggests that they are not as bottlenecked as would be expected
    - KoRV is low prevalence clinically
  - This is contentious, with the status quo being disagreement
    - Still have southern phenotype
    - Are still of low genetic diversity
    - Primary drivers of low koala numbers in north are chlamydial disease and habitat degradation and loss
  - Whole genome studies are pending but may be informative
- How should koalas be evaluated prior to export from Australia?
  - Evaluate pedigree
    - Choose from good family line—low disease and high longevity
    - Institutions that have this data are less likely to be exporting overseas
    - Larger populations have less data about pedigree and health status
  - Test for viral load

High viral load linked to development of disease

Evaluation of viral load may require longitudinal testing

Viral loads may change with stress

- Test for KoRV diversity
  - More diversity suggests more likelihood of disease (uncertain if causation or association)
  - Minimizing diversity in KoRV provides best opportunity to keep viral load in check
    - Minimize number of variants to endogenous KoRV-A only
    - Prefer KoRV-A only breeding females
  - *env* gene amplicon deep sequencing
    - Chappell lab at University of Queensland have done this for a couple of zoos
- Herpesvirus
  - No evidence base in koalas but can be strong immunomodulators
  - May be acting synergistically with KoRV

Who to test?

Males—KoRV profile less important, based on current data

Focus on individual

Females of breeding age—try for KoRV-A only breeding females

Sequence for diversity of KoRV

Focus on population

Southern

Whether they have KoRV-A or not

Northern

Diversity and load of non-KoRV-A subtypes

Challenges

Everyone will want KoRV-A only koalas but where will they come from?

Will likely limit to southern provenance koalas

Not every institution can pay for testing

Not every institution wants to know the profile of their animals because they might fall out of favour for exports and lose income

Testing before export/import does not guarantee that

All offspring will have healthy outcome

All transported animals will have healthy outcome

Animals will never have neoplasia or other KoRV-related disease

Recommendations / Suggestions

Australian export standards

Does not require testing for KoRV

Should we propose this to be changed—federal Department of Agriculture, Water and the Environment manages the export requirements

We should influence a revision

Highly successful from chlamydia perspective

(G) Role of stress and movement in KoRV infection and disease expression

Key issues

Some KoRV-B positive animals do well for long time, then die shortly after transfer

Discussion points

Stress is hard to quantify

Assays need to be validated and standardized. Not all metabolites are useful indicators (Santamaria et al, 2023).

Stress hormone changes need longitudinal testing to be useful

Stress indirectly linked to retrovirus loads

Stress is likely to increase load and initiate a feedback loop

Stress increases, virus escapes immunological control, virus increases

Viral load is also linked to diversity

Testing diversity and focusing on KoRV-A only animals may provide higher resilience. Better to cope with stress of translocation

Recommendations / Suggestions

Continue studies looking at relationships between faecal stress hormones, immune parameters and a range of coinfection loads including KoRV, herpesvirus, and Chlamydia (and immune parameters)

To evaluate health and resilience

Considering KoRV as a parasite

Maybe KoRV load is an indicator of underlying stressors

If not causative, viral load may be an indicator

(H) Treatment: anti-retrovirals

Key issues

No anti-retroviral preventive or therapeutic options available at this time

Discussion points

Not helpful once animal already has cancer

Might be helpful to prevent new integrations that could lead to cancer—prevent expression of disease

Integrase inhibitor—not useful to prevent transmission from dam to offspring

Need to know which drugs will accomplish what

Safety study? Pharmacokinetics?

In vitro first but in-vivo trials needed eventually

Worth exploring more

Target reducing transmission from dam to joey

Recommendations / Suggestions

Explore options for investigation

In vitro >> in vivo

(I) Gammaherpesviruses

Discussion points

Herpesviruses are huge manipulators of the immune system so there could be a synergy between KoRV and herpesvirus in co-infected animals

Could be causing some of the clinical disease seen in koalas

It might be worth adding herpesvirus testing to general testing

Recommendations / Suggestions

Consider making recommendation for gammaherpesvirus testing

(J) Biobanking

Key issues

Currently lack coordinated effort to bank biological samples for diagnostic and research work

Need standardized recommendation

Sample type, volume, handling, method of preservation

Discussion points

Two types of biobanking

Disease investigation biobanking

Retrospective analysis of pedigree biobanking

Standardize biological samples for banking

Vary by size and type—what can each sample type be used for

Random samples vs requested samples (type, volume) with focus on disease testing

- Make disease associations—need paired healthy and neoplastic tissue from given animal
- RNA studies
  - Sample type
    - Whole blood—no heparin, only EDTA
  - RNA stability—How long can RNA be kept without degrading? Varies
    - Longer PCRs—RNA degrades pretty fast if not frozen
    - Fragmented RNA stays pretty stable for a while under certain storage conditions
    - KoRV titres retrospectively with high throughput sequencing—doesn't matter if degraded a bit, as long as it is not completely gone
    - Rough rule of thumb (without preservatives) is one year at -20°C, 10+ years for -80°C
  - RNA preservation
    - Snap frozen for RNA (avoiding RNAlater)—followed by -80°C freezer storage or liquid nitrogen.
    - RNAlater
      - Some problems quantifying KoRV load
      - Lacking solid evidence that it really works
      - With some extraction protocols, RNAlater also decreases yield
      - Viruses remain infectious
- DNA studies
  - DNA is stable (for integration sites and retrospective pedigree work)
  - Snap frozen is good
  - Formaldehyde/formalin stored samples are poor samples for nucleic acid so are to be avoided

Recommendations / Suggestions

- Biobanking Working Group
  - Statement of intent
    - Opportunity for institutions to support research
  - Options
    - Physical biobank—single location
    - Virtual biobank—log inventory into shared database for all to know what is where
  - Samples
    - Protocol for sample type, size, processing, preservation
  - Where to bank
    - North America
      - San Diego Zoo Wildlife Alliance
    - Australia
      - State museums—great repository
        - NSW—Australia Museum
    - Europe
      - EAZA
  - Cost investment—where would funding come from
    - Storage space
    - Sample management—inventory, distribution
      - Database—ZIMS?
    - Transfer sample
    - Approve release of samples
- Damien Higgins and David Alquezar involved with NSW govt
  - Identifying issues with banking and sharing samples and data
  - Might be good starting point
  - Looked at different models for biobanking and data sharing

Protocols on KHH website (koalahealthhub.org.au)

Revisit and revise

References

Santamaria, F., R. Schlagloth, L. Valenza, R. Palme, D. de Villiers, and J. Henning. 2023. The effect of disease and injury on faecal cortisol metabolites, as an indicator of stress in wild hospitalized koalas, endangered Australian marsupials. *Veterinary Science* 10(1): 65. https://doi.org/10.3390/vetsci10010065

DAY 3

Synthesis of Discussions KoRV 2021 Workshop Day 3: Applied Management—Wild Populations

Damien P. Higgins, Amy Robbins, and Philippa A. McKay

Facilitators: Larry Vogelnest, Geoffrey W. Pye, Amber K. Gillett, and Laura Chao

Goals

To bring the discussion back to practical applications of the knowledge that we have whilst acknowledging that we are very far away from knowing everything about KoRV

Draw out differences with the captive situation

Revisit some shared issues after reflecting since previous sessions

Develop a consensus on what is known, what we should do, and level of certainty

Topics discussed

A—Background to management of free-ranging koalas

B—Seek consensus on risk (and certainty)

C—Seek consensus on management (and certainty)

D—Explore Diagnostics again in new context and after some reflection

E—Explore targeted research strategy priorities and strategies

(A) Background to management of free-ranging koalas

Multiple threats: extreme climatic events, climate change, habitat degradation, loss and fragmentation, trauma (cars and dogs), disease (especially oxalate nephrosis in SA, chlamydiosis in NSW, Qld)

Victoria and SA—koalas hunted almost to extinction late 1800s to early 1900s

- Reintroduced from limited stock—genetically fairly homogenous
- Widespread overpopulation issues
- Few valuable remnant populations remain in Gippsland/Strathbogies

NSW ACT and Qld—also hunted but have been left to recover

- Significant pressures—cars, dogs, chlamydial disease, underlying issues of land clearing, fragmentation, climate change
- Listed as Vulnerable under Federal EPBC Act in 2012 (update—Endangered 2022)
- To date no threatened species management plan (update-in progress)
- Large number of local area koala management plans

Currently increase in management activity in all states

- State and federal koala strategies—iconic status
- 2019–2020 bushfires and preceding drought/heat

Formal Federal disease risk analysis underway (update completed 2022)

- Need for this on state and federal levels to inform monitoring and management strategies
- Challenging due to knowledge gaps

(B) Consensus on risk posed by KoRV to wild populations

Discussion points

Southern populations

Break in KoRV dynamics appears to be at NSW/Victorian border

Victorian populations fragmented with low diversity, high inbreeding, and associated defects, and a lot of mange (more than other states), Chlamydia present but rarely see ocular disease. Low rate of neoplasia (minimal risk and only a 1.5–2% lymphoma rate in South Australian koalas, low prevalence in captive koalas). More general surveillance and disease risk assessment needed.

Where population numbers are strong or overpopulated, significance probably low and any impacts mostly related to welfare

Possible exception of valuable remnant Victorian populations with greater genetic diversity: East Gippsland and maybe some remnants in Beechworth/Snowy River Valley, maybe Strathbogie—limited work done on KoRV in these but appears Strzlecki and Strathbogies have similar or possibly lower prevalence of intact KoRV to rest of Vic.

South Australia—most prevalent disease is oxalate nephrosis. Very genetically restricted, very low rate of lymphoma, only some have intact KoRV. KoRV profile of one likely terminal case looked like a Qld koala, with sharp increase in replication and diversity, though host genetics consistent with SA koala.

Kangaroo Island has seen a significant increase in prevalence over the past 15 years to 42% around 2017, which shows the potential for rapid spread in the southern animals

There is disagreement whether non-KoRV-A subtypes exist in Vic and SA, and suggestion that there may be some Qld animals in SA. If non-KoRV-A are present they are never seen without KoRV-A so may not be a critical question in terms of disease impact (virology yes, disease impact probably not)

Significance of recKoRVs is a knowledge gap:

> Findings through PCR/qPCR have shown consistent results across *gag* and *env* gene sites; with central genome sites (mid *gag*, *pol*, to early *env*) are negative in "negative" koalas (ie amplification of recKoRV) and all positive in "positive" koalas (amplification of competent KoRV-A); consistent with nanopore and unbiased RNAseq data.
>
> If very highly expressed (transcribed) recKoRV could still affect gene expression; LTR still active.
>
> The recKoRVs are different among populations and individuals and they are as insertionally polymorphic as KoRV. Some of the tumour specific integrations are recKoRV, as are some joey specific integrants; so they are still behaving like KoRV due to piggy backing
>
> Could also hypothetically affect receptor expression via epigenetic silencing or by stimulating intracellular defence pathways

Northern populations

Risks may vary on a north-south cline from Qld to Sth NSW, in association with differing subtype diversity and proviral loads. This is consistent with anecdotal evidence from field work and koala hospitals: severe chlamydial disease still occurs in central and southern NSW but there appears to be less putative KoRV-associated disease (PKAD)—though there is a real need to standardize evaluation for comparisons.

Neoplasia higher prevalence than southern states but still probably low-impact on populations (3% of SE Qld hospital admissions over 16 years)

PKAD: 8.33% of admissions, however, joey ill-thrift and mortality a concern in zoos (16–33%) and the cases frequently seen in care are likely the tip of the iceberg given likely low detectability of abandonment, morbidity or mortality in back or pouch young. If associated with KoRV integration sites (IS), prevalence and presentation may be patchy (IS are not fixed and so vary between individuals and sub-populations, but may see more fixing, homozygosity and therefore impact in some fragmented populations due to inbreeding—there is some observation of local pockets of ill-thrift/PKAD to support this)

> If non-response to chlamydial treatment or severe chlamydial disease are considered PKAD, then impacts will be much higher.

Mechanisms/evidence for KoRV role in disease

Preliminary evidence for interference of genes or their control regions by IS, retrotransposition of oncogenes, interactions of the immunosuppressive domain with immune cells, or direct disturbance of function of infected cells (e.g., cytotoxicity) resulting in immunomodulation, though none are conclusively proven

Interactions between KoRV, herpesviruses and Chlamydia spp. not fully investigated but precedents exist for interactions.

Competent KoRV load (*pol* gene) and proportion attributed to exogenous subtypes appear to be strongest correlate to disease (association—causation not shown)

Recommendations

Await risk evaluation in National Koala Disease Risk Analysis and revise as new information emerges.

Continue research on KoRV—koala relationships to investigate causality of existing associations of KoRV with disease.

(C) Consensus on principles for management

Discussion points

Free-ranging populations

Active control not warranted at this stage—no known treatment, no consistent KoRV trait to target for control

Due to heritability of IS, should be thinking of it partially like a genetic condition

> Apply the same general management principals as we do now of maintaining genetic diversity and habitat connectivity as much as possible

KoRV is a relatively recent introduction and is still co-evolving with its host. Reduce other threats to allow co-evolution to occur and factor in associated mortality: In most populations if we control habitat, chlamydia infection, dogs and cars, populations appear to thrive.

Should try not to disrupt natural co-evolution or make things worse by introduction of new types, or introduction/concentration of deleterious IS through management interventions such as translocation (precautionary approach):

> Avoid crossing biogeographical barriers and moving over large distances
>
> When re-establishing habitat corridors, consider the time the populations have been separated (has there been enough time for differentiation?)
>
> Subtype screening with deep sequencing should be a minimum requirement if moving over larger distances or crossing biogeographical barriers (over rivers, more than 50 km?)
>
> Large distances—consider moving these koalas into unoccupied habitat
>
> If translocating inbred koalas into another population for genetic rescue, would also be good to screen insertion sites in target recipient population, which will minimize the risk of these insertion sites becoming homozygous and fixed
>
> After translocating for genetic rescue, recommend ongoing testing to see what impact management interventions have
>
> Maintain good biosecurity practices for transfaunation / blood transfusion, artificial insemination—precautionary as transmission modes unknown

For southern populations, prevent the southern populations from becoming like the populations in the north KoRV-wise (control competent KoRV by screening for KoRV *pol* gene).

Exogenous KoRV transmission during transport / stress events

Basic biology not known for any transmission routes.

Lineage studies indicate transmission most likely to be mother to young

Can’t rule out other routes.

> Gammaretrovirus vary in transmission routes—FeLV saliva, FIV bites, only one respiratory (sheep).
>
> KoRV RNA observed in respiratory mucosa by *in situ* hybridization

Stress increases shedding of many viruses. Well known in herpesviruses, which may hypothetically interact with KoRV and chlamydia.

Sensible approach is probably to control possible dam to joey transmission and then use basic precautions for other routes.

Recommendations

Continue research on KoRV—koala relationships to investigate causality of existing associations of KoRV with disease and determine/confirm transmission pathways.

(D) Diagnostics and screening

Intent of screening is like that for captive management—mitigating risks of animal movement

Animals with traits that allow retroviral escape and amplification

Introduction of novel subtypes

Discussion points

Southern populations

KoRV *pol* qPCR to avoid introducing replication competent KoRV

In recKoRV the central region (including *pol*) is absent but initial half of *gag* and then *env* is present

In southern populations a panel of *env*, *pol*, *gag*, LTR could be useful as, if very highly expressed, recKoRV could still affect gene expression as the LTR is still active.

Northern populations

Screening for IS not viable as IS differ too much among animals/regionally

Those that are uncommon can only be found through slow and expensive sequencing and further work to determine impact

Those that are common are less likely to be deleterious

Scat DNA unlikely to be useful due to fragmentation

Requires more work to understand IS as a mechanism of pathogenesis

Potential to screen pathway end-points? (immunological or cell growth traits)

Longitudinal health data for source populations/lineages—likelihood of deleterious KoRV traits based on population/lineage

Screening for subtypes of value to avoid introduction of novel subtypes

Impact unknown but precautionary

Requires amplicon deep sequencing—expensive and slow but suited to large batches

Subtypes can be detected in scats though sensitivity not quantified

Proviral/Viral load (or transcript load)

Need to carefully consider target: *env*? *pol*? other?

Likely of value as reflects

Escape—animal has undesirable traits (KoRV-associated, heritable, or other) that allow retroviral escape

Greater potential for pathogenesis

Are proviral/transcription or plasma viral loads best? Viral/transcription probably more dynamic but in practice proviral and viral appear to correlate well and DNA much easier for clinical purposes.

Very difficult to differentiate leaked transcripts from packaged virus—RNA work could reflect either.

Other assays to be considered for development

Transduced oncogene PCR

CETTG motif

Differentiate exogenous from endogenous.

Ratio A3001/2 vs 3003—virulent CETTG

qPCR design difficult (minor sequence change only). Need to quantify to develop a ratio, as multiple types in individuals and populations

Based on Eiden, cell culture work shows different replication efficiency.

Recommendations

Technically speaking we are in a position to deliver testing needs but need working group to:

Develop consensus and standardization.

Develop test validation and quality assurance standards as well as workflows and charging/handling processes.

(E) Research strategy over the next 5–10 years

Discussion points

Key questions

Relationship between KoRV traits, Chlamydia, herpesviruses, stress and disease

relative contributions

mechanisms for pathogenesis: what KoRV traits or biomarkers are significant

better tests/criteria for animal selection/management strategies

Many association studies equivocal: KoRV is regional so profiles and diseases may vary tremendously

need to compare across multiple populations using standardized approaches

need for longitudinal studies to show causation

need for more necropsy data to definitively determine outcomes (requires timely mortality detection)

need for in-vitro work to understand mechanisms

need to stratify disease classification—especially group of koalas with chlamydiosis that are refractile to treatment

Need new frameworks for study of these questions in endemic disease.

Baselines/comparison populations or animals needed—difficulty where all animals positive.

May compare northern koalas to southern as a control, though need to recognize that they differ in ways other than KoRV dynamics.

Regional differences within northern populations

Longitudinal studies of individuals

Use of treatment or vaccination as a manipulation

limited KoRV work happening in Vic, though opportunities are emerging through DELWP and new Victorian koala strategy, which includes disease risk analysis and surveillance to inform future translocation programs and other interventions

main issue is in integrating population and research effort (and to get some picture of population level problems over time). Needs:

protocols for sample collection and preservation that mean samples are usable for later work (see Koala Health Hub protocols; koalahealthhub.org.au)

researcher-manager engagement well before, to incorporate disease study requirements in planning

Herpes—overlaps

What is the status of herpesvirus infection in koalas in the north?

Any information on prevalence in northern populations would be useful right now. Might be particularly useful in those koalas with chlamydiosis that fail to respond to treatment.

if it is contributing to immune modulation and is being transmitted horizontally we need to mitigate risk in hospitals

- Can anyone offer koala herpesvirus testing in a clinically useful timeframe?
  - Resolved to get TWIST or LAMP based POC testing for herpes, as well as lab based for quantification
- Significance of introducing different (novel) subtype variants between regions
  - Is this really an issue or is one as good/bad as another?
  - How different is important?

Biobanking and collaboration

- Wildlife hospitals and research teams with veterinarians and ecologists can collect a lot
  - issues
    - storage, costs at collection and storage and time to catalogue and label, standardization from the beginning.
    - Confused about best sample to collect and what can be collected. Especially for field researchers without a centrifuge, -80°C freezer, etc.
    - Permits—especially across states—animal ethics, state government scientific, and interstate export-import
    - PhD student management—large, complex, integrated multivariate studies often beyond scope of a PhD, and requirement for independent research in PhDs can impede close collaboration
  - solutions
    - tiered approach to sampling based on question priorities/simplicity
      - Basic DNA—subtype diversity in area
      - RNA, virus—need -80°C storage (RNAlater second best), fresh bodies (< 6h, definitely less than 24h)
      - Focus on sampling animals with good metadata
      - Neoplasia—diseased and non-affected tissue from animals
    - Protocols online (https://koalahealthhub.org.au/sampling-protocols/)
    - Pre-labelled kits
    - Stronger links and feedback between researchers and clinicians and government people. Disease risk analyses are planned and working groups from those may be useful for integration of research.
    - Develop strategies for
      - Cross jurisdictional permitting
      - Management of Intellectual property for data and samples (agreements and communication)
      - Resourcing opportunistic sampling and secure biobanking
      - Overcoming systemic fragmentation—e.g., PhD student independence
      - Obtaining support for above (possibly federal government, possibly bushfire response regarding development of rapid diagnostics and risk assessment)

Recommendations

- Maintain ongoing communication after this seminar via a collaborative platform so projects can crystallise.
  - Establish diagnostics working group
  - Establish biobanking working group
- Establish stronger links between foundational science and management-oriented people
  - National Koala Disease Risk Analysis Sept 2021–2022
- Integration of research into monitoring and management actions
  - National Koala Monitoring Program
  - NSW Koala Monitoring Framework
  - Individual population studies
- Support integrated studies with:
  - protocols for sample collection and preservation that mean samples are usable for later work (see Koala Health Hub protocols; koalahealthhub.org.au)

researcher-manager co-design of studies, to incorporate disease study requirements in planning

Research approaches:

need to compare across multiple populations using standardized approaches

need for longitudinal studies to show causation

need for more necropsy data to definitively determine outcomes (requires timely mortality detection)

need for in-vitro work to understand mechanisms

need to stratify disease classification—especially group of koalas with chlamydiosis that are refractile to treatment

need new frameworks for study of these questions in endemic disease.

Establish TWIST or LAMP based POC testing for phascolarctid herpesvirus, as well as lab based for quantification to establish northern distribution and associations of load with disease

Establish biobanking working group to progress solutions to issues (see discussion).

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ORCID iD of authors and contributors

David E. Alquezar-Planas	https://orcid.org/0000-0001-5360-5263	Philippa A. McKay	https://orcid.org/0000-0003-2432-4811
Michaela Blyton	https://orcid.org/0000-0002-6112-5320	Lee McMichael	https://orcid.org/0000-0002-7675-4859
Rosie Booth		Joanne Meers	https://orcid.org/0000-0001-9305-0469
Keith Chappel		Saba Mottaghinia	https://orcid.org/0000-0002-9409-3302
Jo Devlin		Baptiste Mulot	https://orcid.org/0000-0001-8793-5850
Lucie Etienne	https://orcid.org/0000-0002-8585-7534	Olusola Olagoke	https://orcid.org/0000-0002-4093-2086
Amber K. Gillett	https://orcid.org/0000-0002-7163-1761	Geoffrey W. Pye	
Alex D. Greenwood	https://orcid.org/0000-0002-8249-1565	Michael Pyne	https://orcid.org/0000-0003-2562-0073
Volker Grün		Bonnie L. Quigley	https://orcid.org/0000-0003-3787-0993
Chris Hamlin-Andrus		Amy Robbins	https://orcid.org/0000-0001-9231-0636
Md Abul Hashem	https://orcid.org/0000-0001-6224-2986	Alfred L. Roca	https://orcid.org/0000-0001-9217-5593
Damien P. Higgins	https://orcid.org/0000-0003-1215-6523	Cora L. Singleton	https://orcid.org/0009-0007-1856-4073
Tetsuya Imanishi	https://orcid.org/0000-0003-0087-9214	Natasha Speight	https://orcid.org/0000-0001-5367-0241
Briony A. Joyce	https://orcid.org/0000-0001-7713-1648	Andrew Stent	https://orcid.org/0000-0002-4432-8185
Mark Krockenberger	https://orcid.org/0000-0002-8168-4795	Rachael E. Tarlinton	https://orcid.org/0000-0003-3325-2311
Jeffrey D. Lifson	https://orcid.org/0000-0002-9494-0268	Peter Timms	https://orcid.org/0000-0002-1682-0025
Gayle McEwen	https://orcid.org/0000-0001-5134-1380	Kimberly Vinette Herrin	https://orcid.org/0000-0002-8063-0541