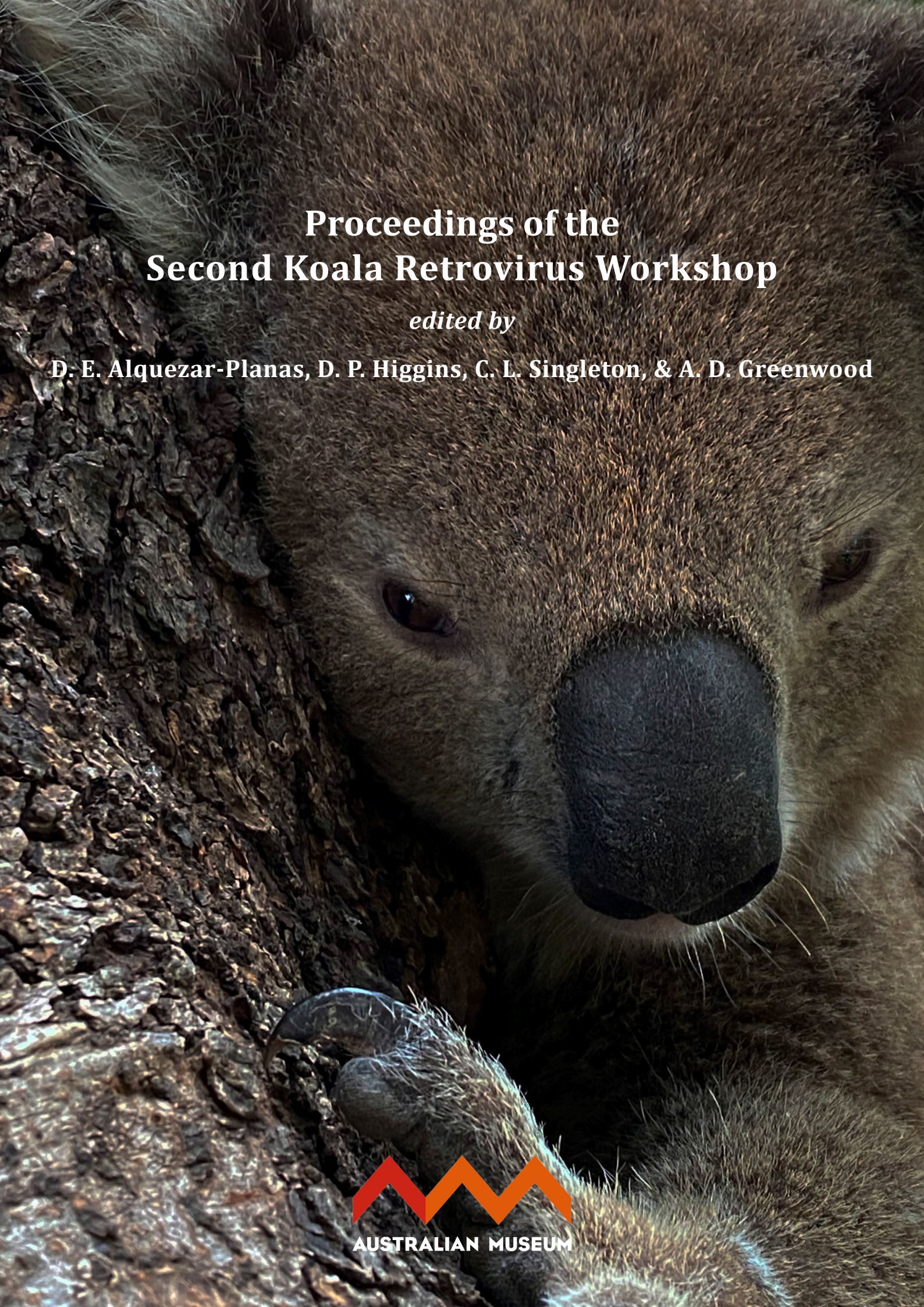
Proceedings of the Second Koala Retrovirus Workshop

edited by

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



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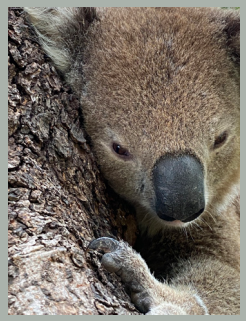

Cover photo
by Damien P. Higgins

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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Synthesis of Discussions of the Second Koala Retrovirus Workshop, 2021

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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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Keywords: koala retrovirus, KoRV, koala, free living koalas, zoological gardens
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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

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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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