The Koala and its Retroviruses:
Implications for Sustainability and Survival

edited by

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Koala Retrovirus in Free-Ranging Populations—Prevalence

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Abstract. The prevalence of koala retrovirus (KoRV) provirus (DNA) and the average number of proviral insertions per cell vary in different free-ranging koala *(Phascolarctos cinereus)* populations across Australia. Populations in the northern states of Queensland and New South Wales have 100% proviral prevalence and mean proviral copy number of 140–165 per cell. In contrast, the proviral prevalence in the southern states of Victoria and South Australia differs among populations, with a mean prevalence in these states' mainland populations of 73% and 38%, respectively and with the prevalence on southern island populations ranging from 0–50%. The proviral load in southern populations is comparatively low, with some populations having an average of less than 1 proviral copy per cell. The KoRV RNA load in plasma shows a similar discordance between northern and southern populations, with consistently high loads in northern koalas (103 to 1010 RNA copies per ml plasma), and loads ranging from 0 to 102 copies per ml in southern KoRV provirus-positive koalas. The variation in KoRV proviral prevalence and the disparity in proviral and viral loads between northern and southern koalas may reflect different types of infection in the two populations (endogenous versus exogenous). Alternatively, it is possible that KoRV has been present for a longer time period in northern populations resulting in differences in the host-virus relationship.

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Koala retrovirus (KoRV) is a gammaretrovirus of koalas *(Phascolarctos cinereus)* that possesses features of both endogenous and exogenous viruses. In previous work, we demonstrated that KoRV is truly an endogenous virus in koalas in south-east Queensland, with proviral DNA present in every animal tested and also present in single sperm cells. We also showed evidence of specific proviral insertion inheritance in Queensland koalas (Tarlinton *et al.*, 2006). However, KoRV is clearly not endogenous in all koala populations in Australia because our early work demonstrated mixed KoRV presence in some southern populations (Tarlinton *et al.*, 2006). Despite its endogenous nature in Queensland koalas, KoRV also displays exogenous virus characteristics in these populations, with high levels of viral RNA present in the blood of every animal tested, indicating active transcription of the KoRV proviral elements (Tarlinton *et al.*, 2005). There is also considerable variation in the number and sites of KoRV proviral insertions in individual koalas, which again is not typical for an endogenous virus where the conservation of a proviral

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integration pattern is expected (Tarlinton *et al.*, 2006). Taken together, these findings suggest that the virus is behaving as both an exogenous and endogenous virus in different koala populations, with transmission of the virus possibly occurring by inherited and/or horizontal routes.

KoRV is closely related to gibbon ape leukaemia virus (GALV), a pathogenic exogenous virus of gibbons (Hanger *et al.*, 2000; Tarlinton *et al.*, 2008). The genetic similarity between KoRV and GALV led to speculation that the two viruses diverged only recently (Bromham, 2002). The sequence similarity between KoRV and GALV is so close across the complete proviral genome that either recent cross-species transmission of virus between koalas and gibbons, or transmission from an intermediate host species is likely (Hanger *et al.*, 2000; Martin *et al.*, 1999). Given that koalas and gibbons do not exist in the same geographical locations in nature and that to date GALV has only been detected in captive gibbons, direct cross-species transmission in the wild appears unlikely. The most probable explanation for the close genetic similarity between KoRV and GALV is that they each were derived from a common virus hosted by a third species of animal whose distribution encompasses that of both gibbons and koalas.

To better understand the endogenous/exogenous nature of KoRV infection in different koala populations, and potentially to gain insight into the origins of the virus, we are conducting on-going studies into the prevalence of KoRV infection across the species' range, including investigation of KoRV proviral DNA load and viral RNA load in these different populations. The samples tested in our studies are collected from a range of sources including koalas presented to veterinary clinics because of illness or trauma, koalas trapped in other research projects, archival samples stored by other researchers and koalas undergoing sterilisation procedures on Kangaroo Island. Related to the diversity in source of samples, the sample type also covered a spectrum including blood, internal organs from euthanized animals, and ear punch biopsies.

Methods used to detect and quantify KoRV proviral DNA and viral RNA comprise standard PCR using *pol* gene primers (Tarlinton *et al.*, 2006), nested PCR using internal primers (Simmons *et al.*, 2012), real-time PCR (qPCR) (Tarlinton *et al.*, 2005), and reverse transcriptase real-time PCR (RT-qPCR).

Prevalence of KoRV provirus in free-ranging koala populations

The prevalence of KoRV provirus in different koala populations ranges from 100% in the northern states of Queensland and New South Wales (NSW) to 0% on one of the southern off-shore islands (Phillip Island) (Simmons *et al.*, 2012). Previously published and recent work has shown that the prevalence in populations on mainland Victoria and South Australia falls between these two extremes, with a mean prevalence on mainland Victoria and South Australia of 73% and 38%, respectively (Simmons *et al.*, 2012; Jones, unpublished data). The prevalence on other southern islands varied considerably, with 50% of koalas tested being provirus-positive on Snake Island, 35% on Raymond Island, 21% on French Island and 15% on Kangaroo Island (in 2007), although the small number of samples tested from some of these islands limits the strength of these data (Simmons *et al.*, 2012). In contrast, the KoRV proviral prevalence on islands off the coast of Queensland is 100% (Jones, unpublished data).

To investigate an apparent change in the dynamics of KoRV infection on Kangaroo Island, we are conducting a temporal and spatial study of KoRV proviral prevalence on the island, which is off the coast of the state of South Australia. In our initial study of blood samples collected from koalas on the island in 2004, none of 26 koalas was provirus-positive (Tarlinton *et al.*, 2006). Twenty-four of 162 (15%) blood samples collected in 2007 were provirus-positive (Simmons *et al*., 2012), and 19 of 50 (38%) and 10 of 38 (26%) samples collected in 2009 and 2011, respectively were provirus-positive (Jones, unpublished data). There does not appear to be a clear geographic segregation of KoRV provirus-positive and –negative animals on Kangaroo Island. However, koalas in the central region of the island are over-represented in each of the four years of our sample collection, thus interpretation of these data must be guarded. The samples used in our study have been collected from koalas caught for a government-funded sterilisation program, which aims to address the over-abundance of the introduced koala population on the island. In the years we obtained samples, this program was focused on the central region of the island, with only limited trapping activities in the western and eastern parts of the island. To date, the small number of animals tested from the western part of the island has been KoRV provirus-negative, but further samples from that part of the island should be tested to confirm these findings.

KoRV proviral and viral loads

We established methods to quantify KoRV proviral DNA and viral RNA loads in cells and plasma, respectively. Initially we used these methods to investigate relationships between either proviral or viral load and disease in koalas, in particular lymphoid neoplasia and chlamydial disease. We found a significant association between KoRV viral load in plasma and the presence of lymphoid neoplasia (Tarlinton *et al.*, 2005). Although there was a trend towards increasing viral load and severity of chlamydiosis, the relationship was not significant.

Using qPCR, we then investigated the levels of KoRV proviral load amongst different koala populations in Australia. The proviral copy number per cell was estimated either from the quantification of DNA concentration in the sample or from comparison to beta-actin copy number (Delta-Delta Ct). Using the first of these approaches, the proviral copy number per cell from DNA extracted from ear punch biopsies of Queensland koalas was markedly higher than that of koalas in southern states, with a mean of 165 copies per cell in Queensland koalas compared to means of 1.5, 0.00153 and 0.000129 copies per cell in three different populations of Victorian koalas (Simmons *et al.*, 2012). Similarly, using the second approach, the means of proviral copies per cell from DNA extracted from blood cell pellets varied from 140 in Queensland koalas, 10 in Kangaroo Island koalas and 1.3 in South Australian mainland koalas (Jones, unpublished data).

KoRV viral RNA levels were determined using RT-qPCR on plasma samples. The viral load in plasma of Queensland koalas is consistently high, ranging from 4.3×10^3 to 8.2×10^{10} copies/ml plasma (Simmons, 2011). In contrast, plasma samples from koalas on Kangaroo Island range from only 1.1×10^2 to 4.3×10^2 copies/ml plasma (Jones, unpublished data) and not all KoRV provirus-positive koalas have detectable viral RNA in plasma.

Discussion

Our data clearly show a marked difference in KoRV proviral prevalence between the northern states of Queensland and NSW and the southern states of Victoria and South Australia. The proviral prevalence declines even further on the southern off-shore islands. Possible interpretations of these data include the northern introduction of the virus with subsequent spread to the south, an inherent genetic resistance to infection or to endogenization of the virus in southern koala populations, or the absence of some kind of environmental factor or vector in the south that limits transmission of the virus. With the current state of knowledge on KoRV, there is insufficient evidence to provide convincing support for any one of these possible interpretations over others.

The large variation between KoRV proviral load of northern koalas in comparison to southern koalas may imply a different type of infection between the two populations. The consistently high copy number of KoRV provirus in individual northern koalas is indicative of endogenous infection, and confirms our earlier evidence for likely endogenous infection of animals in this region. In contrast, the low proviral load in southern populations, with some koalas having less than one provirus copy per cell is clearly not consistent with endogenous infection and more likely reflects exogenous transmission of the virus in these regions.

Despite the probable endogenous infection of Queensland koalas, the KoRV viral RNA load in plasma of these koalas is very high, suggesting that these animals have little control over KoRV transcription and viral replication. In contrast, our findings from the small number of southern koalas on Kangaroo Island tested to date reveal relatively low levels of KoRV RNA in plasma, with some provirus-positive individuals having no detectable viral RNA in their plasma. Coupled with these findings of comparatively low proviral and viral loads, we have also detected a genetic variant of KoRV in koalas on Kangaroo Island, which has a different variable region A (VRA) of the receptor binding domain of the viral *env* gene. Since the VRA is thought to provide specificity for cell surface receptor binding and viral entry, the *env* variant may vary in receptor binding affinity or indeed receptor usage compared to the KoRV A variant found in Queensland. It is unknown whether this genetic variation of the virus is involved in producing the different manifestations of KoRV infection and prevalences between northern and southern populations. Recent studies have reported on similar *env* variants in koalas from zoos in the US (Xu *et al.*, 2013) and Japan (Shojima *et al.*, 2013; Shimode *et al.*, 2014), with the *env* variant from Kangaroo Island showing closest identity to the variant designated KoRV-C from a koala in the Kobe Zoo (Shimode *et al.*, 2014; Young, 2014). Further research is required to better understand the distribution, prevalence and pathogenicity of these *env* variants of KoRV.

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