Koala retrovirus (KoRV) is a newly described endogenous retrovirus and is unusual in that inserts comprise a full-length replication competent genome. As koalas are known to suffer from an extremely high incidence of leukaemia/lymphoma, the association between this retrovirus and disease in koalas was examined. Using quantitative real-time reverse transcriptase PCR it was demonstrated that KoRV RNA levels in plasma are significantly increased in animals suffering from leukaemia or lymphoma when compared with healthy animals. Increased levels of KoRV were also seen for animals with clinical chlamydiosis. A significant positive association between viral RNA levels and age was also demonstrated. Real-time PCR demonstrated as much as 5 log variation in KoRV proviral DNA levels in genomic DNA extracted from whole blood from different animals. Taken together these data indicate that KoRV is an active endogenous retrovirus and suggests that it may be causally linked to neoplastic disease in koalas.

Koalas (Phascolarctos cinereus) suffer from an extremely high incidence of leukaemia and lymphoma, with reputed rates at necropsy of 3–5% in the wild and up to 60% in some captive colonies (Canfield, 1991; Hanger et al., 2000). As a species they are also particularly susceptible to chlamydiosis (Brown et al., 1987; Cockram & Jackson, 1981), a disease that is commonly associated with immunosuppression in other species (Contini et al., 2003; O’Dair et al., 1994). This spectrum of disease is known to be caused by retroviruses in other species, for example feline leukaemia virus (FeLV) in cats (Couto, 1992).

Retroviruses are positive-stranded RNA viruses that insert a DNA proviral copy of their genome into the genomic DNA of the infected cell as part of their life cycle. Proviruses can become inherited if inserted into germ line cells and are then referred to as endogenous retroviruses (Vogt, 1997). Up to 8% of the human genome is estimated to be retroviral in origin, though no active human endogenous retroviruses have so far been found. All vertebrate species examined to date have endogenous retroviruses (Bock & Stoye, 2000), with most being inactive through mutations and deletions (Boeke & Stoye, 1997; Martin et al., 1999). However, several species, including mice and pigs, have been shown to possess full-length replication competent endogenous retroviruses (Akiyoshi et al., 1988; Baillie & Wilkins, 2001; Bartman et al., 1995; Sacco et al., 2001). Some endogenous retroviruses are known to be involved in diseases either through reactivation (as in AKR mice) (Ceccarelli & Rozengurt, 2002) or recombination with related exogenous forms (as in FeLV infection of cats) (Hoover & Mullins, 1991). There have been many reports of increased production of endogenous retroviral products in a variety of human diseases, including leukaemia, lymphoma, breast cancer and multiple sclerosis, although definitively linking endogenous retroviruses to disease has proved elusive (Depil et al., 2002; Patzke et al., 2002; Perron et al., 2001; Wang-Johanning et al., 2003).

Koala retrovirus (KoRV) is a recently discovered endogenous gammaretrovirus of koalas (Hanger et al., 2000). It is present in the koala genome as a full-length, replication competent genome. It is actively transcribed and type C retroviral particles are produced by stimulated peripheral blood mononucleocyte cultures. KoRV is also unusual in that its sequence is most closely related to Gibbon ape leukaemia virus, a pathogenic exogenous virus of gibbons, raising the possibility of a recent species jump (Hanger et al., 2000).

To examine whether KoRV is linked to leukaemia and lymphoma in koalas we used real-time PCR to measure
DNA proviral copy number in koala blood cells and KoRV genomic RNA in cell-free plasma as a marker of viraemia. Real-time PCR primers and probes were designed for a unique region of the KoRV pol gene based on an alignment of 48 gammaretroviral sequences (Martin et al., 1999). The primer sequences were: 5′-TTGGAGGAGGAATACCGATTACAC-3′ (sense) and 5′-GCCAGTCCCATACCTGCTT-3′ (antisense). The Taqman probe sequence was 5′-FAM-TCGACCCGTCATGGC-MGBNFQ-3′ (Applied Biosystems).

Reaction mixtures for real-time DNA PCR consisted of two times mastermix (12 μl; ABGENE), 100 nM probe, 300 nM each primer, 3 μl water and 5 μl template. Reaction mixes for real-time RNA PCR consisted of two times master mix (12 μl), 0.625 μl multiscribe (Taqman one-step RT-PCR MasterMix Reagent kit; Applied Biosystems), 300 nM each primer, 100 nM probe, 3 μl DEPC-treated water and 5 μl template. Cycling conditions were: 48 °C for 30 min (omitted for DNA), 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification and detection was carried out on an ABI 7700 sequence detection system (Applied Biosystems). Plasmid DNA and RNA transcripts of KoRV were prepared using standard methods from a pGEM-T Easy vector containing the complete KoRV proviral genome (GenBank accession no. AF151794) (Hanger et al., 2000). Standards were stored at −80 °C and a 10-fold dilution series prepared just prior to use. Standard curves were prepared for each run using these DNA or RNA standards, and results for each run normalized against these standard curves.

Blood samples from an eastern grey kangaroo (Macropus giganteus), ringtail possum (Pseudocheirus peregrinus) and carpet python (Morelia spilota) were used as negative controls.

DNA extraction from whole blood samples was performed using the Qiagen DNA mini kit. The assay for DNA proviral copy number was performed on 62 koalas from the Dreamworld colony (Gold Coast, Australia). Given the absence of adequate koala genome sequence data necessary for the development of a reporter gene control in the quantitative PCR, DNA copy number was compared with the input DNA concentration as determined by optical density reading at 260 nm. Proviral DNA was detected in all koalas tested but not from other animal species, as expected. Proviral copy number per ng of DNA varied markedly between individuals ranging from $4 \times 10^2$ to $4.5 \times 10^7$. No significant associations were found between proviral DNA levels and disease, age, body mass, sex or viral RNA levels (data not shown).

The assay for viral RNA levels in cell-free plasma was performed on 90 animals comprising 63 captive koalas from the Dreamworld colony and 27 wild koalas presented to veterinarians at Dreamworld or the Queensland Parks and Wildlife Service Koala Hospital. Two to 4 ml of blood was collected in EDTA anticoagulant from each koala. Blood for RNA samples was centrifuged at 11 200 g for 120 s and 200 μl plasma was added to 300 μl RNA later Stabilization agent (Qiagen) within 15 min of collection as per the method of Lee et al. (2002). Viral RNA was then extracted using the Qiagen Viral RNA mini kit with an ‘on column’ RNase free DNase (Qiagen) step. Viral RNA levels were normalized against an RNA standard curve as described above.

Four koalas (aged 6–11 years) were diagnosed with leukaemia or lymphoma during the study, three from the Dreamworld colony and one wild koala. Diagnosis was based on a combination of gross and/or cytological pathology (Fig. 1a) and a white cell count increased to $> 20 \times 10^9 \text{ l}^{-1}$ (normal range: $3–10 \times 10^9 \text{ l}^{-1}$) (Canfield et al., 1989). Thin-section electron microscopy of bone

![Fig. 1.](image-url)
KoRV is associated with disease in koalas

The variation in KoRV proviral copy number indicates that KoRV is not a fixed element within the koala genome.

It could be argued that viral RNA levels were increased in leukaemic animals as a side effect of the increased number of nucleated cells present in the blood. However, there was no significant association between white cell count and viral RNA levels [Spearman’s rank order correlation (jmp)] (data not shown) for 52 healthy captive koalas.

Ten captive animals from the Dreamworld colony, five with high viral RNA levels and five with low levels (matched for age and sex), were selected for follow up testing of viral RNA levels over 18 months. There was some variation in RNA levels between samples for individual koalas, particularly for those in the high level RNA group. Three of the animals from the high level RNA group died during this study and were found to have neoplasia on post-mortem examination. However, none of the animals in the low level RNA group died or were diagnosed with neoplasia. This translates to animals in the high viral RNA group being at higher risk of dying from neoplasia with an odds ratio of 15.4 (Graph Pad Instat). During the course of this study, one animal from the ‘high RNA’ monitored group as well as two other koalas in the colony, showed a marked increase in viral RNA levels between samples taken prior to the development of disease and at euthanasia. This increase in viral load appears to occur concurrently with the onset of the disease. However, it is difficult to determine whether it occurs as a cause or a consequence of disease.

All koalas tested in this study were shown to be viraemic. Furthermore, our data show a clear association between KoRV RNA plasma levels and leukaemia/lymphoma. Similar links have also been made with human endogenous retroviral elements and leukaemia or breast cancer (Depil et al., 2002; Wang-Johanning et al., 2003). However, it remains difficult to determine whether the expression of endogenous retroviruses is the cause or result of oncogenesis.

A positive association between log viral RNA levels and age (P<0.003) was also found (Fig. 3) but no significant association with body mass or sex (data not shown). Wild koalas (n=27, mean=5.3×10⁵ copies ml⁻¹ plasma) had higher log viral RNA levels than captive animals (n=63, mean=1.2×10⁵ copies ml⁻¹ plasma) (P<0.008). However, this may simply reflect the fact that the wild koalas examined in our study were a biased population of sick and injured animals presented to veterinarians.

Surprisingly, all koalas tested showed at least some level of viral RNA in plasma, suggesting active viraemia in all animals. As with proviral DNA, viral RNA levels in plasma varied markedly between individuals, ranging between 1.7×10⁵ and 3.6×10⁹ copy numbers per ml. Analysis of variance (Statistica) was used to examine relationships between log viral RNA levels, age, body mass, sex, origin and disease status. There was a significant (P<0.0007) increase in viral RNA levels in animals with leukaemia and lymphoma (n=4, mean=1.6×10⁹ copies ml⁻¹ plasma) compared with healthy animals (n=67, mean=7.7×10⁷ copies ml⁻¹ plasma) (Fig. 2). While animals with clinical chlamydiosis, including one animal with lymphoma (n=20, mean=6.9×10⁸ copies ml⁻¹ plasma), had higher mean log viral RNA levels than healthy animals (n=67, mean=7.7×10⁷ copies ml⁻¹ plasma) this difference was not statistically significant (Fig. 2).

A positive association between log viral RNA levels and age (P<0.003) was also found (Fig. 3) but no significant association with body mass or sex (data not shown). Wild koalas (n=27, mean=5.3×10⁵ copies ml⁻¹ plasma) had higher log viral RNA levels than captive animals (n=63, mean=1.2×10⁵ copies ml⁻¹ plasma) (P<0.008). However, this may simply reflect the fact that the wild koalas examined in our study were a biased population of sick and injured animals presented to veterinarians.

Marrow from a leukaemic koala revealed typical type C retrovirus particles similar to those described previously (Canfield et al., 1988; Fig. 1b). All koalas were euthanized within a week of diagnosis. Twenty of the 27 wild koalas (including one koala with lymphoma) were assessed by a veterinarian as suffering from chlamydiosis. (including one koala with lymphoma) were assessed by a veterinarian as suffering from chlamydiosis. All koalas tested in this study were shown to be viraemic. Furthermore, our data show a clear association between KoRV RNA plasma levels and leukaemia/lymphoma. Similar links have also been made with human endogenous retroviral elements and leukaemia or breast cancer (Depil et al., 2002; Wang-Johanning et al., 2003). However, it remains difficult to determine whether the expression of endogenous retroviruses is the cause or result of oncogenesis.

The variation in KoRV proviral copy number indicates that KoRV is not a fixed element within the koala genome.

Fig. 2. Comparison of plasma viral load for healthy and diseased koalas. White columns, healthy animals; black columns, leukaemic/lymphomatous diseased animals and grey columns, clinically diagnosed chlamydiosis diseased animals.

Fig. 3. Positive association between plasma viral RNA levels and age (P<0.0003, ANOVA). A logarithmic trend line is shown for viral RNA levels of the total koala population studied (both captive and wild koalas, n=90).
This may be the consequence of new insertions accumulating over the life of the animal as would be expected for an exogenous virus. However, given the lack of a correlation between proviral copy number and either age or viral RNA levels, this appears unlikely. The relationship between viral RNA levels and age may instead indicate that while individual koalas are born with a fixed cohort of inserts, repression of transcription may decrease with age as has been proposed for increased production of endogenous retroviral transcripts in some strains of laboratory mice (Gaubatz et al., 1991; Ono et al., 1989; Wada et al., 1993).

The association between KoRV and chlamydioidosis is less clear. This disease occurs at an extremely high incidence in koalas and is regarded as the primary disease threat to the species (Booth & Blanshard, 1999). Viral load may be a factor in the severity of chlamydioid infection. Exogenous retroviruses are known to cause immunosuppression in other species, and chlamydioidosis is regarded as a disease of immunosuppressed individuals (Contini et al., 2003; O’Dair et al., 1994). The link we have established between KoRV and clinical chlamydioidosis provides a possible explanation for the unusual susceptibility of this species to Chlamydia, although this needs further investigation to be clarified.

KoRV is a replication competent complete viral genome (Hanger et al., 2000), suggesting that it may be a recent insertion into the koala genome. Our findings of a variation in proviral copy number and viral genomic RNA levels in plasma of individual animals indicate ongoing activity of KoRV. The presence of an active insertional mutagen within the koala genome may be the key to the relatively high incidence of leukaemia and lymphoma in the koala population.

As all koalas examined in this study were viraemic with KoRV to some degree; there may be some other effect or trigger that determines which animals develop disease. Further studies will focus on determining if there are differences in viral sequence or insertion site that are related to the development of disease, as both of these are determinants of disease progression in other species infected with exogenous viruses (Rosenburg & Jolicouer, 1997).

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References


