Highlights

- Koalas from European zoos as well as from Australia carried koala retrovirus KoRV-A, in a few cases also KoRV-B, but had no antibodies against the KoRV-A.
- Therefore koalas are tolerant against the KoRV-A and this has implications for future vaccination studies.
- These data correlate with data showing that pigs do not produce antibodies against their porcine endogenous retrovirus (PERV), in particular when immunised with a protein of PERV.
Virus Research

Short communication

Lack of antiviral antibody response in koalas infected with koala retroviruses (KoRV)

Uwe Fiebig\textsuperscript{a}, Martina Keller\textsuperscript{a}, Annekatrin Möller\textsuperscript{a}, Peter Timms\textsuperscript{b}, Joachim Denner\textsuperscript{a,*}

\textsuperscript{a}Robert Koch Institute, HIV and other retroviruses, Nordufer 20, 13353 Berlin, Germany

\textsuperscript{b}University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, Queensland, 4558 Australia

*Corresponding author

Email addresses:

UF: UweFiebig@gmx.de

MK: KellerM@rki.de

AM: AnneMoeller@gmx.de

PT: ptimms@usc.edu.au

JD: DennerJ@rki.de

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ABSTRACT

Many wild koalas are infected with the koala retrovirus, KoRV, some of which suffer from lymphoma and chlamydial disease. Three subgroups, KoRV-A, KoRV-B and KoRV-J, have so far been described. It is well known that other closely related gammaretroviruses can induce tumours and severe immunodeficiencies in their respective hosts and a possible role for KoRV infection in lymphoma and chlamydial disease in koalas has been suggested. In many wild koalas, KoRV-A has become endogenised, i.e., it is integrated in the germ-line and is passed on with normal cellular genes. In this study, sera from koalas in European zoos and from wild animals in Australia were screened for antibodies against KoRV-A. These naturally infected animals all carry endogenous KoRV-A and two zoo animals are also infected with KoRV-B. The antibody response is generally an important diagnostic tool for detecting retrovirus infections. However, when Western blot analyses were performed using purified virus or recombinant proteins corresponding to KoRV-A, none of the koalas tested positive for specific antibodies, suggesting a state of tolerance. These results have implications for koala vaccination, as they suggest that therapeutic immunisation of animals carrying and expressing endogenous KoRV-A will not be successful. However, it remains unclear whether these animals can be immunised against KoRV-B and immunisation of uninfected koalas could still be worthwhile.

Koala retroviruses (KoRV) have been isolated from wild and captive koalas in Australia as well as from koalas housed in zoos in other countries (Hanger et al., 2000; Fiebig et al., 2006; Xu et al., 2013; Miyazawa et al., 2011). They are members of the genus gammaretrovirus and are most closely related to gibbon ape leukaemia virus (GaLV), feline leukaemia virus (FeLV), murine leukaemia virus (MuLV), and
porcine endogenous retrovirus (PERV) (Hanger et al., 2000; Cui et al., 2012; Denner and Young, 2013). As well as leukaemia, GaLV, MuLV, and FeLV induce immunodeficiency in their respective hosts, leaving them susceptible to numerous opportunistic infections (Moloney, 1964; Rosenberg and Jolicoeur, 1997; Hardy, 1985; Gallo et al., 1978). As is the case with virus loads and AIDS in HIV-1 infected humans, there is a correlation between KoRV RNA levels in the plasma and neoplastic disease in koalas (Tarlington et al., 2005; Mellors et al., 1997). KoRVs are likely the result of a relatively recent trans-species transmission from rodents or bats (see Denner and Young, 2013). At present, three KoRV subgroups have been described: KoRV-A, KoRV-B and KoRV-J (Hanger et al., 2000; Fiebig et al., 2006; Miyazawa et al., 2011; Xu et al., 2013). Interestingly, KoRV-A is present in the germline of some koalas, e.g., the virus is endogenised (Tarlington et al., 2006; Stoye, 2006), whereas KoRV-B and KoRV-J remain exogenous viruses.

Antibody responses are commonly observed in individuals infected with exogenous retroviruses and they are therefore of high diagnostic value (Daskalakis, 2011). To study the situation in koalas naturally infected with exogenous KoRV-B, and/or carrying endogenous KoRV-A sequences, sera from 16 koalas were tested for antibodies against the virus using viral lysates and recombinant viral proteins corresponding to KoRV-A. Surprisingly, none of the 16 koalas tested positive (Table 1), suggesting that the animals are tolerant against this virus. These data are in agreement with the lack of antibodies against PERV in normal pigs and in pigs immunised with the transmembrane envelope protein p15E of PERV (Keller et al., 2014), but differ from the situation in humans where antibodies against the human endogenous retrovirus HERV-K have been found in tumour patients and pregnant women (see below).
Sera taken from koalas housed at the Duisburg Zoo in Germany, the Antwerp Zoo in Belgium and from a wild population just south of Brisbane, Australia (Table 1) were analysed by Western blot. Provirus integration in DNA isolated from serum, peripheral blood mononuclear cells (PBMCs), ascites or tumour tissues was tested by PCR using primers specific for KoRV-A and KoRV-B (Table 2). Although all animals carried KoRV-A, two animals, one from the zoo in Duisburg (Kambara) and one from Antwerp (Coolongalook), both originally from the San Diego Zoo, were also found to be infected with KoRV-B (Fig. 1A). Interestingly, a recent study failed to find additional KoRV-B infected animals at the San Diego Zoo, whereas a number were identified at the Los Angeles Zoo (Xu et al., 2013). This suggests that the animals were either infected in Europe or that KoRV-B is the result of a recombination between KoRV-A and other endogenous sequences. The wild animals came from a region of Australia in which all are at least infected with KoRV-A, although the prevalence of KoRV-B remains unknown. As expression of KoRV-B has recently been described in wild Australian koalas (Hobbs et al., 2014), KoRV-B infection of the animals tested here appears likely. Replication-competent KoRV-A in European zoo koalas was demonstrated by the presence of virus particles in tumour tissue taken from the animal Coolongalook (see Table 1) (Fiebig et al., 2006). Furthermore, infectious virus able to replicate in human cells and cells from other species and able to infect rats in vivo was isolated from the animals Coolongalook, Alora and Birubi (see Table 1) (Fiebig et al., 2006). The viruses corresponded to KoRV-A and were able to replicate, for example, on human 293 cells (Fig. 1B). To summarise, viruses able to replicate in vitro and in vivo were released from the PBMCs of three koalas (Coolongalook, Alora and Birubi).
For Western blot analysis, either a lysate of virus particles (KoRV-A grown in human 293 cells and pelleted by ultracentrifugation) or recombinant virus proteins rp15E and gp70/rp52, which were produced as previously described (Fiebig et al., 2006; Denner, 2014), were used. These recombinant proteins corresponded to KoRV-A. KoRV-B differs from KoRV-A in the receptor-binding site of the surface envelope protein gp70 by 35 amino acids, although the sequence of p15E is identical (Xu et al., 2013). Positive control sera against the KoRV-A recombinant viral proteins were generated in goats (serum 33 against rp27Gag, serum 31 and 46 against rp15E, serum 61 against gp70/rp52) (Fiebig et al., 2006; Denner, 2014). As secondary antibodies, an anti-goat antiserum (Dako, polyclonal rabbit antibodies, peroxidase conjugated), and a combination of a sheep anti-koala antiserum (Kollipara et al., 2013) and an anti-sheep antiserum (Biomol, Rockland, donkey anti-sheep IgG, peroxidase conjugated) were used.

Whereas all goat control sera reacted with the KoRV-A lysate, indicating that the antigens had been successfully blotted, none of the koala sera reacted, indicating that koalas carrying KoRV-A in their genome did not naturally produce antibodies against the virus (Fig. 2A, Table 1). The results of the Western blot analysis using the KoRV-A recombinant proteins rp15E (Fig. 2B, Table 1) and gp70/rp52 (not shown) confirmed the results using virus lysate. It is interesting that those animals carrying KoRV-A and also infected with exogenous KoRV-B also failed to produce antibodies against KoRV-A, although it is unclear whether they had antibodies against the receptor-binding site of KoRV-B gp70, the only sequence different from that of KoRV-A. To demonstrate the functionality of the secondary antibody, a combination of sheep anti-koala and donkey anti-sheep antibodies, sera from animals infected with chlamydia or immunized with the recombinant major outer membrane protein
(MOMP) of chlamydia were shown to react in a Western blot analysis against MOMP (Kollipara et al., 2013). Furthermore, the combination of the sheep anti-koala and the anti-sheep antibodies reacted with purified koala immunoglobulins in a Western blot assay (Fig. 1B). The negative Western blot results therefore suggest that the koalas are tolerant against KoRV-A, at least with regard to antibody production.

In order to analyse whether the koalas nevertheless generated neutralising antibodies (unlikely in the absence of binding antibodies), koala sera were analysed in a neutralisation assay that measures provirus integration by real-time PCR, as described for other retroviruses (Behrendt et al., 2009; Kaulitz et al., 2011). Briefly, 100 μl of a cell suspension containing 1x10^3/ml human 293T cells were plated into 96-well flat bottom microtiter plates. One day later, cells were infected with 80 μl cell-free KoRV-A virus stock (1x10^{2.66} TCID_{50}/ml), preincubated for 15 min with 20 μl of heat-inactivated (60°C, 40 min) koala serum at different 2-fold dilutions (1:10 to 1:320). After 65 h incubation (37°C, 5 % CO_2) a quantitative real-time PCR was performed on triplicate 3 μl samples of proteinase K treated cell lysates using specific primers and a probe (Table 2) in an MX3005 real time cycler (Agilent Technologies) (50 cycles, annealing at 56°C, 30 sec elongation phase). Significant neutralisation was defined as over 50% reduction in the quantity of proviral DNA. No reduction in the degree of infection was observed in the presence of koala sera, whereas goat sera specific for rp15E and gp70/rp52 significantly reduced infection (Figure 2C) and the goat serum against rp27Gag did not. This indicates that - as expected - no neutralising antibodies were found in the koala sera, whereas the goat sera against the envelope proteins of KoRV-A neutralised this virus.

The result that koalas are tolerant against KoRV-A, at least, are in agreement with recently published findings that pigs are tolerant against their endogenous
retroviruses, PERVs (Keller et al., 2014). Antibodies specific for PERV were absent in all untreated pigs investigated, and in pigs immunised with the recombinant viral proteins rp15E, (Keller et al. 2014), gp70/rp52 and rp27Gag (unpublished data). However, in contrast to our observations in koalas and pigs, antibodies against endogenous retroviruses have been described in cats, humans and mice. The most convincing example for antibody production against endogenous retroviruses in a host species is the antibody response against the human endogenous retrovirus HERV-K in humans. Antibodies against HERV-K, particularly against the TM protein, were detected in the sera of patients with germ cell tumours, melanomas and in some pregnant women, correlating with the expression of HERV-K in these tumours as well as in the placenta (Muster et al., 2003; Büscher et al., 2005; 2006; Löwer et al., 1996). Presumably, the endogenous retroviral sequences are not expressed in humans during ontogenesis, which allows antibody responses to occur upon later exposure. In pigs and koalas, PERVs and KoRV might, in contrast, be expressed at an early stage of development, leading to recognition of the endogenous retroviral proteins as “self-antigen”. The situation with the FeLV, which is closely related to PERV and KoRV, is more difficult. All cats immunised with FeLV p15E developed binding and neutralising antibodies (Langhammer et al., 2005; 2006; 2011a;b), even though the feline genome contains retroviral sequences related to the p15E sequence used for immunisation, particularly in the highly conserved epitope region, suggesting that cats are not tolerant to their endogenous retroviruses. The situation in mice, which are often characterised by strong expression of their endogenous retroviruses, is as complicated as it is in cats. Whereas young mice appear to be tolerant (Huebner et al., 1971), older animals and also younger animals of some mouse strains developed antibodies against all viral proteins (Oldstone et al., 1972; 1976; Hanna et al., 1975;
Ihle et al., 1973). In some cases, the antibodies were complexed with the corresponding viral antigen in the kidneys, causing glomerulonephritis (Huebner et al., 1971; Oldstone et al., 1976). The mechanism by which the immune system produces antibodies against endogenous retroviruses in mice and cats remains unclear. Expression of infectious or non-infectious recombinant polytropic viruses after the establishment of immunologic tolerance to endogenous retroviral antigens that are expressed earlier in life may play some role in the escape from tolerance and the stimulation the immune system in mice and cats (Sheets et al., 1992; Roy-Burman, 1995; Miyazawa et al., 1994). In this context, it is noteworthy that a soluble and truncated envelope protein of an endogenous retrovirus (FeLIX) present in the serum of domestic cats mediates infection of a pathogenic variant of feline leukaemia virus (Sakaguchi et al., 2014).

These findings potentially impact future efforts to develop prophylactic and therapeutic vaccines for koalas. Goats, rats, mice, hamsters and guinea pigs immunised with the recombinant envelope protein rp15E of PERV all developed binding and neutralising antibodies specific this transmembrane protein (Fiebig et al., 2003; Kaulitz et al., 2011; Denner et al., 2012; Waechter et al., 2013). However, it was impossible to induce such antibodies in pigs, even when immunising with the same batches of PERV rp15E (Keller et al., 2014) and gp70/rp52 (unpublished data). Although binding and neutralising antibodies against KoRV-A rp15E and gp70/rp52 was readily induced by immunising goats, mice and rats (Fiebig et al., 2006, Denner, 2012, Denner, 2014), it is presently unknown whether immunisation of koalas with recombinant KoRV proteins will result in the production of antibodies neutralising KoRV-A and KoRV-B. It is also unclear whether koalas that are tolerant to KoRV-A are able to produce antibodies to KoRV-B, based on the differences in the receptor-
binding site in gp70. However, uninfected koalas should be able to mount a strong neutralising immune response upon immunisation and this, in addition to isolation or quarantine, may be an effective way to prevent infection of these animals.

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Legends

Fig. 1
A, Results of the PCR analysis using primers specific for KoRV-A and KoRV-B. “A” indicates the amplicon using primers for KoRV-A, “B” for KoRV-B. DNA from the following koalas was tested: 1-Irwin, 2-Goonderah, 3-Coolongalook, 4-Alora, 5-Birubi, 6-Kambara, 7-Kangulandai. B, electron microscopy of human 293 cells producing KoRV particles. Infectivity was demonstrated in parallel infection assays.

Fig. 2
A, Western blot analysis using a lysate from KoRV purified by ultracentrifugation. Controls include goat sera specific for recombinant gp70/rp52, recombinant p15E (recognises p15E and p12E) and recombinant p27Gag (recognises Gagp27 and the precursor proteins preGag60 and preGag55). All koala sera (1-Alora, 2-Birubi, 3-Kangulandai, 4-Posh spice, 5-Minkey, 6-Neil, 7-Irwin, 8-Bubbles, 9-Matt, 10-Karen, 11-Setch, 12-Emma, 13-Clay) were negative. In the last lane, koala IgG purified by affinity chromatography was blotted after SDS PAGE and developed with sheep anti-koala antiserum and anti-sheep antiserum. The Peqlab protein marker IV was used. B, Western blot analysis using recombinant KoRV p15E. A molecular weight marker is shown (left, Peqlab protein marker IV). The goat control sera 31 and 46 react with recombinant p15E, whereas all koala sera (1-Minkey, 2-Bubbles, 3-Karen, 4-Matt, 5-Setch, 6-Emma, 7-Clay) did not. C, Absence of neutralising antibodies in the sera of koalas carrying endogenous KoRV-A (two of which are also infected with KoRV-B).
Inhibition of provirus integration at different serum dilutions is shown: the goat sera specific for the envelope proteins p15E and gp70 neutralised KoRV-A, whereas the serum specific for p27Gag did not.
### Table 1

Animals analysed in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>KoRV</th>
<th>Material analysed</th>
<th>Antibodies specific for Virus</th>
<th>Recombinant proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goonderra</td>
<td>Duisburg</td>
<td>A</td>
<td>Blood/Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Coolongalook*</td>
<td>Antwerp</td>
<td>A+B</td>
<td>Blood/Serum/Tumour</td>
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<td>no</td>
</tr>
<tr>
<td>Alora</td>
<td>Duisburg</td>
<td>A</td>
<td>Blood/Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Birubi*</td>
<td>Duisburg</td>
<td>A</td>
<td>Blood/Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Kambara*</td>
<td>Duisburg</td>
<td>A+B</td>
<td>Blood/Serum/Ascitis</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>Kangulandai*</td>
<td>Duisburg</td>
<td>A</td>
<td>Blood/Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Irwin</td>
<td>Duisburg</td>
<td>A</td>
<td>Blood/Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Posh spice</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
<td>no</td>
<td>n.t.</td>
</tr>
<tr>
<td>Neil</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
<td>no</td>
<td>n.t.</td>
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<tr>
<td>Minkey</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Bubbles</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
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<td>no</td>
</tr>
<tr>
<td>Matt</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Karen</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
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<td>no</td>
</tr>
<tr>
<td>Setch</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
<td>no</td>
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</tr>
<tr>
<td>Emma</td>
<td>Australia</td>
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<td>Serum</td>
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<tr>
<td>Clay</td>
<td>Australia</td>
<td>+</td>
<td>Serum</td>
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</table>

* died; n.t., not tested due to lack of material; +, the animals are from a region in Australia where all are infected with KoRV-A at least
### Table 2

Primers used for detection of the corresponding KoRV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
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<tr>
<td>KoRV-A forward</td>
<td>CTAATAAAAGGGCCCATAGA</td>
<td>Hanger et al., 2000</td>
</tr>
<tr>
<td>KoRV-A reverse</td>
<td>GTTGAACCATCCCTCGTACC</td>
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<tr>
<td>KoRV-B forward</td>
<td>CGGTGAAGGTTGACGTTATT</td>
<td>Xu et al., 2013</td>
</tr>
<tr>
<td>KoRV-B reverse</td>
<td>ACCCCAAGGTTCCATAGCTC</td>
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</tr>
<tr>
<td>KoRV-A real-time</td>
<td>CTAATAAAAGGGCCCATAGA</td>
<td></td>
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<tr>
<td>KoRV-A real-time</td>
<td>GTTGAACCATCCCTCGTACC</td>
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<tr>
<td>probe</td>
<td>Fam-CCATGGGATACAGACCTTAGGGCCC-BHQ1</td>
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</tr>
</tbody>
</table>
Fiebig et al., Figure 1

A  

bp  

1000  

500  


1  2  3  4  5  6  7  

KoRV-B  

KoRV-A

B
Inhibition of provirus integration [%] for various serum dilutions.