Koala retrovirus (KoRV) subtypes and their impact on captive koala (*Phascolarctos cinereus*) health

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Abstract

Koala retrovirus (KoRV), a major pathogen of koalas, exists in both endogenous (KoRV-A) and exogenous forms (KoRV-B to J). However, the impact of infection with multiple subtypes is not well understood. Accordingly, in this study, we surveyed a representative sample from a Japanese zoo population to determine the infection status for three KoRV subtypes (KoRV-A, B, and C) and to investigate the proviral and RNA load profiles in animals with single- and multiple-subtype infections, using peripheral blood mononuclear cells (PBMCs) and plasma. Six koalas were evaluated in the study; all were infected with KoRV-A, and two koalas were coinfected with non-A subtypes (KoRV-B and/or KoRV-C). The highest KoRV total RNA and viral loads in PBMCs and plasma were found in a koala infected with multiple subtypes (KoRV-A, -B and -C). The other koala infected with multiple subtypes (KoRV-A and B) showed the highest proviral PBMC load but the lowest RNA copy number in PBMC and plasma. PBMCs from this animal were cultured for further investigation, and KoRV RNA was detected in the cells and culture supernatant after 7 and/or 14 days. The koalas harboring multiple subtypes had a higher white blood cell count than those harboring only KoRV-A and were judged to be leukemic, and they subsequently died due to lymphoma. Accordingly, we conclude that coinfection with multiple KoRV subtypes may be linked to more-severe disease. In a sequence alignment, the detected KoRV-A env gene showed 100% sequence identity to the reference gene, whereas the KoRV-B and -C env genes varied from their reference sequences.

Introduction

Koala retrovirus has captured the interest of many virologists and, interestingly, exists in both endogenous and exogenous forms. Its evolutionary history has been well described elsewhere [1–3]. Briefly, koalas may have first contracted this retrovirus – a relative of gibbon ape leukemia virus [4], feline leukemia virus (FeLV) [5], Melomys burtoni retrovirus (MbRV) [6], and porcine endogenous retrovirus – through transspecies transmission, with the process of endogenization beginning about 49,000 years ago, which is relatively recent in evolutionary terms [7]. Endogenization appears to be complete in wild populations in northern Australia, where a prevalence of 100% has been established, but is still ongoing in some wild populations in southern Australia [1, 2, 8]. Health issues for koalas associated with KoRV have been reported elsewhere [5, 9–12] and include neoplasia and chlamydial diseases [3], which have been seen in both wild and zoo animals. Understanding KoRV is thus more than a subject of academic interest for virologists; such knowledge will have very real practical applications in the field of conservation.

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The current knowledge about KoRV includes the fact that it exists in endogenous and exogenous forms, but further extensive investigation about the variant subtypes is required. KoRV-A, the endogenous form, is the most widely researched subtype, and it accounts for the 100% prevalence in north Australian populations. However, in addition to the endogenous subtype, another nine exogenous subtypes are known to exist (KoRV-B to J). Of these, KoRV-B is the best documented, and it is also believed to be the most pathogenic [5, 10, 11]. We have previously found cases where KoRV-A represented less than 100% of the total KoRV (sometimes the combined KoRV-A and -B load was also below 100% of the total load), indicating that some koalas are infected with multiple subtypes. In another finding in our study, IL-6 expression was significantly greater in PBMCs from koalas infected with multiple subtypes than in those from koalas infected with KoRV-A alone [13]. There may be epidemiological implications with regard to these subtypes. Wild koalas in or from northern Australia and their zoo-reared descendants will all harbor KoRV-A from birth, but exposure to other variants may put them at risk of multiple-subtype infection and subsequent poor health outcomes. The zoo population targeted in our previous studies (at Hirakawa Zoological Park, Kagoshima, Japan) comprises koalas from a north Australian population and may thus represent a group at risk of infection with multiple subtypes. KoRV has been fully sequenced recently; it has a positive-sense, single-stranded RNA genome of 8.4 kb with gag, pol, and env genes, and long terminal repeats (LTRs) at the 5' and 3' ends [4, 9, 14].

Accordingly, in this study, we set out to determine the infection status for the endogenous subtype (KoRV-A) and the two most common exogenous subtypes (KoRV-B and KoRV-C) in a representative sample of captive koalas in a Japanese zoo. We aimed to establish profiles based on proviral and RNA loads in plasma and peripheral blood mononuclear cells (PBMCs) and to make a preliminary assessment of health status for the different profiles based on white blood cell (WBC) count and mortality. Furthermore, we set out to align env gene sequences for any subtype identified in our study with reference sequences. The ultimate aim of this study is to further understanding of KoRV and its subtypes in ways that will benefit both virologists and conservationists.

Materials and methods

Study animals and sample collection

Adult koalas housed at Hirakawa Zoological Park, Kagoshima, Japan, were chosen for blood collection.

EDTA-treated whole blood samples were collected from koalas by venipuncture. The white blood cell (WBC) count was determined at the time of blood collection, and all procedures were in accordance with the regulations and bylaws of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University.

Extraction of DNA and RNA

Genomic DNA (gDNA) was extracted from EDTA-treated whole blood samples using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

Viral RNA and total RNA were extracted using an ISO-GEN-LS Kit (Nippon Gene, Japan) and an RNeasy Plus Mini Kit (QIAGEN, Germany), respectively, according to the manufacturer's instructions. To remove any contaminating DNA, extracted RNA was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. The concentration and purity of each extracted DNA and RNA sample were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). RNA samples were stored at -80°C until analysis.

Peripheral blood mononuclear cells (PBMC) culture

Koala PBMCs were cultured from a whole blood samples from one animal, using a previously described method [15] with some modifications [16]. PBMCs were added to two wells of a 6-well plate at 1×10^5 cells/well. The wells contained 1 mL of RPMI 1640 supplemented with 20% heat-inactivated FCS, 1 mM sodium pyruvate (SIGMA), 50 μM β-mercaptoethanol, 1% MEM non-essential amino acids (Invitrogen), penicillin (100 units/mL), and streptomycin (100 μg/mL). The plate was then incubated at 37°C in a 5% CO_2 incubator overnight, and 1 mL of RPMI 1640 complete growth medium was added to each well the following day. At seven days after the start of incubation, the 1 mL of medium was replaced with fresh medium. Culture supernatants and cells were harvested at days 7 and 14 of culturing. DNA was extracted from cultured cells using a Wizard Genomic DNA Purification Kit (Promega). Total RNA and viral RNA were also extracted from the cells and culture supernatants using an RNeasy Plus Mini Kit (QIAGEN, Germany) and an ISOGEN-LS Kit (Nippon Gene, Japan), respectively, following the manufacturer's instructions.

Subtyping of KoRV

Using gDNA as a template, a polymerase chain reaction (PCR) was performed to detect KoRV subtypes (KoRV-A, -B or -C), using subtype-specific primers targeting the env gene (Table 1) under reaction conditions described previously [8, 17]. To confirm the KoRV subtypes, PCR fragments were subcloned into pCR2.1 TOPO vector (Invitrogen) and sequenced.

Measurement of KoRV provirus and RNA copy numbers

The gDNA isolated from koala PBMCs was used as a template for the measurement of total KoRV proviral, KoRV-A, KoRV-B, and KoRV-C DNA copy numbers by real-time PCR as described previously [17], using subtype-specific primers (Table 1) with Brilliant-III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Amplification and detection were carried out using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The specificity of the PCR was confirmed by melt curve analysis. Standards were generated from pre-quantified plasmids containing the sequence of the target gene. Koala β-actin was used as an endogenous control for the normalization of the KoRV proviral DNA copy numbers. The primers used for detection of koala β-actin are shown in Table 1.

The RNA copy numbers in the RNA isolated from koala PBMCs or plasma were also quantified by real-time PCR as described previously [17], using gene-specific primers (Table 1) as described above. Koala β-actin was used as an endogenous control for the normalization of the KoRV total RNA copy numbers.

Multiple sequence alignment

KoRV-A, -B and -C env genes were isolated from koalas and sequenced. The sequence data were submitted to the GenBank database (GenBank accession nos. MT951447-MT951453). A multiple sequence alignment of KoRV env gene nucleotides was performed using MEGA7 [18].

Results

Study population, KoRV status, WBC count, and mortality

Six adult captive koalas were included in this study (animal nos. KH1 to KH5 and H10): one male and five females; age range, 1 to 12 years). All six koalas were found to be positive for KoRV (Table 2).

Two koalas (KH2 and KH3) showed a high WBC count and were accordingly regarded as leukemic (Table 2). These two leukemic koalas were reported to have died before the end of the experiment, but their deaths occurred independently of any procedure or observation in this study. Zoo veterinarians regarded lymphoma as the cause of death for these two animals. The other four koalas (KH1, KH4, KH5, and H10) survived and were regarded as clinically healthy.

Subtyping of koala retrovirus

KoRV subtypes were determined by PCR with gDNA as a template using subtype-specific primers (Table 1). Four koalas were found positive for KoRV-A only (KH1, KH4, KH5, and H10). One koala (KH2) was positive for KoRV-A, -B, and -C, and one koala (KH3) was positive for KoRV-A and -B (Fig. 1A-C).

Determination of KoRV proviral DNA and RNA copy numbers

KoRV proviral DNA and total RNA copy numbers were measured using real-time PCR and normalized against koala β-actin. We found variations in the amount of KoRV proviral DNA in the koala genome (Fig. 2). We measured total KoRV proviral loads as well as KoRV-A, -B, and -C

Table 1 Primers used in this study for identification of KoRV and KoRV subtypes

Target gene	Forward	Reverse	Amplicon size (bp)	Reference
Pol (KoRV)	TTGGAGGAGGAATACCGATTACAC	GCCAGTCCCATACCTGCCTT	523	[14]
Env KoRV-A	TCCTGGGAACTGGAAAAGAC	GGGTTCCCCAAGTGATCTG	321	[11]
Env KoRV-B	TCCTGGGAACTGGAAAAGAC	GGCGCAGACTGTTGAGATTC	271	[10]
Env KoRV-C	TCCTGGGAACTGGAAAAGAC	AAGGCTGGTCCCGCGAAGT	290	[17]
β-actin	AGATCATTGCCCCACCT	TGGAAGGCCCAGATTC	123	[14]

Table 2 Study population, subtyping, and koala health information

Koala ID	Sex	Age	Place of origin	Detection of KoRV subtypes by PCR		Identified subtypes	WBC (10^2/µL)	Clinical determination
				In gDNA of PBMCs	In plasma viral RNA			
KH1	Female	2 Y	Hirakawa Zoo, Japan	Positive	Positive	KoRV-A	102	Healthy
KH2	Male	2 Y	Hirakawa Zoo, Japan	Positive	Positive	KoRV-A, -B, and -C	**4000**	**Leukemic**
KH3	Female	12 Y	Tama Zoo, Japan	Positive	Negative	KoRV-A and -B	**2275**	**Leukemic**
KH4	Female	1 Y	Hirakawa Zoo, Japan	Positive	Positive	KoRV-A	52	Healthy
KH5	Female	1 Y 10 M	Hirakawa Zoo, Japan	Positive	Positive	KoRV-A	58	Healthy
H10	Female	6 Y 6 M	Dreamworld Theme Park, Australia	Positive	Positive	KoRV-A	125	Healthy

Y, years; M, months; WBC, white blood cell

Fig. 1 Subtyping of KoRV by PCR. Results of PCR analysis using primers specific for KoRV-A (A), KoRV-B (B), and KoRV-C (C) in koalas are shown. In the gel run image, KH1 to KH5 are the designations of the individual koalas, NTC is the no-template control, and 'M' indicates the 100-bp marker (TAKARA). The bands specific for KoRV-A, -B, or -C are indicated by an arrow.
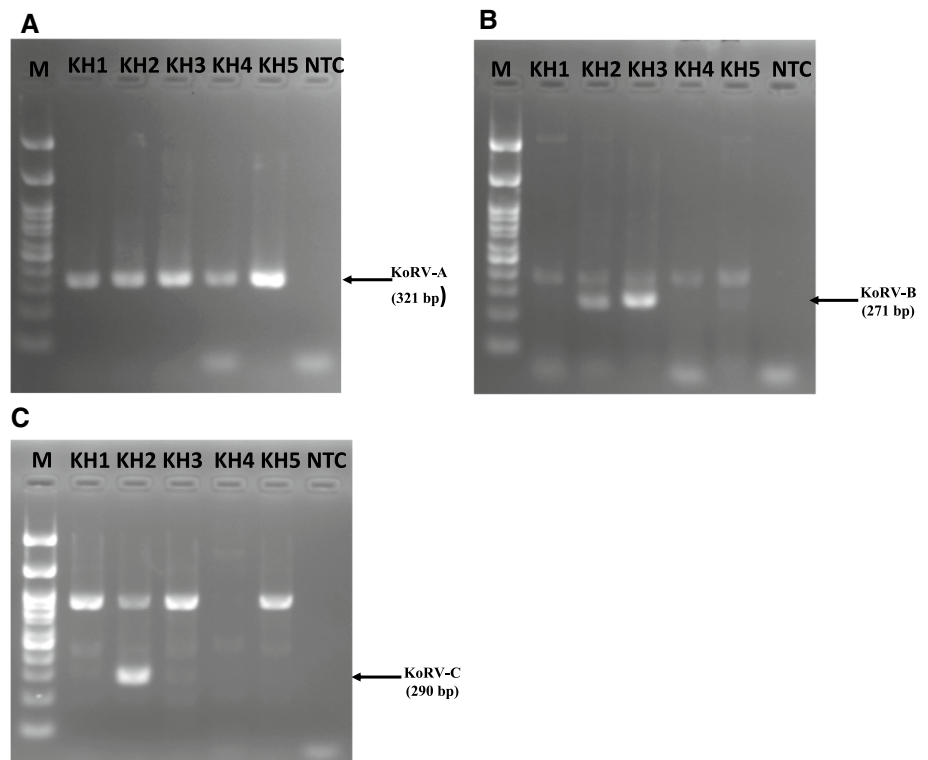
subtype-specific proviral loads. The highest total KoRV proviral load was found in koala KH3, and the lowest KoRV proviral load was found in koala H10 (Fig. 2). All koalas tested positive for KoRV-A, with koala KH2 additionally testing positive for KoRV-B and -C and koala KH3 additionally testing positive for KoRV-B.

We also determined the total KoRV RNA copy number targeting the KoRV pol gene and the KoRV-A, -B,
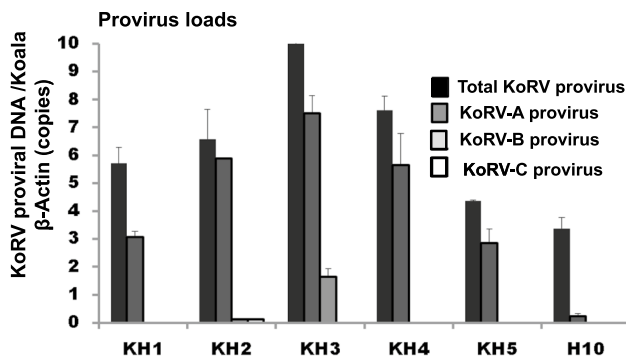
Fig. 2 Quantitation of KoRV provirus in koala PBMCs. The total KoRV, KoRV-A, -B, and -C provirus DNA copy numbers in gDNA isolated from koalas PBMCs are indicated. The provirus copy numbers were measured by real-time qPCR and normalized with the corresponding koala β-actin in PBMCs. Vertical bars indicate SD.
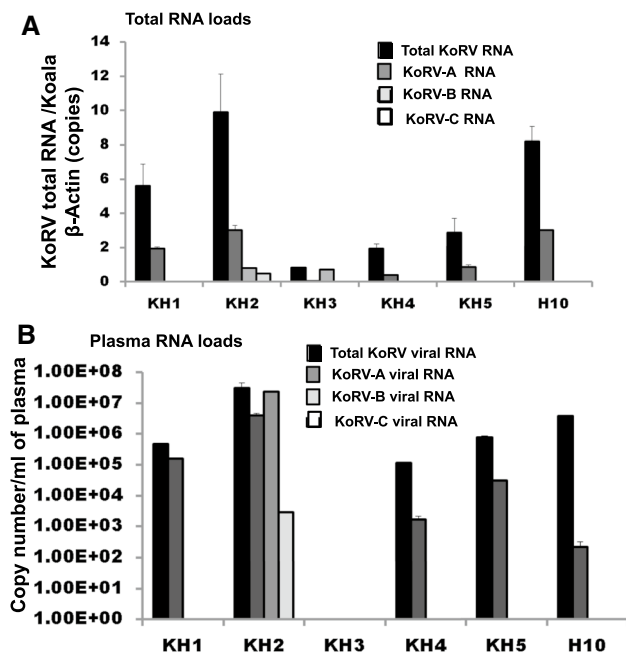

Fig. 3 Quantitation of KoRV RNA in koala PBMCs and plasma. (A) Total RNA copy numbers in koala PBMCs. RNA copies were quantified by real-time RT-qPCR, and PBMC RNA copies were normalized to koala β-actin. (B) Total KoRV, KoRV-A, -B, and -C RNA copies per mL plasma. Vertical bars indicate SD.

and -C subtype-specific RNA copy numbers targeting KoRV env gene in RNAs isolated from koala PBMCs (Fig. 3A) and plasma (Fig. 3B). Both total and subtype-specific KoRV RNA were higher in one of the kolas with

a multiple-subtype infection (KH2: KoRV-A, -B, and -C positive). However, koala KH3 (KoRV-A and -B positive) showed lower KoRV RNA copy numbers than other koalas (Fig. 3A).

We then measured RNA loads for total KoRV, KoRV-A, KoRV-B, and KoRV-C in plasma from each animal (Fig. 3B). The highest plasma KoRV RNA load was in koala KH2 (KoRV-A, -B, and -C positive), but no KoRV was detected in the other koala with a multiple-subtype infection (KH3: KoRV-A and -B positive). The other four koalas had detectable levels of total KoRV and KoRV-A RNA in their plasma, albeit at lower levels than KH2 (Fig. 3B).

Determination of the KoRV copy number in koala PBMC culture

The koala with undetectable plasma KoRV RNA (KH3) was targeted for further investigation with cell cultures of its isolated PBMCs. We measured the KoRV proviral load in the koala gDNA, as well as the total RNA and viral RNA load in the cultured cells and supernatant, respectively (Fig. 4A, B, and C). We detected KoRV proviral DNA in the genome and total KoRV RNA in cultured PBMCs at days 7 and 14 days of culturing. The culture supernatant contained detectable total KoRV RNA, KoRV-A, and -B RNA levels at day 7 of culturing, but not at day 14 of culturing (Fig. 4C).

Sequence alignment

We sequenced the KoRV-A, -B, and -C partial env genes (GenBank accession nos. MT951447-MT951453) from the koalas in this study and performed sequence alignment with KoRV-A, -B, and -C env gene reference sequences (GenBank accession nos.: KoRV-A, AF151794; KoRV-B, KC779547; KoRV-C, AB828005.1). The KoRV-A partial env gene sequence in the study animals was 100% identical to the reference sequence (Fig. 5A). The KoRV-B and KoRV-C partial env gene sequences in the study animal showed differences from the corresponding reference sequence in two and four nucleotides, respectively (enclosed by black boxes, Fig. 5B and C).

Discussion

In this study, we determined KoRV profiles (for total KoRV and subtypes KoRV-A, -B, and -C) in six captive koalas in a Japanese zoo and made a preliminary assessment of the effects of multiple-subtype infection on koala health, based on WBC counts.
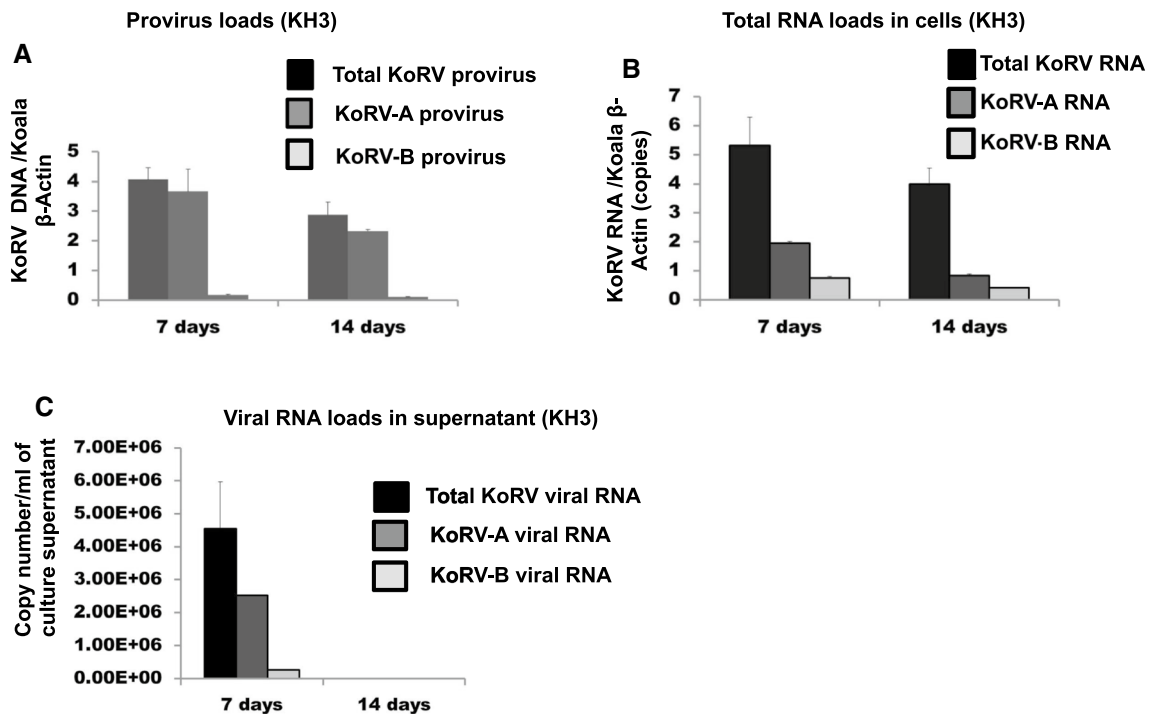
Provirus loads (KH3)

Total RNA loads in cells (KH3)

Viral RNA loads in supernatant (KH3)

Fig. 4 Distribution and quantity of KoRV subtypes in cultured koala PBMCs isolated from koala KH3. The koala PBMCs were cultured, and gDNA and RNA were purified from the cells and supernatant. (A) Total KoRV, KoRV-A, and KoRV-B provirus copy number in gDNA at days 7 and 14 post culture. (B) Total KoRV, KoRV-A, and KoRV-B RNA copy numbers in koala PBMCs at days 7 and days 14 post culture. Provirus copies were measured by real-time PCR and normalized to the corresponding koala β-actin. (C) RNA copy number per mL of koala PBMC culture supernatant at days 7 and days 14. Vertical bars indicate SD.

In our study population, two koalas had multiple-subtype infection (KoRV-B and/or -C in addition to KoRV-A), and four koalas had a single-subtype infection, carrying only the fully endogenized form (KoRV-A). Notably, the koalas with multiple subtypes had poorer health outcomes, showing elevated WBC counts indicative of leukemic status at the time of blood sampling. On the other hand, the four koalas with a single subtype (KoRV-A) appeared clinically healthy at the time of blood sampling.

Our findings are consistent with a number of previous reports on KoRV and koala health. A recent study suggested that KoRV-A does not superinfect koalas harboring endogenized KoRV-A [7], which was substantiated by the apparently clinically healthy status of the four koalas in our study with KoRV-A as a single-subtype infection. Associations between KoRV-B and lymphoma have been reported in both captive and wild populations [3, 10], findings which are reflected in the deaths due to lymphoma of the two KoRV-B-positive koalas in our study. Increased IL-6 expression has also been reported previously in koalas with multiple-KoRV-subtype infection [13], which may be linked to associations of KoRV with immune suppression and neoplasia [10, 13].

We found that all of the koalas had detectable KoRV provirus, but with inter-individual variations in the proviral load. Our findings on proviral load are consistent with those of a previous study [9] in which such

variations were speculated to result from the ongoing endogenization of KoRV into the koala genome. The plasma viral RNA load has been advanced as an indicator of the stage and progression of other retroviral diseases in other species, with previous studies covering HIV1-infected humans [19] and feline-immunodeficiency-virus-infected cats [20]. Tarlinton et al. reported that a higher plasma viral load may be associated with neoplasia [9]. The koala with the highest plasma load in our study died from lymphoma, but interestingly, KoRV could not be detected in the case of the other lymphoma-related death.

The two koalas with multi-subtype infection showed an apparently worse health status that the single-subtype-infected koalas, and they died due to lymphoma. Although one or the other of these koalas showed the highest value for each viral load parameter we examined, there were cases where koalas with single subtype (KoRV-A only) infection showed a higher proviral, total RNA, or plasma viral load than one of the koalas with a multi-subtype infection. Furthermore, the total load exceeded that of KoRV-A only for each koala designated as having single-subtype infection, suggesting that they may have harbored one of the subtypes (KoRV-D to J) not evaluated in this study.

During genotyping of the KoRV-C subtype with subtype-specific primers, we observed a larger amplicon for all of the koalas except KH4. This result is indicative of an extraneous origin for the sequence. Accordingly, we sequenced larger fragments and found one DNA fragment from KH3 containing an N-terminal sequence (length: 181 nt) that was similar to that of the KoRV-C subtype envelope gene (GenBank accession no. MT951447.1), but the downstream sequence did not show any significant similarity to sequences in the GenBank database. This finding suggests that proviral recombination with KoRV-C might have occurred.

The koala with undetectable plasma KoRV RNA was chosen for further investigation with cell cultures of its isolated PBMCs. The culture supernatant contained detectable RNA levels at day 7 of culturing, but not at day 14. This finding may reflect a decrease in available cells releasing KoRV RNA into the culture supernatants at the later time point, as well as the fragile nature of the RNA.

Sequence alignment revealed 100% identity between the KoRV-A partial env gene in the study animals and the KoRV-A reference env gene, suggesting that KoRV-A has been fully endogenized in this population, whereas KoRV-B and -C are continuing to evolve and showed mutations as expected. Alignment of KoRV in our study population with reference sequences suggested that the state of KoRV in this Japanese zoo epidemiologically reflects that in wild koala populations in northern Australia, in which KoRV-A is fully endogenized but other subtypes, while actively replicated, are much less prevalent. We found 100% sequence identity to the reference sequence for KoRV-A but multiple differences in nucleotides for both KoRV-B and KoRV-C. We suggest that KoRV-B and KoRV-C are mutating with circulation in our captive population, as would be expected for virus subtypes that are continuing to evolve. On the other hand, we used only the env genes for sequence alignment, and secure confirmation of a variant's ability to replicate requires identification of its full genome sequence. Further investigations are thus required to test our hypothesis on subtype replication in this captive koala population. Our findings are also consistent with previous reports of greater genetic diversity in the KoRV envelope gene for subtypes other than KoRV-A [3, 21–24].

In conclusion, coinfection with multiple KoRV subtypes may have a negative impact on koala health. We also suggest that single-subtype infection with KoRV-B may be implicated in the development of lymphoma and increased virus loads in plasma. Furthermore, infection with multiple KoRV subtypes may lead to increased KoRV loads in plasma, which could thus be a useful prognostic marker for koala health. For further understanding of disease association in multiple-subtype infection, a large-scale study targeting the full range of KoRV subtypes is warranted for further understanding of this marsupial retrovirus.
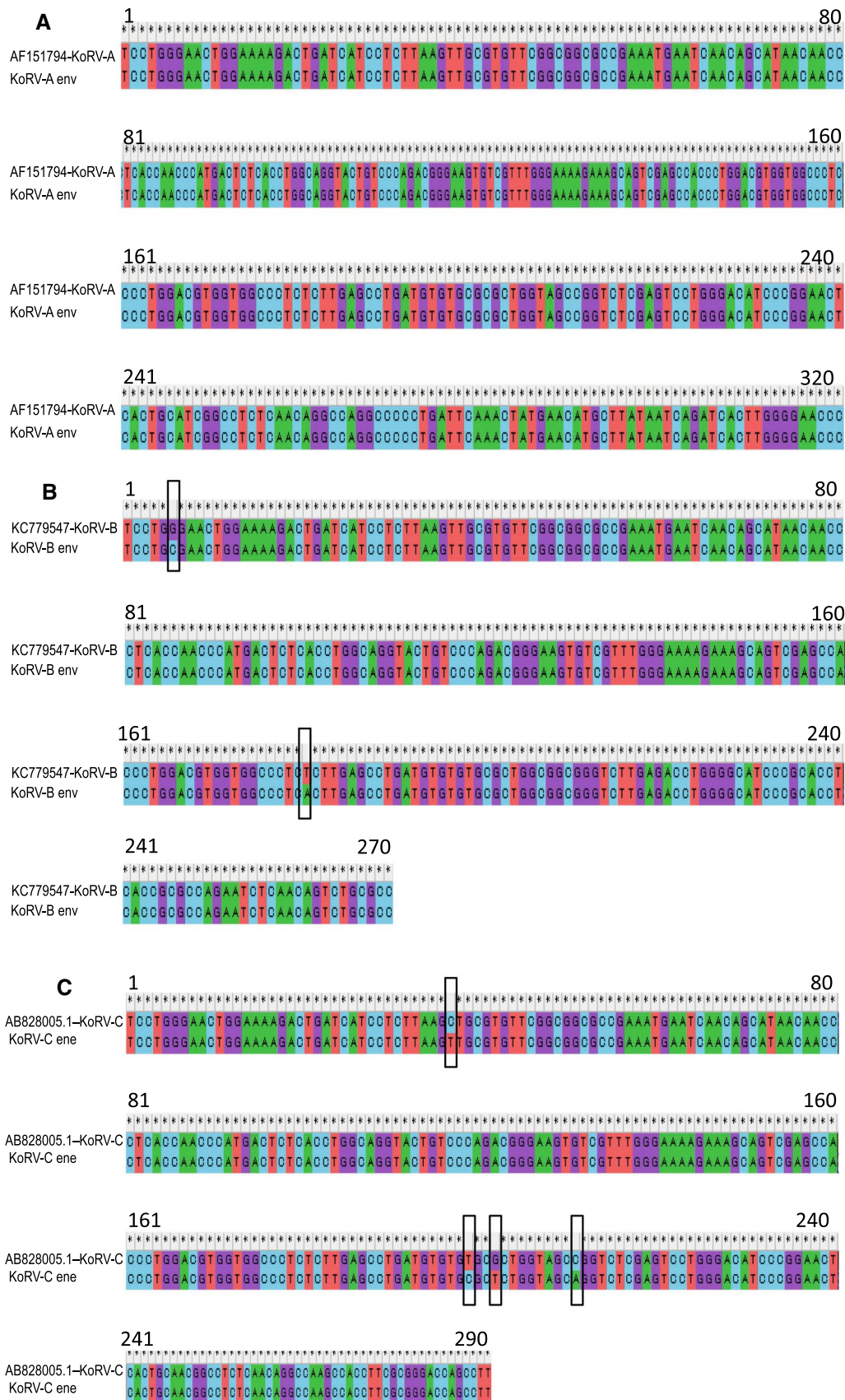
◄**Fig. 5** Sequence alignment partial env genes of KoRV-A (from koala KH1-5), -B (from koalas KH2 and KH 3), and -C (from koala KH2). Boxes indicate the nucleotide difference of isolated KoRV-A, -B, and -C partial env genes from Hirakawa Zoological Park, Kagoshima, Japan, with reference sequences. Reference sequences of KoRV-A (GenBank accession no. AF151794), KoRV-B (GenBank accession no. KC779547), and KoRV-C (GenBank accession no. AB828005.1) are also shown.

Declarations

Conflict of interest The authors declare that they have no competing interest.

Ethical approval This study was performed in accordance with the protocols of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University, Japan.

References

1. Kayesh MEH, Hashem MA, Kohara TK (2020) Koala retrovirus epidemiology, transmission mode, pathogenesis, and host immune response in koalas (Phascolarctos cinereus): a review. Arch Virol 165:2409–2417

2. Quigley BL, Timms P (2020) Helping koalas battle disease – Recent advances in Chlamydia and koala retrovirus 382 (KoRV) disease understanding and treatment in koalas. FEMS Microbiol Rev 44:583–605

3. Zheng H, Pan Y, Tang S, Pye GW, Stadler CK, Vogelnest L, Herrin KV, Rideout BA, Switzer WM (2020) Koala retrovirus diversity, transmissibility, and disease associations. Retrovirology 17:34

4. Hanger JJ, Bromham LD, McKee JJ, O'Brien TM, Robinson WF (2000) The nucleotide sequence of Koala (*Phascolarctos cinereus*) retrovirus: a novel type C endogenous virus related to Gibbon ape leukemia virus. J Virol 74:4264–4272

5. Denner J, Young PR (2013) Koala retroviruses: characterization and impact on the life of koalas. Retrovirology 10:108

6. Simmons G, Clarke D, McKee J, Young P, Meers J (2014) Discovery of a novel retrovirus sequence in an Australian native rodent (*Melomys burtoni*): a putative link between gibbon ape leukemia virus and koala retrovirus. PLoS ONE 9:e106954

7. Ishida Y, Zhao K, Greenwood AD, Roca AL (2015) Proliferation of endogenous retroviruses in the early stages of a host germ line invasion. Mol Biol Evol 32:109–120

8. Hashem MA, Kayesh MEH, Yamato O, Maetani F, Eiei T, Mochizuki K, Sakurai H, Ito A, Kannno H, Kohara KT et al (2019) Coinfection with koala retrovirus subtypes A and B and its impact on captive koalas in Japanese zoos. Arch Virol 164:2735–2745

9. Tarlinton R, Meers J, Hanger J, Young P (2005) Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. J Gen Virol 86:783–787

10. Xu W, Stadler CK, Gorman K, Jensen N, Kim D, Zheng HQ, Tang S, Switzer WM, Pye GW, Eiden MV (2013) An exogenous retrovirus isolated from koalas with malignant neoplasias in a US zoo. Proc Natl Acad Sci USA 110:11547–11552

11. Waugh CA, Hanger J, Loader J, King A, Hobbs M, Johnson R, Timms P (2017) Infection with koala retrovirus subgroup B (KoRV-B), but not KoRV-A, is associated with chlamydial disease in free-ranging koalas (*Phascolarctos cinereus*). Sci Rep 7:134

12. Fabijan J, Woolford L, Lathe S, Simmons G, Hemmatzadeh F, Trott DJ, Speight KN (2017) Lymphoma, koala retrovirus infection and reproductive chlamydiosis in a koala (*Phascolarctos cinereus*). J Comp Pathol 157:188–192

13. Kayesh MEH, Hashem MA, Maetani F, Eiei T, Mochizuki K, Ochiai S, Ito A, Ito N, Sakurai H, Asai T, Kohara KT (2020) CD4, CD8b, and cytokines expression profiles in peripheral blood mononuclear cells infected with different subtypes of KoRV from Koalas (*Phascolarctos cinereus*) in a Japanese Zoo. Viruses 12:1415

14. Tarlinton RE, Meers J, Young PR (2006) Retroviral invasion of the koala genome. Nature 442:79–81

15. Miyazawa T, Shojima T, Yoshikawa R, Ohata T (2011) Isolation of koala retroviruses from koalas in Japan. J Vet Med Sci 73:65–70

16. Kayesh MEH, Yamato O, Rahman MM, Hashem MA, Maetani F, Eiei T, Mochizuki K, Sakurai H, Kohara KT (2019) Molecular dynamics of koala retrovirus infection in captive koalas in Japan. Arch Virol 164:757–765

17. Hashem MA, Maetani F, Kayesh MEH, Eiei T, Mochizuki K, Ito A, Sakurai H, Asai T, Kohara KT (2020) Transmission of koala retrovirus from parent koalas to a joey in a Japanese zoo. J Virol 94:e00019–e00020

18. Kumar SG, Stecher K, Tamura MEGA7 (2016) Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874

19. Piatek M, Saag MS, Yang LC, Clark SJ, Kappes JC, Luk KC, Hahn BH, Shaw GM, Lifson JD (1993) High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 259:1749–1754

20. Diehl LJ, Mathiason-Dubard CK, O'Neil LL, Hoover EA (1996) Plasma viral RNA load predicts disease progression in accelerated feline immunodeficiency virus infection. J Virol 70:2503–2507

21. Xu W, Gorman K, Santiago JC, Kluska K, Eiden MV (2015) Genetic diversity of koala retroviral envelopes. Viruses 7:1258–1270

22. Quigley BL, Ong VA, Hanger J, Timms P (2018) Molecular dynamics and mode of transmission of koala retrovirus as it invades and spreads through a wild Queensland koala population. J Virol 92:e01871-e1917

23. Quigley BL, Phillips S, Olagoke O, Robbins A, Hanger J, Timms P (2019) Changes in endogenous and exogenous koala retrovirus subtype expression over time reflect koala health outcomes. J Virol 93:18

24. Sarker N, Fabijan J, Seddon J, Tarlinton R, Owen H, Simmons G, Thia J, Blanchard AM, Speight N, Kaler J et al (2019) Genetic diversity of Koala retrovirus env gene subtypes: insights into northern and southern koala populations. J Gen Virol 100:1328–1339