

Identification of a Novel Subgroup of Koala Retrovirus from Koalas in Japanese Zoos

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We identified a new subgroup of koala retrovirus (KoRV), named KoRV-J, which utilizes thiamine transport protein 1 as a receptor instead of the Pit-1 receptor used by KoRV (KoRV-A). By subgroup-specific PCR, KoRV-J and KoRV-A were detected in 67.5 and 100% of koalas originating from koalas from northern Australia, respectively. Altogether, our results indicate that the invasion of the koala population by KoRV-J may have occurred more recently than invasion by KoRV-A.

When an exogenous retrovirus integrates into the genome of germ line cells, the provirus becomes permanently embedded into the host genome as an endogenous retrovirus (ERV), resulting in vertical transmission to offspring (1). Although most ERVs are replication defective due to mutations and deletions, endogenous koala retroviruses (KoRVs) are replication competent and can be horizontally transmitted as an exogenous virus. Tarlinton et al. reported that KoRV proviruses are absent in the genomes of some populations of koalas in southern Australia but present in those of koalas in northern Australia (2). These observations indicate that the invasion by KoRV into the koala genome occurred recently and suggest that KoRV is a unique retrovirus in the midst of becoming an endogenous retrovirus (2).

Koala retrovirus belongs to the genus *Gammaretrovirus* in the family *Retroviridae*, and it is closely related to *Gibbon ape leukemia virus* (GALV) (3, 4). Notably, incidences of leukemia and lymphoma, which are the most common neoplastic diseases caused by gammaretrovirus infections, are extremely high in koalas. It has been reported that 3 to 5% of wild koalas and up to 80% of captive koalas die due to these diseases in Australia (3, 5–8). Similarly, captive koalas in zoological parks in Japan suffer from leukemia and lymphoma at a relatively high rate (approximately 10%). A correlation between these neoplastic diseases and the plasma viral load of KoRV, the suspected causative agent, has been reported (6, 7). In addition, many koalas suffer from opportunistic infectious diseases, such as chlamydiosis and cryptosporidiosis, indicating that KoRV may also induce immunosuppression (3, 8–10). To clarify the relationship between KoRV and these diseases, it is necessary to accumulate more epidemiological and virological data.

KoRV infection is mediated by Pit-1, a sodium-dependent phosphate symporter that also functions as a receptor for feline leukemia virus (FeLV) subgroup B (FeLV-B) and GALV (11–13). Previously, we succeeded in isolating several KoRV strains (OJ-1 to OJ-5) from Queensland koalas reared in Kobe Municipal Oji Zoo (Hyogo, Japan) (14). By utilizing an interference assay, we found that one KoRV isolate (strain OJ-4) did not interfere completely with FeLV-B, indicating that the isolate may contain an additional gammaretrovirus or a KoRV variant that utilizes a different receptor than Pit-1 (data not shown). In this study, we identified a new KoRV subgroup in the isolate and found that this subgroup, named KoRV-J, uses thiamine transport protein 1 (THTR1), which is known to be a receptor for FeLV-A.

To investigate the genetic variability of the KoRV *env* of strain OJ-4 (14), full-length *env* was amplified by PCR using *Pfu* Ultra II (Stratagene) with a primer set corresponding to the 3' terminus of *pol* and the 3' untranslated region (GenBank accession number AF151794) (Table 1). Genomic DNA of HEK293T cells chronically infected with KoRV strain OJ-4 (14) was used as a PCR template. The PCR fragments were subcloned into pCR Blunt II TOPO (Invitrogen), and the resulting *env* clones were then digested for restriction enzyme mapping. The clones whose restriction patterns were different from that of the original KoRV *env* (pcindy) (3) were subsequently subjected to sequencing analysis. We identified four unique KoRV *env* sequences (clones 11-1, 11-2, 11-4, and 11-5). Two of the clones (clones 11-4 and 11-5) were functional in the pseudotype virus infection assay, described below. The nucleotide identity of clone 11-5 *env* with that of pcindy was 99.7% (data not shown). The amino acid sequence alignment of pcindy (KoRV subgroup A [KoRV-A]) and clone 11-4 (KoRV-J) is shown in Fig. 1A. The identity of amino acid sequences between the two subgroups was 87.6%, and the variability was mainly found in variable region A (VRA), which is the major determinant of receptor usage (17), and the proline-rich region (PRR) of the envelope glycoprotein (Env). The CETTG motif, which is involved in fusion activity (18), was disrupted by mutations in both KoRV-A and KoRV-J. KoRV-J had a replacement of Gln with Arg in the PHQ motif, which is important for the fusion activity (19–22). Other important motifs that influence the functions of Env and/or the viral infectivity were highly conserved between the two subgroups.

Phylogenetic analysis of *env* using the maximum likelihood approach (23) revealed that KoRV isolates (Cindy [3], 522 [24], and KoRV-J [clone 11-4]) and GALV clustered together, but they were distinct from the cluster that consists of FeLVs, murine leukemia viruses (MLVs), and porcine endogenous retroviruses

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TABLE 1 Primers used in this study

Assay	Target	Orientation	Sequence	Position (nucleotides)	Amplicon size (bp)
Cloning	KoRV <i>env</i>	Forward	5'-ATGCTTCTCATCTCAAACCC-3'	1-20	1,983
		Reverse	5'-TTAAAGTTATCGGCGTTGT-3'	1964-1983	
Differential PCR	KoRV <i>pol</i>	Forward	5'-CCTTGGACCACCAAGAGACTTTTGA-3'	2710-2734	1,051
		Reverse	5'-TCAAATCTTGGACTGGCCGA-3'	3741-3760	
	KoRV-A	Forward	5'-TATGAACATGCTTATAATCAGATCAC-3'	6242-6267	723
		Reverse	5'-ATAATGACTTGGCCTGGAGTTGCG-3'	6940-6964	
	KoRV-J	Forward	5'-AAATGGCTGTGGGGTGTCTTATAGTCAG-3'	303-330	547
		Reverse	5'-TAGGGAGCGTGGGGTAGTACTGGTG-3'	824-849	
Real-time PCR	KoRV-A	Forward	5'-AACTATGAACATGCTTATAATCAGATCACTT-3'	6239-6269	101
		Reverse	5'-GGGCACACGTAGAACTGGGA-3'	6320-6339	
	KoRV-J	Forward	5'-TGCTTTATAGTCAGGTCGGCAG-3'	317-338	101
		Reverse	5'-CTGAGATTGAGCCAGTCTGTCC-3'	395-417	
	Koala β -actin	Forward	5'-GAGACCTTCAACACCCACG-3'	373-392	111
		Reverse	5'-GTGGGTCACACCATCACCAG-3'	464-483	

(PERVs) (Fig. 1B). A similar topology of the phylogenetic tree was obtained using the transmembrane region of *env* (data not shown). KoRVs and GALVs are distantly related to PERVs. Similarities of the Env amino acids among the KoRV-A isolates (Cindy,

522, and OJ-4 [clone 11-5]) were shown to be high, and the degree of diversity between KoRV-A and KoRV-J was less than those of the FeLV and PERV subgroups (data not shown).

We next performed a LacZ pseudotype assay to characterize

A)

KoRV-A	MLLISNPRHLGHPMSPGNWKRLIILLSCVFGGAEMNQHHNPHQPMTLTWQVLSQTGSVV
KoRV-JR.....
	SP PHQ
KoRV-A	WEKKAVEPPTWPPSLEPDVCAVAGLESWDIPELTASASQQARPPDSNYEHAYNQITWG
KoRV-J	...E.....A...T.G..H..VPE...SA..NGC-GVL.S.VGR.
	VRA
KoRV-A	-----TLGCSYPRARTRIARSQFYVCPDRGSLSEARRCGGLESLYCKEWGCEGTAGT
KoRV-J	LVRQSSYG...RC..D.N.L.Q.....W.....F.....
	CETT
KoRV-A	AYWQPRSSWDLITVGGHPTGTCERTGWCNPLKIEFTEPGKRFRNWLQGRTWGLRFYVTG
KoRV-JR.....
	VRB
KoRV-A	HPGVQLTIRLVITSPPPVVVGGPDEVLAEQGPERRKIFELPRVVPVPTLSPASP-IPTVQAS
KoRV-JL.....L..V...L.G.M.S...A..T...RSL.SL.....
	PRR
KoRV-A	PEAPSTPSEPTGDRFLGVLQGAFLALNATNPEATESCWLCALGPPYEGGIATPGQVTVYA
KoRV-JV..V.....L.....V.....S.V.....F.....T.....
	CXXC
KoRV-A	STDSQCRWGGKGLTLTEVSGGLGICIGKVPPTHQHLNLTIPLNASHTHKYLSPNSRNSW
KoRV-JT.....R...SS.V.....S.....H...
	SU TM
KoRV-A	ACNSGLTPCLSTSVFNQSNDFCIQIQLVPRIIYHPDGTLLQAYESPHSRNKRREPVSLLTA
KoRV-JP.....
	RX(K/R)R
KoRV-A	VLLGLGVAAGIGTGSTALIKGPIDLQGLTSLQIAMDTDLRALQDSISKLEDSLTSLSSEV
KoRV-JP.....K.....Y.....
	CXC6
KoRV-A	YEGWFNRSPWLTTLTSLALAGPLLLLLLLLTLGPCVINKLVQFINDRVSAVRIIVLRHKYQ
KoRV-JM.....I.....V.....F.....
	SU TM
KoRV-A	TLDNEDNL
KoRV-J	..N.A...

B)

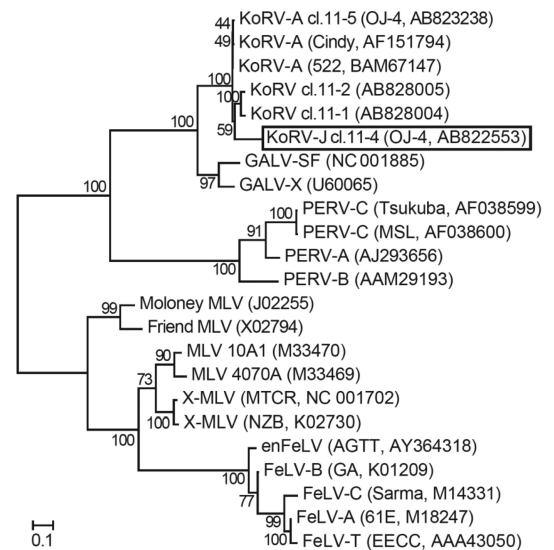


FIG 1 Genetic analysis of KoRV-A and KoRV-J. (A) Comparison of amino acid sequences of KoRV subgroups A and J. Dots indicate the same amino acids. VRA, VRB, and PRR are shaded in gray. Abbreviations: SP, signal peptide; PHQ, PHQ motif; CETTG, CETTG motif; CXXC, CXXC motif; SU, surface unit; TM, transmembrane; RX(K/R)R, furin cleavage site; CXC6, CXC6 motif. (B) Maximum likelihood tree of the entire amino acid sequences of *env* genes of KoRV isolates and other gamma-retroviruses. Numbers at the nodes indicate the percentage of rapid bootstrap values (1,000 replicates). Amino acid sequences used for the analyses were retrieved from the GenBank database, and the accession numbers are shown in parentheses. Abbreviations: X-MLV, xenotropic MLV; enFeLV, endogenous FeLV.

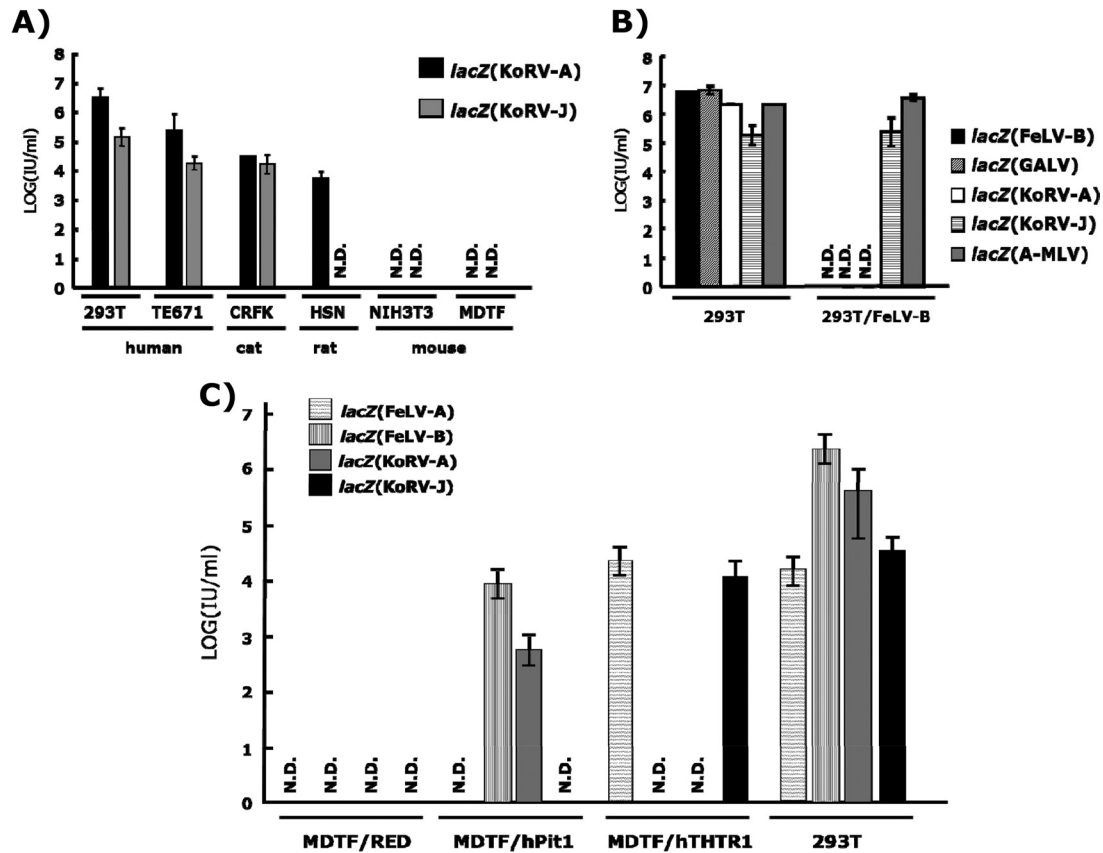


FIG 2 Receptor usage of KoRV-A and KoRV-J. (A) Host ranges of KoRV subgroups. *lacZ* pseudotypes were inoculated into the indicated cells, and titers of the viruses were determined in a LacZ assay. Data were obtained from three independent experiments, and the values are expressed as means \pm 1 standard deviation (SD). N.D., not detected. (B) Interference assay of KoRV subgroups. *lacZ* pseudotypes were inoculated onto naive HEK293T cells and HEK293T cells chronically infected with FeLV-B strain Gardner-Arnstein, and titers of the viruses were determined in a LacZ assay. Data were obtained from three independent experiments, and the values are expressed as means \pm 1 SD. (C) Infectivity of KoRV subgroups in MDTF cells expressing human Pit-1 (MDTF/hPit1) and human THTR1 (MDTF/hTHTR1). cDNAs of human Pit1 and THTR1 were cloned into a RetroQDsRed monomer vector (Clontech) to produce pRetro-hTHTR-DsRed. The resultant plasmid was transfected into MDTF cells, selected by puromycin, and subjected to a *lacZ* pseudotype infection assay. *lacZ* pseudotypes were inoculated onto MDTF/hPit1, MDTF/hTHTR1, and MDTF cells transfected with an empty expression vector (MDTF/RED) and HEK293T cells. Titers of the viruses were determined in a LacZ assay. Data were obtained from three independent experiments, and the values are expressed as means \pm 1 SD.

biological properties of KoRV-J Env. The *env* expression plasmids, termed pFBFeLV-A (25), pFBFeLV-B (26), and pFBASALF (16), were used for the expression of Envs of FeLV-A (strain Glasgow-1), FeLV-B (strain Gardner-Arnstein), and amphotropic MLV (A-MLV, strain 4070), respectively. KoRV-A (clone 11-5), KoRV-J (clone 11-4), and GALV *env* genes were cloned into pFB envelope expression plasmids to produce pFBKoRV-A, pFBKoRV-J, and pFBGALV, respectively (25). The GALV *env* was cloned from the genomic DNA of TELCeB/GAF cells (26). These *env* expression plasmids were transfected into TELCeB6 cells, which express large amounts of MLV core particles, and an MF-GnlLacZ vector to produce pseudotype viruses (16). After selection of the transfectants with 50 μ g/ml of phleomycin, virus supernatants were harvested from the pooled phleomycin-resistant cell populations, filtered through 800-nm Millipore filters, and used immediately in LacZ pseudotype assays. Titration of LacZ pseudotypes was performed as described previously (27). The LacZ pseudotype virus bearing the KoRV-J Env [*lacZ*(KoRV-J)] could infect HEK293T cells, although the titer was approximately 10-fold lower than that for *lacZ*(KoRV-A) (Fig. 2A). These data suggest that the KoRV-J Env is functional. Interestingly, substitu-

tions in the PHQ motif and deletions in the vicinity of the motif did not influence the fusion activity.

We then examined the host cell ranges of KoRV-A and -J by using target cells derived from human (HEK293T and TE671), cat (CRFK), rat (HSN), and mouse (NIH 3T3 and MDTF) cells (Fig. 2A). Consistent with previous reports (11, 24, 28), *lacZ*(KoRV-A) showed the same host cell range as FeLV-B and GALV, which utilize Pit-1 as a receptor. On the other hand, *lacZ*(KoRV-J) could not infect rat HSN cells (Fig. 2A) but efficiently infected HEK293T cells chronically infected with FeLV-B (Fig. 2B). In addition, the stable expression of human Pit-1 rendered MDTF cells susceptible to *lacZ*(KoRV-A), but not to *lacZ*(KoRV-J) (Fig. 2C). These data clearly show that KoRV-J utilizes an unidentified receptor distinct from Pit-1. To further characterize the receptor usage of KoRV-J, we conducted a receptor interference assay using six gammaretroviruses that utilize different receptors, namely, FeLV-A, -B, or -C, RD-114 virus, xenotropic murine leukemia virus (X-MLV), and A-MLV (Table 2). We found that *lacZ*(KoRV-J) interfered with FeLV-A on FEA cells (feline fibroblasts). The receptor for FeLV-A is known to be THTR1 (29, 30). As shown in Fig. 2C, *lacZ*(KoRV-J) and *lacZ*(FeLV-A) infected MDTF cells expressing

TABLE 2 Interference between gammaretroviruses

Virus	Receptor interference between the two viruses in the indicated cell type ^a						
	FeLV-A in FEA	FeLV-B in HEK293T	FeLV-C in HEK293T	A-MLV in TE671	X-MLV in TE671	RD-114 in TE671	KoRV OJ-4 in 293T
<i>lacZ</i> (KoRV-A)	–	+	–	–	–	–	+
<i>lacZ</i> (KoRV-J)	+	–	–	–	–	–	+
<i>lacZ</i> (FeLV-A)	+	–	–	–	–	–	+
<i>lacZ</i> (FeLV-B)	–	+	–	–	–	–	+

^a +, interference observed; –, no interference.

human THTR1 (hTHTR1) but not naive MDTF cells. These data indicate that KoRV-J utilizes THTR1 as a receptor.

To investigate the prevalence of KoRV subgroups in koalas reared in Japanese zoos, we collected heparinized blood samples from 40 northern koalas (Queensland, New South Wales, and hybrids of Queensland and New South Wales koalas) and 11 Victorian koalas from 9 zoological parks in Japan and performed differential PCR analysis using subgroup-specific primer sets. The forward primer specific to the *env* of each subgroup and the reverse primer common to all subgroups were used (Fig. 3A; Table 1). The *pol* gene was detected by a *pol*-long primer set (6) as a positive control for KoRV infection, and the β -actin primer set was used for the quality check of the genomic DNAs. These genes were amplified by using Ex *Taq* DNA polymerase (TaKaRa, Ohtsu, Shiga, Japan) under the following cycling conditions: 1 cycle of denaturing (2 min at 94°C), followed by 30 cycles of chain reaction (30 s at 94°C, 30 s at 60°C, and 1 min at 72°C), and an additional extension (4 min at 72°C). KoRV-A was detected in all northern koalas tested and in 4 out of 11 Victorian koalas (Fig. 3B and Table 3), consistent with previous reports that KoRV had become endogenous in koalas in northern Australia (6, 7, 9, 10). In contrast, KoRV-J was detected in 67.5% of northern koalas but not in southern (Victorian) koalas (Fig. 3B and Table 3). These data indicate that the prevalence of KoRV-J is more limited than

KoRV-A, and the invasion of KoRV-J into the koala population may have occurred more recently than KoRV-A.

To determine whether KoRV-J is exogenous or endogenous, we determined the copy numbers of each subgroup in the genomes of different tissues in individual animals. Copy numbers of each subgroup in tissues of Queensland koalas numbers 211 and 133 (KoRV-A positive, KoRV-B positive) were measured by quantitative real-time PCR. Quantitative real-time PCR was performed using the SYBR green detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Aliquots (100 ng) of each genomic DNA sample extracted from lymph node, liver, spleen, and whole blood of the koalas were used as PCR templates with primers specific to KoRV-A, KoRV-J, and the β -actin gene of koalas (Table 1). Amplification and signal detection were carried out using an ABI 7000 real-time PCR system (Applied Biosystems). The standard curves were generated by using serially diluted pFBKoRV-A, pFBKoRV-J, or a plasmid containing a partial sequence of the koala β -actin gene. Copy numbers of each subgroup per cell were normalized to the copy numbers of the β -actin gene. Approximately 3 to 6 copies of KoRV-A per cell were present in the tissues tested (Fig. 4A). In contrast to the relatively constant copy numbers of KoRV-A among tissues, the copy numbers of KoRV-J were less than 1 copy per cell and varied in tissues in both koalas (Fig. 4B). These data suggest that KoRV-J is not an ERV, at least not in these two koalas.

In this study, we identified a new subgroup of KoRV, named KoRV-J, which utilizes THTR1 as a receptor for entry into target cells. KoRV-A has become endogenous in northern koalas but not in most southern koalas in Australia (2, 8, 10). In koalas reared in Japanese zoos, KoRV-J was found only in northern koalas originating from Queensland and New South Wales (Table 1). There-

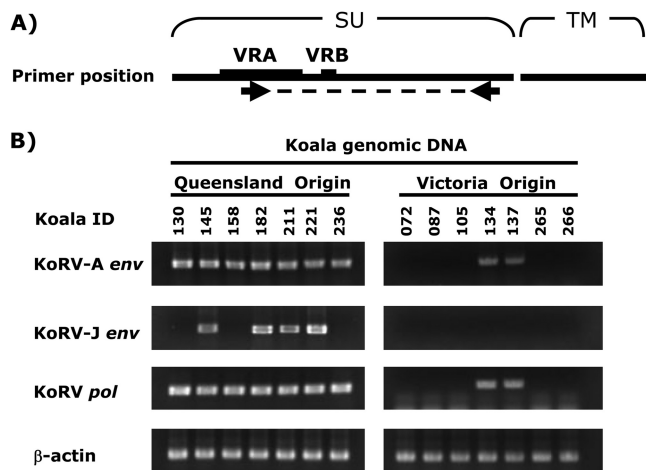


FIG 3 Prevalence of KoRV subgroups in captive koalas in Japanese zoos. (A) Primer positions. Forward and reverse primers were designed in the VRA and the C terminus of surface unit of Env, respectively (see Table 1). Abbreviations: SU, surface unit; TM, transmembrane; VRB, variable region B. (B) Infection status of KoRV subtypes in koalas in Japanese zoos. Subgroup-specific differential PCR was conducted as described in the text. Representative data are shown.

TABLE 3 Prevalence of KoRV subgroups in koalas in Japanese zoos

Source of animals ^a	Infection rate ^b (%)		
	KoRV-pol	KoRV-A	KoRV-J
Northern koalas	40/40 (100)	40/40 (100)	27/40 (67.5)
QLD	31/31 (100)	31/31 (100)	20/31 (64.5)
QLD × NSW hybrid	3/3 (100)	3/3 (100)	2/3 (66.7)
NSW	6/6 (100)	6/6 (100)	5/6 (83.3)
Southern koalas			
VIC	4/11 (36.4)	4/11 (36.4)	0/11 (0)
Total	44/51 (86.3)	44/51 (86.3)	27/51 (52.9)

^a Animals were obtained from northern or southern Australia. QLD, Queensland; NSW, New South Wales; VIC, Victoria.

^b The infection rate is the number of positive animals per the total number tested.

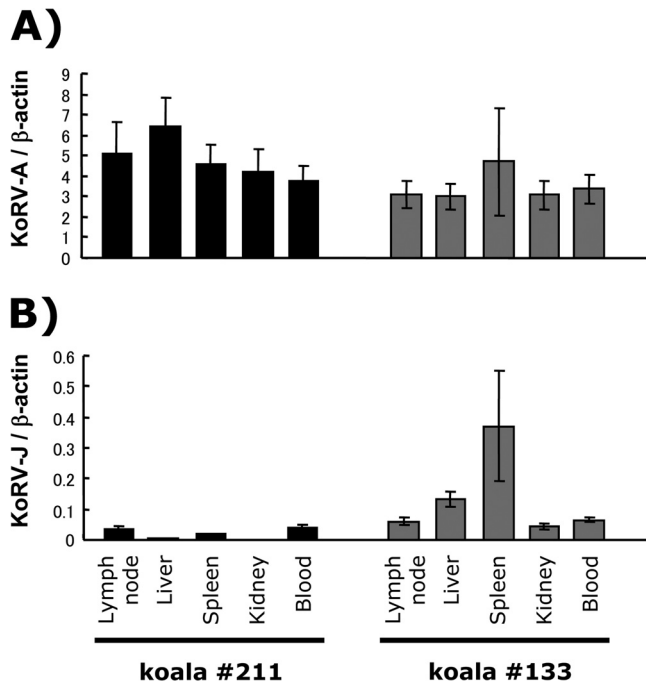


FIG 4 Real-time PCR to detect KoRV-A (A) and KoRV-J (B) *env* genes. Genomic DNA samples from several tissues and blood were isolated from koalas numbers 211 and 133 and subjected to real-time PCR. Data were obtained from three independent experiments, and the values are expressed as the mean \pm 1 standard deviation.

fore, it is plausible that a KoRV-J-infected northern koala(s) was introduced into a Japanese zoo(s) rather than the virus being derived from other animals within the facilities, especially given that koalas are kept separately from other animals except humans.

The origin of KoRV-J is unknown at present. The low amino acid similarity on the surface of Env was not simply caused by nucleotide insertions and/or deletions (data not shown). Furthermore, it is unlikely that KoRV-J was generated from KoRV-A due to an accumulation of nucleotide mutations. KoRV-J could have been prevalent in an unknown host species in Australia that infected a population of northern koalas quite recently. It is also possible that KoRV-J may be the result of a recombination event between KoRV-A and another KoRV-related gammaretrovirus. Thus far, we have been unable to find any KoRV-J VRA-like sequences in the NCBI nr/nt database, indicating that further studies are needed to elucidate the origin of the virus.

Different receptor usage of KoRV subgroups may explain the wide range of diseases seen in koalas. In FeLV, different pathogenicities of FeLV subgroups have been identified (31). The commonly isolated FeLV-A viruses are generally of low to moderate pathogenicity, but FeLV-B viruses are more common in leukemic cats than in FeLV-A-infected healthy cats, and FeLV-B may contribute to the leukemogenic process. It is interesting that megaloblastic anemia has been linked with mutations in THTR1 in humans (32). KoRV-J may disrupt thiamine transport function and cause anemia, which is seen in some captive koalas in Japan. Because the number of koalas is limited in Japan, it is difficult to draw any conclusions regarding differences in the pathogenicities between KoRV-A and -J at this stage. Further long-term surveillance is needed.

Nucleotide sequence accession number. The sequences of the KoRV *env* variants in this study were deposited in GenBank and assigned accession numbers AB822553 (clone 11-4), AB823238 (clone 11-5), AB828004 (clone 11-1), and AB828005 (clone 11-2).

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