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Development of a real-time reverse-transcription-PCR method for detection of RD114 virus in canine vaccines

Rie Narushima [a,*], Tomoaki Shimazaki [b], Toshio Takahashi [c]

[a] National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185-8511, Japan
[b] Animal Health Division, Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan
[c] Department of Veterinary Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan

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ABSTRACT

It is known that certain feline cell lines, such as the Crandell-Rees feline kidney cell, produce an RD-114-like virus. As a feline endogenous retrovirus, RD114 virus, exists in the genome of all cats, it can be assumed that contamination with the virus in feline and canine live vaccines manufactured by culturing cells of feline origin occurs.

To detect an infectious RD114 virus *in vitro*, a LacZ marker rescue assay has recently been established. In feline and canine live vaccines approved in Japan, feline cell lines are widely used to produce vaccines, especially those containing canine parvovirus components. The LacZ marker rescue assay detects infectious viral particles, but the real-time reverse-transcription-PCR detects both infectious and defective viruses. The canine live vaccines manufactured in cells of feline origin showed positive results for the *env* gene by the real-time reverse-transcription-PCR, including all of the 8 vaccines produced in feline cell lines that were negative in the LacZ marker rescue assay. In conclusion, the present investigation suggests that the newly developed method has the advantages of shorter time requirements and can be applied as a valuable screening method to detect RD114 viral RNA in vaccines.

1. Introduction

Endogenous retroviruses (ERVs) are retroviruses that have been integrated into germ line cells [1]. At least two ERVs, endogenous FeLV and RD114 virus, are present in cat genome [2]. The RD114 virus is a recombinant comprising a *gag-pol* gene from a gammaretrovirus and an *env* gene from a betaretrovirus [3]. Infectious RD114 virus and RD114-like virus are known to be produced from several feline cell lines, such as CRFK cells derived from feline kidney cells [4], MCC cells derived from feline large granular lymphoma [5], and FER cells derived from feline fetal fibroblast cells [2]. ERVs are not generally pathogenic in their original hosts, although some ERVs induce diseases for new host [6,7]. To detect infectious RD114 virus, a LacZ marker rescue assay has recently been established [8]. Recently Miyazawa et al. [9] found that certain live attenuated vaccines for cats and dogs contained infectious RD114 viruses by means of this method. Most feline and canine live vaccines approved in Japan are produced in feline cell lines, especially those containing canine parvovirus components.

The LacZ marker rescue assay detects infectious viral particles, but the real-time reverse-transcription-PCR detects both infectious and defective viruses. The real-time reverse-transcription-PCR developed can give rapid, sensitive and specific results for the detection of virus infection [10,11]. To detect RD114 virus, several assays [8,12] have been developed; however there has been no report on real-time reverse-transcription-PCR. In addition, the newly developed technique employing minor groove binder (MGB) probes can enlarge the difference in Tm values between the target template and other non-specific nucleic acid amplification products, to obtain high specificity [13]. The MGB probe can also improve hybridization stability and reduce the fluorescence background to obtain higher sensitivity [13]. Therefore, we attempted to develop a real-time reverse-transcription-PCR method to detect RD114 virus in canine live vaccines.

2. Materials and methods

2.1. RNA extraction from vaccines and reverse-transcription reaction

The principal canine live vaccines approved in Japan were submitted in present investigation prior to their expiration. Viral

* Corresponding author. Tel.: +81 42 321 1841; fax: +81 42 321 1769.
E-mail address: narusima@nval.maff.go.jp (R. Narushima).

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RNA was extracted from each vaccine using spin columns (QIAamp Viral RNA Mini Kit™, Qiagen, CA, USA) as described by the manufacturer. A final volume of 60 μl of viral RNA was extracted from each vaccine. RNA templates (12 μl) were incubated with 4 μl of a mixture consisting of 2 μl gDNA wipeout buffer and 2 μl RNase free water at 42 °C for 2 min to remove contaminating genomic DNA. Following the incubation, cDNA was synthesized using reverse transcriptase (RT) (Quantitect Reverse Transcription™: Qiagen, CA, USA) at 42 °C for 30 min. The reaction mixture (total 6 μl) consisted of 0.5 μl antisense primer, 5′-gtaccggataagacttggac-3′ (10 pmol/μl), 0.5 μl RNase free H_2O, 4 μl 5 × RT buffer and 1 μl RT was added to DNase treated RNA templates. DNase and RT were inactivated at 95 °C for 3min.

2.2. Primers and probe design

Sequence data of the *env* gene of the RD114 virus was obtained from GenBank (accession No; AB559882). The specificity of the primers and probe for the RD114 virus used in this protocol was evaluated by performing a BLAST search, and no significant matches were found to sequences other than that for the RD114 virus. Specific primers and the TaqMan-MGB fluorescent probe were designed with Primer Express Software Version 3.0 (Applied Biosystems, Tokyo, Japan). The probe was labeled with the fluorescent reporter FAM at the 5′ end; with the non-fluorescent quencher at the 3′ end. The sequences of the primers and probe were as follows: sense primer, 5′-ccattcctgccattgatcatta-3′; antisense primer, 5′-ggtgattcccagtccagctagt-3′; probe, 5′-FAM-tacatagacctaaacgagctgt-3′MGB. The amplified fragment was 83 bp in length.

2.3. Real-time PCR

The reaction was conducted in a total volume of 20 μl, consisting of 10 μl master mix (TaqMan Gene Expression Master Mix™, Applied Biosystems, Tokyo, Japan), 1.8 μl forward primer (10 pmol/μl), 1.8 μl reverse primer (10 pmol/μl), 1.0 μl TaqMan probe(5 pmol/μl), 4.4 μl distilled water and 1 μl each template. Real-time PCR was performed in a 96-well format using the StepOnePlus™ system (Applied Biosystems, Tokyo, Japan) and employed the following thermal cycler

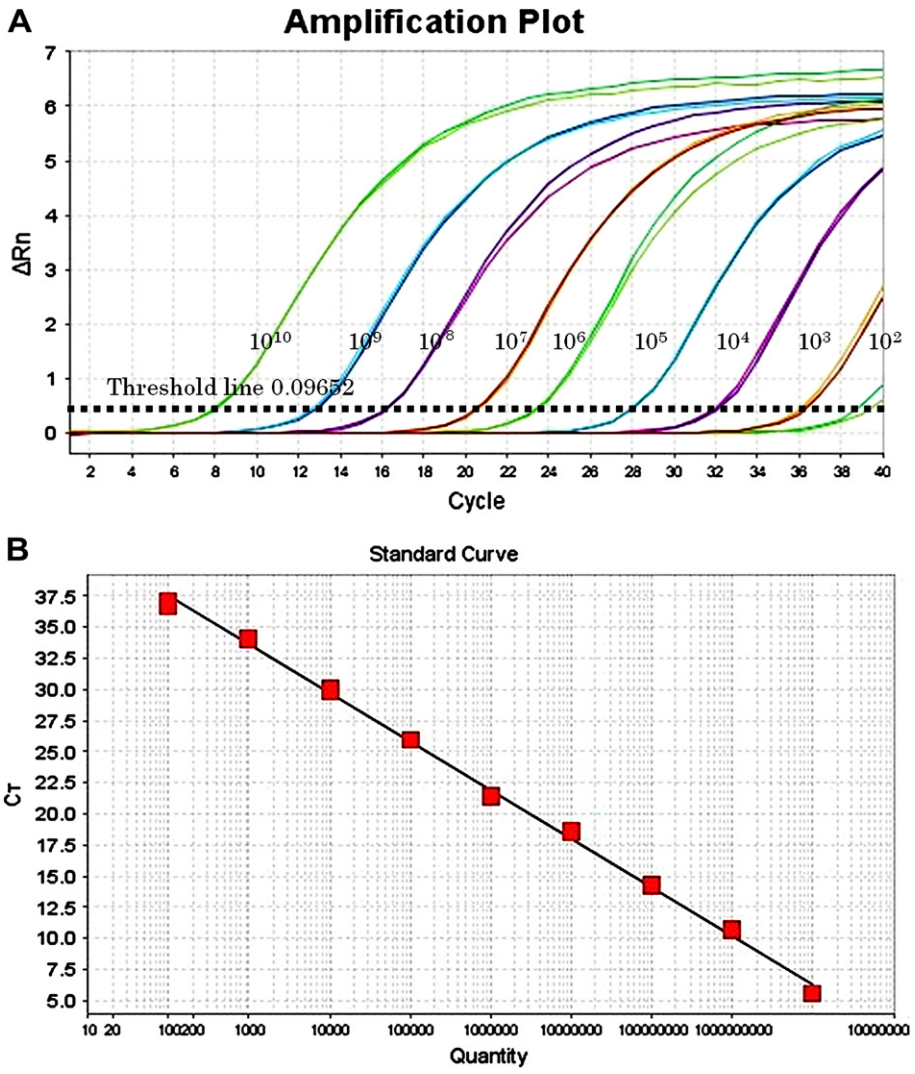

Fig. 1. Quantitative analysis. A – Amplification profile for detection of the *env* gene of the RD114 virus. From left to right, the amplification contained 10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies of cDNA, respectively. The threshold for detection was determined as 10^2 copies with the Ct value under 36.8. B – Standard curve created by analysis of known copies of the target *env* gene. Reactions with copy numbers of target gene from 10^{10} to 10^1 were used to create the standard curve. The standard curve was plotted by the log concentration of copy numbers against Ct values ($R^2 = 0.998$).

settings based on the TaqMan standard mode guidelines: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each assay had at least two negative controls (distilled water) instead of cDNA templates. The fluorescence data was analyzed after completion of the 40 cycle scheduled.

2.4. Quantitative analyses

The real-time PCR assay was run on a standard curve of the Preparative Purification by Gel Electrophoresis (PAGE) refined synthesis cDNA containing the RD114 virus target sequence (93 bp). The 10-fold serial dilutions ranging from 10^1 to 10^{10} RD114 virus copies of cDNA were used to create the standard curve for quantitative analysis. Each sample was run in duplicates or triplicates and threshold cycle number was determined.

2.5. Specificity of TaqMan-MGB real-time reverse-transcription-PCR and RT efficiency

For the RT efficiency test, the RD114 virus inoculum was prepared from the culture supernatant of RD114 virus persistently infected cells (TE671/RD114 cells). RD114 stock virus suspension (titer: approximately 10^5 infectious dose/ml as determined by the LacZ marker rescue assay) was diluted in 10-fold steps ranging from 10^0 to 10^{-10} with medium before RNA isolation to test the specificity and RT efficiency.

2.6. LacZ marker rescue assay

The LacZ marker rescue assay was used to detect infectious RD114 viruses in feline and canine live vaccines [8] by using TE671 cells (human rhabdomyosarcoma) susceptible to canine parainfluenza virus. To prevent infection of cells with parainfluenza virus, the vaccines were neutralized with anti-canine parainfluenza virus antibody. After neutralization with the antibody at 37 °C for 1 h in a 5% CO_2 incubator, samples containing canine parainfluenza virus were inoculated into TE671 cells transduced with LacZ marker gene [8].

3. Results

3.1. Quantitative analysis

The data of 10 standard samples were used for plotting the standard curve of the Ct value and copy number. The threshold for detection was determined as 10^2 copies with the Ct value under 36.8. No amplification was found in negative controls. The amplification results and standard curve are shown in Fig. 1A and B and the copy number in each vaccine in Table 1. The present real-time reverse-transcription-PCR method showed positive results in more vaccines than the LacZ marker rescue assay did (Table 1).

3.2. Specificity of TaqMan-MGB real-time reverse-transcription-PCR and RT efficiency

Equine infectious anemia virus was used for the specificity test, and amplification was not found (data not shown). For the RT efficiency test, standard curve created by the analysis of diluted RD114 stock virus suspension showed a correlation co-efficient of 0.993 on the basis of relationship between virus concentration and Ct values (Fig. 2A and B).

4. Discussion

In the absolute quantification of viral RNA, level of reverse-transcription efficiency is considered to be extremely significant. Present data of Fig. 2 showed more than 0.990 of a correlation co-efficient between Ct value and the initial virus concentration indicating optimum conditions of a newly established real-time reverse-transcription-PCR.

Table 1
Comparison of the LacZ marker rescue assay method and real-time PCR method using each canine live vaccine approved in Japan.

Product (Vaccine)	Composed of viral agents[a]	Production cell for canine parvovirus	LacZ marker rescue assay method	Real-Time PCR (copy number) per one vial
A	D/Ad2/PI/P	Feline, Fibroblast	−	166,567
B	D/Ad2/PI/P/L	Feline, Fibroblast	−	100,138
C	D/P	Feline, Fibroblast	−	79,166
D	D/Ad2/PI/P	Feline, Kidney	−	299,425
E	D/Ad2/PI/P/C/L	Feline, Kidney	−	219,820
F	D/Ad2/PI/P	Canine, Kidney	−	<100
G	D/Ad2/PI/P/C	Canine, Kidney	−	<100
H	D/Ad2/PI/P/C/L	Canine, Kidney	−	<100
I	D/Ad2/PI/P/L	Feline, Kidney	+	56,133,603
J	D/Ad2/PI/P/C/L	Crandell feline kidney	+	115,608,530
K	D/Ad2/PI/P/C/L	Crandell feline kidney	+	158,325,451
L	D/Ad2/PI/P/C	Crandell feline kidney	+	146,739,904
M	D/Ad2/PI/P/L	Mink, Lung	+	1,352,784,971
N	D/Ad2/P	Mink, Lung	+	395,363,668
O	D/Ad2/PI/P/C	Canine, Kidney	−	<100
P	D/Ad2/PI/P/C/L	Canine, Kidney	ND[b]	<100
Q	P	Canine, Kidney	ND[b]	<100
R	D/Ad2/PI/P	Crandell feline kidney	−	17,521,335
S	D/Ad2/PI/P/L	Crandell feline kidney	−	7,526,241
T	P	Crandell feline kidney	−	1,880,984

D; canine distemper virus.
Ad2; canine adenovirus (CAdV-2).
PI; canine parainfluenza virus.
P; canine parvovirus.
C; canine coronavirus.
L; leptospira.
[a] Code.
[b] Not done because of cytotoxicity.

The real-time reverse-transcription-PCR and the LacZ marker rescue assay detect viral RNA and infectious retroviruses, respectively. In other words, the former detects both defective and infectious viral particles, but the latter detects only infectious virus particles. In any case, only the LacZ marker rescue assay method has been established for detection of infectious RD114 virus in vaccines until now. But this method is time consuming, requiring more than 12 days after samples are inoculated into the TE671 cells transduced with the LacZ marker gene. The real-time reverse-transcription-PCR method has advantages over the LacZ marker rescue assay in several ways; ease of use, speed, applicability to general laboratories and particularly the multi-specimen material processing performance. PCR methods also generally detect defective viral particles, thereby providing a very useful aid for a screening test. Furthermore, a threshold of only 100 copies could be detected. The canine live vaccines manufactured in cells of feline origin showed positive results for the *env* gene by the real-time reverse-transcription-PCR, including all of the 8 vaccines produced in feline cell lines that were negative in the LacZ marker rescue assay.

Canine live vaccines manufactured by culturing cells of mink origin were positive in both assays. The results show that mink cells or canine parvovirus seed stocks may be contaminated with the RD114 virus. Since Mv1Lu (mink lung) cells are susceptible to a variety of retroviruses, they have been extensively used for isolation and propagation of endogenous primate retroviruses [14]. This might cause an enormous increase in RD114 virus contamination of the vaccine. The other canine live vaccines manufactured by culturing cells were negative by the real-time reverse-transcription-PCR method, except for those of cat origin. However, it is possible that contamination with low copy numbers of the RD114 virus was not detected. The results of the real-time reverse-transcription-PCR demonstrate that contamination with RD114 virus in

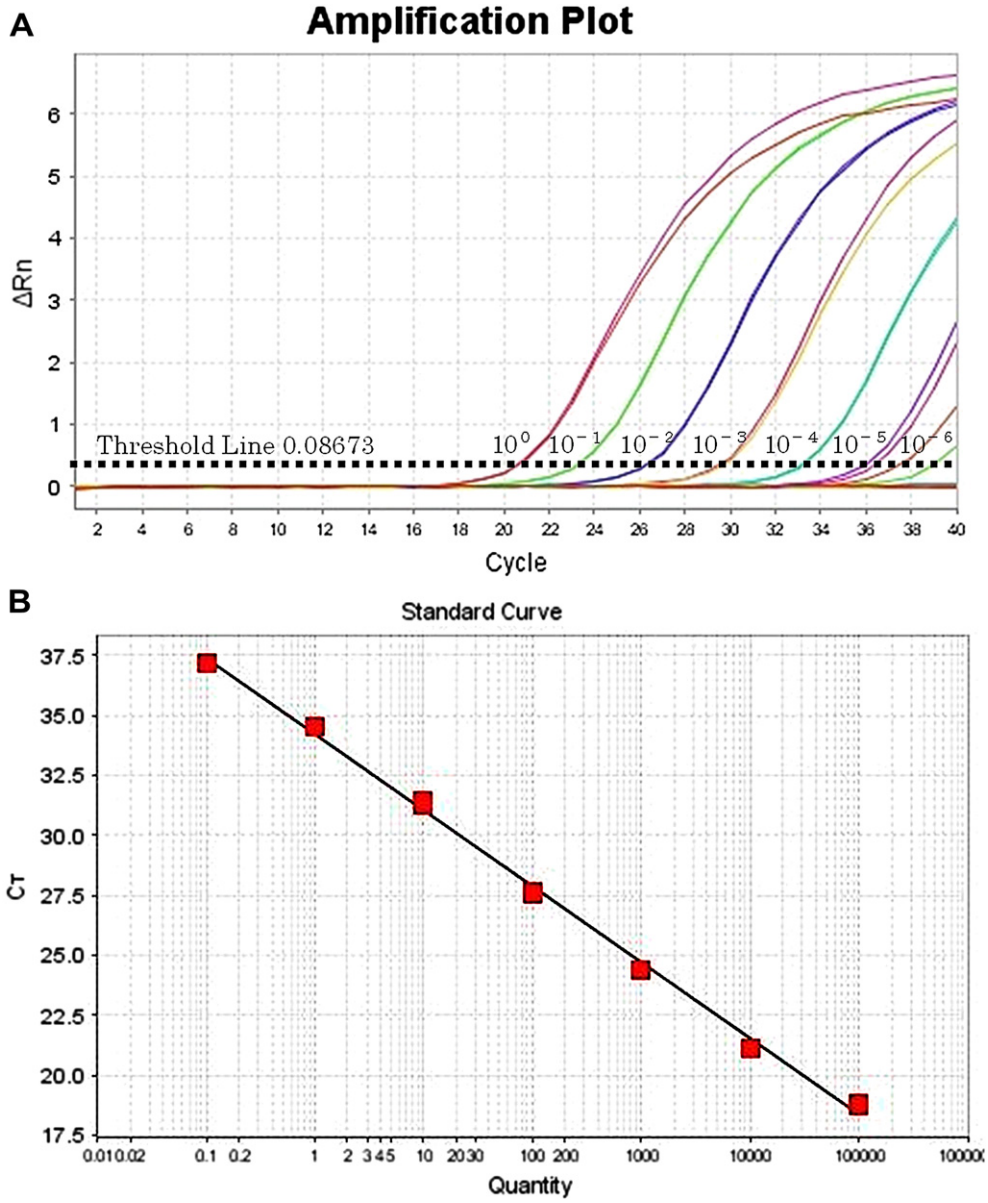

Fig. 2. Specificity and RT efficiency. In LacZ marker rescue assay method, virus concentration ranging from 10^0 to 10^{-5} could be detected. A – RD114 stock virus liquid was diluted in a 10-fold serial dilution ranging from 10^0 to 10^{-10} with medium. B – Standard curve created by the analysis of diluted RD114 stock virus liquid. Ct values obtained from virus concentration 10^0 to 10^{-6} ($R^2 = 0.993$).

canine live vaccines predominantly originates in vaccines manufactured in cells of feline origin. This is the first report demonstrating the presence of RD114 virus in canine live vaccines using a real-time reverse-transcription-PCR method with TaqMan MGB probe.[1] RD114 virus productively infects cells from cats and dogs, and may be transmitted to non-feline species *in vivo* because of the xenotropic features of the virus [2]. As we demonstrated in Table 1, all feline cell lines produce viral particles which contain RD114 viral RNA. Presumably, feline cell lines used to produce vaccines produce RD114 virus irrespective of the infectivity. At present, a complete risk assessment would be impossible because the minimum infectious dose and pathogenicity, to our knowledge, is still unclear.

In conclusion, the present investigation suggests that the newly developed method has the advantages of shorter time requirements and can be used as a screening method to detect RD114 viral RNA in vaccines; however, the assays to detect infectious viral particles are still needed. Although the risks caused by contamination of RD114 virus are unknown at present, we suppose that infectious RD114 viruses should be eliminated from live vaccines at least. As an appropriate regulatory tool, the present real-time reverse-transcription-PCR method will greatly improve the quality control of the canine live vaccine for minimizing the occurrence of unexpected risk such as adverse events in vaccinated dogs.

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References

[1] Gifford R, Tristem M. The evolution, distribution and diversity of endogenous retroviruses. Virus Genes 2003;26:291–315.
[2] Miyazawa T. Endogenous retrovirus as potential hazards for vaccines. Biologicals 2010;38:371–6.
[3] Van der Kuyl AC, Dekker JT, Goudsmit J. Discovery of a new endogenous type C retrovirus (FcEV) in cat: evidence for RD-114 being an $FcEV^{Gag\text{-}pol}$/baboon endogenous virus $BaEV^{Env}$ recombinant. J Virol 1999;73:7994–8002.
[4] Baumann JG, Gunzburg WH, Salmons B. CrFK feline kidney cells produce an RD114-like endogenous virus that can package murine leukemia virus-based vectors. J Virol 1998;72:7685–7.
[5] Cheney CM, Rojko JL, Kociba GJ, Wellman ML, Di Bartola SP, Rezanka LJ, et al. A feline large granular lymphoma and its derived cell line. Vitro Cell Dev Biol 1990;26:455–63.
[6] Nowinski RC, Hays EF. Oncogenicity of AKR endogenous leukemia viruses. J Virol 1978;27:13–8.
[7] Tarlinton R, Meers J, Young P. Biology and evolution of the endogenous koala retrovirus. Cell Mol Life Sci 2008;65:3413–21.
[8] Sakaguchi S, Okada M, Shojima T, Baba K, Miyazawa T. Establishment of a LacZ marker rescue assay to detect infectious RD114 virus. J Vet Med Sci 2008;70:785–90.
[9] Miyazawa T, Yoshikawa R, Golder M, Stewart H, Palmarini M. Isolation of an infectious endogenous retrovirus in a proportion of live attenuated vaccines for pets. J Virol 2010;84:3690–4.
[10] Van Elden LJ, Nijhuis M, Schipper P, Schuurman R, Van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 2001;39:196–200.
[11] Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, et al. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg Infect Dis 2004;10:311–6.
[12] Sakaguchi S, Baba K, Ishikawa M, Yoshikawa R, Shojima T, Miyazawa T. Focus assay on RD114 virus in QN10S cells. J Vet Med Sci 2008;70:1383–6.
[13] Lu YY, Yan JY, Feng Y, Xu CP, Shi W, Mao HY. Rapid detection of H5 avian influenza virus by TaqMan-MGB real-time RT-PCR. Lett Appl Microbiol 2008;46:20–5.
[14] Ahmed M, Yeh J, Stephens R, Holden HE, Schlom J, Drohan W, et al. Characterization of a retrovirus isolated from normal mink cells co-cultivated with a dog mammary tumour. J Gen Virol 1979;42:179–84.

[1] Recently, this journal published a paper regarding detection of RD114 viral RNA by real-time reverse transcription (RT)- PCR (Yoshikawa et al., Contamination of infectious RD-114 virus in vaccines produced using non-feline cell lines).