

1 **Isolation of an Infectious Endogenous Retrovirus in a Proportion of Live**
2 **Attenuated Vaccines for Pets**

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Abstract

The genomes of all animal species are colonized by endogenous retroviruses (ERVs). Although most ERVs have accumulated defects that render them incapable of replication, fully infectious ERVs have been identified in various mammals. In this study, we isolated a feline infectious ERV (RD-114) in a proportion of live attenuated vaccines for pets. Isolation of RD-114 was made in two independent laboratories using different detection strategies and using vaccines for both cats and dogs commercially available in Japan or the United Kingdom. This study shows that the methods currently employed to screen veterinary vaccines for retroviruses are inadequate and should be re-evaluated.

1 **Main Text**

2 During evolution, the genomes of all animal species have been colonized by
 3 endogenous retroviruses (ERVs). ERVs are derived from the integration of the
 4 retrovirus genome (“provirus”) into the host germline and are transmitted vertically
 5 between generations like any other Mendelian gene (3). Most ERVs have accumulated
 6 mutations and/or deletions that render them unable to complete their replication cycle.
 7 However, there are several examples of ERVs that have been shown to be fully
 8 infectious. These replication competent ERVs, especially those that have co-evolved
 9 with their host for long evolutionary periods, can be considered in many ways to be in
 10 equilibrium with their host species which have adopted a variety of strategies to
 11 suppress and control viral expression and/or replication.

12 Regardless of their replication potential, ERVs in general can be considered non-
 13 pathogenic for their host, otherwise they would have been counter-selected during
 14 evolution (1, 11). However, transmission of infectious ERVs to an animal species
 15 different from the one in which they originally integrated (“cross-species
 16 transmission”) could have unpredictable outcomes. For this reason, the potential
 17 transmission of pig ERVs to humans is one of the blocks hampering
 18 xenotransplantation (26).

19 Here, we wanted to evaluate the possibility that live attenuated vaccines could contain
 20 replication-competent ERVs and act as a potential source of retroviral cross-species
 21 transmission. We investigated commercially available vaccine preparations for cats
 22 and dogs. The cat genome contains an infectious ERV known as RD-114, a member of
 23 the *gammaretrovirus* genus, to which other mammalian oncogenic viruses such as
 24 feline leukemia virus and murine leukemia virus belong (6, 14, 17, 23). Some feline
 25 cell lines such as CRFK (Crandell-Rees feline kidney) commonly used to grow feline

1 and canine viruses express variable amounts of RD-114 (2). We attempted to isolate
2 RD-114 from vaccines commercially available in different continents. These vaccines
3 are routinely used to prevent common infections in cats and dogs caused by viruses
4 such as feline herpesvirus, feline calicivirus, feline panleukopenia, canine adenovirus,
5 canine distemper virus, canine parvovirus, canine coronavirus and canine
6 parainfluenza virus (15). For this study, each vaccine sampled was assigned an
7 anonymized code (e.g. J-Aa1, UK-Aa4 etc.). The first letter before the dash indicates
8 the country where the vaccine was acquired (i.e. J- for Japan and UK- for the United
9 Kingdom). The capital letter after the dash indicates the manufacturer. The letter in
10 lower case indicates the specific type of vaccine while numbers are used to
11 differentiate between different batch numbers. Thus, vaccine J-Aa1 and UK-Aa4 are
12 two different batches of the same vaccine acquired from either Japan (J-Aa1) or the
13 UK (UK-Aa4). All the data obtained in this study is summarized in Table 1.

14 Initially, 15 samples of 11 vaccines acquired from Japan (from 7 different
15 manufacturers) were tested for the presence of RD-114 using a LacZ marker rescue
16 assay as previously described (19) (Fig. 1A-F). Briefly, vaccines (one vial per each
17 vaccine) were passaged for 4 weeks (dog vaccines) or 2 weeks (cat vaccines) into
18 TE671(LacZ) cells which are derived from the human rhabdomyosarcoma cell line
19 TE671 transduced with a gammaretroviral vector expressing the *lacZ* gene.
20 Supernatants from the vaccine-inoculated TE671(LacZ) cells were collected and used
21 to infect naïve TE671 cells. The LacZ marker rescue assay showed the presence of a
22 replication competent gammaretrovirus in 6 of the 15 samples tested (Fig. 1A, C). On
23 the other hand, the same vaccine preparations were not able to infect TE671-RD, a
24 cell line chronically infected with RD-114 (Fig. 1B, D). TE671-RD cells were
25 infectable by a retroviral vector pseudotyped with the feline leukemia virus type B

1 (FeLV-B) Env, demonstrating that these cells can be infected by a retrovirus that uses
 2 a different receptor from RD-114 (Fig. 1E-F). We estimated the infectious titers of
 3 RD-114 in some of the vaccines tested above by an endpoint dilution assay. Briefly,
 4 TE671(LacZ) cells were exposed with serial dilutions of vaccines J-Aa2, J-Cd1, J-
 5 Co2 and J-De1. Viral titers were calculated using the formula of Reed and Munch
 6 (16). Titers were as low as 1.8 TCID₅₀/ vial in vaccines J-Aa2 and J-De1. Higher titers
 7 were reached in vaccines J-Co2 (1000 TCID₅₀) and J-Cd1 (1800 TCID₅₀). Note that
 8 the titers indicated above are an estimate that may contain a certain degree of
 9 variability because most of the dog vaccines tested contains viruses that are cytopathic
 10 in TE671 cells. Thus, fresh TE671(LacZ) cells were continuously added to the
 11 cultures exposed to the pet vaccines in order to allow sufficient time for RD-114
 12 replication.

13 A single step PCR assay employing RD-114 specific primers was also employed on
 14 genomic DNA (200 ng/reaction) extracted from TE671(LacX) infected with the
 15 vaccines described above (Fig. 1G). Primers employed were designed to amplify the
 16 RD-114 *env* region using the following oligonucleotide primers: 5'-
 17 ccctcgatactaagagagt-3' and 5'-acttcagctaacgagtctac-3'. We were able to amplify RD-
 18 114 *env* sequences in all samples that tested positive in the LacZ marker rescue assay,
 19 confirming the presence of RD-114. On the other hand, vaccines, which tested
 20 negative in the LacZ marker rescue assay, were also found to be PCR negative for
 21 RD-114. Electron microscopy on human TE671(LacZ) cells infected with vaccine J-
 22 Aa1 and passaged for four weeks showed the presence of viral particles with retroviral
 23 morphology (Fig. 1H).

24 We then isolated RD-114 in another laboratory, using pet vaccines commercially
 25 available in the United Kingdom and using a different strategy. Eight different

1 samples (two vials of each vaccine in each experiment), representing 6 vaccines from
2 6 different manufacturers were passaged for 3 to 6 weeks on TE671 cells and culture
3 fluids were tested for presence of RD-114. Two of the 6 vaccines tested were found to
4 contain RD-114 as assessed by western blotting employing an antiserum raised
5 against the RD-114 major capsid protein (CA) and by a reverse transcriptase (RT)-
6 assay (Fig. 2 and Table 1). In general, cells exposed to vaccines containing RD-114
7 had to be passaged for 3 weeks before RT-activity could be detectable (Fig. 2C).
8 Interestingly, the same vaccine brands were found to contain RD-114 in the UK (UK-
9 Aa and UK-De) and in Japan (JAa and J-De). Note that in some of the vaccine brands
10 we were not able to detect consistently RD-114 in all the batches tested (J-Aa; UK-
11 Aa) or in all the vials from the “contaminated” vaccine batches (i.e. J-Aa1; UK-De3;
12 UK-Aa4) (Table 1). As mentioned above, this is likely due to the small amount of RD-
13 114 present in the vaccine seeds and/or to inherent variability of hte tests due to the
14 presence of viruses in the vaccines formulations that are cytopathic to TE671 cells. A
15 small number of experiments were performed also on human 293T cells. We were
16 able to isolate RD-114 in 293T from vaccine UK-De3. However, these cells were
17 more susceptible than TE671 cells to the cytopathic effect induced by viruses
18 contained in the vaccine preparations and therefore we used the latter for the complete
19 experimental set relative to the UK samples. Overall, it is possible that our data under-
20 represent the number of vaccines where RD-114 can be isolated from.

21 Collectively, our data show unequivocally that RD-114 is present in live attenuated
22 vaccines commonly used in dogs and cats from different continents and produced by
23 three different manufacturers. Future studies will be necessary to determine whether
24 RD-114 has any negative impact in cats or dogs. The risks posed by a low level
25 exposure to RD-114 for pets are likely extremely small. ERVs are in general non-

1 pathogenic for their host species and RD-114 replication has been found to be
2 restricted in some cat cell lines (4, 5, 12). However, cat cell lines fully susceptible to
3 RD-114 replication have also been described (7). Thus, one would expect that
4 exposure to RD-114 will be of little consequence to cats, given that RD-114 is an ERV
5 of this species. However, it is impossible to rule out any consequences at all in the pet
6 population as a whole. Infectious ERVs have the same biological properties and
7 pathogenic potential of exogenous horizontally transmitted retroviruses, once the co-
8 evolutionary mechanisms that have shaped the interaction with their natural hosts
9 cease to exist. In this regard, the large scale exposure to RD-114, particularly of the
10 dog population, may have effects that are impossible to predict even if successful RD-
11 114 transmission was an extremely rare event. Millions of puppies are vaccinated
12 annually worldwide and they may be more susceptible to RD-114 infection than cats
13 as the dog genome does not harbor RD-114. Also wild cats do not harbor RD-114 and
14 they are regularly vaccinated in zoos with the same vaccines used for pets. These
15 “contaminated” vaccines have been used extensively for many years without major
16 acute effects on vaccinated animals but retroviruses rarely induce acute diseases.
17 Therefore, it is impossible to rule out chronic effects, especially as we were able to
18 grow RD-114 very efficiently in dog cell lines (data not shown), confirming older
19 published studies (18).

20 To our knowledge this is the first identification of an infectious ERV in commercially
21 available vaccines. In the 1960s, it was shown that yellow fever live attenuated
22 vaccines prepared in chicken embryo fibroblasts were contaminated with avian
23 leukemia virus (ALV). However, no increased risk for cancer was shown in vaccinated
24 individuals and this was expected considering that ALV does not grow efficiently in
25 mammalian cells (8, 24, 25). Avian endogenous retroviruses genomes and RT activity

1 have been detected more recently in yellow fever, measles and mumps vaccines by RT
2 and RT-PCR assays (9, 21) but no evidence of infectious viruses was presented in
3 these studies. As expected, avian endogenous retroviruses were not detected in the
4 vaccine recipients.

5 A recently identified novel human retrovirus (xenotropic murine leukemia virus-
6 related retrovirus, XMRV) has been found in some forms of prostate cancers and
7 chronic fatigue syndrome in man (13, 20, 22) although causal association has not been
8 proven yet. XMRV is almost undistinguishable from an ERV present in mice and it
9 will be important to investigate how this virus passed into the human population,
10 regardless of its pathogenic potential. Interestingly, the current methods used for
11 screening human vaccines for retroviral contaminants include extremely sensitive
12 PCR-based RT assays (not required for veterinary vaccines) that are much more
13 sensitive than conventional RT assays. Thus, contamination of human vaccines with
14 XMRV, would not pass undetected with the currently available technology although
15 this may not be necessarily true for vaccines produced in previous decades.

16 Finally, although the risks posed by RD-114 are seemingly small, it would be
17 appropriate to produce live attenuated vaccines in cells that do not express this
18 endogenous retrovirus. To this end, cells of dog origin may be better suited to produce
19 pet vaccines than cat cell lines, although not all cat cell lines express RD-114 (2, 10).
20 Contamination of sub-unit or inactivated vaccines by infectious agents in general
21 (including ERVs) is obviously less of a concern.

22 In conclusion, our study suggests that the presence of infectious endogenous
23 retroviruses should be taken into consideration when assessing the purity of live
24 attenuated veterinary vaccines. However, the methods currently employed to screen
25 veterinary vaccines for retroviruses are inadequate and need to be reassessed.

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References

1. **Arnaud, F., M. Caporale, M. Varela, R. Biek, B. Chessa, A. Alberti, M. Golder, M. Mura, Y.-p. Zhang, L. Yu, F. Pereira, J. C. DeMartini, K. Leymaster, T. E. Spencer, and M. Palmarini.** 2007. A paradigm for virus-host coevolution: sequential counter-adaptations between endogenous and exogenous retroviruses. *PLoS Pathogens* **3**:e170.
2. **Baumann, J. G., W. H. Gunzburg, and B. Salmons.** 1998. CrFK feline kidney cells produce an RD114-like endogenous virus that can package murine leukemia virus-based vectors. *J Virol* **72**:7685-7687.
3. **Boeke, J. D., and J. P. Stoye.** 1997. Retrotransposons , endogenous retroviruses and the evolution of retroelements, p. 343-436. *In* J. M. Coffin, S. H. Hughes, and H. E. Varmus (ed.), *Retroviruses*. Cold Spring Harbor Laboratory Press, Plainview, NY.

- 1 4. **Dunn, K. J., C. C. Yuan, and D. G. Blair.** 1993. A phenotypic host range
2 alteration determines RD114 virus restriction in feline embryonic cells. *J Virol*
3 **67**:4704-4711.
- 4 5. **Fischinger, P. J., P. T. Peebles, S. Nomura, and D. K. Haapala.** 1973.
5 Isolation of RD-114-like oncornavirus from a cat cell line. *J Virol* **11**:978-985.
- 6 6. **Gardner, M. B., S. Rasheed, R. W. Rongey, H. P. Charman, B. Alena, R. V.**
7 **Gilden, and R. J. Huebner.** 1974. Natural expression of feline type-C virus
8 genomes, prevalence of detectable felv and RD-114 GS antigen, type-C
9 particles and infectious virus in postnatal and fetal cats. *Int J Cancer* **14**:97-
10 105.
- 11 7. **Haapala, D. K., W. G. Robey, S. D. Oroszlan, and W. P. Tsai.** 1985.
12 Isolation from cats of an endogenous type C virus with a novel envelope
13 glycoprotein. *J Virol* **53**:827-833.
- 14 8. **Harris, R. J., R. M. Dougherty, P. M. Biggs, L. N. Payne, A. P. Goffe, A. E.**
15 **Churchill, and R. Mortimer.** 1966. Contaminant viruses in two live virus
16 vaccines produced in chick cells. *J Hyg (Lond)* **64**:1-7.
- 17 9. **Hussain, A. I., J. A. Johnson, M. Da Silva Freire, and W. Heneine.** 2003.
18 Identification and characterization of avian retroviruses in chicken embryo-
19 derived yellow fever vaccines: investigation of transmission to vaccine
20 recipients. *J Virol* **77**:1105-1111.
- 21 10. **Jarrett, O., and J. P. Ganiere.** 1996. Comparative studies of the efficacy of a
22 recombinant feline leukaemia virus vaccine. *Vet Rec* **138**:7-11.
- 23 11. **Jern, P., and J. M. Coffin.** 2008. Effects of retroviruses on host genome
24 function. *Annu Rev Genet* **42**:709-732.
- 25 12. **Livingston, D. M., and G. J. Todaro.** 1973. Endogenous type C virus from a

- 1 cat cell clone with properties distinct from previously described feline type C
- 2 virus. *Virology* **53**:142-151.
- 3 13. **Lombardi, V. C., F. W. Ruscetti, J. Das Gupta, M. A. Pfost, K. S. Hagen,**
- 4 **D. L. Peterson, S. K. Ruscetti, R. K. Bagni, C. Petrow-Sadowski, B. Gold,**
- 5 **M. Dean, R. H. Silverman, and J. A. Mikovits.** 2009. Detection of an
- 6 infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue
- 7 syndrome. *Science* **326**:585-589.
- 8 14. **McAllister, R. M., M. B. Gardner, M. O. Nicolson, R. V. Gilden, and N.**
- 9 **Davidson.** 1978. RD-114 virus: characterization and identification. *Prog Exp*
- 10 *Tumor Res* **21**:196-215.
- 11 15. **Murphy, F. A., E. P. J. Gibbs, M. C. Horzineck, and M. J. Studderts.** 1999.
- 12 *Veterinary Virology*, 3rd ed. Academic Press, San Diego.
- 13 16. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty
- 14 percent endpoints. *Am J Hyg* **27**:493-497.
- 15 17. **Reeves, R. H., and S. J. O'Brien.** 1984. Molecular genetic characterization of
- 16 the RD-114 gene family of endogenous feline retroviral sequences. *J Virol*
- 17 **52**:164-171.
- 18 18. **Roth, M. G., R. V. Srinivas, and R. W. Compans.** 1983. Basolateral
- 19 maturation of retroviruses in polarized epithelial cells. *J Virol* **45**:1065-1073.
- 20 19. **Sakaguchi, S., M. Okada, T. Shojima, K. Baba, and T. Miyazawa.** 2008.
- 21 Establishment of a LacZ marker rescue assay to detect infectious RD114 virus.
- 22 *J Vet Med Sci* **70**:785-790.
- 23 20. **Schlaberg, R., D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh.**
- 24 2009. XMRV is present in malignant prostatic epithelium and is associated
- 25 with prostate cancer, especially high-grade tumors. *Proc Natl Acad Sci U S A*

- 1 **106:16351-16356.**
- 2 21. **Tsang, S. X., W. M. Switzer, V. Shanmugam, J. A. Johnson, C. Goldsmith,**
3 **A. Wright, A. Fadly, D. Thea, H. Jaffe, T. M. Folks, and W. Heneine.** 1999.
4 Evidence of avian leukosis virus subgroup E and endogenous avian virus in
5 measles and mumps vaccines derived from chicken cells: investigation of
6 transmission to vaccine recipients. *J Virol* **73:5843-5851.**
- 7 22. **Urisman, A., R. J. Molinaro, N. Fischer, S. J. Plummer, G. Casey, E. A.**
8 **Klein, K. Malathi, C. Magi-Galluzzi, R. R. Tubbs, D. Ganem, R. H.**
9 **Silverman, and J. L. Derisi.** 2006. Identification of a novel gammaretrovirus
10 in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS*
11 *Pathog* **2:e25.**
- 12 23. **van der Kuyl, A. C., J. T. Dekker, and J. Goudsmit.** 1999. Discovery of a
13 new endogenous type C retrovirus (FcEV) in cats: evidence for RD-114 being
14 an FcEV(Gag-Pol)/baboon endogenous virus BaEV(Env) recombinant. *J Virol*
15 **73:7994-8002.**
- 16 24. **Waters, T. D., P. S. Anderson, Jr., G. W. Beebe, and R. W. Miller.** 1972.
17 Yellow fever vaccination, avian leukosis virus, and cancer risk in man. *Science*
18 **177:76-77.**
- 19 25. **Weiss, R. A.** 2001. Adventitious viral genomes in vaccines but not in
20 vaccinees. *Emerg Infect Dis* **7:153-154.**
- 21 26. **Wilson, C. A.** 2008. Porcine endogenous retroviruses and xenotransplantation.
22 *Cell Mol Life Sci* **65:3399-3412.**
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Table 1. Detection of RD-114 in commercially available vaccines for dogs and cats: summary of the results obtained in this manuscript.

Vaccine Code	Target Species [†]	LacZ [#]	PCR [‡]
J-Aa1	Cats	3/6 [§]	2/2
J-Aa2	Cats	1/1	1/1
J-Aa3	Cats	0/2	0/2
J-Bc1	Cats	0/3	0/3
J-Bc2	Cats	0/3	0/3
J-Cd1	Dogs	2/2	2/2
J-Co1	Dogs	1/1	1/1
J-Co2	Dogs	1/1	1/1
J-De1	Dogs	1/1	1/1
J-Ef1	Dogs	0/1	0/1
J-Gk1	Dogs	0/1	0/1
J-Ab1	Dogs	0/1	0/1
J-Gl1	Dogs	0/1	0/1
J-Em1	Cats	0/1	0/1
J-Hn1	Cats	0/3	0/1
		Western blotting^{††}	RT[‡]
UK-Aa4	Cats	3/4	2/3
UK-Aa5	Cats	0/2	0/2
UK-Bi1	Dogs	0/2	0/2
UK-De2	Dogs	2/2	2/2
UK-De3	Dogs	1/4	1/3
UK-Eg1	Dogs	0/1	nt
UK-Gk2	Dogs	0/2	0/2
UK-Eh1	Dogs	0/2	0/2
TE671	-	0/4	0/2
RD114 (+)	-	4/4	2/2

*Codes used to anonymize the vaccines used. The first letter before the dash indicates the country where the vaccine was acquired (J- for Japan or UK- for the United Kingdom). The capital letter after the dash indicates the manufacturer. The letter in lower case indicates the specific type of vaccine while numbers are used to differentiate between different batches. Grey shadows for vaccines J-Aa, J-De, UK-De and UK-Aa highlight the same vaccine brands (but different batches) purchased either in Japan (J- series) or the UK (UK- series). RD-114(+) represents the positive control obtained from supernatants of feline FER cells (2). TE671 are the mock-

- 1 infected cells used as negative control.
- 2 [†]Animal species for which each specific vaccine is targeted to.
- 3 [#]Data relative to the LacZ rescue assay using one vial of the various vaccines in each
- 4 assay.
- 5 [§]Values in the whole table refer, for each vaccine, to the number of samples that tested
- 6 positive for RD-114 compared to the total number of vials tested.
- 7 [¶]Results of RD-114 *env* PCR on TE671 cells exposed to the indicated vaccines.
- 8 ^{††}Data relative to the western blotting analysis of supernatants of TE671 cells exposed
- 9 to the indicated vaccines (two vials for each assay).
- 10 [‡]Vaccines were tested for the presence of reverse transcriptase activity using C-type
- 11 RT activity kit (Cavidi) as described by the manufacturer.
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1 **Legends**

2

3 **Fig. 1. Detection of RD-114 in pet vaccines.** In total 14 vaccine brands acquired

4 from 7 different vaccine manufacturers were tested (see also Table 1 for a complete

5 summary of the data obtained). Six of the 14 vaccines tested both in Japan and the UK

6 showed evidence of RD-114 in at least one of their batches. Two of the vaccines (J-Cd

7 and J-Co) from where RD-114 was isolated were commercially available only in

8 Japan while the remaining four were present in both markets (J-Aa, J-De and UK-Aa,

9 UK-De). Consequently, the presence of RD-114 was revealed in two different

10 laboratories in the same vaccines (J-Aa/ UK-Aa and J-De/ UK-De), using independent

11 samples, reagents and detection strategies. **A-E.** LacZ marker rescue assays were

12 performed as already described (19). Panels **A** and **C** show representative examples of

13 TE671 cells exposed to a vial of vaccines J-De1 and J-Co1 respectively. The same

14 vaccine preparations were not able to infect TE671-RD, a cell line chronically

15 infected with RD-114, as shown in panels **B** and **D**. As a control both TE671 and

16 TE671-RD were infected by a MLV-based vector pseudotyped with the FeLV B Env

17 (Panels E and F). **G.** RD-114 provirus was amplified from genomic DNA (200ng/

18 reaction) extracted from TE671 cells infected with supernatants of TE671(LacZ) cells

19 exposed to the indicated vaccines. Reactions were carried out using the primers

20 indicated in the text in a 25µl standard PCR reaction. PCR cycles employed were

21 94°C for 10min, and 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 90 s, with a

22 final extension of 72 °C for 10 min. Specific PCR products were obtained only from

23 those samples that resulted positive in the LacZ assay as indicated schematically in

24 the figure. PCR products were sequenced and confirmed to represent RD-114 *env*. **H.**

25 Visualization of retroviral particles by electron microscopy. TE671(LacZ) infected

26 with vaccine J-Aa1 were passaged for 4 weeks. Cells were then fixed, dehydrated,

1 embedded, and sectioned for electron microscopy by using standard methods.
2 Microphotograph shows mature viral particles with a retroviral morphology in the
3 vicinity of the cell membrane (bar = 100µm).

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5 **Fig. 2. Detection of RD-114 by western blotting and reverse transcriptase (RT)**

6 **assay. A.** Vaccines obtained from the UK were passaged in TE671 cells for 3-6 weeks.
7 Samples represented in the figure were taken at the end of the experiment at 6 weeks
8 after the initial exposure of TE671 cells to the indicated vaccines. TE671 cells were
9 exposed to two vials of vaccines in each experiment. Approximately 10ml of culture
10 supernatants were collected, filtered through a 0.45 µm filter and ultracentrifuged at
11 100,000g for 1h. Virus pellets were resuspended in 50 µl TE buffer. 12µl were then
12 analyzed by western blotting using a goat antiserum against the RD-114 major capsid
13 protein (serum # 72-S-781 from the US National Cancer Institute). Note the presence
14 of RD-114 CA in supernatants of cells infected with vaccines UK-De2 and UK-Aa4.
15 As positive control (lanes labelled “RD-114”) we utilized supernatants obtained from
16 the feline cell line FER that expresses RD-114 (2). Specificity of the antibody used
17 was also tested by transfecting 293T cells transfected with an infectious molecular
18 clone of RD-114 as previously described (13).

19 **B-C.** Reverse-transcriptase (RT) assays. RT-assays were performed using the C-type
20 RT activity kit (Cavidi) as recommended by the manufacturer. Panel B show
21 representative data from some of the vaccines tested. Values are normalized against a
22 a Moloney murine leukemia virus (MuLV) recombinant RT standard and expressed in
23 milliunits (mU) per ml. Note that culture supernatants were taken either undiluted
24 (“neat”) or diluted as indicated in the graph. All samples that contained detectable
25 RD-114 by western blotting had also detectable RT activity. Panel C shows RT-assays

1 of supernatants of TE671 cells infected with vaccine UK-Aa4 and collected at
 2 different time post-infection. Note that RT-activity is above the detection limit only
 3 after three weeks post-infection. An aliquot of the same sample collected at day 41
 4 post-infection is the Uk-Aa4 sample shown also in panel B.

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