A novel simian retrovirus subtype discovered in cynomolagus monkeys (Macaca fascicularis)

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A new simian retrovirus (SRV) subtype was discovered in China and the USA from Cambodian-origin cynomolagus monkeys. Histopathological examination from necropsied animals showed multifocal lymphoplasmacytic and histiocytic inflammation. The complete genome sequences demonstrated that the US virus isolates were nearly identical (99.91–99.93%) and differed only slightly (99.13–99.16% identical) from the China isolate. Phylogenetic analysis showed that the new virus isolates formed a distinct branch of SRV-1 through -7, and therefore were named this subtype, SRV-8. This SRV-8 variant was also phylogenetically and serologically more closely related to SRV-4 than any other SRV subtype.

INTRODUCTION

Simian retrovirus (SRV) is a type D retrovirus within the genus Betaretrovirus of the family Retroviridae. It is a single-stranded, enveloped RNA virus, wherein the RNA transcribes to DNA and integrates into the host genome to become proviral DNA. Both endogenous and exogenous SRVs are found in non-human primates, but no disease is caused by endogenous SRV. Exogenous SRV-infected primates may or may not display symptoms; however, development of severe illness, such as simian acquired immune deficiency syndrome (SAIDS) or malignant thrombocytopenia, can be fatal (Lerche & Osborn, 2003; Okamoto et al., 2015).

Exogenous SRVs are denoted as SRV-1 through -7 based on their neutralization or molecular genetic analysis. The prototype of SRV, Mason-Pfizer monkey virus (MPMV/SRV-3), was first recovered in 1970 from a rhesus macaque (Macaca mulatta) with a spontaneous mammary carcinoma at the Mason Research Institute (Worcester, MA, USA) (Jensen et al., 1970). In 1984, SRV-1 and -2 were both isolated, with SRV-1 isolated from Taiwanese rock macaques (Macaca cyclopis) with SAIDS at the New England Primate Research Center, and rhesus monkeys at the California National Primate Research Center (NPRC) (Daniel et al., 1984; Marx et al., 1984). SRV-2 was isolated from several macaques (Macaca nemestrina, Macaca mulatta, Macaca fuscata and Macaca fascicularis) with SAIDS at the Washington NPRC (Stromberg et al., 1984). In the same year (1984), SRV-4 was isolated at the California NPRC from a cynomolagus monkey (Macaca fascicularis) housed at the California Public Health Laboratory (Berkeley, CA, USA) (Preston A. Marx; unpublished results). In 1987, SRV-5 was isolated at the Oregon NPRC from rhesus macaques exported from China (Axthelm et al., 1987). The molecular characteristics of SRV-1 through -5 have been well studied, and their complete genomes sequenced (Power et al., 1986; Marracci et al., 1999; Sonigo et al., 1986; Zao et al., 2010; Takano et al., 2013). However, SRV-6 and SRV-7 are less well characterized. SRV-6 was isolated from wild-caught Hanuman langurs (Semnopithecus entellus) in India (Nandi et al., 2000), and SRV-7 was isolated from wild rhesus monkeys in India (Nandi et al., 2006).

To diagnose SRV infection, serological tests to detect anti-SRV IgG are performed to verify previous infection. Owing to serological cross-reactivity among different SRV subtypes, routine serological methods such as ELISA, dot-blot immunoassay, multiplex immunoassay, Western blotting (WB) or immunofluorescent assay (IFA) (Lerche et al., 1994) cannot differentiate between SRV subtypes found in SRV-infected animals. As for molecular diagnosis, a generic PCR method (White et al., 2009) is often applied to detect all SRV subtypes. Therefore, it is possible that unknown
SRV subtypes besides SRV-1 through -7 remain undiscovered. In this study, we investigated a new SRV subtype based on characterization of complete viral-genome sequences and named this new subtype SRV-8.

RESULTS

SRV diagnosis

A potential new SRV subtype was detected from two separate groups of cynomolgus monkeys: one group in China in May 2012 and the other in the USA in February 2015. We hypothesized that this represented a new SRV subtype (SRV-8) based on sequence analysis of SRV PCR fragments from positive samples that did not match any published sequence for SRV-1 through -5 subtypes, although serological testing by WB showed cross-reactivity with SRV-1 through -5 (Fig. 1). Virus isolation was performed to obtain the complete genome sequence for analysis. The virus was successfully isolated from two infected animals in China and eight infected animals in the USA by Raji cell culture (Fig. S1a, b, available in the online Supplementary Material) and confirmed to be SRV based on IFA results (Fig. S1c, d).

Pathology

All SRV-infected animals found in China and the USA appeared healthy and lacked symptoms. Complete blood-count results showed that 12 of the 15 infected animals in the USA exhibited higher-than-normal values for either white blood cell, lymphocyte and/or basophil counts, whereas 13 of the 15 animals exhibited slightly higher-than-normal values for either red blood cell count, haemoglobin, haematocrit, mean corpuscular volume and/or mean corpuscular haemoglobin concentration. Tissue samples collected from the abnormal organs of two necropsied animals showed interstitial nephritis, hepatitis, pneumonia and moderate diffuse lymphoid hyperplasia in the spleen. No significant findings were observed in the heart.

Complete genome sequences

One virus isolate (SRV8/SUZ/2012) collected from China and three isolates (SRV8/TEX/2015/V1–V3) collected from the USA were chosen for genetic analysis. The complete genome sequence of the SRV8/SUZ/2012 isolate (Genbank accession no. KU605777) obtained from genomic RNA extracted from Raji-infected cell media was 8126 bp long and 100% identical to sequences obtained from proviral DNA extracted from Raji-infected cells. The full-length genome of the three US isolates were longer, with both SRV8/TEX/2015/V1 (KU605778) and SRV8/TEX/2015/V2 (KU605779) at 8138 bp and SRV8/TEX/2015/V3 (KU605780) at 8134 bp. The Chinese isolate exhibited a 12 nt deletion between position 6268 and 6279 of the first two US isolates, which resulted in deletion of 4 aa between...
residues 140 and 143 of the encoded envelope protein. The third US isolate (SRV8/TEX/2015/V3) exhibited deletion of 4 nt between positions 5832 and 5835 of the first two US isolates, but these deletions were within the non-coding region.

Comparisons between the complete genome nucleotide sequences of different SRVs are listed in Table 1. Three US isolates were nearly identical (99.91–99.93 %) and differed slightly (99.13–99.16 % identical) from that of the China isolate (Table 1). Comparisons of the protein sequences showed that the Gag, Prt and Pol proteins from the China isolate were highly similar (99.24–99.68 % identical) to sequences for the same proteins from the three US isolates, but exhibited greater variation (96.01–96.18 % identical) in the Env protein sequence (Tables S1 and S2).

**LTRs**

All of the new virus isolates exhibited typical SRV genomic organization, including a 5′-LTR-gag-prt-pol-env-3′-LTR. The first and last 340 bp of the genome consisted of separate 5′- and 3′-LTRs. All conserved regions involved in viral DNA synthesis and transcription within 5′- and 3′-LTR of other SRVs, such as unique sequence blocks U3 and U5, tRNA binding sites, polyurine tracts and poly-A signals, were also found in the four virus isolates.

**Comparison of viral ORFs and their translated products**

The first ORF of the virus isolates ranges from nucleotides 488 to 2467 and encodes a 659 aa (Gag), which is a polyprotein precursor of six virion core structural proteins: p10, pp24/pp18/pp16, p12, p27, p14 and p4/p6. The deduced amino acid sequence of the Gag protein from all four new virus isolates shared 100 % sequence identity to the corresponding sequence of SRV-1 through -5. Furthermore, the amino acid sequence of the protease from each of the four new isolates was almost identical (99.68–100 %) (Table S1).

The second ORF, ranging from nucleotides 2284 to 3228, encodes a 314 aa protease. Given the presence of a putative protease active site (Asp-Thr-Gly at position 188–190), this implied its potential role as an aspartyl protease, similar to those found in other SRVs. The amino acid sequence of the protease from the new virus isolate SRV8/TEX/2015/V1 was 83.12 % to 86.31 % identical to the corresponding sequence of the protease from SRV-1 through -5. Furthermore, the amino acid sequence of the protease from each of the four new isolates was almost identical (99.68–100 %) (Table S1).

The third ORF, ranging from nucleotides 3207 to 5807, encodes the 866 aa Pol protein, which is one N-terminal residue shorter than the sequences of SRV-1, -2, -3 and -5. Although the length of the N-terminal Pol proteins from the isolates was the same as that of SRV-4 and baboon endogenous retrovirus type D (SERV; GenBank accession no. U83505), they lacked an extended four-residue sequence at the C terminus of the SRV-4 and SERV Pol proteins. The Pol proteins of all four new isolates contained conserved reverse-transcriptase and endonuclease domains similar to other retroviral Pol proteins and shared 99.54 % to 100 % sequence identity.

The fourth ORF within each of the three US virus isolates encodes a 580 aa Env protein. A potential proteolytic cleavage site at residue position 389 can result in an outer membrane surface gp70 domain in the N-terminal region and a transmembrane gp20 domain in the C-terminal region. Additional amino acid sequence variability was observed in the gp70 domain as compared with that observed in the gp20 domain (Table S3). The sequence identity of the gp70 domain of SRV8/TEX/2015/V1 Env as compared with the gp70 domain of Env from SRV-1 through -5 ranged from 60.58 % to 69.35 %, whereas the sequence identity of the Env gp20 domain from the four isolates as compared with that from the Env of SRV-1 through -5 ranged from 83.25 % to 90.26 %. Additionally, the sequences of the Env gp70 domain between the three US isolates were 100 % identical and shared 94.53 % sequence identity with the China isolate. Within the gp70 domain, the T-cell activation sequence located between residue 229 and 245 of the US isolates shared 100 % sequence identity to the corresponding region of the China isolate.

**Table 1. Complete genome nucleotide sequence-identity percentage comparison between SRVs**

<table>
<thead>
<tr>
<th>Virus</th>
<th>SRV-1</th>
<th>SRV-2</th>
<th>SRV-3</th>
<th>SRV-4</th>
<th>SRV-5</th>
<th>SRV8/SUZ</th>
<th>SRV8/TEX/V1</th>
<th>SRV8/TEX/V2</th>
<th>SRV8/TEX/V3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRV-1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SRV-2</td>
<td>76.06</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SRV-3</td>
<td>91.42</td>
<td>76.59</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SRV-4</td>
<td>77.77</td>
<td>75.16</td>
<td>77.52</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>SRV-5</td>
<td>77.08</td>
<td>74.75</td>
<td>76.94</td>
<td>75.86</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SRV8/SUZ</td>
<td>77.73</td>
<td>74.79</td>
<td>77.84</td>
<td>77.64</td>
<td>74.45</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>SRV8/TEX/V1</td>
<td>77.77</td>
<td>74.83</td>
<td>77.77</td>
<td>77.73</td>
<td>74.46</td>
<td>99.14</td>
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<td>SRV8/TEX/V2</td>
<td>77.75</td>
<td>74.79</td>
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<td>74.46</td>
<td>99.13</td>
<td>99.91</td>
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<tr>
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<td>74.53</td>
<td>99.16</td>
<td>99.93</td>
<td>99.91</td>
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</tbody>
</table>
Phylogenetic analysis

The phylogenetic relationships between the four virus isolates and SRV-1 through -5 were analysed based on the availability of their complete genome sequences (Fig. 2a). Three of the US virus isolates were grouped together with the China virus isolate and formed an independent branch. Although this new branch differed from those of SRV-1 through -5, it exhibited greater similarity to that of SRV-4 than to SRV-1, -2, -3 and -5. To further analyse the relationship of the four new virus isolates to SRV-6 and -7, phylogenetic trees were constructed based on the corresponding sequences of the partial SRV-6 env gene (701 bp; AY598468; Fig. 2b) and the partial SRV-7 pol gene (452 bp; AY594212; Fig. 2c). Our results indicated that the new virus isolates belonged to neither SRV-6 nor SRV-7; therefore, we named the new isolates SRV-8, using the next number in the SRV-subtype series.

Similarity between SRV-8 and SRV-4

Phylogenetic analysis showed that SRV-8 was more closely related to SRV-4 than any other SRV subtypes, despite the complete genome identity between SRV-8 and SRV-4 being at 77.64–77.73 %, which was similar to SRV-8 sequence identity with SRV-1, -2, -3 and -5 (74.46–77.84 %; Table 1). The main genomic similarities between SRV-8 and SRV-4 included the Pol protein being 1 aa short at the N terminus. Additionally, an immunosuppressive peptide sequence (residues 452–483) in the Env protein included a conserved amino acid substitution of Ala to Thr at position 480, which differed from other SRV subtypes. Furthermore, we observed strong serological cross-reactivity of the SRV-8 antigen reacting with anti-SRV4, as well as anti-SRV-8 reacting with the SRV-4 antigen, according to WB results (Fig. 1), suggesting a close relationship between these two viruses.

DISCUSSION

Here, we report the discovery of a novel SRV subtype named SRV-8, based on its genetic relatedness to other SRVs. Non-human primates with an SRV infection may result in confounding variables when used for biomedical research, owing to immune modifications, haematological abnormalities, histological lesions, neoplasia and increased morbidity/mortality (Lerche & Osborn, 2003). Cynomolgus macaques are commonly used in non-human primate research and are susceptible to SRV-2 and SRV-4 infection. Newly discovered SRV-8 isolated from naturally infected cynomolgus monkeys is an additional SRV subtype capable of infecting cynomolgus macaques and is found to be closely related to SRV-4. SRV-8 was first isolated from a Chinese breeding colony, which was established by importing cynomolgus monkeys from a Cambodian colony populated with wild cynomolgus monkeys. In 2014, we performed SRV-antibody screening of 1821 serum samples collected from the Cambodian colony and determined a 37.9 % positive rate (unpublished data). Therefore, we suspect that SRV-8 likely originated from cynomolgus monkeys in Cambodia and then spread into the captive-breeding colonies. SRV-4 origin remains unclear, although it was originally isolated from cynomolgus monkeys in California, Japan (Hara et al., 2005) and Texas, having Indochinese and Indonesians/Indochinese mixed ancestry backgrounds (Zao et al., 2010). While SRV-4 was detected in SRV PCR-positive cynomolgus monkeys in Indonesia (unpublished data), no SRV-4-positive cases have been found in Cambodia. Phylogenetic similarity between SRV-8 and SRV-4 implies that the geographic origin of SRV-4 might be in close proximity to Cambodia and within Southeast Asia.

SRV-8 appears to cause little or no harm to cynomolgus macaques, which are its natural hosts. We observed no common features of SRV infection, such as anaemia, neutropenia, diarrhoea or weight loss (Lerche & Osborn, 2003) in SRV-8-positive animals. Although histological results showed mixed inflammation in multiple tissues, with some infected animals exhibiting increased white blood cell, lymphocyte, and/or basophil counts, the cause was not evident. These findings could be the result of SRV-8 infection, or secondary infections due to immunocompromise secondary to chronic viral infection. However, the multifocal lymphoplasmacytic and histiocytic inflammation was consistent with previous studies of asymptomatic SRV-4-infected cynomolgus monkeys (Zao et al., 2009). To avoid severe diseases developed by SRV-8 infection, different macaque species should not be housed together with SRV-8-positive cynomolgus macaques. Infections across different macaque species could be an issue, similar to SRV-4 transmission from asymptomatic natural host cynomolgus monkeys to Japanese macaques (Macaca fuscata), which caused lethal severe acute thrombocytopenia (Okamoto et al., 2015).

Analysis of the genetic diversity between SRV-8 isolates collected from China and those from the US revealed a 0.9 % difference in the complete genome sequences. Despite deletions in the Env gp70 domain and other non-conserved or conserved amino acid substitutions dispersed throughout all ORFs, these alterations did not appear to affect protein structure or function within the China isolate, which grew at a similar rate as all three US isolates in vitro. The anti-SRV-8 antibodies generated from SRV-8-infected cynomolgus monkeys located in either China or the USA exhibited similar WB reactivity patterns (data not shown). Additionally, observed genetic variations caused by naturally occurring mutations within different isolates implied that SRV-8 might have evolved into different lineages within Cambodia, given that these viruses were isolated from Cambodian-origin cynomolgus monkeys.

It is unclear how SRV-8 was transmitted to the SRV-8-positive animals detected by routine screening; however, all of these SRV-8-positive animals had been housed together. SRVs can be present in bodily fluids, such as blood, saliva, urine and faeces; and tissues, such as those from salivary glands, lymph nodes, spleen and thymus. (Lackner et al., 1988; Hara et al., 2007). Because close physical contact is required for SRV transmission (Lerche et al., 1987), contact
with saliva or blood through bites could be a route for animals to contract SRV-8 from healthy carriers of infectious viruses. Such healthy SRV-8 carriers can be identified by PCR screening, with or without serological testing. Virus shedding may not be persistently observed in SRV-8-infected animals, given that we observed some SRV-8-positive animals returning negative results from PCR screening, but strong positive results from serological testing during the later stage of infection. Therefore, to remove SRV-8-positive animals from the colony, both serological testing and molecular diagnosis play key roles in maintaining an SRV-free colony.

Fig. 2. Phylogenetic analysis. The percentages of replicate trees in which the associated taxa clustered together following the bootstrap test (100 replicates) are shown next to the branches (only bootstrap values >70% that indicate strong support are shown). Each tree is drawn to scale, with branch lengths represented in the units of the number of base substitutions per site. (a) Phylogenetic tree based on the complete genome from various SRVs. The tree with the highest log likelihood (−41997.5) under the generalized time-reversible model (Tavaré, 1986) is shown. (b) Phylogenetic tree based on the partial sequence of the SRV-6 env gene (701 bp; AY598468) and its corresponding position within other SRV genomes. The tree with the highest log likelihood (−4941.8) under the generalized time-reversible model is shown. (c) Phylogenetic tree based on the partial SRV-7 pol gene (452 bp; AY594212) and its corresponding position within other SRV genomes. The tree with the highest log likelihood (−2544.3) under the generalized time-reversible model is shown. Bars indicate 0.05 (a, c) and 0.1 (b) nucleotide substitutions per site.
In conclusion, we isolated a new SRV subtype and named it SRV-8, based on its complete genome sequencing and phylogenetic analysis. Additionally, we observed evolutionary divergence between the different viral isolates collected from naturally infected, Cambodian-origin cynomolgus monkeys. The close phylogenetic relationship discovered between SRV-8 and -4 suggests that these two viruses may have originated from countries in close geographic proximity.

METHODS

Animal history. In May 2012, serum samples collected from 22 of 287 juvenile cynomolgus monkey (2–3 years of age) located in a breeding colony in China tested positive for SRV antibodies according to dot-blot immunoassay (positive rate: 22/287=7.7 %) (Heberling et al., 1988). Among the 265 SRV antibody-negative animals, EDTA whole-blood samples were collected for further screening using a generic SRV PCR (White et al., 2009), resulting in five SRV PCR-positive animals. In subsequent years (December 2013–February 2016), the SRV-positive rate for serum samples received from the same colony varied, with up to a 40.2 % positive rate for groups consisting of adults –10 years of age.

In February 2015, a group of 197 Cambodian-origin cynomolgus monkeys exported from China to the USA were diagnosed as SRV infected, according to ELISA, WB and PCR screening (positive rate: 15/197=7.6 %). Three typical SRV-serological and -PCR patterns were observed within these positive animals: (1) four animals were SRV-serology and -PCR positive; (2) six animals were SRV-serology positive, but -PCR negative; and (3) five animals were SRV-serology negative, but -PCR positive (all five animals were seroconverted to positive in the follow-up samples).

Western blot analysis. SRV antigens were resolved on 4 % to 12 % polyacrylamide gels and transferred to PVDF membranes. The membranes were stained with sera containing anti-SRV antibodies and incubated with a secondary antibody/alkaline-phosphatase conjugate (Promega). Following substrate development, WB patterns were determined based on the criteria that reactivity with at least one gag-encoded antigen (p27, p24/p18/p16, p12, p10 or p4/p6) and at least one env-encoded antigen (gp70 or gp20) constituted a WB-positive result. The antigen (p27, p24/p18/p16, p12, p10 or p4/p6) and at least one indeterminate results (Lerche et al., 2001).

Histopathology. Tissues collected from two necropsied animals were fixed in 10 % neutral-buffered formalin and sent to Texas Veterinary Pathology Associates (San Antonio, TX, USA) for examination.

Complete genome sequencing. Viral RNA was extracted from virus-infected Raji cell media using a TIANamp virus DNA/RNA kit (Tiangen) and reverse transcribed into cDNA using a Reverse Transcriptase M-MLV (RNase H minus) kit (TAKARA). To amplify the full-length viral genome, 5'–RACE, 3'–RACE, and primer walking were performed using a SMARTer® RACE 5'3' kit (TAKARA). Provir DNA preparation from whole blood or virus-infected Raji cells was performed using a NucleoSpin blood kit (Macherey-Nagel). PCR was performed using Platinum PCR SuperMix (Invitrogen) with primers designed from known conserved SRV/D sequences for primer walking (available upon request). PCR products were purified using a QIAquick gel-extraction kit (Qiagen), and 50 ng per sample was used for DNA sequencing on an ABI 3130xl genetic analyzer (Applied Biosystems; Nucleic Acids Core Facility, University of Texas Health Science Center, San Antonio, TX, USA). All sequence data in FASTA format were aligned and compared using CLUSTAL OMEGA (McWilliam et al., 2013).

Phylogenetic analysis and GenBank deposition. Phylogenetic trees were constructed using SeaView 4.6 (Gouy et al., 2010) and the PhyML algorithm (Guindon & Gascuel, 2003). The transition/transversion ratio was calculated from each alignment, with four rate categories across sites. Evolutionary distances were calculated under all available evolutionary models, and the most likely model for each data set was selected. A starting tree was constructed for each dataset by the BIONJ method, and the nearest-neighbour interchange tree-search algorithm was applied to find the most likely tree. The bootstrap consensus tree (Felsenstein, 1985) was then inferred from 100 replicates of the most likely tree. The SERV sequence was used as an outgroup in the phylogenetic analysis.

The complete genomes of newly isolated SRV8/SUZ/2012, SRV8/TEX/2015/V1, SRV8/TEX/2015/V2 and SRV8/TEX/2015/V3 were deposited into GenBank, administered by the National Center for Biotechnology Information, under accession numbers KU605777, KU605778, KU605779 and KU605780, respectively. Reference sequences for SRV-1 (M11841), SRV-2 (AF126467), SRV-3 (M12349), SRV-4 (FJ971077), SRV-5 (AB617077), SRV-6 env (AY598468), SRV-7 pol (AY934212) and SERV (U85505) were included for comparison.

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