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Isolation and Characterization of Simian Retrovirus Type D from *Macaca fascicularis* and *M. nemestrina* in Indonesia

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Simian type D retroviruses (SRVs) are one of the causative agents of simian acquired immunodeficiency syndrome (AIDS) in Asian macaques. In the past, SRV isolates from macaques had only been identified at the US primate centers, outside the country of origin and after the animals had been introduced into a new environment. In this study, we report the first isolation, cultivation and molecular characterization of the type D simian retrovirus naturally infecting wild caught macaques in their natural habitats in the country of origin, in this case, Indonesia. When peripheral blood mononuclear cells (PBMC) from *Macaca fascicularis* (Mf) and *M. nemestrina* (Mn) were co-cultured with Raji human B-cell line, syncytia were observed microscopically and confirmed by immunofluoresence assay using antibody to SRV-2. Immunoblot analysis of purified Mf-ET1006 from cell culture supernatants demonstrated that the viral core and envelope proteins reacted with rabbit anti-SRV. Sequence analysis of Mf isolates in the viral envelope region revealed high homology to SRV-2 (94-96%). On the other hand, the homologies in the envelope region of Mn isolates were less than 80% to SRV-1, SRV-2, SRV-3 and Mf isolates. This study suggests that the isolate from Mn may be different from any other published SRV isolates..

Key words: type D simian retrovirus, *Macaca fascicularis*, *Macaca nemestrina*

For many years, the Retroviridae family has been studied primarily because of the ability of these viruses to transform mammalian cells and cause naturally occurring tumors in many animal species. It was recognized that this virus family could also cause non-oncogenic disease, including immunosuppresive disorders such as AIDS (Gardner *et al.* 1988). Among family Retroviridae, the lentivirus (simian immunodeficiency virus, SIV) and exogenous type D retroviruses (simian retrovirus, SRVs) have been identified as the etiologic agents of infectious immunodeficiency diseases in several macaque species that have some clinical similarities to human AIDS (Lerche *et al.* 1995).

SRV infection is endemic and for the first time was reported in *Macaca* sp. populations at some Primate Research Centers in the United States in the early 1980s (Daniel *et al*. 1984; Gardner *et al*. 1988; Pamungkas *et al*. 1991). Until 1988, five serotypes of SRV had been isolated and identified from macaques (SRV-1 to SRV-5). However, from these five serotypes, only four (SRV-1, SRV-2, SRV-3 and SRV-4) had been completely sequenced and reported to GenBank (Daniel *et al.* 1984; Marx *et al.* 1984; Gardner *et al.* 1988; Li *et al*. 2000). SRVs have emerged as significant pathogens in captive macaques, although infection appears to be sporadically prevalent in Asian macaques at breeding facilities and these species are probably the natural hosts of SRV, but prevalence of infection in feral macaques remains undetermined (Daniel *et al.* 1984; Gardner *et al.* 1988; Pamungkas *et al*. 1991). Serological studies of *M. fascicularis, M. nemestrina,* and *Pongo pygmeus* in Indonesia show variable prevalence of SRV-2 leading to the assumption that the animals have been infected with SRV (Iskandriati *et al.* 1998a; Iskandriati *et al.* 1998b; Warren *et al.* 1998).

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SRV isolates, found to date from macaques at primate centers out of the animals' original country, have been obtained after the animals were introduced to the new environment. According to Grant (1995), the isolates obtained over many years from the Washington Primate Center have a very high homology level, which shows that these SRV isolates probably came from the same source or a small number of source animals. However, the characteristics of virus growth and genetic sequence variability from wild type isolates have not been described yet. Until today there has been no report on findings of wild isolate in its natural population. Since SRV infection has negative impact on the management of macaques breeding facility, it is very important that we be able to identify and characterize new SRV isolates in the animals' original population.

The objective of this research was to identify SRV from *M. fascicularis* and *M. nemestrina* in Indonesia using virus isolation technique, and to identify and characterize provirus DNA from those isolates. The resulting amino acid sequences obtained were then compared with those from other type D retroviruses to describe their genetic relationship.

MATERIALS AND METHODS

Sample Collection and Processing. Fifty-three blood samples from Lampung and Palembang, Indonesia and 76 blood samples from *M. nemestrina* in Palembang were obtained from several breeding facilities in Indonesia. *M. fascicularis* and *M. nemestrina* with a total number of 129 were placed in individual cages during the screening process. Blood samples were taken within the first week of the animals' arrival at the breeding facilities. This was carried out to get accurate data about the animals' condition. If the animals were infected by SRV, it can confirm that the

infection occurred not at the breeding facilities, but in their places of origin. The peripheral blood mononuclear cells (PBMCs) were recovered from samples on a Ficoll gradient (Organon Teknika, USA) as described in Hunt (1987). The cell suspensions were washed in phosphate-buffered saline (PBS) and aliquoted. The cells were further used for virus isolation and polymerase chain reaction (PCR) of proviral DNA.

Virus Isolation. Raji cells (ATCC CCL-86) were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum, 100 IU mL^{-1} penicillin and 100 ug mL^{-1} streptomycin and used for isolation. The cells were subcultured every four days for the duration of three weeks for syncytium induction as described previously (Rosenblum *et al.* 2000). To prove that syncytia formed on infected Raji cells were caused by SRV, immunofluorescence assay was carried out using rabbit anti-SRV serum and detected by a *FITC*-labeled goat anti-rabbit immunoglobulin antibody.

Virus Purification and Immunoblotting. The supernatant from infected Raji cells was inoculated into A549 cells (ATCC CCL-185). Virus was harvested from culture supernatant using ultrafiltration and ultracentrifugation techniques and purified into chromatography column using Sepharose-CL4B as described by Grant *et al.* (1995b). Immunoblotting was modified from Harlow and Lane (1988). Purified virus was electrophoresed on a 7.5-17.5% gradient SDS-PAGE and transferred onto a nitrocellulose membrane. Individual cut strips were incubated with various rabbit anti-SRV polyclonal sera. Bound antibodies were detected by an alkaline phosphatase-labeled goat anti-rabbit immuno-globulin antibody (Sigma, USA) with BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium-nitroblue tetrazolium) substrates (Sigma, USA).

Immunofluorescence Technique (Immunofluorescence Assay, IFA). Infected Raji cells were fixated on object slide using methanol and cold acetone with 1:1 ratio for 5 min. Then the object slides were washed with PBS and dried in room temperature (22°C). Cell blood smear was then incubated with rabbit anti-SRV serum and washed with PBS. Flourescein isothiocyanate-conjugated anti-rabbit immunoglobulin G was added to cell blood smear, incubated for 45-60 min and re-washed with PBS. Then, object slide was given counter staining Evans blue and expelled with mounting medium PBS- glycerol 90% before closing it with glass lid. Cell blood smear was observed by using fluorescent microscope to see color luminescent from cytoplasm.

DNA Extraction and PCR Amplification. Total DNA was extracted using DNA purifying kit (Qiagen, USA). Approximately 1800 bp fragment from this DNA was amplified with a primer set consisting of an upstream primer '5-CACCTCTYTRCTYAYAGAGCTGA-3' and a downstream primer 5'-GAAACTGCGCCTGTCT-3' located in the *env* gene (Grant 2004, personal communication). PCR amplification were performed by following protocol: denaturation for 2 min at 92°C, amplification for 10 cycles consisting of 10 sec at 92°C, 30 sec at 60°C and 2 min at 68°C continued with amplification for 20 cycles consisting of 15 sec at 92°C, 30 sec at 60°C and 2 min at 68°C, with time prolongation of 20 sec for each cycle and ended with an extension at 68°C temperature for 7 min. The PCR amplified DNA fragments were cloned into the TA TOPO vector (Invitrogen, San Diego, California) and sequenced.

DNA Sequencing and Analysis. Sequencing of nucleotides was carried out in Automated DNA sequencer ABI-PRISM Model 310 version 3.7 (Applied Biosystem, Foster City, California) using M13 primers. Alignment of the sequences was done with ClustalW (EMBL-EBI). The phylogenetic analyses for protein were done by the neighbor-joining (NJ) method using MEGA package software version 4.0 (Kumar *et al.* 2008). Distances were estimated by Kimura's two parameter method with one thousand bootstrap replicates. GeneBank accession numbers of the comparison sequences were M11841 (*SRV-1*), M16605 (*SRV-2*), M12349 (*MPMV/SRV-3*), D10032 and AF142988 (Baboon endogenous virus, type C retrovirus), and AF284693 (*Trichosurus vulpecula* type D endogenous retrovirus) and endogenus retrovirus of *Macaca* (Grant, data not yet published).

RESULTS

Out of 129 PBMCs samples, there were only four samples (Mf-ET1006, Mn-5055, Mn-5281, and Mn-5378) showing retrovirus infection, as shown by the appearance of multinucleated giant cells (syncytial formation) on day 7 after inoculation, a typical cytopathic effect (CPE) resulted from the fusion of infected cells. Fig 1B shows the appearance of multinucleated giant cells on Raji cells inoculated with PBMCs from *M. fascicularis* ET1006 compared with normal uninoculated Raji cells (Fig 1A); while Fig 1C shows smaller size syncytia on Raji cells inoculated with PBMCs from *M. nemestrina* 5378. The other two *M. nemestrina* (5055 and 5281) PBMCs produced syncytia with similar size as shown by *M. nemesterina* 5378. Lack of retrovirus infection was shown by the absence of syncytial formation on the remaining 125 PBMCs samples after co-cultured with Raji cells. From the four samples showing syncytial formation on Raji cells, *M. fascicularis* ET1006 gave the most obvious CPE positive result with larger size of giant cells, while isolates from the *M. nemestrina* gave smaller syncytial formation on Raji cells.

IFA was carried out to confirm that the syncytia or multinucleated giant cells formed on Raji cells were caused by SRV infection, not by other retroviruses. Foamy virus, a member of the Retroviridae family from the genus of Spumavirus also shows the appearance of syncytial formation when co-cultured with peripheral blood mononuclear cells *in vitro*. The positive IFA result was indicated by the greenish fluoresceinated color under a fluorescence microscope resulted from the reaction of antigen/ viral protein bond with anti-SRV-2 antibodies produced in rabbit (rabbit anti SRV-2 serum) which later reacted with anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (FITC-anti-rabbit IgG conjugate). From the four samples showing syncytial formation on Raji cells, *M. fascicularis* ET1006 gave the most obvious fluorescein iluminescence (Fig 2B) indi-

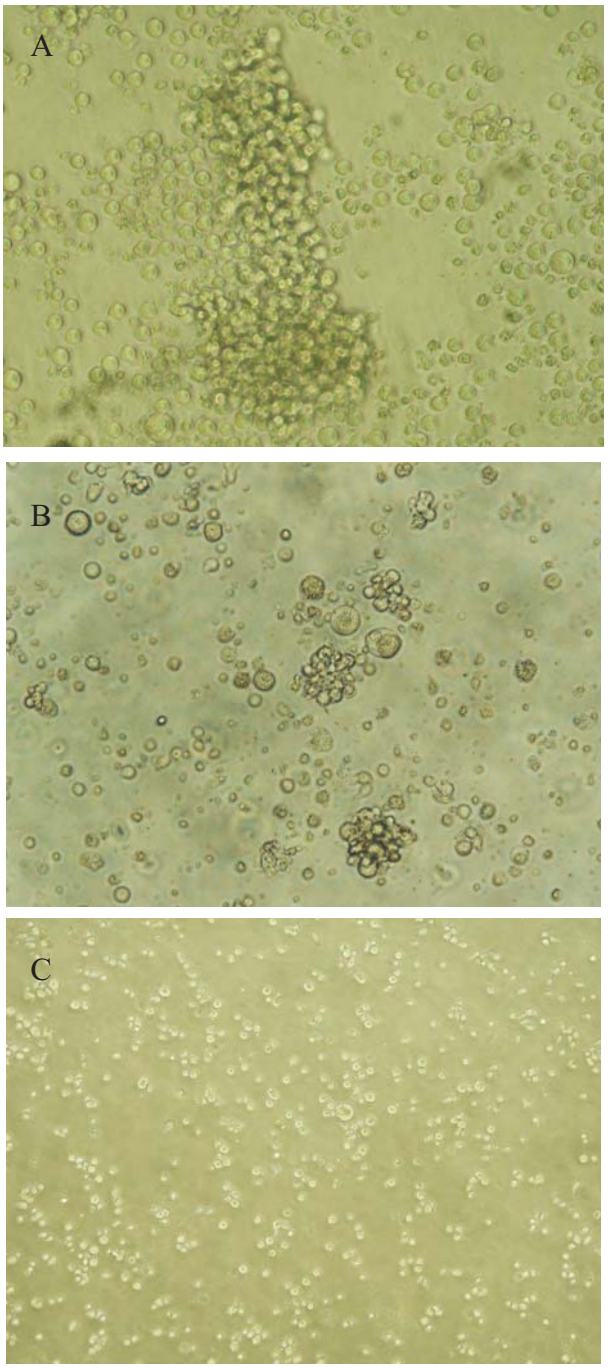

Fig 1 Co-culture of monkey PBMCs with Raji cells: A, uninoculated Raji cells; B, Raji cells inoculated with PBMCs from *Macaca fascicularis* ET1006; C, Raji cells inoculated with PBMCs from *M. nemestrina* 5378.

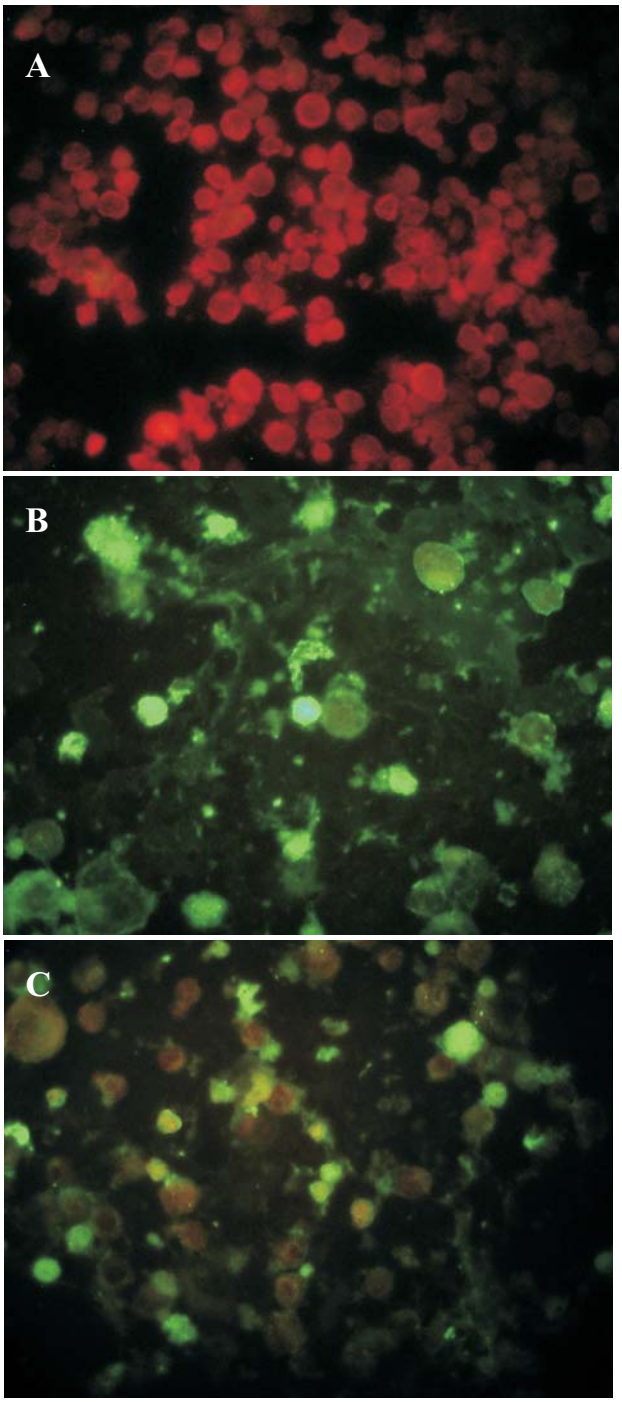

Fig 2 IFA of Raji inoculated with monkey PBMCs: A, uninoculated Raji cells; B, Raji cells inoculated with PBMCs from *Macaca fascicularis* ET1006; C, Raji cells inoculated with PBMCs from *M. nemestrina* 5378.

cating a strong positive result, while isolates from the *M. nemestrina* gave weak positive result (Fig 2C). Uninoculated normal Raji cells were used as negative control. (Fig 2A).

Due to the limited syncitial formation shown by all Mn isolates on Raji cells, further multiplication on A549 cells could only be carried out on Mf-ET1006 sample. To see the relationship between antigens ET1006 and reference SRV-2, immunoblotting analysis was carried out by using antibody panel of some reference simian retroviruses as samples. Fig 3A shows the results of immunoblotting from ET1006 antigen reacted with polyclonal antibody of SRV-1, SRV-2, SRV-3, SRV-5, retrovirus type C from baboon, SRV-Pc and SMRV. As a standard for comparison, immuno-blotting of SRV-2 antigen was carried out (Fig 3B). Most of proteins which are typical property of SRV-2 such as *env* protein (gp70 and gp22) and *gag-pol* protein (p45, p27, p14 and p10) were seen in the Mf-ET1006 strips reacted with antibodies to SRV-1, SRV-2 and SRV-3 with different gp70 intensity levels. Lack of gp22 protein was seen when the Mf-ET1006 strip was reacted with antibody to SRV-5, while reaction to p27 protein was seen in all samples. The same immunoblotting analysis on ET1006 antigen showed cross reactivity in all polyclonal antibodies to simian retroviruses as shown by SRV-2 antigen. However, *env* gp70

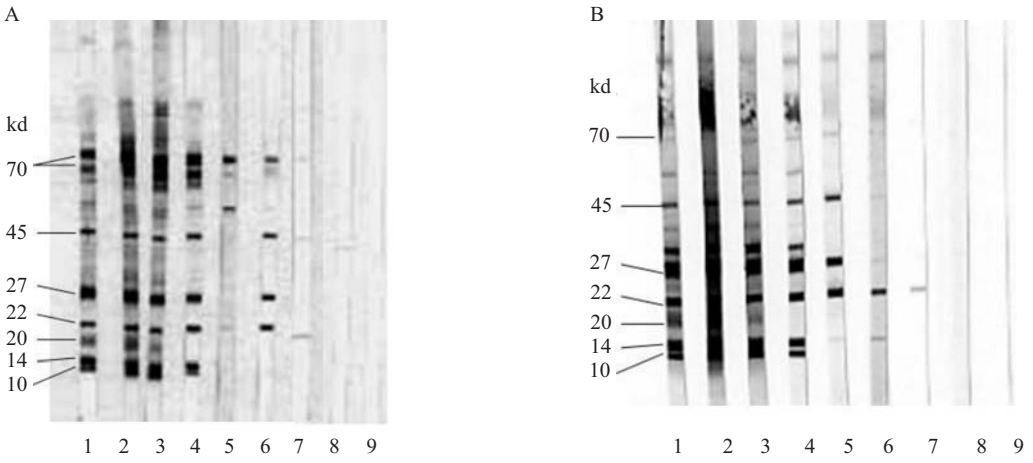

Fig 3 Results of ET1006 immunoblotting: A, and SRV-2; B, with rabbit polyclonal antibody against many SRVs. 1, antibody against SRV-1; 2, antibody against SRV-2; 3, antibody against SRV-3; 4, antibody against SRV-5; 5; antibody against SRV Pc; 6, antibody against type C retrovirus; 7, antibody against SMRV; 8, negative control; 9, conjugate control.

protein of ET-1006 showed a double band instead of one thick band.

A fragment of 500 bp from *env* gp70 gene of SRV-2 was amplified in 18 isolates. However, there was no amplification of 600 bp fragment from *env* gp70 gene of SRV-1 and SRV-3 in any of the samples tested. The results of PCR analyses of the *env* region gp70 and the whole *env* region are shown in Fig 4.

Phylogenetic tree (Fig 5) shows that Mf-ET1006 isolate is very close to SRV-2 with amino acid homology level 95-96%. On the other hand, isolate obtained from Mn has lower homology (less than 80%), to either Mf-ET1006 isolate or other SRV isolates (SRV-1, to SRV-3). Both Indonesian isolates have no similarities to either primate or non-primate endogenous virus.

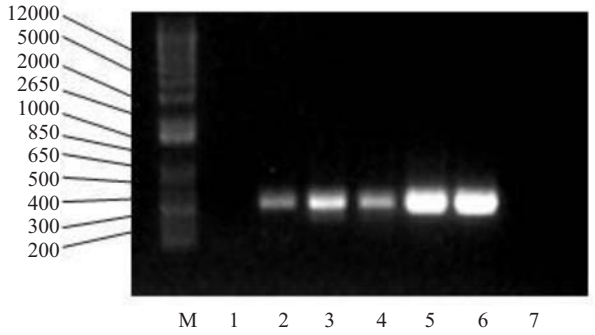

Fig 4 Results of provirus DNA amplification against SRV-2 measuring 500 base pairs. M is a marker measuring 1000 base pairs; 1, reagent control; 2, Mn 5281; 3, Mn 5378; 4, Mn 5505; 5, Mf ET1006; 6, positive control SRV-2 from Mf; 7, reagent control.

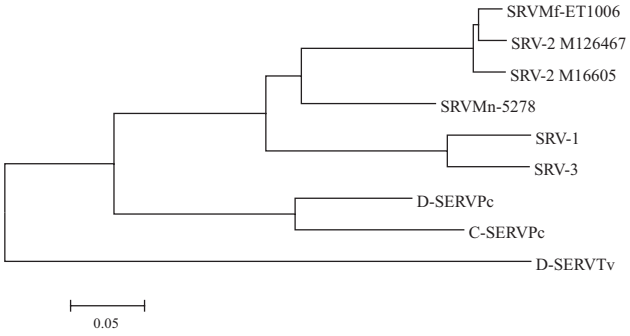

Fig 5 Phylogenetic tree based on amino-acid sequences using Kimura two-parameter and the neighbor-joining method.

DISCUSSION

The appearance of syncytial formation on Raji cells inoculated with PBMCs from *M. fascicularis* ET1006 and from *M. nemestrina* 5378, 5055 and 5281 samples shows the illustration of cytopathic effect (CPE) as an indication that the viruses replicated in Raji cells. The difference of the syncytial formation on Raji cells caused by *M. fascicularis* and *M. nemestrina* isolates is thought to be caused by serotype difference of both viruses that probably have different receptor on the target cells. *M. fascicularis* isolate replicates very well in Raji cells that were originally B lymphocytes. Due to the limitation of cell lines availability in the laboratory, virus isolation was not carried out on T lymphocytes cell lines, so it is unknown whether Mn isolate would be able to replicate better in T lymphocytes. The illustration of IFA results from the *M. fascicularis* and Mn isolates showed different degree of reactivity to SRV-2 antibody. *M. fascicularis*-ET1006 gave a very strong positive reaction, indicating that Mf-ET1006 virus proteins had a high specificity or homology to bind with its antibody. It was also supported by immunoblotting result on the Mf-ET1006 virus protein strip reacted with some antibodies to simian retroviruses. The illustration of Mf-ET1006 protein profile reacting with its antibody was very identical with protein profile illustration of SRV-2. This phenomenon supports an assumption that the Mf-ET1006 isolate has a closer genetic relationship with SRV-2 than the Mn isolate.

Relatively high genome variation is found at *env* gene (Grant *et al*. 1995a; Li *et al*. 2000; Nandi *et al*. 2003) in SRV. Some retrovirus isolates including HIV, MuLV, FeLV, SRV-1, SRV-2 and SRV-3 show that sequence variation on the surface unit or N-terminal domain of *env* gene is higher than

that of the other area in its genome. The surface unit of this *envelope* is binding the target of antibody resulted from adoptive host immune cells in the frame of neutralizing virus. To deceive the immune system attack of host, virus changes by mutation mechanism especially on the envelope surface, resulting high variation in this area (Nandi *et al*. 2003). Some aspects may be influenced by *env* gene change, among others are host, pathogenesis and antigenic characteristics.

The phylogenetic tree shows that Mf-ET1006 isolate has a very close relatedness with SRV-2 with homology level reaching 96%. In contrast, the isolate from Mn has much lower homology (less than 80%) to either Mf-ET1006 isolate or other SRV isolates (SRV-1 to SRV-3). Based on this and some other information showing the differences in both microscopic cytopathic effects and IFA illustration, we conclude that the isolate from *M. nemestrina* is indicated to be a new serotype of type D retrovirus, which is quite different from the other published SRVs.

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