Measurement of Reverse Transcriptase of Feline Immunodeficiency Virus by Poly A-Linked Colorimetric Assay

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ABSTRACT. The method of the poly A-linked colorimetric reverse transcriptase assay (PAC-RTA) was developed and evaluated for the measurement of Mg\(^{2+}\)-dependent reverse transcriptase (RT) activity of feline immunodeficiency virus (FIV). PAC-RTA was first evaluated for the detection of RT activity in the culture supernatant of FIV Petaluma strain. The detection limit of RT activity by PAC-RTA was about 10-fold better than that by the conventional non-radioisotopic RT assay kit. Then, PAC-RTA was evaluated for the indication of FIV isolation from cats naturally infected with FIV. FIV was isolated from peripheral blood mononuclear cells of 9 FIV-seropositive cats. The time course appearance of RT activity measured by PAC-RTA corresponded with the analysis of FIV antigen expression by indirect immunofluorescence. Finally, PAC-RTA evaluated the drug susceptibility of FIV. MYA-1 cells (feline T-lymphoblastoid cells) were infected with FIV and were cultured in the presence of various concentrations of anti-human immunodeficiency virus agents such as azidothymidine (AZT) or dextran sulfate. An inverse relationship between the RT activities and the concentrations of these agents in the culture supernatant was confirmed by PAC-RTA. PAC-RTA is easy to perform without using radioactive materials, and one plate can handle 96 samples at one time. By monitoring the RT activity, this assay is a useful method for FIV studies such as viral replication and drug susceptibility. — KEY WORDS: drug susceptibility, feline immunodeficiency virus, reverse transcriptase assay.

Feline immunodeficiency virus (FIV) was first isolated in 1986 from cats exhibiting an acquired immunodeficiency syndrome (AIDS)-like disease [7]. This virus is similar to the human immunodeficiency virus (HIV) in morphological features and protein structure [12]. Since FIV is thought to induce an AIDS-like disease in cats, FIV infection is a promising animal model for studies of HIV infection [2]. The measurement of reverse transcriptase (RT) activity is very useful for the detection and quantification of FIV. However, the conventional isotopic RT assay requires labor-intensive procedures and is becoming increasingly restrictive due to the use of radioactive materials. Recently, alternative methods for the RT assay using non-isotopic agents were developed [8, 9, 11]. We used the poly A-linked colorimetric reverse transcriptase assay (PAC-RTA) described by Suzuki et al. [9] for measurement of HIV RT activity. Since the reverse transcriptase of FIV is very similar to the HIV RT in template specificity and requirements for Mg\(^{2+}\) [6], we applied the method of PAC-RTA to FIV studies.

MATERIALS AND METHODS

Poly A-linked colorimetric RT assay (PAC-RTA): PAC-RTA was performed using the method described by Suzuki et al. [9] with slight modifications. Briefly, wells were prepared prior to the assay by adding 50 \(\mu\)l of 80 \(\mu\)g/ml poly A (Boehringer Mannheim, Mannheim, Germany) in 12.7 mM N-hydroxysulfo-succinimide (Pierce, Illinois, U.S.A.) to 50 \(\mu\)l of 10 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) per microtiter well (Sumitomo Bakelite, Tokyo, Japan), incubated for 14–18 hr at room temperature, and then stored at 4°C. Wells were washed 3 times with the wash buffer (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA and 0.01% Tween 20) just prior to use. Enzyme activity was measured by adding 10 \(\mu\)l of supernatant from FIV cultures to 50 \(\mu\)l of reaction buffer [4.2 \(\mu\)M biotin-dUTP (Boehringer Mannheim), 8.4 \(\mu\)M TTP, 25 mM KCl, 6.25 mM MgCl\(_2\), 62.5 mM Tris pH 7.8, 1.25 mM dithiothreitol, and 2.5 \(\mu\)g/ml oligo dT\(_{12-18}\) (Pharmacia LKB, Uppsala, Sweden)] in microtiter wells. The reaction was carried out at 37°C for 1–15 hr and then washed 3 times with the wash buffer. After the washings, 100 \(\mu\)l of horseradish peroxidase conjugated streptavidin (GIBCO BRL, Gaithersburg, Maryland, U.S.A.) diluted 1:5,000 in TBSE buffer (10 mM Tris pH 7.5, 0.15 M NaCl, 1 mM EDTA and 1% BSA) was added to each well. After the plate was incubated for 20 min at 37°C, the free conjugate was removed by washing 4 times with the wash buffer. As a final step, 50 \(\mu\)l of 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard and Perry Laboratories, Inc., U.S.A.) was added to each well. The plate was incubated at room temperature for 15 min. The reactions were stopped by the addition of 50 \(\mu\)l of 1N H\(_2\)SO\(_4\). The plate was read at 450 nm with a plate reader. All assays were performed in duplicate.

Virus: FIV Petaluma strain was used in this study. Crandell feline kidney (CrFK) cells persistently infected with the Petaluma strain (CrFK/Petaluma) [12] were grown...
in Eagle’s minimum essential medium supplemented with 10% inactivated fetal calf serum (FCS) and antibiotics. MYA-1 cells (feline T-lymphoblastoid cells) [5] were inoculated with the culture supernatant of CrFK/Petaluma and cultured in RPMI 1640 growth medium supplemented with 10% FCS, antibiotics, 50 µM 2-mercaptoethanol, 2 µg/ml polybrene, and 100 units/ml recombinant human interleukin-2 at 37°C in a humidified atmosphere of 5% CO2 in air [5]. These culture supernatants were used for measuring RT activity.

**Virus isolation:** Blood samples were taken from cats brought to private animal hospitals in Tokyo. These plasma samples were tested by enzyme-linked immunosorbent assay (Pet Check ELISA; IDEXX Corp., Portland, Maine, U.S.A.) and indirect immunofluorescence assay (IFA). The peripheral blood mononuclear cells (PBMCs) collected from the cats were separated by Ficoll-Hypaque density gradient centrifugation. The PBMCs (1 × 10^6/ml) were cocultured with MYA-1 cells (1 × 10^6/ml) in the RPMI 1640 growth medium described above with addition of concanavalin A (Sigma) 10 µg/ml. The growth medium was replaced by fresh medium every 3–5 days [5]. RT activity in the cell-free culture supernatants was measured by PAC-RTA, and the culture cells were examined for the expression of FIV antigen by IFA.

**Drug susceptibility assay of FIV:** One hundred µl of the growth medium containing various concentrations of 3'-azido-3'-deoxythymidine (AZT; Sigma) or dextran sulfate (molecular weight of 5,000; Sigma) were added to each well of a 96-well microtiter plate. Then MYA-1 cells were suspended in growth medium containing the FIV Petaluma strain and were seeded into each well. The culture supernatants were collected to measure RT activity by PAC-RTA.

**Conventional non-radioisotopic reverse transcriptase assay:** A conventional non-radioisotopic (RI) RT assay (Boehringer Mannheim) was used according to the manufacturer’s recommendations. Briefly, 10 µl of supernatant from the cultured cells was added to the 30 µl of provided lysis buffer in an eppendorf micro test tube. The lysis reaction was incubated for 30 min at room temperature and then added to 20 µl of reaction mixture. The reaction mixture contained the RT template: primer (poly A: oligo-dT) and two types of labeled nucleotides (digoxigenin-dUTP and biotin-dUTP). The solution was incubated for 1–15 hr at 37°C. The total sample volume of 60 µl was then transferred to the streptavidin-coated microtiter plate and incubated for 1 hr at 37°C to allow binding of the biotinylated nucleotide. Unbound material was removed by washing the plate 5 times. Anti-digoxigenin-peroxidase conjugate was added to the well and incubated for 1 hr at 37°C to allow the formation of nucleic acid labeled between both biotin and digoxigenin. Unbound conjugate was removed by 5 additional washings of the plate. The peroxidase substrate was added to each well and incubated for 15 min at room temperature. The plate was read at 405 nm with a plate reader.

**RESULTS**

**Detection of RT activity of FIV:** PAC-RTA was compared with the conventional non-RI RT assay kit to determine sensitivity. In this comparison, the same serial dilutions of FIV culture supernatant were measured by both RT assays after the same RT reaction step for 15 hr. A cut-off was defined two times the value for the negative control as well as conventional non-RI RT assay. The detection limit for PAC-RTA was about 10-fold better than that of conventional non-RI RT assay (Table 1).

Then, the culture supernatants of FIV Petaluma strain were taken at 0 (1 hr), 1, 3, 5, 7 and 9 days after the beginning of cultivation, and those RT activities were measured by PAC-RTA.

Figure 1A shows RT activity in the supernatants of CrFK cells persistently infected with Petaluma strain. Virus replicated readily at the beginning of the cell culture. Simultaneously, RT activity increased gradually with time in the culture supernatants.

Figure 1B shows RT activity in the supernatants of FIV Petaluma strain inoculated to MYA-1 cells. RT activity did not appear until the virus infected the cells and replicated. Then, RT activity first appeared in the culture supernatant after 5 days of cultivation.

These data suggested that PAC-RTA could detect actual virions of FIV based on the activity of its RT.

**FIV isolation from cats naturally infected with FIV:** The PAC-RTA was evaluated for the indication of FIV isolation from 9 Japanese domestic cats naturally infected with FIV. All 9 FIV-seropositive cats showed large amounts of RT activity after 21 days from the beginning of cocultivation for virus isolation. The time course appearance of RT activity measured by PAC-RTA showed three patterns (Fig. 2).

<table>
<thead>
<tr>
<th>Serial dilution of FIV</th>
<th>PAC-RTA 0.120</th>
<th>Conventional non-RI RT 0.234</th>
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<tr>
<td>2^0</td>
<td>2.5</td>
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<td>2^-1</td>
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<td>2^-2</td>
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<td>2^-9</td>
<td>0.066</td>
<td>0.114</td>
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<tr>
<td>Negative control</td>
<td>0.060</td>
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Cut-off=Negative control × 2. a) 0.120. b) 0.234.
Three of 9 isolates showed none or small amounts of RT activity at 7 days, followed by large amounts of RT activity at 14 days. Two of 9 isolates showed RT activity increasing gradually with time, and large amounts of RT activity were detected in the culture supernatants at 21 days after cocultivation. The remaining 4 of 9 isolates demonstrated no RT activity until the 14th day, but large amounts of RT activity were detected after 21 days. RT activity was not detected in the culture supernatants from two FIV-seronegative cats throughout the culture period (Fig 2).

The time course appearance of RT activity measured by PAC-RTA corresponded with the expression of FIV antigen determined by IFA (Table 2).

These data showed that PAC-RTA was available for the indication of FIV isolation from cats naturally infected with FIV.

Drug susceptibility assay of FIV: Figure 3 shows the time course appearance of RT activity in the culture supernatants of FIV Petaluma strain which were cultured in the absence/presence of AZT (0.01, 0.1, 1.0 and 10.0 µM) or dextran sulfate (0.1,1.0,10.0 and 100.0 µg/ml).

RT activity in the culture supernatant at the concentration of 0.01 µM AZT increased gradually beginning on the 5th day of cultivation. However, the amount of RT activity was less than that found in the absence of AZT. RT activity did not increase until the 5th day, but small amounts of RT activity were detected at 7 days after cultivation in 0.1 µM of AZT. RT activity was not detected in the culture supernatant at concentrations above 1.0 µM of AZT (Fig. 3A).

RT activity in the culture supernatants at concentrations as low as 1.0 µg/ml dextran sulfate increased similarly to those in the absence of dextran sulfate, but the amounts of RT activity were slightly less than those detected in the absence of dextran sulfate. RT activity in the culture supernatant with 10.0 µg/ml of dextran sulfate increased slightly. RT activity did not increase in the culture supernatant with 100.0 µg/ml of dextran sulfate (Fig. 3B).

Figure 4 shows the RT activity of FIV Petaluma strain in the presence of AZT (A) or dextran sulfate (B), which were serially diluted twofold from 10 to 0.005 µM and from 100 to 0.05 µg/ml, respectively. RT activity of the culture supernatants was measured by PAC-RTA after 7 days of cultivation. PAC-RTA clearly showed an inverse relationship between the RT activities and the concentrations of these agents in the culture supernatant.

DISCUSSION

Measurement of RT activity is very useful and necessary for studying FIV. The RT assay using an isotopic agent is widely used for studies of retroviruses such as HIV or FIV. However, an isotopic assay is time-consuming and restricted to the use and disposal of radioactive material. Recently, alternative methods for measuring RT activity of HIV were developed using non-isotopically labeled nucleoside triphosphates such as BrdUTP or biotin-dUTP [8, 9, 11]. In
comparing PAC-RTA and the conventional non-RI RT assay kit with the same RT reaction time, PAC-RTA measured the detection limit of FIV Petaluma strain RT activity about 10 times better than the conventional kit. The difference of sensitivity between PAC-RTA and conventional non-RI RT assay may be due to the different formats for the incorporation step of labeled nucleotide. The solid phase format used in PAC-RTA probably increased the efficiency of capture the labeled nucleotide compared to the solution phase used in the conventional non-RI RT assay. Moreover, PAC-RTA took less time to carry out than the conventional non-RI RT assay. PAC-RTA could measure RT activity of the sample which was transferred directly from the cell culture plate to the RT reaction plate. PAC-RTA was developed for the detection of FIV RT activity and for the indication of isolation from cats naturally infected with FIV.

For the drug susceptibility assay of FIV, two anti-HIV agents (AZT and dextran sulfate) were used. FIV Petaluma strain was susceptible to both AZT and dextran sulfate [10], whose mechanisms of anti-HIV activity are different. AZT inhibits the reverse transcriptase of HIV [4], but dextran sulfate prevents the early stage of the HIV replication cycle, presumably virus adsorption process [1, 3]. The drug susceptibility assay used in this study demonstrated that FIV Petaluma strain was susceptible to both anti-HIV agents. An inverse relationship between the RT activities and the concentrations of these agents in the culture supernatants was confirmed by PAC-RTA. This method using FIV may be available for screen testing anti-HIV materials without using potentially hazardous HIV.

PAC-RTA is easy to perform without using radioactive materials, and one plate can handle 96 samples at one time. By monitoring the RT activity, this assay is a useful method for FIV studies such as viral replication and drug susceptibility.

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REFERENCES


