Quantification of viral ribonucleic acid in plasma of cats naturally infected with feline immunodeficiency virus

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Objective—To assess plasma viral RNA concentration in cats naturally infected with feline immunodeficiency virus (FIV).

Animals—28 FIV-infected cats.

Procedure—Cats were categorized into 1 of the 3 following stages on the basis of clinical signs: asymptomatic (nonclinical) carrier (AC; n = 11), acquired immunodeficiency syndrome-related complex (ARC; 9), or acquired immunodeficiency syndrome (AIDS; 8). Concentration of viral RNA in plasma (copies per ml) was determined by use of a quantitative competitive polymerase chain reaction (QC-PCR) assay. Total lymphocyte count, $CD4^+$ cell and $CD8^+$ cell counts, and the $CD4^+$ cell count-to-$CD8^+$ cell count ratio were determined by use of flow cytometry.

Results—Plasma viral RNA concentration was significantly higher in cats in the AIDS stage, compared with cats in AC and ARC stages. Most (5/7) cats in the AIDS stage had low total lymphocyte, $CD4^+$ cell, and $CD8^+$ cell counts.

Conclusions and Clinical Relevance—Concentration of plasma viral RNA is a good indicator of disease progression in FIV-infected cats, particularly as cats progress from the ARC to the AIDS stage. Determination of $CD4^+$ and $CD8^+$ cell counts can be used as supportive indicators of disease progression. (*Am J Vet Res* 2000;61:1609–1613)

Feline immunodeficiency virus (FIV) infection in domestic cats results in an **acquired immunodeficiency syndrome (AIDS)**[1,2] similar to that caused by **human immunodeficiency virus (HIV)** infection in humans. There are 5 clinical stages during disease progression in FIV-infected cats; namely: the **acute phase (AP)**, **asymptomatic (nonclinical) carrier (AC)**, **persistent generalized lymphadenopathy (PGL)**, **AIDS-related complex (ARC)**, and AIDS stages.[3,4] These stages are similar to those described for HIV-infected humans.[5]

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In HIV-infected humans, clinical staging is done on the basis of symptoms and $CD4^+$ cell counts in blood samples.[5] Clinical staging in FIV-infected cats has generally been done on the basis of clinical signs alone. A progressive decrease in $CD4^+$ cell counts and the **$CD4^+$cell count-to-$CD8^+$ cell count ratio ($CD4^+$:$CD8^+$)** in blood have been detected in FIV-infected cats,[6-11] but the correlation between clinical stages and lymphocyte subsets is not clear, especially in cats naturally infected with FIV. Although histologic features of lymphoid tissues[12-14] and depletion of $CD4^+$ and $CD8^+$ cells in lymph nodes[15] provide more accurate information on disease progression, examination of lymph node biopsy specimens is not a practical method for monitoring disease progression in cats.

In HIV-infected humans, viral RNA copy number (ie, virion-associated RNA concentration) in plasma, determined by use of **quantitative competitive polymerase chain reaction (QC-PCR)**, has been correlated with disease progression and survival time. Thus, plasma viral RNA concentration represents a useful marker for clinical staging.[16,17] The effect of antiviral therapy in HIV-infected patients also has been assessed by quantification of plasma viral RNA concentrations.[18-20] Feline immunodeficiency virus RNA has been detected by viral culture of plasma[21] and by use of a nonquantitative **reverse transcription- (RT-)** PCR.[22] Recently, the QC-PCR method was used to measure plasma viral RNA concentration in cats experimentally infected with FIV.[23-25] Results of those studies indicated that viral kinetics during transition from the AP to the AC stage of FIV infection are similar to those of HIV infection. However, to our knowledge, the correlation between plasma viral RNA concentration and disease progression has not been studied during the ARC and AIDS stages or in cats naturally infected with FIV.

The purpose of the study reported here was to measure plasma virion-associated RNA concentrations (copies per milliliter) in cats during the AC, ARC, and AIDS stages of a natural FIV infection. Virion-associated RNA concentrations also were compared with total lymphocyte count, $CD4^+$ cell and $CD8^+$ cell counts, and the $CD4^+$:$CD8^+$.

Materials and Methods

Cats and clinical samples—Twenty-eight cats naturally infected with FIV and referred to the Veterinary Medical Center of the University of Tokyo for diagnosis and treatment were examined in the study. All cats were seropositive for FIV-specific antibody and seronegative for FeLV antigen.[a] Blood was collected into tubes containing sodium citrate for

flow cytometry and determination of total lymphocyte count. Plasma samples obtained by centrifugation of blood samples at 700 × g for 10 minutes were centrifuged again at 700 × g for 10 minutes to remove cells and cellular debris. Cell-free plasma samples were stored at –80 C for later determination of viral RNA concentration. Clinical status of each cat was evaluated at the time of blood collection, and the clinical stage was determined, using reported criteria.[3]

Flow cytometry—Cells in whole blood were stained directly at room temperature for 15 minutes with fluorescein isothiocyanate-labeled monoclonal antibodies against feline CD4 and CD8 molecules.[b] Whole blood then was incubated with an erythrocyte lysing solution[c] at room temperature for 15 minutes. Total number of lymphocytes, percentages of each subset (ie, $CD4^+$ and $CD8^+$ cells), and absolute number of $CD4^+$ and $CD8^+$ cells were determined by use of flow cytometry.[d]

Preparation of virion-associated RNA—Cell-free plasma samples (140 µl) from FIV-infected cats were extracted, using a commercially available RNA extraction kit,[e] to obtain samples of virion-associated RNA. Extracted RNA samples were stored at –80 C until used as wild-type template in the QC-PCR.

Synthesis of the FIV competitor RNA template—A 315-**base pair (bp)** fragment of the FIV *gag* gene, which is highly conserved among divergent viral isolates,[26] was obtained by PCR amplification of the standard FIV clone pFIV14[27,28] with primers S1 (5'-TGGAGCACCACAGTATG-TAG-3'), corresponding to nucleotides 1050 to 1069 of the pFIV14 clone representing the entire FIV genome,[27,28] and R2 (5'-TCTGCTTGTTGTTCTTGAGTT-3'), corresponding to nucleotides 1364 to 1344. The amplified fragment was shortened by restriction endonuclease digestion with *Alu*I, resulting in deletion of 99 bp corresponding to nucleotides 1163 to 1261 of pFIV14. The resultant 216-bp FIV *gag* gene fragment was inserted into a plasmid vector[f] to create plasmid pGAGΔ100. In the pGAGΔ100 plasmid, the 216-bp *gag* fragment was placed under control of the T7 promoter to allow for in vitro generation of an RNA transcript for use as a competitive template during QC-PCR (**Fig 1**). In vitro transcription was performed, using a commercially available RNA transcription kit.[g] Concentration of the competitor RNA template was determined by measuring absorbance at 260 nm in a spectrophotometer.[h] Aliquots of the template were stored at –80 C.

QC-PCR technique—Quantitative competitive polymerase chain reactions were performed, using a commercially available kit,[i] essentially in accordance with the method described by Piatak et al.[29] A 20-µl reaction mixture was used for reverse transcription of FIV RNA. Each reaction contained 2 µl of virion-associated RNA (equivalent to the amount of RNA in 5.6 ml of plasma), 1 µl of a known concentration of competitor RNA template, 2.5 m*M* random primers, 50 units of **murine leukemia virus reverse transcriptase (MuLV RT)**, 10 m*M* tris-HCl, pH 8.3, 5 m*M* MgCl, 50 m*M* KCl, 1 m*M* dNTP, and 20 units of RNase inhibitor. Separate reactions were performed with serial dilutions of competitor RNA template (10^3 to 10^8 copies/ml). Reaction mixtures were incubated at 42 C for 45 minutes, and MuLV RT was subsequently inactivated at 99 C for 5 minutes. After reverse transcription, the sample (20 µl) was added to 80 µl of PCR solution containing 2.5 units of *Taq* DNA polymerase, 1 µg each of primers S1 and R2, 10 m*M* tris-HCl (pH 8.3), 2 m*M* MgCl, and 50 m*M* KCl. The S1 and R2 primers were expected to allow for amplification of a 315-bp fragment from cDNA reverse transcribed from wild-type FIV RNA and a 216-bp fragment from cDNA reverse transcribed from the RNA competitor template. Amplification was performed in a thermocycler[j]; an initial denaturation step (94 C for 2 minutes) was followed by 40 cycles of denaturation (94 C for 1 minute) and primer annealing and extension (60 C for 1 minute) and a final extension step (72 C for 7 minutes).

One-tenth of the volume of each QC-PCR mixture was electrophoresed in a 3% agarose gel and stained with ethidium bromide (0.5 µg/ml) for detection of amplified bands under UV light (**Fig 2**). Band density was analyzed by densitometry. The intensity of the fluorescence of the 315-bp band derived from wild-type FIV and the 216-bp band derived from the competitor RNA template were analyzed and compared. Concentration of viral RNA in each plasma sample was calculated by use of standard regression analysis of the known concentration of competitive RNA template versus the molar equivalent of the wild-type amplified band.

To ensure repeatability of the results of the QC-PCR, plasma viral concentration in each sample was measured at least 3 times. The QC-PCR was repeated until the variation

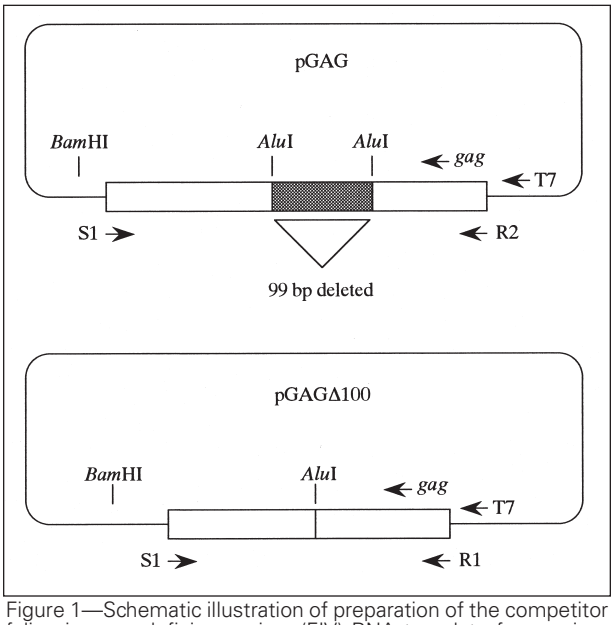

Figure 1—Schematic illustration of preparation of the competitor feline immunodeficiency virus (FIV) RNA template for use in a quantitative competitive polymerase chain reaction (QC-PCR) assay. Ninety-nine base pairs (bp) of a 315-bp portion of the FIV *gag* gene (in the pGAG plasmid) were deleted by restriction endonuclease digestion with *Alu*I. The resultant 216-bp competitor fragment was cloned into a vector under the control of the T7 promoter (plasmid pGAGΔ100). The RNA transcript derived from this fragment was used as a competitor RNA template for QC-PCR.

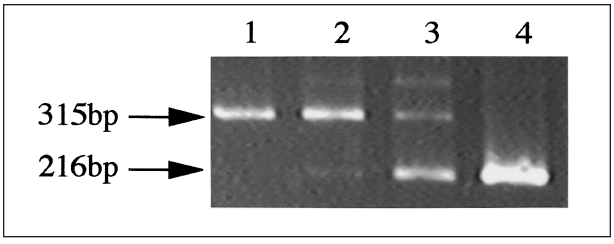

Figure 2—Ethidium bromide-stained 3% agarose gel of QC-PCR amplification of a portion of the FIV *gag* gene. Each lane contains the amplified product from RNA isolated from 5.6 ml of plasma from an FIV-infected cat (expected size, 315 bp) and the amplified product from a known amount of competitor RNA (expected size, 216 bp; lane 1, 5×10^4 copies; lane 2, 10^5 copies; lane 3, 10^6 copies; lane 4, 10^7 copies). Competitor RNA at a concentration of 10^5 copies/reaction is equivalent to 1.79×10^7 copies of wild-type RNA/ml of plasma. *See* figure 1 for key.

of results among analyses was less than 2-fold. Amplification of cDNA reverse-transcribed from serially diluted competitor RNA template and amplification of reactions that did not contain template were used as positive- and negative-control samples, respectively.

Statistical analyses—Mean lymphocyte counts were compared between each group by the use of unpaired Student *t*-tests. Plasma concentration of viral RNA was compared between groups by use of the Mann-Whitney rank sum test. Correlations between plasma viral RNA concentration and total lymphocyte, $CD4^+$ and $CD8^+$ cell counts, and $CD4^+$:$CD8^+$ were analyzed, using the Spearman rank-order correlation test. Significance was set at $P \leq 0.05$.

Results

Clinical findings—Eleven of 28 (39%) FIV-infected cats did not have clinical signs associated with FIV infection; these cats were in the AC stage. Cats in this stage were between 6 months and 16 years old. Eight were males (5 castrated, 3 sexually intact), and 3 were females (2 spayed, 1 sexually intact). Nine (32%) of the infected cats (6 males [3 castrated, 3 sexually intact], 3 females [2 spayed, 1 sexually intact]) had recurrent infections; these cats were in the ARC stage. Cats in this stage were between 3 and 15 years old. The most common clinical signs were chronic stomatitis and gingivitis with or without weight loss (n = 7). One of these 7 cats also had tonic convulsions. Moderate neutropenia (1,500 to 2,000 cells/µl) was detected in 1 of the 9 cats with ARC, and 1 cat was being treated because of recurrent pyoderma. Of 28 cats, all 8 (29%) categorized with AIDS had clinical signs attributable to profound immunodeficiency. Cats in this group were between 3 and 12 years old. Five were castrated males, and 3 were females (2 spayed, 1 sexually intact). Severe stomatitis that was refractory to treatment and myelosuppression (ie, pancytopenia) was detected in 3 of these 8 cats. One of the 8 cats with AIDS had an abdominal mass and suppurative peritonitis, 1 had multiple abscesses, 1 had pulmonary cryptococcosis, 1 had diarrhea, severe weight loss, abnormal neurologic signs, and intermittent tonic convulsions, and 1 had lymphoma and a periocular mass.

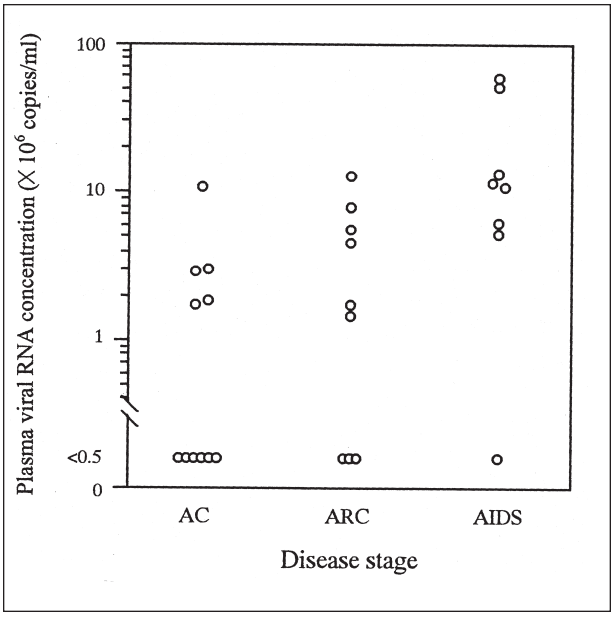

Figure 3—Concentration of viral RNA, determined by use of a QC-PCR assay, in 28 cats naturally infected with FIV. Disease stages were assigned on the basis of clinical signs. Each point represents the value for 1 cat. The threshold sensitivity for the QC-PCR assay was 5×10^5 copies/ml of plasma. AC = Asymptomatic (nonclinical) carrier. ARC = Acquired immunodeficiency syndrome-related complex. AIDS = Acquired immunodeficiency syndrome. *See* Figure 1 for remainder of key.

Total and differential lymphocyte counts—Five of the 8 cats with AIDS were lymphopenic (ie, total lymphocyte count less than the lower limit of the reference range for healthy cats[30] [1,500 to 7,000 lymphocytes/µl]), and 2 were not; lymphocyte counts were not determined for 1 cat with AIDS. On the other hand, 10 of the 11 AC cats and 7 of the 9 cats with ARC had total lymphocyte counts within the reference range. However, mean (± SD) total lymphocyte counts were not significantly different among the 3 clinical stages (AC stage, 2,590 ± 1,330 cells/µl; ARC stage, 2,220 ± 1,180 cells/µl; and AIDS stage, 1,360 ± 1,040 cells/µl). The $CD4^+$ cell counts for cats in the AC, ARC, and AIDS stages were 671 ± 432, 277 ± 136, and 147 ± 103 cells/µl, respectively. Cats with AIDS had a significantly lower mean $CD4^+$ cell count than AC cats. Additionally, $CD8^+$ cell counts were significantly decreased in cats with AIDS (162 ± 70.3 cells/µl), compared with AC cats (492 ± 314 cells/µl) and cats with ARC (653 ± 416 cells/ml). The $CD4^+$:$CD8^+$ decreased during the ARC stage (0.476 ± 0.159), compared with the AC stage (1.45 ± 0.527), and increased again during the AIDS stage (0.868 ± 0.255); differences in $CD4^+$:$CD8^+$ differed significantly between each pair of groups.

Plasma viral concentration—We evaluated several plasma samples that contained detectable amounts of viral RNA and determined that the threshold sensitivity of QC-PCR was 2.5×10^3 copies/5.6 µl of plasma. This corresponded to 5×10^5 copies/ml of plasma. Plasma viral RNA was detected in 18 of 28 (64%) naturally infected cats but was below the detection limit in 10 (36%) cats (**Fig 3**). Viral RNA was detected in the plasma of 7 of 8 cats in the AIDS stage (range, 5.20×10^6 to 5.80×10^7 copies/ml), 6 of 9 cats in the ARC stage (range, 1.45×10^6 to 1.28×10^7 copies/ml), and 5 of 11 cats in the AC stage (range, 1.71×10^6 to 1.07×10^7 copies/ml). Plasma viral RNA concentrations in cats in the AIDS stage were significantly higher than those in cats in the AC ($P = 0.006$) and ARC ($P = 0.042$) stages. There was not a significant correlation between plasma viral RNA concentration and total lymphocyte, $CD4^+$cell count, $CD8^+$ cell count, or $CD4^+$:$CD8^+$ ($r^2 < 0.1$).

Discussion

Evaluation of plasma viral RNA concentration is known to be useful for predicting clinical outcome and determining treatment efficacy in humans infected with HIV.[16,20,29] Results of reports indicate that the kinetics of plasma viral load during FIV infection resembled those during HIV infection; plasma FIV RNA concentration reached an initial peak in the AP stage and

gradually decreased in the AC stage in cats experimentally infected with FIV.[20,25,31] Plasma viral RNA concentration was expected to increase again as cats progressed to AIDS. Results of the study reported here supported this expectation. We found that the mean plasma viral RNA concentration in cats in the AIDS stage was significantly higher than those in cats in the AC and ARC stages. The range in viral RNA concentration (5×10^6 to 6×10^7 copies/ml) detected in cats in the AIDS stage was comparable to those in HIV-infected humans with AIDS.[16,17,20,32-35] Furthermore, within 1 year after the study reported here was completed, disease progressed rapidly in 4 cats with ARC; plasma viral RNA concentration (range, 1.45×10^6 to 8.02×10^6 copies/ml) was high in all 4 cats. These findings indicated that, similar to HIV-infected humans,[17,36] plasma viral RNA concentration increases in naturally FIV-infected cats as disease progresses from ARC to AIDS.

We could not detect viral RNA in plasma of 6 of 11 cats in the AC stage. Sensitivity of the QC-PCR method used in our study was approximately 5×10^5 copies/ml of plasma, which was less than that reported in other studies.[23,25,29] Vahlenkamp et al[25] detected low plasma viral RNA concentrations ranging from 10^3 to 10^5 copies/ml in cats experimentally infected with FIV that did not have clinical signs of disease, but their data cannot easily be compared with ours. Number of plasma RNA copies obtained in several laboratories are not always identical because of differences in methods used to assess RNA concentration.[37] From data obtained in the study reported here, it is conceivable that FIV RNA concentration is less during the AC stage than the AP, ARC, and AIDS stages. To better evaluate the actual plasma viral RNA concentration in naturally FIV-infected cats during the AC stage, more sensitive methods such as the real-time quantitative PCR should be used.

Five of 11 cats in the AC stage had plasma viral RNA concentrations higher than 10^6 copies/ml, although their immunologic status was apparently not severely impaired. In humans infected with HIV, viral concentrations in those individuals whose disease rapidly progresses (ie, rapid progressors) were considerably higher than those in long-term survivors.[38] In cats infected with FIV, a high plasma viral concentration during the early infection stage predicts rapid disease progression.[24] Differences in host immune responses in FIV-infected cats have not been clearly identified. However, plasma viral concentrations are lower after virus challenge in cats vaccinated against FIV than in nonvaccinated cats, indicating the effects of host immune responses on viral replication.[39] Results of another study suggest that reduced $CD8^+$ cell counts may influence neurovirulence of FIV.[9] On the other hand, pathogenic potential may be primarily a result of viral strain.[40-42]

Five of 7 cats with AIDS were lymphopenic. Lymphopenia is a common hematologic abnormality in FIV-infected cats, especially in cats with long-term natural infection.[43,44] In cats in the ARC and AIDS stages, histologic examination revealed the involution or depletion of lymph nodes.[12-14] Endo et al[15] identified that a decrease in $CD4^+$ and $CD8^+$ ymphocytes in lymph nodes was associated with disease progression to AIDS. Although lymphocyte depletion has been detected in cats in advanced clinical stages of FIV infection,[12,14] there was not a significant correlation between total lymphocyte count and clinical stage of disease (Student *t*-test) or plasma viral RNA concentration (Spearman rank-order correlation).

In the study reported here, $CD4^+$ cell and $CD8^+$ cell counts in cats with AIDS were significantly less than those in AC cats. A selective loss of $CD4^+$ cells has been reported during FIV and HIV infections.[6,10,11] In humans infected with HIV, a decrease in $CD4^+$ cell count was frequently correlated with high viral concentrations and disease progression.[16,17,20] A decrease in viral concentration as a result of treatment resulted in an increase in $CD4^+$ cell count.[20,45] In FIV-infected cats, results of reports have indicated a decrease in the percentage and absolute number of circulating $CD4^+$ cells with or without an increase in the $CD8^+$ cell count.[6,10,11] Our data agree with those results. The $CD4^+$ and $CD8^+$ cell counts decreased in cats in the AIDS stage, and the $CD8^+$ cell count increased slightly in cats in the ARC stage, although a close correlation between these variables and plasma viral RNA concentration was not detected.

The $CD4^+$:$CD8^+$ decreased during the ARC stage, compared with the AC stage, and increased again during the AIDS stage. In cats experimentally infected with FIV, Ackley et al[6] detected a decrease in $CD4^+$ cell count and an increase in $CD8^+$ cell count, compared with noninfected cats, whereas Torten et al[10] detected a decrease in $CD4^+$ cell count without an increase in $CD8^+$ cell count. In another study,[46] we did not find a significant difference in $CD4^+$:$CD8^+$ between cats naturally infected with FIV (primarily in the ARC stage) and cats seronegative for FIV-specific antibody. In the study reported here, low $CD4^+$ and high $CD8^+$ cell counts in cats in the ARC stage resulted in a decrease in $CD4^+$:$CD8^+$, compared with cats in the AC stage, and low $CD4^+$ and $CD8^+$ cell counts in cats in the AIDS stage resulted in an increase in $CD4^+$:$CD8^+$, compared with cats in the ARC stage. In these naturally FIV-infected cats, $CD4^+$ and $CD8^+$ cell counts in AIDS stage were significantly lower, compared with those in AC stage (unpaired Student *t*-test), but values varied among cats, especially those in the AC stage. Although lymphocyte subset counts do not always represent clinical stage in FIV-infected cats, they can be good indicators of clinical stage and prognosis when considered in combination with clinical signs and plasma viral RNA concentration.

[a]Snap FeLV/FIV combo, IDEXX Laboratories, Portland, Me.
[b]Mouse monoclonal anti-CD4, CD8 (FITC-labeled), Southern Biotechnology Associates, Birmingham, Ala.
[c]Cal-Lyse Lysing Solution, Caltag Laboratories, Burlingame, Calif.
[d]Cyto-ACE 150, Japan Spectroscopic, Tokyo, Japan.
[e]QIAamp viral RNA kit, Qiagen, Studio City, Calif.
[f]pCR 2.1, Invitrogen, San Diego, Calif.
[g]RNA transcription kit, Stratagene, La Jolla, Calif.
[h]UV-160A, Shimadzu, Kyoto, Japan.
[i]RNA PCR kit, Perkin Elmer, Norwalk, Conn.
[j]Takara Thermal Cycler MP, Takara, Kyoto, Japan.

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