JOURNAL OF CLINICAL MICROBIOLOGY, May 2010, p. 1667–1672 Vol. 48, No. 5
0095-1137/10/$12.00 doi:10.1128/JCM.00227-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Dual-Emission Fluorescence Resonance Energy Transfer (FRET) Real-Time PCR Differentiates Feline Immunodeficiency Virus Subtypes and Discriminates Infected from Vaccinated Cats▽§

Chengming Wang,† Calvin M. Johnson, Sudhir K. Ahluwalia, Erfan Chowdhury, Yihang Li, Dongya Gao, Anil Poudel, K. Shamsur Rahman, and Bernhard Kaltenboeck*

Department of Pathobiology, College of Veterinary Medicine, Auburn University, 270 Greene Hall, Auburn, Alabama 36849-5519

Received 3 February 2010/Returned for modification 5 March 2010/Accepted 15 March 2010

Feline immunodeficiency virus (FIV) is among the most common infectious agents of cats. Five well-characterized FIV subtypes, A, B, C, D, and E, are recognized worldwide. As in HIV diagnosis, serum antibodies against FIV classically serve as an indicator of infection status. After the introduction of an inactivated FIV vaccine, this approach has become problematic, since antibodies generated by vaccination are indistinguishable from antibodies in response to infection. However, PCR detection of host-cell-integrated FIV DNA will differentiate infection-derived antibody from vaccination-derived positivity because presumably the RNA of inactivated vaccine virus will not integrate into the host genome. In this study, we established a *gag* gene-based dual-emission fluorescence resonance energy transfer (FRET) real-time PCR that amplifies single-target copies of all known FIV strains and differentiates five FIV subtypes. All blood samples from experimentally FIV-infected cats ($n = 5$) were antibody positive and highly positive in the FIV PCR. In contrast, nine cats became antibody positive after FIV vaccination but remained negative in the FIV PCR. Of 101 FIV antibody-positive feline blood specimens submitted for FIV PCR diagnosis, 61 were positive (60%). A total of 23 of the positive PCRs identified subtype A, 11 identified subtype B1, 11 identified subtype B2/E, and 16 identified subtype C. FIV subtype D was not detected in any submitted specimens even though 13 blood specimens were from cats known to have received the FIV vaccine, which contains FIV subtype A and D inactivated virions. Therefore, this PCR quantitatively identifies FIV subtypes and unambiguously discriminates between FIV-vaccinated and FIV-infected cats.

Feline immunodeficiency virus (FIV), first reported in 1987 after isolation from a cat in California (17), has been recognized as a common feline retroviral pathogen worldwide (6, 8, 10, 11, 13, 17). FIV is also the only lentivirus that causes an immunodeficiency syndrome resembling that of HIV infection in the natural host, and it has served as a powerful model for HIV infection (23). The envelope (*env*) gene encompasses the epitopic determinants for cellular tropism and neutralization of FIV (14, 15). Based on *env* gene polymorphisms, FIV isolates have been divided into five well-characterized subtypes worldwide (14, 15, 20). Recent studies have demonstrated, however, that the phylogenetic trees constructed from the capsid (*gag*) and *env* genes reveal a virtually identical subtype classification (19, 22).

Isolation of FIV from peripheral blood lymphocytes is a reliable and definitive diagnostic test, but this test is too cumbersome, expensive, and impractical for routine use (8, 9, 11). FIV serology such as Western blot analysis detects antibodies recognizing viral structural proteins and is considered the "gold standard" for FIV diagnosis (7, 8, 9, 11). One recent challenge in FIV diagnostics is that a veterinarian cannot rely on the presence of antibody as an indicator of infection status in vaccinated cats (11). For presumable protection against FIV, the Fel-O-Vax FIV vaccine (Fort Dodge Animal Health, Fort Dodge, IA) has been marketed since 2002 in the United States and several other countries. This is a formalin-inactivated whole-virus vaccine containing partially purified FIV subtype A (Petaluma) and subtype D (Shizuoka) virus. Antibodies generated by vaccination are indistinguishable from antibodies in response to infection.

One alternative to serological diagnosis of FIV infection is detection by PCR, a sensitive and specific assay to identify FIV proviral DNA integrated into the genome of infected cells. In this study, we developed a novel *gag*-targeted dual-emission fluorescence resonance energy transfer (FRET) PCR that amplifies the target sequence of all FIV strains and differentiates FIV subtypes A, B1, C, D, and B2/E (which have identical target sequence). Furthermore, application of this PCR to blood samples from vaccinated and experimentally infected cats demonstrated that the blood samples of FIV-vaccinated cats remain fully negative by FIV PCR, while they become highly positive in infected cats. This FIV PCR reliably detects and quantifies FIV infection, identifies the infecting FIV subtypes, and thus unambiguously discriminates between FIV-vaccinated and FIV-infected cats. In light of the potential future vaccination against AIDS (18, 23), similar problems may develop in HIV diagnosis, and an approach similar to the one presented in this investigation may also be applicable.

MATERIALS AND METHODS

Experimental animals, FIV vaccination, and FIV challenge infection. All animal procedures in this study were approved by the Auburn University Institu-

* Corresponding author. Mailing address: Auburn University, Department of Pathobiology, College of Veterinary Medicine, 270 Greene Hall, Auburn, AL 36849-5519. Phone: (334) 844-2665. Fax: (334) 844-2652. E-mail: kaltebe@auburn.edu.

† Present address: School of Veterinary Medicine, Ross University, P.O. Box 334, Basseterre, St. Kitts, West Indies.

§ This is publication number 9 from the Auburn University Molecular Diagnostics Laboratory.

▽ Published ahead of print on 24 March 2010.

TABLE 1. Oligonucleotide primers and probes used in this study

Primer/probe	Sequence (5′→3′)[a]
Upstream primer	ATGGGGAAYGGACAGGGGCGAGA
Downstream primer	TCTGGTATRTCACCAGGTTCTCGTCCTGTA
F1-ABCE	(6-FAM)-TACTCTTSSCCCCTACTCCTACAGCA-(6-FAM)
F1-D	(6-FAM)-TACTTCGTTGTCCCGTACCTACAGCA-(phosphate)
F2-ABCE	(LC Red 640)-CATTACTACATCTYTTWATGGCCAYTTTCCA-(phosphate)
F3-D	CCATCTAAAATTTCCTTCCCCGAACTTC-(Cy5.5)

[a] Y represents C or T, R represents A or G, S represents C or G, and W represents A or T.

tional Animal Care and Use Committee. Cats were anesthetized for blood collection with an intramuscular injection of medetomidine (Domitor, 0.02 mg/kg) and butorphanol tartrate (Torbugesic, 0.2 mg/kg).

Nine specific-pathogen-free (SPF) cats received three doses of the licensed Fel-O-Vax FIV vaccine (Fort Dodge Animal Health, Wyeth, Madison, NJ) at 2-week intervals by subcutaneous injection on the dorsolateral aspect of the thorax. Whole-blood samples were collected from each cat by jugular vein puncture prior to the first vaccination and 2 weeks after the third vaccination.

FIV challenge infection in cats was performed as described previously (3). Five cats were challenged intraperitoneally with 200 μl of cell culture supernatant containing 10^4 50% tissue culture infective doses ($TCID_{50}$) of virions of the wild-type FIV clone JSY3 (3). In addition, five cats were administered a sham inoculum consisting of 200 μl sterile saline solution. Presence or absence of FIV infection was confirmed by reisolation of the virus from peripheral blood on the feline $CD4^+$-lymphoblast CRL-2417 cell line (3). Blood was collected before the injection and 9 weeks after the challenge inoculation.

Detection of antibodies against FIV by SNAP assay. The SNAP FIV/feline leukemia virus (FeLV) combo test (IDEXX Laboratories, Westbrook, ME) was performed according to the manufacturer's instructions (http://idexx.com/view/xhtml/en_us/smallanimal/inhouse/snap/feline-combo.jsf?selectedTab=Resources#tabs) with plasma specimens.

Design of primers and probes for FIV real-time PCR. All oligonucleotides were designed by use of the Vector NTI software (Invitrogen Corporation, Carlsbad, CA). All available FIV nucleotide sequences with designated FIV subtypes were obtained from GenBank (16, 19, 22). The complete set of sequences was aligned as nucleotide as well as translated amino acid sequences for identification of maximally conserved regions of the FIV genome. This approach clearly identified the *gag* gene, in particular its upstream region, as the least polymorphic region of the FIV genome and, thus, as the best target for PCR detection of all FIV variants. In the final analysis, the first 164 bp of the *gag* coding sequence provided the optimal target, and the final primer sequences were designed for maximum statistical fit based on the polymorphisms of all available FIV *gag* sequences (Table 1 and Fig. 1). Comparison of the nucleotide alignments of all FIV *gag* sequences and of those for which the subtype information was available revealed identical polymorphisms within the PCR target region. Thus, the known subtypes were representative of overall FIV wild-type polymorphisms in the *gag* target region. These unavoidable nucleotide substitutions presented a challenge in probe design but also an opportunity for differentiation of the amplification products. Steinrigl and Klein (19) and Weaver et al. (22) recently showed conclusively that *gag* polymorphisms are concordant with subtype-determining polymorphisms of the FIV *env* gene (19, 22). Thus, variations in the *gag* amplification target could serve as nucleotide signatures for FIV subtypes in mismatch-dependent differential melting profiles of FIV-*gag* FRET probes (Fig. 1).

The probes were designed in the noncoding sense region to avoid interference with amplification, since they are located close to the 5′ end of the amplification product. All probes were synthesized by Integrated DNA Technologies (Coralville, IA). LC Red 640- and Cy5.5-labeled probes were high-performance liquid chromatography (HPLC) purified; 6-carboxyfluorescein (6-FAM)-labeled probes were used unpurified. Since FIV subtype D is substantially different from all other subtypes, a two-channel probing approach was used to detect subtypes A, B, C, and E by LC Red 640 fluorescence in channel 2 (F2, λ = 640 nm), generated by probes F1-ABCE and F2-ABCE (Fig. 1). F1-ABCE is 6-FAM

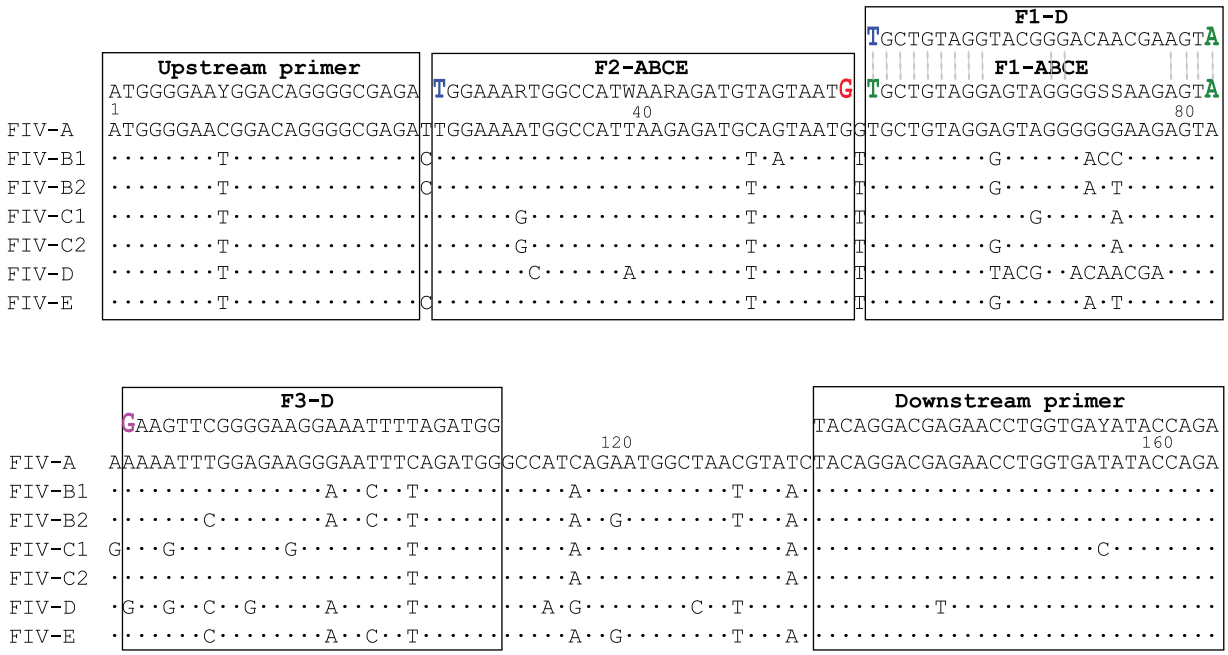

FIG. 1. Alignment of *gag* amplification targets of FIV subtypes A to E. Dots indicate nucleotides identical to those of FIV-A. Primers and probes are shown as uppermost boxed sequences. The upstream primer is used as shown, while the downstream primer and all probes (F1-ABCE, F1-D, F2-ABCE, and F3-D) are used as antisense oligonucleotides. The upstream and downstream primers were designed to hybridize at the 5′ end of the *gag* gene, the most conserved region of the FIV genome, while the probes hybridize to the polymorphic regions of the *gag* amplification target and were chosen for maximum differentiation of FIV subtypes. Y represents C or T, R represents A or G, S represents C or G, and W represents A or T. Nucleotides in blue denote the attachment of a phosphate group to the 3′ terminus of a probe, nucleotides in green indicate 6-FAM (= F1) attachment at the 3′ or 5′ terminus, red indicates LC Red 640 (= F2) attached to the 5′ terminus, and purple indicates Cy 5.5 (= F3) attached to the 3′ terminus.

labeled at both the 5′ and 3′ ends and thus also generates a FRET signal with the Cy 5.5-labeled downstream F3-D probe in fluorescence channel 3 (F3, λ = 705 nm). This probe also generates a signal for subtype D with 6-FAM-labeled probe F1-D that hybridizes at the 58°C fluorescence acquisition temperature only with subtype D and not any other subtype because of 10 or 11 mismatches to all other subtypes (Fig. 1). F1-ABCE is designed to hybridize with the homologous sequence of all other subtypes but has 11 mismatches to D; thus, the probes are sufficiently different so as not to compete with each other.

Extraction of nucleic acids. Total nucleic acid extraction was performed by glass fiber matrix binding and elution with the High-Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, IN) as described before (5). For each sample, 400 μl EDTA-whole blood was mixed with an equal volume of binding buffer (6 M guanidine-HCl, 10 mM urea, 20% [vol/vol] Triton X-100, 10 mM Tris-HCl, pH 4.4), and eluted in 40 μl elution buffer.

Real-time PCR, color compensation, and melting curve analysis. Nucleotide fragments representing the first 164 bp of the *gag* regions of FIV subtypes A, B1, B2/E, C1, C2, and D were synthesized and inserted in the pIDTSMART cloning vector (Integrated DNA Technologies, Coralville, IA). For use as standard templates, the plasmid was restricted with HindIII (Promega, Madison, WI), followed by inactivation of the restriction enzyme at 65°C for 20 min. DNA was quantified by PicoGreen DNA fluorescence assay (Molecular Probes, Eugene, OR) for preparation of quantitative standards (Fig. 2).

The FIV copy number was determined by FRET-PCR performed on a LightCycler 1.5 real-time PCR platform with software version 3.53 (Roche Molecular Biochemicals, Indianapolis, IN) (5, 21). The amplification of single target molecules was verified by limiting dilution of the targets. At nominal single copies per PCR, a Poisson sampling error was observed because only about 70% of the PCRs gave positive results. For the positive PCRs, the linear portion of the amplification curve showed the same slope as that for the PCRs with high template input. These data established that the PCR was equally effective at single-copy input and verified the robust ability of the PCR to detect single-target copies. The melting curve for the annealing of the PCR product was determined by monitoring the fluorescence from 35°C to 75°C with a temperature transition rate of 0.2°C per second (12). The fluorescence ratios F2/F1 and F3/F1 were analyzed, and the first derivatives of F2/F1 and F3/F1 were evaluated for determination of probe T_m (Fig. 3). Fluorescence signals were corrected for spillover from shorter-wavelength emission by a color compensation file that was created according to LightCycler 1.5 instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Specificity of the PCRs was confirmed by amplification of the synthesized target genes of FIV subtypes A, B1, C1, C2, D, and B2/E (Fig. 2) and by amplification of nucleic acids extracted from the Fel-O-Vax FIV vaccine, which contains subtypes A and D. PCR products were verified by automated DNA sequencing of both strands at the Genomic Sequencing Laboratory (Auburn University, Auburn, AL) using the amplification primers (Table 2).

Clinical specimens for FIV diagnosis. Between 2008 and July 2009, 101 specimens from 67 veterinary clinics/hospitals in 24 U.S. states were submitted to the Molecular Diagnostics Laboratory for FIV detection (Table 1). These samples were shipped as EDTA-whole blood at room temperature without prior freezing or refrigeration.

Data analysis. All statistical analyses were performed with the Statistica 7.1 software package (StatSoft, Inc., Tulsa, OK). FIV target numbers in PCR were logarithmically transformed. Data are shown as means ± 95% confidence intervals (CI).

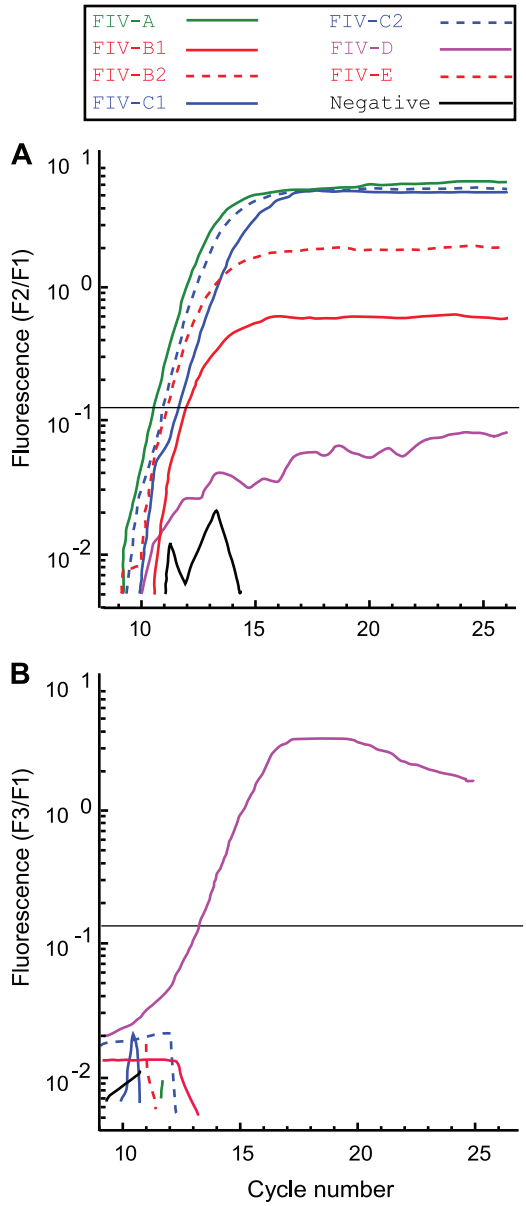

FIG. 2. PCR amplification curves of FIV subtypes. Each subtype template (100 to 1,000 copies) was amplified in a background of 200 ng salmon sperm DNA. Fluorescence channel F2 normalized by F1 (F2/F1) shows only the amplification curves of FIV subtypes A, B, C, and E (A), while fluorescence F3/F1 shows only the FIV-D amplification curve (B).

RESULTS

Establishment of a generic and differential FIV PCR. Our optimized FIV PCR was able to detect single copies of FIV subtypes A, B1, B2/E, C1, C2, and D. The amplification curves of subtypes A, B1, B2/E, C1, and C2 appeared only in fluorescence channel F2 (or as a ratio of results for channels F1 and F2 [F2/F1]; Fig. 2A), while the amplification curve of FIV-D appeared only in channel F3 (or F3/F1; Fig. 2B). Melting curve analysis following the completion of the PCR provided a higher level of differentiation. In the display of F2/F1, the mismatches between probes F1-ABCE and F2-ABCE and the PCR amplicon resulted in higher T_ms of subtypes A and C1 (T_m = 60°C) and C2 (T_m = 58°C) than of subtypes B1 and B2/E (T_m = 54°C) (Table 1; Fig. 1; Fig. 3A). In F3/F1, the mismatches between probes F1-D and F3-D and amplicons resulted in distinct T_ms of subtypes A (45°C), B1 (49°C), B2/E (52°C), C1 (54°C), C2 (54.5°C), and D (68°C) (Table 1; Fig. 1; Fig. 3B).

FIV antibody positivity and *gag* copies in FIV-vaccinated and FIV-challenged cats. Prior to vaccination or challenge infection, all cats were negative for serum antibodies against FIV (Fig. 4A) and also negative in the FIV PCR (Fig. 4B). FIV antibodies were detectable after vaccination or challenge infection of cats but not after sham inoculation with saline (Fig. 4A). The FIV PCR became positive after challenge infection of cats but not after vaccination or sham inoculation (Fig. 4B).

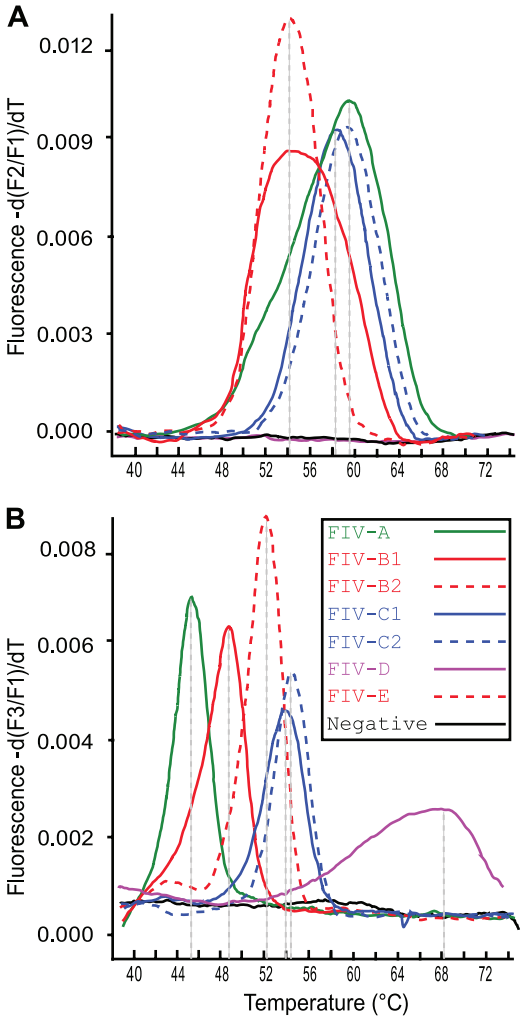

FIG. 3. T_m discrimination of FIV subtypes. After completion of the PCR, the melting temperature (T_m) of probe hybridization to the targets was determined by melting curve analysis as the peak of the second derivative of the fluorescence released during a temperature increase from 35°C to 75°C. (A) In channel F2/F1, subtypes A and C can be discriminated by their higher T_ms (58°C to 60°C) from those of the B subtype (T_m = 54°C) and the D subtype (no melting peak). (B) Fluorescence F3/F1 demonstrates distinct T_m differences between FIV-A (45°C), FIV-B1 (49°C), FIVB2/E (52°C), FIV-C (55°C), and FIV-D (68°C) subtypes.

PCR showed approximately 3×10^7 FIV copies per dose of vaccine. The melting curve analysis demonstrated distinct dual peaks for subtypes A and D in the FIV vaccine. Sequencing results of the PCR products confirmed the subtyping results obtained by melting curve analysis. Collectively, these data unambiguously prove that blood samples from FIV-vaccinated cats remain negative by PCR assay of blood samples. Thus, positivity in the FIV PCR of whole-blood samples indicates FIV infection but not FIV vaccination.

PCR diagnosis of clinical specimens. Between January 2008 and July 2009, 101 whole-blood samples from 67 veterinary clinics/hospitals in 24 U.S. states were submitted to the Auburn University Molecular Diagnostics Laboratory for FIV diagnosis (Table 2). All samples were submitted because the patient-side SNAP test for FIV antibody detection had been positive, and PCR was required to verify the FIV infection status of the cats. In the FIV PCR, 61 of these samples (60%) were confirmed as positive. Among the FIV-positive samples, 23 (38%) contained subtype FIV A, 11 (18%) contained subtype B1, 11 (18%) contained subtype B2/E, and 16 (26%) contained subtype C (Table 2). The data further indicate an easily detectable, very high copy number of FIV targets in all positive specimens that did not differ significantly for individual FIV subtypes (Table 2). FIV-D was not detected in any submitted

TABLE 2. Summary of FIV-PCR data of clinical specimens

Subtype	No. of positive specimens[a]	No. of FIV copies/ml blood[b]
FIV-A	23	40,422 ± 81,129
FIV-B1	11	42,653 ± 227,442
FIV-B2/E	11	78,439 ± 281,108
FIV-C	16	21,301 ± 47,908
FIV-D	0	

[a] Of 101 submitted clinical specimens, 61 (60.4%) were FIV positive.
[b] Shown as geometric mean ± 95% CI. The copy numbers per ml blood do not differ significantly.

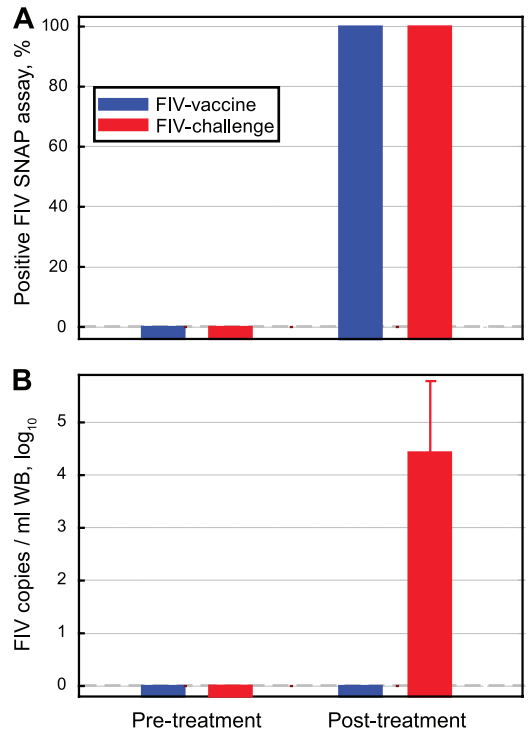

FIG. 4. FIV antibody positivity and *gag* copies in FIV-vaccinated and FIV-challenged cats. SPF cats received three FIV vaccinations at 2-week intervals (n = 9) or were intraperitoneally challenged with 10^4 $TCID_{50}$ of the wild-type FIV clone JSY3 (n = 5). Whole-blood (WB) specimens were collected from all animals before and 9 weeks after treatment. (A) The SNAP assay result for detection of FIV antibodies was negative in all cats prior to treatment and became positive in all nine FIV-vaccinated cats and five FIV-challenged cats. (B) The FIV-PCR for detection of the FIV-*gag* fragment was negative in all cats prior to treatment and remained negative in all FIV-vaccinated cats but became highly positive in all five FIV-challenged cats (26,953 ± 163,296 copies/ml blood, 95% CI).

specimens even though 13 of the submitted samples were known to be from cats that had received the Fel-O-Vax FIV vaccine containing FIV-D. These results confirm the experimental data indicating that FIV-vaccinated cats remain negative for FIV genomes integrated into host DNA. In addition, this FIV subtype survey of infected cats demonstrates that there is no dominant FIV subtype but that subtypes A to C infect cats in the United States in approximately equal proportions. The lack of a difference between copy numbers of FIV targets further suggests that these FIV subtypes are similarly host adapted and confirms that no subtype dominates the epidemiological picture of FIV infection in the United States.

DISCUSSION

PCR has been pursued as the main alternative test to confirm FIV infection in cats, since the standard antibody-dependent diagnosis does not discriminate between FIV-vaccinated and -infected cats. To evaluate the sensitivity and specificity of commercially available FIV PCR tests, Crawford et al. (4) collected blood samples from cats that were either confirmed free of FIV infection ($n = 42$) or naturally or experimentally FIV infected ($n = 41$). The infection status was confirmed by virus isolation, and blinded samples were submitted to three reference diagnostic laboratories in the United States and Canada. For the noninfected cats, three of four PCR tests were FIV positive in 19% of the submitted samples (81 to 100% specificity). Of the infected cats, 41 to 93% were FIV positive (41 to 93% sensitivity) in the four different PCR tests. Thus, the combined specificity and sensitivity of currently available FIV PCR tests result in a predictive accuracy that is essentially equivalent to flipping a coin. Differences in PCR design and quality of PCR execution or prevention of carry-over contamination may explain the markedly varied specificity and sensitivity of these FIV PCR tests that are clearly not suitable for diagnostic use.

This investigation describes a real-time PCR method for all known FIV strains that identifies FIV genotypes by differential melting temperatures of partially mismatched FRET probes at two fluorescence emission wavelengths. This strategy is superior to that of multiplex PCR, which typically results in reduced sensitivity. In clinical specimens, the ratio of multiple targets is usually unknown, and significant reduction of PCR sensitivity is seen when the copy number of one target is much higher or lower than that of another target (21).

The specificity and sensitivity of a diagnostic PCR system is largely determined by the quality of the primer and probe design. This is particularly critical for viruses with a high mutation rate, such as FIV. The primers should be designed to amplify all FIV strains, which show as much as 26% polymorphism in their *env* and *gag* genes (1, 19, 22). To amplify all FIV strains, it is extremely critical to design primers that anneal to the conserved sequences. BLAST searches identified 76 FIV hits for the upstream and 80 hits for the downstream primer, both with at most one mismatch. The next-best matches were from other organisms but had at least five mismatches at the 3′ end. This result allows the conclusion that the primers as designed for this PCR, with degenerate positions at the site of potential FIV mismatches, can amplify all known FIV strains.

In real-time detection by hybridization probes, it is equally critical to design these probes such that they tolerate several mismatches within their target sequences inside the amplicon. In this study, FIV subtype A showed a T_m of 45°C in F3/F1 even though there are six nucleotide mismatches between the 28-bp F3-D probe and the corresponding FIV-A amplicon region (Fig. 1; Table 1). Thus, even if no real-time signal during thermal cycling is observable in F3/F1 because the probe does not anneal at the 58°C acquisition temperature (Fig. 2B), the specific amplification product is revealed and differentiated in F3/F1 by melting curve analysis (Fig. 3B).

The commercial FIV vaccine is composed of inactivated whole virus, and presumably vaccine-derived FIV RNA will be removed quickly from circulation without integration as DNA into the host genome. FIV vaccination will therefore not result in PCR amplification. This hypothesis was unambiguously confirmed in the current study with the survey of experimentally vaccinated and infected cats (Fig. 4). Furthermore, not a single case in the epidemiological survey was positive for FIV subtype D, excluding the possibility that detection of vaccine virus resulted in a false-positive diagnosis of FIV infection. Limited medical records, particularly the FIV vaccination history, were available for most clinical samples in this study. The lack of information is actually the main rationale for submission for FIV PCR diagnosis after a positive FIV antibody test. However, out of the 13 known FIV-vaccinated cats in the epidemiological study, 7 were positive for FIV subtypes A to C but not for subtype D, again confirming the absence of vaccine-derived FIV sequences in the blood of FIV-positive cats. Thus, the PCR assay can conclusively differentiate FIV antibody-positive cats with natural infection from cats that acquired antibody positivity by vaccination, and it therefore detected FIV infection in seven of the FIV-vaccinated cats.

FIV and HIV share many distinct characteristics with regard to their genomic structure, molecular characterization, and pathogenesis, as well as diagnosis. The diagnostic strategy described in this investigation applies equally to PCR genotyping of HIV subtypes and the discrimination of HIV wild-type strains from HIV mutants, such as those with the nonnucleoside reverse transcriptase inhibitor-resistant mutation (2). At times when vaccines of limited efficacy are in use against retroviral infections in cats and vaccine may be used in humans (18), only negative results of patient-side rapid retroviral antibody tests are specific if the vaccination status of the patient is unknown. The approach described in this study may also be applicable to determination of the infection status of HIV-seropositive patients, separating HIV seropositivity by passive maternal antibody transfer or after HIV vaccination from seropositivity elicited by actual HIV infection.

ACKNOWLEDGMENTS

We gratefully acknowledge the staff of the Molecular Diagnostics Laboratory for their contributions to this study and the excellent animal care provided by the staff of the division of Laboratory Animal Health.

REFERENCES

1. **Bachmann, M. H., C. Mathiason-Dubard, G. H. Learn, A. G. Rodrigo, D. L. Sodora, P. Mazzetti, E. A. Hoover, and J. I. Mullins.** 1997. Genetic diversity of feline immunodeficiency virus: dual infection, recombination, and distinct evolutionary rates among envelope sequence clades. J. Virol. **71:**4241–4253.
2. **Carr, J. M., T. Green, D. Shaw, L. Daly, W. Hart, R. Ratcliff, G. Higgins, C. J. Burrell, P. Li, and M. Qiao.** 2009. Application of an allele-specific PCR

to clinical HIV genotyping samples detects additional K103N mutations in both therapy naïve and experienced patients. J. Med. Virol. **81:**1983–1990.

3. **Carreño, A. D., A. Mergia, J. Novak, N. Gengozian, and C. M. Johnson.** 2008. Loss of naïve (CD45RA+) CD4+ lymphocytes during pediatric infection with feline immunodeficiency virus. Vet. Immunol. Immunopathol. **121:**161–168.
4. **Crawford, P. C., M. R. Slater, and J. K. Levy.** 2005. Accuracy of polymerase chain reaction assays for diagnosis of feline immunodeficiency virus infection in cats. J. Am. Vet. Med. Assoc. **226:**1503–1507.
5. **DeGraves, F. J., D. Gao, H. R. Hehnen, T. Schlapp, and B. Kaltenboeck.** 2003. Quantitative detection of *Chlamydia psittaci* and *C. pecorum* by high-sensitivity real-time PCR reveals high prevalence of vaginal infection in cattle. J. Clin. Microbiol. **41:**726–729.
6. **Gleich, S. E., S. Krieger, and K. Hartmann.** 2009. Prevalence of feline immunodeficiency virus and feline leukaemia virus among client-owned cats and risk factors for infection in Germany. J. Feline Med. Surg. **11:**985–992.
7. **Hartmann, K., P. Griessmayr, B. Schulz, C. E. Greene, A. N. Vidyashankar, O. Jarrett, and H. F. Egberink.** 2007. Quality of different in-clinic test systems for feline immunodeficiency virus and feline leukaemia virus infection. J. Feline Med. Surg. **9:**439–445.
8. **Hosie, M. J., D. Addie, S. Belák, C. Boucraut-Baralon, H. Egberink, T. Frymus, T. Gruffydd-Jones, K. Hartmann, A. Lloret, H. Lutz, F. Marsilio, M. G. Pennisi, A. D. Radford, E. Thiry, U. Truyen, and M. C. Horzinek.** 2009. Feline immunodeficiency. ABCD guidelines on prevention and management. J. Feline Med. Surg. **11:**575–584.
9. **Jordan, H. L., J. Howard, R. K. Sellon, D. E. Wildt, W. A. Tompkins, and S. Kennedy-Stoskopf.** 1996. Transmission of feline immunodeficiency virus in domestic cats via artificial insemination. J. Virol. **70:**8224–8228.
10. **Kawaguchi, Y., J. Norimine, T. Miyazawa, C. Kai, and T. Mikami.** 1992. Sequences within the feline immunodeficiency virus long terminal repeat that regulate gene expression and respond to activation by feline herpesvirus type 1. Virology **190:**465–468.
11. **Levy, J., C. Crawford, K. Hartmann, R. Hofmann-Lehmann, S. Little, E. Sundahl, and V. Thayer.** 2008. American Association of Feline Practitioners' feline retrovirus management guidelines. J. Feline Med. Surg. **10:**300–316.
12. **Li, Y., C. Wang, K. E. Allen, S. E. Little, S. K. Ahluwalia, D. Gao, D. K. Macintire, B. L. Blagburn, and B. Kaltenboeck.** 2008. Diagnosis of canine *Hepatozoon* spp. infection by quantitative PCR. Vet. Parasitol. **157:**50–58.
13. **Novak, J. M., P. C. Crawford, H. M. Kolenda-Roberts, C. M. Johnson, and A. Mergia.** 2007. Viral gene expression and provirus load of Orf-A defective FIV in lymphoid tissues and lymphocyte subpopulations of neonatal cats during acute and chronic infections. Virus Res. **130:**110–120.
14. **Pancino, G., S. Castelot, and P. Sonigo.** 1995. Differences in feline immunodeficiency virus host cell range correlate with envelope fusogenic properties. Virology **206:**796–806.
15. **Pancino, G., I. Fossati, C. Chappey, S. Castelot, B. Hurtrel, A. Moraillon, D. Klatzmann, and P. Sonigo.** 1993. Structure and variations of feline immunodeficiency virus envelope glycoproteins. Virology **192:**659–662.
16. **Pecoraro, M. R., K. Tomonaga, T. Miyazawa, Y. Kawaguchi, S. Sugita, Y. Tohya, C. Kai, M. E. Etcheverrigaray, and T. Mikami.** 1996. Genetic diversity of Argentine isolates of feline immunodeficiency virus. J. Gen. Virol. **77:**2031–2035.
17. **Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto.** 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science **235:**790–793.
18. **Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J. G. McNeil, D. P. Francis, D. Stablein, D. L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. L. Robb, N. L. Michael, P. Kunasol, J. H. Kim, and MOPH-TAVEG Investigators.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. **361:**2209–2220.
19. **Steinrigl, A., and D. Klein.** 2003. Phylogenetic analysis of feline immunodeficiency virus in Central Europe: a prerequisite for vaccination and molecular diagnostics. J. Gen. Virol. **84:**1301–1307.
20. **Verschoor, E. J., L. A. Boven, H. Blaak, A. L. van Vliet, M. C. Horzinek, and A. de Ronde.** 1995. A single mutation within the V3 envelope neutralization domain of feline immunodeficiency virus determines its tropism for CRFK cells. J. Virol. **69:**4752–4757.
21. **Wang, C., D. Gao, A. Vaglenov, and B. Kaltenboeck.** 2004. One-step real-time duplex reverse transcription PCRs simultaneously quantify analyte and housekeeping gene mRNAs. Biotechniques **36:**508–519.
22. **Weaver, E. A., E. W. Collisson, M. Slater, and G. Zhu.** 2004. Phylogenetic analyses of Texas isolates indicate an evolving subtype of the clade B feline immunodeficiency viruses. J. Virol. **78:**2158–2163.
23. **Yamamoto, J. K., R. Pu, E. Sato, and T. Hohdatsu.** 2007. Feline immunodeficiency virus pathogenesis and development of a dual-subtype feline-immunodeficiency-virus vaccine. AIDS **21:**547–563.