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Detection of feline infectious peritonitis virus infection in cell cultures and peripheral blood mononuclear leukocytes of experimentally infected cats using a biotinylated cDNA probe

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ABSTRACT

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A dot blot hybridization assay, using a biotinylated cDNA probe, was able to detect feline infectious peritonitis virus (FIPV) RNA in *Felis catus* whole fetus (fcwf-4) cells infected with the FIPV isolates DF2, 79-1146, UCD1, and UCD2. The probe cross-hybridized in the dot blot assay with nucleic acid of a closely related feline coronavirus, feline enteric coronavirus (FECV)-79-1683. To construct the probe, a 2.5 kilobase cDNA, prepared from FIPV-DF2 genomic RNA, was molecularly cloned. The recombinant cDNA clone was digested with the restriction endonuclease Rsa I, and an 870 basepair Rsa I fragment was isolated from vector DNA by agarose electrophoresis and glassmilk purification. This fragment was complementary to the 3′ three fourths of the nucleocapsid gene. The hybridization probe was prepared by random primed labeling in the presence of biotin-11-dUTP. Using an avidin-alkaline phosphatase conjugate and chemiluminescent substrate detection system, virus could be detected in as few as 3000 infected cells.

In an in vivo study, the probe was used to detect FIPV RNA in peripheral blood mononuclear leukocytes (PBML) isolated at various post-infection days (PID) from cats experimentally infected with the FIP-producing coronavirus isolate FIPV-79-1146 or FIPV-DF2. Viral RNA could be detected in as few as 12 000 PBML isolated from cats at PID 7 and in 50 000 PBML at PID 22. There was no consistent pattern, however, between hybridization results and prognosis or severity of disease at the time of sampling. Despite some cross-hybridization with FECV RNA, this probe should be useful for diagnosis of FIP, because cats infected with FECV most likely do not become viremic.

INTRODUCTION

The coronavirus, feline infectious peritonitis virus (FIPV), induces a progressive and usually fatal contagious disease of domestic and exotic cats

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worldwide (Horzinek et al., 1977; Horzinek and Osterhous, 1979). FIP in cats occurs in two clinically distinct forms, effusive or noneffusive. The effusive form is characterized by the accumulation of fluid in one or more of the body cavities, while the noneffusive form is characterized instead by development of granulomatous lesions in various organs (Pedersen, 1987b). The noneffusive form of FIP can be difficult to diagnose clinically, because many feline diseases present in a similar manner (August, 1984). The FIPV is related antigenically to and crossreacts serologically with feline enteric coronavirus (FECV), porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV), and the human coronavirus 229E (Pedersen et al., 1978; Horzinek et al., 1982; Pedersen and Black, 1983). Because cats become infected subclinically with various coronaviruses, serological crossreactions between FIPV and other coronaviruses complicate diagnosis of FIP using standard antibody tests (Barlough, 1984; 1985). Homologous reactions after subclinical infections by FIPV may also complicate the interpretation of serological assays.

The FIPV genome consists of a positive-sense, single-stranded, infectious RNA of at least 27 kilobases (kb) (Siddell et al., 1983; DeGroot et al., 1987b). The 9.6 kb at the 3′ end of the genome encode for three structural proteins: a 205 kilodalton (kDa) peplomer glycoprotein (S), a 45–50 kDa nucleocapsid protein (N), and a 25–32 kDa integral membrane protein (M) (DeGroot et al., 1987a; Tupper et al., 1987; Spaan et al., 1990). The remainder of the genome is believed to encode RNA polymerase and other non-structural proteins (Spaan et al., 1990).

Virulent FIPV isolates preferentially infect and replicate within monocytes and macrophages, while avirulent coronaviruses are considerably less able to infect mononuclear phagocytes (Pedersen, 1976; Stoddart and Scott, 1989). Weiss and Scott (1981a) demonstrated by animal inoculation studies that either whole blood or peripheral blood mononuclear leukocytes (PBML) from cats with FIP are infectious to other cats and can transmit FIP. Experimental infection of SPF cats with the non-FIP producing enteric virus, FECV, results in a limited distribution of virus from the primary site of infection, the mature columnar epithelial cells in the upper third of the villi in the small intestinal mucosa. Virus is demonstrated occasionally in monocytic cells within the medullary sinuses of the mesenteric lymph nodes (Pedersen et al., 1981; 1984; Pedersen, 1987; Evermann et al., 1988); in contrast to FIP, there is no evidence that FECV can induce a systemic disease (Pedersen et al., 1981).

Apart from laparotomy and biopsy in noneffusive FIP, or clinical diagnosis of effusive FIP in the terminal stage, there are presently no specific antemortem diagnostic tests for FIP. A test which can detect FIPV directly in the blood or other clinical sample would be a valuable diagnostic tool. Direct detection of FIPV antigen in blood components with antibodies generally has not been successful, possibly because of low levels of viremia. Virus isolation is not

useful for diagnosis because field isolates grow poorly in cell culture (Pedersen and Floyd, 1985).

In this study, we describe a dot blot hybridization procedure, using a biotinylated recombinant cDNA probe complementary to a major portion of the N protein gene, to detect FIPV in feline cell cultures and PBML isolated from cats infected experimentally with the virulent FIP virus isolate 79-1146 or DF2.

MATERIALS AND METHODS

Cats

Seven of the 9 cats used in these studies were healthy, 10–12 month-old specific-pathogen-free (SPF) males and females. The cats were purchased from a commercial breeder (Liberty Laboratories, Liberty Corners, NJ) and were FeLV test-negative (by ELISA) and feline coronavirus antibody-negative prior to the studies. Two other cats (Z10, Z12) were 18 month-old males and were healthy and feline coronavirus antibody-positive prior to the studies. These cats originally were SPF kittens that had survived a previous experimental challenge exposure with FIPV. The cats were housed separately in cages located in an isolation facility of the Scott-Ritchey Research Center and were tested and cared for according to humane standards as set forth in the "Guide for the Care and Use of Laboratory Animals" (Publication No. 85-23, National Institutes of Health, Bethesda, MD). All experimental protocols were approved by a University animal welfare committee prior to the studies.

Cell cultures and virus

Feline coronaviruses used in this study included three virulent (i.e., FIP-producing) isolates (FIPV-DF2, FIPV-79-1146, FIPV-UCD1) and two avirulent strains (FIPV-UCD2, FECV-79-1683,). The FIPV-79-1146, FIPV-UCD1, FIPV-UCD2 and FECV-79-1683 strains (McKeirnan et al., 1981; Pedersen and Floyd, 1985) were supplied by Dr. N.C. Pedersen, University of California, Davis. The FIPV-DF2 strain (Evermann et al., 1981) was obtained from the American Type Culture Collection, Rockville, Md.; No. VR-743. *Felis catus* whole fetus (fcwf-4) cells were supplied by Dr. N.C. Pedersen and were used to propagate all the viruses. The fcwf-4 cells were grown in Eagle's minimal essential media supplemented with 10% heat-inactivated fetal bovine serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2.5 μg of amphotericin/ml, along with 2 mM L-glutamine and 1% nonessential amino acids. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of air with 5% CO_2.

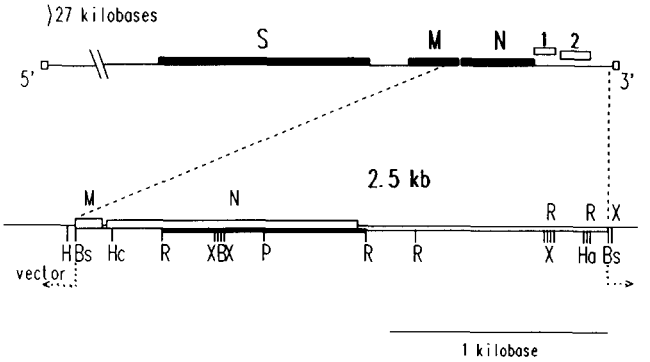

Fig. 1. The upper diagram depicts the structure of the FIPV genome showing relative positions of structural protein genes. Open boxes indicate open reading frames. This map is an adaptation of the one published by Vennema et al. (1990). The lower portion depicts a restriction endonuclease map of the FIPV-DF2 cDNA clone HlA3. The Rsa I frament used as the probe is indicated as a bold line. Restriction sites are indicated; Bs = BstX I, Hc = HincIII, R = RsaI, X = XbaI, B = BgIII, P = PstI, Ha = HaeIII.

cDNA probe preparation

The FIPV-DF2 polyadenylated RNA was isolated from virus-infected fcwf-4 cell pellets (10^8 cells) using the Fast Track System (Invitrogen, San Diego, CA) following the manufacturer's protocol. Four micrograms of FIPV-DF2 RNA were converted into RNA-cDNA hybrids by reverse transcriptase, using oligodeoxythymidine for priming. Resulting hybrids were converted into double-stranded DNA by RNase H together with DNA polymerase I and *Escherichia coli* DNA ligase and blunt-ended by T4 DNA polymerase. The double-stranded cDNA was ligated to BstX I linkers, inserted into the BstX I site of the pcDNAII phagemid (Invitrogen), and propagated in *E. coli* INV1 alpha F'cells (Invitrogen). One recombinant cDNA clone, H1A3, was characterized by restriction enzyme analysis and partial DNA sequencing. This clone represents 2500 bases near the 3′ end of the FIPV-DF2 genomic RNA (Fig. 1). The recombinant phagemid containing H1A3 was digested with Rsa I and the fragments separated by agarose gel electrophoresis. An 870 bp fragment complementary to the 3′ three fourths of the N protein gene (Fig. 1) was excised and purified from the gel using glassmilk (Geneclean II, Bio 101, La Jolla, CA). This fragment was labeled with biotin by random primer extension using hexanucleotide primers and Klenow enzyme in the presence of biotin-11-dUTP (Tropix, Bedford, MA) to make the hybridization probe, FIPV-Rsa I.

Preparation of infected fcwf-4 cells

Confluent monolayers of 3 day-old fcwf-4 cell cultures (150 cm^2) were infected with virus at a multiplicity of infection of 1. At 8 hours post-infection

(PI), cells were harvested by trypsinization. The cells were pelleted, washed once with phosphate-buffered saline (PBS) and then re-pelleted. The cell pellets were frozen and stored at −80°C until use. An uninfected fcwf-4 culture was harvested as described and served as a negative control (uninfected fcwf-4 cellular RNA). Before use, the cells were thawed quickly, resuspended in PBS, and counted on a Neubauer hemocytometer. The cell concentration was adjusted to 10^6 cells/ml in PBS.

Isolation of PBML

Whole blood was collected at various intervals by jugular venipuncture from SPF cats that had been injected intraperitoneally previously with either FIPV-79-1146 or FIPV-DF2 (Table 1). Heparinized whole blood was diluted 1:1.67

TABLE 1

Correlation of clinical signs with results of dot blot hybridization assay

Cat	Infecting virus	PID[1]	Viral RNA detected[2]	Clinical signs
E1	FIPV-1146	7	+	None
		14	++	Icteric, depressed, dehydrated Died on PID 18.
E2	FIPV-1146	0	−	None
		7	++++	None
		14	+++	Icteric, depressed, dehydrated Died on PID 18.
E3	FIPV-1146	0	−	None
		7	+	None
		14	+++	Icteric, depressed, dehydrated
		21	+	None
D1	FIPV-DF2	22	+	Uveitis, anemia Died on PID 39
B6	FIPV-DF2	22	+	Anemia
D5	FIPV-DF2	22	−	Uveitis, fever, anemia Died on PID 33
C1	FIPV-DF2	25	−	Moribund, died the same day
Z10	FIPV-DF2	> 1 yr	−	None
Z12	FIPV-DF2	> 1 yr	−	None

[1]PID; post-infection day

[2]−; no signal above negative control
+; signal detected in 50,000 PBML
++; signal detected in 25,000 PBML
+++; signal detected in 12,000 PBML
++++; signal detected in 6,000 PBML

in calcium and magnesium-free Hank's balanced salt solution (HBSS, pH 7.2) and layered onto Histopaque-1077 (Sigma, St. Louis, MO). After centrifugation for 30 min at 400 *g*, cells banded at the interface were collected, washed 2 times in HBSS, and then resuspended in RPMI-1640. Cells were counted in a hemocytometer and the cell suspensions adjusted to a final concentration of 10^6 cells/ml. Cells were either used immediately or were frozen in a solution of 10% fetal calf serum and 10% DMSO in RPMI-1640 and were stored at −20°C until use.

Dot blot hybridization assays

Preparation of cell dot blots was done as described by Paeratakul et al. (1988). Two-fold serial dilutions of each cell suspension, ranging from either 10^6 to 3×10^4 cells/ml for infected fcwf-4 cells or from 2×10^6 to 1.5×10^4 cells/ml for PBML, were made in PBS. A total of 100 μl of each cell dilution was applied to a 0.45 micron nylon membrane filter (Tropilon, Tropix), using a dot blot apparatus (BioDot, Bio Rad, Richmond, CA). The filters were fixed for 1 h at 4°C in 1% glutaraldehyde in a solution of 3% NaCl, 10 mM NaH_2PO_4, and 40 mM Na_2HPO_4 (pH 7.4). The filters were washed 3 times in proteolytic buffer (50 mM EDTA, 0.1 M Tris-HCL, pH 8.0) and were incubated for 30 min at 37°C in 10 ml of proteolytic buffer containing 20 μg/ml proteinase K (Promega, Madison, WI). After washing once in proteolytic buffer, the filters were air-dried and stored at room temperature until use.

The membrane filters were prehybridized for 1 h at 65°C in hybridization buffer (0.5 % polyvinyl pyrrolidone, 1 mM EDTA, 1 M NaCl, 50 mM Tris, pH 7.5, 5% dextran sulphate and 2.0% sodium dodecyl sulphate) and were then hybridized for 18 h at 65°C in fresh hybridization buffer containing 100 ng of heat-denatured FIPV-Rsa I probe. For chemiluminescent detection, post-hybridization washing and visualization were performed according to the manufacturer's instructions, using a commercial kit (Southern-Light Chemiluminescent Detection System; Tropix, Bedford, MA). Following application of the chemiluminescent substrate, AMPPD, the filters were wrapped in plastic wrap and kept at room temperature for 1 h. Kodak XAR-5 X-ray film was exposed to the wrapped filter for 6 to 10 min and then developed in an automatic processor. In some experiments, colorimetric detection was performed on the hybridized membrane after chemiluminescent detection. For colorimetric detection, post-hybridization washing and visualization were performed according to the manufacturer's instructions, using a commercial kit (BluGENE Nonradioactive Nucleic Acid Detection System; Bethesda Research Labs, Gaithersburg, MD). To minimize contamination by ribonucleases, all solutions were treated initially with diethyl pyrocarbonate (Sigma, St. Louis, Mo.) and all glassware was autoclaved and then baked at 250°C for at least 4 hr prior to use.

RESULTS

Specificity and sensitivity of FIPV probe in infected cell culture

The 2.5 kb FIPV cDNA clone, H1A3, was oriented by restriction enzyme analysis (Fig. 1) and partial sequencing, using the sequence of the 3.2 kb at the 3′ end of genomic RNA of FIPV-79-1146 (DeGroot et al., 1988; Vennema et al., 1990) as a reference. The position of the 870 bp Rsa I probe is indicated by the bold line in the lower diagram of Fig. 1. Complementarity of the 870 bp Rsa I probe to the 2.5 kb FIPV cDNA insert was confirmed by Southern blotting (data not shown).

The FIPV-Rsa I probe hybridized to RNA in cells infected with each of the 5 feline coronaviruses tested (Fig. 2). Viral RNA was detected in as few as 3000 cells for FIPV-DF2, FIPV-79-1146 and FECV-79-1683. Viral RNA was detected in 25 000 cells for FIPV-UCD2 and in 100 000 cells for FIPV-UCD1. The probe did not hybridize above background levels to RNA isolated from normal uninfected feline cells.

Sensitivity of FIPV probe in infected PBML

The FIPV-Rsa I probe hybridized to RNA in PBML isolated from cats that had been infected with either FIPV-79-1146 or FIPV-DF2. Cats E1, E2, and E3 were infected with FIPV-79-1146. The strongest hybridization signal occurred in cat E2 at PID 7 (Fig. 3). The signal was detectable in as few as 6000 PBML. At PID 14, 25 000 PBML were required to produce a similar signal

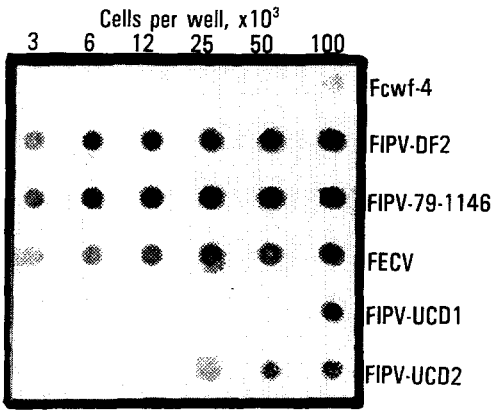

Fig. 2. Autoradiograph of a dot blot hybridization assay of virus-infected fcwf-4 cells. A biotinylated cDNA probe, FIPV-Rsa I, complementary to the FIPV-DF2 nucleocapsid gene, was hybridized to fcwf-4 cells infected with the virulent feline coronaviruses FIPV-DF2, FIPV-79-1146 and FIPV-UCD1 and the avirulent FECV and FIPV-UCD2. Uninfected fcwf-4 cells were included as a negative control. Signal was detected using a chemiluminescent substrate as described in Materials and Methods. The dot blot autoradiograph was computer-scanned to remove background luminescence.

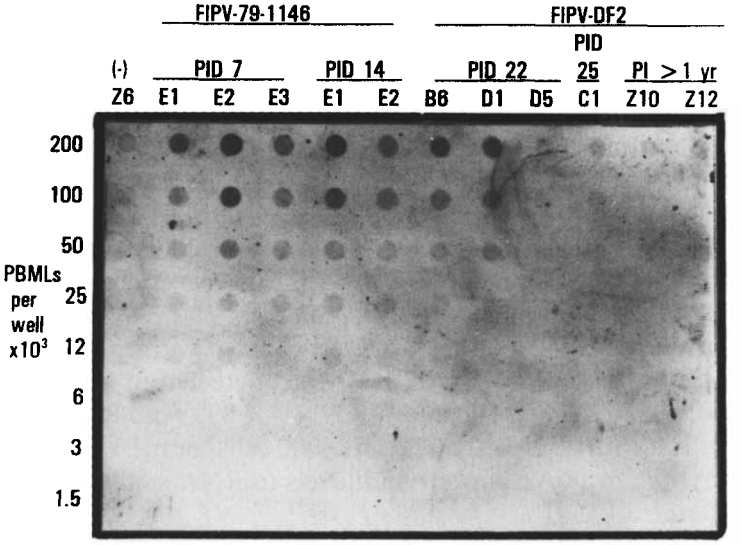

Fig. 3. Dot blot hybridization assay of peripheral blood mononuclear leukocytes (PBML) isolated from cats experimentally infected with FIPV-79-1146 or FIPV-DF2. The biotinylated cDNA probe FIPV-Rsa I was hybridized to glutaraldehyde-fixed PBML isolated from infected SPF cats at the post-infection day (PID) indicated. (Numbers at the top correspond to cat numbers in Table 1.) PBML from an uninfected cat, Z6, are included as a negative control. Signal was detected using a colorimetric detection system as described in Materials and Methods.

TABLE 2

Indirect fluorescent antibody (IFA) and virus-neutralizing (VN) antibody titers in FIPV-DF2 infected cats

Cat	PID[1]	IFA Titer	VN Titer
D1	22	40	800
B6	22	80	200
D5	22	40	1600
C1	25	80	3200
Z10	> 1 yr	ND[2]	6400
Z12	> 1 yr	ND	3200

[1]PID; post-infection day
[2]ND; Not done

by E2. In contrast, hybridization of PBML from cat E1 showed the opposite pattern, i.e. the signal intensified at PID 14 compared to PID 7. Both cats succumbed to FIP at PID 18. PBML from cat E3 had a hybridization pattern similar to E1 at PID 7 and 14; at PID 21, 50 000 cells were required to produce a signal (Table 1). At PID 21, cat E3 was no longer clinically ill, and the cat subsequently recovered.

The PBML from cats infected with FIPV-DF2 were tested at PID 22 and 25. Two (D1, B6) of 3 cats tested at PID 22 showed a positive hybridization signal (Fig. 3). At the time of testing, cat D1 had symptoms consistent with noneffusive FIP, and this cat died at PID 39. Cat B6, however, was only mildly anemic at the time of testing and presently is clinically normal (5 months post-infection). Cat D5, which tested negative at PID 22, had symptoms of noneffusive FIP and later died at PID 33 (Table 1). A hybridization signal above the negative control was not detected in cat Z10 or Z12; both cats had been infected with FIPV-DF2 for at least a year prior to testing and were clinically healthy. A11(6/6) of the FIPV-DF2-infected cats, including Z10 and Z12, had virus neutralizing (VN) antibody titers against FIPV-DF2. Four of these cats (D1, B6, D5, C1) were tested for serum FIPV antibody titers by indirect fluorescent antibody (IFA) assay, and all were positive (Table 2).

DISCUSSION

The FIPV-Rsa I recombinant cDNA probe hybridized to fcwf-4 cells infected with each of several different feline coronavirus isolates. These isolates represented diverse pathotypes: the FIP-producing DF2, 79-1 146 and UCD1, and the avirulent UCD2 and FECV-79-1683. As shown in Fig. 2, the probe hybridized with equal intensity to both FIPV-DF2 and FIPV-79-1146. This finding was not unexpected, because the RNA sequence near the 3′ end of the genome of these 2 isolates is very similar, if not identical (unpublished data). The decreasing intensity of signals seen for FECV-79-1683, FIPV-UCD1 and FIPV-UCD2, compared to FIPV-DF2 or 79-1146, may have been due either to less homology between nucleotide sequences or possibly to a lower number of viral copies per cell, particularly with virus strains, such as UCD1, that replicate more slowly in cell culture (data not shown).

Experiments performed using less stringent hybridization conditions (hybridization and post-hybridization washes at 55°C) did not show significantly altered signal intensities. Due to functional constraints, there are other areas of the viral genome that may show greater sequence homology among isolates. Conceivably, the putative RNA polymerase gene at the 5′-end of the genome contains sequences that are widely conserved among coronaviruses. Comparison of amino acid sequences derived from the RNA polymerase gene of TGEV to amino acid sequences derived from the RNA polymerase gene of infectious bronchitis virus (IBV) revealed a high level of homology; this is in contrast to the low levels of homology between TGEV and IBV structural proteins (Britton, et al., 1990; Wesley, 1990). Possibly, a probe constructed from the RNA polymerase gene can hybridize with more consistent intensity to the different coronavirus isolates tested.

Additionally, we wanted to determine if the probe would detect viral RNA in PBML isolated from cats experimentally infected with the FIP-producing

isolates, FIPV-79-1146 or FIPV-DF2. As shown in Fig. 3, the probe did hybridize to PBML isolated at various intervals from cats infected previously with either of the two isolates. Although the hybridization signal was stronger in cats infected with FIPV-79-1146, the samples were obtained at an earlier PID than were samples from cats infected with FIPV-DF2. All 3 cats infected with FIPV-79-1146 were positive at PID 7, which was during the incubation period for the experimental disease (Pedersen et al., 1984). In general, the signal intensity decreased with time. In the FIPV-DF2-infected animals, results were variable at PID 22 and were negative in the single cat (C1) tested at PID 25. Overall, hybridization results were not correlated with prognosis or severity of disease at the time of sampling.

Cats Z10 and Z12 were included to determine if the probe would detect viral RNA in PBML from cats that had recovered from clinical FIP and possibly were latent carriers of the disease (Pedersen, 1987a). A signal was not detected in PBML from these animals. If indeed they were latent carriers, then either the cDNA probe hybridization assay system was too insensitive, or the virus was not actually circulating in leukocytes but was instead sequestered within macrophages in lymph nodes or bone-marrow, as suggested previously by Pedersen and Floyd (1985).

The results obtained with cats D5 and C1 suggest that the dot blot hybridization format is not sensitive enough to detect the very low levels of viremia which may occur in this disease. Viremia in FIP is primarily cell-associated (Weiss and Scott, 1981a). Conceivably, only very few circulating leukocytes may be infected, whereas macrophages may replicate FIPV very efficiently (Weiss and Scott, 1981b). To increase test sensitivity, we are currently evaluating two different methods to detect FIPV RNA in PBML: (1) a fluorescent in situ hybridization (FISH) of PBML cytospun onto glass slides and tested with biotinylated probes, and (2) reverse transcription of viral RNA isolated from PBML, followed by a polymerase chain reaction (PCR) technique, using oligonucleotide primers specific for FIPV to amplify the virus. Preliminary results obtained with FISH showed greater sensitivity than dot blot hybridization; PBML from D5 and C1, which were previously evaluated to be virus-negative, tested positive (data not shown).

In addition to studying FIP viremia, we are evaluating PBML isolated from cats infected with the non-FIP-producing coronaviruses, FECV-79-1683, FIPV-UCD2 and CCV. Previous studies indicate that cats infected with these viruses most likely have only a localized enteric infection and do not have a viremia (Pedersen et al., 1981; Barlough et al., 1984; Pedersen and Floyd, 1985). PBML will be probed by FISH using our FIPV-cDNA probes to determine whether or not coronaviral RNA is present. If it can be shown that viral nucleic acids are in PBML of cats with FIP and are not in PBML from cats infected with non-FIP-producing coronaviruses, cDNA probes which cross-hybridize between FIPV and FECV may still be valuable diagnostic tools.

CONCLUSIONS

Based on hybridization of a recombinant biotinylated FIPV-cDNA probe to PBML from FIPV-infected cats, an assay has been developed which can detect virus infection directly in blood samples. It is our opinion that this assay can be used to diagnose acute FIPV infections, particularly in viremic cats. Tests that can detect virus or virus-associated components (e.g., proteins or nucleic acids) are preferable to diagnostic tests based on serological reactions. This is particularly true in disease states such as FIP, where anti-coronavirus antibody crossreactivity or homologous reactions after subclinical infections by FIPV complicate the interpretation of serological assays. Further refinements in sensitivity and specificity of the in situ hybridization assay should facilitate specific detection of FIPV RNA and result in a clinically useful diagnostic test for FIP.

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