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Persistence and Evolution of Feline Coronavirus in a Closed Cat-Breeding Colony

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Feline coronavirus (FCoV) persistence and evolution were studied in a closed cat-breeding facility with an endemic serotype I FCoV infection. Viral RNA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in the feces and/or plasma of 36 of 42 cats (86%) tested. Of 5 cats, identified as FCoV shedders during the initial survey, 4 had detectable viral RNA in the feces when tested 111 days later. To determine whether this was due to continuous reinfection or to viral persistence, 2 cats were placed in strict isolation and virus shedding in the feces was monitored every 2–4 days. In 1 of the cats, virus shedding continued for up to 7 months. The other animal was sacrificed after 124 days of continuous virus shedding in order to identify the sites of viral replication. Viral mRNA was detected only in the ileum, colon, and rectum. Also in these tissues, FCoV-infected cells were identified by immunohistochemistry. These findings provide the first formal evidence that FCoV causes chronic enteric infections. To assess FCoV heterogeneity in the breeding facility and to study viral evolution during chronic infection, FCoV quasispecies sampled from individual cats were characterized by RT-PCR amplification of selected regions of the viral genome followed by sequence analysis. Phylogenetic comparison of nucleotides 7–146 of ORF7b to corresponding sequences obtained for independent European and American isolates indicated that the viruses in the breeding facility form a clade and are likely to have originated from a single founder infection. Comparative consensus sequence analysis of the more variable region formed by residues 79–478 of the S gene revealed that each cat harbored a distinct FCoV quasispecies. Moreover, FCoV appeared to be subject to immune selection during chronic infection. The combined data support a model in which the endemic infection is maintained by chronically infected carriers. Virtually every cat born to the breeding facility becomes infected, indicating that FCoV is spread very efficiently. FCoV-infected cats, however, appear to resist superinfection by closely related FCoVs.

INTRODUCTION

Coronaviruses (genus *Coronavirus,* family *Coronaviridae,* order *Nidovirales*), common pathogens of mammals and birds, are enveloped RNA viruses with an unsegmented genome 27–32 kb in size (for reviews see Siddell, 1995; de Vries *et al.,* 1997). The 5′ two-thirds of the viral genome are taken up by the pol gene encoding the POL1a and POL1b polyproteins from which the viral polymerase is derived by proteolytic cleavage. During replication, a 3′-coterminal nested set of mRNAs that codes for the structural proteins S, E, M, and N and for a number of presumptive nonstructural proteins is produced. Each of these mRNAs contains a short nontranslated 5′ leader sequence derived from the 5′ end of the genome.

Although generally associated with acute, self-limiting enteric and respiratory infections (McIntosh, 1990), coronaviruses can establish persistent infections both *in vitro* and *in vivo.* During persistent infection of tissue culture cells, replication-defective viruses often accumulate. Presumably, these moderate viral dissemination through the culture (Stohlman *et al.,* 1979; Hirano *et al.,* 1981; Holmes and Behnke, 1981; Mizzen *et al.,* 1983; Hingley *et al.,* 1994). An alternative mechanism for coronavirus persistence *in vitro* involves the selection of resistant host cells, with viral replication being supported by a small percentage of susceptible cells (MacIntyre *et al.,* 1989; Hofmann *et al.,* 1990; Sawicki *et al.,* 1995). Persistent coronavirus infections *in vivo* have mostly been studied using mouse hepatitis virus (MHV) as a model system. Suckling rodents intracranially inoculated with a sublethal dose of neurotropic MHV variants develop chronic demyelination with viral replication in the central nervous system (Sorensen *et al.,* 1980; Knobler *et al.,* 1982; Jackson *et al.,* 1984; Parham *et al.,* 1986; Perlman *et al.,* 1988; Morris *et al.,* 1989; Fleming *et al.,* 1994). From such animals, virus has been isolated as late as 1 year after inoculation (Knobler *et al.,* 1982). Few studies have addressed the role of viral persistence during natural coronavirus infection.

Feline coronaviruses (FCoVs) generally cause mild enteric infections but also cause a rare, fatal immune-mediated disease called feline infectious peritonitis (FIP; for a review see de Groot and Horzinek, 1995). The "enteric" FCoVs and the disease-causing FIP viruses are genetically very closely related (Herrewegh *et al.,* 1995), and it appears

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that the latter are virulence variants arising spontaneously in FCoV-infected hosts (Vennema *et al.,* 1995; Poland *et al.,* 1996). FCoVs can be allocated to two serotypes on the basis of *in vitro* neutralization (Pedersen *et al.,* 1984; Hohdatsu *et al.,* 1991a,b). The type II FCoVs are thought to have originated from RNA recombination events during which the spike gene of canine coronavirus was incorporated into FCoV type I genomes (Herrewegh *et al.,* 1995; Vennema *et al.,* 1995; Motokawa *et al.,* 1996).

Epidemiological studies suggest that an FCoV carrier state exists and that asymptomatic FCoV-infected cats may spread the infection to susceptible kittens, presumably via the fecal–oral route. Some of these kittens develop FIP subsequently (Addie and Jarrett, 1992). Best evidence for a carrier state has come from an experiment in which cats were infected with a sublethal dose of tissue culture grown FIPV and kept in isolation (Pedersen, 1987). To induce FIP, the cats were superinfected with the immunosuppressive feline leukemia virus at various times after isolation. From this work, it appeared that FIPV could persist in the experimentally infected host for at least 4 months (Pedersen, 1987).

Feline coronaviruses are notoriously difficult to isolate and to grow in tissue culture. To identify asymptomatic FCoV carriers and to monitor virus shedding, we therefore developed a nested RT-PCR assay targeted to the highly conserved 3′ nontranslated region (NTR) of the FCoV genome. Using this assay, viral RNA was detected in the feces, tissues, and body fluids of cats with FIP (Herrewegh *et al.,* 1995; Egberink *et al.,* 1995; Addie *et al.,* 1996; Fehr *et al.,* 1996). Interestingly, FCoV RNA was also found in the feces, and occasionally in the serum, of asymptomatic cats, consistent with the notion that clinically healthy cats may shed FCoV. Here, we have studied the natural history and evolution of FCoV in a closed cat-breeding facility. By placing animals in isolation and by genetic analysis of FCoV shed in their feces, we have obtained formal evidence for viral persistence. Furthermore, we show that FCoV is subject to immune selection during chronic infection and that chronically infected cats may shed virus for at least 7 months.

MATERIALS AND METHODS

Animals and clinical specimens

Domestic short-hair cats (*Felis silvestris felis catus*) were bred and housed in the closed breeding colony of the Central Animal Facility at Medical School Hannover, Germany. This colony was free of ecto- and endoparasites, feline leukemia virus, and feline immunodeficiency virus. Cats were vaccinated against infection with feline herpesvirus, feline calicivirus, and feline parvovirus. They were housed in groups of 2–20 animals and could roam freely. The rooms were environmentally controlled and personnel entering the cattery were required to wash their hands and to wear overshoes and a gown. Cats were fed commercial diets and water was provided *ad libitum.* The two cats that were placed in isolation were housed on different floors in a separate building and tended by different animal caretakers. The isolation regime included the wearing of gown, head cover, face mask, overshoes and gloves. Plasma and fecal samples were collected of individual cats and stored at −20° until analysis.

Virus strains and sequence data

FCoV strains FIPV UCD1 and FECV 79-1683 were provided by N. Pedersen and J. Evermann, respectively, and grown in fcwf-D (*felis catus* whole fetus) cells as described previously (de Groot *et al.,* 1987b). Anti-FCoV type I serum 701 and type II serum G73 were obtained from cats experimentally infected with FCoV strain FIPV

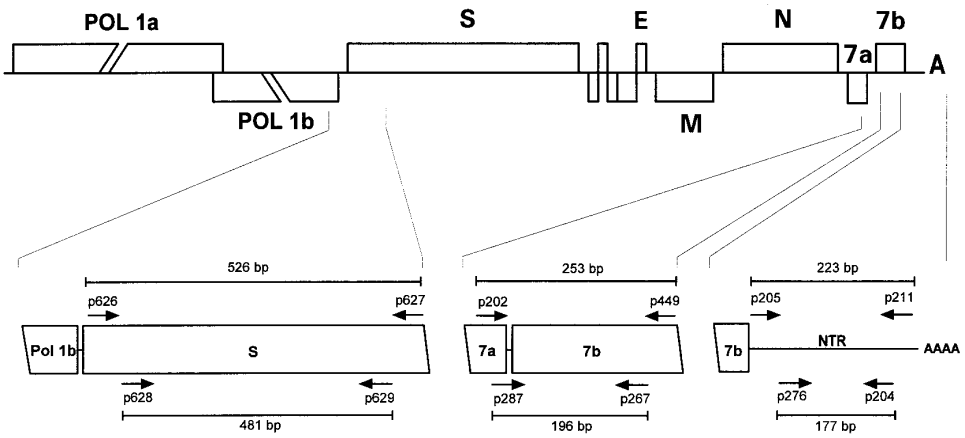

FIG. 1. Genomic organization of FCoV and an outline of the strategies used for amplification of specific genomic regions. The top shows a schematic representation of the FCoV genome with the various genes represented by boxes. The genes for the polymerase (POL1a, POL1b), the spike protein (S), the envelope protein (E), the membrane protein (M), the nucleocapsid protein (N), and the nonstructural proteins 7a and 7b are indicated. The bottom gives a schematic outline of the RT-PCRs targeted to the 5′ end of the spike gene, the 5′ end of the 7b gene, and the 3′ NTR. The positions and orientations of the oligonucleotides on the FCoV genome are shown, as are the lengths of the products of the first and the nested PCRs.

TABLE 1

Oligonucleotide Primers Used for RT-PCR and Sequence Analysis

Primer	Sequence (5′ to 3′)	Orientation
p202	CCTGCTATACATTGTTGGGTGC	Sense
p267	GATCCAAGCGGTAGTGCTAAGA	Antisense
p287	ATTGTCGGCCGCATTTATTTTAACATCATGATT	Sense
p449	CAGGCCATGTGCAATTAAAACC	Antisense
p510	ACGCGTTGTCCCTGTGTGGCCAT	Antisense
p511	CTTTTGGAAGGTTCATCTCCCCA	Antisense
p527	ACTTTAACTAGCCTTGTGCTAG	Sense
p626	AGCATTGCTAGGTCTGAAGATG	Sense
p627	TCCTCATGGTGTTACTCTACC	Sense
p628	CAAACATTGGCCTCCATCTCC	Antisense
p629	CCAATGAGAATCTCTGGTTAAAC	Antisense
p525	TAAAGTGAGTGTAGCGTGGC	Sense

UCD1 (Pedersen *et al.,* 1981) and FCoV strain FIPV 79-1146 (Vennema *et al.,* 1990), respectively. Previously published nucleotide sequences from FCoV strains Wellcome, UCD3, UCD1, TN406, Dahlberg, and UCD4 (Herrewegh *et al.,* 1995), FIPV 79-1146 (de Groot *et al.,* 1987a, 1988), FECV 79-1683 (Vennema *et al.,* 1992; Lewis, 1996), FIPV Ku2 (Motokawa *et al.,* 1995), CCV Insavc-1 (Horsburgh *et al.,* 1992), CCV K378 (Vennema *et al.,* 1992; Wesseling *et al.,* 1994), TGEV Purdue (Kapke and Brian, 1986), and TGEV-TFI (Chen *et al.,* 1995) were used for phylogenetic studies. The FCoV sequences C2490, C2490, and C2494 were obtained from FCoV present in ascitic fluid samples of cats with naturally occurring FIP (A. A. P. M. Herrewegh and R. J. de Groot, unpublished). The sequences from FECV-RM (H. Vennema *et al.,* in preparation) and CB02, CB03, and CB07 (A. A. P. M. Herrewegh and R. J. de Groot, unpublished) were obtained from FCoV present in fecal samples of cats from breeding facilities with a natural FCoV infection.

Serotype characterization and antibody detection

Sera and plasma samples were heat-inactivated by incubation at 56° for 10 min prior to use in virus neutralization (VN) assay. In duplicate 96-well microtitration plates, serial fivefold dilutions of plasma and serum were mixed with 50 $TCID^{50}$ units of FCoV strain FIPV UCD1 (serotype I) or FECV strain 79-1683 (serotype II) and incubated at 37° for 60 min. Subsequently, freshly trypsinized fcwf-4 cells were added and the plates were incubated for 4 days at 37°. The VN antibody titer was expressed as the reciprocal of the highest dilution that completely inhibited viral cytopathic effect. FCoV-specific antibodies were detected by immunofluorescence, as described previously (Herrewegh *et al.,* 1995).

Detection of FCoV antigens in tissues

Organ samples were snap frozen in liquid nitrogen immediately after removal and stored at −80° until use. Cryostat sections (8 μm, cut at −20°) were fixed in acetone containing 0.02% H_2O_2 for 10 min and preincubated for 20 min at room temperature with normal goat serum (diluted 1/40 to reduce nonspecific binding). Sections were examined for the presence of FCoV antigen using horseradish peroxidase-coupled polyclonal anti-FCoV antibodies (Tammer *et al.,* 1995) as a conjugate. Briefly, the slides were washed in PBS and incubated with the conjugated antibodies diluted 1:100 in PBS for 1 hr at room temperature. Subsequently, the slides were washed in PBS and peroxidase

TABLE 2

Detection of FCoV RNA in Feces and Plasma

			RT-PCR	
Cat	Age[a]	IFA[b]	Feces	Plasma
H304	1 Y	20	+	+
H308	1 Y	20	+	+
H320	1 Y	40	+	+
H322	1 Y	40	+	+
H324▶	10 M	40	+	+
H326▶	10 M	20	+	+
H328◆	10 M	<20	+	+
H330	10 M	40	+	+
H337	10 M	20	+	+
H349▼	8 M	160	+	+
H419■	3 M	<20	+	+
H005	1 Y	320	+	−
H039	6.5 Y	20	+	−
H158	2.5 Y	40	+	−
H177	2.5 Y	<20	+	−
H269	1.5 Y	<20	+	−
H305■	1 Y	160	+	−
H307▲	1 Y	40	+	−
H309	1 Y	80	+	−
H314◀	1 Y	80	+	−
H318	1 Y	80	+	−
H319◀	1 Y	20	+	−
H323▶	10 M	40	+	−
H329◆	10 M	80	+	−
H331◆	10 M	20	+	−
H336	10 M	20	+	−
H338	10 M	320	+	−
H340	9.5 M	40	+	−
H343▲	8 M	80	+	−
H350▼	8 M	40	+	−
H356	6 M	20	+	−
H170	4.5 Y	<20	−	+
H195	2.5 Y	<20	−	+
H276	3.5 Y	80	−	+
H351	8 M	<20	−	+
H704	9 Y	160	−	+
H267	1.5 Y	<20	−	−
H303	1 Y	<20	−	−
H341	9.5 M	<20	−	−
H359	3 M	<20	−	−
H360	3 M	<20	−	−
H361	3 M	<20	−	−

Note. Cats marked with identical symbols are born to the same queen

[a] Age of the cats at the time the fecal and plasma samples were taken Y, years; M, months.

[b] IFA, Immunofluorescence assay.

activity was detected using 0.003% H_2O_2 and 0.5% 3,3′-diaminobenzidine in 0.05 *M* Tris–HCl buffer (pH 8.3). The preparations were counterstained with hematoxylin and mounted. Organ sections of an SPF cat, tested FCoV-negative by serology and RT-PCR, were processed alongside to serve as negative controls.

Detection of FCoV RNA in feces, plasma, and tissues

The presence of FCoV in fecal, plasma, and tissue samples was demonstrated by detection of viral RNA using a reverse transcriptase nested PCR (RT-nPCR) assay targeted to the 3′ NTR of the viral genome, as described by Herrewegh *et al.* (1995) and outlined in Fig. 1. For RT-PCR detection of FCoV nucleocapsid mRNA, total RNA was extracted from various organ samples as described (Herrewegh *et al.,* 1995). The RT reaction was primed with p511 (Table 1), followed by cDNA amplification using p525 and p511. Subsequently, a seminested or a nested PCR was performed with primer pairs p527/p511 or p527/p510, respectively. The seminested RT-PCR

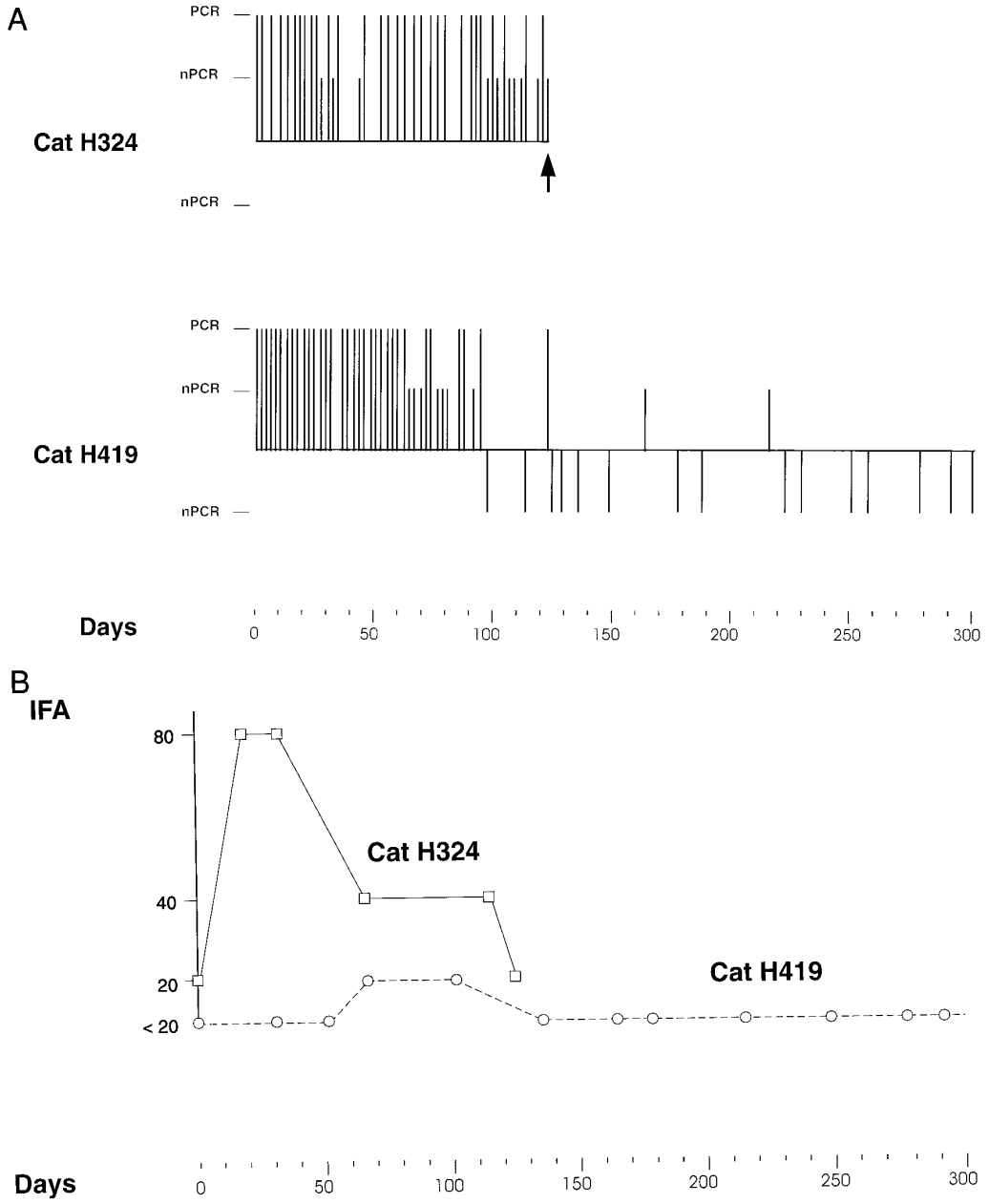

FIG. 2. Chronic shedding of FCoV as monitored by RT-PCR. (A) Detection of viral RNA in feces by RT-PCR. Cats H324 and H419 were placed in strict isolation at Day 0, and fecal samples were collected and processed for RT-PCR amplification of the 3′ nontranslated region of the FCoV genome. The results are shown in a graph with the horizontal axis representing the number of days in isolation. Long and short bars above the horizontal axis indicate detection of FCoV RNA by single or nested RT-PCR, respectively. The instances in which viral RNA was detected neither by single nor by nested RT-PCR are represented by short bars below the *x* axis. Cat H419 was kept in isolation for 300 days. Cat H324 was sacrificed at Day 124 as indicated by an arrow. (B) Antibody titers in the plasma of cat H324 and H419 during isolation. FCoV-specific antibody titers of cat H324 (squares) and H419 (circles) were determined by immunofluorescence assay.

products from colon and rectum were cloned in the pGEM-T vector (Promega Corp., Madison, WI) and sequenced. The variable 5′ regions of the spike and 7b gene were reverse transcribed using primer p627 and p449, respectively. cDNA of the spike gene was amplified using primers p626 and p627 for the first rounds of amplification followed by a nested step using primers p628 and p629. The variable region of the 7b gene was amplified with primers p202 and p449 followed by a nested PCR using primers p287 and p267. The PCR products were directly sequenced using the AmpliCycle sequencing kit (Perkin–Elmer/Roche, Branchburg, NJ).

Sequence alignment and phylogenetic analysis

Multiple alignments of nucleic acid and amino acid sequences were performed using the PileUp program (University of Wisconsin), which scores the similarity between every possible pair using a method similar to the one described by Higgins and Sharp (1989). Pairwise genetic distances between nucleic acid sequences were estimated using the DnaDist program and the two-parameter model of Kimura (1980). Pairwise genetic distances between amino acid sequences were estimated with the ProtDist program using maximum likehood estimates based on the Dayhoff PAM matrix (Dayhoff, 1979). Unrooted phylogenetic trees (cladograms) were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987). Bootstrap resampling of the data was performed using the SeqBoot program (Felsenstein, 1985) with 100 iterations. Potential antigenic sites were calculated using the method described by Jameson and Wolf (1988). The antigenic index threshold was set at $\geqslant$1.3.

RESULTS

Occurrence of FCoV in a closed breeding colony

The closed cat-breeding facility at the Medical School Hannover houses between 65 and 126 animals. In 1981, FCoV was inadvertently introduced. During the next 12 years, 60 cats died of FIP, 31 (50%) of which were between 4 and 5 months of age. The yearly incidence of FIP was approximately 5%. In the past 3.5 years, no cases of FIP have occurred.

To study whether FCoV was still present in the colony, a serologic survey was performed on 42 clinically healthy cats, ranging in age from 3 months to 9 years. Of these, 29 (71%) were seropositive as determined by IFA (Table 2). Sera taken from cats H324 and H338 neutralized FCoV strain UCD1 (serotype I) but not strain 79-1683 (serotype II), indicating that the cats had been infected by a type I FCoV.

To identify cats with an ongoing FCoV infection, samples of the feces and the serum were screened by RT-PCR targeted to the highly conserved 3′ NTR of the FCoV genome (Fig. 1; Herrewegh *et al.,* 1995). Of the 42 selected cats, 20 (48%) had FCoV RNA in the feces, 5 (12%) in the plasma, and 11 (26%) in the feces and plasma (Table 2). There was no apparent correlation between virus shedding and the antibody titer or the age of the cats.

Persistence of FCoV infection

Interestingly, when cats H304, H324, H326, H330, and H337 were examined 3 months after the initial analysis, all but H337 (i.e., 80%) again tested positive for viral RNA in the feces (not shown). This finding could be explained either by recurrent infections or by viral persistence. To distinguish between these possibilities, cats H324 and H419 were each placed separately and kept in strict isolation. Samples were taken from stools and plasma and analyzed for the presence of FCoV RNA. The results are summarized schematically in Fig. 2A. Viral RNA was readily detected in the feces during the first 4 months of isolation. Initially, a single PCR using primers p205 and p211 (Fig. 1) was sufficient to detect viral RNA in the

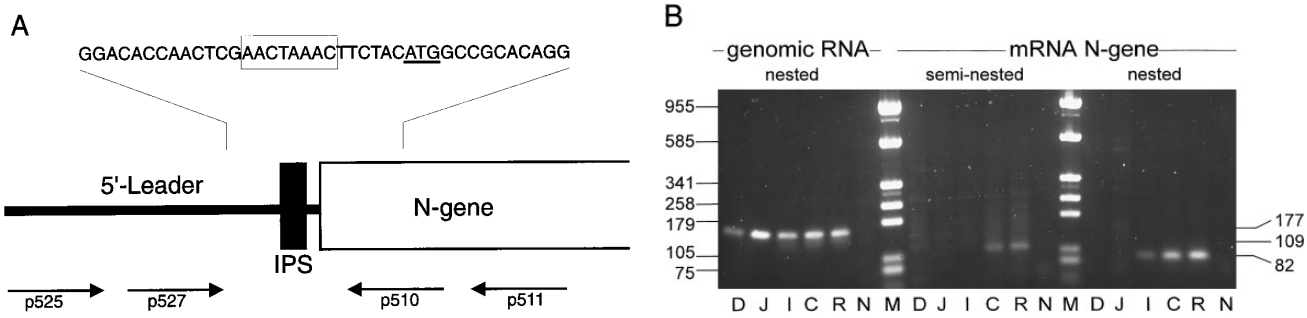

FIG. 3. Detection of FCoV mRNA in tissues of a chronically infected cat. (A) Outline of the RT-PCR amplification of the 5′ end of mRNA 6 encoding N. The structure of the 5′ end of mRNA 6 is depicted schematically with the 5′ leader sequence indicated by a black line, the intergenic promoter sequence (IPS) by a black box, and the N gene by an open box. The positions and orientations of the oligonucleotides used in the RT-PCR are indicated by arrows. Also presented is the nucleotide sequence flanking the IPS, AACTAAAC (boxed). (B) Detection of genomic RNA and mRNA 6 in tissue samples from cat H324 collected at Day 124 postisolation. Total RNA was extracted and subjected either to the RT-nPCR targeted to the 3′ NTR (Fig. 1), yielding a product of 177 bp, or to a nested and seminested RT-PCR targeted to the N mRNA (A), yielding products of 82 and 109 bp, respectively. Products were separated in 2% agarose gels, and *Sau*3AI-digested pUC 18 DNA was used as a molecular weight marker (lanes M). Sizes are given in basepairs. The origins of the tissue samples are indicated by the following abbreviations: D, duodenum; J, jejunum; I, ileum; C, colon; R, rectum; N, negative control.

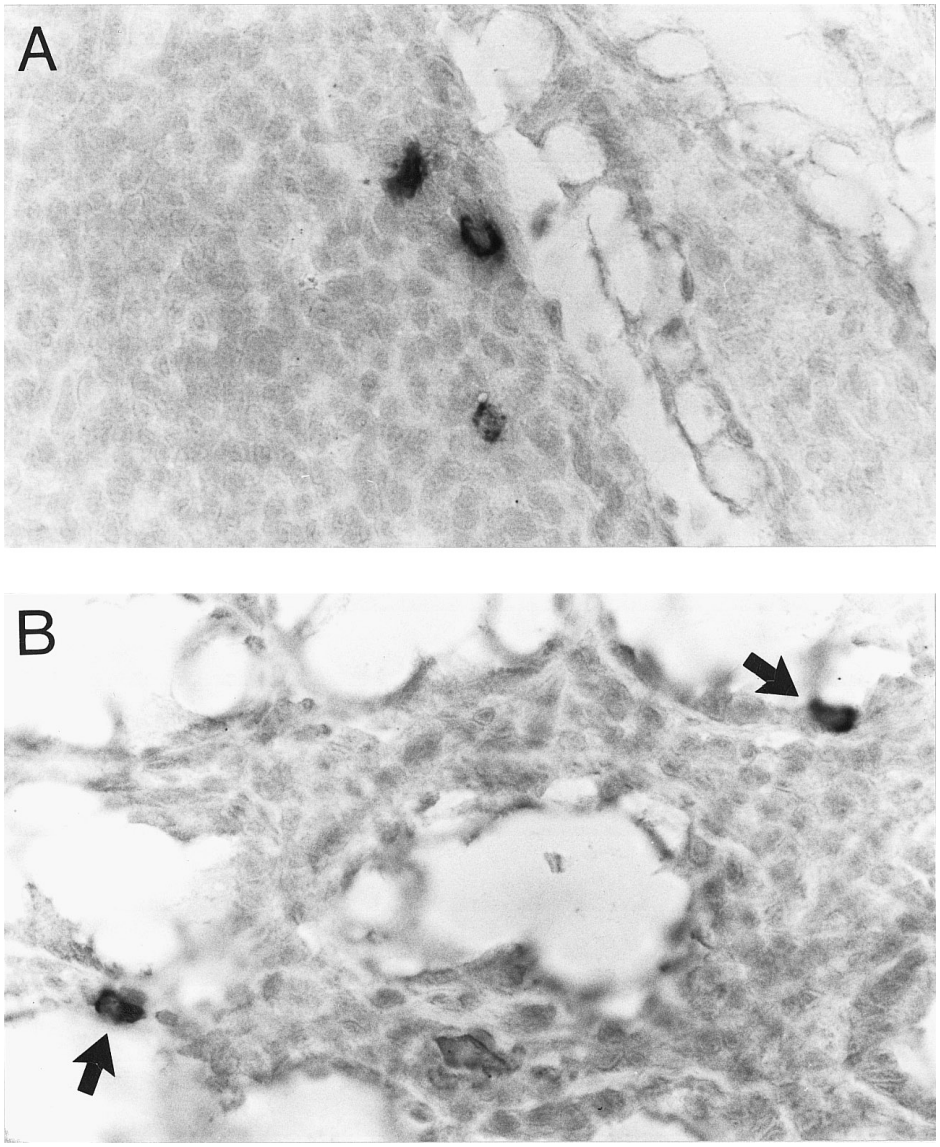

FIG. 4. Immunohistochemical detection of FCoV antigens in tissue sections of the chronically infected cat H324. Cells containing FCoV antigens were identified using a horseradish peroxidase-conjugated FCoV-specific antiserum. (A and B) Tissue sections of the ileum and colon, respectively. In A, infected cells are located at the periphery of a Peyer's patch. B shows a section of the glands of Lieberkühn with infected cells facing the luminal side of the tubules.

feces and all samples were FCoV positive. Later, the more sensitive RT-nPCR was required and viral RNA was detected less frequently. For cat H419, the FCoV infection appeared to wane after 4 months of isolation, but through RT-nPCR viral RNA was detected in the feces even after 7 months of isolation. Neither of the cats had detectable levels of viral RNA in their plasma at any time during the isolation period nor showed any sign of disease.

Viral persistence in the gastrointestinal tract

Our findings indicate that FCoV can indeed establish asymptomatic chronic infections. To determine the site of

FIG. 5. Comparative sequence analysis of residues 7–146 of ORF 7b. (A) Alignment of the consensus sequences of the FCoV quasispecies shed by 11 cats from the breeding facility (see Table 2). Fecal samples were collected and processed for RT-nPCR. Nucleotides −26–170 of ORF7b were amplified as indicated in Fig. 1 and the PCR products were sequenced directly by cycle sequence analysis. Only those nucleotides differing from the overall consensus sequence (Cons.) are depicted. Nucleotide changes leading to amino acid substitutions are boxed. [a,b] and [c,d] represent the consensus sequences of the FCoV quasispecies in fecal samples taken from cats H324 and 419, respectively. Animals were placed in isolation on Day 0. Fecal samples were collected on Days −111,[a] +113,[b] 0,[c] and +94.[d] (B) Unrooted phylogenetic tree illustrating the evolutionary relationships of the FCoVs shed by the cats in the breeding facility to the FCoV laboratory isolates TN406, UCD1, UCD3, UCD4, Dahlberg, Wellcome, 79-1146, and 79-1683; the American field strain FECV RM; and the Dutch field strains C2461, C2490, C2494, CB02, CB07, and CB03. The tree was obtained using the neighbor-joining algorithm on the basis of nucleotide distances. H324 represents the overall consensus sequence from A.

A

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          7
H324[a]
H324[b]
H419[c]
H419[d]
H158
H307
H314
H328
H331
H349
H350                      g
H039                                                          a     c
H320
Cons.  GTTGTATTCCTTGTGTGTGTTTTCTTGGCTACTGGAATCAAAGCTACCACTGTGCAACTTGACCTTCATG

                                                                                146
H324[a]
H324[b]
H419[c]
H419[d]
H158
H307
H314
H328
H331
H349
H350
H039       t   g   t
H320                                                                  g   g
Cons.  AACACCCAGTCCTTACCTGGGACTTGTTGCAACATTTCATAGGACACACTCTTTACATCACAACACATCA
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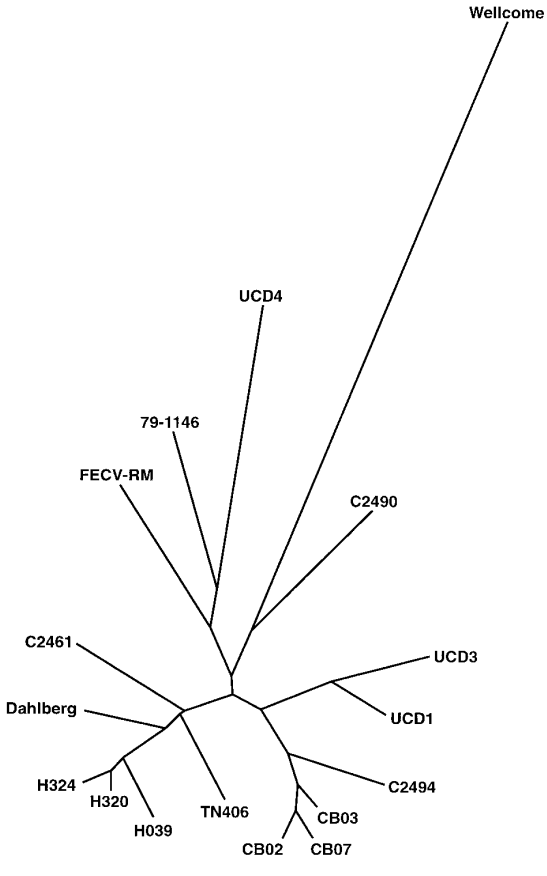
B



viral persistence, cat H324 was sacrificed after 124 days of isolation and tissue and organ samples were collected. Using the nested RT-PCR, FCoV RNA was detected in several organs, including kidney, lungs, brain, tonsils, salivary glands, and bone marrow. However, viral RNA could be detected by single PCR only in the samples of duodenum, jejunum, ileum, colon, and rectum, suggesting that the virus was most abundant in these tissues.

Evidently, the RT-nPCR assay targeted to the 3′ NTR does not differentiate between the viral genome and the mRNAs (Fig. 1). To test for FCoV replication, we performed a semi-nested RT-PCR specific for the mRNA encoding the nucleocapsid protein (N), using oligonucleotide primers p525, p527, and p511, which were designed after the FCoV 5′ common leader sequence and the N gene (Table 1, Fig. 3A; Vennema *et al.,* 1992; R. J. de Groot, unpublished). An RT-PCR product of the predicted size of 109 bp was obtained only for samples taken from the colon and rectum (Fig. 3B). Sequence analysis of this product yielded the sequence of the leader-body fusion region of the mRNA for N (not shown), confirming the specificity of the RT-PCR assay. Using a more sensitive RT-nPCR (Fig. 3A), we could detect N mRNA also in the ileum but not in other tissues (Fig. 3B).

The presence of N mRNA strongly suggested that viral replication occurs in cells of the lower gastrointestinal tract. To corroborate our findings, cryostat tissue sections of organ samples were tested for the presence of FCoV-infected cells by immunohistochemistry using a horseradish peroxidase-conjugated FCoV-specific antiserum. Tissue sections taken from FCoV-negative specific pathogen-free cats served as negative controls. Cells containing FCoV antigens were found only in the ileum, colon, and rectum of cat H324. In sections of the ileum, these cells were located at the periphery of the Peyer's patches (Fig. 4A), whereas in sections of the large intestine, cells containing FCoV antigens were observed facing the luminal side of the crypts of Lieberkühn (Fig. 4B). The combined findings of RT-PCR and histochemistry were consistent with viral persistence in the lower part of the intestinal tract.

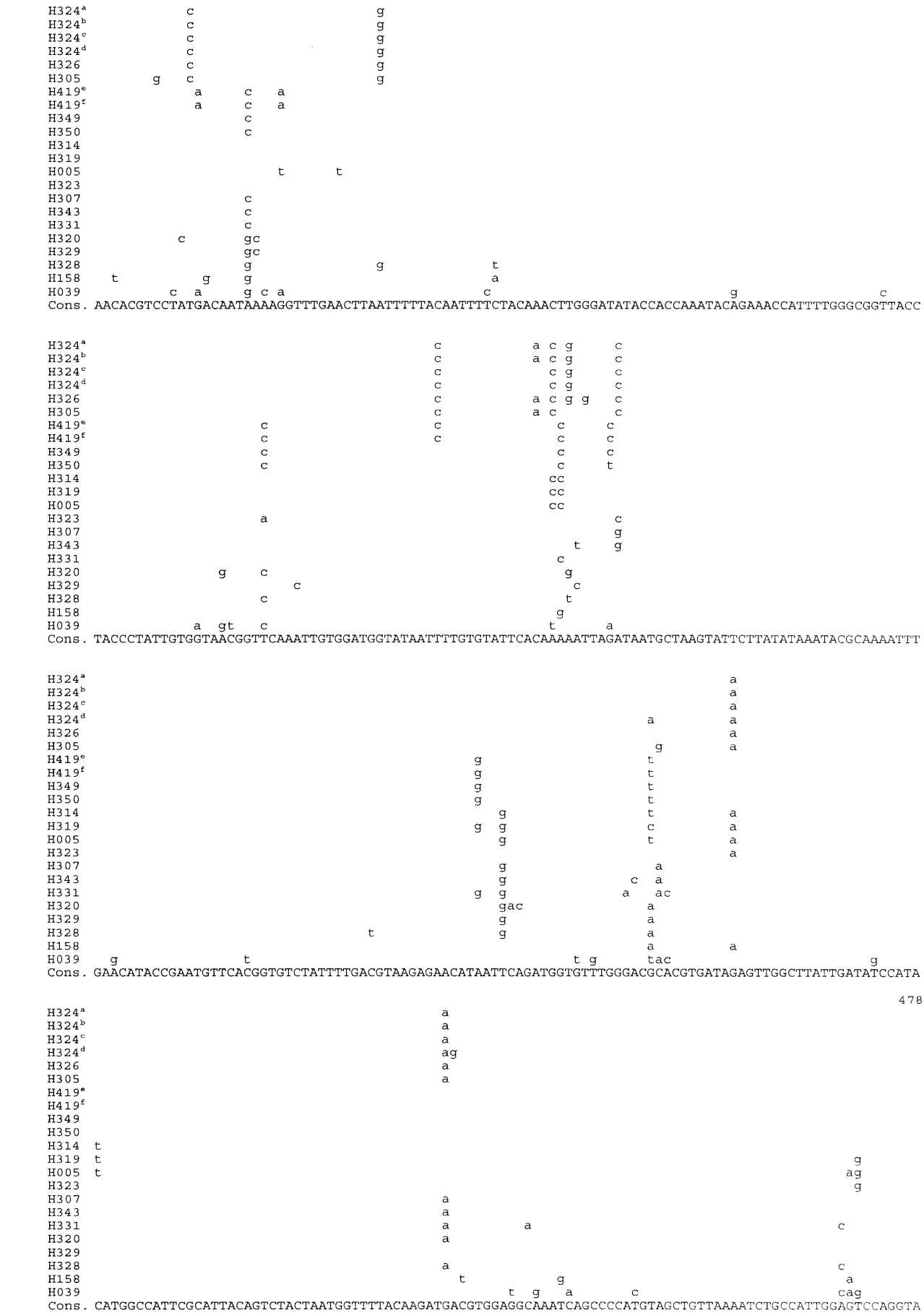
Genetic diversity and evolution of the FCoV population in the breeding facility

To further our understanding of FCoV epidemiology, we performed a genetic analysis of the viruses present in the breeding facility and compared the viruses shed by cats H419 and H324 to those shed by other cats in the colony. Another objective of this set of experiments was to obtain genetic evidence for FCoV persistence and to exclude the possibility that the extended virus shedding during the isolation period was caused by accidental reinfections. Viral RNA was extracted from fecal samples, and sequences derived from the 5′ ends of the S and 7b genes (Fig. 1) were amplified by RT-nPCR. The PCR products were directly sequenced using cycle sequence analysis, thus yielding the consensus sequence of the FCoV quasispecies shed by each individual animal. The nucleotide sequences and deduced amino acid sequences were compared by multiple sequence alignment and subjected to phylogenetic analysis (Figs. 5 and 6).

A previous genetic comparison of 11 FCoV isolates from various origins revealed 83–94% overall nucleotide sequence identity in ORF7b, with most sequence variation occurring in the 5′-most 150 residues (Herrewegh *et al.,* 1995). Ten of eleven FCoVs sampled from cats in the breeding facility shared 99–100% sequence identity in this region. The FCoV shed by cat H039 appeared to be somewhat more distant, displaying 96% sequence identity to the consensus sequence (Fig. 5A). A comparison to various FCoV laboratory isolates (Herrewegh *et al.,* 1995) and European field strains (A. A. P. M. Herrewegh and R. J. de Groot, unpublished) showed that the FCoVs circulating in the breeding facility form one clade (Fig. 5B). FCoVs sampled from another, commercial, breeding facility also clustered and showed a similar degree of sequence variation (CB02, CB03, and CB07; Fig. 5B).

Among the FCoVs in the breeding facility, the sequence variation in ORF7b was limited. We therefore examined another region of the FCoV genome, i.e., the 5′ end of the S gene (Fig. 1). Comparative sequence analysis of various coronaviruses has shown that this gene segment is highly variable (de Groot *et al.,* 1987c; Cavanagh, 1995). Oligonucleotide primers designed after the S sequences of the type I FCoV strain KU2 (Motokawa *et al.,* 1995) and the type II strain 79-1146 (de Groot *et al.,* 1988; Table 1) were used for RT-nPCR to amplify a 481-bp DNA corresponding to nucleotides 51–531 of the S-gene of FCoV KU2. A comparison of the FCoVs shed by 18 cats, including H419 and H324, revealed considerable sequence variation in this region with sequence identities ranging from 90.5 to 99.7% (Fig. 6A). The average percentage nucleotide substitutions is 2.3, and 91% of these substitutions results in an amino acid change (Fig. 7A). The amino acid substitutions were not randomly

FIG. 6. Comparative sequence analysis of nucleotides 79–478 of the S gene. (A) Alignment of the consensus sequences of the FCoV quasispecies shed by 17 cats from the breeding facility (see also Table 2). FCoV RNA extracted from fecal samples was subjected to RT-nPCR to amplify residues 51–531 of the S gene as outlined in Fig. 1. The PCR products were sequenced directly and the nucleotides 79–478 were aligned. [a,b,c,d] and [e,f] represent the consensus sequences of the FCoV quasispecies in fecal samples taken from cats 324 and 419, respectively. The animals were placed in isolation on Day 0. In the case of cat 324, samples were collected on Days −111,[a] +2,[b] +8,[c] and +113.[d] Those from cat 419 were collected on Days 0[e] and +94.[f] Only those nucleotides differing from the overall consensus sequence (Cons.) are shown. (B) Unrooted phylogenetic tree based on comparison of the S nucleotide sequences illustrating the evolutionary relationship of the FCoVs isolated from the cats in the breeding facility. The tree was obtained using the neighbor-joining algorithm on the basis of nucleotide distances.


A
79
H324[a]
H324[b]
H324[c]
H324[d]
H326
H305
H419[e]
H419[f]
H349
H350
H314
H319
H005
H323
H307
H343
H331
H320
H329
H328
H158
H039
Cons. AACACGTCCTATGACAATAAAAGGTTTGAACTTAATTTTTACAATTTTCTACAAACTTGGGATATACCACCAAATACAGAAACCATTTTGGGCGGTTACC
H324[a]
H324[b]
H324[c]
H324[d]
H326
H305
H419[e]
H419[f]
H349
H350
H314
H319
H005
H323
H307
H343
H331
H320
H329
H328
H158
H039
Cons. TACCCTATTGTGGTAACGGTTCAAATTGTGGATGGTATAATTTTGTGTATTCACAAAAATTAGATAATGCTAAGTATTCTTATATAAATACGCAAAATTT
H324[a]
H324[b]
H324[c]
H324[d]
H326
H305
H419[e]
H419[f]
H349
H350
H314
H319
H005
H323
H307
H343
H331
H320
H329
H328
H158
H039
Cons. GAACATACCGAATGTTCACGGTGTCTATTTTGACGTAAGAGAACATAATTCAGATGGTGTTTGGGACGCACGTGATAGAGTTGGCTTATTGATATCCATA
478
H324[a]
H324[b]
H324[c]
H324[d]
H326
H305
H419[e]
H419[f]
H349
H350
H314
H319
H005
H323
H307
H343
H331
H320
H329
H328
H158
H039
Cons. CATGGCCATTCGCATTACAGTCTACTAATGGTTTTACAAGATGACGTGGAGGCAAATCAGCCCCATGTAGCTGTTAAAATCTGCCATTGGAGTCCAGGTA

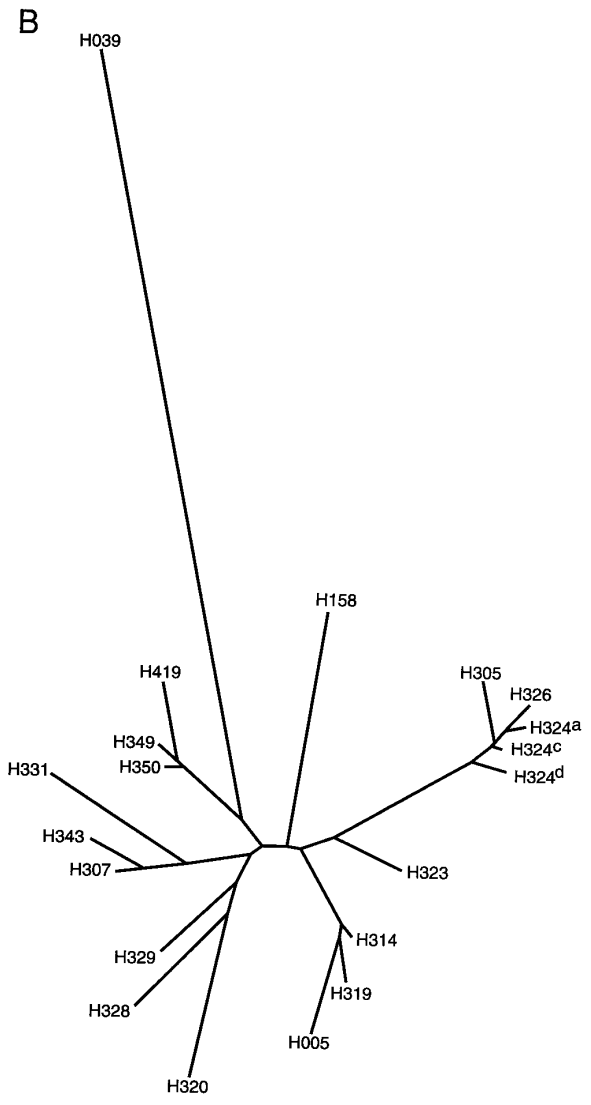


FIG. 6 — *Continued*

scattered throughout the gene segment but rather occurred at seven sites, six of which coincided with potential antigenic sites as predicted by a Jameson–Wolf analysis (Jameson and Wolf, 1988; Fig. 7B).

As illustrated by the cladogram in Fig. 6B, several clusters of more closely related FCoVs can be distinguished. In most cases, these viruses were sampled from littermates, such as cats H349 and H350, cats H319 and H314, and cats H324 and H326 (Fig. 6B; Table 2). As shown in Fig. 6A, the consensus sequences of the FCoV quasispecies shed by cat 419 at Days 0 and 94 postisolation were identical. In the case of cat H324, the consensus sequence of the virus shed at 111 days before isolation was identical to that of the quasispecies sampled at Day 2 postisolation. At Day 8 postisolation, a single point mutation was found resulting in an $Asn^{52} \rightarrow$ His substitution at site D (Fig. 7A), whereas by Day 113 postisolation, two additional nucleotide substitutions were detected, resulting in substitutions of Ala^{90} and Asn^{115} to Thr and Ser, respectively. In a phylogenetic analysis, the FCoVs sampled from cat H324 cluster and, with the exception of FCoV sampled from cat H326, are more closely related to each other than to the FCoVs sampled from other cats. Given the overall genetic diversity in this region of the S gene among the FCoVs in the cattery, these findings support our conclusion that cats H419 and H324 carried an asymptomatic chronic FCoV infection and argue against accidental reinfections during the isolation period.

DISCUSSION

Coronavirus epidemiology, persistence, and evolution were studied in a closed cat-breeding facility with an endemic FCoV infection. Serological and genetic analysis (Fig. 8) revealed that the virus involved was a serotype I strain, closely related to the FCoV isolate Dahlberg (Fig. 5B). An initial survey showed that 86% of the cats had an ongoing FCoV infection, as demonstrated by RT-PCR detection of viral RNA in feces and/or plasma. When tested 100 days later, four of five cats that had previously been identified as virus shedders still had detectable viral RNA in the feces. One explanation was that the cats in the breeding facility were subject to frequent reinfections. Alternatively, as previously speculated by us and others (Pedersen, 1987; Addie and Jarrett, 1992; Herrewegh *et al.,* 1995; de Groot and Horzinek, 1995), FCoV may cause chronic infections resulting in prolonged virus shedding. We now provide the first direct evidence for FCoV persistence. By placing cats in strict isolation and by comparative sequence analysis of excreted virus, it was shown that naturally infected asymptomatic cats may remain infected and shed FCoV in their feces for at least 7 months.

Through the use of a highly sensitive RT-nPCR targeted to the 3′ NTR (Herrewegh *et al.,* 1995), FCoV RNA was detected in several tissues of the chronically infected cat H324. The lower part of the gastrointestinal tract was identified as a major site of viral replication, as indicated by RT-PCR detection of viral mRNA and by immunohistochemical detection of FCoV-infected cells. The failure to detect viral mRNAs or infected cells in organs other than the intestinal tract does not exclude viral replication in these tissues. Rather, the number of infected cells may have been below detection level. In the large intestine, infected cells were found lining the luminal side of the crypts of Lieberkühn. Conceivably, these infected enterocytes could represent a main source of FCoV present in the feces. In the ileum, FCoV-infected cells were located at the periphery of the Peyer's patches, a mucosa-associated lymphoid tissue. FCoV may have gained access to these sites via the so-called M cells, which are in close contact with intestinal lymphoid tissue and mediate the transepithelial transport of macromolecules, particles, and microorganisms (for a review see Neutra *et al.,* 1996). Presumably, the infected

cells seen in the Peyer's patches were monocytes. Enterocytes and cells of the monocyte lineage are generally considered the main host cells of FCoV. Aminopeptidase N (APN), a protein expressed on the apical membrane of enterocytes, was recently identified as a receptor for FCoV (Tresnan *et al.,* 1996). Monocytes also express APN (Griffin *et al.,* 1981; Look *et al.,* 1989; Delmas *et al.,* 1992) and support replication of both pathogenic and nonpathogenic FCoVs *in vitro* (Pedersen, 1976b; Jacobse-Geels and Horzinek, 1983; Stoddart and Scott, 1989).

To determine the relationship between the FCoV types in the breeding facility and to study coronavirus evolution during chronic infection, a genetic analysis was performed on FCoV samples taken from individual cats. According to the quasispecies concept, (Eigen and Schuster, 1979; Eigen, 1971; Eigen and Biebricher, 1988; reviewed in Holland *et al.,* 1992; Duarte *et al.,* 1994; Domingo *et al.,* 1996), each FCoV sample does not constitute a genetically homogeneous virus population but rather a cloud of variants with related, yet nonidentical genomes. Direct sequence analysis of RT-PCR-amplified cDNA yields the consensus sequence of the quasispecies, which often but not always coincides with the predominant viral genome, the master sequence. This approach has the added advantage that sequence errors generated during reverse transcription or amplification will not be detected. Two regions of the genome were selected for analysis: residues 7–146 of ORF7b, which appeared highly conserved among the FCoVs in the breeding facility, and residues 79–478 of the S gene, which represent a hypervariable region (HVR). A phylogenetic comparison to ORF7b sequences of independent FCoV isolates and field strains demonstrated that the FCoVs in the breeding facility form a separate clade, consistent with the notion that these viruses originated from a single founder infection. Formally, however, multiple introduction of genetically related viruses cannot completely be excluded.

In contrast to ORF7b, the 5′ HVR of the S gene showed considerable sequence heterogeneity, and each cat in the facility appeared to carry a genetically distinct quasispecies. The vast majority of the nucleotide differences (91%) were nonsynonymous. Sequence variation was not randomly distributed but confined to seven sites, six of which coincided with regions identified as potential antigenic sites by computer-assisted analysis of the polypeptide sequence. The combined data suggest that the sequence heterogeneity in the 5′ HVR of the S gene results from antigenic drift. In cat H419, no changes occurred in the quasispecies over a 94-day period of chronic shedding. Similarly, the FCoV quasispecies of cat H324 remained invariant in this region of the genome over a period of 111 days prior to isolation. However, during the subsequent isolation period, three nonsynonymous nucleotide changes occurred. The consecutive accumulation of nonsynonymous nucleotide substitutions in the 5′ HVR of S suggests a sequential emergence of variants, each time replacing the preexisting population, apparently as a result of immune selection. This is reminiscent of the genetic variation occurring in the HVR I of hepatitis C virus (Kurosaki *et al.,* 1994; van Doorn *et al.,* 1995) and in the variable regions of the envelope protein (Env) of the human immunodeficiency virus (Holmes *et al.,* 1992; Strunnikova *et al.,* 1995) and feline immunodeficiency virus (FIV) (Rigby *et al.,* 1993; Sodora *et al.,* 1994) during chronic infection.

The endemic infection in the facility seems to be maintained by chronically infected asymptomatic carriers, rather than through the repetitive occurrence of novel FCoV variants that escape herd immunity. In fact, it would appear that infected animals develop some resistance against superinfection. Prior to isolation, cat H324 was exposed to other infected, virus-shedding cats over a period of at least 111 days. Yet, the H324 quasispecies was maintained, indicating that effective superinfection by one of the other, genetically distinct, FCoVs in the cattery had not occurred. Similarly, the 6-year-old tom cat H039 has been exposed repeatedly to different FCoV-shedding queens. Nevertheless, the FCoV shed by this cat is quite unique and genetically farthest removed from the FCoVs sampled from the other cats, both in the ORF7b and in the S regions. Conceivably, the considerable sequence divergence observed for H039 may have resulted from antigenic drift during prolonged chronic infection. Resistance to superinfection would also explain why littermates, which most likely were infected as kittens, still harbor genetically related FCoV populations long after weaning. The data would fit a model in which the FCoV-specific immune response, though inadequate to clear the infection, is vigorous enough to enforce immune selection and to prevent effective infection by other, antigenically related, FCoVs. Antibodies cross-neutralizing type I FCoV strain UCD1 were detected in the sera of cats H324 and H338. It is of note, however, that FCoV-specific antibody titers were modest and remained low throughout the chronic infection as monitored by immunofluorescence assay (Fig. 2B).

The relationship between the asymptomatic carrier state and the development of feline infectious peritonitis remains to be resolved. Genetic evidence implies that the avirulent FCoVs, which have been designated "feline enteric coronaviruses," and the FIP viruses are not separate species but merely virulence variants of the same virus (Herrewegh *et al.,* 1995; de Groot and Horzinek, 1995; Vennema *et al.,* 1995; Poland *et al.,* 1996). Actually, apathogenic FCoVs readily give rise to disease-causing variants in hosts, immunosupressed by an FELV or FIV superinfection (Pedersen, 1987; Poland *et al.,* 1996). In the field, FIP is a rare disease, predominantly occurring in cats younger than 1 year (Pedersen, 1976a; Addie and Jarrett, 1992; Kass and Dent, 1995). Of the cats that died of FIP in the Hannover breeding facility, 50% were 4–5 months of age. Apparently, these animals were unable to mount a protective immune response. Failure to control FCoV replication may increase viral load, thus

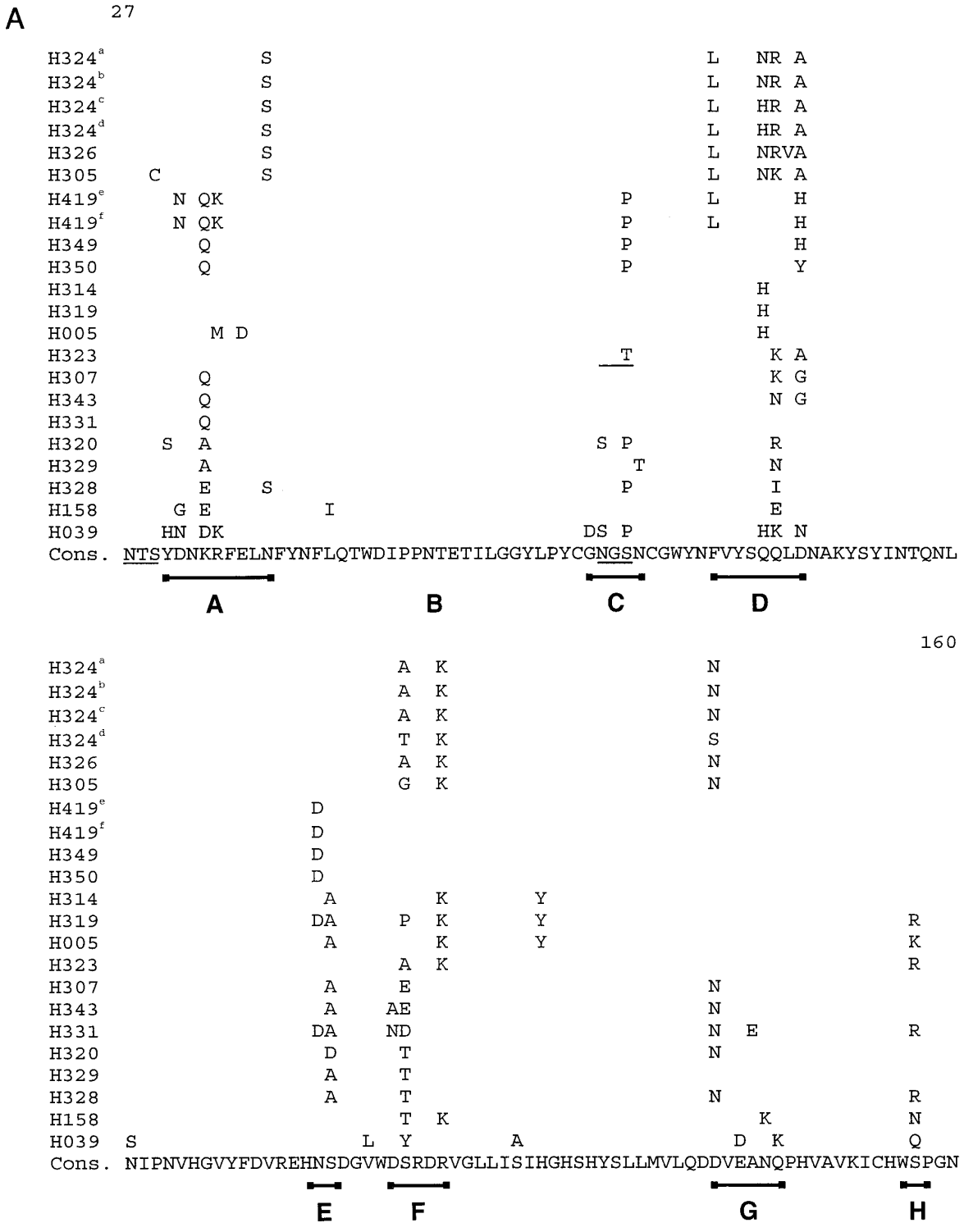


FIG. 7. Amino acid sequence variation in the N-terminal hypervariable region of S. (A) Alignment of amino acid residues 27–160 of the spike protein. Amino acid sequences were deduced from the nucleotide sequences in Fig. 6A. Only those residues differing from the overall consensus sequence (Cons.) are presented. [a,b,c,d,e,f] are as in Fig. 6. Potential N-glycosylation sites are underlined. (B) Jameson–Wolf prediction of potential antigenic sites in the N-terminal hypervariable region of S. The overall consensus sequence of the region formed by residues 27–160 of the S protein was analyzed using the Jameson–Wolf algorithm (Jameson and Wolf, 1988). The results are plotted in a two-dimensional representation of the predicted secondary structure of this region with α helices indicated by a sine wave, β sheets by a sharp sawtooth wave, turns with 180° turns, and coils with a dull sawtooth wave. Regions with an antigenic index exceeding 1.3 are indicated by circles. The size of each circle is proportional to the value of the antigenic index.

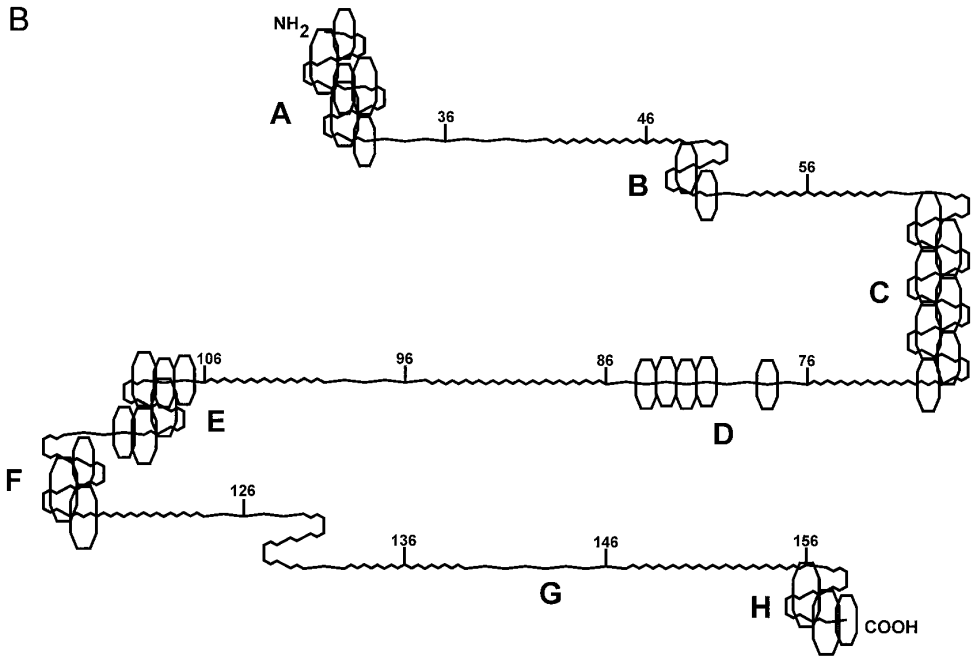


FIG. 7 — *Continued*

raising the odds that a pathogenic mutant is generated. In this view, both host and viral factors determine the outcome of an FCoV infection.

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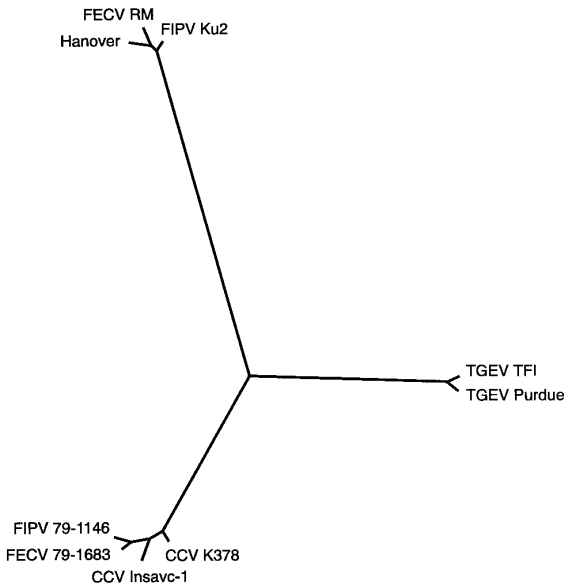

FIG. 8. Unrooted phylogenetic tree illustrating the relationship of the FCoVs in the breeding facility to type I and type II FCoVs and to CCV and TGEV. The tree was constructed using the neighbor-joining algorithm, comparing the overall consensus sequence in the N-terminal S HVR of the FCoVs present in the Hannover breeding colony (see Fig. 7A) to that of the FCoV type I strains FECV RM and FIPV Ku2, the type II strains FIPV 79-1146 and FECV 79-1683, the CCV strains Insavc-1 and K378, and the TGEV strains TFI and Purdue.

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