Feline coronavirus (FCoV) is endemic in feral cat populations and cat colonies, frequently preceding outbreaks of fatal feline infectious peritonitis (FIP). FCoV exhibits 2 biotypes: the pathogenic disease and a benign infection with feline enteric coronavirus (FECV). Uncertainty remains regarding whether genetically distinctive avirulent and virulent forms coexist or whether an avirulent form mutates in vivo, causing FIP. To resolve these alternative hypotheses, we isolated viral sequences from FCoV-infected clinically healthy and sick cats (8 FIP cases and 48 FECV-asymptomatic animals); 735 sequences from 4 gene segments were generated and subjected to phylogenetic analyses. Viral sequences from healthy cats were distinct from sick cats on the basis of genetic distances observed in the membrane and nonstructural protein 7b genes. These data demonstrate distinctive circulating virulent and avirulent strains in natural populations. In addition, 5 membrane protein amino acid residues with functional potential differentiated healthy cats from cats with FIP. These findings may have potential as diagnostic markers for virulent FIP-associated FCoV.

Feline infectious peritonitis (FIP) is an uncommon, fatal, progressive, and immune-augmented disease of cats caused by feline coronavirus (FCoV) infection. Although FCoV is common in most domestic, feral, and nondomestic cat populations worldwide (seroprevalence 20%–100%), FIP will develop in <10% of FCoV seropositive cats (1–4). FIP tends to occur most frequently in cats <2 years of age or, less commonly, in geriatric cats (4, 5). The clinical manifestations of FCoV infection can be either a pathogenic disease, FIP (cases infected with feline infectious peritonitis virus [FIPV]) and, more commonly, a benign, or mild enteric infection (feline enteric coronavirus [FECV] asymptomatic) (6,7). Specific genetic determinants of these clinical outcomes have yet to be discovered. There is no effective treatment, vaccine, or diagnostic protocol that can discriminate the avirulent FECV from FIPV.

FIP pathology is characterized typically by severe systemic inflammatory damage of serosal membranes and widespread pyogranulomatous lesions, which occurs in the lungs, liver, lymph tissue, and brain (8). Evidence suggests that the host immune system is crucial in this pathogenesis; profound T-cell depletion from the periphery and lymphatic tissues and changes in cytokine expression are observed in end-stage FIP (9,10). The clinical finding of hypergammaglobulinemia-associated FIP is indicative of virus-induced immune dysregulation (11).

Viral genetic determinants specifically associated with FIPV pathogenesis have yet to be discovered. An in vivo mutation transition hypothesis postulated that de novo virus mutation occurs in vivo, giving rise to virulence (12,13). The precise nature of the mutation responsible for pathogenesis has not been identified, although studies have suggested sequence differences in the spike protein (14), nonstructural protein (NSP) 7b, and NSP3c (13) as disease determinants. Together with in vitro studies describing the FIPV strains affinity for macrophages in contrast to FECV strains (15), the hypothesis was extended to propose that the enteric coronavirus (FECV) undergoes a mutational shift in the gastrointestinal system, thus allowing infection of macrophages, systemic dissemination, and fatal disease manifestation (12,13). However, attempts to use engineered chimeric viruses designed to identify the operative virulence determinants have been unsuccessful (16). Furthermore, circulating FCoVs found in different tissues of FCoV-infected asymptomatic cats were indistinguishable (17,18).
The in vivo mutation hypothesis of FIPV pathogenesis is widely cited, although it has never been explicitly confirmed. Mutational transition of HIV-1 has been demonstrated in AIDS patients, in which mutation of envelope residues alters coreceptor use from CCR5 to CXCR4, a prelude to the collapse of the CD4-bearing lymphocyte population (19). Similarly, key amino acid changes in the porcine coronavirus spike gene lead to increased virulence in the coronavirus transmissible gastroenteritis virus, a fatal disease causing high rates of illness and death in young pigs (20–22).

An alternative circulating virulent-avirulent FCoV hypothesis of viral pathogenesis suggests that distinctive benign and pathogenic strains of FCoV circulate in a population, and that the disease will develop only in those persons infected by the virulent strains. Dengue virus may offer an example because it has been shown that 4 distinctive viral strains circulate worldwide, and severe hemorrhagic fever develops in persons exposed sequentially to distinct strains (23). Zoonotic equine Venezuelan encephalitis virus also displays circulating virulent and avirulent strains, which through interaction with ecologic and epidemiologic factors, contribute to or constrain the disease incidence (24).

This study aimed to systematically test evolutionary predictions of the in vivo mutation hypothesis versus the circulating virulent/avirulent hypothesis in the pathogenicity of FIP in the cat. We developed a study of naturally occurring FECV and FIPV using molecular genetic tools by collecting samples from FIPV (cases) and FECV-positive but asymptomatic cats (controls). Cases were infected with feline coronavirus (FCoV) and had the clinical disease of feline infectious peritonitis (FIP). Controls were also infected with FCoV, but were clinically asymptomatic (FECV-asymptomatic). The prediction was that phylogenetic analysis of viral gene sequences would demonstrate paraphyly for FIPV case-cats and FECV-asymptomatic cats if the in vivo mutation hypothesis was supported, and monophyly of the 2 if the circulating virulent/avirulent hypothesis was supported (Figure 1). Additionally, we surveyed the viral genetic diversity and dynamics and determined genetic signatures associated with pathogenesis in FIP.

Materials and Methods

Sampling
A total of 56 live, euthanized, or recently deceased domestic cats were clinically examined and sampled in Maryland veterinary hospitals during 2004–2006 (online Appendix Table 1, available from www.cdc.gov/EID/content/15/9/1445-appT1.htm). Blood (3–6 mL) was collected routinely by venipuncture from manually restrained or anesthetized domestic cats. Feces were obtained from the rectum by cotton swab and frozen in 0.5 mL of phosphate-buffered saline. Cats from 1 (Weller Farm) of 6 farms were micro-chipped (AVID, Folsom, LA, USA) for identification for repeat sampling of individual cats. Samples were collected in full compliance with specific federal permits (Convention on International Trade in Endangered Species; Endangered and Threatened Species) issued to the National Cancer Institute by the US Fish and Wildlife Service of the Department of the Interior.

For euthanized and recently deceased cats, gross necropsy examination and sample collection were performed within 2 hours of death. Samples from liver, spleen, mesenteric lymph node, kidney, jejunum, and colon were taken, fixed in 10% buffered formalin, and routinely embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE). Tissues were also flash frozen in liquid nitrogen (–220°C) for RNA extraction and stored at either −220°C or −70°C.

Clinical Hematologic and Biochemical Analysis
For complete blood counts, fresh (<12 hours) whole-blood samples were assessed by Antech veterinary diagnostic laboratory by using an automated cell counter (Avid Cell-Dyn 3500; Abbott Laboratories, Abbott Park, IL, USA). Biochemical analysis (Hitachi 717 Clinical Chemistry Analyzer; Roche Diagnostics, Indianapolis, IN, USA) and ELISA for feline immunodeficiency virus (FIV; Petchek FIV ELISA, Idexx Laboratories, Westbrook, ME,
USA), and coronavirus (Virachek CV, Synbiotics Corp., San Diego, CA, USA) antibodies were also performed.

Pathologic and Immunohistochemical Analysis

HE-stained slides of spleen, liver, lymph node, intestine, and kidney sections were evaluated for evidence of granulomatous and pyogranulomatous lesions (National Cancer Institute Laboratory Animal Sciences Program, Frederick, MD, USA). Formalin-fixed sections (3 μm thick) were cut from paraffin blocks and placed on glass slides for immunohistochemical (IHC) testing, as previously described, with CoV p56, a cross-reacting antibody for the demonstration of feline coronavirus (FECV and FIPV biotypes) (9,10) (Washington Animal Disease Diagnostic Laboratory, Pullman, WA, USA) (Figure 2).

RNA Extraction and Reverse Transcription

RNA from 160 μL ascites fluid or frozen feces suspended 10% in phosphate-buffered saline was extracted by using the QIAamp virus RNA mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer’s instructions. RNA from tissue was extracted from ≈60 mg of frozen liver, lung, spleen, colon, jejunum, or lymph node by using RNAeasy (QIAGEN) following the manufacturer’s instructions. Extracted RNA was eluted in 35 μL of RNase-free water and stored at −70°C. cDNA was reverse transcribed using 9 μL of eluted RNA (10 pg–5 μg) in an initial 12-μL reaction mixture containing 50 ng of random hexamers and 0.5 mmol/L of dNTPs. After incubation at 65°C for 5 min to denature the RNA, 10 mmol/L of dithiothreitol, 5× Synthesis Buffer, 40 U of RNaseOUT, and 15 units of Thermostable RT were added on ice (Invitrogen, Carlsbad, CA, USA). Reaction mixtures were incubated in thermocycler at 25°C for 10 min, followed by 50°C for 30 min. cDNA was stored at −20°C.

PCR

Primers amplifying 7b (736 bp), membrane protein (575 bp), polymerase (386 bp), and spike-NSP3 (1,017 bp) (Figure 3) were designed based on available feline coronavirus sequence (1,12,13). PCR was performed by using 2 μL of cDNA in a 50-μL reaction containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.25 mmol/L concentrations of dNTPs (dATP, dCTP, dGTP, and dTTP), 2 mmol/L concentrations of each primer, and 2.5 units of Platinum Taq DNA polymerase (Invitrogen). PCR was conducted on a geneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following touchdown conditions: 2 min at 94°C then a touchdown, always starting with 20 sec at 94°C, then 30 sec at 60°C (3 cycles), 58°C (5 cycles), 56°C (5 cycles), 54°C (5 cycles), 52°C (22 cycles), and then 1 min at 72°C for extension, and with a final extension at 72°C for 7 min and a hold at 4°C. PCR products were visualized by

Figure 2. A) Histopathologic and immunohistochemical (IHC) results from 23 necropsied cats positive for antibodies against feline coronavirus. Liver, lung, spleen, colon, jejenum, stomach, heart, kidney, lymph node were evaluated by IHC. Feline infectious peritonitis (FIP) cases are highlighted in gray. Pos, positive; Neg, negative; ND, not done. B) Representative tissues from cat no. FCA-4653, spleen (histopathologic) showing granuloma (arrow); magnification ×20. C) Representative tissues from cat no. FCA-4590, small intestine (IHC); magnification ×20. D) Red staining indicates binding of coronavirus antibody (CoV p56, arrow); magnification ×100.
electrophoresis on a 1% agarose gel and primers and unincorporated dNTPs were removed by using Microcon YM (Millipore, Billerica, MA, USA).

**Cloning and Sequencing**

Representative PCR products were cloned and sequenced (Figure 3, panel B). Cloning was performed by using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer’s instructions. Plasmid DNA was isolated from 1–47 clones from each reaction product (Agencourt CosMCPrep; Agencourt Bioscience Corporation, Beverly, MA, USA). The presence of the correct sized insert was verified by restriction enzyme digestion (EcoRI), and sequences were obtained from clones with the correct insert by using standard ABI BigDye terminator reactions (Applied Biosystems). Anticontamination measures were taken at all steps of reverse transcription–PCR (RT-PCR) amplification and post-PCR processing.

**Phylogenetic Analysis**

Sequences from pol 1a, spike-NSP3, membrane, and NSP7b were analyzed separately. Nucleotide sequences were compiled and aligned for subsequent phylogenetic analysis by using ClustalX (25) and verified visually (26). Analyses involved producing a phylogenetic tree of viral gene sequences based upon the following approaches: minimum evolution, maximum parsimony, and maximum likelihood in PAUP (27). Modeltest was used to estimate the optimal model of sequence evolution, and these settings were incorporated into subsequent analyses (28). Minimum evolution trees were constructed from models of substitution specified by Modeltest, with starting trees obtained by neighbor joining followed by application of a tree-bisection-reconnection (TBR) branch-swapping algorithm during a heuristic search for the optimal tree. Maximum parsimony analysis employed a heuristic search of starting trees obtained by stepwise addition following by TBR. Maximum-likelihood parameters specified by Modeltest selected the general time-reversible model of substitution, included empirical base frequencies, and estimated rate matrix and corrected for among-site rate variation (gamma distribution). A bootstrap analysis using 1,000 iterations was performed for maximum parsimony and minimum evolution and 100 iterations by using the nearest neighbor interchange branch-swapping algorithm for maximum likelihood. Amino acid residue alignments were generated using MacClade 3.05 (26) and ClustalX (www.softpedia.com/get/Science-CAD/Clustal-X.shtml).

Variable sites and parsimoniously informative sites were computed in MEGA version 3.0 (29). Pairwise comparisons of genetic distances were performed in PAUP and the mean and range of genetic distances were calculated in Excel (Microsoft, Redmond, WA, USA). The sequences of FCoV pol 1a, membrane, NSP 7b, and spike-NSP3 were deposited in GenBank under accession nos. EU663755–EU664317.

**Results**

During 2004–2006, fifty-six domestic cats with suspected FIP or exposure to infected FIP cats from Maryland farms and veterinary hospitals were sampled (online Appendix Table 1). All samples producing RT-PCR products were from cats positive for antibodies against FCoV (titers >25). Thirty-six sampled cats were from the Weller farm where several individual cats were sampled once per year for the 3-year study period. Healthy and recently deceased or euthanized cats were included from the Ambrose farm (n = 7), Palmer Veterinary Hospital (n = 3), Frederick County Animal Shelter (n = 7), Seymour farm (n = 1), and the New Market Animal Hospital (n = 2). Fca-4590 from the Weller farm is an important FIP case because samples were obtained on May 20, 2004, when the cat was clinically healthy (predisease) and again on December 22, 2004, when FIP developed in the cat and it died (postdisease).

Necropsies were performed on 23 cats that died or were euthanized due to suspected FIP. Most of the necropsied cats were FCoV antibody positive (online Appendix Table 1). Eight cats were classified as FIP cases based on histopathologic findings (Fca-4549, Fca-4566, Fca-4590, Fca-4618, Fca-4653, Fca-4662, Fca-4663, and Fca-4664)
The presence of pyogranulomatous lesions at histology evaluation was sufficient for designation of an FIP case. Additionally, 5 of the 8 FIP cases were evaluated by IHC testing. Multiple tissues were positive by IHC in each of these cats. One cat (Fca-4561) was IHC positive only in the jejunum and negative by histopathologic analysis on all tissues, therefore it was classified as FECV asymptomatic. The FCoV-seropositive necropsied cats with no characteristic FIP histopathologic changes and IHC lesions were classified as FECV asymptomatic (online Appendix Table 1; Figure 2). Healthy cats were classified as FECV asymptomatic if they had normal results on physical examinations, were FCoV antibody positive (titer >25) but not lymphopenic (<1.5 cells/μL), or were monitored until 2007 and known to be free of FIP disease (online Appendix Table 1).

RT-PCR was attempted with 4 primer pairs designed from FCoV genes for all cats (Figure 3, panel B). Of the 82 samplings from 56 cats, 42 samplings amplified virus with at least 1 primer pair yielding a 51% rate of recovery of viral sequence (online Appendix Table 1). From 8 cats with clinical FIP and 23 FECV-asymptomatic cats, amplification from the 4 viral regions produced 735 cloned viral gene segments that resulted in 501 unique gene sequences (online Appendix Table 2, available from www.cdc.gov/EID/content/15/9/1445-appT2.htm; Figure 3, panel B).

Phylogenetic analysis of the cloned virus sequences from 3 Maryland locales sampled during 2004–2006 showed specific patterns of viral dynamics. First, gene sequences from healthy cats infected with FECV displayed a monophyletic cluster pattern that was generally distinctive from cats diagnosed with FIP in the membrane, NSP 7b, and spike-NSP3 gene segments (Figure 4). For example, every FCoV gene sequence for the membrane gene from FIP cases fell within a major cluster consisting of 3 principal clades (Figure 4). By contrast, 127/154 (82%) virus gene sequences from FECV-asymptomatic cats sorted in 2 separate clades that were distinct (100 bootstrap statistical support) from the viral gene sequences of FIP cases (Figure 4). Similar reciprocal monophyly of 140 NSP7b sequences was obtained for FIP cases versus FECV-asymptomatic cats (Figure 4). A consistent disease driven phylogeographic sorting was also observed for the 1,017-bp sequence spanning the spike-NSP3 genes, albeit with less statistical resolution, likely because of evolutionary constraints on gene divergence in this region (Figure 4). Together the remarkable reciprocal monophyly in these 3 genes supports the predictions of the circulating virulent-avirulent strain hypothesis illustrated in Figure 1.

Samples from 1 cat, Fca-4590, were particularly informative. The virus was isolated from the cat predisease, and then again 7 months later postdisease. Fca-4590 was asymptomatic but infected with FECV in May 2004. FCoV sampling from that month showed strong (high bootstrap) affiliation with the FECV-asymptomatic clades for the membrane and the NSP7b genes. However, virus isolated
7 months later in December 2004 after FIP developed in Fca-4590 fell within the FIP-case clades (also with high bootstrap), and was indistinguishable from FCoV isolated from other cats with FIP. This finding suggested that the pathogenic FIP-case type of FCoV infected this cat subsequent to its infection with an avirulent FECV and apparently replaced it.

Tissue-specific differentiation within each cat was minimal (Figure 4). By contrast, there were notable locale-specific distinctions within the sick and healthy cats (Figure 4). For example, the FECV strains in asymptomatic cats from the Weller household were associated together in a major FECV subclade; strains in cats from the Frederick Animal Shelter were classified in a different subclade, nestled within the FECV-asymptomatic clade (Figure 4). The archival FCoV virulent strain (Aju-92), isolated from cheetahs in Oregon in 1982 (30), defined a phylogenetic lineage distinctive from the FIP and FECV-asymptomatic clades resolved in the Maryland domestic cats (Figure 4).

Nucleotide sequences of membrane and NSP 7b generated in this study were translated to amino acid sequences (online Appendix Figure 1, available from www.cdc.gov/EID/content/15/9/1445-appF1.htm). Relative to pathogenesis, 5 informative amino acid sites were found in the membrane protein at positions 108, 120, 138, 163, and 199 (based on reference sequence for TGEV GenBank no. NP058427) (22), giving rise to 6 composite genotypes potentially diagnostic of FIP cases versus FECV-asymptomatic cats (online Appendix Table 2). Among the 8 cats with FIP, 19 FECV-asymptomatic cats, and 1 cheetah with FIP, 6 composite genotypes were identified based on these 5 diagnostic sites (online Appendix Table 2).

All domestic cats with FIP diagnosed by pathologic or immunohistochemical changes displayed the amino acid signature of either YIVAL (I) or YIILAL (II); infected cats without clinical FIP had the HIIVI (III), HIIVL (IV), HVI-

Infection with FCoV is common in cats throughout the world, although in most cats the virus causes no clinical signs. However, in some cats, FCoV infection is associated with the development of the progressive and fatal disease manifestation of FIP. This disease is among the most serious viral infections in cats, not only because of its fatal nature, but also because of the difficulties in diagnosing FIP antemortem and controlling the spread of FCoV. We have presented a molecular virologic study of naturally occurring FCoV infection and phylogenetic analysis of the cloned virus sequences obtained from the membrane, NSP 7b, spike-NSP3, and pol 1a genes isolated from domestic cats located in Maryland households infected with FCoV during 2004–2006. We observed predominately monophyletic clustering of strains correlating with disease phenotype in membrane and NSP 7b genes consistent with the circulating virulent/avirulent strain hypothesis of FIP pathogenesis, which calls into question the in vivo mutation hypothesis.

The amino acid alignments presented in online Appendix Figure 1 clearly demonstrate that in the FIPV cases included in this study the genotypes correlated with disease phenotype are ancestrally derived and not the result of a few de novo mutations. Given the clear genetic differentiation between viruses from FIP cases and FECV asymptomatic cats in multiple gene segments, we infer that cats become reinfected with new strains of FCoV from external sources, rather than by in vivo mutations. Cats in our study were not co-infected with multiple strains of FECV and FIPV at the same time and were generally infected with one predominant virus strain. Two exceptions to this finding in our study were cats with cases of FIPV (Fca-4662 and 4664) that from which distinct gastrointestinal (feces or intestine) and systemic (liver and/or ascites fluid) viral isolates were obtained, which indicates that in vivo superinfection does occur (Figure 4; online Appendix Table 2).

A role of the membrane protein in FIP pathogenesis seems likely, given its known functions in other coronaviruses. The membrane protein is the most abundant structural protein with important functions in virus budding and with cell-mediated host immunity (31). The specific functions of the membrane protein amino acid sequences have been determined in severe acute respiratory syndrome (SARS)–CoV (32). Aligning the sequences from this study with SARS-CoV, the first diagnostic amino acid site 108...
aligns to a site just upstream from the second transmembrane helix (online Appendix Figure 1). A tyrosine at position 108, which is found in all FIPV biotypes and shared among SARS-CoV, has a neutral polarity (in contrast to a histidine there, found in most FECV biotypes, which have a positive polarity) and may play a role in the stability of the virus within the membrane. Site 120 aligns within the third transmembrane helix, site 138 aligns just downstream to the transmembrane helice, site 163 aligns within the C-terminus, which projects inside the virus particle, and site 199, also within the C-terminus domain, aligns within a defined SARS-immunodominant epitope (32) (Figure 5).

The demonstration of 6 naturally occurring composite genotypes based on 5 variable sites in the membrane protein amino acid alignment that are highly correlate with disease phenotype (online Appendix Table 2) offers specific opportunities for developing diagnostics and for the preventive management of this disease. By extending this study to additional cat populations in disparate geographic locations, designing chimeric FCoV challenge experiments, and investigating host genetic correlations with pathogenesis, we will be able to further discern the causative factors in FIP pathogenesis. Fca-4594, which was infected with the disease-associated genotype composite without succumbing to FIP, suggests additional requirements for viral pathogenesis. As has been suggested in the outbreak of FIP in a colony of captive cheetahs (33), host immune genetics may play a role.

Both the viral strain and host immune genes contribute to disease progression and virus-related death, such as AIDS progression in HIV infection. With the recent publication of the full cat genome sequence (34) and the viral genotype composites described here, new genomic tools are now available to proceed with both viral and host genetic association studies in the pathogenesis in FCoV infection, a model for coronavirus infection in humans, such as SARS-CoV.

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Dr Brown completed this work while a member of the Laboratory of Genomic Diversity at the National Cancer Institute. Currently, she is a postdoctoral research fellow at University College Dublin Veterinary Sciences Centre, Ireland. Her research interests include infectious disease studies in the cat animal model.

References


Figure 5. Diagram of membrane protein containing 3 transmembrane helices, an external N terminus and an internal carboxy terminus. Approximate position of 5 variable diagnostic amino acid sites (see Table 2) as determined by sequence comparison to severe acute respiratory syndrome coronavirus (32). Amino acid residue, polarity, and hydrophobicity or hydrophilicity is stated.
Borna disease virus
[bor′nə]
Borna disease virus was named after the town of Borna in Saxony, southeastern Germany, where in 1885 many horses in a German cavalry regiment died of a fatal neurologic disease. The ill horses exhibited abnormal behavior—running about excitedly, walking into walls, being unable to chew food. A similar disease had been observed in horses, sheep, and cattle for more than 100 years. The causative agent was later found to be a negative-stranded RNA virus, which may also be a human pathogen.