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Prevalence and Genetic Pattern of Feline Coronaviruses in Urban Cat Populations

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SUMMARY

The prevalence and phylogeny of feline coronaviruses were studied in urban cat populations by sampling of 113 clinically healthy cats. Rectal swab samples were subjected to a nested reverse-transcription polymerase chain reaction, specific for the conservative nucleocapsid region of the virus genome. More than 30% of the sampled animals proved positive for the presence of feline coronaviruses. The nucleotide sequences of amplified 440 bp products were determined, aligned and the phylogenetic analysis revealed noticeable genetic clusters among the prevalent feline coronaviruses in the surveyed geographic area. These findings will hopefully contribute to the elucidation of the epidemiology of feline infectious peritonitis. © 2000 Harcourt Publishers Ltd

KEYWORDS: Feline; coronavirus; RT-PCR; prevalence; phylogeny.

INTRODUCTION

Feline coronaviruses (FCoV) are members of the family *Coronaviridae* in the newly established Order *Nidovirales* (de Vries *et al.*, 1997). The family *Coronaviridae* is divided into three distinct antigenic groups (Siddell *et al.*, 1983; Horsburgh *et al.*, 1992; Motokawa *et al.*, 1996); FCoV belongs to the group which contains a human respiratory coronavirus (HCV 229E), transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCV), porcine epidemic diarrhoea virus (PEDV), and canine coronavirus (CCV). Feline coronaviruses are enveloped viruses with at least 20kb long, single-stranded, positive-sense RNA genomes. In addition to non-structural genes, the genome encodes four structural proteins termed S (spike or peplomer), E (envelope), M (matrix), and N (nucleocapsid; de Groot *et al.*, 1987; Luytjes, 1995).

Two serotypes of FCoV have been discriminated (Hohdatsu *et al.*, 1991a,b) which differ in their *in vitro* growth characteristics and in their prevalence in the field (Pedersen, 1987b; Hohdatsu *et al.*, 1992). The replication of coronaviruses is characterized by a high frequency of RNA recombination (Lai *et al.*, 1985; Makino *et al.*, 1986; Keck *et al.*, 1988; Baric *et al.*, 1990; Kusters *et al.*, 1990; Kottier *et al.*, 1995; Lai, 1996). Recent findings suggest that type II feline coronaviruses have arisen from a recombination event between CCV and a type I FCoV (Herrewegh *et al.*, 1998).

Based on pathogenicity, FCoV has been divided into two biotypes, termed feline enteric coronavirus and feline infectious peritonitis virus (FECV and FIPV, respectively; Pedersen, 1987a).

Feline infectious peritonitis virus (FIPV) is an important pathogen of cats, causing death among young animals, especially those originating from

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pure-bred catteries (Pedersen, 1987a; Foley *et al.*, 1997a). The disease has either a classical wet/effusive or a dry/non-effusive clinical manifestation. Both forms are progressive and ultimately lethal (de Groot & Horzinek, 1995). However, initial signs are not pathognomonic; the affected cats show anorexia, chronic fever and malaise. Occasionally, ocular and neurological disorders occur. The pathogenesis of the disease includes type III and IV hypersensitivity reaction of the immune system (Paltrinieri *et al.*, 1998). The diagnosis and control is rather complicated and serological data are not always conclusive (Olsen, 1993). Although a commercially available vaccine has had promising results (Fehr *et al.*, 1997), some other experimental vaccines seem not to be of great help; furthermore, by antibody-dependent enhancement of infection, early death may be provoked with their use (Vennema *et al.*, 1990; de Groot & Horzinek, 1995; Harvey *et al.*, 1996).

In contrast, feline enteric coronaviruses are not an important cause of morbidity in cats. They may produce mild enteritis, but most cases of experimental and natural infections remain subclinical. FECV can establish persistent infection in the large intestine (Herrewegh *et al.*, 1997). By shedding the virus in the faeces, the asymptomatic FECV-infected carrier cats spread the infection to susceptible animals via the faecal-oral route. Although it is not considered a highly pathogenic agent, FECV presents a considerable health risk to cats because it is the parent of FIPV, which arises as a result of genetic alterations of FECV during the intestinal replicative phase of infection (Poland *et al.*, 1996; Foley *et al.*, 1997b; Vennema *et al.*, 1998). The resultant FIPV causes systemic infection and, consequently, various clinical forms of the disease may develop (Evermann *et al.*, 1991; Herrewegh *et al.*, 1997). It has been suggested that the incidence of FIP in a cat population is more correlated to the FECV → FIPV mutation rate than to the presence of FIPV variants (Vennema *et al.*, 1998). Thus, it is recommended that a population be monitored for the prevalence of asymptomatic FECV carriers in order to estimate the probability of the occurrence of FIPV and the chances for development of the disease. Since the detection of FCoV by virus isolation is difficult, the application of reverse-transcription polymerase chain reaction (RT-PCR) has been proposed for surveying asymptomatic FCoV carriers (Herrewegh *et al.*, 1995; Foley *et al.*, 1997a).

The aim of this study was to determine the prevalence of asymptomatic FCoV carriers and to investigate the phylogeny of coronaviruses in urban cat populations.

MATERIALS AND METHODS

Cat populations

The 113 sampled cats lived in urban areas of Eastern Hungary. Approximately 60% of the animals were allowed outside and 40% were kept indoors. The majority of the cats (88 animals) are kept alone in a household. Two breeding stocks were involved in the investigations, comprising 4 and 11 breeding Persians, respectively. The age distribution of the examined animals from either gender varied between 6 months and 10 years. Altogether, 71 mixed-bred (63%), 34 Persian (30%), and eight Siamese (7%) cats were sampled.

Clinical specimens and sample preparation

The clinically healthy cats, presented to private veterinary clinics for routine examination, vaccination, or castration were sampled by rectal swabbing as described by Foley *et al.*, (1997b). The RNA extraction was carried out according to Boom *et al.* (1990) and Cheung *et al.* (1994), using size-fractionated silica and guanidine thiocyanate based buffers.

Reverse transcription

The FCoV cDNA synthesis was performed in 28-μL reaction mixtures. A preliminary mixture containing 5 μL RNA, 5 μL diethyl pyrocarbonate-treated water, and 1 μL random hexamers (0.02 U; Pharmacia Biotech) was incubated at 65°C for 5 min to denature the RNA. Subsequently, tubes were placed on ice, and 17 μL premixture was added containing 2.5 μL of each deoxynucleotide triphosphate (2 mM; Pharmacia), 5 μL of 5 X reaction buffer [0.25 M Tris-HCl (pH 8.3), 0.375 M KCl, 15 mM $MgCl_2$], 1 μL RNAguard (24 U; Pharmacia), and 1 μL Moloney murine leukaemia virus reverse transcriptase (200 U; Gibco BRL). The reactions were incubated at 37°C for 90 min, followed by incubation at 98°C for 5 min to inactivate the enzyme.

Primer selection

Primers were selected from the highly conserved nucleocapsid (N) region of the FIP virus strain 79–1146 genome (Vennema *et al.*, 1991; GenBank Acc. No: X56496) using the OLIGO 4.0 program (National Biosciences Inc.). The sequences of the primers are shown in Table I.

Table I
Primers used for the RT-PCR of feline coronaviruses

Primer	*Sequence (5′–3′)*	*5′ position*[a]
OFIP1A(F)	ATTTT GGAAT TTATG TCCGA GAGA	990
OFIP2A(R)	CTAGC ACCAT AGAAA GTTGT CACA	1598
OFIP3(F)	CGCTG AGAGG TGGTT CTTTT ACTTC	1110
OFIP4(R)	CTTCC AGGTG TGTTT GTTGG CATTC	1554

F, forward primer; R, reverse primer.[a], nucleotide positions in the nucleocapsid coding region, according to Vennema *et al.* (1991).

PCR

The PCR assays were carried out in 50-μL volumes. The reaction mixtures contained 5 μL of 10 X GeneAmp PCR buffer II (Perkin-Elmer Cetus), 15 pmol of each primer (OFIP1A and OFIP2A in the first reaction), 2.5 μL of each deoxynucleotide triphosphate (10 mM; Pharmacia), 2.5 mM $MgCl_2$, AmpliTaq DNA polymerase (1 U; Perkin-Elmer Cetus), and 5 μL of cDNA. Two drops of mineral oil (Sigma) were added to prevent evaporation. The second (nested) PCR was performed under similar conditions to the first PCR, using 15 pmol of each of the primers OFIP4 and OFIP3 and 2 μL of the first PCR product as target. Distilled water was used as negative control.

During the 35 cycles of the first round, the temperature profile was: 94°C for 45 s for denaturation, 45°C for 60 s for primer annealing, (50°C for 60 s for the first five cycles), and 72°C for 2 min for synthesis. For the second round of amplification 96°C for 45 s, 52°C for 1.5 min, and 72°C for 3 min was applied. This cycle profile was repeated 30 times followed by a final extension step at 72°C for 7 min.

The PCR products were detected by agarose gel-electrophoresis and ethidium bromide staining.

Nucleotide sequencing and phylogenetic analysis

A total of 36 nested PCR products were sequenced in both directions with primers OFIP3 and OFIP4, using an ABI PRISM sequencing device, based on the incorporation of fluorescent labelled dideoxynucleotide terminators. The nucleotide sequences were aligned by the MegAlign program of the DNASTAR multiple program package (DNASTAR Inc.) using the Clustal method (Higgins *et al.*, 1992).

RESULTS

PCR positive cats

By running the nested PCR, 36 (31.8%) out of the 113 examined animals were found to be positive. The PCR positivity of the pure-bred and the mixed-bred animals was 45.2 and 23.9%, respectively. The virus was most frequently detected among the Persians (52.9%), and the two Persian breeding catteries showed 100% (all the four animals being positive) and 72.7% (eight positives out of the eleven cats) of prevalence.

The PCR positivity was evenly distributed through the age categories, exemplified by 34.6% positive animals in cats younger than 1 year of age, 31.6% among animals aged 1–5 years, and 35.3% among cats older than 5 years.

Phylogenetic analysis of the amplified sequences

Figure 1 shows the dendrogram prepared from the sequence alignments and reveals the marked clustering of the detected viruses. Furthermore, FCoV originating from the two Persian breeding stocks showed a very close similarity to each other (with one or two nucleotide differences) and displayed separate clusters with some distance (due to four to six nucleotide alterations) from the majority of the examined viruses. In this major group there is an apparent cluster comprising cats Nos. 72, 8, 48, 58, 49, 69P, and 36 (see Fig. 1). The overall difference between the most disparate strains originating from the examined cats remained below 10 out of the examined 440 nucleotides.

DISCUSSION

In the present study a representative group of animals was investigated to estimate the prevalence of asymptomatic carriers and the genetic diversity of feline coronaviruses occurring in urban cat populations. Although other regions of the genome have been amplified by various groups (Li & Scott, 1994; Herrewegh *et al.*, 1995; Gamble *et al.*, 1997; Gunn-Moore *et al.*, 1998), we applied an RT-PCR assay which targeted the highly conserved N region of the FCoV genome in order to detect all possible

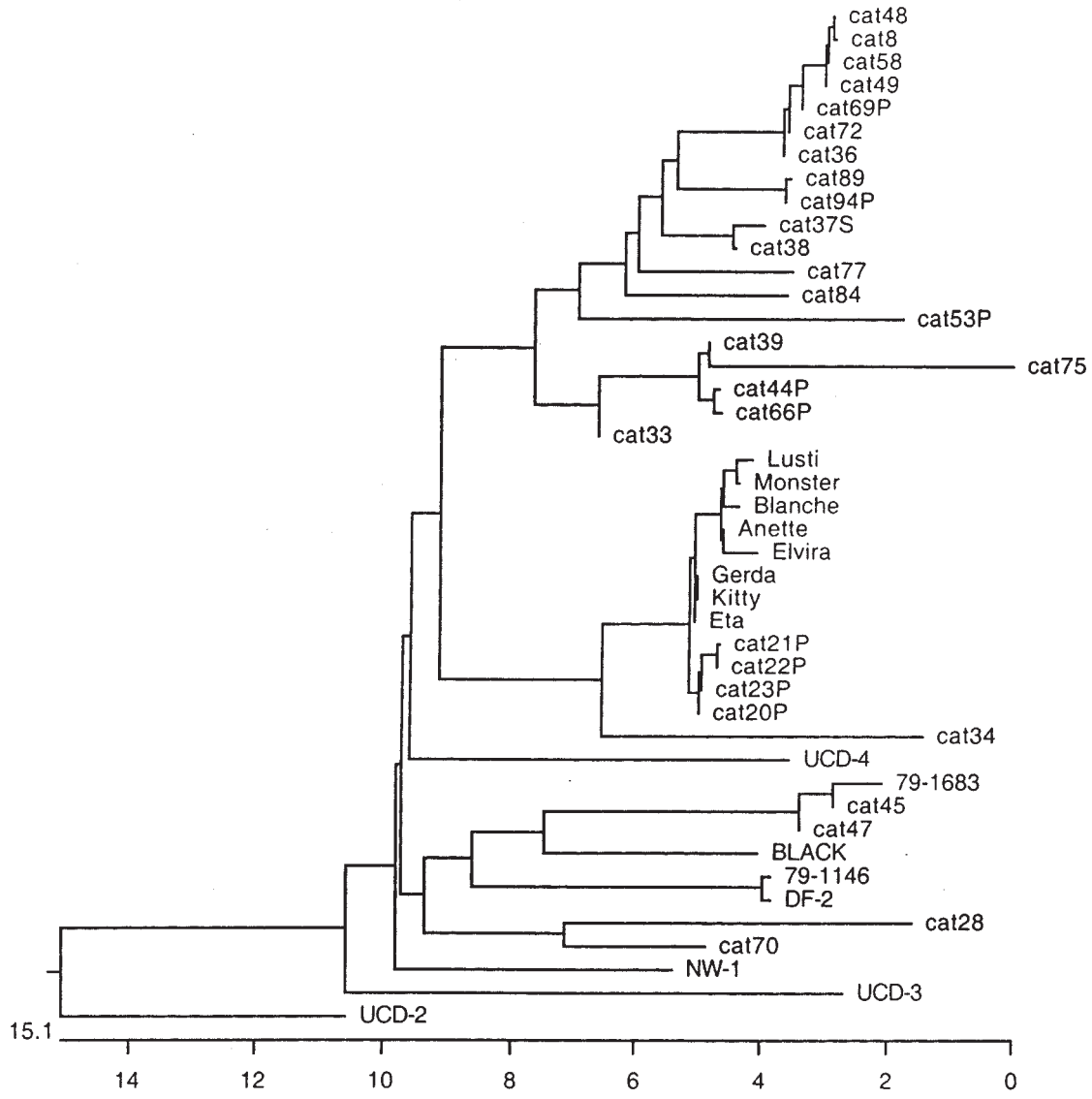

Fig. 1. Phylogenetic tree obtained with the nucleocapsid coding region (N) sequences amplified from FCoV reference strains (UCD-2, UCD-3, UCD-4, NW-1, DF-2, BLACK, 79-1146, 79–1683), and from the rectal swab samples of the clinically healthy cats in this study. The scale below measures the evolutionary distances between the sequences. Units indicate the number of substitution events. P, Persian; S, Siamese cats; unlabeled animals were mixed-bred carriers. Samples represented by names of the cats and by Nos 20P–23P represent two Persian breeding stocks.

variants of the virus. Using this assay, we found that approximately 30% of the clinically healthy cats carried FCoV in the monitored area. The seroprevalence has not been determined in this study; however, 80–90% seropositivity against FCoV has been described in catteries and 10–50% of cats in single cat households (Pedersen, 1976; Addie & Jarrett, 1992a,b). In accordance with previous studies (Foley *et al.*, 1997a), there was no significant correlation between positivity and the gender of the animals. However, the pure-bred animals showed higher rate of positivity than the mixed-bred groups, supporting the observation that pure-bred cats are more susceptible to FCoV infections (Foley & Pedersen, 1996). Since the positivity in the examined groups of age was evenly distributed,

the detection of some chronically infected asymptomatic carrier animals is assumed, in agreement with previous reports (Herrewegh *et al.*, 1997).

It is known that FCoV, CCV, and TGEV are antigenically related. However, based on the deduced amino acid sequences of N and M proteins, separation of FCoV from the other members of group I coronaviruses was shown (Motokawa *et al.*, 1996). Considering antigenicity and pathogenicity, FCoV have been divided as two serotypes and two biotypes, respectively (Pedersen, 1987a, b; Hohdatsu *et al.*, 1992). Based on antigenicity, the separation of the two serotypes is clearly demonstrated (Hohdatsu *et al.*, 1992). However, the discrimination between the two biotypes is complicated, although considerable research has been carried out to investigate the hypothesized evolution of FIPV from FECV (Herrewegh *et al.*, 1997; Vennema *et al.*, 1998). For a better understanding of this process, large populations of clinically healthy cats should be screened and the presence of FCoV should be estimated. Subsequently, the genomes of the detected FCoV variants should be analysed in order to investigate the alterations leading to the switch of the biotype.

The present study aimed at the first main task, i.e. to survey populations of healthy urban cats of various breeds to reveal the prevalence of FCoV. Further, we performed a pilot phylogenetic analysis based on N gene sequences in order to investigate the genetic heterogeneity of the coronaviruses detected in the cat populations. We demonstrated an interesting clustering of the feline coronaviruses: the two Persian breeding stocks composed separate clusters different from those of the prevalent viruses (see Fig. 1). Since it was hypothesized that cats infected with FCoV develop resistance against superinfection by other coronaviruses (Herrewegh *et al.*, 1997), the viruses carried by the Persian breeding animals could represent a unique variant of FCoV. Visiting exhibitions and mating abroad must have contributed to the infection of these otherwise closed stocks of Persian cats. Another noteworthy fact is that, since the establishment of the stocks (which was more than 10 years ago), the cats have not been affected seriously by viral diseases and the suspicion of FIP has never arisen. Thus, either the FCoV variants present in these Persian cats lack the ability to evolve into FIP virus, or these particular cats have a unique resistance against the circulating viruses. The investigations of further genomic regions of the viruses (ORF3c, ORF7b; Vennema *et al.*, 1998) will hopefully provide more information to answer these questions. An additional cluster, consisting of seven viruses (cats Nos. 8, 36, 48, 49, 58, and 69P, see Fig. 1.) may also have the same source of infection since these cats are kept in a restricted area.

In conclusion, the nucleocapsid-based nested RT-PCR assay proved to be a useful tool for monitoring clinically healthy cat populations and to detect asymptomatic FCoV shedding. The obtained sequence data allowed the grouping of the viruses, even so, the differences remained below 10 nucleotides. Thus, the approaches of molecular virus detection and genetic characterization provide effective novel means to study the spread of FCoV variants. Hopefully, these assays and the collected genomic data will contribute in the understanding of the molecular events responsible for the development of infectious peritonitis in cat populations.

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Book Review

Companion Animal Death
Stewart, M.F., Oxford, Butterworth-Heinemann, 1999. 188pp. £14.99 (soft) ISBN 0750640766

'A unique new book for the veterinary surgeon and other members of the veterinary practice team …' is how this title is described on the back cover. It is interesting that, while euthanasia of pets is a constant and common part of practice, it is only recently that much has been written and discussed about the subject. Mary Stewart brings a warm and instructive approach to this rather sad event.

The book observes the whole subject of euthanasia and death of a pet, but also looks further to the bereavement that follows. As veterinary students, we are taught how to euthanase animals competently; what is often lacking, however, is the skill to deal with owners at a time when they may be unbearably distressed. Many vets do not fully understand the depth of feeling that some owners have for their animals. Mary Stewart sets out to educate both the veterinary practice staff and students by discussing the nature of the human/companion animal bond, which is the basis for the very powerful emotion that can overcome owners at the death of a pet.

She discusses in detail the process of bereavement which, once understood, will give veterinary staff a much better chance of handling the situation competently and with compassion. Information on how to break bad news and helping clients to come to a decision (to euthanase) is well presented by the use of notes and bullet points. The procedures that follow euthanasia (support for the owner, options for disposal of the body and practice sympathy correspondence) are clearly laid out and may give new ideas to help the practice achieve a caring attitude. There is also acknowledgement, and discussion, of the stress and sadness felt by the veterinary team. Many owners forget, in the throes of their own grief, that the staff may be upset to lose an old practice 'friend'. In the section 'Stress in veterinary practice' there is detailed information on the factors (unrelated to euthanasia) that can cause anxiety in practice, and some excellent advice on how to avoid becoming stressed, which should be read by all veterinarians.

The emphasis of the book throughout is on communication, sensitivity and attitude. The consequences of poor client management at the time of death or euthanasia of a pet are stated unequivocally as causing troublesome, unhappy clients who will 'spread the word' and may want to 'take the matter further'. This leads to vets who feel guilty, angry and misunderstood, thereby creating general tension and low morale within the practice. I would encourage all veterinary practices to study this immensely readable book and to adopt some of Mary's sound advice.

F. SCOTT-PARK