This is to certify that the
thesis entitled

REAL-TIME RT-PCR OF FELINE CALICIVIRUS
AND OPTIMIZATION FOR DETECTION OF
VIRUS IN FELINE URINE

presented by

BRIAN ALAN SCANSEN

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Small Animal Clinical Sciences

John M. Kerry

Major Professor's Signature

MAY 3RD, 2004

Date

REAL-TIME RT-PCR OF FELINE CALICIVIRUS AND OPTIMIZATION FOR DETECTION OF VIRUS IN FELINE URINE

By

Brian Alan Scansen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Small Animal Clinical Sciences

2004

ABSTRACT

REAL-TIME RT-PCR OF FELINE CALICIVIRUS AND OPTIMIZATION FOR DETECTION OF VIRUS IN FELINE URINE

By

Brian Alan Scansen

Investigating the role of feline calicivirus (FCV) in idiopathic cystitis may be facilitated by a reverse-transcriptase polymerase chain reaction (RT-PCR) assay optimized for detection of FCV urinary tract infections. Two FCV RT-PCR assays were developed; a p5.6 gene-based qualitative assay and a p30 gene-based quantitative real-time SYBR® Green I assay. The p5.6 gene assay was highly sensitive and specific, but was not efficiently adapted to real-time RT-PCR. The real-time p30 gene assay was sensitive, specific, and linear over a wide range of template concentrations, and had a reaction efficiency of 95%. The p30 gene assay detected all 51 North American FCV field isolates tested. To optimize detection of FCV in urine by RT-PCR, viral RNA was prepared from urine by dilution and thermal inactivation, polyethylene glycol precipitation, isolation with oligo$(dT)_{25}$-coated magnetic beads, or extraction with two silica gel-based columns. The FCV real-time p30 gene assay performed significantly better when using RNA isolated from feline urine with either of the silica gel-based columns.

This work is dedicated to my wife, Kimberly, for her constant support and unwavering praise and for showing me how to succeed in medicine,

And,

To my parents for continually reaffirming the value of a strong education and for instilling in me the desire to achieve all of my goals.

ACKNOWLEDGEMENTS

This work would not have been possible without the guidance of my major professor, Dr. John Kruger. He deserves much of the credit for making the combined DVM/MS program a reality and for motivating me throughout the research. For designing courses, improving my methodology, editing manuscripts, and dealing with administrative issues, this work is as much a reflection of his ability as a mentor as it is of mine as a student.

Dr. Roger Maes also deserves acknowledgement for his guidance in virological methodology and continued support of my research from inception to finish. His knowledge of diagnostic virology was seemingly limitless and was crucial to the success of the project. Similarly, Dr. Pat Venta's understanding of genetics was invaluable in dealing with the intricacies of molecular diagnostics and in troubleshooting problems as they arose. Dr. John Baker also deserves recognition for his guidance, suggestions, and input throughout the program. A significant amount of assistance was also provided by Dr. Annabel Wise and I am indebted to her for her expertise and assistance in diagnostic virology.

This work was generously funded by the Michigan State University Companion Animal Fund and the Center for Feline Health and Well-Being as well as by the Geraldine R. Dodge Foundation in concert with the Kenneth A. Scott Charitable Trust.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ANOVA	–	Analysis of Variance
BHV4	–	Bovine Herpesvirus-4
cDNA	–	Complementary Deoxyribonucleic Acid
CFI	–	California Feline Isolate
CPE	–	Cytopathic Effect
C_t	–	Threshold Cycle
DNA	–	Deoxyribonucleic Acid
DT	–	Dilution and Thermal Inactivation
dTMB	–	Oligo(dT) Magnetic Beads
FCV	–	Feline Calicivirus
FCV-R	–	Feline Calicivirus, Respiratory Strain
FHV	–	Feline Herpesvirus
FIV	–	Feline Immunodeficiency Virus
IC	–	Idiopathic Cystitis
FAT	–	Fluorescent Antibody Test
IHC	–	Immunohistochemistry
KCDV	–	Kidney Cell Degenerating Virus
HPF	–	High Power Field
MAP	–	Magnesium Ammonium Phosphate
MSU-DCPAH	–	Michigan State University's Diagnostic Center for Population and Animal Health

NV	–	Norwalk Virus
ORF	–	Open Reading Frame
PCR	–	Polymerase Chain Reaction
PEG	–	Polyethylene Glycol
QA	–	Silica Gel-Based Extraction Column (Qiagen QIAamp Viral RNA Mini Kit®)
RHDV	–	Rabbit Hemorrhagic Disease Virus
RN	–	Silica Gel-Based Extraction Column (Qiagen RNeasy Mini Kit®)
RNA	–	Ribonucleic Acid
RT	–	Reverse-Transcriptase
SMSV	–	San Miguel Sea Lion Virus
SPF	–	Specific Pathogen Free
SV	–	Sapporo Virus
$TCID_{50}$	–	Tissue Culture Infective Dose
TCM	–	Tissue Culture Medium
TNTC	–	Too Numerous To Count
USG	–	Urine Specific Gravity
URTD	–	Upper Respiratory Tract Disease
VESV	–	Vesicular Exanthema of Swine Virus
VI	–	Virus Isolation

INTRODUCTION

The family *Caliciviridae* comprises a group of small non-enveloped viruses known to cause a myriad of clinical syndromes in humans, cats, dogs, rabbits, pigs, cattle, and marine mammals. Several genera of calicivirus have been identified and these have been associated with a wide variety of disorders including vesicular lesions, stomatitis, rhinitis, pneumonia, arthropathies, abortion, gastroenteritis, and hemorrhagic systemic disease (Murphy et al. 1999).

One of the earliest identified members of *Caliciviridae* was feline calicivirus (FCV), an agent that induces upper respiratory tract disease primarily afflicting young cats (Fastier 1957, Crandell & Madin 1960). Although best characterized as a respiratory pathogen, FCV infection has also been associated with lameness (Pedersen et al. 1983), oral ulcerations (Tenorio et al. 1991), abortion (Ellis 1981), and a severe and highly fatal hemorrhagic-fever-like syndrome (Pedersen et al. 2000). Studies have also implicated FCV as a potential etiologic agent in feline idiopathic cystitis (IC), a common urinary disorder of cats resulting in hematuria, pollakiuria, strangury, and urethral obstruction (Rich & Fabricant 1969, Kruger et al. 1996, Rice et al. 2002).

The role of FCV in the pathogenesis of feline IC is uncertain. The inability to establish a causative link between FCV and the development of IC thus far may have been the result of insensitive or inappropriate methods of virus detection

(Kruger et al. 1996). Detection of the virus in urine by standard methods, such as virus isolation, requires viable virus and the absence of substances that are inhibitory to viral replication or that are toxic to cell culture (Sykes et al. 2001, Rice et al. 2002). FCV is extremely sensitive to the effects of urine, storage temperature, and freeze-thaw cycles and feline urine may be toxic to standard cell lines used for feline virus isolation (Appendix A, Komolate et al. 1976, Sykes et al. 1998, Rice et al. 2002).

Diagnosis of FCV has historically relied upon detection of viable virus by virus isolation in cell-culture or detection of FCV neutralizing antibodies in serum (Bittle et al. 1960, Gillespie & Scott 1973). Virus isolation is time consuming, labor intensive and requires viable virus in a substrate that is not toxic to standard cell culture lines (Rice et al. 2002). Serologic detection of FCV antibodies only indicates exposure and can be confounded by vaccination status or prior viral exposure (Kruger et al. 1996). Other reported methods of FCV diagnosis include direct identification of FCV antigens by fluorescent antibody testing (Gillespie et al. 1971) and immunohistochemistry (Dick et al. 1989), or virions by electron microscopy (Studdert 1978). These methods are similarly laborious and may yield false negative and/or false positive results.

Molecular diagnostic techniques have become increasingly popular in the diagnosis of viral infections. In particular, the polymerase chain reaction (PCR), or its variant, the reverse-transcriptase PCR (RT-PCR), have been increasingly

used in diagnostic virology for direct detection of viral DNA and RNA respectively in a broad range of clinical samples. Compared to conventional methods, PCR-based diagnostic assays have the advantages of enhanced specificity and sensitivity, increased scalability, more rapid throughput, and lower per-sample cost (Murphy et al. 1999). In addition, direct sequencing of PCR products allows for rapid detection of genomic variation between viral strains. Several RT-PCR assays have been reported for FCV, of which the majority amplify regions of the capsid protein gene (Seal 1994, Radford et al. 2000). Fewer FCV RT-PCR assays have been developed specifically for diagnostic purposes, and fewer still report sensitivity, specificity, and diagnostic range. Nearly all previously reported FCV RT-PCR assays rely on gel electrophoresis for verification of nucleic acid amplification.

A relatively new development in molecular detection of viral nucleic acids is real-time RT-PCR (Mackay et al. 2002). Real-time RT-PCR offers the advantage over traditional RT-PCR of increased sensitivity, faster throughput, increased reproducibility, decreased risk of laboratory contamination, and better quantification (Mackay et al. 2002). Real-time RT-PCR also eliminates the need for post-PCR processing for detection of amplification products by ethidium bromide staining and agarose gel electrophoresis. A p30 gene-based real-time RT-PCR assay for FCV has recently been reported that was comparable in sensitivity to virus isolation and that had a broad diagnostic range when used to detect FCV isolates from the United Kingdom (Helps et al. 2002).

Molecular diagnostics, such as RT-PCR, may circumvent many of the difficulties associated with detecting FCV urinary tract infections in cats. While molecular diagnosis of FCV infection has been reported (Seal 1994, Radford et al. 2000), the substrate on which testing is performed can play an important role in assay performance. Urine, in particular, is known to inhibit PCR amplification of viral and bacterial agents (Khan et al. 1991, Behzadbehbahani et al. 1997, Echavarria et al. 1998, Biel et al. 2000). Consequently, preparation of nucleic acids becomes a critical step that not only serves to concentrate and purify nucleic acids, but also to remove or inactivate PCR inhibitors. Studies comparing RNA isolation methods for their ability to remove or inactivate RT-PCR inhibitors and preserve FCV RNA integrity in feline urine specimens have not been reported.

The overall goal of the following studies was to develop a more sensitive and rapid means of detecting FCV urinary tract infections in cats. We hypothesized that a real-time RT-PCR assay for FCV could circumvent many problems associated with conventional urine virus isolation and RT-PCR methods, and facilitate clinical and laboratory investigations into the causative role of FCV in feline IC. The specific aims of the following studies were to develop a real-time quantitative RT-PCR assay and optimize viral RNA preparation methods for detection of FCV nucleic acids in feline urine.

LITERATURE REVIEW

Overview

The family *Caliciviridae* consists of numerous viruses of veterinary and human medical importance. The caliciviruses are organized into four genera, which are *Vesivirus*, *Lagovirus*, *Norovirus* (previously known as small round structured viruses or Norwalk-like viruses), and *Sapovirus* (previously known as classical caliciviruses or Sapporo-like viruses) (Büchen-Osmond 2003). All caliciviruses share a similar structure with a single linear positive-sense RNA genome 7.4-8.3kb in length. The genome has a covalently attached protein (VPg) at the 5' end and a polyadenylated tail at the 3' end. The nucleic acid is embedded within a 35-40nm non-enveloped icosahedral capsid. The name calicivirus is derived from the Latin *calyx,* for cup, and reflects the 32 cup-shaped indentations visible on the capsid surface (Green et al. 2000).

Numerous species of calicivirus have been identified and these are associated with a wide variety of disorders including vesicular lesions, stomatitis, rhinitis, pneumonia, arthropathies, abortion, gastroenteritis, and hemorrhagic-like systemic disease (Murphy et al. 1999). The prototypical caliciviruses include vesicular exanthema of swine virus (VESV, genus *Vesivirus*), San Miguel sea lion virus (SMSV, genus *Vesivirus*), Norwalk virus (NV, genus *Norovirus*), Sapporo virus (SV, genus *Sapovirus*), rabbit hemorrhagic disease virus (RHDV, genus *Lagovirus*), and feline calicivirus (FCV, genus *Vesivirus*).

Historical Perspectives

Vesicular Exanthema of Swine Virus

The first reported outbreak of disease caused by a calicivirus occurred in 1932 at swine farms in southern California (Traum 1936). Clinical signs associated with the initial outbreak, and subsequent similar outbreaks over the next three years, were characterized by pyrexia and vesicle formation around the snout, mouth, and digits and were clinically indistinguishable from foot-and-mouth disease or vesicular stomatitis. However, the disease was found to be distinct from foot-and-mouth-disease and vesicular stomatitis, based on its pattern of infectivity in test animals, and was therefore designated VESV (Traum 1936). VESV outbreaks occurred sporadically in California until 1952 when it was diagnosed for the first time in Nebraska and then throughout 42 states by September of 1953 (Fenner et al. 1993). Implementation of a slaughter program and enforcement of garbage cooking laws resulted in eradication of the disease by 1956. It has not been detected anywhere in the world since that time. Although VESV was believed to be viral in origin, it was not until 1968 that the agent was confirmed to be a calicivirus (although still classified as a picornavirus at that time) and found to be structurally similar to FCV (Wawrzkiewicz 1968).

Feline Calicivirus

The discovery of feline calicivirus was an unintended event. While attempting to grow feline panleukopenia virus in cell culture, a new and unrelated agent was isolated which produced severe cytopathogenic changes in kitten kidney cells

(Fastier 1957). The agent was described at that time as the kidney cell-degenerating virus (KCDV). KCDV growth *in vitro* was supported by a wide range of feline tissues. When inoculated intravenously into kittens, KCDV induced only mild diarrhea and anorexia approximately one week after exposure. However, seroconversion was noted on paired sera. The inability of the virus to produce any overt clinical disease prompted Fastier (1957) to label KCDV a virus "in search of a disease." KCDV was later determined to be FCV by serum neutralization using antiserum from feline viral isolates from cats with upper respiratory infection (Bittle et al. 1960) and feline "picornavirus" isolates (Takahashi et al. 1971). The discovery of KCDV in an animal co-infected with feline panleukopenia, and the inability of KCDV to produce notable clinical disease suggested that this strain was relatively avirulent.

The first report in the literature of a feline virus consistent with what we now recognize clinically as FCV-induced disease occurred in 1960 (Crandell & Madin 1960). Designated the California feline isolate (CFI), the virus was isolated from blood and oropharyngeal secretions obtained from a nine-week-old female kitten from the San Francisco Bay area that presented with fever, tachypnea, rales, anorexia, lethargy, and a stiff gait. The CFI isolate showed characteristic cytopathogenic changes in kitten kidney cell culture. Furthermore, upper respiratory clinical signs and pyrexia were reproducible in other kittens exposed to the virus, both intranasally and via contact with experimentally infected cats. Serologic cross-neutralization did not occur between CFI and feline

panleukopenia virus, feline rhinotracheitis virus, or feline pneumonitis (now designated *Chlamydophila felis*). Throughout the next decade, similar agents were isolated from cats with upper respiratory tract disease (URTD) in other areas of the United States and from Europe, Australia, and Japan (Gillespie & Scott 1973, Takahashi et al. 1971).

San Miguel Sea Lion Virus

In 1972, a series of abortions was studied among sea lions on San Miguel Island, California. From one of the aborting animals a virus, indistinguishable from VESV, was isolated and termed SMSV (Smith et al. 1973). The similarity in structure of SMSV to VESV coupled with the ability of SMSV to cause vesicular lesions when injected intradermally into the lips of swine led Smith et al. (1973) to theorize that the outbreaks of VESV in California in the 1930's were the result of transmission of SMSV from its natural marine host to swine via contaminated garbage feeding. Since the discovery of SMSV, similar viral agents have been isolated from a variety of other marine mammals (Fenner et al. 1993).

Norwalk Virus

The first human disease attributed to a calicivirus occurred at a school in Norwalk, Ohio in October 1968. Fifty percent of the students and teachers developed gastroenteritis, primarily characterized by vomiting and nausea, for which no etiological agent was determined (Kapikian 2000). Four years later, immune electron microscopy detected 27nm viral particles in infectious stool

filtrates derived from the Norwalk outbreak (Kapikian et al. 1972). The Norwalk virus (NV), although of appropriate size, did not have the typical appearance of the caliciviruses and came to be classified with a number of other small round structured viruses. However, detection of a single major structural protein, a feature of the *Caliciviridae*, was compelling evidence that the NV was a calicivirus (Greenberg, et al. 1981). Finally, sequencing of the NV genome confirmed its placement in *Caliciviridae* (Jiang et al. 1990). NV is considered the predominant cause of acute gastroenteritis in humans, accounting for an estimated 23 million cases annually, or roughly half of all foodborne outbreaks (CDC 2004). Research on NV has been hindered due to the inability to grow the virus in tissue culture. Current trends indicate that FCV may be a convenient model for NV due to the ease with which FCV can be cultivated in the laboratory, even though the two viruses are in different genera (Bidawid et al. 2003).

Sapporo Virus

In contrast to the small round structured viruses such as NV, the first human viral agents having the prototypical cup-shaped depressions of caliciviruses were isolated in 1976 from the fecal samples of children in Glasgow, Scotland (Madeley & Cosgrove 1976). Further work on the "human caliciviruses", as they came to be known, occurred as a result of an outbreak of gastroenteritis at an orphanage in Sapporo, Japan in 1977 (Chiba et al. 1979) and subsequent outbreaks between 1977 and 1982 (Nakata et al. 1985). Viral particles from these outbreaks were morphologically identical to known animal caliciviruses,

such as FCV, and came to be called Sapporo Virus (SV) (Chiba et al. 2000). SV has now been shown genetically to be a member of the *Caliciviridae*, although distinct from NV, and is now the archetypal virus of the genus *Sapovirus* (Numata et al. 1997). Although closer in morphologic appearance to FCV than to NV, SV has proven resistant to cultivation in the laboratory.

Rabbit Hemorrhagic Disease Virus

Rabbit hemorrhagic disease (RHDV) was detected as an emerging epizootic beginning in China in 1984 and extending to Western Europe and Africa by 1988 (Murphy 1999). The outbreaks of this disease were severe with morbidity approaching 100% and mortality rates of 90% in adult animals, with pathologic signs of systemic hemorrhage (Fenner et al. 1993). The causative agent of the disease was determined to be a calicivirus by electron microscopy and physical properties consistent with *Caliciviridae* (Parra & Prieto 1990, Ohlinger et al. 1990). A similar disease causing large-scale deaths of brown hares, European brown hare syndrome, was described at the same time as rabbit hemorrhagic disease and determined to be caused by a calicivirus as well (Chasey & Duff 1990). Although both viruses are in the genus *Lagovirus*, the etiologic agents of rabbit hemorrhagic disease and European brown hare syndrome are believed to be distinct species.

Other Caliciviruses

The caliciviruses described above are the best characterized of the family *Caliciviridae*. Numerous other caliciviruses have been identified in a variety of species. Bovine enteric calicivirus, in the genus *Norovirus*, has now been described as an agent of calf diarrhea (Liu et al. 1999). Yet to be adequately classified, other known caliciviruses include canine calicivirus, mink calicivirus, porcine enteric calicivirus, walrus calicivirus, lion calicivirus, chicken calicivirus, and other caliciviruses of birds (Murphy et al. 1999). As a family, members of *Caliciviridae* have fairly restricted host ranges, but a broad range of target organ systems and tissues.

Biology of Feline Calicivirus

Physical Properties

Much of the early work on FCV detailed the virus's physico-chemical properties. The virion size was estimated to be 37-40nm by filtration and electron microscopy (Bürki 1965, Zwillenberg & Bürki 1966, Gillespie & Scott 1973). The virus is non-enveloped and the capsid is comprised of 180 copies of a single structural protein assembled as 90 homodimers in a T=3 icosahedral symmetry (Prasad et al. 1994). The sedimentation coefficient of FCV was determined to be 154S in sucrose gradients (Studdert 1978), with a virion density in cesium chloride estimated to be 1.37-1.38 g/ml based on the density of VESV (Wawrzkiewicz et al. 1968). Due to its morphology as an unenveloped virus, FCV is resistant to lipid solvents such as ether and chloroform and remains

partially stable in acidic environments to pH 4, but is almost completely inactivated at pH 3 (Gillespie & Scott 1973). FCV is heat inactivated at 50C for 30 minutes and $MgCl_2$ does not stabilize FCV against heat inactivation (Studdert 1978). FCV is fairly resistant in the environment, able to persist for a week or more, but is susceptible to hypochlorite (bleach) solutions (Gaskell & Dawson 1998). FCV displays no hemagglutinating activity (Studdert 1978). The physical and chemical properties of FCV and the other members of *Caliciviridae* were sufficient to separate these viruses from their original classification as members of the *Picornaviridae* into a new family of viruses, the *Caliciviridae*. Genomic analyses, beginning in the 1990s, would confirm this division (Green et al. 2000).

Molecular Biology

Complete sequencing of the genome of the FCV-F9 strain, the prototypical vaccine strain, was accomplished by primer extension cloning in 1992 (Carter et al. 1992). The complete genome of several other strains of FCV, from differing geographic locale and year of isolation, have subsequently been sequenced (Table 1).

The FCV genome consists of nearly 7.7kb of positive sense RNA with a covalently-linked protein (VPg) at the 5' end (Herbert et al. 1997) and a poly(A) tail at the 3' end (Carter et al. 1992). Three open reading frames (ORF) are present in the FCV genome (Figure 1A) (Green et al. 2002). ORF1 encodes nonstructural proteins labeled according to their sequence similarity to

picornaviruses, or to protein expression studies. These nonstructural proteins, which are cleaved by the viral proteinase, include p5.6; p32; p39, a 2C-like nucleoside triphosphatase; p30, a 3A-like protein; 3B (VPg); a 3C-like cysteine protease; and a 3D-like RNA-dependent RNA polymerase (Figure 1B) (Glenn et al. 1999, Green et al. 2002, Sosnovtsev et al. 2002). ORF2 encodes the single capsid protein, which is subdivided into 6 regions based on strain variation with regions C and E being hypervariable and regions A, B, D and F showing greater conservation between strains (Figure 1C) (Seal et al. 1994). The third ORF encodes a protein, labeled VP2, of unknown function that is incorporated with mature virions (Green et al. 2002). This genomic organization is shared with the *Vesivirus* and *Norovirus* genera and is in contrast to the *Lagovirus* and *Sapovirus* genera in which there exist only two ORFs – the first comprising the nonstructural polyprotein together with the capsid and the second containing VP2 (Green et al. 2000).

Table 1. Complete FCV Sequences. FCV strains for which the complete genome sequence is available on GenBank with their accession number, year and country of isolation, and the corresponding reference. NR = Not Reported.

Strain	Accession Number	Year of Isolation	Country of Isolation	Reference
FCV-F9	M86379	1960	USA	Carter et al. 1992
FCV-F4	D31836	1971	Japan	Oshikamo et al. 1994
FCV-CFI/68	U13992	1960	USA	Neill 1994
FCV-Urbana	NC_001481	late 1960's	USA	Sosnovtsev & Green 1995
FCV-F65	AF109465	1990	UK	Glenn et al. 1999
FCV-2024	AF479590	NR	NR	Thumfart & Meyers 2002

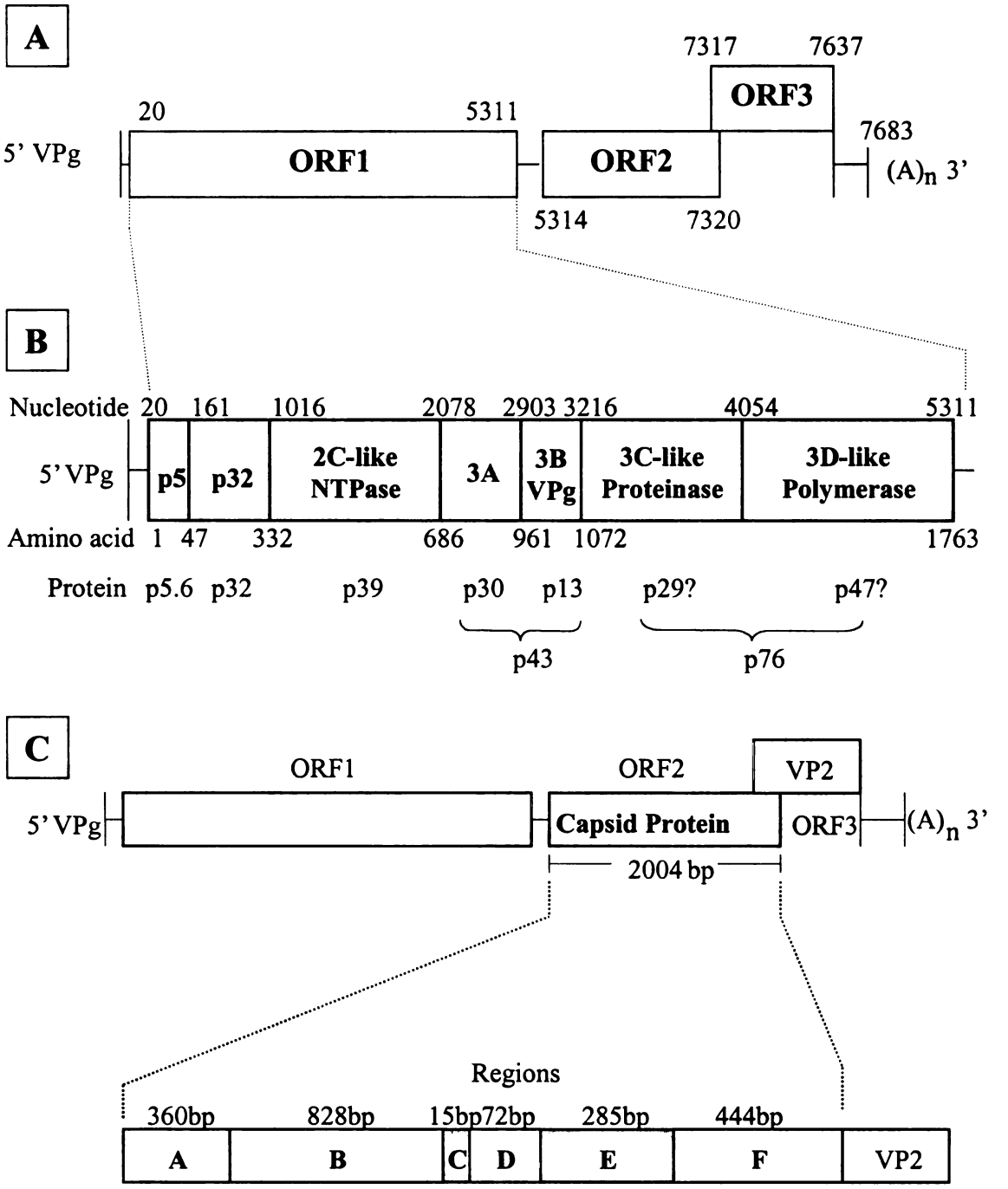


Figure 1. Schematic diagram of the genomic organization of FCV. (A) The three open reading frames (ORF) of FCV (numbers correspond to nucleotides of the FCV-Urbana strain) with protein VPg at 5' end and poly(A) tail at 3'; (B) the nonstructural proteins of ORF1; and (C) the organization of the capsid gene (ORF2) into 6 regions with C and E being hypervariable (adapted from Green et al. 2002, Sosnovtsev & Green 2002).

Replication

FCV is a lytic virus that replicates in the cytoplasm of host cells (Fenner et al. 1993). Production of both the complete genome transcript as well as a subgenomic transcript corresponding to the 3' end of the genome occurs following infection (Fenner et al. 1993). The polyprotein translated from the complete genome transcript is cleaved to yield the individual viral proteins, while the subgenomic transcript serves as a template for translation of the capsid protein precursor (Neill & Mengeling 1988). The capsid protein precursor is a 75-kDa protein that is processed posttranslationally to generate the mature 62-kDa capsid protein after removal of 124 amino acids from the N terminus (Sonovtsev et al. 1998, Geissler et al. 1999).

Antigenic Relationships

Numerous strains of FCV have been isolated with widely varying serologic relationships (Bittle et al. 1960, Burki 1965, Takahashi et al. 1971). The first serologic survey of FCV reported two distinct groups of FCV: one group of eight isolates that were antigenically identical to the first American FCV isolate (CFI) (Crandell & Madin 1960) and a second group of 15 isolates that were antigenically unrelated (Bittle et al. 1960). Evaluation of seven additional isolates resulted in identification of five provisional FCV serotypes with little cross-reactivity (Burki 1965). Finally, 27 Japanese strains of FCV were compared serologically and divided into five serologic subgroups. When these 27 Japanese FCV strains were compared to 15 American FCV isolates, only two of

the American strains were neutralized by Japanese strain antisera (Takahashi et al. 1971). These results suggested wide antigenic variation between FCV strains and discouraged initial hopes for a vaccine. However, it was later shown that although significant variability existed between strains, most isolates could be neutralized with antiserum derived from the FCV-F9 strain (Kalunda et al. 1975). This led to the development of an intramuscular FCV vaccine based on the FCV-F9 strain. Vaccination began in earnest in the mid-1970's. Although the FCV vaccine was apparently effective at reducing clinical signs, infection still occurred and viable FCV could be isolated from vaccinated cats after virulent challenge (Bittle & Rubic 1976, Kahn & Hoover 1976b). This was the first evidence that persistently infected, asymptomatic carrier cats (vaccinated or unvaccinated) likely played a critical role in the pervasiveness of FCV.

Transmission

Transmission of FCV is via close contact with the nasal, oral, or ocular secretions from an infected animal, either symptomatic or an asymptomatic animal persistently shedding the virus (Sykes 2001). Environmental contamination and fomite transmission are also possible means of viral spread (Sykes 2001). Direct aerosol transmission is not believed to occur over distances greater than 4 feet (Hurley et al. 2004).

Pathogenesis

Kahn and Gillespie (1971) further characterized the clinical syndrome induced by FCV. Aerosol exposure to FCV resulted in a biphasic temperature response in some kittens, characterized by a transient rise in temperature 24 hours after exposure and a second rise nearly one week later. Clinical signs persisted for seven to ten days and included conjunctivitis, rhinitis, pneumonia in some cases, and oral ulcerations on the tongue and hard palate. Pulmonary lesions were believed to result from cytolytic infection of the respiratory epithelium, particularly within the terminal bronchi and alveoli, with alveolar edema and seropurulent exudates following due to secondary bacterial pneumonia (Kahn & Hoover, 1976a). Mortality was 30% with all deaths occurring within two weeks of viral exposure and 64% of deaths occurring within the first 5 days (Kahn & Gillespie 1971). Virus was isolated from the lungs, pharynx, conjunctival sac, nasal turbinates, third eyelid, trachea, and, infrequently, from the blood. Virus was consistently isolated from the tonsils until 34 days after exposure suggesting persistent shedding. Later studies confirmed an asymptomatic carrier state associated with chronic FCV infection that was characterized by persistent viral shedding up to 2.5 years following natural exposure (Povey et al. 1973). Because pathogenicity may vary considerably between FCV strains, the clinical picture of FCV-induced URTD can range from mild or subclinical infection to severe disease characterized by oral ulceration, anorexia, pneumonia, and even peracute death in young kittens (August 1984). Although most cats recover and

cease to shed FCV, 50% of cats will still be shedding the virus 75 days after infection and some will become persistent asymptomatic carriers, continuing to perpetuate the disease in susceptible feline populations. The percentage of asymptomatic cats shedding FCV is estimated to be 40% of colony cats, 25% of show cats, and 8% of household pets (Gaskell & Dawson 1998).

Although primarily believed to affect the oral mucosa and respiratory tract, FCV has been isolated from the spleen, blood, intestine, feces, muscle, joints, kidney, urinary bladder, conjunctiva, tonsils, oral mucosa, and lungs of affected cats (Kahn & Gillespie 1971, Kahn & Hoover 1976a, Truyen et al. 1999). The effect of FCV on these non-respiratory tract tissues is largely unknown as pulmonary and oral/respiratory mucosal lesions predominate during experimental and natural infection (Kahn & Hoover 1976a). FCV-induced URTD is often self-limiting and the course of infection typically lasts 5 to 7 days, if uncomplicated by secondary bacterial infection (Kahn & Hoover, 1976a). Hematological abnormalities usually involve a lymphopenia at 2-4 days post-infection. Resolution of clinical signs coincides with development of FCV neutralizing antibodies at day five post-infection (Truyen et al. 1999).

FCV is widely recognized as a cause of URTD. However, the variability in tissue tropism of FCV coupled with the ability of the virus to mutate rapidly is a cause for concern and makes future, as yet unknown, manifestations of FCV infection possible.

FCV-Induced Clinical Diseases

Upper Respiratory Tract Disease

The prevalence of FCV infection was first estimated to be 40% of all cases of URTD in cats, with FCV and feline herpes virus (FHV) combining equally to account for 80% of all cases (Kahn & Hoover 1976a). However, a retrospective study from the United Kingdom showed a much higher frequency of FCV isolation compared to FHV isolation from 1980 to 1989 (Harbour et al. 1991). In this analysis of 6,866 oropharyngeal swabs, FCV was isolated from 1,364 (19.9%) while FHV was isolated from 285 (4.2%) suggesting a ratio of FCV to FHV infection of 4.8:1 and giving a proportion of FCV among positive isolates of 82.7%. Furthermore, of the 1,180 cats with acute URTD, 348 (29.5%) were shedding FCV compared to 162 (13.7%) shedding FHV. Lastly, of the 963 cats positive for FCV for which vaccination history was known, 417 (43.3%) were fully vaccinated. This data led Harbour and coworkers to conclude that the increased prevalence of FCV compared to FHV was likely due to widespread use of respiratory vaccines, which reduced the shedding of FHV while having little impact on the shedding of FCV (Harbour et al. 1991).

Today, FCV remains an important and common agent in feline upper respiratory tract disease. Recent reports of the prevalence of FCV showed 33% of cats with respiratory disease were FCV positive by virus isolation from oropharyngeal swabs; 21% of healthy animals were similarly positive (Binns et al. 2000). Despite extensive FCV vaccination over the last 3 decades, these results are

comparable to estimates of FCV prevalence prior to and for 10 years after introduction of a FCV vaccine (Kahn & Hoover 1976a, Harbour et al. 1991). The fact that FCV incidence is unchanged in the face of vaccination can be explained by one or more of the following possibilities: (1) FCV has evolved resistance to the vaccine, (2) the vaccine (live forms) may cause disease or chronic shedding, or (3) vaccination prevents clinical symptoms, but not the carrier state (Pedersen & Hawkins 1995). To evaluate the relative contribution of each scenario to the persistence of FCV, Pedersen & Hawkins (1995) evaluated the degree of protection afforded, the persistence of shedding, and the pathogenicity of three current vaccine strains as well as several field isolates of FCV. This study found that immunization with the original FCV-F9 strain resulted in production of antibodies that neutralized most field strains. It was also apparent that oral administration of vaccine strains could cause mild to moderate disease and persistent shedding of virus. Furthermore, this study demonstrated that cats exposed to field or vaccine strains of FCV developed less severe disease when secondarily challenged, but were not protected against the carrier state. They therefore concluded that the persistence of FCV as a cause of feline URTD could not be explained by the evolution of vaccine resistant strains and that the vaccine strain may contribute to acute infection and the development of a persistent carrier state (Pedersen & Hawkins 1995). Future research is needed to develop effective strategies to reduce the prevalence and eventually eradicate this enigmatic pathogen of the feline respiratory system.

Arthropathy

Although well recognized as a respiratory pathogen, FCV has been shown to be the cause of several other clinical syndromes. In the early 1980's, FCV was isolated from two kittens from separate catteries (California and Ontario) with a similar presentation of high fever and shifting limb lameness (Pedersen et al. 1983). Studies of these viruses showed that clinical signs induced by the Californian strain could be prevented by vaccination, whereas clinical signs induced by the Canadian strain could not. Both isolates induced clinical disease characterized by pyrexia, depression, anorexia, reluctance to move, and limping in specific-pathogen-free (SPF) cats after oral or oronasal inoculation (Pedersen et al. 1983). However, only mild oral ulceration was observed after resolution of the lameness; signs of upper respiratory disease were not observed. FCV was cultured directly from synovial fluid obtained from a 12-week-old kitten with signs of anorexia and severe shifting-limb lameness (Levy & Marsh 1992). This kitten, and three littermates, also had signs of URTD, which were not seen in the field or experimental cases of Pedersen et al. (1983). In all reported cases of FCV-associated lameness, the clinical signs resolved within two to four days with no known recurrence.

Abortion

FCV has also been associated with abortion in a small number of cats. FCV-related abortion was first observed when FCV was isolated from autolysed fetuses recovered during necropsy of a one-year-old Siamese queen in Australia

(Ellis 1981). Interestingly, two other cats in the same household as the aborting Siamese had recently recovered from URTD. More recently, FCV was isolated from pooled organs of a fetus from an unvaccinated queen with a bloody vaginal discharge and four dead fetuses with petechial hemorrhage on the skin along their backs (van Vuuren et al. 1999). Experimental studies of FCV-infected pregnant queens have not been performed so the exact pathogenesis of FCV-induced abortion uncertain. However, these two reports suggest that FCV can be transmitted transplacentally and may be abortigenic.

Chronic Gingivitis

Dual infection with FCV and feline immunodeficiency virus (FIV) has been implicated as a cause of chronic gingivitis and pharyngitis in cats (Tenorio et al. 1991). In this study of 226 cats from a veterinary teaching hospital, a shelter, and a purebred cattery, oral cavity disease was present in 43% to 100% of the animals from each group. Chronic FCV shedding was detected in roughly 20% of the animals in each group. Cats infected with FCV alone did not have a greater risk of oral lesions. However, cats that were FIV positive, and coinfected with FCV, had greater prevalence and severity of oral disease than cats solely infected with FIV. This finding of more severe disease in the presence of FCV suggests that although FCV may not play a primary role in chronic feline oral disease, it may enhance the severity of disease in immunocompromised individuals (Tenorio et al. 1991).

Virulent Systemic Disease

An apparently new clinical manifestation of FCV infection has begun to emerge in the last decade. Initially described as a hemorrhagic-like fever (Pedersen et al. 2000), several outbreaks of highly pathogenic strains of FCV have been reported with a high mortality rate and systemic symptoms (Schorr-Evans et al. 2003, Hurley et al. 2004). The first outbreak occurred in Northern California among six cats with exposure to a private veterinary practice and was transmitted to four other healthy adult cats via personnel in an isolation ward (Pedersen et al. 2000). Clinical manifestations in these cats included cutaneous edema of the face and limbs, cutaneous lesions with crusting, erythema, and epilation on the face and pinnae, fever, signs consistent with URTD, and pancreatitis. Mortality rates in field cases, experimentally infected cats, and inadvertently infected cats ranged from 33% to 50%. Prior FCV vaccination did not appear to be effective for field cases, but did lessen the severity of signs associated with experimentally-induced disease. Similar outbreaks of highly virulent FCV have been subsequently reported in Southern California (Hurley et al. 2004), Pennsylvania, Tennessee, Nevada, and Massachusetts (Schorr-Evans et al. 2003). In these outbreaks, FCV infection was associated with severe edema, ulceration, icterus, pancreatitis, and often death. The Southern California strain was shown to be genetically and serologically distinct from the FCV strain involved in the original outbreak in Northern California, and also from the vaccine strain FCV-F9, with nucleotide homology of 73.4-76.5% for a portion of the hypervariable region of

the viral capsid gene (Hurley et al. 2004). All reported outbreaks were self-limiting and did not spread far beyond the index cases.

Lower Urinary Tract Disease

Disorders of the feline lower urinary tract have long been recognized as resulting in clinical signs of hematuria, periuria (urination in inappropriate locations), dysuria, and pollakiuria (Kirk 1925, Osbaldiston & Taussig 1970). Known causes of these clinical signs include uroliths, urethral plugs, infections (bacterial, fungal, or parasitic), anatomic abnormalities (congenital or acquired), and iatrogenic causes (Kalkstein et al. 1999b). Unfortunately, the cause of lower urinary tract signs cannot be determined in many cases, and these cats are diagnosed as having idiopathic lower urinary tract disease or, alternatively, idiopathic cystitis (IC). Idiopathic cystitis accounts for roughly two-thirds of all feline patients presenting for signs of hematuria, pollakiuria, and dysuria (Kruger et al. 1991, Buffington et al. 1997). In cats with urethral obstruction, a cause could not be identified in 29% of cases (Kruger et al. 1991). Nonobstructive IC has no sex or breed predilection and is typically encountered in young to middle-aged cats (mean age: 3.5 years, range of 0.5 to 17.5 years) from multi-cat households (Kalkstein et al. 1999a). In contrast, obstructive IC is encountered almost exclusively in male cats and is most likely related to formation of crystalline-matrix urethral plugs that more readily occlude the narrow penile urethra (Osborne et al. 1997). In the majority of cats with nonobstructive IC, clinical signs persist for five to ten days and then spontaneously resolve with or without

treatment (Osborne et al. 2000). This biological behavior has resulted in the speculation that feline IC may have a viral etiology (Kruger & Osborne 1990).

The notion of a viral etiology for feline IC is not a new one. Hemorrhagic cystitis in people has been associated with a number of viral agents including adenovirus, herpes simplex, herpes zoster, cytomegalovirus, polyomavirus, influenza A, and human immunodeficiency virus (Kruger et al. 1996). In cats, a viral etiology for IC was first proposed in 1969 after urethral obstruction was experimentally produced in male cats following bladder inoculation of centrifuged, filtered, and bacteriologically sterile urine from male cats with naturally occurring obstruction (Rich & Fabricant 1969). Shortly thereafter, a FCV was isolated from a Manx cat with spontaneous urethral obstruction and this isolate induced urethral obstruction in 80% of conventionally reared cats following urinary bladder, aerosol, or contact exposure (Rich et al. 1971). The role of FCV in cystitis was questioned, however, when (1) FCV could not be reisolated beyond the fourth day post-infection, (2) a second virus (feline synctia-forming virus) was isolated from all obstructed cats, and (3) no significant serum-neutralizing antibody response was detected in experimentally infected cats (Fabricant 1984). The role of FCV in viral-induced cystitis then came to be considered secondary, perhaps being able to incite latent herpesviruses into producing cellular injury and producing the clinical syndrome. This was supported by the isolation of a gamma herpesvirus, bovine herpesvirus-4 (BHV4), from a kitten who died of spontaneous urethral obstruction and who also experienced FCV-induced URTD

one month previously (Fabricant 1973, Kruger et al. 1989). Subsequent experimental studies were also supportive of this theory as urethral obstruction and cystitis were induced in specific-pathogen-free (SPF) male cats after urinary bladder or intravenous inoculation with BHV4 alone or BHV4 in combination with FCV (Fabricant 1977). However, other investigators were unable to reproduce cystitis and urethral obstruction in SPF cats infected with the same BHV4 isolate (Kruger et al. 1990).

The role of FCV in feline IC came increasingly into question when other investigators were unable to isolate the virus from cats with spontaneous forms of lower urinary tract disease (Kruger & Osborne 1990). However, these studies relied primarily on standard cell-culture-inoculation techniques. More recently, FCV-like particles were detected by electron microscopy in 35 of 92 (38%) crystalline-matrix urethral plugs (Kruger et al. 1996). This observation led to the notion that virus isolation by conventional means may not be a uniformly sensitive method of detecting FCV in feline urine. Virus isolation requires the presence of viable virus and the absence of substances that are toxic to cell culture or that inhibit viral replication (Sykes et al. 2001, Rice et al. 2002). Furthermore, FCV is extremely sensitive to the effects of urine, storage temperature, and freeze-thaw cycles and feline urine may be toxic to standard cell lines used for feline virus isolation (Appendix A, Komolate et al. 1976, Sykes et al. 1998, Rice et al. 2002).

The development of a modified virus isolation technique designed to circumvent some of the difficulties of FCV detection in feline urine has recently enabled the isolation of two novel FCV strains from cats with IC (Rice et al. 2002). Designated FCV-U1 and FCV-U2, phylogenetic analyses of the capsid protein genes revealed that these strains were sufficiently different from standard vaccine strains to preclude the possibility that they represent urinary shedding of vaccine virus (Rice et al. 2002). They may, however, represent coincidental urine shedding of a non-pathogenic wild-type FCV strain or they may represent true uropathogens. Further studies are required to determine if FCV-U1 or FCV-U2 play a causative role in the pathogenesis of feline IC.

The isolation of two new FCV strains, coupled with the finding of FCV-like particles in a large proportion of cats with obstructive IC, justifies reexamination of the potential role of FCV in feline IC. Although conventional viral diagnostic techniques have proven inconsistent for detection of FCV urinary tract infections, the ability of molecular diagnostics to detect virus in a wide range of biologic substrates makes PCR-based detection methods an attractive means of reexamining the role of FCV in feline IC.

Diagnosis of FCV Infection

Virus Isolation

Viral isolation has historically been the primary means of establishing a specific diagnosis of FCV infection. FCV grows rapidly in standard feline cell lines (i.e.

Crandell-Reese feline kidney cells), exerting a rapid cytopathic effect in 1 to 3 days (Gillespie & Scott 1973). While very useful for FCV-induced URTD, standard cell-culture techniques may be less optimal for isolation of FCV from other biological substrates, such as feline urine. Virus isolation requires the presence of viable virus and the absence of substances that are toxic to cell culture or that inhibit viral replication (Sykes et al. 2001, Rice et al. 2002). FCV is extremely sensitive to the effects of urine, storage temperature, and freeze-thaw cycles and feline urine may be toxic to standard cell lines used for feline virus isolation (Appendix A, Komolate et al. 1976, Sykes et al. 1998, Rice et al. 2002). Virus isolation also has the drawbacks of being expensive and labor intensive, and may require days to weeks for final results (Fenner et al. 1993).

Serology

Detection of viral neutralizing antibodies offers an indirect method of identifying FCV infection and was one of the first methods employed to characterize FCV isolates (Bittle et al. 1960). In particular, a 4-fold increase in antibody titers in paired sera from the acute and convalescent phases of infection is indicative of recent exposure (Fenner et al. 1993). However, because of the often asymptomatic and persistent nature of FCV infection, a cause-and-effect relationship between viral antibody titer and a specific disease state should be made with caution (Kruger et al. 1996). Furthermore, the widespread use of FCV vaccines makes interpretation of serological data difficult in determining the role of FCV in a given disease (Kruger et al. 1996).

Electron Microscopy, Fluorescent Antibody, and Immunohistochemistry

Electron microscopy offers direct morphologic identification of FCV virions due to the characteristic spherical shape, 37-40nm diameter virion size, and 32 dark spots visible due to negative stain filling the cup-shaped depressions on the surface of the virion (Studdert 1978). While electron microscopy offers good specificity, it lacks sensitivity as it requires greater than 5 logs of virus per milliliter for detection (Castro 1992).

The fluorescent antibody test (FAT) is another diagnostic tool employed for the direct detection of FCV antigens in tissues or fluids. Antiviral antibody bound to FCV antigens is detected by a fluorescein-conjugated immunoglobulin derived from cats hyperimmunized to FCV (Fenner et al. 1993). FAT has been described for FCV (Gillespie et al. 1971). Unfortunately, FAT testing is also labor-intensive and costly, and is less sensitive than virus isolation (Sykes 2001). FAT testing also may be associated with false positive results due to nonspecific fluorescing debris (Sykes 2001).

Immunohistochemistry (IHC) has also been used for direct identification of FCV antigens in tissues. Immunohistochemistry is similar to FAT testing in that both methods utilize a primary anti-FCV antibody. However, IHC relies on an enzyme-labeled antiviral antibody and the production of a colored insoluble precipitate in the presence of the enzyme (Fenner et al. 1993). IHC has been used in the detection of FCV-infected tissues in conjunction with virus isolation

(Dick et al. 1989). Like IFA, immunohistochemistry may be associated with false-positive results (Fenner et al. 1993).

Molecular Techniques

Molecular diagnostic techniques such as nucleic acid hybridization and the polymerase chain reaction (PCR) have become increasingly popular for diagnosis of viral infections. DNA or RNA hybridization using labeled probes has proven to be a sensitive, specific, and fairly rapid method of identifying viral nucleic acids in cells, tissue sections, or membrane fixed nucleic acids (Fenner et al. 1993). A dot blot DNA hybridization assay using a radiolabeled cDNA probe specific for the capsid protein of FCV has been reported (Dawson et al. 1994). This assay was found to be nearly twice as sensitive as conventional virus isolation in detecting FCV in tissues of infected cats (Dawson et al. 1994).

PCR has become increasingly useful in the field of diagnostic virology due to this test's sensitivity, specificity, scalability, and low cost (Fenner et al. 1993). In particular, PCR amplification allows for the direct detection of viral DNA. Direct sequencing of PCR products allows for detection of variations in genomic sequences between strains. PCR methods also can be employed to detect RNA viruses by incorporating a preliminary step in which the enzyme reverse transcriptase converts viral RNA to cDNA, which is then amplified by the PCR. Reverse transcription PCR (RT-PCR) assays have been developed for a wide variety of RNA viruses, including FCV.

RT-PCR assays for FCV initially focused on analysis of the capsid protein gene and the genotypic determination of antigenic variability between FCV strains. The first reported primers for RT-PCR amplification of FCV were those given by Seal (1994), whose design was based on the FCV-CFI/68 strain. These primers amplified a 670bp portion of the capsid protein gene (Table 2) beginning at the C-terminus of region B and ending at the N-terminus of region F (Figure 2). Twelve different FCV isolates were amplified using this assay and all amplifications were verified by agarose gel electrophoresis, including amplification of 7 isolates that did not cross-hybridize with any of 5 cDNA capsid gene clones from known strains of FCV (Seal 1994). This assay was an effective means to evaluate nucleotide variation in the hypervariable region of the capsid gene among divergent FCV strains. However, neither the sensitivity nor specificity of the assay was reported and the diagnostic range was limited to evaluation of only 12 FCV isolates.

In 1996, a FCV polyprotein gene-based RT-PCR assay was reported (Doyle et al. 1996). This assay amplified a 477bp region of the p5.6 and p32 genes of ORF1 at the 5' end of the genome (Figure 2, Table 2). This assay detected 21 of 24 (88%) field isolates of FCV and sensitivity was not reported. Although this assay was based in a highly conserved region of the FCV genome, its diagnostic utility was limited by its inability to detect all isolates of FCV that it was tested against.

One of the most frequently cited FCV RT-PCR assays is the nested RT-PCR of Radford and coworkers (Radford et al. 1997). This assay also amplified a portion of the FCV capsid protein gene with the outer primers delineating a 529bp product spanning from region B to region F, and with the inner primers delineating a 235bp product covering the 3' end of region D and the 5' portion of region E (Table 2, Figure 2). A variation of this assay has also been reported, which altered the combination of primers to increase the size of the final amplicon to 393bp (Table 2) and create a semi-nested RT-PCR assay (Radford et al. 2001). This assay and the nested RT-PCR have been repeatedly used to evaluate antigenic variation in genes encoding portions of the capsid protein, which form neutralizing antibody epitopes (hypervariable region E of the capsid protein gene). Epidemiologically related isolates were typically less than 5% distant and epidemiologically unrelated isolates were 20-40% distant from each other (Radford et al. 2000). There are no published reports of this assay's sensitivity, specificity, or diagnostic range. The assay did, however, amplify RNA from 15 wild-type FCV isolates and 3 FCV vaccine strains (Radford et al. 1997). This assay was also used by Pedersen and coworkers (2000) to detect FCV RNA from an outbreak of highly virulent FCV, indicating that the diagnostic range of the assay is sufficiently broad to cover a new disease phenotype. The diagnostic range of this assay cannot, however, be quantitatively determined based on the data available. A nested RT-PCR provides greater sensitivity, but can be hampered by a higher probability of environmental contamination and false positive results, and requires more time and labor to perform.

Table 2. Feline calicivirus RT-PCR assays. This table comprises all previously reported FCV RT-PCR assays, together with the two assays reported here. Listed are the primers given by the individual authors (labeled as in the corresponding reference), the primer sequence (5'-3'), the size of the amplicon, the region of the FCV genome amplified (see Figure 2), the type of RT-PCR assay (gel-based or real-time), the diagnostic range of the assay as reported in the corresponding reference (listed as number of positive isolates / number tested), the corresponding nucleotides of strain FCV-Urbana (GenBank Accession number = NC_001481) covered by the assay, and the reference in which the assay was reported.

* This paper utilized a semi-nested RT-PCR with P1 and P2 in the first round of amplification and P2 and P4 in the second round of amplification. The size of this amplicon was then 393bp.

NR = Not reported

Primer Designation	Sequence (5' - 3')	Amplicon Size	Target Gene	Gel-based or Real-time	Diagnostic Range	Target Region (bp)	Reference
Sense	TTCGGCCTTTTGTGTTCC	670 bp	Capsid	Gel-based	12/12	6401-7071	Seal 94
Antisense	TTGAGAATTGAACACATC						
Forward	GCTTAAAACTCACAGTGTCC	477 bp	p5.6 - p32	Gel-based	21/24	43-519	Doyle 96
Reverse	AATTTCTCCCTCCATTCC						
P1	CCCTTTGTGTTCCAAGCAAATCG	529 bp	Capsid	Nested, Gel-based	NR	6406-6925	Radford 97 Radford 98
P2	CCTCTCCGATACCAGTGTATCC						
P4	TTGCAACTGATTATATTGTTCCTGG	235 bp				6533-6767	Radford 00 Radford 01*
P5	GCAGTGTTGGATATTTTCTTGTCACC						
FCV-F4	ATGTGCCAACCTGCGCTAA	2303 bp	Spanning Capsid	Gel-based	13/13	5314-7607	Geissler 97
FCV-F2	TCTAATTGCATTTAATTGATCGTCA						
CALCAPF	TTCGGCCTTTTGTGTTCC	673 bp	Capsid	Gel-based	13/13	6401-7071	Sykes 98 Sykes 01
CALCAPR	TTGAGAATTGAACACATCAATAGATC						
FCVCAPFOR	TACACTGTGATGTGTTCGAAGTTTGAGC	2190 bp	Spanning Capsid	Gel-based	5/5	5286-7567	Baulch-Brown 99
FCVCAPREV	GTGTATGAGTAAGGGTCAACCC						
F4-s	CATTTCGACTTTAACCAAGA	478 bp	Capsid	Gel-based	23/36	6430-6926	Tohya 97 Horimoto 01
F4-a	TCCTCGCCAATCCCAGTGTA						
CFI-s	TTCGGCCTTTTGTGTTCC	508 bp			12/13	6401-6926	
F4-a	TCCTCGCCAATCCCAGTGTA						
Forward	ACAATGTCTCAAACTCTGAGC	118 bp	p5.6	Gel-based	11/11	17-134	Scansen 02
Reverse	GCYTGTTRTARADATACTGAA						
Forward	TAATTCGGTGTTTGATTTGGCCTGGGCT	83 bp	p30	Real-time	60/60	2452-2534	Helps 02
Reverse	CATATGCGGCTCTGATGGCTTGAAACTG						
Forward	TGGATGAACTACCCGCCA	126 bp	p30	Real-time	51/51	2415-2540	Scansen 04
Reverse	GCACATCATATCCGGCTC						

Assays that amplify the entire capsid protein gene have also been reported (Geissler et al. 1997, Baulch-Brown et al. 1999). Geissler and coworkers amplified a 2,303bp product, which began at the start of the capsid protein gene and extended well into ORF3 (Table 2, Figure 2). This assay amplified 13 FCV isolates that were associated with varying disease manifestations including chronic stomatitis, acute stomatitis, acute URTD, and lameness. The sensitivity and specificity of the assay were not reported. However, too few isolates were assessed to evaluate diagnostic range. The assay of Baulch-Brown and coworkers similarly amplified a ~2,190bp product spanning all of ORF2 and the 5' portion of ORF3 (Table 2, Figure 2). This assay was used to compare the capsid protein gene sequence of 5 Australian FCV isolates to the prototypical F9 strain. However, specificity, sensitivity, and diagnostic range of the assay were not comprehensively evaluated. In addition, diagnostic utility of the assay may be compromised by the size of its amplicon. Assays which amplify the entire capsid protein gene are hindered by a large product size, making transformation to a real-time format more difficult (Mackay et al. 2002).

A variation of Seal's (1994) assay was described by Sykes and coworkers in 1998. The primers used by Sykes covered the same region of the capsid protein gene as those of Seal (1994), with the exception of an extra 8 base pairs on the 3' end of the antisense primer (Table 2). The product of this assay was 673bp in size and was detected by standard agarose gel electrophoresis. This study evaluated the diagnostic range of the assay and was one of the first to compare

molecular diagnostics to conventional virus isolation protocols in experimentally infected cats. The assay detected 13 different FCV strains including 12 field isolates spanning 18 years. Conjunctival swabs of experimentally infected cats were positive in 19 of 144 swabs by RT-PCR and in 16 of 144 swabs by virus isolation. This FCV RT-PCR assay was then incorporated into a multiplex assay, which also detected FHV and *Chlamydia psittaci* (Sykes et al. 2001). The multiplex assay did detect FCV in field cases of feline URTD, but no controls were employed nor was virus isolation used to validate these results. Unfortunately, it is difficult to assess the diagnostic range of the assay of Sykes and coworkers (1998) due to the small number of isolates tested. Primers were designed to span the hypervariable region E of the capsid protein gene, but the resulting amplicon was large and may therefore be unsuitable for real-time assay (Mackay 2002).

Amplification of the capsid protein gene from Japanese FCV isolates has also been reported (Tohya et al. 1997, Horimoto et al. 2001). Two primer sets were used to amplify a portion of the capsid protein gene from 36 different Japanese FCV isolates. The first primer set was based on the capsid protein gene sequence of the Japanese prototype strain FCV-F4 (Tohya et al. 1997) and yielded a 478bp product that spanned from the 3' end of region B to the 5' portion of region F (Table 2, Figure 2). In a survey of 36 Japanese FCV isolates, this assay amplified only 23 of 36 (64%) isolates (Horimoto et al. 2001).

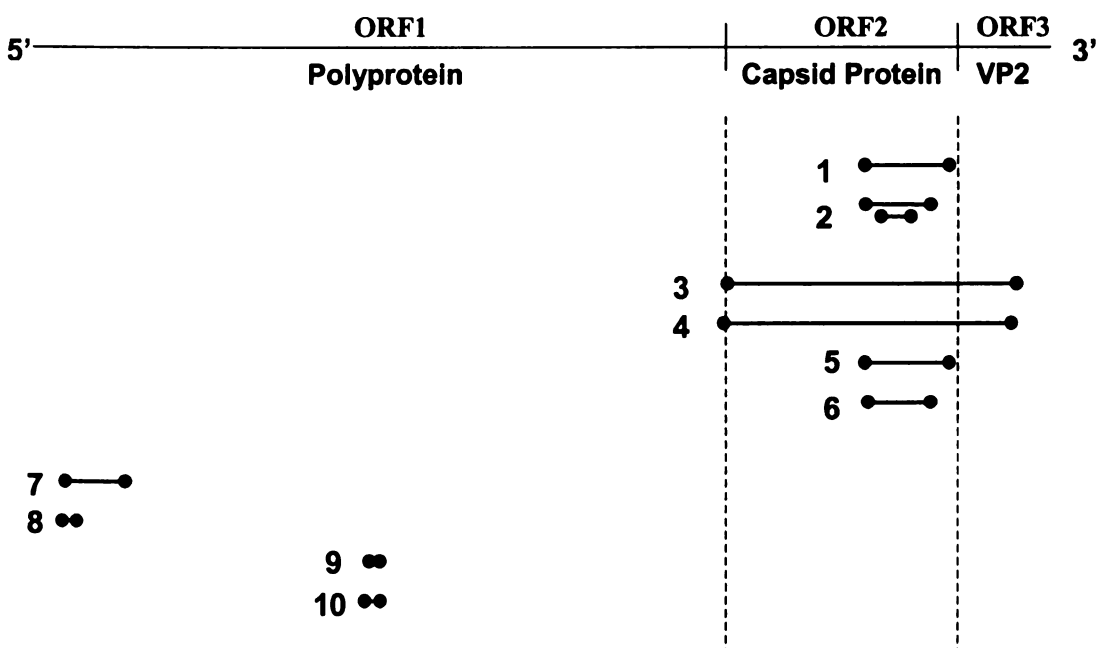


Figure 2. Genomic location of reported FCV RT-PCR assay targets. The target location of the reported FCV RT-PCR assays are shown in relation to the complete genome and its three open reading frames (ORF1, ORF2, ORF3). Products shown are: (1) Seal 1994, (2) Radford et al. 1997, (3) Geissler et al. 1997, (4) Baulch-Brown et al. 1999, (5) Sykes et al. 1998, (6) Horimoto et al. 2001, (7) Doyle et al. 1996, (8) Scansen et al. 2002, (9) Helps et al. 2002, (10) Scansen et al. 2004.

The assay was then altered to include the sense primer of Seal (1994) together with the same antisense primer of Tohya and coworkers (1997). This combination of primers added 30bp to the 5' end of the amplicon and amplified 12 of the 13 (92%) isolates that were not amplified with the first primer set (Horimoto et al. 2001). The sensitivity and specificity of this assay were not reported. The diagnostic range of the assay with the FCV-F4 based primers was poorer than other FCV RT-PCR assays, although it could be improved by switching the sense primer. Furthermore, only Japanese FCV isolates were evaluated, which limits extrapolation of its effective diagnostic range to FCV strains from other geographic regions.

In 2002, a group in the United Kingdom published the first real-time RT-PCR assay for FCV (Helps et al. 2002). This real-time assay amplified a small 83bp fragment of the p30 gene (Table 2, Figure 2). The real-time feature of this assay was accomplished by incorporating the fluorescent dye SYBR® Green I into the PCR reaction. SYBR® Green I non-specifically binds to double-stranded DNA and fluoresces once bound. Post-amplification melt curve analysis was performed to discriminate true product from nonspecific amplification. This assay was linear over a 10^6 dilution range and had a reaction efficiency of 82%. The lower detection limit of this assay was 10^{-8} dilution of stock virus and was comparable to that determined by virus isolation for the same stock virus. The diagnostic range of this assay was excellent, amplifying RNA from 60 of 60 (100%) FCV isolates representing 25 years of sampling. All FCV isolates tested

were from the United Kingdom. However, strains of FCV originating from the same geographic area tend to be genetically related (Rice et al. 2002). Genetic clustering of isolates by geographic region could therefore result in over-estimation of the effective diagnostic range of the assay. Assay specificity was verified by sequencing PCR products from 17 isolates. Although minor genetic variation between strains existed, the sequences were consistent with other published FCV p30 gene sequences. Amplification of non-feline calicviruses was not attempted in this study.

In summary, several RT-PCR assays for detection of FCV have been reported in the last 10 years. The majority of these assays have focused on the amplification of a portion of the capsid protein gene, most commonly the hypervariable region E, for studies of antigenic variation between strains. Fewer FCV RT-PCR assays have been developed specifically for diagnostic purposes, and fewer still report sensitivity, specificity, and diagnostic range. The assay of Sykes and coworkers (1998) was promising as a gel-based assay, although evaluation of the diagnostic range was limited to only 13 isolates. There are also no reports in the literature on the use of these primers in a real-time RT-PCR assay. Real-time RT-PCR offers the advantage over traditional RT-PCR of increased sensitivity, faster throughput, increased reproducibility, decreased risk of laboratory contamination, and better quantification (Mackay et al. 2002). Real-time RT-PCR also eliminates the need for post-PCR processing such as ethidium bromide staining and agarose gel electrophoresis. The first reported real-time

RT-PCR assay for FCV utilized p30 gene-based primers and the fluorescent dye SYBR® Green I. This real-time assay was comparable to virus isolation in terms of sensitivity and had a broad diagnostic range (Helps et al. 2002). However, evaluation of the diagnostic range of this assay was limited to FCV isolates from the United Kingdom.

Detection of FCV in Urine

Virus isolation has been the "gold standard" for FCV detection. However, virus isolation is time consuming, expensive, and requires viable virus in the specimen and absence of substances that are toxic to cell culture or that inhibit viral replication (Sykes 2001). Molecular diagnostic methods, such as reverse-transcriptase polymerase chain reaction (RT-PCR) circumvent many of the difficulties associated with conventional virus isolation methods and are increasingly being used for rapid detection of FCV (Sykes et al. 1998, Helps et al. 2002, Mackay et al. 2002). Molecular diagnostic methods have been shown to be comparable to virus isolation in sensitivity and diagnostic range, and offer the advantages of reduced sample size, faster throughput, and better quantitation (Sykes et al. 1998, Helps et al. 2002, Mackay et al. 2002).

While molecular diagnosis of FCV infection is effective, the substrate on which testing is performed can play an important role in assay performance. Human urine, blood, fecal matter, sputum, and vitreous fluid have been reported to contain substances that have an inhibitory effect on the polymerase chain

reaction (Khan et al. 1991, Wiedbrauk et al. 1995, Forbes & Hicks 1996, Hale et al. 1996, Klein et al. 1997). Consequently, preparation of nucleic acids becomes a critical step that not only serves to concentrate and purify nucleic acids, but also to remove or inactivate PCR inhibitors.

Urine, in particular, is well known to inhibit PCR amplification of viral and bacterial agents (Khan et al. 1991, Behzadbehbahani et al. 1997, Echavarria et al. 1998, Biel et al. 2000). Urinary substances, in general, may affect PCR-based assays in one of two major ways: (1) urine constituents may cause viral lysis and degradation or capture of viral nucleic acid, or (2) urine components may inhibit PCR by inactivating enzymes (reverse-transcriptase, *Taq* DNA polymerase, etc.) or chelating necessary cations such as magnesium (Behzadbehbahani et al. 1997). Studies on human urine specimens indicate that the nucleic acid preparation method significantly influences the ability of PCR-based assays to detect viruses in urine and other complex biological specimens (Demmler et al. 1988, Kahn et al. 1991, Behzadbehbahani et al. 1997, Echavarria et al. 1998, Biel et al. 2000). Unfortunately, the optimal method of nucleic acid preparation varies depending on the nature the specimen, the type and quantity of inhibitory substances present in the sample, the physical, biochemical, molecular, and antigenic properties of the virus, and the susceptibility of individual PCR assay components to inhibition.

Numerous methods of nucleic acid extraction and sample preparation have been employed on human urine samples to mitigate the inhibitory effects of urine on PCR-based detection of viral and bacterial agents. These preparation methods include dialysis (Chang et al. 1996), polyethylene glycol precipitation (Vinogradskaya et al.1995, Behzadbehbahani et al.1997), sample dilution (Mahony et al. 1998, Toye et al. 1998, Biel et al. 2000), thermal inactivation (Verkooyen et al. 1996, Biel et al. 2000), oligo$(dT)_{25}$-coated magnetic beads (Chiodi et al. 1992, Kingsley & Richards 2001), and silica gel-based extraction columns (Echavarria et al. 1998). Unfortunately, there is limited knowledge regarding optimal methods for preparation of nucleic acids from veterinary urine specimens. Results of one study in which thermal inactivation was used to neutralize inhibitory substances in fecal specimens for the detection of canine parvovirus, suggested that the efficacy of thermal inactivation is species-dependent (Uwatoko et al. 1996). It is plausible that species differences exist between feline urine and human urine samples as well. Studies comparing RNA isolation methods for their ability to remove or inactivate RT-PCR inhibitors and preserve FCV RNA integrity in feline urine specimens have not been reported.

p5.6 GENE-BASED RT-PCR ASSAY

A portion of this work was presented as an abstract at the 20th Annual ACVIM Forum, Dallas TX, May 2002 (See Scansen BA, Kruger JM, Venta PJ, Wise AG, Maes RK. Development of a one-step reverse-transcriptase polymerase chain reaction assay for detection of feline calicivirus. Abstract. *Journal of Veterinary Internal Medicine* 2002; 16: 365).

Abstract

Studies investigating the causative role of FCV in feline idiopathic cystitis have been hindered by lack of a sensitive means of detecting FCV urinary tract infections. The purpose of this study was to develop a sensitive and specific FCV reverse-transcriptase polymerase chain reaction (RT-PCR) assay for rapid detection of FCV infections. Multiple cDNA sequences corresponding to the FCV p5.6 gene were obtained from GenBank. Oligonucleotide primers delineating a conserved 118 bp region of the p5.6 gene were synthesized at a commercial laboratory. The reverse primer was degenerate in 4 nucleotide positions. Total RNA was extracted from FCV-infected tissue-culture cell preparations. Viral RNA was reverse transcribed into cDNA and amplified using a one-step RT-PCR and FCV p5.6 gene-specific primers. The 118 bp RT-PCR products were identified by agarose and polyacrylamide gel electrophoresis. The qualitative RT-PCR assay was optimized for primer concentrations and cycling conditions. Assay specificity and diagnostic range was evaluated by direct sequencing of RT-PCR products and by amplification of viral RNA from 10 field strains of FCV. Assay sensitivity was determined by extracting and amplifying viral RNA from serial 10-fold dilutions of FCV. The qualitative assay was then adapted to a real-time quantitative RT-PCR format by incorporating (1) a 25 bp oligonucleotide DNA hybridization probe that was dual-labeled with the florescent dye 6-FAM and the quencher BHQ-1, or (2) the nonspecific reporter dye SYBR® Green I, into the RT-PCR reaction. Real-time RT-PCR was performed on a thermal cycler with an integrated real-time optical detection system. The sensitivity of the

quantitative real-time RT-PCR assay utilizing the dual-labeled probe was assessed as described above. The optimal concentration of SYBR® Green I was determined by varying the concentration of dye used in the real-time assay.

Optimal qualitative RT-PCR occurred when the reverse degenerate primer concentration was four times that of the forward primer. Specific FCV amplification product bands were observed for all 10 wild-type FCV strains. The nucleotide sequence of the RT-PCR product was 95% identical to published FCV sequences. Amplification products from the 9.2×10^{-1} $TCID_{50}$ per ml dilution of FCV were detected visually. FCV amplification was detected in real-time using the dual-labeled DNA hybridization probe and this assay yielded a lower detection limit of 1,840 $TCID_{50}$ per sample. A real-time assay utilizing the reporter dye SYBR® Green I showed optimal fluorescence detection at a dye concentration of 1:40,000. However, agarose gel electrophoresis indicated the presence of extraneous nonspecific high molecular weight bands that prevented accurate interpretation of the real-time results.

In conclusion, this p5.6 gene-based one-step qualitative RT-PCR assay appears to be a highly sensitive and specific means of detecting FCV. However, the assay was not efficiently adapted to either a dual-labeled fluorescent probe or the fluorescent dye SYBR® Green I methodologies for continuous monitoring of amplification in real-time.

Introduction

Feline calicivirus (FCV) is best known as an important cause of upper respiratory disease in domestic cats (Baulch-Brown et al. 1997). In addition, there is increasing evidence that FCV may have a causative role in feline idiopathic cystitis (Kruger et al. 1996, Rice et al. 2002). Idiopathic cystitis is the most common disorder of the feline urinary tract and is characterized by bloody and painful urination (hematuria and dysuria), increased frequency of urination (pollakiuria), urination in inappropriate locations, and/or urethral obstruction (Kalkstein et al. 1999a). The disorder is believed to affect a quarter to half a million cats in the United States annually. At this time, there is no effective therapy (Kalkstein et al. 1999b). Isolation of a FCV from a Manx cat with urethral obstruction, and experimental induction of urethral obstruction in 80% of conventionally reared cats following urinary bladder, aerosol, or contact exposure with this virus directly supported the concept of a viral etiology (Rich & Fabricant 1969, Rich et al. 1971). In subsequent studies, calicivirus-like particles were observed by electron microscopy in 38% of 92 urethral plugs obtained from male cats with obstructive idiopathic cystitis (Kruger et al. 1996). More recently, an improved virus isolation technique enabled isolation of two new FCV strains (designated FCV-U1 and FCV-U2) from urine obtained from cats with idiopathic cystitis (Rice et al. 2002). Genetic analyses revealed that both new urinary FCV strains were genetically distinct from vaccine and other wild-type strains (Rice et al. 2002). In a subsequent pilot study, specific-pathogen-free cats were infected with both urinary strains via the oronasal and urinary bladder routes, and

compared to noninfected controls and control cats infected with a respiratory strain of FCV (Kruger et al. 2002). All three strains of FCV induced persistent hematuria via the oronasal or urinary bladder routes. Persistent hematuria was not observed in any of the noninfected control cats that were mock infected and sampled in an identical fashion. Despite persistent hematuria in the majority of infected cats, urine shedding of FCV was detected by virus isolation in only 1 cat infected oronasally with the urinary strain FCV-U1, and one cat infected via the urinary bladder with the urinary strain FCV-U2 (Kruger et al. 2002).

Although clinical and experimental evidence support a potential causative role for FCV in idiopathic cystitis, large scale epidemiologic studies and studies of experimentally induced FCV urinary tract disease have been hindered by lack of a sensitive and rapid means of detecting FCV urinary tract infections. Previous studies have relied upon recovery of live virus from urine using tissue culture-based virus isolation methods (Rice et al. 2002, Rich & Fabricant 1969). As with all isolation techniques, isolation of FCV requires the presence of live viruses in specimens and absence of substances that are toxic to cell culture or inhibit viral replication (Sykes et al. 2001). Our observations, and those of others, indicate that FCV is extremely sensitive to the effects of urine, storage temperature, and freeze-thaw cycles (Komolafe 1979, Kruger & Doyle 1997, Sykes et al. 2001). Compounding the problem, feline urine appears to be highly toxic to standard cell-lines used for feline virus isolation (Kruger & Doyle 1997). Although virus isolation has the advantage of recovering viable viruses from urine, the fact that

they are time consuming, labor intensive, expensive, and have limited sensitivity for detecting low levels of FCV in urine, limit their usefulness for large scale clinical or experimental studies of idiopathic cystitis.

Rapid molecular diagnostic methods, such as reverse-transcriptase polymerase chain reaction (RT-PCR) assays, have been shown to complement conventional virus isolation and identification techniques for detection of FCV (Sykes et al. 1998, Sykes et al. 2001). Furthermore, real-time RT-PCR offers the advantage over traditional RT-PCR of increased sensitivity, faster throughput, increased reproducibility, and better quantification (Lanciotti et al. 2000, Leutenegger et al. 1999). Real-time RT-PCR assays incorporate a fluorescent reporter DNA hybridization probe or a nonspecific reporter dye (e.g. SYBR® Green I) into the RT-PCR reaction that allows dynamic quantitative detection of hybridization events (Livak et al. 1995). The DNA hybridization probe is a dual-labeled oligonucleotide, having a fluorescent reporter dye at one end and a fluorescence quencher at the other. If the target is present during RT-PCR, the probe anneals specifically between the forward and reverse primer sites. The 5' nuclease activity of the DNA polymerase during PCR cleaves the probe from the target, resulting in an increase in fluorescence intensity of the reporter dye. SYBR® Green I is a dye that nonspecifically bind to double stranded DNA and fluoresces once bound. Optical detectors quantify the increase in fluorescence intensity in real-time without further post-PCR analysis. Consequently, real-time RT-PCR assays eliminate the need for ethidium bromide staining and agarose gel

electrophoresis to detect amplification products. Because both amplification and detection processes are contained within a closed system, real-time RT-PCR substantially reduces risks of laboratory contamination and false positive results.

The purpose of this study was to develop and optimize a sensitive and specific conventional qualitative FCV RT-PCR assay, and to adapt the assay to a quantitative real-time format. Information gained from this study might allow us to better characterize the potential causative role of FCV in feline idiopathic cystitis. A sensitive real-time RT-PCR optimized for urine would be ideal for large-scale epidemiologic surveys or experimental studies designed to investigate the pathogenic role of FCV in idiopathic cystitis. Furthermore, a rapid real-time quantitative RT-PCR would have broad diagnostic applications for detection of FCV infection in non-urinary tract disorders (e.g. upper respiratory disease, stomatitis, abortion, and arthritis).

Materials and Methods

Viruses

Samples of 10 FCV strains (including urinary strains FCV-U1 and FCV-U2) isolated from 1991 to 2001 were provided by the Virology Section of the Michigan State University Diagnostic Center for Population and Animal Health. FCV strain #2351774 (designated FCV-R), previously characterized in our laboratory, was used as the reference strain for assay optimization.

Conventional Qualitative RT-PCR Assay

Multiple FCV cDNA sequences corresponding to the FCV p5.6 gene were obtained from GenBank. Oligonucleotide (21-mer) primers delineating a 118 base pair region of the p5.6 gene of ORF1 of the FCV genome (nucleotides 17-134 of strain FCV-Urbana, Figure 2) (Green et al. 2002, Sosnovtsev & Green 2002) were synthesized at a commercial DNA synthesis laboratory[a]. The reverse primer was degenerate in 4 nucleotide positions (Table 2). Total RNA was extracted using a commercial silica-gel based extraction column[b]. Viral RNA was reverse-transcribed and amplified using a one-step RT-PCR system[c]. The RT-PCR amplifications were performed in 50μl reaction mixtures containing 5x buffer (a proprietary buffer containing 12.5 mM $MgCl_2$), dNTPs, enzyme mix (a proprietary mixture containing 2 reverse transcriptases and a *Taq* DNA polymerase), the forward primer 5'-ACAATGTCTCAAACTCTGAGC, the reverse primer 5'-GCYTGTTRTARADATACTGAA, 5 μl of total RNA, and RNase-free water. The 118 bp RT-PCR products were identified by agarose (1.5% w/v) and polyacrylamide (10% w/v) gel electrophoresis. Positive and negative reaction controls were included with each set of amplifications. The RT-PCR assay was optimized for primer concentrations and cycling conditions. Assay specificity was evaluated by direct automated sequencing of RT-PCR products. Amplified product was prepared using a commercial PCR product purification kit[d] and sequenced using a commercial radiolabeled sequencing kit[e]. The diagnostic range of the assay was determined by amplification of viral RNA from ten wild-

type FCV strains. Assay sensitivity was determined by extracting and amplifying viral RNA from serial 10-fold dilutions of FCV-R (starting titer = 9.2×10^8 $TCID_{50}$).

Real-Time RT-PCR Assay with a Dual-Labeled DNA Hybridization Probe

A 25bp oligonucleotide DNA hybridization probe corresponding to nucleotides 45-69 of the FCV-Urbana Strain was synthesized and dual labeled with the florescent dye 6-FAM and the quencher BHQ-1 at a commercial laboratory[f]. Reverse transcription of viral RNA and subsequent cDNA amplification were performed in 50μl reaction mixtures as described above with the addition of a dual labeled reporter hybridization probe 5'-/56-FAM/TTAAAA CTCACAGTGTCCGCAAGGA/3BHQ-1/-3' at a concentration of 400 nM. Primer concentrations were 0.3 μM for the forward primer and 1.2 μM for the reverse. Positive and negative reaction controls were included with each set of amplifications. Thermocycling and real-time quantification of amplification products were performed in a commercial thermocycler with attached optical detection system[g]. Amplification of cDNA was continuously monitored in real-time by quantifying the amount of fluorescence emitted at 530nm at each annealing step. Samples with fluorescence signals exceeding the baseline fluorescence were determined to be FCV-specific product, while samples not exceeding the baseline fluorescence were determined to be the result of non-specific background fluorescence. To confirm the size of real-time RT-PCR products, all amplification products were visualized by electrophoresis in an agarose gel (2% w/v) with ethidium bromide staining. Assay sensitivity was

determined by extracting and amplifying viral RNA from serial 10-fold dilutions of FCV-R (titer = 9.2×10^8 $TCID_{50}$ per ml).

Real-Time RT-PCR Assay with SYBR® Green I

SYBR® Green I dye was obtained from a commercial vendor[h] and diluted in RNase-free water. Reverse transcription of viral RNA and subsequent cDNA amplification were performed in 50μl reaction mixtures as described above for qualitative RT-PCR with the addition of varying concentrations of SYBR® Green I dye and primer concentrations of 0.3μM for the forward primer and 1.2μM for the reverse primer. Amplification of cDNA was continuously monitored in real-time by quantifying the amount of fluorescence emitted at 530nm at each annealing step. Amplicon melt temperatures were determined by raising the temperature in 0.5C increments from 55C to 95C. Post-PCR analysis was performed using software supplied with the real-time thermocycler[g]. The RT-PCR assay was optimized for dye concentrations and cycling conditions. To confirm the size of real-time RT-PCR products, all amplification products were visualized by electrophoresis in agarose gels (2% w/v), following ethidium bromide staining.

Results

Conventional Qualitative RT-PCR Assay

Optimal RT-PCR amplification occurred when the concentration of the forward conserved primer was 2 to 4 times less than that of the reverse degenerate primer (Figure 3). For all subsequent amplifications, a forward primer

concentration (0.3μM) one fourth of the reverse primer concentration (1.2μM) was chosen. Optimal cycling conditions were determined to be reverse transcription at 50C for 45 minutes, an initial denaturation step of 95C for 15 minutes, and then 40 cycles of 94C for 30s, 48C for 30s, and 72C for 30s, followed by a final extension step of 72C for 7 minutes. Specific FCV amplification product bands were observed for all 10 wild-type FCV strains. The nucleotide sequence of the RT-PCR product was 95% identical to published FCV sequences (data not shown), indicating that the product amplified was the targeted gene. Amplification products from the 9.2×10^{-1} $TCID_{50}$ per ml dilution of FCV were detected visually (Figure 4).

Real-Time RT-PCR Assay with a Dual-Labeled DNA Hybridization Probe

An increase in fluorescence above the baseline was observed at the 10^{-4} and lower dilutions (Figure 5). This corresponded to a detection limit of 1,840 $TCID_{50}$ per sample using the dual-labeled DNA hybridization probe. Bands of a size consistent with real product from the 10^{-6} and lower dilutions were visualized on an agarose gel, but not sequenced (data not shown). Further optimization of this assay was not performed.

Real-Time RT-PCR Assay with SYBR® Green I

No increase in fluorescence was detected in samples with SYBR® Green I at a concentration greater than or equal to 1:25,000 (Figure 6). Optimal detection of fluorescence occurred at a SYBR® Green I concentration between 1:30,000 and

1:40,000 (Figure 6). At concentrations less than 1:40,000, a fluorescent signal was not detected and, likewise, at SYBR® Green I concentrations greater than or equal to 1:25,000, an increase in fluorescence did not occur even at high template concentration (data not shown). Agarose gel electrophoresis indicated the presence of extraneous nonspecific high molecular weight bands (data not shown) that prevented accurate interpretation of the real-time results and, therefore, the sensitivity of this assay was not determined.

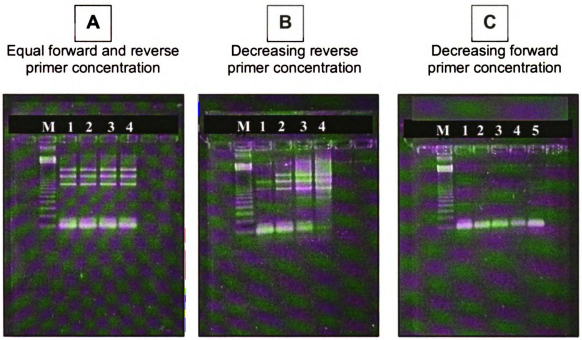


Figure 3. Agarose gel (1.5% w/v) electrophoresis of amplification products showing the effect of variable primer concentration on performance of a FCV p5.6 gene-based RT-PCR assay. Varying the concentration of the primers indicated optimal assay performance when the forward (conserved) primer concentration was decreased in comparison to the reverse (degenerate) primer concentration: (A) Lanes 1-4 contain equal concentration (0.6μM) of the forward and reverse primers; (B) Lanes 1-4 contain 0.6μM of the forward primer and 0.6μM (lane 1), 0.3μM (lane 2), 0.15μM (lane 3), and 0.06μM (lane 4) of the reverse primer; (C) Lanes 1-4 contain 0.6μM of the reverse primer and 0.6μM (lane 1), 0.3μM (lane 2), 0.15μM (lane 3), and 0.06μM (lane 4) of the forward primer, and lane 5 contains 0.6μM of the forward primer and 1.2μM of the reverse primer. The amplicon size of the assay was 118bp. Lane M = Molecular size marker (123bp DNA ladder).

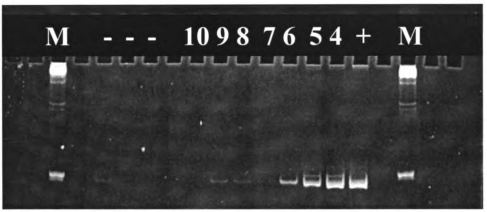


Figure 4. Polyacrylamide gel (10% w/v) electrophoresis of amplification products from serial dilutions of FCV (titer = 9.2 x 10^8 $TCID_{50}$/ml) reverse-transcribed into cDNA and amplified by a p5.6 gene-based RT-PCR assay. Dilutions shown here include the 10^{-4} (lane 4), 10^{-5} (lane 5), 10^{-6} (lane 6), 10^{-7} (lane 7), 10^{-8} (lane 8), 10^{-9} (lane 9), and 10^{-10} (lane 10). The highest detectable dilution was 10^{-9}, corresponding to a $TCID_{50}$ of 9.2 x 10^{-1} per ml. The amplicon size was 118bp. Lane M = Molecular size marker (123bp DNA ladder), lane + = positive control, lane - = negative control.

Discussion

The FCV p5.6 gene is an effective site for detection of FCV by qualitative RT-PCR amplification. Interestingly, the performance of the assay was substantially improved when concentration of the reverse (degenerate) primer was four times that of the forward (nondegenerate) primer. If the degenerate primer resulted in a greater prevalence of nonspecific binding, as might be expected, then the assay would be expected to improve with lower concentrations of this degenerate

primer. However, the opposite occurred and is most likely due to forward primer excess relative to the quantity of target and to the quantity of specific degenerate oligonucleotide reverse primer required for amplification of a given FCV strain.

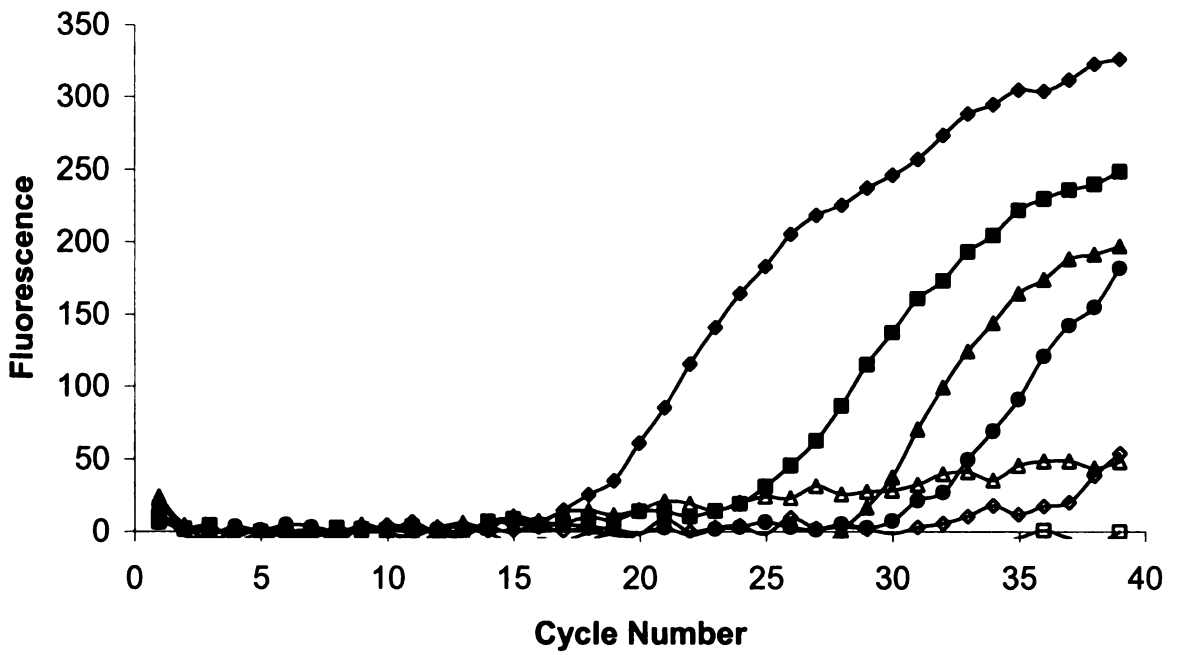


Figure 5. Lower detection limit of the p5.6 real-time RT-PCR assay for FCV with the dual-labeled DNA hybridization probe. A graph of fluorescence vs. cycle number illustrating amplification of serial FCV dilutions with the threshold cycle varying inversely with virus concentration. Serial log dilutions of virus were made starting from a stock with a titer of 9.2×10^8 $TCID_{50}$/ml (◆) progressing through 10^{-2} (■), 10^{-3} (▲), 10^{-4} (●), 10^{-5} (◇), 10^{-6} (□) dilutions, and a negative control (△). All samples were amplified in duplicate.

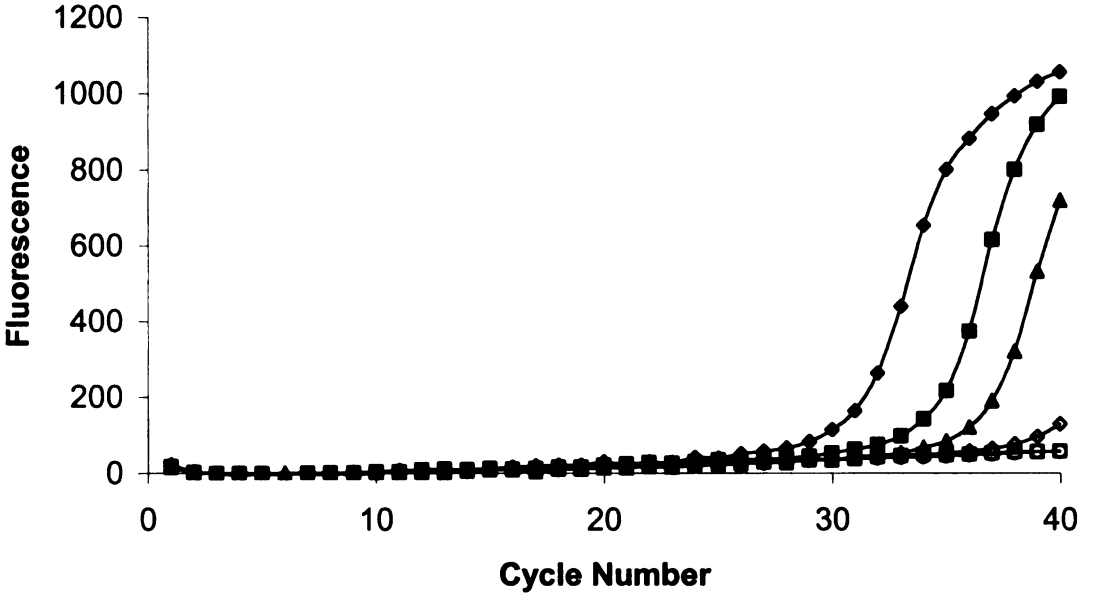


Figure 6. Optimization of SYBR® Green I dye concentration. A graph of fluorescence vs. cycle number indicating optimal amplification at dilute dye concentrations and inhibitory effects of higher concentrations. SYBR® Green I dye was added to the p5.6 gene-based assay at variable concentrations: 1:40,000 (◆), 1:35,000 (■), 1:30,000 (▲), 1:25,000 (○), 1:20,000 (◇), and 1:15,000 (□).

These results indicate that the p5.6 gene-based qualitative RT-PCR assay is approximately 10 to 100 times more sensitive than virus isolation and previously reported FCV RT-PCR assays (Sykes et al. 1998, Sykes et al. 2001). In addition, the new assay appears to be specific for FCV based on direct sequencing of RT-PCR products and has a broad diagnostic range.

Despite standard laboratory precautions, the qualitative assay appeared to be very susceptible to contamination and false positive results. This is most likely due to the small amplicon size and need for post-amplification agarose gel electrophoresis to identify FCV products.

Real-time quantitative RT-PCR offers the advantage over traditional gel-based RT-PCR assays of faster throughput, increased reproducibility, and better quantification, and reduced risk of false positive results (Leutenegger et al. 1999, Lanciotti et al. 2000, Mackay et al. 2002). Despite the fact the qualitative FCV p5.6 gene-based RT-PCR assay was sensitive and specific, the assay was not easily adapted to either a dually-labeled fluorescent probe or to the fluorescent dye SYBR® Green I methods for continuous monitoring of amplification in real-time. The dual-labeled fluorescent probe assay did offer real-time monitoring of FCV amplification, but was 2 to 3 logs less sensitive than the qualitative assay. This may have been due to secondary structure present in the portion of the genome to which the probe annealed. Analysis of probable secondary structure formation within the FCV genome indicated the presence of a hairpin loop at the location of probe binding (data not shown), which may have decreased the sensitivity of this detection system.

The sensitivity of the SYBR® Green I assay was not adequately determined due to the persistence of extraneous high molecular weight bands that prevented accurate interpretation of the real-time results. The finding of assay inhibition in

the presence of high concentrations of SYBR® Green I is not new. Other authors have reported inhibition at a 1:7,000 and higher dilution (Wittwer et al. 1997), much more concentrated than our finding of 1:25,000 and higher. The finding of optimal real-time detection at a SYBR® Green I dilution of between 1:30,000 and 1:40,000 is similar to other reports (Ririe et al. 1997, Morrison et al. 1998).

In conclusion, the FCV p5.6 gene appears to be a suitable site for a conventional qualitative RT-PCR assay. However, the assay was very susceptible to environmental contamination with PCR product due to small product size and the need for post-reaction analysis by agarose gel electrophoresis. Furthermore, the p5.6 gene was not an optimal site for real-time RT-PCR amplification of FCV. Development of a sensitive and specific FCV real-time RT-PCR assay with a broad diagnostic range may require identification of other highly conserved regions of the FCV genome more amenable to dual-labeled fluorescent probes or SYBR® Green I methodologies. However, this may be difficult due the highly variable nature of the FCV genome.

Footnotes

a – Michigan State University Molecular Structure Facility, East Lansing, MI

b – Qiagen RNeasy Mini Kit®

c – Qiagen OneStep RT-PCR Kit®, Qiagen Incorporated, Valencia, CA

d – Qiaquick PCR Purification Kit®, Qiagen Incorporated, Valencia, CA

e – Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit®, USB Corporation, Cleveland, OH

f – Integrated DNA Technologies, Incorporated, Skokie, IL

g – iCycler™ iQ® System with detection softare v2.3B, Bio-Rad Laboratories, Hercules, CA

h – SYBR® Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO, Molecular Probes, Eugene, OR.

p30 GENE-BASED RT-PCR ASSAY

Scansen BA, AG Wise, JM Kruger, PJ Venta, RK Maes. 2004. Evaluation of a p30 gene-based real-time reverse transcriptase polymerase chain reaction assay for detection of feline caliciviruses. *Journal of Veterinary Internal Medicine*; 18(1): 135-138.

Evaluation of a p30 Gene-Based Real-Time Reverse Transcriptase PCR Assay For Detection of Feline Caliciviruses

Brian A. Scansen, BS[1]

Annabel G. Wise DVM, PhD[2,3]

John M. Kruger DVM, PhD[1]

Patrick J. Venta PhD[1,2]

Roger K. Maes DVM, PhD[2,3]

Key Words: feline calicivirus, real-time RT-PCR, p30 gene, SYBR Green I®, RNA, cDNA, quantitative

Running Title: Feline Calicivirus Real-Time RT-PCR

From the Departments of Small Animal Clinical Sciences[1], Microbiology and Molecular Genetics[2] and the Diagnostic Center for Population and Animal Health[3], Michigan State University College of Veterinary Medicine, East Lansing MI. An abstract of this work was presented at the 21st Annual Veterinary Medical Forum, Charlotte, NC, June, 2003. This study was supported by the Michigan State University Companion Animal Fund and Center for Feline Health and Well-Being, and by the Geraldine R. Dodge Foundation.

Reprint Requests: John M. Kruger DVM, PhD, Department of Small Animal Clinical Sciences, Room D208 Veterinary Clinical Center, Michigan State University, East Lansing, MI 48824-1314; (517) 355-6568; e-mail: kruger@cvm.msu.edu.

Abstract

This report describes a feline calicivirus (FCV) p30 gene-based real-time SYBR Green I® reverse transcriptase PCR (RT-PCR) assay that is capable of detecting low virus concentrations and a broad range of FCV isolates. The assay consisted of a one-step RT-PCR reaction with primers delineating a 126 bp region of the FCV p30 gene. Sensitivity of the RT-PCR assay was determined to be equivalent to a FCV titer of 1.2×10^1 to 1.2×10^2 $TCID_{50}$/ml. The assay was linear over a wide range of template concentrations and had a reaction efficiency of 95%. Specific FCV amplification products were detected from 51 wild-type FCV isolates, whereas specific products were not detected from a canine calicivirus, a rabbit calicivirus, and a bovine calicivirus. The primers used in this study amplified a large number of North American FCV isolates and further confirm the diagnostic utility of p30 gene-based real-time RT-PCR for detection of FCV.

Feline caliciviruses (FCV) have been associated with a variety of clinical manifestations including rhinitis, conjunctivitis, stomatitis, ulcerative glossitis, faucitis, pneumonia, enteritis, lameness, abortion, and cystitis (Baulch-Brown et al. 1997, Rice et al. 2002). Historically, detection of FCV infection has relied upon virus isolation. Although sensitive, the clinical utility of virus isolation for detection of FCV is limited by expense, low availability, and slow throughput. Rapid molecular diagnostic methods, such as the reverse transcriptase polymerase chain reaction (RT-PCR) assay, have been shown to complement conventional virus isolation and identification techniques for detection of FCV (Sykes et al. 1998). Real-time quantitative RT-PCR assays incorporate a fluorescent reporter DNA hybridization probe or a nonspecific reporter dye into the RT-PCR reaction that allows dynamic quantitative detection of hybridization events (Mackay et al. 2002). Optical detectors quantify increases in fluorescence intensity in real-time and negate the need for post-PCR analysis of amplification products by agarose gel electrophoresis. Real-time RT-PCR offers the advantage over other diagnostic techniques of increased sensitivity, higher throughput, increased reproducibility, better quantitation, and reduced risk of laboratory contamination and false positive results (Mackay et al. 2002).

The FCV genome is approximately 7.7 kb in size and contains 3 open reading frames (ORF) (Baulch-Brown et al. 1997). ORF1 encodes nonstructural proteins including p5.6, p32, a nucleoside triphosphatase, p30, VPg, a cysteine protease, and a polymerase; ORF2 encodes the capsid protein and ORF3 encodes a minor

structural protein (Baulch-Brown et al. 1997, Green et al. 2002). Previous FCV RT-PCR assays have targeted the p5.6, p32, and polymerase genes of ORF1 (Sykes et al. 1998).[a,b] and portions of the capsid protein gene of ORF2 (Sykes et al. 1998, Horimoto et al. 2001, Radford et al. 2001). However, the high degree of variability inherent in the FCV genome has limited effective diagnostic application of these assays (Baulch-Brown et al. 1997). A real-time RT-PCR assay targeting an 83 base pair region of the FCV p30 gene (nucleotides 2454-2536 of the Urbana strain) was recently reported from the United Kingdom and was found to be sensitive and capable of detecting a wide range of clinical isolates from that region (Helps et al. 2002). We have independently developed a real-time RT-PCR assay that targets a generally conserved 126 base pair portion of the FCV p30 gene. The assay incorporates the fluorescent dye SYBR Green I® to dynamically quantify product formation in the PCR reaction. The assay is sensitive and capable of detecting a large number of North American FCV isolates. Our results further confirm the diagnostic utility of p30 gene-based real-time RT-PCR detection of FCV.

Multiple FCV cDNA sequences corresponding to the FCV p30 gene were obtained from GenBank. Oligonucleotide (18-mer) primers delineating a 126 base pair region of the p30 gene of ORF1 of the FCV genome (nucleotides 2417-2542 of the Urbana strain) (Baulch-Brown et al. 1997, Green et al. 2002) were synthesized at a commercial DNA synthesis laboratory. Total RNA was extracted using a commercial silica-gel based extraction column.[c] Viral RNA was reverse-

transcribed and amplified using a one-step RT-PCR system.[d] The RT-PCR amplifications were performed in 50μl reaction mixtures containing 25μl of 2x buffer (a proprietary buffer containing *Taq* DNA polymerase, SYBR Green I, dNTPs, and 5.0 mM $MgCl_2$),[d] 0.5μl of mixed reverse transcriptases,[d] 0.5 μM of the forward primer 5'-TGGATGAACTACCCGCCA, 0.5 μM of the reverse primer 5'-GCACATCATATGCGGCTC, 5 μl of total RNA, and RNase-free water. Real-time RT-PCR amplification was performed in a thermal cycler with an integrated real-time optical detection system.[e] Cycling conditions consisted of reverse transcription at 50C for 30 minutes and a preliminary denaturation step at 95C for 15 minutes, followed by 38 cycles of 94C for 30s, 53C for 30s, and 72C for 60s. Amplification of cDNA was continuously monitored in real-time by quantifying the amount of fluorescence emitted at 530nm at each annealing step (53C). Following a post-amplification step at 55C for one minute, amplicon melt temperatures were determined by raising the temperature in 0.5C increments from 55C to 95C. Post-PCR analysis was performed using iCycler detection software.[e] Samples giving peak fluorescence between 80.5C and 84.5C were determined to be FCV-specific product, while samples with peaks below 76C were determined to be the result of non-specific amplification. To confirm the size of real-time RT-PCR products, all amplification products were visualized by electrophoresis in an agarose gel (2% w/v) and ethidium bromide staining.

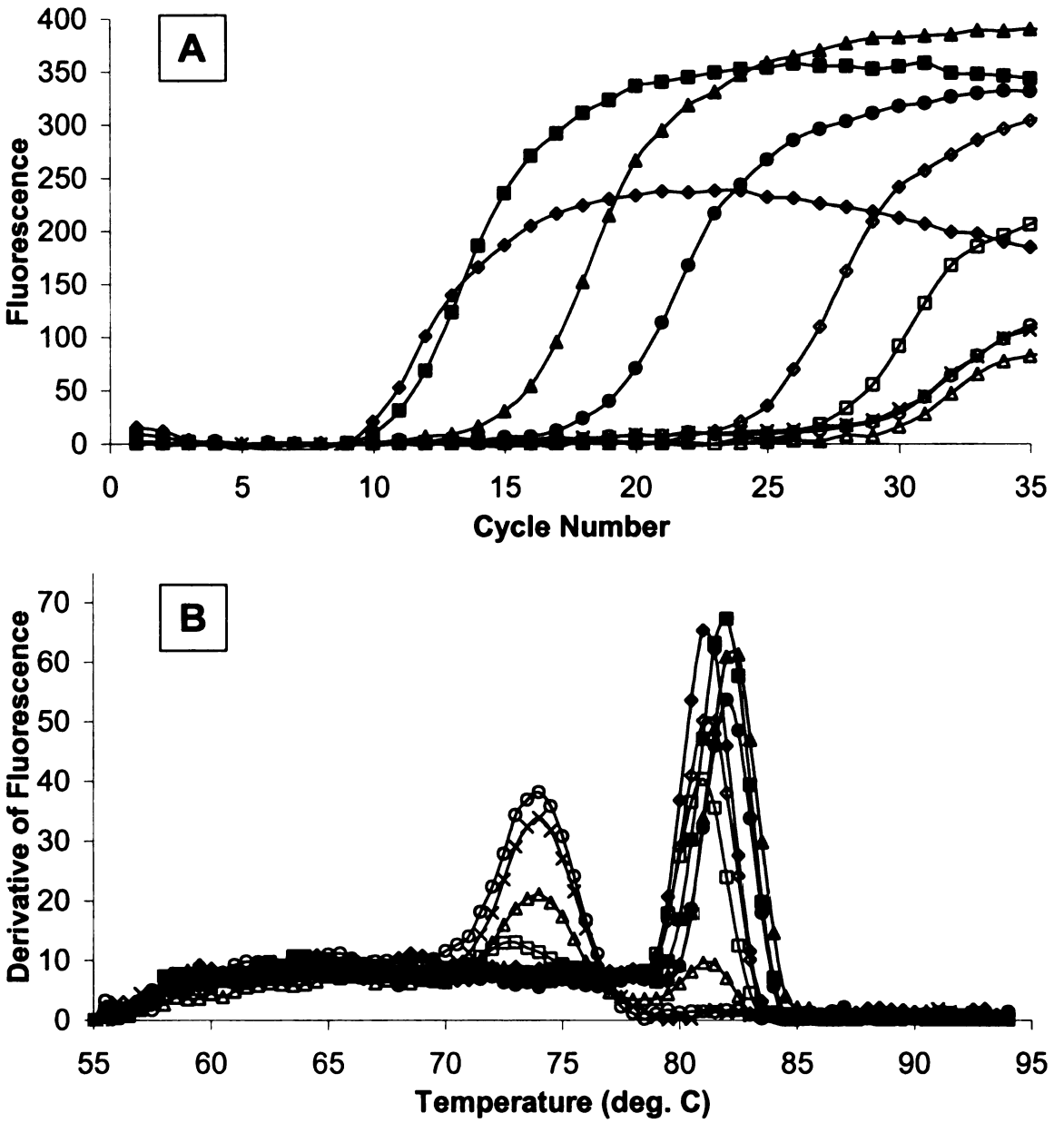


Figure 7. Lower detection limit of the real-time RT-PCR assay for FCV. Serial log dilutions of virus were made starting from a stock with a titer of 5.2 x 10^8 $TCID_{50}$/ml (◆) progressing through 10^{-1} (■), 10^{-2} (▲), 10^{-3} (●), 10^{-4} (◇), 10^{-5} (□), 10^{-6} (△), 10^{-7} (○), and 10^{-8} (X) dilutions. All samples were amplified in triplicate (**A**) A graph of fluorescence vs. cycle number illustrating amplification of serial FCV dilutions with the threshold cycle varying inversely with virus concentration. (**B**) The melting temperature curve illustrating the difference in melting temperature between specific FCV amplification products (~82C) and non-specific products (<76C). Specific FCV products were observed with amplification of the 10^{-6} dilution.

Assay sensitivity was determined in triplicate by extracting and amplifying viral RNA from serial 10-fold dilutions of a FCV respiratory disease strain (FCV-R; starting titer = 5.2×10^8 $TCID_{50}$/ml) isolated in the virology section of the Michigan State University Diagnostic Center for Population and Animal Health (MSU-DCPAH). Specific FCV amplification products from all triplicate samples from the 10^{-1} to 10^{-5} serial FCV-R dilutions were detected optically and visualized on agarose gels (Figure 7). One of three samples was positive for specific FCV amplification products at the 10^{-6} FCV-R dilution; none of the reactions at the 10^{-7} dilution gave a positive result. The threshold cycle (C_T) of each dilution with specific product varied inversely with virus concentration when plotted against the dilution factor ($R^2 = 0.974$; data not shown). The lower detection limit of the RT-PCR assay corresponds to a FCV-R titer of between 1.2×10^1 to 1.2×10^2 $TCID_{50}$/ml. Our results are similar to those of previous studies of p30 gene- and capsid protein gene-based assays which suggest that RT-PCR assays are comparable in sensitivity to conventional virus isolation (Sykes et al. 1998, Helps et al. 2002).

Assay linearity and efficiency were determined by amplifying serial 10-fold dilutions of FCV RNA in triplicate (Helps et al. 2002, Meijerink et al. 2001). The assay appeared linear over a dilution range of 6 logs, with a regression coefficient of 0.978 and a reaction efficiency of 95% (Figure 8). The RT-PCR reaction efficiency was higher than that reported for a recently described FCV p30 gene-based real-time RT-PCR assay (Helps et al. 2002). Increased reaction

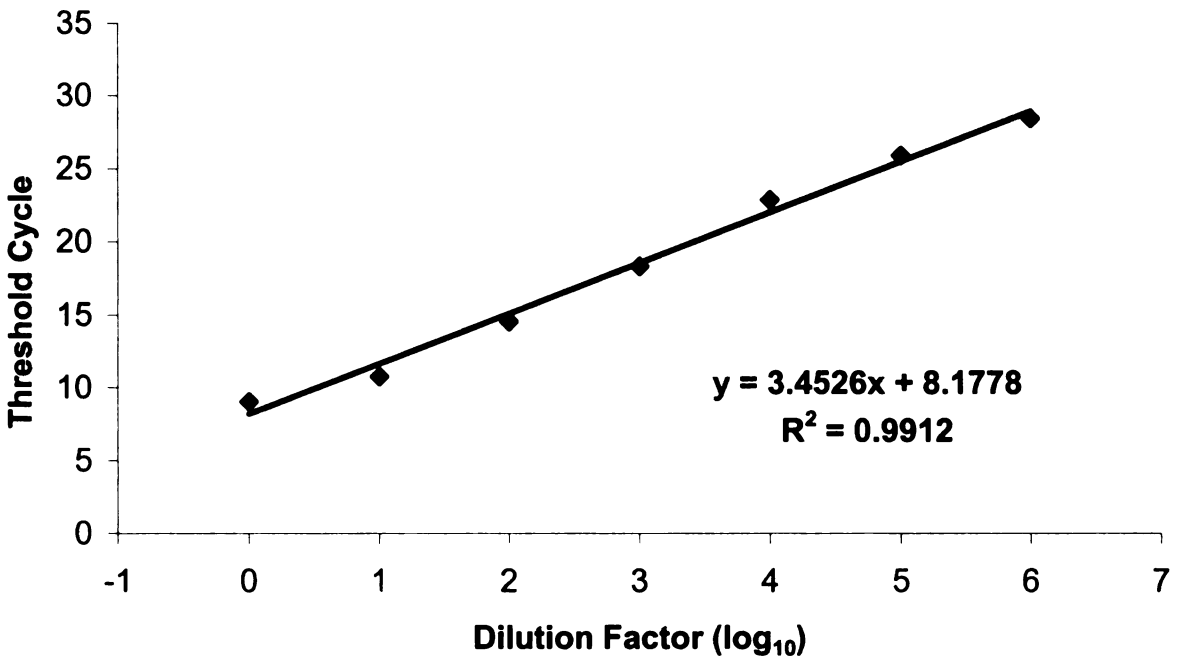


Figure 8. Linearity of the FCV p30 gene-based real-time RT-PCR. Serial 10-fold dilutions of FCV RNA were amplified in triplicate. The C_T was calculated for each reaction, plotted against the dilution factor (expressed as the $\log_{10}$ of the dilution), and evaluated by linear regression analysis.

efficiency lowers the C_T value, which may increase sensitivity in reactions with low template concentrations (Meijerink et al. 2001).

Assay specificity was verified by bidirectional automated fluorescent sequencing of 8 FCV amplification products. Tissues infected with canine calicivirus (genus *Vesivirus*), a rabbit calicivirus (genus *Lagovirus*), and a bovine calicivirus (genus *Norovirus*) obtained from the MSU-DCPAH were used as additional specificity controls. The diagnostic range of the assay was evaluated by duplicate extraction and amplification of RNA from 51 randomly selected wild-type FCV strains isolated between 1990 and 2002 in the virology section of MSU-DCPAH. The cDNA sequences of 8 FCV isolates had greater than 93% identity with one or more published FCV p30 gene sequences. When compared to consensus sequence, nucleotide substitutions were identified in 13 positions that were nearly identical to those described for 17 FCV isolates from the United Kingdom (Helps et al. 2002). However, deduced amino acid sequences were highly conserved with substitutions in only two positions (S for T in 4 isolates and A for T in 2 isolates in positions corresponding to amino acid residues 826 and 828 of the Urbana strain respectively). Specific FCV amplification products were detected optically and visualized on agarose gels for all 51 North American FCV isolates; specific amplification products for the canine, rabbit, or bovine calicivirus isolates were not detected. Amplicon melt temperatures of the FCV isolates ranged from 80.5C to 84C (mean 82.2C $\pm$ 0.77). Specific FCV amplification products were easily distinguished from nonspecific products by examination of

the RT-PCR product melt curves (Figure 7). The diagnostic range of our assay exceeds that of previously reported FCV RT-PCR gel-based assays (Sykes et al. 1998, Horimoto et al. 2001, Radford et al. 2001) and is similar to that of a recently described real-time RT-PCR assay in which p30 gene-specific primers amplified RNA from 60 FCV field isolates collected in the United Kingdom (Helps et al. 2002). In our study, we evaluated specimens obtained from cats residing in the upper Midwestern United States. Strains of FCV originating from the same geographic area tend to be genetically related (Rice et al. 2002). Genetic clustering of isolates by geographic region could result in over-estimation of the effective diagnostic range of the assay. However, our results, and those from the United Kingdom, suggest that p30 gene-based RT-PCR assays have a broader diagnostic utility compared to other FCV RT-PCR assays. Additional studies of FCV isolates obtained from diverse geographic locations and representing different disease phenotypes are indicated to further assess the diagnostic utility of FCV p30 gene-based RT-PCR assays.

Variation in FCV amplicon melting temperature amongst clinical isolates was an intriguing finding in this study. Similar variation in FCV p30 gene amplicon melting temperatures has been observed by others (Helps et al. 2002). The shape and position of the cDNA melting curve is dependent on amplicon GC content, length, and sequence (Ririe et al. 1997). Slight variation in p30 gene nucleotide sequences observed between FCV isolates observed by us and others could alter the binding energy of the amplified cDNA and most likely

accounts for the minor differences observed in specific FCV product melting temperatures.

In conclusion, the primers used in this study amplified a large number of North American FCV isolates and further confirm the diagnostic utility of p30 gene-based real-time RT-PCR for detection of FCV. The p30 gene-based real-time SYBR Green I® RT-PCR assay described in this study is considered to be a rapid, accurate, and affordable method of detection of FCV infections.

Footnotes

[a] Doyle SA, MD Sussman, JM Kruger, RK Maes. 1997. A reverse-transcriptase polymerase chain reaction assay for detection of feline caliciviruses. Abstract. *Journal of Veterinary Internal Medicine*; 11:148.

[b] Scansen BA, JM Kruger, PJ Venta, et al. 2002. Development of a one-step reverse-transcriptase poly-merase chain reaction assay for detection of feline calicivirus. Abstract. *Journal of Veterinary Internal Med*icine; 16:365.

[c] RNeasy Mini Kit®, Qiagen, Incorporated, Valencia, CA

[d] QuantiTect™ SYBR Green RT-PCR Kit, Qiagen, Incorporated, Valencia, CA

[e] iCycler™ iQ® System with detection software V2.3B, Bio-Rad Laboratories, Hercules, CA

COMPARISON OF RNA PREPARATION METHODS

Scansen BA, JM Kruger, AG Wise, PJ Venta, P Bartlett, RK Maes. Comparison of RNA preparation methods for detection of feline calicivirus in urine by RT-PCR. *Journal of Veterinary Internal Medicine*; submitted May 3rd, 2004.

Comparison of RNA Preparation Methods for Detection of Feline Calicivirus in Urine by RT-PCR

Brian A. Scansen, BS[1]

John M. Kruger DVM, PhD[1]

Annabel G. Wise DVM, PhD[2]

Patrick J. Venta PhD[1,2]

Paul Bartlett DVM, PhD, MPH[3]

Roger K. Maes DVM, PhD[2]

Key Words:

Running Title: Comparison of RNA Preparation Methods

From the Departments of Small Animal Clinical Sciences[1], Microbiology and Molecular Genetics[2], Michigan State University College of Veterinary Medicine, East Lansing MI. An abstract of this work was presented at the 22nd Annual Veterinary Medical Forum, Minneapolis MN, June 2004. This study was supported by the Michigan State University Companion Animal Fund and Center for Feline Health and Well-Being.

Reprint Requests: John M. Kruger DVM, PhD, Department of Small Animal Clinical Sciences, Room D208 Veterinary Clinical Center, Michigan State University, East Lansing, MI 48824-1314; (517) 355-6568; e-mail: kruger@cvm.msu.edu.

Abstract

Investigating the causative role of feline calicivirus (FCV) in idiopathic cystitis may be facilitated by PCR-based diagnostic methods for detection of FCV urinary tract infections. The purpose of this study was to compare methods of RNA preparation from feline urine for amplification with a FCV p30 gene-based real-time reverse-transcriptase PCR (RT-PCR) assay. Urine and blood were obtained from 6 specific-pathogen-free cats. Unaltered and centrifuged urine, and urine with added whole or hemolyzed blood, from each cat were spiked with FCV, and serially diluted in urine. FCV serially diluted in tissue culture medium served as positive controls. Viral RNA was prepared for RT-PCR by dilution and thermal inactivation (DT), polyethylene glycol precipitation (PEG), isolation with oligo$(dT)_{25}$-coated magnetic beads (dTMB), or extraction with two silica gel-based columns (RN and QA). All RT-PCR amplifications were performed in duplicate. Lower detection limit and mean RT-PCR threshold cycle (C_t) values for each RNA preparation method and for each sample type were compared with a mixed-effects model ANOVA. Because RNA prepared with DT yielded negative results, it was eliminated from final analyses. The lower detection limits (expressed as $TCID_{50}$/sample) for the assay in urine were 1,950 for PEG, 104 for dTMB, 11 for RN, and 7 for QA. Mean C_t values for RN and QA were similar and significantly lower than those for dTMB and PEG ($p<0.01$). Urine modifications did not affect performance when samples were prepared with dTMB, RN, or QA. In conclusion, results of the RT-PCR assay were significantly better for RNA isolated from feline urine with either RN or QA. (257)

Introduction

Feline caliciviruses (FCV) have been associated with a variety of clinical manifestations including, rhinitis, conjunctivitis, stomatitis, ulcerative glossititis, faucitis, pneumonia, enteritis, lameness, and abortion (Baulch-Brown et al. 1997, Sykes 2001). In addition, there is evidence supporting a potential causative role for FCV in the pathogenesis of feline idiopathic cystitis. Isolation of FCV from urine from cats with nonobstructive idiopathic cystitis, and observation of FCV-like particles in 38% of 96 urethral plugs obtained from male cats with obstructive idiopathic cystitis supported the concept of a viral etiology (Rich & Fabricant 1969, Kruger et al. 1996, Rice et al. 2002). However, large-scale epidemiologic studies and studies of experimentally induced FCV urinary tract disease have been hindered by lack of a sensitive and efficient means of detecting FCV urinary tract infections. Previous studies have relied upon recovery of live virus from urine using tissue culture-based virus isolation methods (Rich & Fabricant 1969, Kruger & Osborne 1990, Rice et al. 2002). Although virus isolation has been the "gold standard" for FCV detection, virus isolation is time consuming, expensive, and requires viable virus in the specimen and absence of substances that are toxic to cell culture or that inhibit viral replication (Sykes et al. 2001). Molecular diagnostic methods, such as reverse-transcriptase polymerase chain reaction (RT-PCR) circumvent many of the difficulties associated with conventional virus isolation methods and are increasingly being used for rapid detection of FCV (Sykes et al. 1998, Helps et al. 2002, Mackay et al. 2002). We recently reported development of a p30 gene-based real-time RT-PCR assay for detection of FCV

(Scansen et al. 2004). The p30 gene-based FCV RT-PCR assay was comparable to virus isolation in sensitivity and diagnostic range, and offered the advantages of reduced sample size, faster throughput, and better quantitation (Scansen et al. 2004).

Although RT-PCR assays are sensitive and rapid methods for virus detection, it is also recognized that that urine is a particularly difficult substrate for amplification of nucleic acids (Demmler et al. 1988, Kahn et al. 1991, Behzadbehbahani et al. 1997, Echavarria et al. 1998, Biel et al. 2000). Urinary substances may compromise RT-PCR assay performance most likely by interfering with enzymatic reverse-transcription of viral RNA or cDNA amplification by DNA polymerase. Consequently, preparation of nucleic acids becomes a critical step that not only serves to concentrate and purify nucleic acids, but also to remove or inactivate PCR inhibitors. Studies on human urine specimens indicate that the nucleic acid preparation method significantly influences the ability of PCR-based assays to detect viruses in urine and other complex biological specimens (Demmler et al. 1988, Kahn et al. 1991, Behzadbehbahani et al. 1997, Echavarria et al. 1998, Biel et al. 2000). Unfortunately, the optimal method of nucleic acid preparation varies, depending on the nature of the specimen, the type and quantity of inhibitory substances present in the sample, the physical, biochemical, molecular, and antigenic properties of the virus, and the susceptibility of individual PCR assay components to inhibition. Studies investigating amplification of cytomegalovirus

DNA from human urine indicated that the inhibitory effects of urine on PCR performance could be effectively removed by simple ultrafiltration (Kahn et al. 1991). However, results of pilot studies in our laboratory revealed that use of a similar ultrafiltration device[a] to remove urea and concentrate FCV in feline urine, resulted in concomitant concentration of unidentified substances that substantially inhibited the FCV RT-PCR (Rice et al. 2002). To our knowledge, studies comparing RNA isolation methods for their ability to remove or inactivate RT-PCR inhibitors, and preserve FCV RNA integrity in feline urine specimens have not been reported. Therefore, the purpose of this study to was to evaluate RNA preparation methods for their ability to recover FCV RNA from feline urine for real-time RT-PCR testing.

Materials and Methods

Collection and Preparation of Samples

Urine and whole blood were obtained from each of six 9-month-old specific-pathogen-free female cats[b]. All cats were negative for FCV neutralizing antibodies. Approximately 15 ml of urine was collected aseptically by cystocentesis from each cat. In addition, 5ml of acid citrate dextrose anticoagulated whole blood was collected from each cat by jugular venipuncture. A complete urinalysis was performed on each urine sample. If necessary, 0.75ml to 1.25ml of RNase-free water was added to the urine specimen to obtain a total volume 15.75ml of urine for each cat (Table 3). This volume of urine was required for preparation of serial dilutions of FCV in urine.

Table 3. Characteristics of urine specimens obtained from six 9-month-old female specific-pathogen-free cats used in the study. A small quantity of RNase-free water was added to each urine specimen from each cat to obtain the final volume of 15.75ml per cat required for analyses.

Variable	*Cat Identification Number*					
	1	**2**	**3**	**4**	**5**	**6**
Initial Urine Volume (ml)	15	15	14.75	14.75	15	14.5
Water Added (ml)	0.75	0.75	1	1	0.75	1.25
Final Urine Volume (ml)	15.75	15.75	15.75	15.75	15.75	15.75
USG Predilution	1.031	1.020	1.014	1.026	1.025	1.004
USG Postdilution	1.030	1.019	1.013	1.024	1.023	1.003
Urine pH[a]	7.5	7.0	7.0	7.5	7.5	7.0
Urine Occult Blood[a]	3+	Neg	3+	3+	3+	3+
Urine Protein[a]	1+	Trace	Trace	Trace	Trace	Trace
Urine RBC (per hpf)[b]	100-200	0-2	TNTC	1-4	8-10	2-5
Urine WBC (per hpf)[b]	Occ	0-2	0-5	0-2	1-3	1-3
Urine Epithelial Cells (per hpf)[b]	Occ	0-2	Occ	Occ	Occ	Occ
Urine Crystals (per hpf)[b]	Many MAP	Occ MAP	Neg	Occ MAP	Many MAP	Neg

Hpf = high power (400x) field; MAP = magnesium ammonium phosphate; Neg = negative; Occ = occasional; RBC = red blood cells; TNTC = too numerous to count; USG = urine specific gravity; WBC = white blood cells

a. Semiquantitatively estimated by reagent test strips.
b. Median number per high-power field (400x) from urine sediment.

Urine from each cat was divided into 4 aliquots of 3.75ml each (Figure 9). One urine aliquot was centrifuged at 300 x g for 15min at 4C. The cell-free urine supernatant was removed and used for subsequent analyses. The second urine aliquot was unaltered. To the third urine aliquot, 3.75µl of whole blood from the same cat was added to simulate gross hematuria (Osborne & Stevens 1999). To the fourth urine aliquot, 3.75µl of hemolyzed blood from the same cat was added

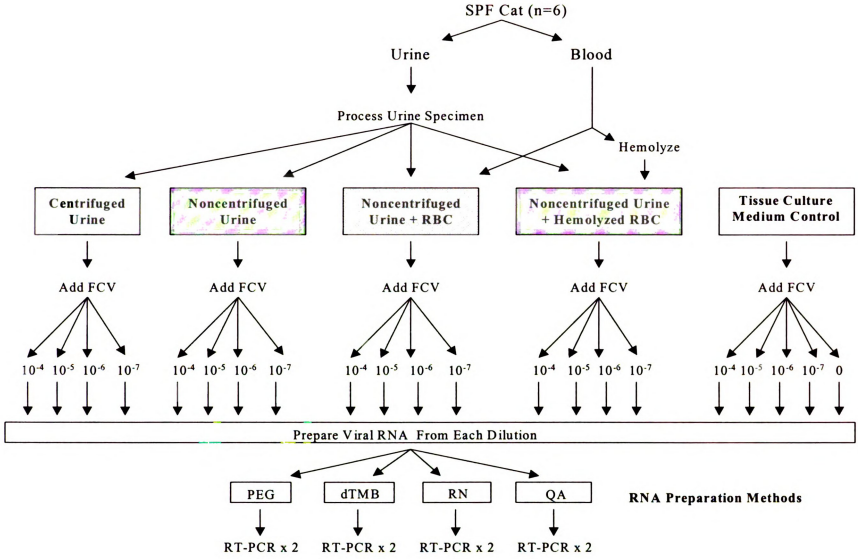
to simulate hemoglobinuria. Hemolyzed blood was prepared by freezing and thawing whole blood 4 times.

Serial 10-fold dilutions (10^{-1} to 10^{-7}) of a FCV respiratory disease strain (FCV-R; titer = 5.2×10^8 $TCID_{50}$/ml), isolated in the Virology Section of the Michigan State University Diagnostic Center for Population and Animal Health, were made in each urine specimen and in tissue culture medium[c] (positive control). Nucleic acids were isolated from each of the 10^{-4} through 10^{-7} dilutions of each urine and tissue culture medium sample using each of the RNA isolation methods described below (Figure 9). A sample of uninfected tissue culture medium served as a negative control for each RNA extraction.

RNA Isolation Methods

Based on studies in other species, 5 methods of preparing viral RNA from feline urine were selected for study. These methods included (1) dilution and thermal inactivation (DT), (Biel et al. 2000) (2) polyethylene glycol precipitation (PEG), (Behzadbehbahani et al. 1997) (3) a commercial mRNA extraction kit[d] incorporating oligo$(dT)_{25}$-coated magnetic beads (dTMB) to capture FCV polyadenylated RNA, (Chiodi et al. 1992, Dynal Handbook 2000) (4) a commercial silica gel-based extraction column method[e] (RN) designed for isolation of total RNA from highly cellular material, (RNeasy Handbook 2001) and (5) a commercial silica gel-based extraction column method[f] (QA) designed for

Figure 9. A schematic representation of study design. Urine and blood specimens were obtained from six 9-month-old female specific-pathogen-free cats. Unaltered urine, urine with added whole or hemolyzed blood, centrifuged urine supernatant, and tissue culture medium were spiked with FCV and serially diluted. Viral RNA was prepared from samples with each isolation method and amplified in duplicate with the FCV RT-PCR assay.


SPF Cat (n=6)
Urine
Blood
Process Urine Specimen
Hemolyze
Centrifuged Urine
Noncentrifuged Urine
Noncentrifuged Urine + RBC
Noncentrifuged Urine + Hemolyzed RBC
Tissue Culture Medium Control
Add FCV
Add FCV
Add FCV
Add FCV
Add FCV
10-4 10-5 10-6 10-7
10-4 10-5 10-6 10-7
10-4 10-5 10-6 10-7
10-4 10-5 10-6 10-7
10-4 10-5 10-6 10-7 0
Prepare Viral RNA From Each Dilution
PEG
dTMB
RN
QA
RNA Preparation Methods
RT-PCR x 2
RT-PCR x 2
RT-PCR x 2
RT-PCR x 2

isolation of viral nucleic acids from body fluids with low-cellularity (Echavarria et al. 1998, QIAamp Handbook 1999).

For DT, urine was diluted 1:10 in RNase-free water, incubated at 95C for 5 minutes, cooled, and used directly for RT-PCR (Behzadbehbahani et al. 1997, Biel et al. 2000). However, RNA prepared from higher dilutions of FCV in urine, but not in tissue culture medium, with DT yielded negative results with the RT-PCR assay (data not shown). Since samples prepared with PEG, dTMB, RN, and QA consistently yielded positive RT-PCR results in urine at higher dilutions, DT was not evaluated in subsequent studies.

Isolation of viral nucleic acids from urine using PEG has been described (Behzadbehbahani et al. 1997). Briefly, 150μl of sample was mixed with 50μl of 30% w/v polyethylene glycol[g] in 3M sodium chloride, incubated for 30 min on ice, and centrifuged at 10,000 x g for 15min. The supernatant was discarded and the pellet resuspended in 20μl of a 10mM Tris-HCl buffer (pH – 7.6) with 0.5% v/v nonionic detergent[h]. The suspension was then incubated for 10 min at room temperature and stored at –80C.

For dTMB, viral polyadenylated RNA was isolated from 100 μl of sample using oligo$(dT)_{25}$-coated magnetic beads[d] according to the manufacturer's instructions for viral poly A^+ RNA isolation (Dynabeads® mRNA DIRECT™ Kit Handbook 2000). Briefly, 100μl of urine sample was mixed with 300μl of lysis/binding buffer

(100mM Tris-HCl, 500mM LiCl, 10 mM EDTA, 1% LiDS, and 5 mM dithiothreitol) and 25μl of preconditioned magnetic beads, and incubated at room temperature for 10min. The beads were then washed twice with 500μl of buffer containing 10mM Tris-HCl, 0.15mM LiCl, 1mM EDTA, and 0.1% LiDS, and twice more with 500μl of buffer containing 10mM Tris-HCl, 0.15mM LiCl, and 1mM EDTA. After a final wash with 50μl of 10mM Tris-HCl, viral RNA was eluted from the magnetic beads by incubation in 25μl of 10mM Tris-HCl at 65C for 2min. Eluted RNA was then stored at –80C prior to use.

For RN, total RNA was isolated from urine samples according to the manufacturer's instructions for animal cells[e] (RNeasy Mini Handbook 2001). Briefly, 200μl of urine sample was mixed with 350μl of a proprietary lysis buffer containing guanidine thiocyanate and β-mercaptoethanol, and vortexed at room temperature for one minute. The lysate was then mixed with an equal volume of 70% ethanol, applied to the silica gel-based extraction spin column, centrifuged, washed once with a proprietary buffer containing guanidine thiocyanate and ethanol, and washed twice with a second proprietary buffer. Total RNA was then eluted in 50μl of RNase-free water and stored at –80C until use.

For QA, viral RNA was isolated from urine samples according to the manufacturer's instructions[f] (QIAamp Viral RNA Mini Kit Handbook 1999). Briefly, 140μl of sample was mixed with 560 μl of a proprietary lysis buffer containing guanidine thiocyanate and 10μg/ml of carrier RNA, and incubated at

room temperature for 10 minutes. The lysate was then mixed with 560 μl of 100% ethanol, applied to the silica gel-based extraction spin column, centrifuged, washed once with a proprietary buffer containing guanidine hydrochloride and ethanol, and washed once with a second proprietary buffer. Viral RNA was then eluted in 60μl of RNase-free water containing 0.04% sodium azide, and stored at –80C until use.

FCV RT-PCR Assay

Viral RNA from each sample was assayed in duplicate using a FCV p30 gene-based real-time RT-PCR protocol and a one-step RT-PCR system[i] (Scansen et al. 2004). Briefly, RT-PCR was performed in a 50μl reaction volume containing 25μl of 2x buffer (a proprietary buffer containing *Taq* DNA polymerase, SYBR® Green I, dNTPs, and 5.0 mM $MgCl_2$),[i] 0.5μl of mixed reverse transcriptases,[i] 0.5 μM of the forward primer 5'-TGGATGAACTACCCGCCA, 0.5 μM of the reverse primer 5'-GCACATCATATGCGGCTC, 5 μl of sample RNA, and RNase-free water. Real-time RT-PCR amplification was performed in a thermal cycler with an integrated real-time optical detection system.[j] Cycling conditions consisted of reverse transcription at 50C for 30 minutes and a preliminary denaturation step at 95C for 15 minutes, followed by 38 cycles of 94C for 30s, 53C for 30s, 72C for 60s, and 78C for 12s. These cycling conditions are a variation from those previously reported (Scansen et al. 2004) with the addition of a data acquisition step at 78C for 12s to reduce the fluorescent signal from non-specific product formation (Mackay et al. 2002). Amplification of cDNA was continuously

monitored in real-time by quantifying the amount of fluorescence emitted at 530nm at each data acquisition step (78C). Following a post-amplification step at 55C for one minute, amplicon melt temperatures were determined by raising the temperature in 0.5C increments from 55C to 95C. Post-PCR analysis was performed using iCycler detection software[j]. Samples giving peak fluorescence between 80.5C and 84.5C were determined to be FCV-specific product, while samples with peaks below 76C were determined to be the result of non-specific amplification. Samples with discordant RT-PCR results were assayed a third time and the majority result used in data analysis.

Data Analyses

The lower detection limit (expressed as $TCID_{50}$/sample) for a given RNA extraction method was defined as the highest sample dilution at which FCV RNA was amplified from 3 or more of the 6 samples. The final lower detection limit of the system was determined by factoring the volume of the initial sample and the proportion of the extracted RNA used in the amplification. In addition, the $\log_2$ of the mean RT-PCR detection threshold cycle (C_t) value for each RNA preparation method and for each urine specimen modification and positive control were determined at the 10^{-4} dilution. The lower detection limits and mean C_t values for each RNA preparation method for each urine specimen modification and positive control were compared with a mixed-effects model ANOVA, with cat as a random effect variable and 3 fixed-effect variables (urine specimen, RNA preparation method, and their two-way interaction) (SAS/STAT 1989).

Results

Urine Specimens

The volume of urine collected from each cat ranged from 14.5 to 15 ml (Table 3). Mean urine specific gravity prior to dilution was 1.020 ± 0.009. Dilution of urine specimens to a final volume of 15.75 ml resulted in a small decrease in urine specific gravity of approximately 0.001. All urine specimens were neutral to slightly alkaline in pH. Reagent test strips were positive for occult blood or hemoglobin in 5 of 6 (83%) urine specimens, and for mild proteinuria in one of 6 (17%) urine specimens. Urine sediment examination revealed microscopic hematuria in 3 of 6 (50%) specimens and struvite crystalluria in 4 of 6 (67%) specimens; pyuria and bacteriuria were not detected any sample.

Effect of RNA Isolation Method on RT-PCR Performance

The lower detection limit of the RT-PCR assay for urine specimens varied significantly by RNA isolation method (mixed-effects ANOVA $p<0.0001$). The detection limit of the RT-PCR assay was lower when RNA was prepared with QA (Table 4). However, the lower detection limits for QA, RN, and dTMB were not statistically different. In contrast, the detection limits for these 3 methods were significantly lower than that of PEG (Mixed-effects ANOVA, $p<0.01$). There was no significant interaction between sample type, isolation method, and the lower detection limit of the RT-PCR assay.

Table 4. Mean lower detection limits of the FCV RT-PCR assay using RNA prepared by four RNA isolation methods. Urine and blood specimens were obtained from six 9-month-old female specific-pathogen-free cats. Unaltered urine, urine with added whole or hemolyzed blood, centrifuged urine supernatant, and tissue culture medium were spiked with FCV and serially diluted. Viral RNA was isolated from samples with each of 4 preparation methods and amplified with the FCV p30 gene-based RT-PCR assay.

RNA Preparation Method	Mean Lower Detection Limit ($TCID_{50}$/sample)[a]				
	Centrifuged Urine Supernatant	Unaltered Urine	Urine + Whole Blood	Urine + Hemolyzed Blood	Tissue Culture Medium
PEG	1950	1950	1950	1950	195
dTMB	104	104	104	104	104
RN*	11	11	11	11	11
QA*	6	6	6	6	6

DTMB = oligo(dT)-coated magnetic beads (Dynabeads mRNA Direct Kit®) PEG = polyethylene glycol precipitation; QA = silica-gel-based extraction column (QIAamp Viral RNA Mini Kit®); RN = silica-gel-based extraction column (RNeasy Total RNA Mini Kit®); $TCID_{50}$- median tissue culture infectious dose.

a. Lower detection limit defined as the highest sample dilution at which FCV RNA was amplified from 3 or more of the 6 samples. The final lower detection limit of the system was determined by factoring the volume of the initial sample and the proportion of the extracted RNA used in the amplification.

Similarly, mean C_t values for the RT-PCR assay varied significantly by RNA isolation method (Mixed-effects ANOVA, $p<0.0001$). The C_t value corresponds to the PCR cycle number at which the fluorescence increases from a low background level to a detectable level, and reflects starting template concentration and amplification efficiency (Mackay et al. 2002). Lower C_t values imply higher starting template concentration, more efficient amplification, or both. Although QA resulted in the lowest mean C_t values over all sample types, there was no significant difference between mean C_t values for samples prepared with

QA or RN (Table 5). Amplification of RNA prepared with dTMB resulted in a significantly lower C_t value compared to PEG, but a significantly higher C_t value compared to QA and RN (mixed-effects ANOVA, $p<0.01$). In addition, analysis of mean C_t values revealed a significant interaction between isolation method, sample type, and mean C_t value (mixed-effects ANOVA, $p<0.0001$).

Effect of Urine Variables on RT-PCR Performance

There was no apparent association between the urine specific gravity of each individual cat's urine sample and the C_t values of their respective amplifications using RNA prepared with QA, RN, and dTMB (Figure 10). With the PEG method, however, C_t tended to increase with increasing USG (linear regression, R^2 = 0.56; Figure 10).

Although sample type did not significantly affect the lower detection limit, sample type significantly affected mean C_t values for the RT-PCR assay (mixed-effects ANOVA, $p<0.0001$). Compared to unmodified urine, modification of urine by centrifugation, or addition of whole or hemolyzed blood did not significantly influence mean C_t values for any of the RNA preparation methods (Table 5). When compared to tissue culture medium positive control preparations, there were no significant effects of urine or any urine modification on mean C_t values for QA, RN and dTMB (Table 5). However, the mean C_t value for the tissue culture medium positive control prepared with PEG was significantly lower than the mean C_t values for any urine specimen similarly prepared (mixed-effect

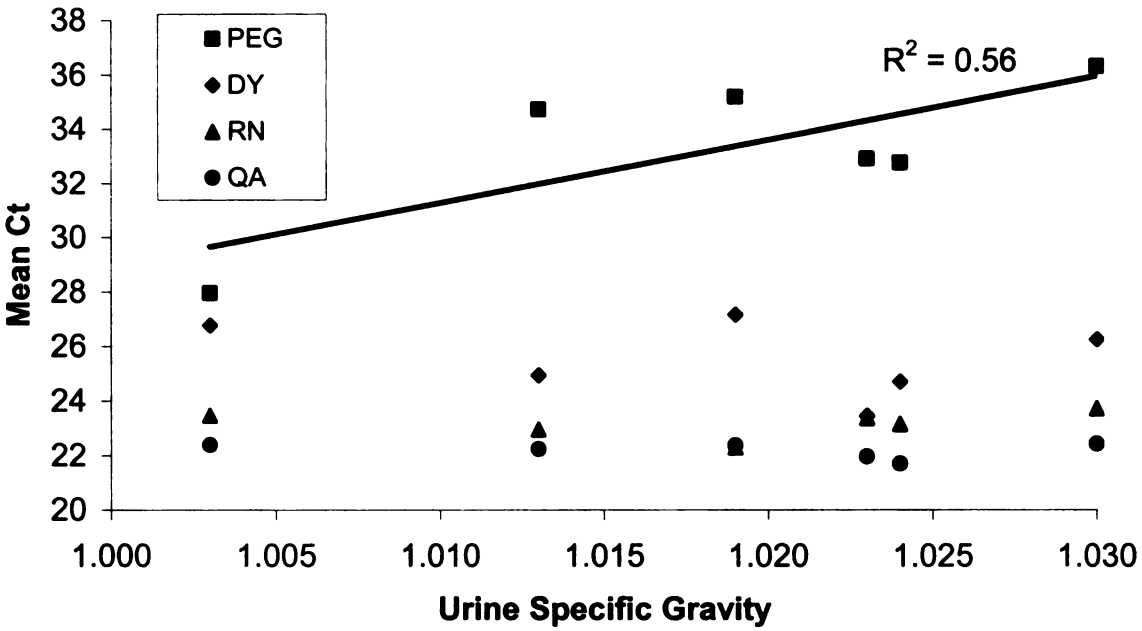


Figure 10. Effect of individual cat urine specific gravity on threshold cycle (C_t). The association between urine specific gravity and C_t was evaluated by linear regression analysis.

ANOVA, $p<0.01$; Table 5). The mean C_t values for the tissue culture control preparation prepared with PEG where 9.4 to 10.6 cycles lower than those for any urine specimen (Table 5). This increase in mean C_t value represents an approximately 1,024-fold decrease in assay performance when amplifying RNA prepared from urine with PEG compared to tissue culture medium positive controls prepared in a similar manner.

Table 5. Geometric mean detection threshold cycle (C_t) of the FCV RT-PCR assay using RNA prepared by four RNA isolation methods. Urine and blood specimens were obtained from six 9-month-old female specific-pathogen-free cats. Unaltered urine, urine with added whole or hemolyzed blood, centrifuged urine supernatant, and tissue culture medium were spiked with FCV and serially diluted. Viral RNA was prepared from samples with each isolation method and amplified in duplicate with the FCV RT-PCR assay.

RNA Preparation Method	Geometric Mean Detection Cycle Threshold (Ct)[a]				
	Centrifuged Urine Supernatant	Unaltered Urine	Urine + Whole Blood	Urine + Hemolyzed Blood	Tissue Culture Medium
PEG	34.8	34.7	35.7	35.9	25.3
dTMB	26.5	25.6	26.5	24.9	24.1
RN	23.3	23.7	22.6	23.1	23.2
QA	22.6	22.5	21.7	21.5	22.6

Ct = detection threshold cycle; dTMB = oligo(dT)-coated magnetic beads (Dynabeads mRNA Direct Kit®); PEG = polyethylene glycol precipitation; QA = silica-gel-based extraction column (QIAamp Viral RNA Mini Kit®); RN = silica-gel-based extraction column (RNeasy Total RNA Mini Kit®)

a. The geometric mean RT-PCR detection C_t value for each RNA preparation method and for each urine specimen modification and positive control were determined at the 10^{-4} dilution.

Discussion

Our results indicate that RNA preparation method significantly influences the ability of the RT-PCR assay to detect FCV in feline urine. Thermal inactivation and dilution of urine has proven effective for enhancing detection of polyomavirus, cytomegalovirus, and *Chlamydia* DNA by PCR from human urine specimens (Vinogradskaya et al. 1995, Mahony et al. 1998, Toye et al. 1998, Biel et al. 2000). In contrast, our initial studies indicated that DT was unable to alleviate the inhibitory effects of feline urine on RT-PCR performance. The inability of DT to enhance FCV detection may be due to species-related differences in urine composition or variations in the susceptibility of the assay to the effects of inhibitory substances. Interestingly, studies examining thermal inactivation for improved viral detection in fecal specimens suggest that the efficacy of thermal inactivation is species-dependent (Uwatoko et al. 1996). It is plausible that such a difference exists between feline urine and human urine samples as well.

Of the four remaining preparation methods, PEG was the most rapid, economical and technically least demanding. However, isolation of viral nucleic acids from urine with PEG resulted in the poorest RT-PCR assay performance. Diminished assay performance may be due to more limited removal or inhibition of urine RT-PCR inhibitors, lower RNA yield, or both. The relative decrease in RT-PCR performance for all PEG-prepared urine specimens, compared to PEG-prepared tissue culture medium specimens, suggests that inhibitory substances present in

feline urine were not effectively removed or inactivated by PEG. Our findings are in contrast to those of other studies, in which PEG appeared to be one of the methods of choice for preparation of urine for PCR-based detection of human cytomegalovirus and polyomavirus DNA (Yamaguchi et al. 1992, Vinogradskaya et al. 1995, Behazadbehbahani et al. 1997). The reason for this discrepancy is unknown. Although feline urine would be expected to contain similar inhibitory substances, it is probable that quantitative and qualitative differences in composition exist between feline and human urine. In addition, it is possible that the reverse transcriptase required in our assay, but not in those for cytomegalovirsus or polyomavirus, was inhibited by urine substances that were not inactivated or removed by PEG.

The poor RT-PCR performance associated with RNA prepared with PEG may also be due to decreased RNA yield. Although RNA yield was not quantified, the observation that assay performance was significantly decreased for tissue culture medium positive controls prepared with PEG compared to other preparation methods, suggests that PEG resulted in a lower RNA yield. It is likely therefore, that a combination of decreased RNA yield and failure to remove or inactivate all urine RT-PCR inhibitors were responsible for poor RT-PCR assay performance with PEG prepared specimens.

The oligo$(dT)_{25}$-coated magnetic bead method of RNA preparation relies on direct base pairing between the poly-adenylated tail of viral genomes and the

oligo dT sequences bound to the surface of magnetic beads (Kingsley & Richards 2001). This method has been successfully used for purification of human calicivirus (Norwalk virus), hepatitis A virus RNA from shellfish (Kingsley & Richards 2001), and HIV RNA from human cerebral spinal fluid specimens (Chiodi et al. 1992). Since the FCV genome is similarly composed of single stranded RNA with a poly-adenylated tail, it was logical to hypothesize that dTMB would be effective for isolation of FCV RNA from urine. Although the magnetic bead method significantly improved RT-PCR performance compared to PEG, isolation of FCV RNA from urine with dTMB was not as effective as either of the silica gel-based column methods. Since the RT-PCR assay performed equally well with RNA prepared from urine or tissue culture medium, the relative decrease in RT-PCR performance associated with samples prepared with dTMB compared to QA and RN, was most likely due to decreased RNA yield. It is possible that RNA yield with magnetic beads could be improved by using alternative nucleic acid capture strategies. Human calicivirus (Norwalk-like) RNA has been successfully prepared from environmental samples for RT-PCR by using magnetic beads coated with virus-specific antibodies (immunomagnetic-bead separation), or streptavidin-coated magnetic beads and biotinylated oligonucleotides (Loisy et al. 2000, Myremel et al. 2000). However, immunomagnetic-beads were not equally effective for isolating all antigenic types of Norwalk-like caliciviruses (Myremel et al. 2000). Antigenic variation among FCV isolates could similarly limit the use of immunomagnetic-beads for isolation of FCV from biological samples (Baulch-Brown et al. 1997). It may be of value to

investigate whether capture with specific biotinylated FCV oligonucleotides, or nonspecific biotinylated oliogo dT, and streptavidin-coated magnetic beads would enhance the performance of the FCV RT-PCR assay.

Preparation methods using silica-gel-based membranes rely on selective nucleic acid binding by silica. These methods have been used extensively for isolation of viral RNA or DNA from urine, feces, CSF, serum, and other complex biological samples (Biel et al. 2000, Echavarria et al. 1998). Since feline urine may contain variable numbers of cells, and since FCV may be intracellular, extracellular, or both, we evaluated the performance of two silica-gel-based membrane methods: RN, designed to isolate total RNA from highly cellular preparations, and QA, designed to maximize recovery of viral RNA from cell-free fluids. Both methods use chaotropic salts to lyse cells and virus particles, and to inactivate RNases prior to membrane binding. However, QA incorporates carrier RNA in the lysis step to improve viral RNA binding and to competitively limit viral RNA degradation due to residual RNase activity. Performance of the RT-PCR was significantly better using samples prepared with QA and RN than with samples prepared with PEG and dTMB. Improved RT-PCR performance associated with QA and RN was most likely due to a combination of higher RNA yield per extracted volume of sample and more effective removal or antagonism of urine RT-PCR inhibitors. Further studies are needed to determine whether RN and QA are equally suitable for preparing viral RNA under all extremes of sample conditions encountered in cats with or without urinary tract disorders.

To the extent that we attempted to modify urine by removal of urine sediment, or addition of whole or hemolyzed blood, we were unable to detect significant differences between dTMB, QA, and RN on RT-PCR performance. Several substances have been shown to inhibit PCR and RT reactions including urea (Kahn et al.1991), red blood cells (Panaccio & Lew 1991), heme compounds, (Byrnes et al. 1975, Tsutsui & Mueller 1987, Levere et al. 1991, Klein et al. 1997, Morata et al. 1998, Al-Soud & Rådström 2001) leukocytes (Morata et al. 1998, Al-Soud & Rådström 2001), immunoglobin G (Al-Soud et al. 2000) and crystalluria (Mahony et al. 1998). Some or all of these inhibiting substances may be present in urine specimens obtained from normal cats or cats with urinary tract disorders. Although there were no apparent sample effects in this study, our results should be interpreted within the context of a limited sample size. A type II statistical error (i.e. failure to reject the null hypothesis) can occur when the sample size is inadequate to detect small, but clinically meaningful, effects. Likewise, small sample size in the present study precluded analysis of cat-specific variables (e.g. urine specific gravity, proteinuria, crystalluria) that may affect RNA isolation and RT-PCR performance. The influence of specific feline urine components on RNA preparation and FCV RT-PCR assay performance requires further investigations.

In conclusion, there are notable differences between RNA isolation methods for recovery of FCV nucleic acids from feline urine. The FCV p30 gene-based RT-PCR assay performed significantly better when using RNA isolated from feline

urine with either of two silica gel-based extraction column methods. Our results underscore the need for species-specific studies to determine the optimal method of nucleic acid preparation from a particular clinical sample for a particular assay system. A RT-PCR assay system optimized for detection of FCV in feline urine may be useful for large-scale epidemiologic and experimental studies of FCV-induced urinary tract diseases.

Footnotes

a – Ultrafree-CL ultrafiltration device, Millipore Corporation, Bedford, MA

b – Harlan Bioproducts, Indianapolis, IN

c – Eagle's minimum essential medium, Gibco BRL, Grand Island, NY

d – Dynabeads mRNA DIRECT Micro Kit®, Dynal Inc., Lake Success, NY

e – RNeasy Mini Kit®, Qiagen, Incorporated, Valencia, CA

f – QIAamp Viral RNA Mini Kit®, Qiagen, Incorporated, Valencia, CA

g – Polyethylene glycol, 8,000 average molecular weight, Sigma-Aldrich, St. Louis, MO

h – Igepal CA-630, Sigma-Aldrich, St. Louis, MO

i – QuantiTect™ SYBR Green RT-PCR Kit, Qiagen, Incorporated, Valencia, CA

j – iCycler™ iQ® System with detection software V2.3B, Bio-Rad Laboratories, Hercules, CA

APPENDICES

APPENDIX A

Effects of Storage Temperature on Detection of FCV in Urine and Tissue Culture Medium by Virus Isolation and RT-PCR.

Material and Methods - Feline calicivirus (FCV; strain FVC-R, starting titer 2.4 x 10^8 $TCID_{50}$/ml) was serially diluted in pooled urine (urine specific gravity 1.057) obtained aseptically by cystocentesis from five 1.25 to 2 year-old female specific-pathogen-free cats, or in tissue culture medium. One aliquot of each 10^{-4} to 10^{-9} dilution was stored at 4C for 24 hours prior to assay. One additional aliquot of each dilution was stored at –70C for 5 days prior to assay. For virus isolation, 200 µl of each 10^{-4} to 10^{-9} dilution was added to 800 µl of pooled urine or tissue culture medium and concentrated approximately 10-fold by centrifugation in an ultrafiltration device (Ultrafree-CI ultrafiltration device, 10,000MW cutoff; Millipore Corp, Bedford MA). Material retained within the filter device was resuspended in 1 ml of tissue culture medium, gently sonicated, and inoculated into 2 tissue culture roller tubes as previously described (Rice et al. 2002). For the FCV RT-PCR assay, viral RNA from 140 µl of each 10^{-4} to 10^{-9} dilution was isolated using a silica-gel-based extraction column (QIAamp Viral RNA Mini Kit®; Qiagen Incorporated, Valencia CA), and amplified in triplicate using a 1-step real-time FCV RT-PCR assay previously described (Scansen et al. 2004). The lowest detection dilution for virus isolation was defined as the highest sample dilution at which FCV CPE was observed in one or more roller tubes. The lowest detection dilution for the RT-PCR was defined as the highest sample dilution at which FCV RNA was amplified from 2 or more of the 3 RT-PCR replicates. The final lower

detection limit of each system was determined by factoring the volume of the initial sample and the proportion of the extracted RNA used in the amplification.

Results- Virus isolation and RT-PCR had similar lower detection limits for detecting FCV in urine and tissue culture medium stored at 4C (Tables 6 and 7). Storage of FCV in tissue culture medium at –70C did not appear to substantially affect virus titer compared to samples stored at 4C (Table 7). In contrast, storage of FCV in urine at –70C resulted in a substantial (greater than 1000-fold) loss of virus titer as determined by virus isolation when compared to tissue culture medium controls (Table 7). Likewise, storage of FCV in urine at –70C resulted in a 10-fold loss of sensitivity of the RT-PCR assay compared to tissue culture controls.

Conclusions - Virus isolation and the RT-PCR assay were similar in their ability to detect FCV in urine and tissue culture medium stored for 24 hours at 4C. However, detection of FCV in urine specimens by virus isolation may be profoundly compromised by storage at –70C. Similarly, but to a lesser extent, detection of FCV in urine by RT-PCR also may be affected by storage of samples at –70C. These results indicate that proper sample handling is an essential prerequisite for detection of low concentrations of FCV in urine by virus isolation and RT-PCR.

Table 6. Number of positive results for virus isolation and RT-PCR for detection of FCV in urine or tissue culture medium stored at 4C or –70C.

Sample	Log of FCV Dilution (starting titer 2.4 x 10^8 $TCID_{50}$/ml)						
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Negative Control
Stored at 4C							
Virus Isolation							
Urine	2/2	2/2	2/2	1/2	neg	neg	neg
TCM	2/2	2/2	2/2	2/2	neg	neg	neg
RT-PCR							
Urine	3/3	3/3	3/3	neg	neg	neg	neg
TCM	3/3	3/3	3/3	1/3	neg	neg	neg
Storage at -70C							
Virus Isolation							
Urine	neg	neg	neg	neg	neg	neg	neg
TCM	2/2	2/2	2/2	1/2	neg	neg	neg
RT-PCR							
Urine	3/3	3/3	neg	neg	neg	neg	neg
TCM	3/3	3/3	3/3	neg	neg	neg	neg

FCV= feline calicivirus; RT-PCR= reverse-transcriptase polymerase chain reaction; TCM= tissue culture medium

Table 7. Final lower detection limits (expressed as $TCID_{50}$/sample) for detection of FCV in urine by virus isolation or RT-PCR after storage at 4C and –70C.

Sample	Lower Detection Dilution	Dilution FCV Conc. ($TCID_{50}$/ml)	Vol. Used for VI or Extraction (ml)	RT-PCR Dilution Factor	Final Detection Limit ($TCID_{50}$/sample)
Stored at 4C					
Virus Isolation					
Urine	1 x 10^{-7}	24	0.2	NA	5
TCM	1 x 10^{-7}	24	0.2	NA	5
RT-PCR					
Urine	1 x 10^{-6}	240	0.14	0.083	3
TCM	1 x 10^{-6}	240	0.14	0.083	3
Stored at –70C					
Virus Isolation					
Urine	>1 x 10^{-4}	>2.4 x 10^4	0.2	NA	>4,800
TCM	1 x 10^{-7}	24	0.2	NA	5
RT-PCR					
Urine	1 x 10^{-5}	2.4 x 10^3	0.14	0.083	28
TCM	1 x 10^{-6}	240	0.14	0.083	3

FCV= feline calicivirus; NA= not applicable; RT-PCR= reverse-transcriptase polymerase chain reaction; TCM= tissue culture medium; VI= virus isolation

APPENDIX B

Results of cDNA Sequencing of the p30 Gene of 8 FCV isolates

Table 8. Descriptions of FCV strains used for genotypic comparisons of cDNA and amino acid sequences of the FCV p30 gene.

Strain	Case No.[a]	Date	Location	Biotype	GenBank[b]	Ref
MSU 2.2	1459461	1994	USA	URTD	NA	Scansen 04
MSU 3	1467733	1994	USA	URTD	NA	Scansen 04
MSU 4	1524133	1995	USA	URTD	NA	Scansen 04
FCV-U1	1903867	1998	USA	ILUTD	NA	Rice 02
FCV-U2	2089726	1999	USA	ILUTD	NA	Rice 02
MSU 7	2611408	2002	USA	URTD	NA	Scansen 04
FCV-R	2351774	2000	USA	URTD	NA	Scansen 04
MSU 9	1027025	1990	USA	URTD	NA	Scansen 04
CFI/68	NA	1960	USA	URTD	U13992	Neill 90 Glen 99
F4	NA	1971	Japan	URTD	D31836	Oshikamo 94
F65	NA	1990	UK	OD-Arthrop	AF109465	Glenn 99
F9	NA	1958	USA	URTD	M86379	Carter 92
FCV2024	NA		Germany		AF479590	Thumfart 02
Urbana	NA	Late 1960s	USA	URTD-OD	L40021	Sosnovtsev 95

Arthrop= arthropathy; ILUTD= idiopathic lower urinary tract disease; NA= not applicable; NR= not reported; OD=oral disease; URTD= upper respiratory tract disease

a. Identification number assigned by the Michigan State University Diagnostic Center for Population and Animal Health.
b. GenBank Accession Number

Table 9. Results of bidirectional automated sequencing of a 90 base pair portion of the p30 (3A-Like) gene of ORF1 of 8 FCV isolates obtained from the Michigan State University Diagnostic Center for Population and Animal Health. The cDNA sequences correspond to nucleotides 2433 to 2522 and to amino acids 806 to 834 of the Urbana reference strain.

Isolate	cDNA Sequences	Deduced Amino Acid Sequences
MSU 2.2	at caa cat gtg gta acc gtt aac tcg gta ttt gat ttg gcc tgg gct ctt cgc cga cat ctg aca cta act ggg cag ttt caa gca atc a	QHVVTVNSVFDLAWALRRH LTLTGQFQAI
MSU 3	at cag cat gtg gta acc gtt aat tcg gtg ttt gat ttg gcc tgg gct ctt cgt cgt cac ctt aca ctg gca gga cag ttc caa gct atc a	QHVVTVNSVFDLAWALRRH LTLAGQFQAI
MSU 4	at caa cat gtg gta acc gtt aat tcg gtg ttt gat ttg gcc tgg gct ctt cgc cgc cac ctt tca cta act gga cag ttc caa gca atc a	QHVVTVNSVFDLAWALRRH LSLTGQFQAI
FCV-U1	at caa cat gtg gta acc gtt aac tcg gtg ttt gat ttg gcc tgg gct ctt cgc cgt cac cta tcg cta act gga cag ttt caa gca atc a	QHVVTVNSVFDLAWALRRH LSLTGQFQAI
FCV-U2	at caa cat gtg gta acc gtt aat tcg gtg ttt gat ttg gcc tgg gct ctt cgc cgc cac ctt acg ctg gca ggg cag ttt caa gcc atc a	QHVVTVNSVFDLAWALRRH LTLAGQFQAI
MSU 7	at caa cat gtg gta acc gtt aat tcg gtg ttt gat ttg gcc tgg gct ctt cgc cga cac ctc acg cta aca gga cag ttt caa gca atc a	QHVVTVNSVFDLAWALRRH LTLTGQFQAI
FCV-R	at caa cat gtg gta acc gtt aat tcg gtg ttt gat ttg gcc tgg gct ctt cgc cgt cac ctc tca cta act gga cag ttc caa gct atc a	QHVVTVNSVFDLAWALRRH LSLTGQFQAI
MSU 9	at caa cat gtg gta acc gtt aac tcg gtg ttt gat ttg gcc tgg gct ctt cgt cgc cac ctg tca cta act gga cag ttt caa gca atc a	QHVVTVNSVFDLAWALRRH LSLTGQFQAI

Table 10. Percent nucleotide identity between the FCV p30 gene cDNA sequences of 8 FCV isolates obtained from the Michigan State University Diagnostic Center for Population and Animal Health and 6 selected FCV reference strains (see Table 8 for isolate descriptions). Comparisons were made using a 90 base pair region of the FCV p30 gene corresponding to nucleotides 2433-2522 of the Urbana reference strain.

	Percent cDNA Identity													
Strain	MSU 2.2	MSU 3	MSU 4	FCV U1	FCV U2	MSU 7	FCV R	MSU 9	CFI 68	F4	F9	F65	FCV 2024	Urb
MSU 2.2														
MSU 3	85													
MSU 4	91	90												
FCV U1	91	86	95											
FCV U2	88	93	89	89										
MSU 7	93	90	93	95	94									
FCV R	90	91	97	95	87	93								
MSU 9	94	88	96	96	87	91	94							
CFI 68	89	90	93	89	87	90	93	91						
F4	88	84	89	90	88	93	99	87	87					
F9	87	91	96	94	91	95	96	94	86	83				
F65	93	91	96	94	91	95	96	94	94	94	88			
FCV 2024	87	88	87	84	93	90	86	85	85	85	87	89		
Urb	87	95	87	89	98	93	87	87	89	87	94	91	91	

URB= Urbana

Table 11. Percent amino acid identity between the FCV p30 protein sequences of 8 FCV isolates obtained from the Michigan State University Diagnostic Center for Population and Animal Health and 6 selected FCV reference strains (see Table 8 for isolate descriptions). Comparisons were made using a 29 amino acid region of the FCV p30 protein corresponding to amino acids 806-834 of the Urbana reference strain.

	Percent Amino Acid Identity													
Strain	MSU 2.2	MSU 3	MSU 4	FCV U1	FCV U2	MSU 7	FCV R	MSU 9	CFI 68	F4	F9	F65	FCV 2024	Urb
MSU 2.2														
MSU 3	97													
MSU 4	97	93												
FCV U1	97	93	100											
FCV U2	97	100	93	93										
MSU 7	100	97	97	97	97									
FCV R	97	93	100	100	93	97								
MSU 9	97	93	100	100	93	97	100							
CFI 68	97	93	93	93	93	97	93	93						
F4	93	90	90	90	90	93	90	90	90					
F9	93	97	90	90	97	93	90	90	90	93				
F65	100	97	97	97	97	100	97	97	97	93	93			
FCV 2024	90	93	86	86	93	90	86	86	86	83	90	90		
Urb	97	100	93	93	100	97	93	93	93	90	97	97	93	

Urb= Urbana

LIST OF REFERENCES

REFERENCES

Al-Soud WA, LJ Jönsson, P Rådström. 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *Journal of Clinical Microbiology*, 38(1):345-350.

Al-Soud WA and P Rådström. 2001. Purification and characterization of PCR-inhibitory components in blood cells. *Journal of Clinical Microbiology*, 39(2):485-493.

August JR. 1984. Feline viral respiratory disease: the carrier state, vaccination, and control. *Veterinary Clinics of North America: Small Animal Practice*; 14(6):1159-1171.

Baulch-Brown C, DN Love, J Meanger. 1997. Feline calicivirus: a need for a vaccine modification? *Australian Veterinary Journal*; 75:209-213.

Baulch-Brown C, D Love, J Meanger. 1999. Sequence variation within the capsid protein of Australian isolates of feline calicivirus. *Veterinary Microbiology*; 68:107-117.

Behzadbehbahani A, PE Klapper, PJ Vallely, GM Cleator. 1997. Detection of BK virus in urine by polymerase chain reaction: a comparison of DNA extraction methods. *Journal of Virological Methods*; 67:161-166.

Bidawid S, N Malik, O Adegbunrin, et al. 2003. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. *Journal of Virological Methods*; 107:163-167.

Biel SS, TK Held, O Landt, et al. 2000. Rapid quantification and differentiation of human polyomavirus DNA in undiluted urine from patients after bone marrow transplantation. *Journal of Clinical Microbiology*; 38(10):3689-3695.

Binns SH, S Dawson, AJ Speakman, et al. 2000. A study of feline upper respiratory tract disease with reference to prevalence and risk factors for infection with feline calicivirus and feline herpesvirus. *Journal of Feline Medicine and Surgery*; 2:123-133.

Bittle JL, CJ York, JW Newberne, M Martin. 1960. Serologic relationship of new feline cytopathogenic viruses. *American Journal of Veterinary Research*; 21:547-550.

Bittle JL and WJ Rubric. 1976. Immunisation against feline calicivirus infection. *American Journal of Veterinary Research*; 37:275-278.

Büchen-Osmond C. (Ed). 2003. 00.012. Caliciviridae. In: *ICTVdB - The Universal Virus Database*, version 3. ICTVdB Management, The Earth Institute, Biosphere 2 Center, Columbia University, Oracle, AZ, USA

Buffington CAT, DJ Chew, MS Kendall, et al. 1997. Clinical evaluation of cats with nonobstructive urinary tract diseases. *Journal of the American Veterinary Medical Association*; 210(1):46-50.

Bürki F. 1965. Picornaviruses of cats. *Archiv für die gesamte Virusforschung*; 15:690-696.

Byrnes JJ, KM Downey, L Esserman, AG So. 1975. Mechanism of hemin inhibition of erythroid cytoplasmic DNA polymerase. *Biochemistry*; 14(4):796-799.

Carter MJ, ID Milton, J Meanger, et al. 1992. The complete nucleotide sequence of a feline calicivirus. *Virology*; 190:443-448.

Castro AE. 1992. Isolation and identification of viruses. In: *Veterinary Diagnostic Virology: A Practitioner's Guide* (AE Castro & WP Heuschele, eds.). Mosby – Year Book, Inc., St. Louis, MO; 3-8.

CDC. 2004. CDC technical fact sheet about noroviruses. From, http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm.

Chang D, M Wang, WC Ou, et al. 1996. A simple method for detecting human polyomavirus DNA in urine by the polymerase chain reaction. *Journal of Virological Methods*; 58:131-136.

Chasey D and P Duff. 1990. European brown hare syndrome and associated virus particles in the UK. *Veterinary Record*; 126:623-624.

Chiba S, Y Sakuma, R Koasaka, et al. 1979. An outbreak of gastroenteritis associated with calicivirus in an infant home. *Journal of Medical Virology*; 4:249-254.

Chiba S, S Nakata, K Numata-Kinoshita, et al. 2000. Sapporo virus: History and recent findings. *Journal of Infectious Diseases*; 18(Suppl 2):S303-308.

Chiodi F, B Keys, J Albert, et al. 1992. Human immunodeficiency virus type-1 is present in the cerebrospinal fluid of a majority of infected individuals. *Journal of Clinical Microbiology*, 30(7):1768-1771.

Crandell RA and SH Madin. 1960. Experimental studies on a new feline virus. *American Journal of Veterinary Research*; 21:551-556.

Dawson S, D Bennett, SD Carter, et al. 1994. Acute arthritis of cats associated with feline calicivirus infection. *Research in Veterinary Science*; 56:133.

Demmler GJ, GJ Buffone, CM Schimbor, RA May. 1988. Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. *The Journal of Infectious Diseases*; 158(6):1177-1184.

Dick CP, RP Johnson, S Yamashiro. 1989. Sites of persistence of feline calicivirus. *Research in Veterinary Science*; 47(3): 367-373.

Doyle SA, MD Sussman, JM Kruger, RK Maes. 1996. A reverse-transcriptase polymerase chain reaction assay for detection of feline caliciviruses. Abstract. *Proceedings of the 7th Annual Phi Zeta Research Day*; East Lansing, MI.

Dynabeads® mRNA DIRECT™ Kit Handbook. 2000. Dynal Incorporated, Lake Success, New York; 10,14-21.

Echavarria M, M Forman, J Ticehurst, et al. 1998. PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. *Journal of Clinical Microbiology*; 36(11):3323-3326.

Ellis TM. 1981. Jaundice in a Siamese cat with in utero feline calicivirus infection. *Australian Veterinary Journal*; 57:383-385.

Fabricant CG. 1973. Urolithiasis: a review with recent viral studies. *Feline Practice*; 3:22-30.

Fabricant CG. 1977. Herpesvirus-induced urolithiasis in specific-pathogen-free male cats. *American Journal of Veterinary Research*; 38:1837-1842.

Fabricant CG. 1984. The feline urologic syndrome induced by infection with a cell-associated herpesvirus. *Veterinary Clinics of North America: Small Animal Practice*; 14(3):493-502.

Fastier, LB. 1957. A new feline virus isolated in tissue culture. *American Journal of Veterinary Research*; 18:382-389.

Fenner FJ, EPJ Gibbs, FA Murphy, et al. 1993. *Caliciviridae*. In: *Veterinary Virology*, 2nd Edition. Academic Press, San Diego, California; 425-430.

Forbes BA & KE Hicks. 1996. Substances interfering with direct detection of *Mycobacterium tuberculosis* in clinical specimens by PCR: effects of bovine serum albumin. *Journal of Clinical Microbiology*; 34(9):2125-2128.

Gaskell R and S Dawson. 1998. Feline respiratory disease. In: *Infectious Diseases of the Dog and Cat* (CE Greene, ed.), 2nd edition. W.B. Saunders; Philadelphia, PA; 97-106.

Geissler K, K Schneider, G Platzer, et al. 1997. Genetic and antigenic heterogeneity among feline calicivirus isolates from distinct disease manifestations. *Virus Research*; 48:193-206.

Geissler K, K Schneider, A Fleuchaus, et al. 1999. Feline calicivirus capsid protein expression and capsid assembly in cultured feline cells. *Journal of Virology*; 73(1):834-838.

Gillespie JH, AB Judkins, DE Kahn. 1971. Feline viruses XIII. The use of immunofluorescent test for the detection of feline picornaviruses. *The Cornell Veterinarian*; 61:172.

Gillespie JH and FW Scott. 1973. Feline viral infections. *Advances in Veterinary Science and Comparative Medicine*; 17:163-200.

Glenn M, AD Radford, PC Turner, et al. 1999. Nucleotide sequence of UK and Australian isolates of feline calicivirus (FCV) and phylogenetic analysis of FCVs. *Veterinary Microbiology*; 67:175-193.

Green KY, T Ano, MS Balayan, et al. 2000. Taxonomy of the caliciviruses. *Journal of Infectious Diseases*; 181(Suppl 2):S322-330.

Green KY, A Mory, MH Fogg, et al. 2002. Isolation of enzymatically active replication complexes from feline calicivirus-infected cells. *Journal of Virology*; 76:8582-8595.

Greenberg HB, JR Valdesuso, AR Kalica, et al. 1981. Proteins of Norwalk virus. *Journal of Virology*; 37:994-999.

Hale AD, J Green, DWG Brown. 1996. Comparison of four RNA extraction methods for the detection of small round structured viruses in faecal specimens. *Journal of Virological Methods*; 57:195-201.

Harbour DA, PE Howard, RM Gaskell. 1991. Isolation of feline calicivirus and feline herpesvirus from domestic cats 1980 to 1989. *Veterinary Record*; 128:77-80.

Helps C, P Lait, S Tasker, D Harbour. 2002. Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR. *Journal of Virological Methods*; 106:241-244.

Herbert TP, I Brierley, TD Brown. 1997. Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. *Journal of General Virology*; 78:1033-1040.

Horimoto T, Y Takeda, K Iwatsuki-Horimoto, et al. 2001. Capsid protein gene variation among feline calicivirus isolates. *Virus Genes*; 23:171-174.

Hurley KF, PA Pesavento, NC Pedersen, et al. 2004. An outbreak of virulent systemic feline calicivirus disease. *Journal of the American Veterinary Medical Association*; 224(2):241-249.

Jiang X, DY Graham, K Wang, et al. 1990. Norwalk virus genome: cloning and characterization. *Science*; 250:1580-1583.

Kahn DE and JH Gillespie. 1971. Feline viruses: pathogenesis of picornavirus infection in the cat. *American Journal of Veterinary Research*; 32:521-531.

Kahn DE and EA Hoover. 1976a. Infectious respiratory diseases of cats. *Veterinary Clinics of North America: Small Animal Practice*; 6(3):399-413.

Kahn DE and EA Hoover. 1976b. Feline caliciviral disease: experimental immunoprophylaxis. *American Journal of Veterinary Research*; 37:279-283.

Khan G, HO Kangro, PJ Coates, RB Heath. 1991. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. *Journal of Clinical Pathology*; 44:360-365.

Kalkstein TS, JM Kruger, CA Osborne. 1999a. Feline idiopathic lower urinary tract disease. Part I: clinical manifestations. *The Compendium on Continuing Education for the Practicing Veterinarian*; 21(1):15-26.

Kalkstein TS, JM Kruger, CA Osborne. 1999b. Feline idiopathic lower urinary tract disease. Part II: potential causes. *The Compendium on Continuing Education for the Practicing Veterinarian*; 21(2):148-154.

Kalunda M, KM Lee, DF Holmes, JH Gillespie. Serologic classification of feline caliciviruses by plaque-reduction neutralization and immunodiffusion. *American Journal of Veterinary Research*; 36:353-356.

Kapikian AZ, RG Wyatt, R Dolin, et al. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *Journal of Virology*; 10(5):1075-1081.

Kapikian AZ. 2000. The discovery of the 27-nm Norwalk virus: An historic perspective. *Journal of Infectious Diseases*; 18(Suppl 2):S295-302.

Kingsley DH and GP Richards. 2001. Rapid and efficient extraction method for reverse transcription-PCR detection of Hepatitis A and Norwalk-like viruses in shellfish. *Applied and Environmental Microbiology*, 67(9):4152-4157.

Kirk H. 1925. Urino-genital diseases. In: *The Diseases of the Cat*. Eger, Chicago, Illinois; 261-273.

Klein A, R Barsuk, S Dagan, et al. 1997. Comparison of methods for extraction of nucleic acid from hemolytic serum for PCR amplification of hepatitis B virus DNA sequences. *Journal of Clinical Microbiology*; 35(7):1897-1899.

Komolafe OO. 1976. Effect of storage on the integrity of purified feline calicivirus particles. *Microbios*; 26:137-146.

Kruger JM and CA Osborne. 1990. The role of viruses in feline lower urinary tract disease. *Journal of Veterinary Internal Medicine*; 4(2):71-78.

Kruger JM, CA Osborne, SM Goyal, et al. 1991. Clinical evaluation of cats with lower urinary tract disease. *Journal of the American Veterinary Medical Association*; 199(2):211-216.

Kruger JM, CA Osborne, PJ Venta, MD Sussman. 1996. Viral infections of the feline urinary tract. *Veterinary Clinics of North America: Small Animal Practice*; 26(2):281-296.

Kruger JM and SA Doyle. 1997. Unpublished observations.

Kruger JM, BA Scansen, RK Maes. 2002. A pilot study of feline calicivirus-induced urinary tract infection in SPF cats. Abstract. In *Proceedings: 12th Annual Phi Zeta Research Symposium*, East Lansing, MI. p. 31.

Lanciotti RS, AJ Kerst, RS Nasci, et al. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *Journal of Clinical Microbiology*; 38:4066-4071.

Leutenegger CM, D Klein, R Hofmann-Lehmann, et al. 1999. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. *Journal of Virological Methods*; 78:105-116.

Levere RD, Y Gong, A Kappas, et al. 1991. Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. *Proceedings of the National Academy of Sciences*; 88:1756-1759.

Levy JK, A Marsh. 1992. Isolation of calicivirus from the joint of a kitten with arthritis. *Journal of the American Veterinary Medical Association*; 201(5):753-755.

Liu BL, PR Lambden, H Günther, et al. 1999. Molecular characterization of a bovine enteric calicivirus: relationship to Norwalk-like viruses. *Journal of Virology*; 73(1):819-825.

Livak KJ, SJ Flood, J Marmaro, et al. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications*; 4:357-362.

Loisy F, P Le Cann, M Pommepuy, F Le Guyader. 2000. An improved method for the detection of Norwalk-like caliciviruses in environmental samples. *Letters in Applied Microbiology*; 31:411-415.

Mackay IM, KE Arden, A Nitsche. 2002. Real-time PCR in virology. *Nucleic Acids Research*; 30:1292-1305.

Madeley CR and BP Cosgrove. 1976. Caliciviruses in man. *Lancet*; 1:199-200.

Mahony J, S Chong, D Jang, et al. 1998. Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity. *Journal of Clinical Microbiology*; 36(11):3122-3126.

Meijerink J, C Mandigers, L van de Locht, et al. 2001. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. *Journal of Molecular Diagnostics*; 3:55-61.

Morata P, MI Queipo-Ortuño, J de Dios Colmenero. 1998. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. *Journal of Clinical Microbiology*; 26(9):2443-2446.

Morrison TB, JJ Weis, CT Wittwer. 1998. Quantification of low-copy transcripts by continuous SYBR® Green I monitoring during amplification. *BioTechniques*; 24:954-962.

Murphy FA, EPJ Gibbs, MC Horzinek, et al. 1999. *Caliciviridae*. In: *Veterinary Virology*, 3rd Edition. Academic Press, San Diego, California; 533-541.

Myremel M, E Rimstad, Y Wasteson. 2000. Immunomagnetic separation of a Norwalk-like virus (genogroup 1) in artificially contaminated environmental water samples. *International Journal of Food Microbiology*; 62:17-26.

Nakata S, S Chiba, H Terashima, et al. 1985. Humoral immunity in infants with gastroenteritis caused by human calicivirus. *Journal of Infectious Diseases*; 152(2):274-279.

Neill JD and WL Mengeling. 1988. Further characterization of the virus-specific RNAs in feline calicivirus infected cells. *Virus Research*; 11:59-72.

Neill JD. 1990. Nucleotide sequence of a region of the feline calicivirus genome which encodes picornavirus-like RNA-dependent RNA polymerase, cysteine protease and 2C polypeptides. *Virus Research*; 17:145-160.

Neill JD. 1994. GenBank Accession No. U13392.

Numata K, ME Hardy, S Nakata, et al. 1997. Molecular characterization of morphologically typical human calicivirus Sapporo. *Archives of Virology*; 142:1537-1552.

Ohlinger VF, B Haas, G Meyers, et al. 1990. Identification and characterization of the virus causing rabbit hemorrhagic disease. *Journal of Virology*; 64(7):3331-3336.

Osbaldiston GW and RA Taussig. 1970. Clinical report on 46 cases of feline urological syndrome. *Veterinary Medicine, Small Animal Clinician*; 65:461-468.

Osborne CA, JM Kruger, JP Lulich, et al. 1997. Feline lower urinary tract disease: The Minnesota experience. *Proceedings of the 15th ACVIM Forum*; 338-339.

Osborne CA and JB Stevens. 1999. Biochemical analysis of urine: indications, methods, interpretation. In: *Urinalysis: A Clinical Guide to Compassionate Patient Care*. Bayer Corporation, Shawnee Mission, KS; 106-109.

Osborne CA, JM Kruger, JP Lulich, et al. 2000. Feline lower urinary tract diseases. In: *Textbook of Veterinary Internal Medicine* (SJ Ettinger & ED Feldman, eds.), 5th Edition. W.B. Saunders, Philadelphia, PA; 1710-1747.

Oshikamo R, Y Tohya, Y Kawaguchi, et al. 1994. The molecular cloning and sequence of an open reading frame encoding for non-structural proteins of feline calicivirus F4 strain isolated in Japan. *Journal of Veterinary Medical Science*; 56(6):1093-1099.

Panaccio M and A Lew. 1991. PCR based diagnosis in the presence of 8% (v/v) blood. *Nucleic Acids Research*; 19(5):1151.

Parra F and M Prieto. 1990. Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits. *Journal of Virology*; 64(8):4013-4015.

Pedersen NC, L Laliberte, S Ekman. 1983. A transient febrile "limping" syndrome of kittens caused by two different strains of feline calicivirus. *Feline Practice*; 13(1):26-35.

Pedersen NC and KF Hawkins. 1995. Mechanisms for persistence of acute and chronic feline calicivirus infections in the face of vaccination. *Veterinary Microbiology*; 47:141-156.

Pedersen NC, JB Elliott, A Glasgow, et al. 2000. An isolated epizootic of hemorrhagic-like fever in cats caused by a novel and highly virulent strain of feline calicivirus. *Veterinary Microbiology*; 73:281-300.

Povey RC, RC Wardley, H Jessen. 1973. Feline picornavirus infection: the in vivo carrier state. *The Veterinary Record*; 92:224-229.

Prasad BV, DO Matson, AW Smith. 1994. Three-dimensional structure of calicivirus. *Journal of Molecular Biology*; 240:256-264.

QIAamp Viral RNA Mini Kit Handbook. 1999. Qiagen Incorporated, Valencia, California; 18-19,25.

Radford AD, M Bennett, F McArdle, et al. 1997. The use of sequence analysis of a feline calicivirus (FCV) hypervariable region in the epidemiological investigation of FCV related disease and vaccine failures. *Vaccine*; 15(2):1451-1458.

Radford AD, PC Turner, M Bennett, et al. 1998. Quasispecies evolution of a hypervariable region of the feline calicivirus capsid gene in cell culture and in persistently infected cats. *Journal of General Virology*; 79:1-10.

Radford AD, S Dawson, C Wharmby, et al. 2000. Comparison of serological and sequence-based methods for typing feline calicivirus isolates from vaccine failures. *Veterinary Record*; 146:117-123.

Radford AD, LM Sommerville, S Dawson, et al. 2001. Molecular analysis of isolates of feline calicivirus from a population of cats in a rescue shelter. *Veterinary Record*; 149:477-481.

Rice CC, JM Kruger, PJ Venta, et al. 2002. Genetic characterization of 2 novel feline caliciviruses isolated from cats with idiopathic lower urinary tract disease. *Journal of Veterinary Internal Medicine*; 16:293-302.

Rich LJ and CG Fabricant. 1969. Urethral obstruction in male cats: transmission studies. *Canadian Journal of Comparative Medicine*; 33:164-165.

Rich LJ, CG Fabricant, JH Gillespie. 1971. Virus induced urolithiasis in male cats. *The Cornell Veterinarian*; 61:542-553.

Ririe KM, RP Rasmussen, CT Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry*; 245:154-160.

RNeasy Mini Handbook, 3rd ed. 2001. Qiagen Incorporated, Valencia, California; 30-35.

SAS/STAT Users Guide: Statistics, Version 8, 4th ed. 1989. SAS Institute Incorporated, Cary, North Carolina.

Scansen BA, JM Kruger, PJ Venta, et al. 2002. Development of a one-step reverse-transcriptase polymerase chain reaction assay for detection of feline calicivirus. Abstract. *Journal of Veterinary Internal Medicine*; 16:365.

Scansen BA, AG Wise, JM Kruger, et al. 2004. Evaluation of a p30 gene-based real-time reverse transcriptase polymerase chain reaction assay for detection of feline caliciviruses. *Journal of Veterinary Internal Medicine*; 18:135-138.

Schorr-Evans EM, A Poland, WE Johnson, NC Pedersen. 2003. An epizootic of highly virulent feline calicivirus disease in a hospital setting in New England. *Journal of Feline Medicine and Surgery*; 5:217-226.

Seal BS, JF Ridpath, WL Mengeling. 1993. Analysis of feline calicivirus capsid protein genes: identification of variable antigenic determinant regions of the protein. *Journal of General Virology*; 74:2519-2524.

Seal BS. 1994. Analysis of capsid protein gene variation among divergent isolates of feline calicivirus. *Virus Research*; 33:39-53.

Smith AW, TG Akers, SH Madin, NA Vedros. 1973. San Miguel sea lion virus isolation, preliminary characterization and relationship to vesicular exanthema of swine virus. *Nature*; 244:108-110.

Sosnovtsev S and KY Green. 1995. RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. *Virology*; 210:383-390.

Sosnovtsev SV, SA Sosnovtseva, KY Green. 1998. Cleavage of the feline calicivirus capsid precursor is mediated by a virus-encoded proteinase. *Journal of Virology*; 72(4):3051-3059.

Sosnovtsev S, M Garfield, KY Green. 2002. Processing map and essential cleavage sites of the nonstructural polyprotein encoded by the ORF1 of the feline calicivirus. *Journal of Virology*; 76:7060-7072.

Studdert MJ. 1978. Caliciviruses: brief review. *Archives of Virology*; 58:157-191.

Sykes JE, VP Studdert, GF Browning. 1998. Detection and strain differentiation of feline calicivirus in conjunctival swabs by RT-PCR of the hypervariable region of the capsid protein gene. *Archives of Virology*; 143:1321-1334.

Sykes JE. 2001. Feline upper respiratory tract pathogens: herpesvirus-1 and calicivirus. *The Compendium on Continuing Education for the Practicing Veterinarian*; 23(2):166-175.

Sykes JE, JL Allen, VP Studdert, GF Browning. 2001. Detection of feline calicivirus, feline herpesvirus 1 and Chalmydia psittaci mucosal swabs by multiplex RT-PCR/PCR. *Veterinary Microbiology*; 81:95-108.

Takahashi E, S Konishi, M Ogata. 1971. Studies on cytopathogenic viruses from cats with respiratory infections. *Japanese Journal of Veterinary Science*; 33:81-87.

Tenorio AP, CE Franti, BR Madewell, NC Pedersen. 1991. Chronic oral infections of cats and their relationship to persistent oral carriage of feline calici-, immunodeficiency, or leukemia viruses. *Veterinary Immunology and Immunopathology*; 29:1-14.

Thumfart JO and G Meyers. 2002. Feline calicivirus: recovery of wild-type and recombinant viruses after transfection of cRNA or cDNA constructs. *Journal of Virology*; 76(12):6398-6407.

Tohya Y, N Yokoyama, K Maeda, et al. 1997. Mapping of antigenic sites involved in neutralization on the capsid protein of feline calicivirus. *Journal of General Virology*; 78:303-305.

Toye B, W Woods, M Bobrowska, K Ramotar. 1998. Inhibition of PCR in genital and urine specimens submitted for *Chlamydia trachomatis* testing. *Journal of Clinical Microbiology*; 36(8):2356-2358.

Traum J. 1936. Vesicular exanthema of swine. *Journal of the American Veterinary Medical Association*; 88:316-327.

Truyen U, K Geissler, J Hirschberger. 1999. Tissue distribution of virus replication in cats experimentally infected with distinct feline calicivirus isolates. *Berliner und Münchener tierärztliche Wochenschrift*; 112:355-358.

Tsutsui K and GC Mueller. 1987. Hemin inhibits virion-associated reverse transcriptase of murine leukemia virus. *Biochemical and Biophysical Research Communications*; 149(2):628-634.

Uwatoko K, M Sunairi, A Yamamoto, et al. 1996. Rapid and efficient method to eliminate substances inhibitory to the polymerase chain reaction from animal fecal samples. *Veterinary Microbiology*; 52:73-79.

van Vuuren M, K Geissler, D Gerber, et al. 1999. Characterisation of a potentially abortigenic strain of feline calicivirus isolated from a domestic cat. *Veterinary Record*; 144:636-683.

Verkooyen RP, A Luijendijk, WM Huisman, et al. 1996. Detection of PCR inhibitors in cervical specimens by using the AMPLICOR *Chlamydia trachomatis* assay. *Journal of Clinical Microbiology*; 34(12):3072-3074.

Vinogradskaya GR, IY Goryshin, YA Berlin, VA Lanzov. 1995. Optimization of PCR-based diagnostics for human cytomegalovirus. *Journal of Virological Methods*; 53:103-112.

Wawrzkiewicz J, CJ Smale, F Brown. 1968. Biochemical and biophysical characteristics of vesicular exanthema virus and the viral ribonucleic acid. *Archiv für die gesamte Virusforschung*; 25:337-351.

Weidbrauk DL, JC Werner, AM Drevon. 1995. Inhibition of PCR by aqueous and vitreous fluids. *Journal of Clinical Microbiology*; 33(10):2643-2646.

Wittwer CT, MG Herrmann, AA Moss, RP Rasmussen. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques*; 22:130-138.

Yamaguchi Y, T Hironaka, M Kajiwara, et al. 1992. Increased sensitivity for detection of human cytomegalovirus in urine by removal of inhibitors for the polymerase chain reaction. *Journal of Virological Methods*; 37:209-218.

Zwillenberg LO and F Bürki. 1966. On the capsid structure of some small feline and bovine RNA viruses. *Archiv für die gesamte Virusforschung*; 19:373-384.