In vitro comparison of RNA preparation methods for detection of feline calicivirus in urine of cats by use of a reverse transcriptase-polymerase chain reaction assay

Brian A. Scansen, DVM, MS; John M. Kruger, DVM, PhD; Annabel G. Wise, DVM, PhD; Patrick J. Venta, PhD; Paul C. Bartlett, DVM, PhD, MPH; Roger K. Maes, DVM, PhD

Objective—To compare 5 methods of preparation of RNA from feline urine samples for use in a feline calicivirus (FCV), p30 gene-based, real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assay.

Sample Population—Urine and blood samples from 6 specific-pathogen-free cats.

Procedures—Aliquots of each urine sample (unmodified, centrifuged, or mixed with whole or hemolyzed blood) were spiked with FCV and serially diluted in urine. Serial dilutions of FCV in tissue culture medium were used as positive controls. Viral RNA was prepared via dilution and thermal inactivation (DT method), polyethylene glycol precipitation (PEG method), isolation with oligo(dT)$_{25}$-coated magnetic beads (dTMB method), or extraction by use of 2 silica gel–based columns (RN or QA method). Lower detection limits and mean RT-PCR threshold cycle (C_t) values associated with each RNA preparation method and sample type were compared.

Results—Because DT-prepared samples yielded negative results via RT-PCR assay, this method was not evaluated. Lower detection limits ($TCID_{50}$/sample) for the assay in urine were 1,950, 104, 11, and 7 for PEG-, dTMB-, RN-, and QA-prepared samples, respectively. For RN and QA preparations, C_t values were similar and significantly lower than those for dTMB and PEG preparations. Overall, urine modifications did not affect FCV RNA detection in dTMB-, QA-, and RN-prepared samples.

Conclusions and Clinical Relevance—Of the methods evaluated, the RN and QA methods of RNA preparation were most appropriate for the FCV RT-PCR assay. An RT-PCR assay optimized for detection of FCV in feline urine may aid investigations of FCV-induced urinary tract diseases in cats. (*Am J Vet Res* 2005;66:915–920)

Feline caliciviruses (FCVs) have been associated with a variety of clinical conditions including rhinitis, conjunctivitis, stomatitis, ulcerative glossitis, faucitis, pneumonia, enteritis, lameness, and abortion.[1,2] In addition, there is evidence supporting a potential causative role for FCV in the pathogenesis of feline idiopathic cystitis. Isolation of FCV from urine of cats with nonobstructive idiopathic cystitis and detection 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.[3-5] However, isolation does not establish causation, and detection of FCV in urine of cats with idiopathic cystitis may represent coincidental virus shedding associated with subclinical FCV infections of the upper respiratory tract.[3,4] Unfortunately, large-scale epidemiologic studies and studies of experimentally induced, FCV-associated urinary tract disease have been hindered by a lack of sensitive and efficient means of detecting FCV infections of the urinary tract in cats. Previous studies[4-6] have involved recovery of live virus from urine samples by use of tissue culture–based virus isolation methods. Although virus isolation has been the gold standard for FCV detection, virus isolation is time-consuming and expensive; the procedure also requires the presence of viable virus in the specimen and absence of substances that are toxic to cell culture or that inhibit viral replication.[2] Molecular diagnostic methods, such as **reverse-transcriptase polymerase chain reaction (RT-PCR)** assays, circumvent many of the difficulties associated with conventional virus isolation methods and are increasingly being used for rapid detection of FCV.[7-9] Recently, we reported[10] development of a p30 gene-based, real-time RT-PCR assay for detection of FCV. The p30 gene-based FCV RT-PCR assay was comparable to virus isolation in sensitivity and diagnostic range and offered the advantages of use of a smaller sample size, faster throughput, and better quantitation.[10,a]

Although RT-PCR assays are sensitive and rapid methods for virus detection, it is also recognized that urine is a particularly difficult substrate for amplification of nucleic acids.[11-15] 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 not only for concentration and purification of nucleic acids but also for removal or inactivation of PCR inhibitors. Results of studies[11-15] involving human urine specimens indicate that the nucleic acid preparation method substantially influences the ability of PCR-based assays to detect viruses in urine and other

Received June 1, 2004.
Accepted July 30, 2004.
From the Departments of Small Animal Clinical Sciences (Scansen, Kruger, Venta), Microbiology and Molecular Genetics (Wise, Venta, Maes), and Large Animal Clinical Sciences (Bartlett), College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824. Dr. Scansen's present address is Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210.
Supported by the Michigan State University Companion Animal Fund and Center for Feline Health and Well-being.
Presented in abstract form at the 22nd Annual Veterinary Medical Forum, Minneapolis, June 2004.
Address correspondence to Dr. Kruger.

complex biological specimens. 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. Findings of a study[15] to investigate amplification of cytomegalovirus DNA from human urine samples indicated that the inhibitory effects of urine on PCR assay performance could be effectively removed by simple ultrafiltration. However, results of pilot studies in our laboratory have indicated that use of a similar ultrafiltration device to remove urea and concentrate FCV in urine specimens from cats resulted in concomitant concentration of unidentified substances that inhibited the FCV RT-PCR assay. To our knowledge, studies comparing the abilities of RNA isolation methods to remove or inactivate RT-PCR inhibitors and preserve FCV RNA integrity in feline urine specimens have not been reported. The purpose of this in vitro study was to compare 5 methods of preparation of RNA from urine samples obtained from cats for use in an FCV, p30 gene-based, real-time RT-PCR assay.

Materials and Methods

Collection and preparation of samples—Urine and whole blood for subsequent in vitro experiments were obtained from each of six 9-month-old specific-pathogen-free female cats.[b] Tests for FCV neutralizing antibodies yielded negative results for all cats. From each cat, approximately 15 mL of urine was collected aseptically via cystocentesis; in addition, 5 mL of blood was collected via jugular venipuncture and placed in tubes containing acid citrate dextrose anticoagulant. A complete urinalysis was performed on each urine sample. If necessary, 0.75 to 1.25 mL of RNAse-free water was added to the urine specimen to obtain a 15.75-mL volume of urine for each cat. This volume of urine was required for preparation of serial dilutions of FCV in urine.

The urine samples collected from the cats were each divided into four 3.75-mL aliquots. For each cat, 1 urine aliquot was centrifuged at 300 × *g* for 15 minutes at 4°C; the cell-free urine supernatant was removed and used for subsequent analyses. The second urine aliquot was not modified. To the third urine aliquot, 3.75 µL of whole blood from the same cat was added to simulate gross hematuria.[16] To the fourth urine aliquot, 3.75 µL of hemolyzed blood from the same cat was added to simulate hemoglobinuria. To prepare the hemolyzed blood, a specimen of whole blood was frozen and thawed 4 times.

Serial 10-fold dilutions (10^{-1} to 10^{-7}) of an FCV respiratory tract disease strain (FCV-R; titer, 5.2×10^8 $TCID_{50}$/mL) that had been isolated at 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 specimens). Nucleic acids were isolated from each of the 10^{-4} through 10^{-7} dilutions of each urine and tissue culture medium sample by use of 5 RNA isolation methods. A sample of noninfected urine and tissue culture medium was used as negative control specimens for each RNA extraction.

RNA isolation methods—On the basis of studies in other species, 5 methods of preparing viral RNA from feline urine were selected for investigation. These methods included dilution and thermal inactivation (DT method)[12]; polyethylene glycol precipitation (PEG method)[11]; use of a commercial mRNA extraction kit[d] incorporating oligo$(dT)_{25}$-coated magnetic beads (dTMB method) to capture FCV polyadenylated RNA[17,18]; use of a commercial silica gel–based extraction column[e] (RN method) designed for isolation of total RNA from highly cellular material[19]; and use of a commercial silica gel–based extraction column[f] (QA method) designed for isolation of viral nucleic acids from body fluids with low levels of cellularity.[14,20]

For the DT method, urine was diluted 1:10 in RNAse-free water, incubated at 95°C for 5 minutes, cooled, and used directly for the RT-PCR procedure.[11,12] However, RNA prepared from higher dilutions of FCV in urine, but not in tissue culture medium, via the DT method yielded negative results with the RT-PCR assay (data not shown). Because samples prepared by use of the PEG, dTMB, RN, and QA methods consistently yielded positive RT-PCR results in urine at higher dilutions, the DT method was not evaluated in subsequent experiments.

Isolation of viral nucleic acids from urine samples by use of the PEG method has been described.[11] Briefly, 150 µL of sample was mixed with 50 µL of polyethylene glycol[g] (30% wt/vol) in 3M NaCl, incubated for 30 minutes on ice, and centrifuged at 10,000 × *g* for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 20 µL of 10mM Tris-HCl buffer (pH, 7.6) with nonionic detergent[h] (0.5% vol/vol). The suspension was then incubated for 10 minutes at room temperature (approx 21°C) and stored at –80°C.

For the dTMB method, viral polyadenylated RNA was isolated from 100 µL of sample by use of oligo$(dT)_{25}$-coated magnetic beads,[d] according to the manufacturer's instructions for viral poly A^+ RNA isolation.[18] Briefly, 100 µL of a urine sample was mixed with 300 µL of lysis-binding buffer (100mM Tris-HCl, 500mM LiCl, 10mM EDTA, 1% LiDS, and 5mM dithiothreitol) and 25 µL of preconditioned magnetic beads and incubated at room temperature for 10 minutes. The beads were then washed twice with 500 µL of buffer containing 10mM Tris-HCl, 0.15mM LiCl, 1mM EDTA, and 0.1% LiDS and washed twice more with 500 µL of buffer containing 10mM Tris-HCl, 0.15mM LiCl, and 1mM EDTA. After a final wash with 50 µL of 10mM Tris-HCl, viral RNA was eluted from the magnetic beads by incubating them in 25 µL of 10mM Tris-HCl at 65°C for 2 minutes. Eluted RNA was then stored at –80°C prior to use.

For the RN method, total RNA was isolated from urine samples according to the manufacturer's instructions for use in animal cells.[19] Briefly, 200 µL of a urine sample was mixed with 350 µL of a proprietary lysis buffer containing guanidine thiocyanate and β-mercaptoethanol and vortexed at room temperature for 1 minute. The lysate was then mixed with an equal volume of 100% ethanol before it was 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 50 µL of RNAse-free water and stored at –80°C until use.

For the QA method, viral RNA was isolated from urine samples according to the manufacturer's instructions.[20] Briefly, 140 µL of a urine sample was mixed with 560 µL of a proprietary lysis buffer containing guanidine thiocyanate and carrier RNA (10 µg/mL) and incubated at room temperature for 10 minutes. The lysate was then mixed with 560 µL of 100% ethanol before it was 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 60 µL of RNAse-free water containing 0.04% sodium azide and stored at –80°C until use.

FCV RT-PCR assay—Viral RNA from each sample was assayed in duplicate by use of an FCV, p30 gene-based, real-time RT-PCR protocol and a 1-step RT-PCR system.[10,i] Briefly, the RT-PCR assay was performed in a 50-µL reaction volume containing 25 µL of 2X buffer (a proprietary buffer containing *Taq* DNA polymerase, SYBR Green I, dNTPs, and 5.0mM $MgCl_2$),[i] 0.5 µL of mixed RTs,[i] 0.5µM of the forward primer 5'-TGGATGAACTACCCGCCA, 0.5µM of the reverse primer 5'-GCACATCATATGCGGCTC, 5 µL of sample RNA, and RNAse-free water. Real-time RT-PCR amplification was performed on a thermal cycler with an integrated real-time optical detection system.[j] Cycling conditions consisted of reverse transcription at 50°C for 30 minutes and a preliminary denaturation step at 95°C for 15 minutes, followed by 38 cycles of 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 60 seconds, and 78°C for 12 seconds. These cycling conditions are a variation from those previously reported[10] with the addition of a data acquisition step at 78°C for 12 seconds to reduce the fluorescent signal from nonspecific product formation.[8] Amplification of cDNA was continuously monitored in real time by quantifying the amount of fluorescence emitted at 530 nm at each data acquisition step (78°C). After a post-amplification step at 55°C for 1 minute, amplicon melt temperatures were determined by raising the temperature in increments of 0.5°C from 55° to 95°C. Post-PCR analysis was performed by use of the thermal cycler detection software.[j] Samples associated with peak fluorescence readings between 80.5° and 84.5°C were determined to be an FCV-specific product, whereas samples with peak readings below 76°C were determined to be the result of nonspecific amplification.[10] Samples with discordant RT-PCR results were assayed a third time, and the result obtained on 2 of 3 occasions was used in data analyses.

Data analyses—The lower detection limit (expressed as the minimum number of $TCID_{50}$ equivalents/sample) for a given RNA extraction method was defined as the lowest virus concentration at which FCV RNA was amplified from 3 or more of the samples from each of the 6 cats. 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 sample was determined at the 10^{-4} dilution. The lower detection limit and mean C_t value for each RNA preparation method for each urine specimen modification and positive control sample 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 2-way interaction).[21] A value of $P < 0.05$ was considered significant.

Results

Urine specimens—Among the 6 cats, the volume of urine collected ranged from 14.5 to 15 mL. Mean ± SD urine specific gravity after dilution was 1.019 ± 0.01. All urine specimens were neutral to slightly alkaline. By use of reagent test strips, 5 of 6 urine specimens yielded positive results for occult blood or hemoglobin; mild proteinuria was detected in 1 of 6 urine specimens. Microscopically, urine sediment examination revealed hematuria in 3 of 6 urine specimens and struvite crystalluria in 4 of 6 urine specimens; pyuria and bacteriuria were not detected in any of the urine samples.

Effect of RNA isolation method on RT-PCR assay results—Mean lower detection limits (expressed as $TCID_{50}$/sample) for the assay in urine were 1,950 for the PEG method, 104 for the dTMB method, 11 for the RN method, and 7 for the QA method. Modification of urine did not affect the mean lower detection limit for any RNA preparation method. Mean lower detection limits for the assay in tissue culture medium were 950 for the PEG method, 104 for the dTMB method, 11 for the RN method, and 7 for the QA method. Although the RT-PCR assay had the lowest detection limit when RNA was prepared via the QA method, the lower detection limits for the QA, RN, and dTMB methods were not significantly different. In contrast, the lower detection limits for these 3 methods were significantly (mixed-effects ANOVA, $P < 0.01$) lower than that of the PEG method. In dilutions containing high concentrations of virus, there were no significant differences in RT-PCR assay performance among RNA preparation methods. Specific FCV amplification products were not detected for any of the negative control preparations. There were no significant 2-way interactions among the sample type, isolation method, and lower detection limit of the RT-PCR assay.

Mean C_t values for the RT-PCR assay varied significantly (mixed-effects ANOVA, $P < 0.001$) by the RNA isolation method. The C_t value corresponds to the PCR cycle number at which the fluorescence associated with a sample increases from a low background level to a detectable level and reflects starting template concentration and amplification efficiency.[8] Lower C_t values imply higher starting template concentration, more efficient amplification, or both. Although the QA method resulted in the lowest mean C_t values among all sample types, there was no significant difference in mean C_t values between samples prepared via the QA or RN methods (**Table 1**). Amplification of RNA prepared by use of the dTMB method resulted in a significantly (mixed-effects ANOVA, $P < 0.01$) lower C_t value, compared with that associated with the PEG method, but a significantly higher C_t value, compared with values associated with the QA and RN methods. In addition, analysis of mean C_t values revealed a significant (mixed-effects ANOVA, $P < 0.001$) interaction between the isolation method and sample type.

Effect of urine variables on RT-PCR assay performance—Although sample type did not significantly affect the lower detection limit, sample type significantly (mixed-effects ANOVA, $P < 0.001$) affected mean C_t values for the RT-PCR assay. The mean C_t value for the tissue culture medium positive control prepared by use of the PEG method was significantly (mixed-effect ANOVA, $P < 0.01$) lower than the mean C_t values for any urine specimen prepared similarly. The mean C_t values for the tissue culture control prepared by use of the PEG method were 9.4 to 10.6 cycles lower than those for any urine specimen (Table 1). This increase in mean C_t value represented an approximately 1,024-fold decrease in assay sensitivity when amplifying RNA prepared from urine via the PEG method, compared with the assay performance associated with analysis of tissue culture medium positive controls prepared in a similar manner. In contrast, there were no significant effects of urine or any urine modification on mean C_t values for the QA, RN, and

Table 1—Geometric mean detection threshold cycle (C_t) values of a feline calicivirus (FCV) reverse transcriptase-polymerase chain reaction (RT-PCR) assay for FCV RNA prepared via 4 RNA isolation methods from modified and unmodified samples of urine (collected from 6 specific-pathogen-free cats) and tissue culture medium samples that had been spiked with FCV and serially diluted.

	Geometric mean detection C_t*				
RNA preparation method†	Centrifuged urine supernatant	Unmodified urine	Urine with whole blood	Urine with 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

*The geometric mean RT-PCR detection C_t value for each RNA preparation method and for each urine specimen modification and tissue culture medium specimen (positive control specimens) were determined at the 10^{-4} dilution. †Viral RNA was prepared from samples via each isolation method and amplified in duplicate with the FCV RT-PCR assay.

PEG = Polyethylene glycol precipitation method. dTMB = Method involving the use of oligo$(dT)_{25}$-coated magnetic beads.[d] RN = Method involving the use of a silica gel–based extraction column[e] designed for isolation of total RNA from highly cellular material. QA = Method involving the use of a silica gel–based extraction column[f] designed for isolation of viral nucleic acids from body fluids with low levels of cellularity.

dTMB methods, compared with C_t values for the tissue culture medium positive control preparations. Compared with mean C_t values for 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.

Discussion

Our results indicated that the RNA preparation method significantly influences detection of FCV in feline urine via the RT-PCR assay. Thermal inactivation and dilution of urine have proven effective for enhancing detection of polyomavirus, cytomegalovirus, and *Chlamydia* DNA by use of a PCR assay from human urine specimens.[12,22-24] In contrast, our initial experiments indicated that the DT method could not counteract the inhibitory effects of feline urine on RT-PCR performance. The inability of the DT method of RNA preparation 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, results of an investigation[25]of the use of thermal inactivation for improved viral detection in fecal specimens have suggested that the efficacy of thermal inactivation is species-dependent. It is plausible that such a difference also exists between urine samples collected from cats and those collected from humans.

Of the 4 remaining preparation methods investigated in the present study, the PEG method was the most rapid, economical, and technically least demanding. However, isolation of viral nucleic acids from urine via the PEG method resulted in the poorest RT-PCR assay sensitivity. Diminished assay sensitivity may be a result of decreased removal or inactivation of urine RT-PCR inhibitors, lower RNA yield, or both. The relative decrease in RT-PCR performance for all PEG-prepared urine specimens, compared with 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[11,24,26] in which PEG appeared to be one of the methods of choice for preparation of urine samples for PCR-based detection of human cytomegalovirus and polyomavirus DNA. This discrepancy may be related to the fact that previous studies evaluating the PEG method did not incorporate commercial silica gel–based columns methods. Alternatively, species differences in PEG performance may be a consequence of quantitative and qualitative differences in composition of feline and human urine. In addition, it is possible that urine substances that were not inactivated or removed via PEG inhibited the RT required in the assay used in our study. Reverse transcriptase inhibitors would not affect cytomegalovirus or polyomavirus assays, as these assays do not require RT.

The poor RT-PCR assay sensitivity associated with RNA prepared via the PEG method may also be a result of low RNA yield. Although RNA yield was not quantified, the observation that assay sensitivity was significantly decreased for tissue culture medium positive control specimens prepared by use of the PEG method, compared with assay sensitivities associated with the positive control specimens prepared via other methods, suggests that the PEG method resulted in a comparatively lower RNA yield. Therefore, it is likely that a combination of low RNA yield and failure to remove or inactivate all urine RT-PCR inhibitors was responsible for the lower RT-PCR assay sensitivity associated with PEG-prepared specimens.

The dTMB method of RNA preparation relies on direct base pairing between the polyadenylated tail of viral genomes and the oligo(dT) sequences bound to the surface of magnetic beads.[27] This method has been successfully used for purification of human norovirus and hepatitis A virus RNA from shellfish[27] and HIV RNA from human CSF specimens.[17] Because the FCV genome is similarly composed of single-stranded RNA with a polyadenylated tail, it was logical to hypothesize

that the dTMB method would be effective for isolation of FCV RNA from urine obtained from cats. Although the dTMB method significantly improved RT-PCR assay sensitivity, compared with that associated with the PEG method, isolation of FCV RNA from urine via the dTMB method was not as effective as either of the silica gel–based column methods. Because the RT-PCR assay performed equally well with RNA prepared from samples of urine or tissue culture medium, the relative decrease in RT-PCR sensitivity associated with samples prepared by use of the dTMB method, compared with sensitivity associated with samples prepared by use of the QA or RN methods, was most likely a result of decreased RNA yield. It is possible that RNA yield by use of magnetic beads could be improved through application of alternative nucleic acid capture strategies. Human norovirus RNA has been successfully prepared from environmental samples for RT-PCR assay by use of magnetic beads coated with virus-specific antibodies (immunomagnetic-bead separation) or use of streptavidin-coated magnetic beads and biotinylated oligonucleotides.[28,29] However, immunomagnetic-beads were not equally effective for isolating all antigenic types of noroviruses.[29] Antigenic variation among FCV isolates could similarly limit the use of immunomagnetic-beads for isolation of FCV from biological samples.[1] It may be of value to investigate whether capture with specific biotinylated FCV oligonucleotides (or nonspecific biotinylated oligo[dT]) and streptavidin-coated magnetic beads would enhance the performance of the FCV RT-PCR assay.

Preparation methods involving 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 specimens of urine, feces, CSF, serum, and other complex biological samples.[12,14] Because urine samples obtained from cats may contain variable numbers of cells, and because FCV may be located intracellularly or extracellularly (or both), we evaluated 2 silica gel–based membrane methods of RNA preparation in our study: the RN method (designed to isolate total RNA from highly cellular preparations) and the QA method (designed to maximize recovery of viral RNA from cell-free fluids). Both methods use chaotropic salts to lyse cells and virus particles and inactivate RNAses prior to membrane binding.[19,20] However, the QA method incorporates carrier RNA in the lysis step to improve viral RNA binding and competitively limit viral RNA degradation due to residual RNAse activity.[20] The sensitivity of the RT-PCR assay was significantly better when using samples prepared by use of the QA and RN methods than with samples prepared by use of the PEG and dTMB methods. Improved RT-PCR assay sensitivity associated with the QA and RN methods was most likely a result of 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 the RN and QA methods are equally suitable for preparing viral RNA under all conditions associated with samples obtained from cats with or without urinary tract disorders.

In the present study, urine samples were modified through removal of urine sediment or addition of whole or hemolyzed blood prior to RNA preparation; however, in experiments involving these modified samples, we were unable to detect significant differences between the dTMB, QA, and RN methods of RNA preparation on RT-PCR assay sensitivity. Several substances have been shown to inhibit PCR and RT reactions, including urea,[15] RBCs,[30] heme compounds,[31-36] leukocytes,[31,35] IgG,[37] and urine crystals.[22] Some or all of these inhibiting substances may be present in urine specimens obtained from clinically normal cats or cats with urinary tract disorders. Although there were no apparent effects of urine sample modification in the present study, our results should be interpreted within the context of a limited sample size. Similarly, the small sample size of the present study precluded analysis of other cat-specific variables (eg, urine specific gravity, proteinuria, and crystalluria) that may affect RNA isolation and RT-PCR assay performance. The influence of specific feline urine components on RNA preparation and FCV RT-PCR assay performance requires further investigation.

Our data have indicated that there are notable differences between RNA isolation methods for recovery of FCV nucleic acids from urine samples collected from cats. The results of the present study 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. An RT-PCR assay system optimized for detection of FCV in feline urine samples may be useful for large-scale epidemiologic and experimental studies of FCV-induced urinary tract diseases in cats.

a. Scansen BA. *Real-time RT-PCR of feline calicivirus and optimization for detection of virus in feline urine.* MS thesis, College of Veterinary Medicine, Michigan State University, East Lansing, MI, 2004.
b. Harlan Bioproducts, Indianapolis, Ind.
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 Inc, Valencia, Calif.
f. QIAamp Viral RNA mini kit, QIAGEN Inc, Valencia, Calif.
g. Polyethylene glycol (average molecular weight, 8,000), Sigma-Aldrich, St Louis, Mo.
h. Igepal CA-630, Sigma-Aldrich, St Louis, Mo.
i. QuantiTech SYBR Green RT-PCR kit, QIAGEN Inc, Valencia, Calif.
j. ICycler iQ system with detection software, version 2.3B, Bio-Rad Laboratories, Hercules, Calif.

References

1. Baulch-Brown C, Love DN, Meanger J. Feline calicivirus: a need for a vaccine modification? *Aust Vet J* 1997;75:209–213.

2. Sykes JE. Feline upper respiratory tract pathogens: herpesvirus-1 and calicivirus. *Compend Contin Educ Pract Vet* 2001;23:166–175.

3. Kruger JM, Osborne CA, Venta PJ, et al. Viral infections of the feline urinary tract. *Vet Clin North Am Small Anim Pract* 1996;26:281–296.

4. Rice CC, Kruger JM, Venta PJ, et al. Genetic characterization of 2 novel feline caliciviruses isolated from cats with idiopathic lower urinary tract disease. *J Vet Intern Med* 2002;16:293–302.

5. Rich LJ, Fabricant CG. Urethral obstruction in male cats: transmission studies. *Can J Comp Med* 1969;33:164–165.

6. Kruger JM, Osborne CA. The role of viruses in feline lower urinary tract disease. *J Vet Intern Med* 1990;4:71–78.

7. Helps C, Lait P, Tasker S, et al. Melting curve analysis of feline calicivirus isolates detected by real-time reverse transcription PCR. *J Virol Methods* 2002;106:241–244.

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

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

10. Scansen BA, Wise AG, Kruger JM, et al. Evaluation of a p30 gene-based real-time reverse transcriptase polymerase chain reaction assay for detection of feline caliciviruses. *J Vet Intern Med* 2004;18: 135–138.

11. Behzadbehbahani A, Klapper PE, Vallely PJ, et al. Detection of BK virus in urine by polymerase chain reaction: a comparison of DNA extraction methods. *J Virol Methods* 1997;67:161–166.

12. Biel SS, Held TK, Landt O, et al. Rapid quantification and differentiation of human polyomavirus DNA in undiluted urine from patients after bone marrow transplantation. *J Clin Microbiol* 2000;38: 3689–3695.

13. Demmler GJ, Buffone GJ, Schimbor CM, et al. Detection of cytomegalovirus in urine from newborns by using polymerase chain reaction DNA amplification. *J Infect Dis* 1988;158:1177–1184.

14. Echavarria M, Forman M, Ticehurst J, et al. PCR method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. *J Clin Microbiol* 1998;36: 3323–3326.

15. Khan G, Kangro HO, Coates PJ, et al. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. *J Clin Pathol* 1991;44:360–365.

16. Osborne CA, Stevens JB. *Urinalysis: a clinical guide to compassionate patient care*. Shawnee Mission, Kan: Bayer Corp, 1999; 106–109.

17. Chiodi F, Keys B, Albert J, et al. Human immunodeficiency virus type-1 is present in the cerebrospinal fluid of a majority of infected individuals. *J Clin Microbiol* 1992;30:1768–1771.

18. *Dynabeads mRNA DIRECT kit handbook*. Lake Success, NY: Dynal Inc, 2000;10:14–21.

19. *RNeasy mini handbook*. 3rd ed. Valencia, Calif: QIAGEN Inc, 2001;30–35.

20. *QIAamp viral mini kit handbook*. Valencia, Calif: QIAGEN Inc, 1999;18–19, 25

21. *SAS/STAT users guide: statistics, version 8*. 4th ed. Cary, NC: SAS Institute Inc, 1989.

22. Mahony J, Chong S, Jang D, et al. 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. *J Clin Microbiol* 1998;36:3122–3126.

23. Toye B, Woods W, Bobrowska M, et al. Inhibition of PCR in genital and urine specimens submitted for *Chlamydia trachomatis* testing. *J Clin Microbiol* 1998;36:2356–2358.

24. Vinogradskaya GR, Goryshin IY, Berlin YA, et al. Optimization of PCR-based diagnostics for human cytomegalovirus. *J Virol Methods* 1995;53:103–112.

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

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

27. Kingsley DH, Richards GP. Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. *Appl Environ Microbiol* 2001;67: 4152–4157.

28. Loisy F, Le Cann P, Pommepuy M, et al. An improved method for the detection of Norwalk-like caliciviruses in environmental samples. *Lett Appl Microbiol* 2000;31:411–415.

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

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

31. Al-Soud WA, Rådström P. Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol* 2001;39:485–493.

32. Byrnes JJ, Downey KM, Esserman L, et al. Mechanism of hemin inhibition of erythroid cytoplasmic DNA polymerase. *Biochemistry* 1975;14:796–799.

33. Klein A, Barsuk R, Dagan S, et al. Comparison of methods for extraction of nucleic acid from hemolytic serum for PCR amplification of hepatitis B virus DNA sequences. *J Clin Microbiol* 1997;35:1897–1899.

34. Levere RD, Gong Y, Kappas A, et al. Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. *Proc Natl Acad Sci U S A* 1991;88: 1756–1759.

35. Morata P, Queipo-Ortuño MI, de Dios Colmenero J. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. *J Clin Microbiol* 1998;26:2443–2446.

36. Tsutsui K, Mueller GC. Hemin inhibits virion-associated reverse transcriptase of murine leukemia virus. *Biochem Biophys Res Commun* 1987;149:628–634.

37. Al-Soud WA, Jönsson LJ, Rådström P. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J Clin Microbiol* 2000;38:345–350.