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Detection of Rodent Coronaviruses by Use of Fluorogenic Reverse Transcriptase-Polymerase Chain Reaction Analysis

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Reverse transcriptase-polymerase chain reaction (RT-PCR) assays have proved useful for the detection of mouse hepatitis virus (MHV) and rat coronavirus (RCV) in acutely infected animals and contaminated biomaterials. Fluorogenic nuclease RT-PCR assays combine RT-PCR with an internal fluorogenic hybridization probe, thereby eliminating post-PCR processing and potentially enhancing specificity. Consequently, a fluorogenic nuclease RT-PCR assay specific for rodent coronaviruses was developed. Primer and probe sequences were selected from the viral genome segment that encodes the membrane (M) protein that is highly conserved among rodent coronaviruses. Use of the fluorogenic nuclease RT-PCR detected all strains of MHV and RCV that were evaluated, but did not detect other RNA viruses that naturally infect rodents. Use of the assay detected as little as two femtograms of in vitro transcribed RNA generated from cloned amplicon, and when compared directly with mouse antibody production tests, had similar sensitivity at detecting MHV-A59 in infected cell culture lysates. Finally, use of the assay detected coronavirus RNA in tissues, cage swipes, and feces obtained from mice experimentally infected with MHV, and in tissues and cage swipes obtained from rats naturally infected with RCV. These results indicate that the fluorogenic nuclease RT-PCR assay should provide a potentially high-throughput, PCR-based method to detect rodent coronaviruses in infected rodents and contaminated biological materials.

Mouse hepatitis virus (MHV) and rat coronavirus (RCV) infections are the most common viral infections detected in contemporary laboratory mouse and rat colonies, respectively (1). Mouse hepatitis virus can induce hepatitis, encephalitis, enterocolitis, and death in susceptible mice, with preweanling and immunocompromised mice most severely affected (2, 3). Subclinical MHV infection can also have a tremendous impact on research that involves use of infected mice, especially immunologic research, since essentially all MHV strains are lymphotrophic (2, 4). Rat coronavirus generally induces high morbidity characterized by rhinitis and sialodacryoadenitis in acutely infected colonies with low mortality, and can complicate numerous types of research that involves use of rats, especially ophthalmologic and respiratory research (5-7). Both viruses are considered to be highly contagious; therefore, rapid diagnosis is essential to prevent transmission of infection throughout a research animal facility. The MHV and RCV also are frequent contaminants of transplantable tumors and other biological materials that are inoculated into mice and rats (8, 9); therefore, identification of viral contamination is critical to prevent entry of these pathogens into research animal facilities via this route.

Several methods are currently used to detect coronavirus infections in rodents and contaminated biological materials. Serologic evaluation for the presence of coronavirus antibodies has typically been used to diagnose MHV and RCV infections in rodents (10-13). However, serologic assays cannot detect coronavirus infections directly in immunodeficient strains of rodents that do not generate a humoral immune response, and the time required for host seroconversion in immunocompetent rodents may prevent rapid definitive diagnosis by serologic testing during an epizootic. As a result, reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been developed and have proven a useful adjunct diagnostic method for detection of MHV and RCV. Specific diagnostic applications include RT-PCR detection of rodent coronavirus RNA in the liver of nude mice naturally infected with MHV (14), in feces of mice infected with enterotropic MHV (15), and in tissues and cage swabs of rats naturally infected with RCV (16). The RT-PCR assays also are an attractive alternative to the rodent antibody production (RAP) test for detection of rodent coronavirus contamination in biological materials (17). Compared with RAP testing, RT-PCR analysis confers the substantial advantages of a greatly reduced turnaround time and cost while also providing an alternative to whole animal testing (18). Despite these advantages, RT-PCR analysis is still labor-intensive and costly, the requirement of post-PCR processing by gel electrophoresis limits its application as a high-throughput diagnostic assay, and the potential for false-positive results secondary to carry-over contamination is substantial.

Fluorogenic nuclease PCR (19), a recently developed technique also known as real time PCR or TaqMan PCR, confers several advantages over gel detection PCR. Like PCR, fluorogenic nuclease PCR amplifies DNA between two specific oligonucleotide primers by thermocycling in the presence of *Taq* polymerase. However, included in the fluorogenic nuclease PCR reaction mix is an internal fluorogenic hybridization probe with covalently linked fluorogenic and quencher dyes in close proximity. The *Taq*

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polymerase nucleolytically cleaves the probe during each round of amplification, thereby releasing the fluorogenic dye from the quencher. The increase in fluorescence is measured optically at the end of each thermocycle, these data are transmitted to an attached computer in "real" time, and subsequent computer analysis results in a quantitative, closed-tube detection system for specific PCR products. Fluorogenic nuclease PCR therefore eliminates post-PCR processing and carry-over contamination, potentially imparts improved specificity via the internal probe, and can be used to detect RNA when combined with an initial reverse transcriptase step. The objective of the study reported here was, therefore, to develop and evaluate a fluorogenic nuclease RT-PCR assay specific for rodent coronaviruses.

Materials and Methods

Viruses. The RCV-Parker and RCV-SDA-681 strains were obtained from the American Type Culture Collection (Rockville, Md.). The MHV-A59, MHV-1, MHV-JHM, MHV-S, Theiler's mouse encephalomyelitis virus (TMEV), lymphocytic choriomeningitis virus (LCMV), pneumonia virus of mice (PVM), reovirus-3, Sendai virus, and rotavirus SA-11 were obtained from intramural stocks. The MHV-A59, MHV-1, MHV-JHM, and MHV-S were propagated in murine $A9_{2L}$ fibroblasts (20) in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37°C in 10% CO_2. Similarly, LCMV was propagated in L929 cells (ATCC CCL-1), TMEV, PVM, reovirus 3, and Sendai virus were propagated in BHK-21 cells (ATCC CCL-10), and SA-11 was propagated in MA-104 cells (ATCC CRL-2378). Cell lysates were prepared, and the 50% tissue culture infective dose ($TCID_{50}$) for each was determined as described (21). Permissive cells infected with each cultivated viral stock were then used to prepare indirect fluorescent antibody (IFA) test spot slides and were evaluated, using homologous and non-immune serum to confirm the presence of viable virus. All viral stocks were stored at -70°C until use.

Fluorogenic nuclease RT-PCR assays. Nucleotide sequences that encode the coronavirus M protein were obtained from GenBank for MHV-A59, MHV-JHM, MHV-RI, MHV-Y, MHV-DVIM, RCV-NJ, and RCV-SDA. Sequences were aligned and compared with the ClustalW and Pretty software programs (Genetics Computer Group, Madison, Wis.), and the primers and probe for the rodent coronavirus fluorogenic nuclease RT-PCR assay were identified within a highly conserved region, using the Primer Express software (PE Applied Biosystems, Foster City, Calif.). All fluorogenic nuclease RT-PCR reactions were performed by use of a PE Applied Biosystems GeneAmp 5700 Sequence Detection System, and products were analyzed by use of the accompanying software. Each 25-µl reaction consisted of 2.5 µl of template, 50 n*M* forward primer, 300 n*M* reverse primer, 100 n*M* probe, 1X TaqMan buffer (50 m*M* KCl, 10 m*M* EDTA, 10 m*M* Tris-HCl [pH 8.3], 60 n*M* passive reference), 5.5 m*M* $MgCl_2$, 300 µ*M* dATP, dCTP, and dGTP, 600 µ*M* dUTP, 0.05% gelatin, 0.01% Tween-20, 200 ng of calf liver RNA, 10 U of RNase inhibitor, 0.625 U of Amplitaq Gold polymerase, and 0.625 U of MuLV reverse transcriptase (PE Applied Biosystems). Thermal cycling conditions consisted of reverse transcription at 48°C for 30 min, denaturation and *Taq* polymerase activation at 95°C for 10 min, and 40 cycles of 95°C for 15 sec., followed by 60°C for 1 min. Samples were considered test positive if they had mean fluorescence (Rn) > 0.1 and cycle threshold (Ct) < 40. The baseline Rn value was selected so as to intersect the amplification curve in the middle of the linear amplification phase (as recommended by the manufacturer). The Ct limit was selected to minimize the incidence of false-positive results on the basis of our experience with this technique and the potential for probe degradation to occur at Ct > 40.

Amplicon cloning. Amplicons generated by the fluorogenic nuclease RT-PCR primer set were resolved by use of agarose gel electrophoresis, and the target band was eluted by use of a QIAquick kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions. The 108-basepair (bp) amplicon product was then ligated into the pT7Blue-2 vector (Novagen, Madison, Wis.), amplified in Tuner (DE3) pLacI Competent Cells (Novagen), purified using the Qiagen Plasmid Purification kit, and sequenced by personnel at the University of Arizona's Molecular Core Facility.

Internal RNA positive-control standards were generated by in vitro transcription from the linearized plasmid DNA template, using the RiboMAX system (Promega, Madison, Wis.). The RNA was quantified by use of an MBA 2000 UV-vis spectrophotometer (PE Applied Biosystems), and the sensitivity of the fluorogenic nuclease RT-PCR assay was determined by evaluation of serial dilutions of in vitro transcribed RNA.

Specificity. Assay specificity was determined by evaluation of RNA extracted from viral preparations of MHV-A59, MHV-1, MHV-JHM, MHV-S, RCV-Parker, and RCV-SDA-681, TMEV, LCMV, PVM, reovirus 3, Sendai virus, and SA-11. The viral concentration for each of these preparations ranged from 10^6 to 10^{10} $TCID_{50}$/ml. Viral RNA from one milliliter of each viral preparation was extracted and eluted into a 160-µl total volume, using a Qiagen QIAamp Viral RNA kit, with 2.5 µl of extracted viral RNA/reaction used as template for evaluation by the fluorogenic nuclease RT-PCR assay. It is, therefore, estimated that a minimum of 10^4 $TCID_{50}$ of each virus was evaluated in each fluorogenic RT-PCR reaction for the specificity determination.

Serologic testing. The MHV/RCV IFA test slides were prepared in our laboratory by acetone fixation of a 1:1 suspension of uninfected and MHV-A59 infected $A9_{2L}$ cells to 12-well spot slides. Serum diluted 1:5 (20 µl) and phosphate-buffered saline (PBS)-3% nonfat dry milk (10 µl) were added to each well, incubated for 15 min, rinsed, and air dried. Fluorescein-labeled goat anti-mouse or anti-rat IgG (20 µl; Kirkegaard & Perry, Gaithersburg, Md.) at a concentration of 20 µg/ml was added to each well, incubated for 15 min, rinsed, air dried, coverslipped, and examined, using an Olympus BX60 epifluorescent microscope through a narrow band blue filter. Sera that resulted in green fluorescence in 50% of the cells were considered test positive.

Animals. Four- and six-week-old male Hsd:ICR(CD-1) mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Mice were specified to be free of murine viruses, pathogenic bacteria, and endo- and ectoparasites by the supplier. Each control or experimental group was housed separately in microisolator cages, and all animal manipulations were performed in a class-IIA biological safety cabinet, using standard microisolation technique. Tissues from Sprague Dawley (SD) rats naturally infected with RCV were obtained during an epizootic that occurred at the University of Arizona during February 2000. Uninfected control rats were obtained from intramural SD rat colonies documented to be free of RCV infection and other pathogens on the basis of results of repeated se-

rologic, microbiological, parasitologic, and histologic testing.

Animals were housed at a temperature of 22 to 24°C, humidity of 40 to 60%, 12 to 15 air exchanges/h, and a 12:12 h light:dark cycle. All animal procedures were performed in accordance with federal regulations and were approved by the University of Arizona Institutional Animal Care and Use Committee.

Mouse antibody production test. Six-week-old ICR mice were separated into groups of four animals each and were administered tenfold serial dilutions of MHV-A59 (range, 2.5×10^2 to 2.5×10^{-2} $TCID_{50}$/mouse). Approximately 20% of the viral inoculum was administered oronasally (volume = 10 μl), with the remainder injected intraperitoneally (volume = 50 μl). Dose ranges were established from the endpoint dilution of the rodent coronavirus fluorogenic nuclease RT-PCR assay, along with the addition of tenfold and 100-fold more and less concentrated dilutions. Mice were euthanized by carbon dioxide inhalation at postinoculation (PI) week 4, and blood was collected by cardiocentesis. Serum was diluted 1:5 in PBS and was stored at -20°C until evaluated by use of an IFA serologic assay specific for MHV.

Animal infections. Experimentally induced infections in mice were performed by intranasal inoculation of four-week-old ICR mice with MHV-A59 (volume = 25 μl; dose = 2.5×10^5 or 2.5×10^4 $TCID_{50}$/mouse at PI weeks 1 and 4, respectively) or mock inoculum ($A9_{2L}$ cell lysate; volume = 25 μl). These mice were euthanized at PI weeks 1 and 4 by carbon dioxide inhalation, blood samples were collected by cardiocentesis, and the resulting sera were diluted 1:5 in PBS. Liver was harvested, fixed in buffered 10% formalin, embedded in paraffin, cut into 5-μm-thick sections, stained with hematoxylin and eosin, and evaluated for histopathologic changes.

Approximately 30 mg each of liver, lung, spleen, mesenteric lymph node, and small intestine was harvested from each animal and was snap-frozen in liquid nitrogen for fluorogenic nuclease RT-PCR analysis. Cage swipes (1-in^2 swabs soaked with 70% isopropyl alcohol swiped across all interior cage walls, then were stored in a microcentrifuge tube at -70°C until RNA extraction), and fecal specimens also were collected at PI days 1, 3, 5, 7,10, 14, 21, and 28. Similarly, serum, Harderian gland, submandibular salivary gland, lung, and cage swipes were obtained from uninfected and RCV-infected SD rats after euthanasia by carbon dioxide inhalation. Each tissue specimen was snap-frozen in liquid nitrogen immediately after harvest, and all specimens were stored at -70°C until RNA was extracted.

The same tissues also were collected in buffered 10% formalin and were prepared for histologic evaluation as indicated previously for mice. The RNA was extracted from all frozen tissues, using a Qiagen RNeasy kit, and from cage swipes and feces, using a Qiagen QIAamp Viral RNA kit according to manufacturer's instructions. Specimens from infected and uninfected rodents were harvested and processed simultaneously. The RNA was stored at -70°C until evaluation by use of fluorogenic nuclease RT-PCR analysis, and diluted serum samples were stored at -20°C until evaluation by use of IFA serologic testing.

Results

Rodent coronavirus fluorogenic nuclease RT-PCR. Alignment of rodent coronavirus M gene sequences revealed a highly conserved region that was used to design the primers and probe (Table 1). Each primer had no more than one nucleotide mismatch with any one of the aligned M gene sequences of the evaluated MHV and RCV strains, and the probe was completely homologous to all those MHV and RCV strains. Primers were optimized at a concentration of 50 n*M* for the forward primer and 300 n*M* for the reverse primer through use of checkerboard titrations of 50, 300, and 900 n*M* concentration of each primer with a 200 n*M* probe. Similarly, the probe was optimized at a concentration of 100 n*M* through evaluation of 25, 50, 75, 100, 125, 150, 175, 200, and 225 n*M* concentrations of probe with the optimal primer concentrations. The assay could detect as little as two femtograms of RNA through evaluation of serial dilutions of in vitro transcribed RNA generated from cloned amplicon DNA (Fig. 1).

Table 1. Primer and probe sequences for the rodent coronavirus fluorogenic nuclease reverse transcriptase-polymerase chain reaction (RT-PCR) assay

	Sequence (5'-3')	Position (5'-3')
Forward primer	GGAACTTCTCGTTGGGCATTATACT	153-177[a]
Reverse primer	ACCACAAGATTATCATTTTCACAACATA	260-233
Probe	ACATGCTACGGCTCGTGTAACCGAACTGT	227-199

[a]Nucleotide positions from MHV-A59 sequence (GenBank accession No. X00509).

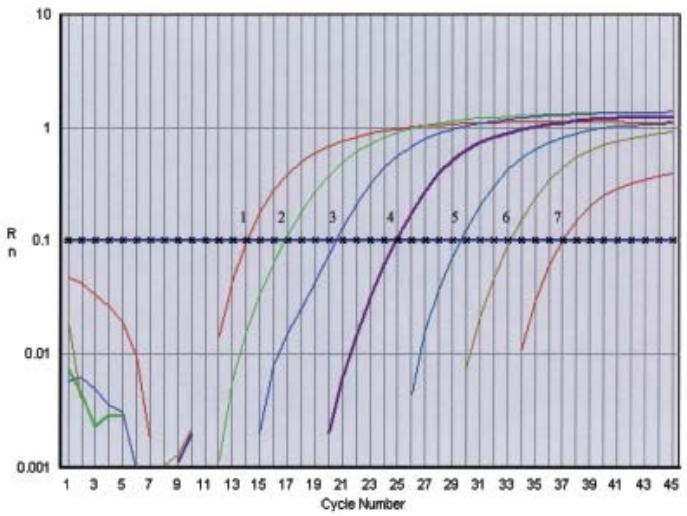

Figure 1. Sensitivity of the rodent coronavirus fluorogenic nuclease reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Serial dilutions of in vitro transcribed RNA complementary to the amplicon sequence were evaluated. Dilutions from left to right on the amplification plot (labeled 1–7) correspond to 1 ng; 100, 10, and 1 pg; and 100, 10, and 2 fg of amplicon cRNA, respectively. All values with mean fluorescence (Rn) > 0.1 and a cycle threshold (Ct) < 40 are considered positive results.

Assay specificity was determined by evaluation of RNA extracted from preparations of MHV-A59, MHV-1, MHV-JHM, MHV-S, RCV-Parker, RCV-SDA-681, TMEV, LCMV, PVM, reovirus 3, Sendai virus, and rotavirus SA-11. The assay detected all evaluated strains of MHV and RCV (Ct values ranging from 12.3 to 15.0), but did not detect TMEV, LCMV, PVM, reovirus 3, Sendai virus, and rotavirus SA-11 (all Ct values > 40).

Comparison of the fluorogenic nuclease RT-PCR assay and mouse antibody production (MAP) test. Relative sensitivities of the fluorogenic nuclease RT-PCR assay and MAP testing were determined. Tenfold dilutions of an MHV-A59 viral preparation were evaluated directly by use of the fluorogenic nuclease RT-PCR assay, and the endpoint detection limit (2.5×10^0

Table 2. Relative sensitivity of rodent coronavirus fluorogenic nuclease RT-PCR assay, compared with that of the mouse antibody production (MAP) test

Viral concentration ($TCID_{50}$)	Fluorogenic nuclease RT-PCR[a]	MAP test[b]
2.5×10^{2}	+	4/4[cd]
2.5×10^{1}	+	0/4
2.5×10^{0}	+	1/4
2.5×10^{-1}	–	0/4
2.5×10^{-2}	–	0/4

[a]Positive result interpreted as a C_t value < 40.
[b]As detected by use of mouse hepatitis virus (MHV) indirect fluorescent antibody (IFA) serologic testing.
[c]No. of mice positive/total No. of mice tested.
[d]Two mice that died prior to four weeks after inoculation were confirmed test positive on the basis of results of histologic examination and MHV RNA detection in the liver.

$TCID_{50}$) was determined. A viral dose equivalent to the endpoint detection limit of the fluorogenic nuclease RT-PCR assay was then administered oronasally (10 μl) and intraperitoneally (50 μl) to each of four 6-week-old ICR mice. Similarly, 10- or 100-fold more and 10- or 100-fold less virus was administered to each of four 6-week-old ICR mice in each of four other groups to provide a range of five logarithmic dilutions of virus. Four weeks after inoculation, serum was harvested from the mice and evaluated by use of IFA serologic testing. At the highest viral concentration evaluated by MAP testing, two mice seroconverted to MHV, and two mice died of MHV infection ten days after inoculation, as determined by observation of severe acute diffuse hepatic necrosis and detection of MHV RNA in the liver by use of the fluorogenic nuclease RT-PCR assay (Table 2). Of the remaining four groups of mice, only one mouse in the endpoint viral dilution group seroconverted to MHV.

Fluorogenic nuclease RT-PCR detection of MHV RNA in experimentally infected ICR mice. Four-week-old ICR mice were inoculated intranasally with MHV-A59 or mock inoculum and were euthanized at PI week 1 or 4. The RNA extracts from the liver, lung, mesenteric lymph node, small intestine, and spleen of each mouse were evaluated by use of the rodent coronavirus fluorogenic nuclease RT-PCR assay. The MHV RNA was detected in all mice at PI week 1 and in six of nine mice at PI week 4 (Table 3). The assay could detect viral RNA in each type of tissue evaluated, although detection was observed most consistently in mesenteric lymph node. All MHV-infected mice were also seropositive on the basis of results of MHV IFA serologic testing at both time points, and necrotizing hepatitis (at PI week 1) or chronic hepatitis with mineralization (at PI week 4) was observed in approximately half of these mice. Viral RNA, anti-viral antibodies, and hepatic lesions indicative of MHV infection were not detected in any of the age- and strain-matched mock-infected mice. The RNA extracted from feces and cage swipes that were collected on PI days 1, 3, 5, 7, 10, 14, 21, and 28 also were evaluated by use of the fluorogenic nuclease RT-PCR assay. The assay could detect viral RNA in the cage swipes at PI days 1 and 3 and in feces at PI day 7 from cages housing MHV-infected mice, whereas viral RNA was not detected in feces or swipe samples after PI day 7. The MHV RNA was not detected from any of the cage swipes and feces collected from mock-infected mice at identical time points.

Fluorogenic nuclease RT-PCR detection of RCV RNA in naturally infected Sprague Dawley rats. During an intramural epizootic of RCV infection, Harderian gland, submandibular salivary gland, lung, and serum were obtained from clinically affected rats, and alcohol swipes were collected from the cages in which these rats were housed. The RNA extracted from each of these specimens was then evaluated by use of the rodent coronavirus fluorogenic nuclease RT-PCR assay. The RCV RNA was detected by the assay in one or more tissues in all rats that had serologic or histologic evidence of RCV infection, with histologic evidence defined as acute to subacute necrotizing dacryoadenitis and/or sialoadenitis (Table 4). The RCV RNA was detected most consistently in Harderian gland, and all rats for which RCV RNA was detected in Harderian gland or salivary gland also had pathognomonic histologic lesions in Harderian gland or salivary gland, respectively. Interestingly, RCV RNA was detected in lung only from seronegative rats and was detected in salivary gland only from seropositive rats. The RCV RNA was detected in all cage swipes collected from cages that contained RCV-infected rats, including one cage that contained RCV-seronegative rats that had histologic evidence of sialodacryoadenitis. Similar specimens obtained from strain-matched, viral antibody-free rats were uniformly test negative on the basis of results of fluorogenic nuclease RT-PCR analysis and IFA serologic testing.

Discussion

A fluorogenic nuclease RT-PCR assay was developed to specifically detect rodent coronaviruses. The primers and probe for the assay were designed from a highly conserved region of the gene encoding the membrane (M) protein, the most highly conserved protein among rodent coronaviruses (22). The assay was able to detect as little as two femtograms of positive-control RNA and detected only rodent coronaviruses when evaluated against a panel of rodent RNA viruses, indicating that the assay was sensitive and specific for rodent coronaviruses.

The fluorogenic nuclease RT-PCR assay was directly compared with the MAP test used to detect MHV contamination in biological materials. The rodent coronavirus fluorogenic nuclease RT-PCR assay had comparable sensitivity to that of the MAP test, although the MAP test yielded intermittent positive results at lower concentrations of MHV-A59 virus that were detected by use of the fluorogenic nuclease RT-PCR assay. Reasons for the intermittent positive MAP test results are unknown, but are possibly the result of viral factors, such as strain or the route of inoculation, and/or host factors, such as use of an outbred stock of mice. The fluorogenic nuclease RT-PCR assay also cannot distinguish infective from non-infective virus, which might explain the intermittent MAP test results. Even so, detection of viral RNA is a definite indication of exposure of the biological material to a rodent coronavirus, and if the material were inoculated into rodents, it would warrant precautionary measures to prevent possible transmission to immune-naïve rodents in a research animal facility. In addition, the fluorogenic nuclease RT-PCR assay confers the advantages of a substantially reduced turnaround time, an alternative to a whole animal bioassay, and a less costly alternative to MAP testing. Given the high potential of rodent coronavirus contamination of cell cultures and other biological materials (8, 9), fluorogenic nuclease assays should provide an accurate, cost-effective, and timely means of screening these materials for MHV and RCV contamination.

Fluorogenic nuclease RT-PCR also offers several advantages as an adjunct diagnostic method for routine rodent health monitoring. The assay could provide a useful diagnostic option for di-

Table 3. Detection of MHV-A59 by use of the rodent coronavirus fluorogenic nuclease RT-PCR assay in experimentally infected ICR mice

Group	Hepatic lesions	Fluorogenic nuclease RT-PCR						MHV IFA
		Liver	Lung	MLN	Intestine	Spleen	Any tissue	
Mock (PI week 1)	0/4[a]	0/4	0/4	0/4	0/4	0/4	0/4	0/4
MHV (PI week 1)	7/11[b]	4/11	6/11	10/11	2/11	7/11	11/11	11/11
Mock (PI week 4)	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
MHV (PI week 4)	4/9[c]	1/9	2/9	5/9	1/9	3/9	6/9	9/9

[a]No. of mice positive/total No. of mice tested.
[b]Acute necrotizing hepatitis observed.
[c]Chronic hepatitis with mineralization observed.
MLN = mesenteric lymph node; MHV IFA = mouse hepatitis virus indicect fluorescent antibody (test).

Table 4. Detection of rat coronavirus (RCV) RNA by use of the fluorogenic nuclease RT-PCR assay in naturally infected Sprague Dawley rats

Group	RCV IFA	Histopathologic changes[a]		Fluorogenic nuclease RT-PCR				
		Harderian	Salivary	Harderian	Salivary	Lung	Any tissue	Cage swipe
Uninfected	0/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	0/3[c]
RCV-infected	0/4	4/4	0/4	3/4	0/4	3/4	4/4	1/1
RCV-infected	10/10	10/10	7/10	9/10	6/10	0/10	10/10	4/4

[a]Acute or subacute necrotizing dacryoadenitis or sialoadenitis observed.
[b]No. of animals positive/total No. of animals tested.
[c]No. of cage swipes positive/total No. of cages tested.
Harderian and salivary refer to the glands of that name.

rectly evaluating acutely infected rodents or immunodeficient rodents that do not generate a detectable humoral immune response. For example, during the intramural RCV epizootic our institution experienced in February 2000, our laboratory was able to definitively diagnose RCV in multiple animal rooms by use of fluorogenic nuclease RT-PCR prior to seroconversion, which enabled us to immediately quarantine those rooms and prevent further transmission to uninfected colonies. A previous report of detection of MHV infection directly in immunocompromised nude mice by use of RT-PCR analysis supports the potential application of the fluorogenic nuclease RT-PCR assay to coronavirus detection in immunodeficient rodents (14). Obviously, sample selection for evaluation is critical and depends on the pathogenesis of the virus suspected in an epizootic situation to prevent false-negative results. In our study, mesenteric lymph node in MHV-infected mice and Harderian gland in RCV-infected rats were the most consistent tissues in which coronavirus RNA could be detected. Although this study represent the pathogenesis of only one strain of MHV and RCV within defined hosts, previous pathogenesis studies of numerous MHV and RCV strains indicate these two tissues would likely be excellent target tissues for diagnostic evaluation by use of fluorogenic nuclease RT-PCR analysis (23, 24). However, as observed in this study, evaluation of multiple tissues from each animal would increase the likelihood of detecting coronavirus RNA within any individual animal and should be considered.

Stage of infection is also a critical factor to consider for immunocompetent rodents, since MHV and RCV generally induce acute infections, with clearance of infective virus within several weeks (3). Interestingly, MHV RNA could be detected at PI week 4 in certain tissues obtained from most mice experimentally infected with MHV-A59. Although infective MHV-JHM has been detected up to one month after infection in the brain of mice inoculated intranasally, persistence of infective virus even for one month has not been observed for other MHV strains (25). More likely, this finding represents detection of residual viral RNA and not infective virus by the assay, because residual viral RNA can remain in certain tissues of mice experimentally infected with MHV-A59 for up to 10 months (26-28). Stage of infection is likely not as critical for immunodeficient rodents since coronaviruses often induce persistent infections in these hosts (3).

Finally, the fluorogenic nuclease RT-PCR assay could potentially be used for ante-mortem sample testing since MHV was detected in fecal pellets and cage swipes obtained from cages of experimentally infected mice, and RCV was detected in cage swipes of naturally infected rats. The MHV RNA detected in cage swipes immediately after inoculation likely reflects residual viral inoculum, whereas the detection of MHV in the feces is more consistent with shedding during acute viral infection. Although the short duration when ante-mortem samples were MHV or RCV positive limits the usefulness of this application, detection within a rodent colony could potentially be accomplished by random sampling or evaluation of pooled samples. A reliable, non-invasive approach toward ante-mortem testing would obviously be advantageous for rodent colony health monitoring, particularly among animals involved in long-term studies.

The rodent coronavirus fluorogenic nuclease PCR assay could also be applied to coronavirus research. The quantitative results generated by fluorogenic nuclease PCR analysis could be useful in coronavirus pathogenesis studies when quantitation of viral load or viral shedding is desired. It could also be applied to coronavirus epizootiology studies to help determine transmission modes and frequency. Finally, the rodent coronavirus assay could be useful at detecting novel rodent coronavirus strains since it indiscriminately detects all known rodent coronaviruses.

In conclusion, the rodent coronavirus fluorogenic nuclease RT-PCR assay provides a sensitive, specific, and quantitative assay for the detection of rodent coronaviruses in laboratory rodents, environmental samples, and contaminated biological materials, with the potential to be useful in rodent coronavirus research. The assay is also amenable to high-throughput diagnostics due to the elimination of post-PCR processing, and its closed tube detection system minimizes the potential for carryover contamination.

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