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Detection of Sendai Virus and Pneumonia Virus of Mice by Use of Fluorogenic Nuclease Reverse Transcriptase Polymerase Chain Reaction Analysis

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Sendai virus may induce acute respiratory tract disease in laboratory mice and is a common contaminant of biological materials. Pneumonia virus of mice (PVM) also infects the respiratory tract and, like Sendai virus, may induce a persistent wasting disease syndrome in immunodeficient mice. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays have proven useful for detection of Sendai virus and PVM in immunodeficient animals and contaminated biomaterials. Fluorogenic nuclease RT-PCR assays (fnRT-PCR) combine RT-PCR with an internal fluorogenic hybridization probe, thereby potentially enhancing specificity and eliminating post-PCR processing. Therefore, fnRT-PCR assays specific for Sendai virus and PVM were developed by targeting primer and probe sequences to unique regions of the Sendai virus nucleocapsid (NP) gene and the PVM attachment (G) gene, respectively. The Sendai virus and PVM fnRT-PCR assays detected only Sendai virus and PVM, respectively. Neither assay detected other viruses of the family Paramyxoviridae or other RNA viruses that naturally infect rodents. The fnRT-PCR assays detected as little as 10 fg of Sendai virus RNA and one picogram of PVM RNA, respectively, and the Sendai virus fnRT-PCR assay had comparable sensitivity when directly compared with the mouse antibody production test. The fnRT-PCR assays were also able to detect viral RNA in respiratory tract tissues and cage swipe specimens collected from experimentally inoculated C.B-17 severe combined immunodeficient mice, but did not detect viral RNA in age- and strain-matched mock-infected mice. In conclusion, these fnRT-PCR assays offer potentially high-throughput diagnostic assays to detect Sendai virus and PVM in immunodeficient mice, and to detect Sendai virus in contaminated biological materials.

Sendai virus and pneumonia virus of mice (PVM) are among the most common viral infections detected in contemporary laboratory mouse and rat colonies, respectively (1). Sendai virus can induce interstitial pneumonia and necrotizing bronchiolitis with mortality in susceptible mice, with suckling and weanling mice most severely affected (2). Subclinical Sendai virus infections can also have a tremendous impact on research that involves use of infected mice, especially immunologic and respiratory research since these systems have been documented to be adversely affected during infection. Pneumonia virus of mice is not associated with clinical disease in immunocompetent mice, but may induce a chronic wasting syndrome in nude mice, as does Sendai virus. Both viruses are readily transmitted between animals, and therefore, rapid diagnosis is essential to prevent transmission of infection throughout a research animal facility. Sendai virus can also contaminate biological materials that are inoculated into mice and rats (3); therefore, identification of viral contamination is critical to prevent entry of this pathogen into research animal facilities via this route.

Several methods are currently used to detect Sendai virus and PVM infections in rodents and contaminated biological materials. Serologic evaluation for presence of anti-viral antibodies has typically been used to diagnose Sendai virus and PVM infections in rodents (4, 5). However, serologic assays cannot detect virus 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 use of 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 the detection of Sendai virus and PVM (6, 7). The RT-PCR assays are also an attractive alternative to the mouse or rat antibody production (MAP/RAP) test for detection of Sendai virus and PVM contamination in biological materials (8). Compared with MAP/RAP testing, RT-PCR analysis confers the substantial advantages of greatly reduced turnaround time and cost while also providing an alternative to whole-animal testing. Despite these advantages, RT-PCR analysis is labor intensive and costly; the requirement of post-PCR detection by use of gel electrophoresis, colorimetric, or chromatographic means limits its application as a high-throughput diagnostic assay, and the potential for false-positive results secondary to carry-over contamination is appreciable.

Fluorogenic nuclease (fn) PCR (fnPCR) (9), a recently devel-

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oped technique also known as real-time PCR or TaqMan PCR, confers several advantages over gel detection PCR. Like PCR, fnPCR amplifies DNA between two specific oligonucleotide primers by thermocycling in the presence of *Taq* polymerase. However, included in the fnPCR reaction mixture is an internal fluorogenic hybridization probe with covalently-linked fluorogenic and quencher dyes in close proximity. The *Taq* 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. The fnPCR, 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 objectives of the study reported here were, therefore, to develop and evaluate fnRT-PCR assays specific for Sendai virus and PVM.

Materials and Methods

Virus. Seed stocks of PVM strain 15, Sendai virus Cantell strain, simian virus-5 (SV-5), mouse hepatitis virus (MHV) A59 strain, lymphocytic choriomeningitis virus (LCMV) Armstrong strain, reovirus type 3 (Reo-3) Dearing strain, rotavirus SA-11, and Theiler's mouse encephalomyelitis virus (TMEV) strain GDVII were kindly provided by the University of Missouri Research Animal Diagnostic Laboratory (Columbia, Mo.). Seed stocks were then expanded by cultivation in Eagle's medium at 37°C with 10% CO_2. The PVM, Sendai and Reo-3 viruses, and TMEV were propagated in BHK-21 cells (ATCC CCL-10); SV-5 was propagated in LLC-MK2 cells (ATCC CCL-7); LCMV was propagated in L929 cells (ATCC CCL-1); MHV was propagated in $A9_{2L}$ cells (ATCC CCL-1.4); 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 (10). Bovine respiratory syncytial virus subgroup A (BRSV-A) and subgroup B (BRSV-B), canine distemper virus (CDV), and parainfluenza 1 (PIV-1) virus were kindly provided by Drs. Jim Collins and Carlos Reggardio, University of Arizona. Rat coronavirus Parker strain (RCV-Parker) was purchased through ATCC, and Sin Nombre virus (SNV) RNA was isolated from lung tissue from SNV-seropositive *Peromyscus maniculatus*. All viral stocks were stored at –70°C until use. The PVM strain 15 and Sendai virus Cantell strain were used in all experiments that required infective virus.

Fluorogenic nuclease RT-PCR assays. Genomic nucleotide sequences were obtained from GenBank for PVM No. 15, PVM J3666 strain, Sendai Ohita strain, and Sendai Z strain. Sequences were aligned by use of the ClustalW and Pretty software programs (Genetics Computer Group, Madison, Wis.), and primer/probe sets specific for PVM and Sendai virus were identified by use of the Primer Express software (PE Applied Biosystems, Foster City, Calif.). All fnRT-PCR reactions were performed, using a PE Applied Biosystems GeneAmp 5700 Sequence Detection System, and results were analyzed by the accompanying software. The reverse transcription step consisted of 1X TaqMan buffer (50 m*M* KCl, 10 m*M* Tris-HCl [pH 8.3]); 5.5 m*M* $MgCl_2$; 500 µ*M* dATP, dCTP, dGTP, and dTTP; 8 U of RNase inhibitor; 25 U of Multiscribe Reverse Transcriptase; 2.5 µ*M* random hexamer, and 2 µl of template RNA in a 20-µl reaction. Thermal cycling conditions consisted of 25°C for 10 min for first-strand cDNA synthesis, reverse transcription at 48°C for 30 min, then 95°C for 5 min to inactivate the reverse transcriptase. The PCR reaction consisted of 1X TaqMan buffer (50 m*M* KCl, 10 µ*M* EDTA, 10 m*M* Tris-HCl [pH 8.3], and 60 n*M* Passive Reference); 5.5 m*M* $MgCl_2$; 200 µ*M* dATP, dCTP, and dGTP; 400 µ*M* dUTP; 0.5 U of AmpliTaq Gold; 0.2 U of uracil-N-glycosylase (UNG); 300 n*M* forward and reverse primers; 100 n*M* probe; and 2 µl of cDNA from reverse transcription reaction in a 20-µl reaction. Thermal cycling conditions consisted of 50°C for 2 min UNG incubation, polymerase activation at 95°C for 10 min, then 45 cycles of 95°C for 15 sec, followed by 60°C for one minute. The reverse transcription and PCR amplification thermocycling parameters for each assay were selected to conform with the universal thermocycling parameters recommended by the manufacturer of the RT-PCR reagents (PE Applied Biosystems.).

Samples were considered test positive if they had mean fluorescence (Rn) > 0.05 and a cycle threshold (Ct) < 45. 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 at < 45 cycles to enable detection to the 10-template copy level to maximize assay sensitivity on the basis of the authors' experience with this technique.

Amplicon cloning. Amplicons generated by each fnRT-PCR assay 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 122-base pair (bp) PVM product and the 123-bp Sendai virus product were then ligated into the pT7Blue-2 vector (Novagen, Madison, Wis.), amplified in Tuner (DE3) pLacI Competent Cells (Novagen), and purified, using the Qiagen Plasmid Purification kit. Inserts were confirmed to be present by sequencing at the University of Arizona's Molecular Core Facility. The concentration of the purified plasmid DNA was determined by use of an MBA 2000 UV-vis spectrophotometer (PE Applied Biosystems). The plasmid DNA concentration and the calculated molecular weight of the plasmid vector with amplicon insert were used to calculate the concentration of each sample in units of template copy number.

Specificity and sensitivity. The specificity of each fnRT-PCR assay was determined by evaluation of RNA extracted from viral preparations of PVM, Sendai virus, BRSV-A, BRSV-B, CDV, PIV-1, SV-5, LCMV, MHV, RCV, Reo-3, SA-11, and TMEV. Viral RNA from each lysate was extracted, using a QIAamp viral RNA kit and following the manufacturer's protocol (Qiagen). In addition, SNV RNA was extracted from lung tissues obtained from naturally infected *P. maniculatus*, using Qiagen's RNeasy kit and following the manufacturer's protocol. Presence of infective virus and/or viral RNA template in each preparation was confirmed by reactivity with virus-specific fluorescent antibodies (PVM, Sendai, BRSV-A, BRSV-B, CDV, PIV-1, SV-5, LCMV, MHV, Reo-3, SA-11, TMEV) or by amplification with virus-specific RT-PCR assays (LCMV, MHV, RCV, Reo-3, TMEV, SNV). The sensitivity of each fnRT-PCR assay was determined through evaluation of 10-fold serial dilutions of viral RNA extracted from cell lysates of PVM and Sendai virus (range, 100

ng to one femtogram), respectively.

Mice. Six-week old male Hsd:ICR(CD-1) mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Four-week-old C.B-17 severe combined immunodeficient (scid) mice were obtained from an intramural breeding colony. All mice were specified to be free of PVM, Sendai virus, parvovirus, TMEV, Reo-3, rotavirus, ectromelia, LCMV, adenovirus, polyomavirus, cytomegalovirus, Hantaan virus, mouse thymic virus, K virus, lactate dehydrogenase-elevating virus, *Mycoplasma pulmonis*, *Helicobacter* spp., *Clostridium piliforme*, fur mites, pinworms, potentially pathogenic protozoa, *Encephalitozoon cuniculi*, and endo- and ectoparasites by the supplier or by routine health monitoring of sentinels exposed to dirty bedding from the SCID mouse colony. Each control or experimental group was housed separately in microisolator cages, and all animal manipulations were performed in a class-II biological safety cabinet, using standard microisolation technique. Animals were housed in a biocontainment facility 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. The University of Arizona's Institutional Animal Care and Use Committee approved all animal procedures.

Mouse antibody production test. Six-week-old ICR mice were separated into groups of four animals each and administered ten-fold serial dilutions of Sendai virus Cantell strain (range, 8×10^3 to 8×10^{-1} $TCID_{50}$). Ten percent of each dose was administered oronasally, with the remainder injected intraperitoneally. Dose ranges were established from the endpoint dilution of the Sendai fnRT-PCR assay, along with the addition of 10-fold and 100-fold more and less concentrated dilutions. At postinoculation (PI) week 4, mice were euthanized by carbon dioxide inhalation, and blood was collected by cardiocentesis. Serum was diluted 1:5 in phosphate-buffered saline and was stored at –20°C until evaluated by use of indirect fluorescent antibody (IFA) serologic assays specific for Sendai virus.

Animal infections. Four-week-old scid mice were inoculated intranasally with 5×10^8 $TCID_{50}$ of PVM, 10^5 $TCID_{50}$ of Sendai virus, or mock inoculum (Eagle's medium containing 10 µg of ciprofloxacin hydrochloride/ml) and were euthanized on PI day 10 by use of carbon dioxide inhalation. Lung and trachea specimens were harvested from all animals, and RNA was extracted from approximately 30 mg of each tissue, using Qiagen's RNeasy kit and following the manufacturer's protocol. Alcohol swipe specimens of the animal cages were taken on PI days 1, 3, 5, 7, and 10, and RNA was extracted, using Qiagen's QIAamp Viral RNA kit.

Results

Sendai virus and PVM fnRT-PCR assays. Sequence alignment of the Sendai virus nucleocapsid protein (NP) and PVM attachment protein (G) gene sequences revealed highly conserved regions specific for each virus that were used to design the primers and probe for each assay (Table 1). Each primer/probe set was completely homologous to the Sendai virus and PVM strains evaluated, with the exception of a single nucleotide mismatch between the Sendai virus probe and Sendai virus Ohita strain. Primers were optimized at a concentration of 300 n*M* for the forward and reverse primers through use of checkerboard titrations of 50, 300, and 900 n*M* concentrations of each primer with 200 n*M* probe. Similarly, the probe was optimized at 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.

Table 1. Primer and probe sequences for Sendai virus and pneumonia virus of mice (PVM) fluorogenic nuclease reverse transcriptase-polymerase chain reaction (fnRT-PCR) assays

Assay	Sequence (5'-3')	Position (5'-3')
PVM		
Forward primer	AGATCACAGAGCCCGTCAAAAT	412-433[a]
Reverse primer	GCATATAACATCCAATACGAGTTTGAA	533-507
Probe	CAACACCTTCAGAGGATCCCTACCAATGCT	436-465
Sendai virus		
Forward primer	CAGAGGAGCACAGTCTCAGTGTTC	210-233[b]
Reverse primer	TCTCTGAGAGTGCTGCTTATCTGTGT	332-307
Probe	TGCATCATCAGTCACACTTGGGCCTAGTA	263-235

[a]Nucleotide positions from PVM No.15 sequence (GenBank accession number D11129).
[b]Nucleotide positions from Sendai virus Z strain sequence (GenBank accession number M30202).

Table 2. Relative sensitivity of the Sendai virus fnRT-PCR assay, compared with the mouse antibody production (MAP) test

Viral dilution ($TCID_{50}$)	FnRT-PCR[a]	MAP test[b]
8×10^3	+	4/4[c]
8×10^2	+	2/4
8×10^1	+	1/4
8×10^0	–	0/4
8×10^{-1}	–	0/4

[a]Positive result interpreted as a C_t value < 45.
[b]As detected by immunofluorescent antibody testing of serum.
[c]Number of mice positive/total number of mice tested.

Specificity and sensitivity. Assay specificity was determined by evaluation of RNA extracted from preparations of PVM, Sendai virus, MHV-A59, RCV-Parker, Reo-3, SV-5, LCMV, TMEV, rotavirus SA-11, SNV, BRSV-A, BRSV-B, CDV, and PIV-1. The Sendai virus assay detected only Sendai virus (Ct = 18.5), and the PVM assay detected only PVM (Ct = 14.8). Neither assay detected MHV-A59, RCV-Parker, Reo-3, SV-5, LCMV, TMEV, rotavirus SA-11, SNV, BRSV-A, BRSV-B, CDV, and PIV-1 (all Ct values > 45). The Sendai virus and PVM assays could detect as little as 10 fg and one picogram of RNA, respectively, through evaluation of serial dilutions of RNA extracts of virus-infected cell cultures (Figs. 1 and 2).

Comparison of fnRT-PCR and MAP tests. The relative sensitivities of the Sendai virus fnRT-PCR assay and MAP testing were determined (Table 2). Ten-fold dilutions of a Sendai viral preparation were evaluated directly by use of the fnRT-PCR assay, and the endpoint detection limit (8×10^1 $TCID_{50}$) was determined. A viral dose equivalent to the endpoint detection limit of the fnRT-PCR was then administered oronasally (20 µl) and intraperitoneally (180 µl) to each of four 6-week-old ICR mice. Similarly, 10- or 100-fold more and 10- or 100-fold less virus were administered to each of four 6-week-old ICR mice in each of four other groups to provide a range of five $\log_{10}$ dilutions of virus. Four weeks after inoculation, serum was harvested from the mice and was evaluated by use of IFA serologic analysis. Sendai virus was detected by fnRT-PCR and MAP testing at the three most concentrated dilutions that were evaluated.

Fluorogenic nuclease RT-PCR detection of Sendai virus and PVM RNA in experimentally infected C.B-17 scid mice. Four-week-old C.B-17 scid mice were inoculated intranasally with Sendai virus, PVM, or mock inoculum, then were euthanized at PI day 10. The RNA extracted from the trachea

Table 3. Fluorogenic nuclease RT-PCR results for four-week-old C.B-17 scid mice inoculated with PVM or Sendai virus

Virus dose ($TCID_{50}$)		Tissue harvest (PID 10) Lung[a]	Trachea[a]	Cage swipe[a]				
				PID 1	PID 3	PID 5	PID 7	PID 10
PVM	0	0/4[b]	0/4	0/1[c]	0/1	0/1	0/1	0/1
PVM	5×10^8	4/4	4/4	1/1	1/1	1/1	1/1	1/1
Sendai	0	0/4[b]	0/4	0/1[c]	0/1	0/1	0/1	0/1
Sendai	10^5	4/4	4/4	0/1	1/1	0/1	0/1	0/1

[a]PVM assay was used for PVM-inoculated mice; Sendai assay was used for the Sendai virus-inoculated mice; both assays were used for mock-inoculated mice.
[b]Number animals positive/number animals tested.
[c]Cage swipe analysis expressed as number of cages positive/number of cages tested.
$TCID_{50}$ = 50% tissue culture infective dose.

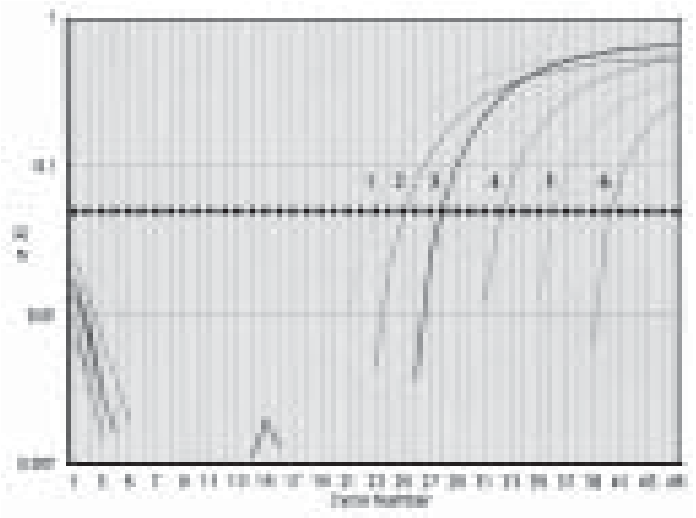

Figure 1. Sensitivity of the pneumonia virus of mice (PVM)-specific fluorogenic nuclease reverse transcriptase-polymerase chain reaction (fnRT-PCR) assay, as demonstrated by an amplification plot of ten-fold serially diluted viral RNA. Dilutions from left to right on the amplification plot (labeled 1 through 6) range from 100 ng to one picogram of total RNA. All values with mean fluorescence (Rn) > 0.05 and a cycle threshold (Ct) < 45 are considered positive results.

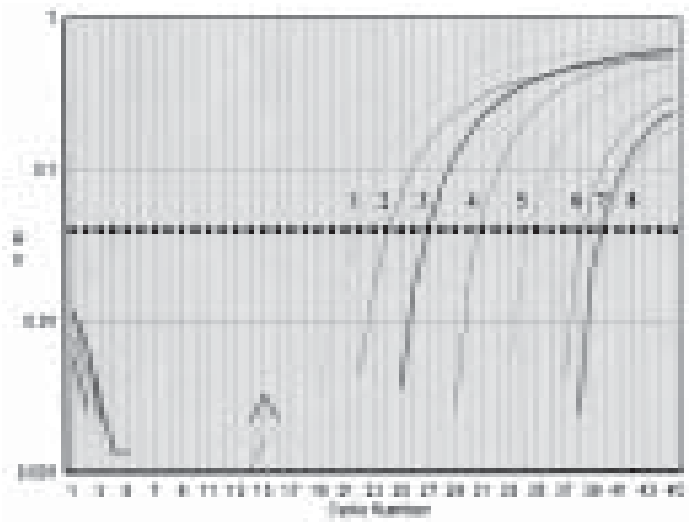

Figure 2. Sensitivity of the Sendai virus-specific fn RT-PCR assay, as demonstrated by an amplification plot of ten-fold serially diluted viral RNA. Dilutions from left to right on the amplification plot (labeled 1 through 8) range from 100 ng to 10 femtogram of total RNA. All values with mean fluorescence (Rn) > 0.05 and a cycle threshold (Ct) < 45 are considered positive results.

and lung of each mouse was evaluated by use of the Sendai virus and PVM fnRT-PCR assays. Sendai virus RNA was detected in the trachea and lung of all mice inoculated with Sendai virus at PI day 10 by use of the Sendai virus fnRT-PCR assay (Table 2). Similarly, PVM was detected in the trachea and lung of all mice inoculated with PVM virus at PI day 10 by use of the PVM fnRT-PCR assay (Table 2). Viral RNA indicative of Sendai virus or PVM infection was not detected in any of the age- and strain-matched mock-infected mice. The RNA extracted from cage swipe specimens that were collected on PI days 1, 3, 5, 7, and 10 also was evaluated by use of the fnRT-PCR assay (Table 3). The PVM fnRT-PCR assay could detect viral RNA in the cage swipe specimens at all time points from cages housing PVM-infected mice. The Sendai virus fnRT-PCR assay detected Sendai viral RNA on the day-3 cage swipe specimen from Sendai virus-infected mice. Viral RNA was not detected by either assay from any of the cage swipe specimens collected from mock-infected mice at identical time points.

Discussion

Fluorogenic nuclease RT-PCR assays were developed to specifically detect Sendai virus and PVM. The primers and probe for each assay were designed from highly conserved regions of the Sendai virus NP gene and the PVM attachment (G) gene, respectively. The assays were able to detect at least one picogram of targeted viral RNA and detected only the targeted virus when evaluated against a panel of rodent RNA viruses, indicating that the assays were sensitive and specific for Sendai virus and PVM, respectively.

When compared directly with the MAP test, the Sendai virus fnRT-PCR had sensitivity comparable to that of the MAP test. Although fnRT-PCR assays do not distinguish between infective and non-infective virions, the detection of virus-specific RNA would reliably indicate biological material exposure to Sendai virus. Fluorogenic nuclease RT-PCR also has substantially reduced turnaround time, provides an alternative to a whole animal bioassay, and is less costly than MAP testing. Given the potential of Sendai virus contamination of biological materials (3), fluorogenic nuclease assays should provide an accurate, cost-effective, and timely means of screening these materials for Sendai virus. Although the PVM assay could also be useful as an alternative to MAP testing, to the authors' knowledge, there have been no published reports of PVM contamination of biological materials; therefore, direct comparison between the PVM fnRT-PCR assay and MAP testing was not performed.

Fluorogenic nuclease RT-PCR offers several advantages as an adjunct diagnostic method for routine rodent health monitoring. Since fnRT-PCR does not rely on an induced immune response

to detect infection, it can be used to directly detect Sendai virus and PVM in tissues obtained from immunocompromised rodents, as indicated by detection of viral RNA in tissues obtained from experimentally infected scid mice in these studies. This could be especially useful when wasting is observed in immunodeficient mice as this syndrome can be observed secondary to a number of viral infections, including Sendai virus and PVM (2). Fluorogenic nuclease PCR may also be suited for ante-mortem diagnostic evaluation. The Sendai and PVM fnRT-PCR assays could detect viral RNA in cage swipe specimens collected from the cages of experimentally inoculated mice, with PVM consistently detected over a 10-day period and Sendai virus only detected at day 3. The PVM RNA was not detected in cage swipe specimens from scid mice administered a lower dose of inoculum, indicating that this lower dose may not have resulted in productive infection and subsequent shedding (data not shown). The detection of Sendai virus only at day 3 suggests this may be residual inoculum or transient low-level shedding, and perhaps a higher dose was needed to induce persistent shedding. Although consistent ante-mortem detection of these viruses in individual cages over long periods might be difficult due to acute and/or intermittent viral shedding, detection within a mouse colony could potentially be accomplished by random sampling or evaluation of pooled samples. A reliable, non-invasive approach toward ante-mortem testing would be advantageous for rodent colony health monitoring, particularly among animals involved in long-term studies.

In conclusion, the Sendai virus and PVM fnRT-PCR assays provide sensitive, specific, and quantitative assays for detection of Sendai virus and PVM in laboratory rodents, environmental samples, and contaminated biological materials. These assays are 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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