Exp. Anim. 68(2), 169–176, 2019

—Original—

A semi-nested RT-PCR assay for detection of norovirus in rat fecal samples

Josélia Cristina de Oliveira MOREIRA[1)], Daniele Masselli RODRIGUES[1)], Rovilson GILIOLI[1)], Fernando Ferreira COSTA[2)] and Dulcinéia Martins ALBUQUERQUE[2)]

[1)]*Cidade Universitária "Zeferino Vaz" Barão Geraldo, 250, 5 de Junho Street, Campinas - São Paulo 13083-970, Brazil*
[2)]*Cidade Universitária "Zeferino Vaz" Barão Geraldo, 480 Carlos Chagas Street, Campinas - São Paulo 13083-878, Brazil*

Abstract: Norovirus is a highly prevalent pathogen that can infect a wide range of host species. Thus far, there have only been two reports of norovirus infection in rats. Diagnostic assays for the detection of norovirus are well established, but a specific molecular assay for the diagnosis of norovirus infection in laboratory rats has not yet been reported. In this study, we describe the development of a sensitive, semi-nested RT-PCR assay for detection of norovirus in fecal samples from *Rattus norvegicus,* reared in animal facilities under different sanitary barrier conditions. Additionally, we describe the first report of the presence of norovirus in rat colonies from Brazilian animal facilities.
Key words: Brazil, norovirus, rats, semi-nested

Introduction

Norovirus (NoV) is a non-enveloped single-strand positive-sense RNA virus, with a 7.5 kb genome that is divided into three open reading frames (ORFs). ORF1 encodes a large polyprotein that includes the viral RNA-dependent RNA polymerase, and ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively [6, 51, 53]. A novel open reading frame overlapping ORF2, designated ORF4, is only present in murine NoV (MNV), and encodes the protein virulence factor 1 (VF1) [27].

NoV is a member of *Caliciviridae* family, which is classified into six genogroups (G) according to their capsid gene sequence [53]. Human NoV (HuNoV) belongs to the groups GI, GII, and GIV [5, 6], porcine NoV belongs to GII genogroup, and bovine NoV belongs to GIII [32, 42]. The feline and canine NoVs are members of the GIV and GVI genogroups, respectively [26, 49], while MNV belongs to GV [15].

Currently, HuNoV is a significant viral agent that is able to cause acute gastroenteritis in people of all ages [37, 39]. However, even though HuNoV is recognized as a worldwide pathogen [1, 2, 8, 12, 16, 22, 50], the establishment of *in vitro* propagation methods have thus far been unsuccessful [23, 43], although recent progress in the cultivation of HuNoV has been described [13, 14]. Additionally, the prototype murine NoV strain, MNV-1, has been used as a model to better understand the molecular mechanisms and pathogenicity of NoVs [45, 52].

MNV was first described by Karst *et al.* in 2003, in a strain of laboratory mice that was both $RAG2^{-/-}$ (recombination activating gene 2) and $STAT1^{-/-}$ (signal transducer and activator of transcription 1) [15]. MNV is a highly prevalent laboratory mouse pathogen [11, 17, 31, 38, 40] that can cause persistent infection in many dif-

(Received 22 March 2018 / Accepted 11 November 2018 / Published online in J-STAGE 7 December 2018)
Address corresponding: J.C.O. Moreira. e-mail: jocris@unicamp.br

ferent mouse strains [10].

Taken advantage of the fact that NoV can infect a broad range of hosts, several studies have been done to identify and characterize NoV in laboratory rats. In 2012, Smith *et al.* were unable to detect MNV related viruses in different rodent species using a molecular and multiple degenerate primers [41]. In attempt to demonstrate infection of rats with NoV, other researchers used serological tests as dot blot assays, multiplexed fluorometric immunoassays (MFIA), and indirect immunofluorescence assays (IFA) to investigate the presence of MNV antigens in a colony of Sprague-Dawley rats. Although the serological tests had been performed, the molecular characterization and virus isolation methods were unsuccessful [11].

The infection of rat strains with NoV was initially reported by Japanese researchers, who demonstrated the NoV infection of wild rodents belonging to the species *Rattus rattus and Apodemus speciosus* [48]. Phylogenetic analysis of the NoV nucleotide sequences in this case showed that different viruses infected two wild rodent species, suggesting that the genetic diversity of NoV is associated with host species and geographic distribution [41]. The complete genome sequences of two novel NoVs, JX486101 and JX486102, isolated from brown rats (*Rattus norvergicus*), were subsequently deposited in GenBank [47]. These two sequences were shown to have 91.4% identity and were approximately 7541 bases in length. This report confirmed that the organization of the rat NoV genome is similar to that of other NoV.

In this way, the current study aimed to develop a sensitive semi-nested RT-PCR assay for detection of NoV in fecal samples from laboratory rats (*Rattus norvegicus*) in Brazilian animal facilities.

Material and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of National Council on Animal Experimentation (CONCEA) from Science and Technology Ministry (MCTI) from Brazil. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Campinas (CEUA - UNICAMP), under the protocol number 2901-1.

Fecal samples

One hundred and eight fecal samples from laboratory rats, from 23 Brazilian animal facilities, were collected in the Laboratory of Animal Health at the Multidisciplinary Center for Biological Research on Laboratory Animal Science (CEMIB). Fecal samples were tested for the presence of NoV as part of a health monitoring program. Seventeen of the 23 facilities kept laboratory rats under conventional conditions, while the other 6 facilities maintained rats in specific pathogen-free (SPF) barrier conditions. Four wild rat fecal samples were also obtained and included in the study.

Prior to nucleic acid extraction, a 20% (w/v) fecal suspension was prepared. Feces were weighed, dissolved in 0.01 M PBS (pH 7.2), and centrifuged at 6,000 g for 10 min. The supernatant was collected and stored at −80°C until use.

RNA extraction

RNA was extracted from the previous prepared 20% fecal suspension using a QIamp Viral RNA mini kit (QIAgen® Inc., Valencia, California) according to the manufacturer's instructions, and stored at −80°C until use.

Primer design

Primer sequences that were complementary to the highly conserved region of the capsid gene (VP1) were designed, using an alignment of the sequences from MNV-1 (GenBank accession: AY228235) [11] and rat NoV (JX486101 and JX486102**) [47]. Primer pairs are summarized in Table 1.

Reverse transcription PCR (RT-PCR) and semi-nested PCR

RT-PCR was performed using a OneStep RT-PCR Kit (QIAgen® Inc., Valencia, California) according to the manufacturer's recommended protocol. Each 25 μl reaction volume included: 0.2 μM of each primer, 1× OneStep RT-PCR buffer, 0.4 mM of dNTPs, 10 U/μl of RNase Inhibitor, 1× Q-solution buffer, and 1 μl of enzyme mix. The cycling conditions were as follow: 50°C for 30 min; DNA polymerase activation at 96°C for 2 min; 40 cycles of amplification, with an initial denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 1 min.

The product of the first reaction was used as template in the semi-nested PCR reaction, which was performed

Table 1. RT-PCR and semi-nested PCR primer sequences

Primer	Sequence (5' - 3')	Position**	Amplicon size
First Reaction	Forward AAATTTTGTCCAGTG*YCCCCTT	5271–5292	199bp
	Reverse AACCACCTTGCCAGCAGTAA	5469–5450	
Semi-Nested PCR	Forward TTCACCGTCTCCCCAAGAA	5299–5317	171bp
	Reverse AACCACCTTGCCAGCAGTAA	5469–5450	

IUB code: *Y=C or T **(JX486101 and JX486102)

Table 2. Reference norovirus sequences used in this study

Prototype strains	Accession No (GenBank)	Genogroup/ species
Rn/GV/HKU_CT2/HKG/2011	JX486101	Rat
Rn/GV/HKU_KT/HKG/2012	JX486102	Rat
Hu/GI.1/CHA6A003_20091104/2009/USA	KF039737	GI (Human)
Swine/GII/OH-QW125/03/US	AY823305	GII (Swine)
Hu/NLV/GII/Neustrelitz260/2000/DE	AY772730	GII (Human)
Bo/Newbury2/1976/UK	AF097917	GIII (Bovine)
cat/GIV.2/CU081210E/USA/2010	JF781268	GIV (Feline)
Hu/GIV.1/LakeMacquarie/NSW268O/2010/AU	JQ613567	GIV (Human)
Mu/NoV/GV/MNV1/2002/USA	AY228235	GV (Murine)
Murine norovirus 2 (MNV-2)	DQ223041	GV (Murine)
Murine norovirus 3 (MNV-3)	DQ223042	GV (Murine)
Murine norovirus 4 (MNV-4)	DQ223043	GV (Murine)
Murine norovirus 5 (MNV-5)	EF650480	GV (Murine)
Murine norovirus 6 (MNV-6)	EF650481	GV (Murine)
Murine norovirus 7 (MNV-7)	GQ180108	GV (Murine)
MNV/Brasilia-DF/Pr+Ob/2012	KF976714	GV (Murine)
Murine norovirus strain TF3PLM capsid protein VP1	JQ408727	GV (Murine)
Murine norovirus strain TF7WM capsid protein VP1	JQ408738	*Apodemus agrarius*
Murine norovirus isolate Mu/NoV/Apo455/UK/2007	JN975491	*Apodemus sylvaticus*
dog/GVI.1/HKU_Ca035F/2007/HKG	FJ692501	GVI (Canine)
Norwalk virus	M87661	Outgroup

using the PCR conditions described above. All the reactions were performed in a GenePro 96G thermal cycler (Hangzhou Bori Technology Co., Ltd., BioER). The PCR amplicons were analyzed by agarose gel electrophoresis, stained with GelRed™ nucleic acid stain (Uniscience, Hayward-CA), and visualized using a Geldoc-It™ imaging system (UVP, Cambridge).

Cloning and sequencing

The amplified products were cloned into a pGEM®-T Easy Vector System (Promega Corporation, Madison-USA). The cloned products were purified, and sequenced in both directions with semi-nested primers by capillary electrophoresis using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Sequencing Analysis Software v5.4 (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

The sequencing chromatograms were visualized using the BioEdit sequence alignment editor program version 7.2.5, [7] and the sequences were analyzed in the NCBI Entrez Nucleotide browser (www.ncbi.nlm.nih.gov/sites/entrez). A consensus sequence was created and assembled using the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). The amplicons obtained in this study were aligned using the ClustalW algorithm within the BioEdit software (1,000 bootstrap replicates) and compared with the reference nucleotide sequences from GenBank that are listed in Table 2.

Results

Using conventional RT-PCR, no positive results were obtained, even with specific primers. A semi-nested RT-PCR protocol, producing an amplicon 171 bp in length, was therefore established. Twelve of the 108 fecal samples (11%), obtained from 6 different animal facili-

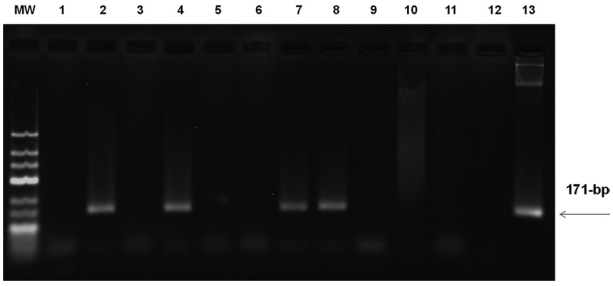

Fig. 1. Detection of Rat NoV in fecal samples by semi-nested RT-PCR. A 2% agarose gel was stained with GelRed™. The 171 bp amplicons from semi-nested RT-PCR are shown. MW 50 bp. Lanes 1–11: amplified products from 11 rat fecal samples. Lane 12: negative control (DEPC-treated water). Lane 13: positive control (plasmid).

ties (33% SPF and 67% conventional) were NoV positive by semi-nested PCR (Fig. 1). Furthermore, all of the wild rat samples were negative for NoV.

All the NoV-positive amplicons were sequenced and given the unique identifiers LCQS Unib1 through 12. Molecular analysis of these amplicons revealed 98% nucleotide identity with two prototype strains of rat NoV (JX486101 and JX486102) and presented higher identity, 38–82% with GV prototypes. In the other NoV genogroups the identity values ranged between 3% and 31%, as shown in the identity matrix (table 3).

The VP1 capsid sequences for all the prototype strains and our amplicons were aligned, and two single base substitutions were revealed in all 12 amplicons. These two substitutions, at positions 5334 (C to T) and 5343 (T to C) did not however represent a structural change in the protein.

A phylogenetic tree was constructed using a member of the *Caliciviridae* family, Norwalk virus (M87661), as outgroup. In constructing the phylogenetic tree the neighbor-joining method (1,000 replicates) was applied in the Molecular Evolutionary Genetic Analysis (MEGA 6) software package [44], with settings to account for nucleotide substitution rates and the pairwise deletion of gaps (Fig. 2).

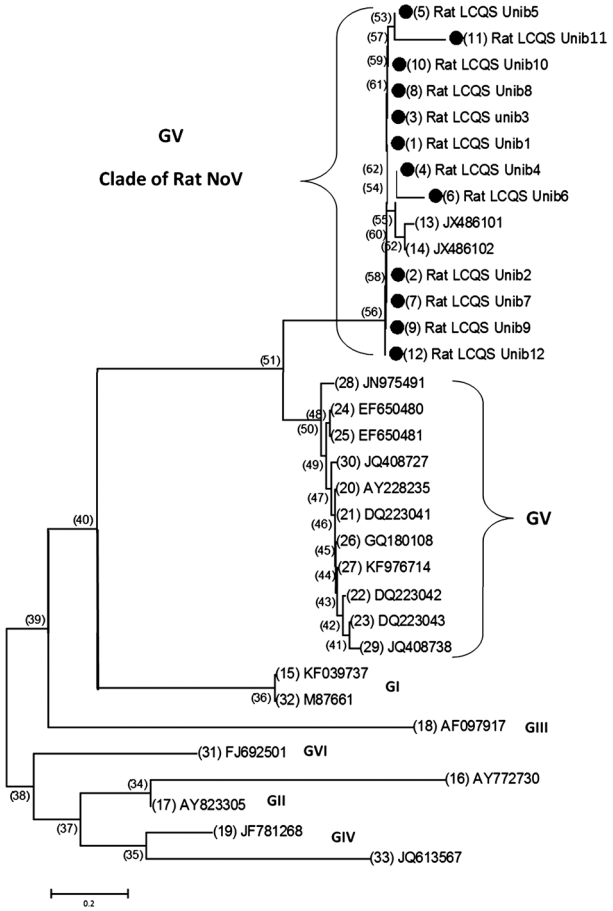

Fig. 2. Phylogenetic tree of NoV. The tree was constructed using 132 nt of the capsid protein VP1 sequences from genogroups GI to GVI and the amplicons Rat LCQS Unib1 to 12. The phylogenetic tree was generated using neighbor joining method in the MEGA 6.06 software package with bootstrap analysis (1,000 replicates). Norwalk virus (M87661) were used as outgroup.

Discussion

Methods for detection NoV have been developed, and are well-established in laboratories of animal health monitoring [3, 4, 36]. MNV has been described as the most prevalent viral agent in mice colonies [10, 11, 17, 20, 21, 25, 40], and NoV infection of laboratory animals has been diagnosed using RT-PCR [11, 28], qRT-PCR [9], enzyme-linked immunosorbent assay (ELISA) [17, 18] and multiplexed fluorescent immunoassay [11]. Currently, nested and semi-nested PCR is the most sensitive diagnostic assay for the detection of low concentrations of RNA virus, and is mainly used for stool and environmental samples [19]. In this study, we have developed a semi-nested RT-PCR protocol for the detection of NoV in fecal samples from laboratory rats.

Although we used specific primers, we obtained a low sensitivity of detection for NoV, probably because of the low concentration of viral RNA in our samples. Conversely, using the semi-nested RT-PCR method, we found that 12 of the 108 fecal samples contained NoV, a positivity rate of 11%. All of the positive samples came

Table 3. Identity matrix

Genogroup	Seq->	Rat LCQS Unib1	Rat LCQS Unib2	Rat LCQS Unib3	Rat LCQS Unib4	Rat LCQS Unib5	Rat LCQS Unib6	Rat LCQS Unib7	Rat LCQS Unib8	Rat LCQS Unib9	Rat LCQS Unib10	Rat LCQS Unib11	Rat LCQS Unib12
	Rat LCQS Unib1	*	100%	100%	98%	56%	89%	100%	100%	99%	99%	93%	95%
	Rat LCQS Unib2	100%	*	100%	98%	56%	89%	100%	100%	99%	99%	93%	95%
	Rat LCQS Unib3	100%	100%	*	98%	56%	89%	100%	100%	99%	99%	93%	95%
	Rat LCQS Unib4	98%	98%	98%	*	56%	91%	98%	98%	98%	98%	92%	94%
	Rat LCQS Unib5	56%	56%	56%	56%	*	48%	56%	56%	56%	56%	52%	57%
	Rat LCQS Unib6	89%	89%	89%	91%	48%	*	89%	89%	88%	89%	84%	85%
	Rat LCQS Unib7	100%	100%	100%	98%	56%	89%	*	100%	99%	99%	93%	95%
	Rat LCQS Unib8	100%	100%	100%	98%	56%	89%	100%	*	99%	99%	93%	95%
	Rat LCQS Unib9	99%	99%	99%	98%	56%	88%	99%	99%	*	98%	93%	95%
	Rat LCQS Unib10	99%	99%	99%	98%	56%	89%	99%	99%	98%	*	93%	95%
	Rat LCQS Unib11	93%	93%	93%	92%	52%	84%	93%	93%	93%	93%	*	89%
	Rat LCQS Unib12	95%	95%	95%	94%	57%	85%	95%	95%	95%	95%	89%	*
Rat NoV	JX486101	96%	96%	96%	98%	54%	88%	96%	96%	95%	95%	90%	92%
Rat NoV	JX486102	97%	97%	97%	98%	55%	89%	97%	97%	96%	96%	90%	92%
GI (Human)	KF039737	6%	6%	6%	5%	3%	5%	6%	6%	6%	6%	5%	5%
GII (Human)	AY772730	5%	5%	5%	5%	3%	5%	5%	5%	5%	5%	5%	5%
GII (Swine)	AY823305	5%	5%	5%	5%	3%	5%	5%	5%	5%	5%	5%	5%
GIII (Bovine)	AF097917	31%	31%	31%	30%	17%	29%	31%	31%	30%	30%	29%	29%
GIV (Feline)	JF781268	5%	5%	5%	5%	3%	5%	5%	5%	5%	5%	4%	4%
GIV Human)	JQ613567	5%	5%	5%	5%	3%	5%	5%	5%	5%	5%	5%	5%
GV (Murine)	AY228235	75%	75%	75%	77%	44%	69%	75%	75%	75%	75%	69%	72%
GV (Murine)	DQ223041	77%	77%	77%	78%	48%	71%	77%	77%	77%	76%	70%	75%
GV (Murine)	DQ223042	63%	63%	63%	64%	38%	62%	63%	63%	63%	62%	58%	61%
GV (Murine)	DQ223043	76%	76%	76%	77%	46%	71%	76%	76%	76%	75%	69%	74%
GV (Murine)	EF650480	77%	77%	77%	77%	48%	71%	77%	77%	77%	77%	71%	76%
GV (Murine)	EF650481	77%	77%	77%	77%	48%	71%	77%	77%	77%	77%	71%	76%
GV (Murine)	GQ180108	79%	79%	79%	79%	47%	72%	79%	79%	79%	78%	72%	77%
GV (Murine)	KF976714	78%	78%	78%	78%	47%	71%	78%	78%	78%	77%	72%	76%
GV (Murine)	JQ408727	80%	80%	80%	82%	45%	75%	80%	80%	80%	80%	74%	76%
GVI (Canine)	FJ692501	5%	5%	5%	5%	3%	5%	5%	5%	5%	5%	5%	5%
A. Sylvaticus	JN975491	67%	67%	67%	68%	40%	66%	67%	67%	67%	66%	61%	65%
A. agrarius	JQ408738	77%	77%	77%	78%	46%	71%	77%	77%	77%	76%	70%	75%
outgroup	M87661	6%	6%	6%	5%	3%	5%	6%	6%	6%	5%	5%	5%

*100% identity

from Brazilian animal facilities that kept animals either under a sanitary protection barrier system or in conventional environmental conditions. These results indicate that NoV is circulating in rat facilities, and that it can infect animals that are maintained in different sanitary conditions.

In the first report about the occurrence of MNV-like virus in wild rodents, Tsunesumi *et al.* found a 14.9% positivity rate from 47 analyzed fecal samples [48]. Conversely, we were unable to detect NoV in wild rat samples, which could be the result of either the geographic localization and distribution of these animals in Brazil, or the small sample size used here as already related by other researchers [41].

Molecular analysis of the sequences these amplicons were also compared with those from the prototype strains from each of the NoV genogroups (GI-GVI), and in all twelve cases single base substitutions from either C to T, or T to C, were observed between our amplicons and rat NoV references. While these base changes were not predicted to lead to amino acid substitutions, these polymorphisms probably describe the independent evolution of NoV strains [30]. The recombination of single-stranded RNA viruses, allowing the emergence of new viral strains, is known to occur often in the *Caliciviridae* family [29]. Furthermore, due to the high frequency of recombination at the ORF1-ORF2 junction in VP1 capsid protein or RNA-dependent RNA polymerase NoV sequences, several researchers recommend the phylogenetic analysis of the complete NoV genome [29, 46].

However, in this study, a phylogenetic analysis was performed based on an alignment of the NoV VP1 sequences. It allowed us to cluster all of the amplicons obtained from the rat samples within a subgroup of geno-

group V. Consistent with previous reports [47, 48, 51], we suggest that rat NoV comprises a new cluster within genogroup V.

This is the first report of the development of a semi-nested RT-PCR assay for the identification of NoV in laboratory rats (*Rattus norvegicus*) in Brazil, and the first evidence of the prevalence of this viral agent in Brazilian animal facilities. Previously, rat NoV had only been described in *Rattus rattus* and *Apodemus speciosus* [48].

At the present time, it is not clear what impact the presence of NoV has on rat colonies, and further studies are required to better understand both the pathogenesis of NoV and its impact on research using rat models.

As already described, laboratory mouse colonies have also a high prevalence of MNV [10, 11, 17, 20, 21, 25, 40]. A previous report of our workgroup has demonstrated that most of the Brazilian animal facilities are dealing with MNV-infected mice [40].

Since it has an impact on the experimental results [33–35] is primordial a regular health monitoring program for this agent. FELASA (Federation for Laboratory Animal Science) has recommended a routine screening for most of the commons mouse and rat infectious agents [24]. Rat NoV is not yet been included in FELASA list and based in our results we strongly recommend that rat NoV should be included in this list and also in all health monitoring programs.

In conclusion, we have developed a semi-nested RT-PCR assay, and have shown this to be an useful method allowing us to detect NoV in fecal samples from laboratory rats.

Acknowledgments

We would like to thank our colleagues at the Laboratory of Animal Health Monitoring in the Multidisciplinary Center for Biological Research on Laboratory Animal Science (CEMIB), University of Campinas, São Paulo, Brazil. In particular, we thank our veterinarian Clarice Yukari Minagawa Issei, our technologist Euma da Cunha Ramos Silva, and our biologists Robson da Silva Pontes, Lenira Aparecida Guaraldo de Andrade and Silvio Rogerio Cardozo dos Santos and Jhenifer Alves de Camargo for technical support. We also thank the team at the Hematology and Hemotherapy Center for assistance with molecular methodologies.

References

1. Centers for Disease Control and Prevention (CDC) 2009. Surveillance for foodborne disease outbreaks - United States, 2006. *MMWR Morb. Mortal. Wkly. Rep.* 58: 609–615. [Medline]
2. Doyle, T.J., Stark, L., Hammond, R. and Hopkins, R.S. 2009. Outbreaks of noroviral gastroenteritis in Florida, 2006-2007. *Epidemiol. Infect.* 137: 617–625. [Medline] [CrossRef]
3. Farkas, T., Fey, B., Keller, G., Martella, V. and Egyed, L. 2012. Molecular detection of murine noroviruses in laboratory and wild mice. *Vet. Microbiol.* 160: 463–467. [Medline] [CrossRef]
4. Goto, K., Hayashimoto, N., Yasuda, M., Ishida, T., Kameda, S., Takakura, A. and Itoh, T. 2009. Molecular detection of murine norovirus from experimentally and spontaneously infected mice. *Exp. Anim.* 58: 135–140. [Medline] [CrossRef]
5. Green, K. and Caliciviridae, Y. The Noroviruses, 5th ed., pp. 949–979. *In:* Field's Virology (Field, B.N., Knipe, D.M. and Howley, P.M. eds) Wolters Kluwer Health, Lippincott Williams and Wilkins, Philadelphia.
6. Green, K.Y., Ando, T., Balayan, M.S., Berke, T., Clarke, I.N., Estes, M.K., Matson, D.O., Nakata, S., Neill, J.D., Studdert, M.J. and Thiel, H.J. 2000. Taxonomy of the caliciviruses. *J. Infect. Dis.* 181:(Suppl 2): S322–S330. [Medline] [CrossRef]
7. Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95–98.
8. Hamano, M., Kuzuya, M., Fujii, R., Ogura, H. and Yamada, M. 2005. Epidemiology of acute gastroenteritis outbreaks caused by Noroviruses in Okayama, Japan. *J. Med. Virol.* 77: 282–289. [Medline] [CrossRef]
9. Hanaki, K., Ike, F., Kajita, A., Yasuno, W., Yanagiba, M., Goto, M., Sakai, K., Ami, Y. and Kyuwa, S. 2014. A broadly reactive one-step SYBR Green I real-time RT-PCR assay for rapid detection of murine norovirus. *PLoS One* 9: e98108. [Medline] [CrossRef]
10. Henderson, K.S. 2008. Murine norovirus, a recently discovered and highly prevalent viral agent of mice. *Lab Anim. (NY)* 37: 314–320. [Medline] [CrossRef]
11. Hsu, C.C., Wobus, C.E., Steffen, E.K., Riley, L.K. and Livingston, R.S. 2005. Development of a microsphere-based serologic multiplexed fluorescent immunoassay and a reverse transcriptase PCR assay to detect murine norovirus 1 infection in mice. *Clin. Diagn. Lab. Immunol.* 12: 1145–1151. [Medline]
12. Huhti, L., Szakal, E.D., Puustinen, L., Salminen, M., Huhtala, H., Valve, O., Blazevic, V. and Vesikari, T. 2011. Norovirus GII-4 causes a more severe gastroenteritis than other noroviruses in young children. *J. Infect. Dis.* 203: 1442–1444. [Medline] [CrossRef]
13. Jones, M.K., Grau, K.R., Costantini, V., Kolawole, A.O., de Graaf, M., Freiden, P., Graves, C.L., Koopmans, M., Wallet, S.M., Tibbetts, S.A., Schultz-Cherry, S., Wobus, C.E., Vinjé, J. and Karst, S.M. 2015. Human norovirus culture in B cells. *Nat. Protoc.* 10: 1939–1947. [Medline] [CrossRef]
14. Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes,

L.R., Grau, K.R., Gonzalez-Hernandez, M.B., Iovine, N.M., Wobus, C.E., Vinjé, J., Tibbetts, S.A., Wallet, S.M. and Karst, S.M. 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. *Science* 346: 755–759. [Medline] [CrossRef]
15. Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. and Virgin, H.W. 4th. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. *Science* 299: 1575–1578. [Medline] [CrossRef]
16. Karst, S.M. 2010. Pathogenesis of noroviruses, emerging RNA viruses. *Viruses* 2: 748–781. [Medline] [CrossRef]
17. Kim, J.R., Seok, S.H., Kim, D.J., Baek, M.W., Na, Y.R., Han, J.H., Kim, T.H., Park, J.H., Turner, P.V., Chung, D.H. and Kang, B.C. 2011. Prevalence of murine norovirus infection in Korean laboratory animal facilities. *J. Vet. Med. Sci.* 73: 687–691. [Medline] [CrossRef]
18. Kitagawa, Y., Tohya, Y., Ike, F., Kajita, A., Park, S.J., Ishii, Y., Kyuwa, S. and Yoshikawa, Y. 2010. Indirect ELISA and indirect immunofluorescent antibody assay for detecting the antibody against murine norovirus S7 in mice. *Exp. Anim.* 59: 47–55. [Medline] [CrossRef]
19. Kitajima, M., Tohya, Y., Matsubara, K., Haramoto, E., Utagawa, E. and Katayama, H. 2010. Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. *Lett. Appl. Microbiol.* 51: 119–121. [Medline]
20. Kitajima, M., Oka, T., Takagi, H., Tohya, Y., Katayama, H., Takeda, N. and Katayama, K. 2010. Development and application of a broadly reactive real-time reverse transcription-PCR assay for detection of murine noroviruses. *J. Virol. Methods* 169: 269–273. [Medline] [CrossRef]
21. Kitajima, M., Oka, T., Tohya, Y., Katayama, H., Takeda, N. and Katayama, K. 2009. Development of a broadly reactive nested reverse transcription-PCR assay to detect murine noroviruses, and investigation of the prevalence of murine noroviruses in laboratory mice in Japan. *Microbiol. Immunol.* 53: 531–534. [Medline] [CrossRef]
22. Koo, H.L., Ajami, N., Atmar, R.L. and DuPont, H.L. 2010. Noroviruses: The leading cause of gastroenteritis worldwide. *Discov. Med.* 10: 61–70. [Medline]
23. Marshall, J.A., Hellard, M.E., Sinclair, M.I., Fairley, C.K.F., Cox, B.J., Catton, M.G., Kelly, H. and Wright, P.J. 2004. Failure to detect norovirus in a large group of asymptomatic individuals. *Public Health* 118: 230–233. [Medline] [CrossRef]
24. Mähler Convenor, M., Berard, M., Feinstein, R., Gallagher, A., Illgen-Wilcke, B., Pritchett-Corning, K., Raspa, M., FELASA working group on revision of guidelines for health monitoring of rodents and rabbits 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab. Anim.* 48: 178–192. [Medline] [CrossRef]
25. Manjunath, S., Kulkarni, P.G., Nagavelu, K., Samuel, R.J., Srinivasan, S., Ramasamy, N., Hegde, N.R. and Gudde, R.S. 2015. Sero-prevalence of rodent pathogens in India. *PLoS One* 10: e0131706. [Medline] [CrossRef]
26. Martella, V., Lorusso, E., Decaro, N., Elia, G., Radogna, A., D'Abramo, M., Desario, C., Cavalli, A., Corrente, M., Camero, M., Germinario, C.A., Bányai, K., Di Martino, B., Marsilio, F., Carmichael, L.E. and Buonavoglia, C. 2008. Detection and molecular characterization of a canine norovirus. *Emerg. Infect. Dis.* 14: 1306–1308. [Medline] [CrossRef]
27. McFadden, N., Bailey, D., Carrara, G., Benson, A., Chaudhry, Y., Shortland, A., Heeney, J., Yarovinsky, F., Simmonds, P., Macdonald, A. and Goodfellow, I. 2011. Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. *PLoS Pathog.* 7: e1002413. [Medline] [CrossRef]
28. Moser, M.J., DiFrancesco, R.A., Gowda, K., Klingele, A.J., Sugar, D.R., Stocki, S., Mead, D.A. and Schoenfeld, T.W. 2012. Thermostable DNA polymerase from a viral metagenome is a potent RT-PCR enzyme. *PLoS One* 7: e38371. [Medline] [CrossRef]
29. Müller, B., Klemm, U., Mas Marques, A. and Schreier, E. 2007. Genetic diversity and recombination of murine noroviruses in immunocompromised mice. *Arch. Virol.* 152: 1709–1719. [Medline] [CrossRef]
30. Obara, M., Hasegawa, S., Iwai, M., Horimoto, E., Nakamura, K., Kurata, T., Saito, N., Oe, H. and Takizawa, T. 2008. Single base substitutions in the capsid region of the norovirus genome during viral shedding in cases of infection in areas where norovirus infection is endemic. *J. Clin. Microbiol.* 46: 3397–3403. [Medline] [CrossRef]
31. Ohsugi, T., Matsuura, K., Kawabe, S., Nakamura, N., Kumar, J.M., Wakamiya, M., Morikawa, S. and Urano, T. 2013. Natural infection of murine norovirus in conventional and specific pathogen-free laboratory mice. *Front. Microbiol.* 4: 12. [Medline] [CrossRef]
32. Oliver, S.L., Dastjerdi, A.M., Wong, S., El-Attar, L., Gallimore, C., Brown, D.W.G., Green, J. and Bridger, J.C. 2003. Molecular characterization of bovine enteric caliciviruses: a distinct third genogroup of noroviruses (Norwalk-like viruses) unlikely to be of risk to humans. *J. Virol.* 77: 2789–2798. [Medline] [CrossRef]
33. Paik, J., Fierce, Y., Mai, P.O., Phelps, S.R., McDonald, T., Treuting, P., Drivdahl, R., Brabb, T., LeBoeuf, R., O'Brien, K.D. and Maggio-Price, L. 2011. Murine norovirus increases atherosclerotic lesion size and macrophages in Ldlr(-/-) mice. *Comp. Med.* 61: 330–338. [Medline]
34. Paik, J., Kwok, F., Seamons, A., Brabb, T., Kim, J., Sullivan, B., Hsu, C., O'Brien, K.D. and Maggio-Price, L. 2015. Effects of murine norovirus on atherosclerosis in ldlr(-/-) mice depends on the timing of infection. *Comp. Med.* 65: 114–122. [Medline]
35. Paik, J., Fierce, Y., Drivdahl, R., Treuting, P.M., Seamons, A., Brabb, T. and Maggio-Price, L. 2010. Effects of murine norovirus infection on a mouse model of diet-induced obesity and insulin resistance. *Comp. Med.* 60: 189–195. [Medline]
36. Pang, X. and Lee, B.E. 2015. Laboratory diagnosis of noroviruses: present and future. *Clin. Lab. Med.* 35: 345–362. [Medline] [CrossRef]
37. Patel, M.M., Hall, A.J., Vinjé, J. and Parashar, U.D. 2009. Noroviruses: a comprehensive review. *J. Clin. Virol.* 44: 1–8. [Medline] [CrossRef]
38. Perdue, K.A., Green, K.Y., Copeland, M., Barron, E., Man-

del, M., Faucette, L.J., Williams, E.M., Sosnovtsev, S.V., Elkins, W.R. and Ward, J.M. 2007. Naturally occurring murine norovirus infection in a large research institution. *J. Am. Assoc. Lab. Anim. Sci.* 46: 39–45. [Medline]

39. Rockx, B., De Wit, M., Vennema, H., Vinjé, J., De Bruin, E., Van Duynhoven, Y. and Koopmans, M. 2002. Natural history of human calicivirus infection: a prospective cohort study. *Clin. Infect. Dis.* 35: 246–253. [Medline] [CrossRef]
40. Rodrigues, D.M., Moreira, J.C.O., Lancellotti, M., Gilioli, R. and Corat, M.A.F. 2017. Murine norovirus infection in Brazilian animal facilities. *Exp. Anim.* 66: 115–124. [Medline] [CrossRef]
41. Smith, D.B., McFadden, N., Blundell, R.J., Meredith, A. and Simmonds, P. 2012. Diversity of murine norovirus in wild-rodent populations: species-specific associations suggest an ancient divergence. *J. Gen. Virol.* 93: 259–266. [Medline] [CrossRef]
42. Sugieda, M. and Nakajima, S. 2002. Viruses detected in the caecum contents of healthy pigs representing a new genetic cluster in genogroup II of the genus "Norwalk-like viruses". *Virus Res.* 87: 165–172. [Medline] [CrossRef]
43. Takanashi, S., Saif, L.J., Hughes, J.H., Meulia, T., Jung, K., Scheuer, K.A. and Wang, Q. 2014. Failure of propagation of human norovirus in intestinal epithelial cells with microvilli grown in three-dimensional cultures. *Arch. Virol.* 159: 257–266. [Medline] [CrossRef]
44. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725–2729. [Medline] [CrossRef]
45. Taube, S., Kolawole, A.O., Höhne, M., Wilkinson, J.E., Handley, S.A., Perry, J.W., Thackray, L.B., Akkina, R. and Wobus, C.E. 2013. A mouse model for human norovirus. *MBio* 4: e00450-13. [Medline] [CrossRef]
46. Thackray, L.B., Wobus, C.E., Chachu, K.A., Liu, B., Alegre, E.R., Henderson, K.S., Kelley, S.T. and Virgin, H.W. 4th. 2007. Murine noroviruses comprising a single genogroup exhibit biological diversity despite limited sequence divergence. *J. Virol.* 81: 10460–10473. [Medline] [CrossRef]
47. Tse, H., Chan, W.M., Lam, C.S., Lau, S.K., Woo, P.C. and Yuen, K.Y. 2012. Complete genome sequences of novel rat noroviruses in Hong Kong. *J. Virol.* 86: 12435–12436. [Medline] [CrossRef]
48. Tsunesumi, N., Sato, G., Iwasa, M., Kabeya, H., Maruyama, S. and Tohya, Y. 2012. Novel murine norovirus-like genes in wild rodents in Japan. *J. Vet. Med. Sci.* 74: 1221–1224. [Medline] [CrossRef]
49. van der Poel, W.H., van der Heide, R., Verschoor, F., Gelderblom, H., Vinjé, J. and Koopmans, M.P. 2003. Epidemiology of Norwalk-like virus infections in cattle in The Netherlands. *Vet. Microbiol.* 92: 297–309. [Medline] [CrossRef]
50. Victoria, M., Carvalho-Costa, F.A., Heinemann, M.B., Leite, J.P. and Miagostovich, M. 2007. Prevalence and molecular epidemiology of noroviruses in hospitalized children with acute gastroenteritis in Rio de Janeiro, Brazil, 2004. *Pediatr. Infect. Dis. J.* 26: 602–606. [Medline] [CrossRef]
51. Vinjé, J. 2015. Advances in laboratory methods for detection and typing of norovirus. *J. Clin. Microbiol.* 53: 373–381. [Medline] [CrossRef]
52. Wobus, C.E., Thackray, L.B. and Virgin, H.W. 4th. 2006. Murine norovirus: a model system to study norovirus biology and pathogenesis. *J. Virol.* 80: 5104–5112. [Medline] [CrossRef]
53. Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. and Monroe, S.S. 2006. Norovirus classification and proposed strain nomenclature. *Virology* 346: 312–323. [Medline] [CrossRef]