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Murine norovirus infection in Brazilian animal facilities

Daniele Masselli RODRIGUES[1)], Josélia Cristina de Oliveira MOREIRA[1)], Marcelo LANCELLOTTI[2)], Rovilson GILIOLI[1)], and Marcus Alexandre Finzi CORAT[1)]

[1)]*Multidisciplinary Center for Biological Research on Laboratory Animal Science (CEMIB), Animal Health Laboratory, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil*
[2)]*Department of Biochemistry, Institute of Biology, University of Campinas, Campinas, São Paulo 13083-970, Brazil*

Abstract: Murine norovirus (MNV) is a single-stranded positive-sense RNA virus of the *Caliciviridae* family. MNV has been reported to infect laboratory mice with the ability to cause lethal infections in strains lacking components of the innate immune response. Currently, MNV is considered the most prevalent infectious agent detected in laboratory mouse facilities. In this study, mice in 22 laboratory animal facilities within Brazil were analyzed for MNV infection. Using primers targeting a conserved region of the viral capsid, MNV was detected by RT-PCR in 137 of 359 mice from all 22 facilities. Nucleotide sequencing and phylogenetic analysis of the capsid region from the viral genome showed identity ranging from 87% to 99% when compared to reported MNV sequences. In addition, RAW264.7 cells inoculated with a mouse fecal suspension displayed cytopathic effect after the fifth passage. This study represents the first report of MNV in mouse colonies in Brazilian laboratory animal facilities, emphasizing the relevance of a health surveillance program in such environments.
Key words: Brazil, mouse, murine norovirus, RT-PCR

Introduction

Noroviruses (NoV) are a group of small, non-enveloped, positive-sense RNA viruses, and are members of the *Caliciviridae* family, which includes five genera: *Sapovirus*, *Lagovirus*, *Vesivirus*, *Norovirus* and *Nebovirus*. NoV can infect a broad range of animals, including cattle, pigs, sheep, lions, cats, dogs, and rodents [13, 34, 46]. The genus *Norovirus* is subdivided into six genogroups (GI–GVI), based on complete sequences of the capsid gene [41, 49]. GI, GII, and GIV include human norovirus strains [5, 13, 20, 41, 49]. GIV is also known to infect felines (a captive lion) [23] and canine species [3, 24, 26]. Porcine viruses belong to GII genogroup [35, 36, 42]. GIII, GV and GVI include bovine, murine and canine norovirus, respectively [14, 20, 30, 41]. The genome of NoV is 7.7 kb in length and organized into three open-reading frames (ORFs). ORF1 encodes a large polyprotein cleaved to produce six non-structural proteins, whereas ORF2 and ORF3 encode two structural proteins, VP1, the major capsid protein, and VP2, a minor structural protein, respectively [5]. For murine norovirus (MNV), an alternative ORF4 that overlaps ORF2 produces protein virulence factor 1 (VF1), which is involved in the regulation of the innate immune responses [13, 25, 39].

MNV infection demonstrated in innate immune system deficient mouse strains such as RAG/STAT$^{-/-}$ (recombination-activating gene and signal transducer and activator of transcription 1) and IFN$\alpha\beta\gamma^{-/-}$ (alpha/beta/gamma interferon receptors) was first described in 2003 by Karst *et al*. [14]. Furthermore, new genotypes have been described and were named MNV-2, MNV-3, MNV-4 [9], MNV-5 and MNV-6 [17]. Although these strains

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Address corresponding: D.M. Rodrigues, Cidade Universitária "Zeferino Vaz", Barão Geraldo. Campinas, Rua 5 de junho, 250, São Paulo 13083-970, Brazil

comprise the GV genogroup, they differ in pathogenicity inducing persistent infection and prolonged fecal shedding, and demonstrating biological and genetic diversity [10, 39, 45].

MNV can infect macrophages, dendritic cells, and B lymphocytes, indicating effects on inflammatory pathways and the immune responses [12, 28, 44].

MNV is reported to be the most prevalent infectious agent in many laboratory mouse facilities in North America, Europe, and Asia [4, 8, 11, 18, 19, 29, 32, 33]. However, MNV infection in Brazilian mouse colonies has not been reported. Hence, it is important to reveal the prevalence of MNV and to identify the prevalent strains in Brazilian mouse facilities. Therefore, we have described the prevalence of MNV infection in laboratory mouse facilities in Brazil. Thereafter, we performed MNV isolation and molecular investigation of the prevalent MNV groups.

Material and Methods

Animals and fecal sample collection

Mice (*Mus musculus*) from 22 animal facilities from 18 Brazilian institutions located in states of different 5 regions of Brazil were included. They were 19 production facilities consisting with 9 universities, 7 research institutes, 2 veterinary private companies and 1 vender, and 3 research facilities. These animals were subjected to a health monitoring program for screening of infectious agents such as bacteria, mycoplasmas, viruses, and parasites in the Multidisciplinary Center for Biological Research on Laboratory Animal Science (CEMIB) at the University of Campinas/UNICAMP, between 2011 and 2015. Mice were euthanized with carbon dioxide and submitted to necropsy for the collection of biological materials. Tested mice (n=359) had different genetic backgrounds, including immunocompetent (n=222), immunodeficient (n=8), hybrids (n=9), genetically modified (n=105), and unknown background (n=15). All mice were aged between 30 days and 1 year. Fecal samples were collected from the rectums and frozen at −80°C until use. A 20% (w/v) suspension was prepared in 0.01 M PBS (pH 7.2), vortexed, and clarified by centrifugation at 5,000 × *g* for 15 min. The supernatant was filtered through a 0.22 μm filter. All animal procedures were approved by the Ethical Committee on Animal Experimentation of the Institute of Biology, University of Campinas, São Paulo, Brazil (protocol number: 2372–1).

Virus isolation

The murine leukemia macrophage cell line RAW264.7 (Fig. 1A) was maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM, Sigma Aldrich Co.), supplemented with 10% bovine fetal serum (Nutricell, Campinas, São Paulo, Brazil), 2.5 mM L-glutamine, and a non-essential amino acids solution (×100) (Sigma Aldrich Co.). Cells were cultured to 80% of confluence in 25 cm^2 cell culture flasks.

One milliliter of a clarified fecal suspension from one transgenic mouse strain was inoculated into RAW264.7 cells and incubated for 1 h at 37°C in a 5% carbon dioxide atmosphere for virus adsorption. Subsequently, the inoculums were replaced with DMEM supplemented with 2% bovine fetal serum. Blind serial passages were performed up to five times, and cells showing cytopathic effect (CPE) were lysed by a cycle of freeze thawing. The cell lysate was obtained by centrifugation at 1,200 × *g* for 30 min at 4°C. Clarified viral suspension was stocked at −80°C.

RT-PCR targeting the MNV VP1 gene

Based on the nucleotide sequences of 4 MNV strains (MNV-1, −2, −3, and −4), a primer sequence targeting the VP1 capsid gene, a conserved region in the MNV genome [9, 10] was designed and used (forward: 5′-AGATCACATGCTTCCCAC-3′; reverse: 5′- AGACCACAAAAGACTCATCAC-3′) to amplify a product of 187 bp. Viral RNA from fecal or cellular suspensions was extracted using an RNeasy Viral RNA Mini Kit (Qiagen® Inc., Valencia, California) or TRIzol (Invitrogen Life Technologies, Carlsbad, California). The cDNAs were synthesized using random hexamer primers (2.5 μM) and Revert-Aid H Minus M-MuLV reverse transcriptase (2.5 U) (Thermo Fisher Scientific, Inc.), and performed the 20 μl scale reaction in following conditions: 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. Synthesized cDNA (2 μl) was added to a 25 μl of PCR reaction as follows: 1× PCR buffer (100 mM Tris-HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.6 μM of each primer, and 5 U *Taq* DNA polymerase (Thermo Fisher Scientific, Inc.). The PCR cycle was completed the following conditions: one cycle of 95°C for 15 min and 40 cycles of 94°C for 30s, 60°C for 30 s, and 72°C for 10 min.

Nucleotide sequencing

PCR products from MNV positive samples were puri-

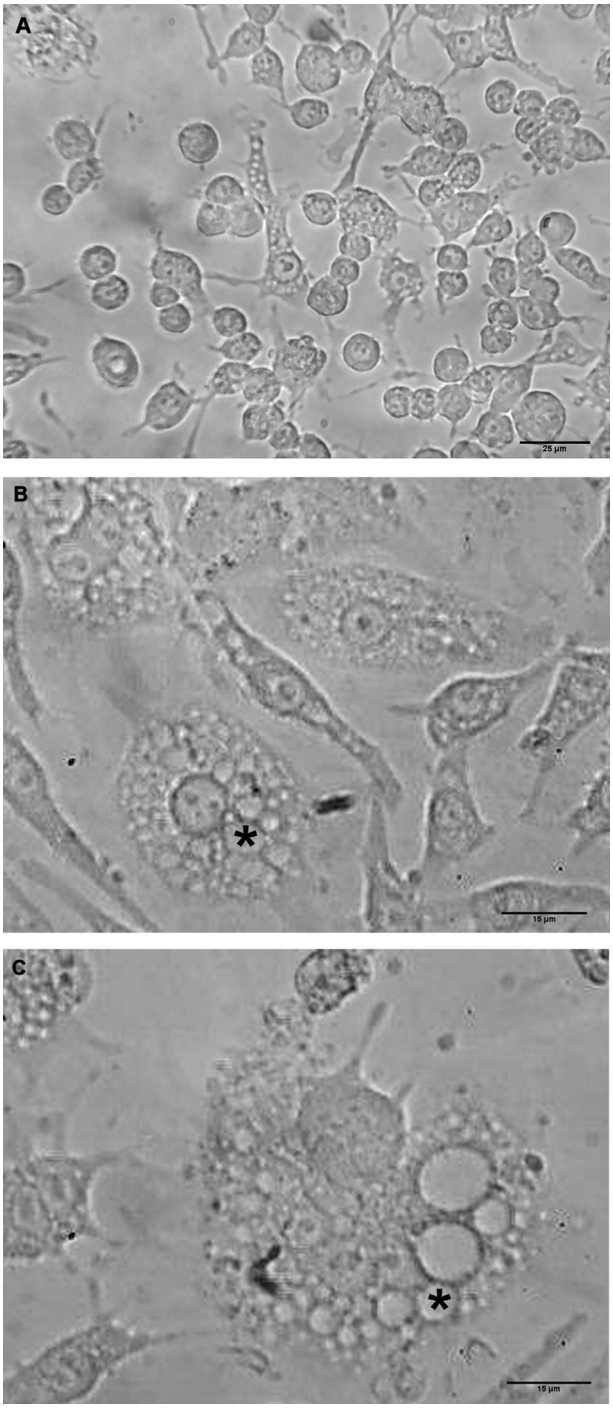

Fig. 1. RAW 264.7 cells infected with MNV. Original magnification ×400. (A) Uninfected RAW264.7 cells. (B) and (C): RAW264.7 cells infected with a fecal sample from a B6.129S7-*Rag1*^{tm1Mom} mouse. CPE at 48 h post-infection, showing a typical vesiculated (*) and rounded cell.

fied and sequenced in both directions, by a commercial laboratory (HELIXXA Bases for Life, Campinas, São Paulo, Brazil) using Sanger dideoxy technology. The chromatograms were visualized in Bioedit Sequence Alignment Editor Program 7.2.5 [6] and sequences were analyzed using NCBI Entrez Nucleotide (www.ncbi.nlm.nih.gov/sites/entrez). A consensus sequence was created and assembled using the Assembler tool software (http://www.hpa-bioinformatics.org.uk/cgi-Bin/assembly_tool/seq_assemble.cgi).

Phylogenetic analysis

Capsid gene nucleotide sequence alignments were performed using Clustal W within the Bioedit software. A phylogenetic tree was generated with Molecular Evolutionary Genetic Analysis (MEGA 6) software [37] based on the neighbor-joining method. Bootstrap analysis was performed with 1,000 replicates using reference sequences from the GenBank database.

Results

MNV surveillance

MNV positive samples were detected in all 22 facilities. The mean positive rate was 38.16% (137/359), according to Table 1.

Virus isolation

MNV was isolated from an RT-PCR positive sample from a B6.129S7-*Rag1*^{tm1Mom} transgenic mouse strain. Infected cells showed CPE evidenced by rounded, shrunken, and vesiculated cells after the fifth passage (Figs. 1B and 1C) and 48 h post-inoculation. This sample was named *LCQS1* and was used for molecular characterization.

Molecular characterization and phylogenetic analysis

A set of 9 sequences from positive samples named *LCQS2*, *LCQS3*, *LCQS4*, *LCQS5*, *LCQS6*, *LCQS7*, *LCQS10 and LCQS11*, were chosen for sequence analysis, in addition to the sequence isolated from cell culture (*LCQS1*). Phylogenetic analysis were performed based on MNV VP1 gene sequences through multiple alignment with other reference MNV sequences available in GenBank as follows: MNV-1: AY228235, MNV2: DQ223041, MNV3: DQ223042, MNV4: DQ223043, MNV5: EF650480, MNV6: EF650481, MNV7: GQ180108, one Brazilian sequence: KF976714.1, isolates from other countries: EF531290.1, DQ911368.1, JN975491.1, JN975524, EU 004674.1, EU 004671.1, EU 004654.1, HQ317203.1, AB435514.1, FJ446719.1

Table 1. Rates of MNV infection in breeding and research facilities

Laboratory mouse facilities	# of positive samples / # of tested mice	Rate of infection
Breeding facilities (n=19)		
Universities (n=9)	51/127	40.15%
Research institutes (n=7)	50/175	28.57%
Veterinary private companies (n=2)	16/20	80%
Vender (n=1)	3/9	33%
Research facilities (n=3)		
Universities (n=3)	17/28	60.71%
Total	137/359	38.16%

and two representative sequences of the *Caliciviridae* family used as outgroups (M87661 and X86560) (Fig. 2). Comparing our sequences with that of the prototype MNV-1 strain, sequence identities ranged from 93% to 96.7% and when comparing to those of other MNV reference strains identities ranged from 83.4% to 98.9%, demonstrating that these isolates are closely related. The JN975491.1 sequence was 83.4%–86.6% identical to that of all other isolates. Viruses included as outgroups were weakly related as expected, including Norwalk virus (M87661) with 7% identity and Sapporo virus (X86560.1) with 13.9% identity. The genetic identities among the samples analyzed in our study ranged from 90.9% to 99.4% (Table 2). This identity matrix analysis led us to generate an MNV phylogenetic tree, using the MEGA6 program based on the neighbor-joining method with *kimura-2* parameters and 1,000 bootstraps. Our cell culture isolate (*LCQS1*) was closely related to MNV-1 (AY228235), MNV5 (EF650480), MNV6 (EF650481), and GQ180108, which is a Brazilian isolate (Fig. 3). *LCQS5* was clustered in a group that was closely related to a virus strain from South Korea (FJ446719.1). Samples *LCQS2, LCQS3,* and *LCQS4* were included and comprised a phylogenetic group of virus strains from Europe (EF531290.1) and China (HQ317203.1). *LCQS6, LCQS7, LCQS10, and LCQS11* correspond to isolates from three distinct animal facilities and, were closely related to the Charles River (CR) strain (EU004674.1). As expected, all samples clustered within the MNV, genogroup V (GV).

Discussion

Our institution has been part of the Laboratory Animal Quality Network of International Council for Laboratory Animal Science (ICLAS) since 2010, which is a program established to certify the diagnostic laboratories for health monitoring of laboratory animals. This work has been indispensable for quality assurance of laboratory animal facilities in Brazil.

MNV, which is associated with subclinical infections in many different mouse strains, appears to be the most frequent infectious agent in mouse colonies as demonstrated by previous reports [8, 10, 11, 18, 29, 34]. The MNV diagnostic method developed by Hsu and colleagues in 2006 [10] which is used to identify a conserved region of the VP1 capsid gene (ORF2) of 4 MNV strains was applied to our work.

MNV positive mice were detected in all 22 facilities, and the mean positive rate was 38.16%. Our results showed that research facilities had higher rates of MNV contamination than animal production facilities having the highest requirements for barrier systems. However, we showed that even with the highest requirements for barrier systems, production facilities were broadly contaminated with MNV.

These data suggest that most of these facilities are dealing with MNV-infected mice, regardless of the adoption of sanitary barriers systems. Kim *et al*. (2011) [18] report similar results, indicating the presence of MNV in mice housed in both barrier facilities and conventional systems. Ohsugi *et al*. (2013) [29] also documented widespread MNV infection in colonies of commercial companies in Japan.

These results suggest that MNV has spread through all the Brazilian laboratory mouse facilities, which is similar to that found in European, Asian, and North American animal facilities [4, 10, 11, 19, 27]. Using a highly sensitive real-time RT-PCR assay, Muller *et al.* (2007) [27] found a positive frequency of 64.3% in German research mice. In another report, Hanaki *et al*. (2014) [7] developed a one-step SYBR Green I real-time

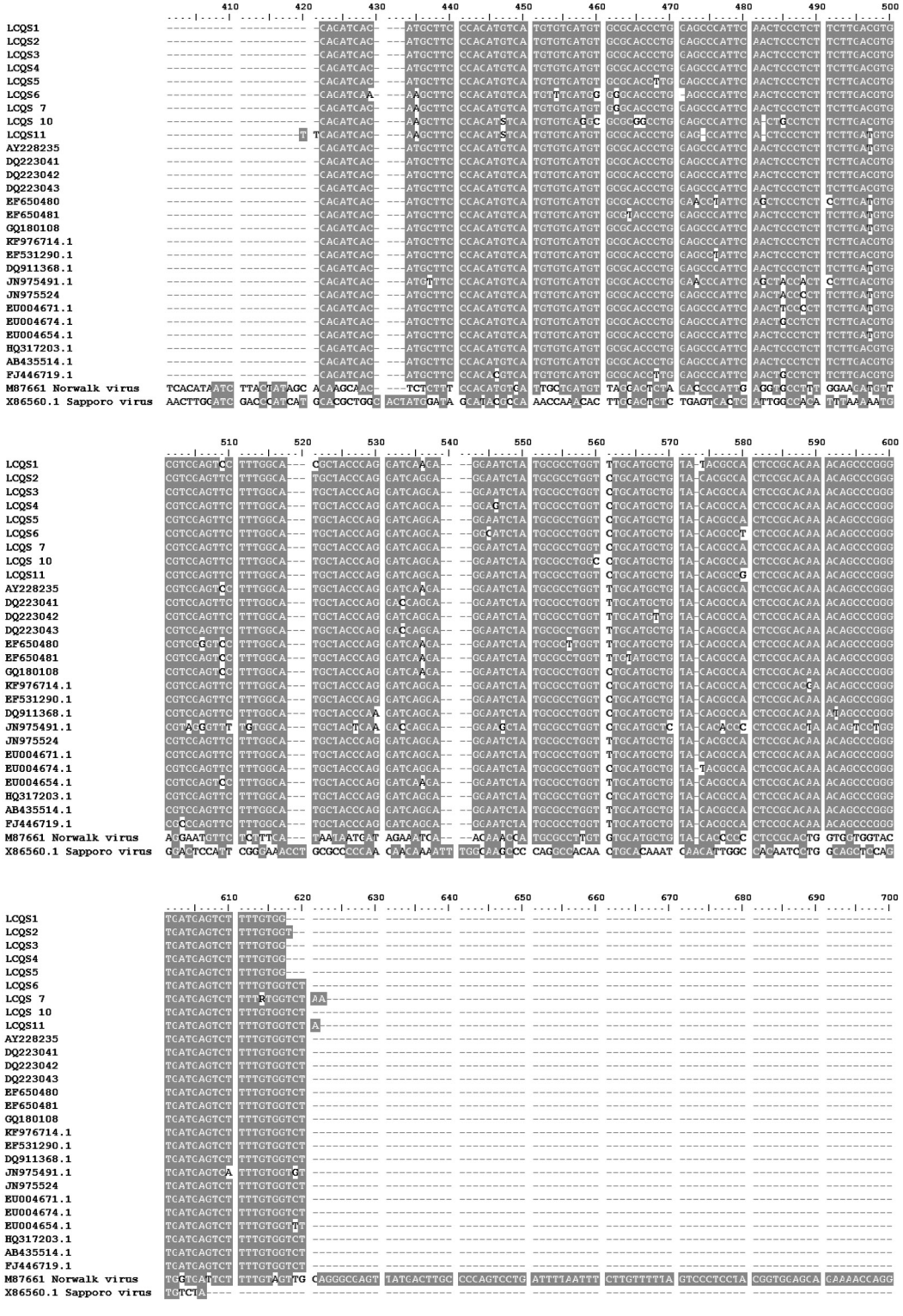

Fig. 2. Nucleotide sequence alignment of the capsid gene (VP1) sequences of MNV. *LCQS1* corresponds to the sequence isolated in cell culture; *LCQS2, LCQS3, LCQS4, LCQS5, LCQS6, LCQS7, LCQS10, and LCQS11* are sequences derived from PCR products of naturally infected mice. MNV reference strains are represented by GenBank accession numbers. Conserved nucleotides are shaded in gray, and variable regions are represented by coloured nucleotides.

Table 2. Sequence identity matrix representing nucleotide percentage identity of conserved regions of the capsid gene among murine noroviruses reference strains and isolates from cell culture (*LCQS1*) and infected animals (*LCQS2, LCQS3, LCQS4, LCQS5, LCQS6, LCQS7, LCQS10 and LCQS11*)

	LCQS1	LCQS2	LCQS3	LCQS4	LCQS5	LCQS6	LCQS7	LCQS 10	LCQS11
LCQS1	*	96.7%	97.2%	96.7%	96.7%	90.9%	93.1%	90.9%	91.0%
LCQS2	96.7%	*	99.4%	98.9%	98.9%	94.1%	96.2%	94.1%	94.2%
LCQS3	97.2%	99.4%	*	99.4%	99.4%	93.5%	95.7%	93.5%	93.6%
LCQS4	96.7%	98.9%	99.4%	*	98.9%	93.0%	95.2%	93.0%	93.1%
LCQS5	96.7%	98.9%	99.4%	98.9%	*	93.0%	95.2%	93.0%	93.1%
LCQS6	90.9%	94.1%	93.5%	93.0%	93.0%	*	94.7%	91.9%	92.1%
LCQS7	93.1%	96.2%	95.7%	95.2%	95.2%	94.7%	*	93.6%	94.7%
LCQS 10	90.9%	94.1%	93.5%	93.0%	93.0%	91.9%	93.6%	*	93.6%
LCQS11	91.0%	94.2%	93.6%	93.1%	93.1%	92.1%	94.7%	93.6%	*
AY228235	96.7%	96.7%	96.2%	95.7%	95.7%	93.0%	95.2%	93.0%	94.2%
DQ223041	95.7%	97.8%	97.3%	96.7%	96.7%	94.1%	96.2%	94.1%	94.2%
DQ223042	95.7%	97.8%	97.3%	96.7%	96.7%	94.1%	96.2%	94.1%	94.2%
DQ223043	95.7%	97.8%	97.3%	96.7%	96.7%	94.1%	96.2%	94.1%	94.2%
EF650480	93.5%	93.5%	93.0%	92.5%	92.5%	89.8%	92.0%	90.3%	91.5%
EF650481	95.7%	95.7%	95.1%	94.6%	94.6%	91.9%	94.1%	91.9%	93.1%
GQ180108	96.2%	97.3%	96.7%	96.2%	96.2%	93.5%	95.7%	93.5%	94.7%
KF976714.1	95.1%	98.3%	97.8%	97.3%	97.3%	94.6%	96.8%	94.6%	94.7%
EF531290.1	95.1%	98.3%	97.8%	97.3%	97.3%	94.6%	96.8%	94.6%	94.7%
DQ911368.1	94.1%	97.3%	96.7%	96.2%	96.2%	93.5%	95.7%	93.5%	94.7%
JN975491.1	83.4%	86.6%	86.0%	85.5%	85.5%	83.4%	84.6%	83.4%	83.6%
JN975524	94.6%	96.7%	96.2%	95.7%	95.7%	93.0%	95.2%	93.5%	94.2%
EU004671.1	94.6%	96.7%	96.2%	95.7%	95.7%	93.0%	95.2%	93.5%	94.2%
EU004674.1	95.7%	97.8%	97.3%	96.7%	96.7%	94.1%	96.2%	95.1%	94.2%
EU004654.1	96.7%	96.7%	96.2%	95.7%	95.7%	92.5%	94.7%	92.5%	93.6%
HQ317203.1	95.7%	98.9%	98.3%	97.8%	97.8%	95.1%	97.3%	95.1%	95.2%
AB435514.1	96.2%	98.3%	97.8%	97.3%	97.3%	94.6%	96.8%	94.6%	94.7%
FJ446719.1	94.1%	96.2%	95.7%	95.1%	96.2%	92.5%	94.7%	93.5%	92.6%
M87661 - Norwalk virus	6.9%	7.1%	7.0%	7.0%	7.0%	6.9%	7.1%	6.8%	7.0%
X86560.1- Sapporo virus	13.5%	13.9%	13.9%	13.7%	13.7%	13.4%	13.6%	13.4%	13.6%

*Represents 100% of identity.

RT-PCR assay and analyzed 158 fecal samples from laboratories and institutes in Japan, and identified 88 MNV-positive samples. Other studies have shown high frequencies of MNV in mouse colonies using serological techniques such as ELISA and IFA [11, 18, 29, 33].

Data regarding the prevalence of infectious agents in laboratory animals have contributed to a better understanding of the pathology and epidemiology of these infections and have assisted in the implementation of an animal health monitoring program. Although there is great concern regarding this pathogen, little is known about its impact on research results or animal models [12, 40]. For example, Paik *et al.* (2010) [31] showed that MNV infection can accelerate the inflammatory bowel disease in Mdr1a$^{-/-}$ mice, which was suggested to be mediated by dendritic cells, T cells, or inflammatory cytokines responses [21], demonstrating that MNV infection can modify antigen presentation in such animal models.

Contamination by infectious viral and bacterial agents in mouse and rat colonies has changed with the passage of time. In the past, results from North America, Europe, Korea, Japan, and Brazil indicated that both SPF and conventional mouse colonies were likely to be contaminated with mouse hepatitis virus, Sendai virus, *Helicobacter* spp and other many pathogens [38]. The prevalence of these agents has recently decreased, whereas some other pathogens have become more prevalent, including mouse parvovirus (MPV) and MNV, even with the implementation of strict barrier systems [33]. Although MPV and MNV have been recently identified, previous data indicate that they have been present in laboratory mice for many years [48]. Several factors can contribute to this prevalence. We assume that this issue could be due to the increasing use of genetically modified animals, as these models are continually exchanged between research institutions. In addition, MNV induces a persistent infection with long periods of virus shedding, and thus increasing the risk of transmission to other animals [10, 43]. Some studies on persistent viral infec-

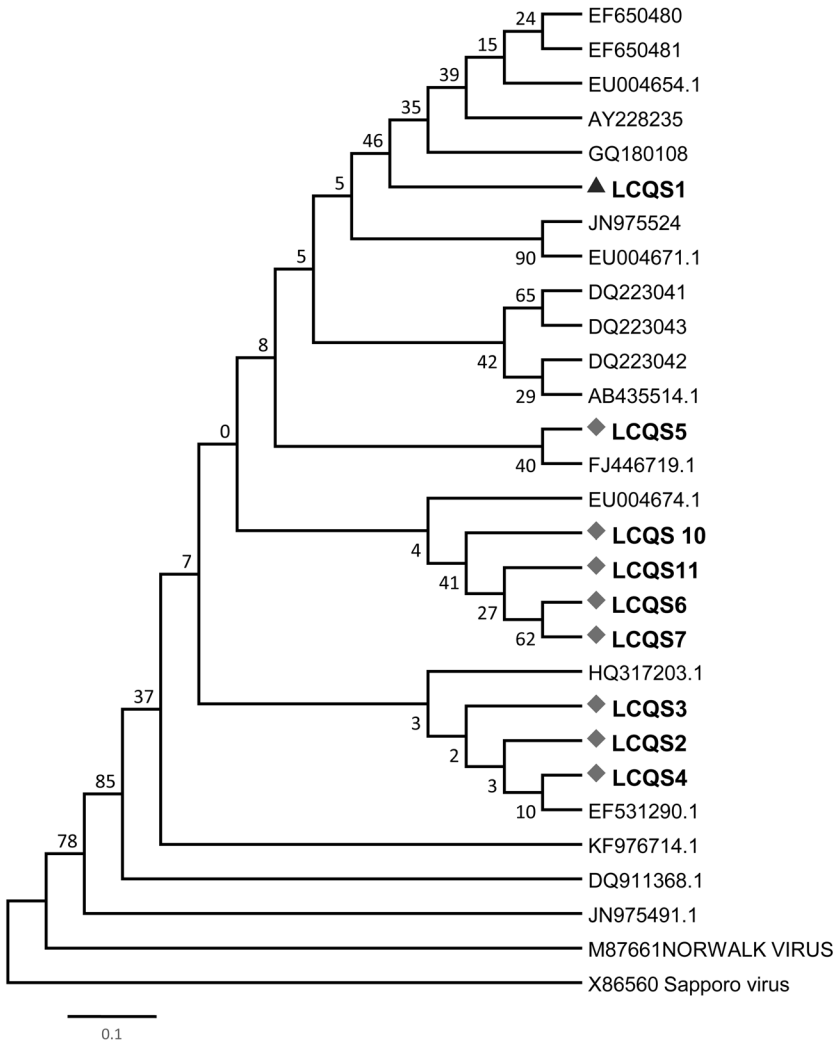

Fig. 3. Phylogenetic analysis based on the nucleotide sequences of the viral protein (VP1) genes from MNV isolates and strains of genogroup V. Norwalk virus (M87661) and Sapporo virus (X86560) sequences were used as outgroups. *LCQS1* refers to the MNV isolate from infected RAW 264.7 cells. *LCQS2, LCQS3, LCQS4, LCQS5, LCQS6, LCQS7, LCQS10, and LCQS11* refer to fecal samples of naturally infected mice from southeast animal facilities. A phylogenetic tree was constructed with MEGA6 software using the Neighbor-Joining method and evolutionary distances was computed using the *Kimura-2* model and bootstrap values defined for 1,000 replicates. The scale bar is units per nucleotide substitution. Brazilian MNV sequences are identified by symbols ▲ and ♦.

tion by MNV have shown a correlation with bacterial microbiota. Data suggest that the enteric microbiome plays an important role in the capacity for the virus to establish a persistent infection, and that antibiotic treatment might modify the pathogenesis of enteric viral infections [1].

In an attempt to isolate MNV, we selected a PCR-positive sample from a B6.129S7-*Rag1*^{tm1Mom} transgenic mouse strain which is reported to be highly susceptible to MNV infection [15]. A previous report has also demonstrated virus isolation from the mesenteric lymph nodes and duodenum of RAG2$^{-/-}$ mice [2]. We isolated MNV in a fecal sample after five passages in cell culture and submitted the sequences for comparative molecular analysis.

Based on a phylogenetic analysis of the capsid protein gene (VP1), the *LCQS1* isolate revealed similarities of 83.4%–96.7% when compared to reference strains in GenBank (AY228235, DQ223041, DQ223042, DQ223043, EF650480, EF650481, GQ180108, KF976714.1, EF532290, DQ911368.1, JN975491.1, JN975524, EU 004674.1, EU 004671.1, EU 004654.1,

HQ317203.1, AB35514.1, and FJ446719.1). This demonstrates a close genetic relationship to these strains. Genetic relatedness among MNV strains has already been reported [10, 27, 39]. Furthermore, even though these strains came from different sources, they share a genetic relationship because of animal exchange between Brazilian animal facilities. It is known that NoV exhibit great diversity, showing approximately 51.1% nucleotide identity between strains in different genogroups [16, 27, 39]. This genetic diversity can be attributed to the genomic recombination which is common in RNA viruses, in addition to the intrinsic high mutation rate associated with RNA-dependent RNA polymerases [15, 47].

In conclusion, this is the first report of the identification of MNV in fecal samples from mouse strains from different Brazilian animal facilities. Our findings are epidemiologically important and show that this virus is prevalent in every animal facility in Brazil, even SPF or conventional mouse colonies. The results also reinforce the need to include this agent in surveillance programs, as recommended by Federation of Laboratory Animal Science Associations (FELASA) (2014) [22], and to track the distribution of this virus in Brazil and worldwide, with the ultimate goal of establishing a global strategy of MNV control.

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