Genetic diversity and recombination of murine noroviruses in immunocompromised mice

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Summary

Murine noroviruses (MNV) are newly identified pathogens which infect laboratory mice. In this study, we found a high prevalence (64.3%) of MNV in various breeding colonies of immunocompromised, transgenic and wild-type mouse lines. All mice survived infection with no signs of clinical disease. Faeces samples were collected from animals housed in two separate laboratory mouse colonies in Berlin, Germany, and screened using quantitative reverse transcription (RT)-PCR. We have determined the complete nucleotide sequences of 3 novel MNV strains. Furthermore, we sequenced two subgenomic regions within open reading frames (ORFs) 1 and 2 that are suitable for genotyping. Sequence analysis of the full-length and partial genomes obtained from naturally infected mice yielded valuable data on genetic diversity of murine noroviruses. The discordance of genotype affiliation of some MNVs shown in ORF1 and ORF2 suggests intertypic recombination events in vivo.

Introduction

The first strain of murine noroviruses (MNV-1) was isolated in 2003 from the brain of an immunodeficient mouse, lacking recombination-activating gene 2 and signal transducer and activator of transcription 1 (RAG2/STAT1−/−), which succumbed to a systemic disease [17, 31]. MNV is the first known norovirus to infect small animals. Sequence analysis designated the virus as a member of the new genogroup V within the genus Norovirus. In contrast to the lack of a culture system for human noroviruses, the murine norovirus replicates in cultured haematopoietic cells. This provides an excellent model system to study norovirus biology and pathogenesis [32, 33].

Noroviruses are positive-sense RNA viruses that belong to the family Caliciviridae. These viruses are the major cause of outbreaks of acute nonbacterial gastroenteritis in humans worldwide. The viral genome of human noroviruses is approximately 7.5 kb in length and contains three overlapping open reading frames. ORF1 encodes the nonstructural polyprotein, which is processed by the viral 3C-like protease into several functional proteins, including NTPase and RNA-dependent RNA polymerase [3, 26]. ORF2 and ORF3 encode the major capsid protein (VP1) and a small basic structural protein (VP2), respectively. The VP1 protein possesses two
domains: the P (protruding, P1 and P2) domain and the S domain, which forms the icosahedral shell of the capsid [27]. Most of the cellular interactions and immune recognition features are thought to be located in the P2 sub-domain, which is exposed at the viral surface and shows the highest degree of sequence diversity within the genome [7, 12]. VP2 is associated with upregulation of VP1 expression in cis and stabilization of VP1 in the virus structure [4]. Although varying in size, the murine norovirus exhibits an identical genomic organisation [30].

Based on RT-PCR and genomic sequencing, the genus Norovirus demonstrates high levels of genetic diversity and can be differentiated into five genogroups (GG). Hence, strains of GGI, GGII and GGIV are found in humans, GGIII and GGV strains are found in cattle and mice, respectively. Currently, 29 genetic clusters are classified within these 5 genogroups: 8 in GGI, 17 in GGII, 2 in GGIII, and 1 each in GGIV and GGV [35]. Recently, three more strains have been described within GGV [15]. The purpose of this study was to get an insight into the prevalence and genetic diversity of murine norovirus circulating among laboratory mouse colonies in Germany.

**Materials and methods**

**Specimens and extraction of viral RNA**

A total of 76 faeces samples were collected from research mice housed in two separate colonies in Berlin, Germany. These specimens were obtained from 28 different mouse lines, including immunocompetent mice, lines with muta-

| Mouse line (genotype) | Real-time PCR +/n | Isolate | Acc. no. ORF1b | Acc. no. ORF2b |
|-----------------------|-------------------|---------|----------------|----------------|
| MPI                   |                   |         |                |                |
| IFN α/β-R−/−          | 4/4               | 6.6 × 10^6 | S6, S33       | EF531578       | EF531588       |
| IFN γ-RI−/−           | 5/7               | 2.4 × 10^7 | S11           | EF531579       | EF531589       |
| Myd 88−/−             | 2/2               | 6.8 × 10^6 | S25, S27, S28 | EF531292^     |
| TLR 2/4−/−            | 4/4               | 1.2 × 10^7 | S29, S30, S31 | EF531581       | EF531596       |
| RAG 1−/−              | 4/4               | 2.6 × 10^6 | S15           | EF531582       | EF531594       |
| CD1 wt                | 2/2               | 1.7 × 10^6 | S37, S38      | EF531583       | EF531597       |
| 129 Casp −/−          | 1/2               | 1.7 × 10^7 | ND            | ND             |
| II 18/1β−/−           | 2/2               | 1.5 × 10^7 | ND            | ND             |
| C57B6/6J-Tyr wt       | 1/2               | 1.2 × 10^6 | ND            | ND             |
| RKI                   |                   |         |                |                |
| Galectin 3−/−         | 2/2               | 2.1 × 10^6 | S73, S74      | EF531584       | EF531592       |
| SRA−/−                | 2/2               | 1.7 × 10^6 | ND            | ND             |
| CD36−/−               | 1/2               | 5.9 × 10^7 | ND            | ND             |
| GFAP-BLC tg           | 2/2               | 7.4 × 10^6 | S90           | EF531585       | EF531595       |
| CAST tg               | 1/2               | 2.8 × 10^7 | ND            | ND             |
| ITAC (A)−/−           | 2/2               | 4.7 × 10^7 | S93, S94      | EF531586       | EF531591       |
| ITAC (B)−/−           | 1/2               | 5.9 × 10^6 | S97           | EF531587       | EF531593       |
| YFPH tg               | 2/2               | 9.2 × 10^7 | S99           | EF531291^     |

Negative tested strains (n)
TLR 2−/− (4), β-TCR−/− (2), RAG2/γ-Chain−/− (5), B6 Casp−/− (2), CD36/SRA−/− (2), CCL3−/− (2), LFA−/− (2), LyZ−/− (2), PSEN tg (2), Bl6 wt (4)

a GE genome equivalents; for more than one positive sample the mean was calculated
b Includes partial sequences of the ORFs (underlined isolates; ORF1, RNA polymerase gene; ORF2, capsid gene)
* Contains both regions
ND sequences not determined; MPI Max Planck Institute; RKI Robert Koch Institute; tg transgene; wt wild-type; TLR toll-like receptor; RAG recombination-activating gene; Casp caspase; SRA scavenger receptor A; GFAP-BLC glial fibrillary acidic protein-B-lymphocyte chemoattractant; CAST calpastatin; ITAC interferon-inducible T-cell alpha-chemoattractant; YFPH yellow fluorescent protein H; CCL3 chemokine, motif CC, ligand 3; LFA-1 lymphocyte function-associated antigen-1; LyZ lysozyme M; PSEN presenilin-1.
tions in a number of immune effector molecules, and trans-genic mouse lines (Table 1). Mice were group-housed in static filter-top cages with up to 10 animals per cage in barrier units with documented SPF status. Viral RNA was extracted from 140 mL of a 10% faecal suspension using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer’s recommended protocol.

Virus detection and quantification

For detection of murine norovirus, a single-tube real-time RT-PCR assay was established by using the TaqMan technology (Applied Biosystems, Foster City, USA). Primers MNV-1.1s and MNV-1.2s and a fluorescence-labeled TaqMan probe MNV-TMP within a conserved MNV-1 ORF2 gene region were selected (Table 2). In the final format, QuantiTect Probe PCR kit reaction mixtures (Qiagen) contained 2 µL of template RNA, each primer at a concentration of 250 nM, and 100 nM probe. The mixture was then subjected to a one-step assay by using the following conditions: RT for 30 min at 50°C, RT inactivation/DNA polymerase activation for 15 min at 95°C, and 45 cycles of 15 s at 95°C, and 30 s at 55°C for amplification. To quantify the number of genomes, an external standard curve was established with a 10-fold series of dilution from 2 x 10² to 2 x 10⁷ copies per reaction using a plasmid that contained the capsid gene of MNV-1.

Virus propagation and determination of complete nucleotide sequences

Viral RNA was extracted from either faecal suspension (isolates M21 and S36) or plaque-purified virus (isolates S99 and S74). However, all four isolates have been propagated on RAW 264.7 cells as described elsewhere [32]. Single-stranded cDNA was synthesized from total RNA using PowerScript™ reverse transcriptase (BD Clontech, Heidelberg, Germany) with a tagged primer complementary to the poly(A) tail of the genome (5'T-aTag 5'-gCC AAC gAC Cgg gAg gCC AgC TTT TTT TTT TTT V-3', tag sequence is underlined). PCR fragments were generated using Phusion DNA polymerase (Finnzymes, Espoo, Finland). The primer pair MNV-1.1s and MNV-1.2s amplifies a 5224 bp product resembling the complete ORF1 and the 5' end of ORF2. Primer pair MNV-1.3s and a reverse primer containing the tag sequence (aTag 5'-gCC AAC gAC Cgg gAg gCC AgC-3'), amplifies ORF2, ORF3 and the 3' UTR. Thus, the complete genomic sequences were obtained by two overlapping PCR fragments, which were cloned using Phusion DNA polymerase (Finnzymes, Espoo, Finland). The primer pair MNV-1.1s and MNV-2.1as amplifies a 5224 bp product resembling the complete ORF1 and the 5'end of ORF2. Primer pair MNV-1.2s and a reverse primer containing the tag sequence, which were cloned using TOPO-XL cloning Kit according to manufacturer’s recommended protocol.

Universal T7 forward and M13 reverse primers were used for sequencing of the plasmids with the Big-Dye terminator cycle sequencing kit (Perkin Elmer, Wellesley, MA), and sequences were determined using an ABI Prism 3100 genetic analyser (Applied Biosystems). Primer walking was applied for subsequent sequencing.

Amplification of partial ORF1 and ORF2 sequences

ORF1 (RNA polymerase region) and ORF2 (capsid) were amplified by nested RT-PCRs. Partial amplification of the polymerase gene was performed using primer pair MNV-1.3s and MNV-1.3as, resulting in a 609-bp product, which

### Table 2. RT-PCR primers and probe

| Primer     | Sequence (5'-3') | Location |
|------------|-----------------|----------|
| **ORF1**   |                 |          |
| MNV-1.1s   | gTg AAA TgA ggA Tgg CAa CgC CAT CTT CT g  | 1–30     |
| MNV-1.2s   | CgC Agg AAC gCT CAg CAg TC                | 5029–5048|
| MNV-1.3s   | CAT gTT Ctg gCg CAC CTC gCC              | 3626–3646|
| MNV-1.3as  | CgC gAA gAT gAA ggg CCC ATC               | 4215–4235|
| MNV-1.4s   | CCG CTT TgA gAA CAC CCT Yg               | 3821–3840|
| MNV-1.4as  | gCA gCT Cgg ATC ATg gTg CC               | 4113–4132|
| **ORF2**   |                 |          |
| MNV-2.1as  | ggA AgA TCC Agg ggT CAa TTT gg           | 5202–5224|
| MNV-2.3s   | TCT TCT TgA TgT gCg Tcg Ag               | 5535–5554|
| MNV-2.3as  | CTC ggg AAT CAC ATC CCT g                | 6258–6276|
| MNV-2.4s   | TgC TAC CCA gga TCA AgA gg              | 5564–5583|
| MNV-2.4as  | CCT AAC TCT gCC ATC CAC Cag             | 6211–6231|
| Real-time RT-PCR |              |          |
| MNV-TM1 (s) | AgA ggA ATC TAT gCg CCT gg         | 5579–5598|
| MNV-TM2 (as) | gAA ggC ggC CAg AgA CCA C     | 5653–5671|
| MNV-TMP    | 6FAM-CgC CAC TCC gCA CAA ACA gCC C    | 5615–5636|

*a Refers to GenBank accession no. AY228235, Murine norovirus-1 complete genome.
then was employed as template for a second round of amplification. Second primer pair MNV-1.4s and MNV-1.4as subsequently amplified a 311-bp product. For the nested RT-PCR of the capsid gene, MNV-2.3s and MNV-2.3as generated a 741-bp product and the nested primer pair MNV-2.4s and MNV-2.4as a 667-bp product. All fragments were analyzed by 1.5% agarose gel electrophoresis and subjected to direct sequencing.

Detection of recombination

Strains that did not cluster with the same group of viruses in ORF1 and ORF2 were considered to be putative recombinant strains. A nested RT-PCR which produces a long genomic fragment (2410-bp) encompassing the 3′ end of the polymerase region and the 5′ end of the capsid was performed using primer pairs MNV-1.3s=MNV-2.3as (first round) and MNV-1.4s=MNV-2.4as (second round). The product was cloned into a plasmid vector as described above. Similarity plots were generated (SimPlot version 3.5 [21]) in order to detect recombination.

Phylogenetic analysis

Sequence alignments and phylogenetic analysis were performed using BioEdit (version 7.0.5 [9]), which includes the Phylogeny Inference Package (PHYLIP [8]). Evolutionary distances were calculated by Kimura’s 2-parameter method [19]. The computed distances were utilized to construct phylogenetic trees by the neighbor-joining method (TreeView version 1.6.6 [24]). To gain an internal estimate of how well the data supported the phylogenetic trees, bootstrap resampling (1000 datasets) of the multisequence alignments was carried out.

Results

In this study, 76 faeces specimens collected from two separate laboratory mouse colonies in Berlin, Germany, were screened for murine norovirus using a newly established one-tube real-time RT-PCR. These specimens were obtained from 28 mouse lines with diverse genetic modifications mainly involving the immune system (Table 1). There was no evidence of clinical symptoms or abnormal behaviour in these mice. Murine norovirus was detected in 18 mouse lines (64.3%), while the mean prevalence within each infected mouse line was 83.7%. A mean viral load of $5 \times 10^7$ copies/ml ($1.3 \times 10^5 – 7.5 \times 10^8$) was quantified. The rate of MNV-positive mouse lines was comparable for both colonies (Max Planck-Institute, MPI 67%; Robert Koch-Institute, RKI 57%).

Complete nucleotide sequences from strains M21 (IFNg-/-), S36 (RAG1-/-), and S99 (YFPhtg) were determined. Sequence data are available in GenBank (Fig. 1). Sequences for M21 and S36 could be successfully determined from original material, whereas S99 and S74 had to be propagated on RAW 264.7 cells and plaque purified before sequence determination.

The phylogenetic tree and distance plot analysis demonstrated that our strains were genetically distinct from strains MNV1–4 (Fig. 1). Regarding full-length genomes, pairwise sequence comparison with the known reference sequences displayed nucleotide identities from 87 to 92% for M21, S36 and S99 (Table 3). The highest identity was found between strains M21 and S36 (94%).

Overall, we found a very high homology within the ORF1-ORF2 junction region of all investigated MNV strains (Fig. 1b), where these strains shared a 100-nucleotide stretch with almost 100% identity. Furthermore, the shell regions of all capsid sequences proved to be highly conserved between the strains investigated in this study. The highest variability was shown for the P2 domain, where up to 33% distance between the sequences was calculated. This observation is consistent with the results of investigations of the human noroviruses [18, 35]. Interestingly, we found a significant difference within the 3′UTR sequence of the strains MNV1-4 and S99 compared to M21 and S36. The latter strains exhibited an insertion of 6 and 7 bases, respectively, at position nt 7356.

For extensive investigation of genetic diversity within the detected murine noroviruses, part of the RNA polymerase and part of the capsid gene were amplified and sequenced. Distance plotting analysis and full-length sequence alignments were used to find optimal regions for amplification. We identified two variable regions that appeared suitable for differentiation of viral strains. These regions were composed of conserved areas which were adequate for primer design. Figure 1b shows an example for distance plotting and primer binding sites. We used a region of 272 bases in length located within ORF1 between nt 3840 and nt 4113. Within ORF2, the region was located between nt 5583 and nt 6211, encompassing 626 bases (Table 2). Represent-
Fig. 1. Phylogenetic tree and SimPlot analysis of full-length genomes from the novel MNV strains M21 (DQ911368), S36 (EF531290) and S99 (EF531291) and the reference strains MNV-1 (AY228235), MNV-2 (DQ223041), MNV-3 (DQ223042) and MNV-4 (DQ223043). a Phylogenetic tree with percental bootstrap values (1000 replicates). b SimPlot analysis. Query sequence: MNV Berlin 05/06/DE Isolate S36; window size, 200 bp; step, 30 bp. The ordinate indicates the genetic distance between the query strain and the three strains M21, S99 and MNV-1, the abscissa indicates the nucleotide positions. Vertical lines indicate the primer positions used for genotyping of subgenomic regions. A genome diagram of MNV drawn to scale is shown above [30].
tative partial genome sequences have been published in GenBank. The accession numbers are listed in Table 1.

Phylogenetic trees were constructed to analyze the relationships of the partial sequences with the corresponding parts of the MNV-Berlin strains (M21, S36 and S99) and the reference strains MNV1-4 (Fig. 2). Strains that share a branch of a phylogenetic tree were classified as different clusters. The term “genetic cluster” was used as defined by Ando et al. [2] and represents a minimum classification unit consisting of strains that reproducibly group together on a distinct branch of a phylogenetic tree and are sufficiently close in nucleotide sequence to be distinguished from strains related to other groups. Isolates within the same cluster showed nucleotide distances between 0 and 0.0766 for ORF1 and 0 and 0.0494 for ORF2 (Fig. 2). Strains that share a branch of a phylogenetic tree were classified as different clusters.

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For most isolates, the phylogenetic clusters observed for ORF1 (Fig. 2a) were reflected by ORF2 (Fig. 2b). However, isolates S25, S27 and S28 are classified in different clusters comparing both phylogenetic trees. Concerning ORF1, these isolates appear to be related to S36, whereas in ORF2 they cluster with M21. These three isolates were obtained from the same mouse line (TLR4/C0) from different cages (Table 1).

To investigate if the different branching of isolates S25, S27 and S28 was due to recombination, sliding window analyses were performed. For this purpose we cloned large fragments (2410 bp) spanning the 3’ end of ORF1 and the 5’ region of ORF2. Compared to each other, the three fragments obtained from S25, S27 and S28 revealed more than 99% nucleotide sequence identities. The fragments were analyzed with SimPlot software by comparing the sequences to potentially parental strains as described elsewhere [21]. Figure 3a shows the results obtained by comparison of the sequence from isolate S28 to M21 and S36 as putative parental strains. S28 is distant from M21 within the ORF1 polymerase region except for the 3’ end, which corresponds to the conserved ORF1-ORF2 junction. S28 showed a closer association to S36 within ORF1, with a similarity of 96–99%, which confirms the clustering shown in Fig. 2. In contrast, the S28 sequence was only distantly related to S36 within the capsid region, while on the other hand, the distance to M21 was less than 5%, supporting the relationship within ORF2.

Furthermore, the reference strain MNV-4, which was phylogenetically divergent in ORF1, clustered with isolates S93, S94 and S99 in ORF2. To confirm the genetic recombination of MNV-4, SimPlot analysis was performed with S99 and M21, considering the 3’ end of ORF1, ORF2, ORF3 and the 3’UTR, respectively (Fig. 3b). While MNV-4 was distant to S99 within ORF1, the sequences were closely related in the complete 3’ part of the genomes. The relationship between the two strains, furthermore, could be confirmed regarding the 3’UTR of S99, which is closely homologous to MNV-4 and also lacks the insertion at position nt 7356. The reference strain M21 and MNV-4 displayed low similarity over the entire fragment except for the junction region.

### Table 3. Percent nucleotide identities of the full-length genomes

|          | M21 (%) | S36 (%) | S99 (%) |
|----------|---------|---------|---------|
| MNV-1    | 87.1    | 87.3    | 87.0    |
| MNV-2    | 89.6    | 90.0    | 90.4    |
| MNV-3    | 90.7    | 91.0    | 91.2    |
| MNV-4    | 89.8    | 90.0    | 91.6    |
| M21      | 100     | 94.1    | 89.9    |
| S36      | 94.1    | 100     | 90.1    |

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Fig. 2. Phylogenetic trees of (a) ORF1 (RNA polymerase, 312-bp) and (b) ORF2 (capsid, 610-bp) fragments. MNV-1 strain was chosen as outgroup for the phylogenetic trees. Corresponding partial sequences of MNV-2, MNV-3, MNV-4, MNV-Berlin 04/06/DE Isolate M21, MNV-Berlin 05/06/DE Isolate S36, and MNV-Berlin 06/06/DE Isolate S99 are included. Strains belonging to the same cluster are enclosed by dashed circles. Putative recombinants S25, S27, S28 and MNV-4 are indicated by closed circles to emphasize their different phylogenetic groupings.
Fig. 3. SimPlot analysis of potential MNV recombinants. a Query: MNV Berlin S28/06 (EF531292). The window size was 200 bp with a step size of 20 bp. The vertical axis indicates the percental genetic similarity between the 2 putative parental strains, M21 and S36, with the recombinant strain S28. b Query: MNV-4. The window size here was 300 bp with a step size of 30 bp. The genetic similarity between the recombinant strain, an unrelated reference strain (M21) and the putative parental strain for ORF2 (S99) is shown.
Discussion

Following the first description of murine norovirus, serological testing in North America showed that MNV is one of the most prevalent pathogens infecting research mice [13]. To investigate the prevalence of MNV in German research mice, we established a highly sensitive real-time PCR assay. The frequency determined in this study (64.3%) was much higher than expected based on the data from serological testing (22.1%) [13]. This fact could be attributed to local endemic differences. In addition, it is possible that not all infected mice develop immunity. Time-related reduction of antibody titers or insufficient cross-reactivity may also play a role. Studies concerning the immunity against human noroviruses have indicated that immunity seems to be short-lived and protection, furthermore, is strain-specific [23, 25].

The potential to induce persistent infections in mice [14], the high degree of environmental stability [6], and the faecal-oral route of transmission [33] all could have contributed to the high prevalence. None of the mice investigated here showed any symptoms of disease, confirming the observations regarding infection of mice with MNV2-4 [14]. This certainly contributed to the unnoticed spread of the virus. In contrast, MNV-1 infection has been shown to be associated with lethal disease in severely immunodeficient mice [17, 31]. It remains unclear if the pathogenesis is dependent on the mouse genotype or specific MNV strains. In this respect, further studies are required to evaluate if MNV infection of laboratory mice affects independent experiments unrelated to research on MNV.

Here, viral infection was independent of the genotype of the mouse. There was no significant difference in the prevalence of MNV in immunodeficient mice compared to wild-type or transgenic mice.

Human noroviruses exhibit great genetic diversity. Molecular methods have become major means of characterizing and understanding the relatedness of different viral strains [2, 18, 35].

Like human noroviruses, the present sequence data indicate that murine norovirus strains are also genetically diverse and can, therefore, be subdivided in genetic clusters. The MNV strains analysed in the present study were classified into 8 genetic clusters. The cluster pattern is comparable to the diversity of the sapoviruses (SV), another genus within the family *Caliciviridae* [11, 29]. These data suggest that MNVs, like SVs, could be evolutionary more stable than human noroviruses. Published MNV1–4 complete nucleotide sequences are prototypes of clusters 1 to 4, whereas M21, S36, S74 and S99 would be the reference sequences for clusters 5 to 8 (Fig. 1a). However, the divergences of partial ORF1 and ORF2 sequences of MNV isolates investigated here reveal a high degree of variability among murine noroviruses.

It is noticeable that the genetic relatedness of MNV isolates is not always equivalent when analyzing different genome regions. The phylogenetic analysis presented here demonstrated that recombination events might have occurred between murine norovirus strains (Fig. 2). Analysis of the sequences obtained from isolate S28 by a nucleotide identity window search indicated a phylogenetic shift (Fig. 3a). Furthermore, reference strain MNV-4 clustered differentially (Fig. 3b). In addition to the high error rate of the viral RNA-dependent RNA polymerase due to the lack of proofreading activity, recombination also contributes to the diversity and the evolution of RNA viruses [34]. Intertypic recombination within human SVs as well as bovine and human NVs has been demonstrated by comparison of complete genome sequences and incongruent clustering of sub-genomic sequence fragments from different regions [1, 5, 10, 11, 18, 28]. Breakpoint analysis of recombinant viruses revealed the ORF1-ORF2 overlap as a recombination site. Our data confirm that a putative crossover point of MNV recombinants (Fig. 3) is located within 100 nucleotides (Fig. 1b) of this region. This junction region is highly conserved, suggesting that the recombination might be driven by homologous RNA interaction, comparable to picornaviruses and coronaviruses, where the “copy choice” model of RNA recombination is preferred [20, 22]. Occurrence of subgenomic RNA during replication of MNV supports this model [17]. Additionally, RNA promoter regions often contain stem-loop structures [16]. We found evidence of such structures within the highly conserved junction of MNV strains.
In conclusion, our study has demonstrated a high prevalence of genetically divergent murine noroviruses in the faeces of different mouse lines. These results confirm recent observations indicating that MNV commonly circulates among laboratory mice. The potential occurrence of recombinant strains has to be taken into account for phylogenetic analysis of MNV strains. Therefore, sequencing of different genome regions is recommended. The PCR methods used in this study provide means for routine diagnosis and molecular characterization of MNV.

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