Diversity of murine norovirus in wild-rodent populations: species-specific associations suggest an ancient divergence

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A survey of wild-rodent populations has revealed that murine norovirus (MNV) is present and diverse in wild-house mice Mus musculus. This virus is genetically similar to MNV infecting show mice and previously described variants circulating in laboratory mice. The detection of MNV in wild-mouse populations suggests that MNV infection of laboratory mice and show mice (from which laboratory mice are derived) derives from contact with or their origins from wild-mouse progenitors. The survey additionally identified frequent infection of wood mice (Apodemus sylvaticus) with genetically divergent variants of MNV. These viruses are distinct from previously described MNV variants, differing by 22–23% over the complete genome sequence compared with a maximum of 13% between M. musculus-derived strains. Comparison with other noroviruses reveals that the Apodemus MNV groups with MNV in genogroup V and shares the same overall genome organization, predicted lengths of proteins encoded by ORFs 1–3 and the existence of a conserved alternative reading frame in VP1 encoding a homologue of the MNV ORF4. Different Apodemus MNV isolates were as variable as MNV isolates and showed evidence for inter-isolate recombination. Our observation of species-specific associations of MNV variants in wild populations suggests that murine noroviruses have an ancient origin, a feature that they may share with other norovirus genogroups.

INTRODUCTION

Murine norovirus (MNV) was first identified in laboratory mice deficient in both the recombination-activating gene 2 (RAG2) and the signal transducer and activator of transcription 1 gene (STAT1) (Karst et al., 2003). MNV infection appears to be widespread in laboratory mice, with serological evidence of infection found in 6–26% of sera (Hsu et al., 2005; Kim et al., 2011) and PCR-positive results obtained in 23% of samples (6–100% in different facilities; Kim et al., 2010). MNV has been identified in a wide variety of inbred and transgenic mouse strains from laboratories across the world.

The origin of MNV in laboratory mouse strains is unknown. Phylogenetic analysis of the complete genome sequences of 26 MNV variants suggests that MNV comprises a single group (genogroup V) of viruses that are more than 87% identical to each other (Thackray et al., 2007). However, genetic subgroupings of MNV variants are associated with the animal facility of origin rather than country or mouse strain (Thackray et al., 2007; Kim et al., 2010; Barron et al., 2011). This could reflect sampling of MNV diversity present in the progenitors of modern laboratory mice, or the repeated introduction of MNV virus variants from unknown local hosts (Barron et al., 2011). Serological evidence for MNV infection has been reported for two of 31 wild mice trapped in Pennsylvania, USA (Parker et al., 2009), but no nucleotide sequence information is available to confirm the identity of these isolates.

A zoonotic relationship between mice and humans for MNV is unlikely as there are no reports of MNV in humans. Zoonotic relationships for other norovirus genogroups are similarly uncertain. Human isolates are confined to genogroups I, II and IV, while genogroups III and V (MNV) are restricted to ruminants and mice, respectively (Zheng et al., 2006). Although genogroups II and IV have been detected in pigs (Mattison et al., 2007) and dogs (Martella et al., 2008), the original direction of transmission...
with respect to humans is unknown. Nevertheless, several authors have raised the possibility that the epidemiology and pathogenicity of noroviruses might be significantly affected by zoonotic relationships (Bank-Wolf et al., 2010; Marshall & Bruggink, 2011).

In order to clarify the origin and zoonotic context of MNV we have studied its presence in wild-rodent populations. We report the detection of MNV in both natural and managed Mus musculus populations, as well as the detection of a divergent MNV-related virus in the wood mouse Apodemus sylvaticus. The observation of species-specific virus variants is consistent with co-speciation of virus and host, a hypothesis which has implications for the zoonotic context of human norovirus infections and the tempo of RNA virus evolution.

## RESULTS

### Detection of MNV in M. musculus populations

We tested faeces from a wide variety of domesticated M. musculus populations for the presence of MNV by PCR in order to establish if MNV is confined to laboratory mice. These samples included cages of laboratory mice, each of a different strain, as a reference population, adult mice being sold as pets or as snake food by pet shops and show mice from different breeders. The cages of laboratory mice each contained more than one individual and 67 % of samples were PCR positive for MNV (Table 1). Virus was also detected in faecal pellets from non-laboratory domesticated mice such as pet mice and mice bred for competitive showing. MNV was not detected in faecal pellets dissected from 25 adult domesticated mice sold as snake food, possibly because these mice represented a single batch from one breeder.

Phylogenetic analysis of the ORF2 sequence (1623 nt) of virus isolated from domesticated mice revealed restricted genetic diversity (Fig. 1). Virus sequences from 23 show mice clustered together (mean nucleotide divergence of 3.8 %, maximum 6.6 %), as also did sequences from four laboratory mice (4.2 % divergent, maximum 4.8 %). An additional laboratory mouse isolate (Lab12) was up to 8.8 % divergent from the other laboratory mouse isolates. Similar phylogenetic relationships were observed when comparisons were made of 10 further laboratory mice isolates for a 739 nt region of ORF2/3 encoding the C-terminal half of VP1 and part of VP2; 13 sequences fell into a single group differing by at most 8.1 % of nucleotide positions, while two additional sequences (Lab12 and Lab32) differed from members of this group and from each other by 9.6–12.4 %. The same phylogenetic groupings were observed when a 790 nt region in ORF1 (positions 1001–1791) of these laboratory mouse virus isolates were analysed, together with another two laboratory isolates (Lab10 and Lab34). The Lab34 sequence clustered with the main group, while the Lab10 sequence grouped separately from this group and also from the Lab12 and Lab32 sequences, being most closely related to the sequence of GenBank accession no. FJ446720 (data not shown). BLAST searches of GenBank with the ORF1 or VP1/2 Lab10, 12 and 32 sequences did not identify any sequences more closely related than the closest complete genome sequence.

In order to test the possibility that MNV of domesticated mice was derived from virus infecting a wild progenitor, we also tested faeces from a variety of different wild-mouse populations in both rural and urban settings. We were unable to detect MNV in M. musculus faeces of unknown age sampled from houses (n = 2) or from an urban stable (n = 38). However, amongst M. musculus faeces sampled from a wider variety of locations in a rural location two of 12 samples were PCR positive. These faecal pellets were also of unknown age, although one of the PCR-positive samples was obtained from the anus of a mouse caught in a trap set in an open-farm building. The other PCR-positive sample was collected from a grain store/vehicle shed located 1.5 miles away and separated from the first site by a river. This is the first report that the MNV genome can be detected in wild-M. musculus populations.

Nucleotide sequence analysis of the 1623 nt ORF2 region revealed that one of these MNV wild-mouse isolates grouped amongst previously described MNV variants (Fig. 1). The other did not group with any of the complete genome sequences, differing at 12 % or more of nucleotide positions. No more closely related sequence was present amongst searches of GenBank with the ORF1 or VP1/2 Lab10, 12 and 32 sequences.

### Table 1. Detection of MNV in M. musculus

<table>
<thead>
<tr>
<th>Source</th>
<th>PCR positive/no. tested</th>
<th>Positive (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory</td>
<td>26/39</td>
<td>67</td>
<td>Fresh – multiple individuals in cage</td>
</tr>
<tr>
<td>Pet shop</td>
<td>5/7</td>
<td>71</td>
<td>Fresh – multiple individuals in cage</td>
</tr>
<tr>
<td>Show mice</td>
<td>24/69</td>
<td>35</td>
<td>Fresh – mixture of individuals and groups</td>
</tr>
<tr>
<td>Snake food</td>
<td>0/25</td>
<td>0</td>
<td>Individually dissected from colon</td>
</tr>
<tr>
<td>Urban houses</td>
<td>0/25</td>
<td>0</td>
<td>Dry faeces of unknown age</td>
</tr>
<tr>
<td>Urban stables</td>
<td>0/38</td>
<td>0</td>
<td>Dry faeces of unknown age</td>
</tr>
<tr>
<td>Rural farm buildings</td>
<td>2/12</td>
<td>17</td>
<td>Dry faeces of unknown age</td>
</tr>
</tbody>
</table>
sequences reported here were sampled from nearby sites, the nucleotide divergence between them (14 %) was slightly greater than the maximum divergence observed for this region amongst 27 distinct MNV complete genome sequences reported from animal facilities around the world (13.3 %), and substantially greater than the mean divergence observed amongst these sequences (9 %).

Screening of other small mammal species for MNV-related viruses

Our finding that MNV can be detected in wild-M. musculus populations led us to screen wild populations of other small mammal species for the presence of MNV or related viruses (Table 2). A variety of sources were screened including faeces collected in stables, farms and sheds, and faeces dissected from rodents collected by trapping or retrieved shortly after being killed by domestic cats. Samples were screened with one or more sets of MNV-specific primers and also with degenerate primers covering a region of ORF1 conserved between MNV and human norovirus isolates and designed so as to detect divergent viruses (Methods).

PCR products were not detected in any of the vole, shrew or rat samples or in RNA extracted from the spleen and jejunum of bank voles (n=4) or a wood mouse (A. sylvaticus). However, 11 of 51 (22 %) dissected faecal samples from wood mice were PCR positive for MNV. Nucleotide sequences were obtained for two different isolates of this virus and compared to those of other published MNV sequences. Comparison with VP1 sequences of representatives of norovirus genogroups I–V revealed that the Apodemus MNV groups closely with genogroup V, a clade consisting of all known MNV variants (data not shown). Phylogenetic analysis of two Apodemus-derived complete genome sequences revealed that this virus was distinct from all previously described MNV sequences (Fig. 2). In contrast, the complete genome sequences we obtained from five

![Fig. 1. Phylogenetic analysis of M. musculus-derived MNV VP1 nucleotide sequences. VP1 nucleotide sequences derived from 27 distinct complete MNV genome sequences were compared with those derived from 23 show mice (S, ○), five laboratory mice (Lab, ■), one pet shop mouse (PSM, ▲) and two wild mice (WM, ●). Bootstrap support of branches (1000 replications) is indicated.](http://vir.sgmjournals.org)

<table>
<thead>
<tr>
<th>Species</th>
<th>Collected faeces (no. PCR positive/no. tested)</th>
<th>Dissected faeces (no. PCR positive/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood mouse (A. sylvaticus)</td>
<td>0/7</td>
<td>11/51</td>
</tr>
<tr>
<td>Field vole (Microtus agrestis)</td>
<td>0/1</td>
<td>0/12</td>
</tr>
<tr>
<td>Bank vole (Myodes galvulus)</td>
<td>0/1</td>
<td>0/12</td>
</tr>
<tr>
<td>Brown rat (Rattus norvegicus)</td>
<td>0/4</td>
<td>–</td>
</tr>
<tr>
<td>Common shrew (Sorex araneus)</td>
<td>–</td>
<td>0/3</td>
</tr>
<tr>
<td>Pygmy shrew (Sorex minutus)</td>
<td>–</td>
<td>0/1</td>
</tr>
</tbody>
</table>
laboratory mice (Lab12, 17, 18, 22 and 26) and a single pet shop mouse (PSM, n=1) formed a cluster with previously described MNV GV isolates. The Apodemus- and Mus-derived MNV isolates differed from each other at 22–23% of nucleotide positions compared with a maximum of 13% between Mus-derived MNV isolates and 6% between the two Apodemus-derived sequences. Comparisons within ORF2 revealed that Apodemus MNV VP1 sequences differed from Mus MNV sequences at 13.5–16.4% of amino acid positions, sequence distances that span the ranges proposed for norovirus strains (0–14.07%) and clusters/groups (14.26–43.78%) (Zheng et al., 2006). Amino acid sequence variation was especially pronounced in the VP1 P2 region where the Apodemus and Mus MNV sequences differed at 27–32% of positions compared with only 6–9% for the VP1 P1 region and 5–9% for the VP1 N/S region.

Analysis of subgenomic regions of 10 additional MNV isolates from Apodemus sampled at two sites 30 miles apart revealed significant diversity between sequences. Four distinct groups were detected from analysis of the region from 1770 to 2018 (part of ORF1) differing by up to 14.5% of nucleotide positions (compared with a maximum of 19% within 27 MNV sequences). Sequences that grouped together derived from animals caught at the same sampling site. Similar groupings were observed in other subgenomic regions, although these were not always congruent. The lack of congruence is consistent with recombination having occurred between distinct Apodemus MNV strains. The diversity observed amongst Apodemus MNV samples in these different subgenomic regions was similar to or less than that documented for MNV isolates from laboratory mice sampled in animal facilities around the world. For example, the divergence amongst eight Apodemus MNV isolates for positions 5078–5718 was 5.6% or less compared with 4.9% or less for MNV, while for the region 6069–6949 the Apodemus sequences differed by a maximum of 10.7% amongst seven isolates compared with 18.3% for MNV. This may reflect the relatively small number of Apodemus MNV samples studied.

Attempts were made to passage Apodemus MNV in RAW264.7 cells, an immortalized macrophage cell line derived from M. musculus. Two Apodemus faecal supernatants (Apo 496 and 1020) that gave strong PCR signals and for which sufficient material was available were passaged four times, as also were faecal supernatants from two show mice (S2 and S20). A cytopathic effect was observed for cells infected with the show mice samples, but not for the Apodemus-derived material after four blind passages. In addition, PCR analysis of lysates of fifth passage cells using VP1-specific primers produced a signal for the cells inoculated with show mice faecal extracts, but not for those derived from Apodemus.

We also screened a small number of samples of faecal matter taken from the bedding of exotic small mammals sampled in Edinburgh pet shops (hamster n=11, guinea pig n=3, gerbil n=1, chinchilla n=1 and rat n=1), but all were PCR negative using either MNV-specific or general norovirus primers.

**Genetic organization of Apodemus MNV**

The Apodemus MNV genome was collinear with that of M. musculus-derived MNV and contained the same ORFs described for MNV, including ORF4. These reading frames were of the same lengths as in MNV with the exception of ORF4 which was seven residues shorter in Apodemus MNV. Analysis of variation in subgenomic regions revealed that the Mus MNV and Apodemus MNV sequences differed at 20–27% of nucleotide positions across most of the genome. Exceptions were the region centred on position 5000 where ORF2 and ORF4 overlap and divergence reached a minimum of 10%, and also around position 6000 corresponding to the P2 region of VP1 where divergence reached a maximum of 33%. These features mirror those observed in comparisons between MNV genomes and also in comparisons between the two Apodemus MNV genomes, although in this second case the relative conservation around position 5000 was less obvious (data not shown).

The region 5013–5076 that is absolutely conserved between MNV genomes, although in this second case the relative conservation around position 5000 was less obvious (data not shown).

The region 5013–5076 that is absolutely conserved between MNV genomes, although in this second case the relative conservation around position 5000 was less obvious (data not shown).
substitutions in the *Apodemus* MNV sequence, but these maintained the proposed pairing in loop Ia. Substitutions were also present in a second conserved region of ORF2/ORF4 (5401–5447; Thackray et al., 2007). The P1 and P1′ amino acid residues of the five proposed cleavage sites in ORF1 (Sosnovtsev et al., 2006) were absolutely conserved. A proposed stem–loop structure at the 5′-end of the MNV genome (positions 29–60; Simmonds et al., 2008) was also present in the *Apodemus* MNV genome, although covariant substitutions had changed U:A pairings to C:G pairings at two positions, while G:C and C:G pairings at either end of the main loop were changed to C:A and G:A mismatches. Another proposed stem–loop at positions 7014–7157 (Simmonds et al., 2008) was also a feature of the *Apodemus* genome sequence despite the presence of numerous substitutions.

**DISCUSSION**

This work provides the first genetic evidence for the presence of diverse variants of MNV in wild-rodent populations. The first isolation of MNV was from laboratory mice (Karst et al., 2003) and most subsequent work has focused on virus isolated from inbred strains of *M. musculus* housed in research laboratories. All these MNV isolates belong to the norovirus genogroup V with limited nucleotide sequence variation documented between isolates (Thackray et al., 2007). The limited variability observed amongst MNV isolates from domesticated mice could be explained by the virus having been introduced as a single event. This may have been within the past 100–200 years, the period during which laboratory mice were derived from show mice (reviewed by Wade & Daly, 2005). Alternatively, the limited diversity of MNV could reflect its limited diversity in the host population, presumably *M. musculus*.

In this study, we have also observed limited sequence diversity amongst 15 *M. musculus*-derived virus isolates from laboratory mice and 23 isolates from show mice. Our finding that the isolates from show mice grouped in a single clade is surprising given their diverse breeds and origins from exhibitors travelling from different parts of the UK to a large regional show. This homogeneity is especially surprising since back-crosses between show mice and laboratory mice are used by breeders of show mice to introduce coat colour variants. Previous subgroupings of strains have been observed amongst isolates from single animal facilities (Thackray et al., 2007; Kim et al., 2010; Barron et al., 2011). While the homogeneity of MNV observed within research facilities may reflect the infrequent introduction of MNV from exogenous sources (Barron et al., 2011), this does not provide a convincing explanation for the lack of diversity observed amongst show mice. A more likely alternative is that the show mice are subject to frequent re-exposure to MNV during handling by judges and other competitors, and also due to less stringent infection control measures applied to the bedding used in transport boxes. As a result there may be severe competition between strains with the consequent elimination of viruses with lower fitness.

We show here that MNV can also be found in wild-*M. musculus* populations, with distinct viruses obtained from samples taken only 1.5 miles apart. The divergence between these viruses was as extreme as the most divergent pair of currently described laboratory isolates. This suggests that the restricted variability observed amongst laboratory-derived MNV isolates may not just reflect the limited time (ca. 100 years), during which laboratory mice have been developed from show mice progenitors.

We were also able to detect a distinct but closely related virus in wild populations of the wood mouse, *A. sylvaticus*. This virus has the same overall genomic organization as MNV, including the presence of ORF4, which has been proposed to be involved in the regulation of the innate immune response (McFadden et al., 2011). The degree of variation observed amongst the *Apodemus* MNV isolates was similar to that observed amongst MNV isolates. Phylogenetic trees of subgenomic regions of these *Apodemus* MNV isolates were not always congruent, consistent with recombination occurring within this genogroup cluster, a circumstance that has previously been reported for MNV (Thackray et al., 2007) and genogroup II and IV isolates (Phan et al., 2007; Martella et al., 2009). We were unable to grow the *Apodemus*-derived MNV in RAW264.7 macrophages under conditions where MNV was successfully cultured. Given the 22% nucleotide divergence between the *Apodemus* and *Mus*-derived viruses and the fact that their respective hosts belong to different genera, it may be that the successful culture of *Apodemus* MNV will require the development of primary cell cultures from *Apodemus* tissues or the development of *Apodemus*-derived immortalized macrophage or dendritic cell lines.

We were unable to detect MNV-related viruses in faeces from a variety of other rodent species (bank vole, field vole, brown rat, hamster, guinea pig, gerbil and chinchilla) using degenerate and specific primers from different regions of the virus genome. We were also unable to detect MNV in more distantly related species, the common shrew and the pygmy shrew. In some instances the faecal samples tested were of unknown age and virus RNA could have become degraded, although in all cases we were still able to detect and sequence host mitochondrial nucleic acid. In other samples faeces were obtained by dissection from animals frozen shortly after death or from fresh litter, making degradation of virus RNA less probable as an explanation for the lack of detection of virus in these cases. We note that even amongst freshly collected faeces from communally housed laboratory mice, a third of samples were PCR negative, while virus levels can vary 100-fold between different PCR-positive samples (Müller et al., 2007).

The finding that wild-rodent populations contain distinct genogroup V noroviruses raises the possibility that these host species–virus associations are long-standing, possibly
dating back to the divergence of their host species. A similar association has been documented for genogroup III viruses that are confined to the ruminant order, having been detected only in cattle (Woode & Bridger, 1978; Liu et al., 1999; Zheng et al., 2006; Mattison et al., 2007) and sheep (Wolf et al., 2009). We note that the Mus and Apodemus MNV VP1 N/S region sequences differ by 15.5%, their host species having diverged about 8 million years ago (mya), while the sheep and bovine isolates differ by 42%, their hosts having diverged 20–30 mya.

The situation with genogroups II and IV is more complex since although the majority of isolates are from humans or environmental samples associated with human sewage, similar viruses have also been found in animal species. Genogroup II viruses have been isolated from farmed pigs around the world. These isolates belong to genogroup II subgroupings (II/11, II.18 and II/19) that have not been observed to infect humans (Wang et al., 2005; Zheng et al., 2006; L’Homme et al., 2009). In addition, genogroup II/4 virus has been detected in pooled pig farm manure, processed pork and cattle farm sewage (Mattison et al., 2007), but it was not demonstrated in these cases that virus derived from infected animals. Genogroup IV viruses have been isolated from both humans and domestic dogs (Martella et al., 2008), but again these belong to distinct subgroupings. A virus similar to the canine genogroup IV subgroup has been isolated from a captive lion (Martella et al., 2007), but it is not known if this virus was acquired during captivity or is present in wild populations of this and possibly other carnivore species. Genogroup I variants have only been isolated from humans or environmental samples where humans are the likely source.

One interpretation of these species-specific associations is that different norovirus genogroups derive from different mammalian orders or suborders, with genogroup II and IV viruses having been transmitted to humans following the domestication of pigs and dogs, respectively. According to this view, the original genogroup associations would have been genogroup I – Primates, genogroup II – suborder Suina, genogroup III – suborder Ruminantia, genogroup IV – Carnivora, genogroup V – Rodentia. These groups diverged from each other about 70–100 mya (Bininda-Emonds et al., 2007). If these associations are supported by further observations, this would suggest that norovirus genogroupings have developed and remained distinct over tens of millions of years. Similar species-specific associations have been documented for other RNA viruses such as GBV-A (Bukh & Apgar, 1997) and arenaviruses (Bowen et al., 1997). Further evidence for an ancient origin of virus lineages comes from the detection of RNA virus-related elements within the genomes of eukaryotes and with phylogenies congruent with those of their hosts (Katzourakis & Gifford, 2010). Norovirus-related elements were not detected by this approach, and we have also failed to find norovirus-related sequences amongst eukaryotic genomes (data not shown). The inferred long-term constraint on RNA virus evolution as implied by the species distribution of noroviruses challenges current conceptions of RNA virus evolution. If RNA viruses can maintain close phylogenetic relationships over long evolutionary timescales, the variation and evolution observed amongst viruses sampled years or decades apart must be subject to different constraints from those observed between viruses isolated from different species.

**METHODS**

**Samples.** Faeces were collected from a range of domesticated mice representing laboratory, pet and show mice as follows: (i) the cages of laboratory mice of 39 different strains at the University of Edinburgh; (ii) from caged mice from Edinburgh pet shops and (iii) from show mice sampled at a competitive show in England. We also sampled faeces from wild-roded populations at a range of urban and rural sites at different locations in the UK. The sample groups were (i) horse stables in Edinburgh and (ii) a variety of farm and garden buildings in a rural area near Haddington, East Lothian, 17 miles from Edinburgh. In addition, we tested faeces dissected from the rectum of small rodents in the following groups: (i) trapped in Scotland (Midlothian, Borders) and England (Cumbria); (ii) from animals trapped in houses in Edinburgh and (iii) from animals caught by domestic cats in a rural area near Haddington, East Lothian. Sampling sites and the species analysed were chosen to represent a range of locations and habitats in order to maximize the possibility of virus detection.

Faecal pellets (2–3) were softened in PBS containing protease inhibitors for 2 h on ice before homogenization. The supernatant obtained after centrifugation at 4000 g for 20 min was passed through a 0.2 μm filter and stored at −20 °C. RNA was purified from 140 μl of these samples using the QIAamp Viral RNA mini kit (Qiagen) following the manufacturer’s protocol.

**RT-PCR and sequencing.** cDNA was generated from 5 μl virus RNA using random hexamer as primer in 20 μl reactions containing 15 U AMV reverse transcriptase with incubation at room temperature or 25 °C for 10 min, followed by 50–60 min at 37–42 °C. PCRs (20 μl) were carried out on 2 μl cDNA with 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s using GoTaq DNA polymerase (Promega). The primers used for screening were set A: MNV capsid (outer sense (OS) 5’-TCAAGCAGTCTTTGTTGAATGGAGG-3’, outer anti-sense (OAS) 5’-TCAAGAAGAGGGAGTTGAATGG-3’), inner sense (IS) 5’-ACCCCAGGTGAAATACCTTTGTTG-3’, inner anti-sense (IAS) 5’-TGCGAAAATAAGTCTTTGAGG-3’; set B: general norovirus ORFI hemi-nested (OS 5’-SCCCMSTCACKCIVAAVTGTAATCG-3’, OAS and IAS 5’-CCYTTSCTCRTNNGGGTTRTT-3’, IS 5’-TGTGTAGYAGRATTGARAAAYAR-3’); set C: hemi-nested capsid (OS 5’-ACCCCAAGTGGAAATTGTTGAGG-3’, OAS and IAS 5’-TGCGAAATAGGGTGTGAATCGGAGG-3’), IS 5’-TGCCACCTTCCTAGCTGATGAC3’T; set D: rodent norovirus capsid (OS 4964 5’-GATGARATGTCGGTGCCACAG-3’, OAS 5510 5’-AC-RTGTGGGAAACTGTGTACGCTG-3’, IS 4998 5’-CTGGCCGTGGGTGCCGTTTGG3’ and IAS 5429 5’-CCACCTTGGCCAGCTAAAGGC-3’). PCRs were separated by electrophoresis through 2% agarose gels containing ethidium bromide and visualized under UV light. Products were sequenced using BigDye reactions separated on agarose gels containing ethidium bromide and visualized under UV light. Products were sequenced using BigDye reactions separated on an ABI 3730 machine (Edinburgh University GenePool). Complete and subgenomic virus sequences were obtained by amplification of overlapping subgenomic fragments using a series of MNV- and *Apodemus*-MNV-specific primers (primer sequences available from authors, Table 3).

The rodent species from which environmental samples of faeces were derived was discovered by phylogenetic analysis of mitochondrial
Table 3. Summary of faecal samples from which virus sequences were obtained

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Species</th>
<th>Year</th>
<th>Source (all UK)</th>
<th>ORF1</th>
<th>GenBank accession numbers</th>
<th>ORF2/3</th>
<th>Genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab12, 17, 18, 22, 26</td>
<td>M. musculus</td>
<td>2007</td>
<td>Laboratory (Edinburgh)</td>
<td>6</td>
<td>JN975495–7</td>
<td>5</td>
<td>JN975522–44</td>
</tr>
<tr>
<td>Lab1, 5, 7, 9, 19, 21, 25, 31, 32, 38</td>
<td>M. musculus</td>
<td>2007</td>
<td>Laboratory (Edinburgh)</td>
<td>10 (759 nt)</td>
<td>JN975499–508</td>
<td>10 (739 nt)</td>
<td>JN975509–18, JN975483–90</td>
</tr>
<tr>
<td>Lab10, 34</td>
<td>M. musculus</td>
<td>2007</td>
<td>Laboratory (Edinburgh)</td>
<td>2 (790 nt)</td>
<td>JN975520–1</td>
<td>2 (1670 nt)</td>
<td>JN975545–6</td>
</tr>
<tr>
<td>Pet shop (Edinburgh)</td>
<td>M. musculus</td>
<td>2007</td>
<td>Pet shop (Edinburgh)</td>
<td>1</td>
<td>JN975489</td>
<td>2</td>
<td>JN975569–70</td>
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<tr>
<td>Show (Honley)</td>
<td>M. musculus</td>
<td>2010</td>
<td>Show (Honley)</td>
<td>6</td>
<td>JN975491–2</td>
<td>6 (858–1984 nt)</td>
<td>JN975475–82, JN975493–7</td>
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<tr>
<td>Rural (East Lothian)</td>
<td>M. musculus</td>
<td>2010</td>
<td>Rural (East Lothian)</td>
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<tr>
<td>PSM1</td>
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<td>8</td>
<td>JN975499</td>
<td>8</td>
<td>JN975475–82, JN975493–7</td>
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</tbody>
</table>

cytochrome b sequences amplified from faecal cDNA products using nested primers OS 5′-CACTAYACATCGAYACAWYAACMG-3′, OAS 5′-TARGGWRAAGGWTATTTCRAGT-3′, IS 5′-CTCCTARAT TCATTCKACTAG-3′, IAS 5′-GTCGAGCCTAAAYTAYGG-3′. Only samples that were PCR positive in this assay were used for MNV screening with the exception of the urban stables samples of which only 2/39 were tested, both of which contained M. musculus mitochondrial sequences.

**Phylogenetic analysis.** Nucleotide sequences were edited and aligned using the SSE package (Simmonds & Smith, 1999). Phylogenetic analysis of these and related sequences deposited in GenBank were carried out using MEGA 4 (Tamura et al., 2007). Phylogenetic trees were produced using the neighbour-joining algorithm using the maximum composite likelihood model of nucleotide substitution and pairwise deletion of gaps or missing data.

**Cell culture.** RAW264.7 macrophages (at <20 passages) were seeded into a 96-well plate in Dulbecco’s modified Eagle’s medium containing 10% FCS, penicillin (100 U ml⁻¹; Invitrogen), streptomycin (100 µg ml⁻¹; Invitrogen) and plasmocin (5 µg ml⁻¹; Source Bioscience). Each well containing 1 × 10⁶ cells in 100 µl volume was then inoculated with 25 µl faecal supernatant that had been passed through a 2 micron filter (VWR International). After 1 h, the medium was topped up to 250 µl total volume per well. Plates were incubated at 37 °C, 5% CO₂. Plates were frozen at −80 °C after 4 days. This passage style was continued four times using freeze-thawed lysate from the previous passage as an inoculum. For the isolation of viral RNA, cells were seeded at 3.75 × 10⁶ cells per well of a six-well dish and 100 µl clarified lysate was used as an inoculum. Post-infection (16 h) RNA was isolated using a Qiagen RNA isolation kit as described previously.

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