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Research paper

A novel reassortant mammalian orthoreovirus with a divergent S1 genome segment identified in a traveler with diarrhea

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ABSTRACT

Mammalian orthoreoviruses with reassortant genomes have recently been detected in various mammals and humans with respiratory, central nervous system, and gastrointestinal symptoms. This study describes the detection of the novel reassortant mammalian orthoreovirus SI-MRV07 in a traveler with gastroenteritis that returned from southeast Asia. The virus was initially detected with electron microscopy in stool, followed by propagation in the epithelial-like monkey kidney Marc145 cell line. Whole-genome sequencing revealed the reassortant nature of the genome segments, whereby the S1 genome segment was the most variable according to known sequences deposited in GenBank. Based on the nucleotide sequence of the S1 genome segment, the isolate clusters to serotype 2, close to the reference strain Jones T2J. The patient's serum showed the highest virus neutralization capacity toward SI-MRV07 and T2J isolates. This study provides additional insight into emerging mammalian orthoreoviruses with reassortant genomes and possible zoonotic potential, which should be carefully monitored in the future.

1. Introduction

Mammalian orthoreoviruses (MRVs) were first detected in the 1950s in human respiratory and gastrointestinal tracts, although they are rarely associated with severe medical conditions (Sabin, 1959). Since then, several studies have been carried out to determine the clinical importance of MRVs in humans. MRVs have been generally recognized as respiratory and enteric orphan viruses. MRV seroprevalence correlates with age, and already amounts to up to 50% in 5-year-old children (Lerner et al., 1962; Leers and Rozee, 1966; Tai et al., 2005). MRVs are double-layered non-enveloped viruses, and thus relatively stable in the environment, allowing not only person-to-person infection, but also indirect infection with contaminated food, water, or surfaces. MRVs are often present in wastewater, and they are commonly used as surrogates to determine fecal pollution of surface or groundwater (Betancourt and Gerba, 2016). The presence of MRVs in wastewater, as well as the possibility of indirect transmission and infection, might explain the high seroprevalence already observed in early childhood.

Mammalian orthoreoviruses are double-stranded viruses with a segmented genome. The whole genome consists of 10 segments, which are designated as large (L, three segments), medium (M, three segments), or small (S, four segments) based on their electrophoretic mobility (Nibert et al., 1990). Neutralization and hemagglutinin activities are restricted to the S1 genome segment (Weiner and Fields, 1977), which encodes the outermost glycoprotein σ1. This is responsible for viral attachment to cellular receptors, and it defines the MRV serotype (Lee et al., 1981). Within the S1 open reading frame (ORF) most of the MRV strains possess another shorter and shifted ORF that encodes the non-structural σ1s protein. The expression of σ1s has been shown to be an important factor in viral pathogenesis (Boehme et al., 2009; Boehme et al., 2011). Having segmented genomes, MRVs can reassort their genomes during coinfections. This is evident from detailed molecular studies, reporting highly divergent and reassortant reovirus strains (Steyer et al., 2013; Lelli et al., 2015; Thimmasandra Narayanappa et al., 2015; Feher et al., 2017). Moreover, MRVs have been found in almost all mammals studied, and many MRVs can cross the species barrier, resulting in a constant source of zoonotic viruses with a potential threat to humans.

Although generally considered bystanders, there have been some recent reports on reovirus infections in animals and humans associated with severe clinical presentations, mainly related to the respiratory, enteric, and central nervous systems (Decaro et al., 2005; Ouattara et al., 2011; Steyer et al., 2013; Thimmasandra Narayanappa et al.,

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2015). Interestingly, the majority of these clinical conditions were caused by reovirus isolates with evident zoonotic transmission.

This report presents a case of acute gastroenteritis caused by the novel reassortant mammalian orthoreovirus SI-MRV07 in an adult traveler returning from southeast Asia. The virus was initially identified in the patient's stool by electron microscopy followed by isolation in epithelial-like monkey kidney cell line and whole-genome shotgun sequencing. Characterization of the isolate SI-MRV07 revealed a zoonotic strain with a reassortant genome. This study provides additional insight into emerging mammalian orthoreoviruses with reassortant genomes and possible zoonotic potential.

2. Case description

A 23-year-old female with recent travel history was examined in an outpatient setting at the Department of Infectious Diseases, Ljubljana University Medical Center (Slovenia) due to acute gastroenteritis. The patient was otherwise healthy, with no regular medications, except atovaquone/proguanil (Malarone), which she had been taking as malaria chemoprophylaxis during her travel to southeast Asia (Myanmar and Thailand). The patient had been traveling for 4 weeks, and she had returned home 5 days prior to her first visit to the clinic. One week before, the patient fell ill with a fever and abdominal pain with cramping and diarrhea. She took one tablet of azithromycin (500 mg) on the first day of symptoms. Due to vomiting, the patient did not take any further tablets.

The symptoms improved for a day or two, but then her diarrhea returned. The patient noted no blood or mucus in her stools. At presentation, she was afebrile, normocardic (pulse 68 per min), with blood pressure of 125/71 mmHg, and eupnoeic. Her abdomen was soft, with no palpatory enlargement of the liver or spleen. Her heart and lungs were normal on auscultation. The patient's skin was warm, with no signs of infection. Laboratory results were within the normal ranges (*e.g.*, C-reactive protein, leucocytes, and electrolytes). The patient recovered completely within 3 days.

A written consent was obtained and the study was explained to the patient prior to sample processing for research purposes.

3. Materials and methods

Two stool samples were collected for microbiological testing, the first at initial clinical evaluation (S_{t0}) and the second at follow-up, on day 46 after her first presentation at the clinic (S_{t46}). In addition, two blood samples (B_{t0} and B_{t46}) were collected for a full blood count, electrolytes, and C-reactive protein.

3.1. Molecular analysis

A 10% stool suspension was prepared in phosphate-buffered saline (PBS), and 200 µl of this suspension was processed for nucleic-acid extraction (MagNA Pure Compact; Roche, Basel, Switzerland). The nucleic-acid extract was used in all of the further molecular testing for several viral (noroviruses GGI, GGII, group A rotaviruses, adenoviruses species F, and human astroviruses), bacterial (*Yersinia* sp., *Campylobacter* spp., *Shigella* sp., *Salmonella* sp., *Plesiomonas* sp., and verotoxin-producing *E. coli*), and parasitic (*Giardia* sp. and *Cryptosporidium* sp.) pathogens, following the previously described workflow of an in-house standard molecular syndrome diagnostics approach (Kotar et al., 2018). Briefly, the molecular tests were performed as multiplex reactions using a multiplex RT-PCR system (Roche), which included specific target primer–probe modular assays (TibMolbiol, Berlin, Germany). In addition, a portion of the stool suspension was centrifuged at 9000 ×*g* for 10 min to obtain a clear suspension for direct electron microscopy examination and for virus isolation in cell cultures. Direct negative-staining electron microscopy was carried out with 2% phosphotungstic acid (pH 4.5), and electron micrograph grids were examined at 80,000× magnification in a transmission electron microscope (JEM 1400 Plus; Jeol, Tokyo, Japan) at an acceleration voltage of 120 kV.

For reovirus confirmation, broad-spectrum RT-PCR was performed that targeted the MRV L3 genome segment using the L3-1 and L3-5 primers (Ouattara et al., 2011), as described previously (Naglic et al., 2018). Briefly, the RNA was denaturated together with the reverse L3-5 primer, followed by RT-PCR amplification. The PCR product (512 bp) was purified and the direct Sanger sequencing method was performed. The sequences were analyzed and the contigs were uploaded to the Basic Local Alignment Search Tool (BLASTn) to determine the highest similarities to sequences in the NCBI GenBank nucleotide database.

3.2. Virus isolation

Next, 100 µl of the clarified stool suspension was transferred to 1.5 ml of Eagle's minimum essential medium with 5% fetal bovine serum. The inoculum was passed through a 0.2 µm filter and transferred to an 80% confluent cell monolayer of the epithelial-like monkey kidney Marc145 cell line (provided by the Department of Virology, IZSLER, Brescia, Italy). The cells were incubated for 1 h at 37 °C and under 5% CO_2 to allow binding of the viruses to the cells. After this incubation, the inoculum was discarded from the cell layer, and 8 ml of fresh Eagle's minimum essential medium with 5% fetal bovine serum was added. The cells were further incubated under the conditions specified above and were observed daily for the development of any cytopathic effect (CPE). After the onset of a CPE, the viruses were harvested through two freeze/thaw cycles of the infected cell cultures and centrifugation of the cell debris; once harvested, the total virus suspension was stored at −80 °C.

3.3. Virus neutralization test

Virus neutralization tests (VNT) were performed on serum samples from the patient that were collected during the acute stage of infection at her first visit to the clinic (B_{t0}), and again with the follow-up serum sample (B_{t46}). Serial two-fold dilutions from 1:5 to 1:640 of the patient's sera were prepared in 25 µl of Eagle's minimum essential medium in 96-well microplates and mixed with an equal volume of 100 tissue culture infection dose 50% ($TCID_{50}$) virus inoculum. Virus back titration of the inoculum was included, as six wells per 10-fold dilution, to confirm the validity of the test results. The plates were incubated for 1 h at 37 °C and under 5% CO_2. After this incubation, 50 µl of the Marc145 cell line at 10^5 cells per ml in Eagle's minimum essential medium containing 10% fetal bovine serum was added to each well. After a 5-day incubation at 37 °C and under 5% CO_2, the wells were scored for CPE and the neutralizing titers were expressed as the reciprocal of the final serum dilution required to neutralize 90% of the inoculated cells. In addition to the MRV isolate of the patient's SI-MRV07, the reference MRV strains of T1L (Lang; ATCC VR-230), T2J (Jones; ATCC VR-231), and T3D (Dearing; ATCC VR-824) were included for positive controls and type specificities for the serum antibody reactivity.

3.4. Indirect immunofluorescence assay

To test the reactivity of the patient's sera against specific reovirus isolates, indirect immunofluorescence (IIF) assays were performed using the infected Marc145 cell line, harvested 24 h post infection. One drop (approximately 20 µl) of infected cell suspension was transferred to each field of a 10-field IIF slide and left to dry overnight in a laminar flow safety cabinet. The IIF slides were then fixed in cold acetone for 10 min at room temperature. Serial two-fold dilutions of the patient's sera were prepared in PBS (from 1:16 to 1:2048) and 15 µl of each dilution was transferred to each field of the IIF slide. Following a 30 min incubation at room temperature in a humid chamber, the slides were carefully washed in PBS and then incubated in PBS for 10 min. The

slides were finally washed in distilled water and left to dry. Then 15 µl of conjugate was transferred to each field of the IIF slides and incubated for 30 min at room temperature in a humid chamber followed by an additional washing step as described above. The slides were examined at 400× magnification under an immunofluorescence microscope (EUROStar II; Euroimmune US, Mountain Lakes, NY).

3.5. Orthoreovirus complete genome sequencing

To obtain the complete genome sequence of the virus isolate, a cell culture supernatant was used for total nucleic-acid extraction. The RNA was depleted from the original suspension with 1 mg/ml working solution of RNase A (Qiagen, Hilden, Germany), for 15 min at room temperature. The samples then proceeded directly to the total nucleic-acid extraction protocol (iPrep Virus DNA/RNA kits; Invitrogen, Thermo Fisher Scientific, Waltham, MA). Seventeen microliters of the extracted RNA were used for reverse transcription, and subsequently for second strand cDNA synthesis, using cDNA Synthesis System (Roche). The cDNA was purified with a High Pure PCR Product Purification kit (Roche) and cDNA concentrations were measured using a Qubit fluorometer (Thermo Fisher Scientific). The whole-genome shotgun sequencing library was prepared using Nextera XT DNA Library Prep kits (Illumina, San Diego, CA), and sequenced using the MiSeq system (Illumina) with MiSeq Reagent kits v3 (Illumina). The raw data were analyzed using Geneious 8.1.8 software (Biomatters Ltd., Auckland, New Zealand).

To obtain the complete genome sequence of the SI-MRV07 isolate, the reads were mapped to the reference MRV genome, which was obtained from GenBank (MRV Lang 1: acc. nos. M24734, AF378003, AF129820, AF461682, AF490617, AF174382, EF494445, L19774, M14325, and M13139), and the individual genome segments were assembled *de novo* using Geneious 8.1.8 (Biomatters Ltd.) software with its default settings. The contigs generated were then compared for similarity against all of the virus sequences deposited in the NCBI GenBank nucleotide database using BLASTn.

To obtain the complete sequence of the S1 genome segment, *de novo* assembly was performed using SPAdes v3.12.0 (Bankevich et al., 2012), following read-space restrictions to the k-mer spectrum (k-mer length = 27 nt), found in the reference MRV S1 genome segments that were obtained from the NCBI nucleotide database (downloaded on December 27th, 2017). Read-space restriction was performed using bbduk (bbtools, https://jgi.doe.gov/data-and-tools/bbtools/). Consensus sequences of the genome segments were analyzed for their ORFs using Geneious 8.1.8. software (Biomatters, Ltd.), and the deduced amino-acid sequences were obtained. Phylogenetic and evolutionary analyses were carried out for all 10 genome segments using MEGA 6.0 software (Tamura et al., 2013). A multiple sequence alignment was obtained using the ClustalW algorithm. The maximum likelihood phylogenetic calculations were carried out using MEGA 6.0 software, based on the Tamura–Nei model (Tamura and Nei, 1993) with 1000 bootstrap replicates.

4. Results

A routine in-house microbiological syndrome-based diagnostics approach developed for patients with severe diarrhea, which includes a range of molecular tests and electron microscopy examination of stool suspensions, was used to determine the etiology of diarrhea in our patient. The molecular panel tested negative for all targeted bacterial, viral, and parasitic pathogens (Supplementary Table 1). However, under electron microscopy examination, 80 nm viral particles with a reovirus capsid morphology were observed (Supplementary Fig. 1). Broad-spectrum MRV RT-PCR was positive, with a strong band at the correct amplicon length (512 bp) of the MRV L3 genome segment. The partial L3 sequence shared 87% identity with the MRV serotype 3 MPC/04 isolate (NCBI GenBank accession number GQ468270). The follow-up stool sample (S_{t46}) tested negative in both electron microscopy and broad-spectrum MRV RT-PCR.

4.1. Virus isolation

After direct inoculation of the filtered stool suspension (S_{t0}) onto the Marc145 cells, a non-specific CPE was noted at 2 days post inoculation. The beginning of the CPE was observed on the 1st day post inoculation, with no observable progress on the 2nd day. The virus was harvested and a second passage of the virus was prepared to exclude the appearance of a CPE due to toxicity of the stool components. During the second passage, the CPE appeared 3 days post inoculation, and the total virus was harvested and stored at −80 °C. Direct electron microscopy examination of the cell culture suspension from both virus passages again revealed the reovirus particles, as observed in the stool suspension (Supplementary Fig. 1). The isolate was designated as SI-MRV07 and was deposited with the European Virus Archive (EVAg; https://www.european-virus-archive.com/; ref. no. 007 V-02722). The virus isolate is available upon request.

4.2. Virus neutralization and indirect immunofluorescence assay

As presented in Table 1, virus neutralization of the initial serum sample (B_{t0}) was only observed for the reference T1L strain at a very low titer (with the titer expressed as the reciprocal of the final serum dilution required to neutralize 90% of the inoculated cells). However, the re-convalescent serum sample (B_{t46}) neutralized the patient SI-MRV07 isolate and the reference T2J strain at high titer (Table 1).

The IIF assay confirmed the VNT results for the B_{t46} sample, which exhibited the highest reactivity of the patient's sera against the SI-MRV07 isolate and the T2J reference strain (Supplementary Fig. 2). In addition, the IIF assay also indicated cross-reactivity in the re-convalescent serum against the reference serotype 1 and 3 strains (Table 1, T1L, T3D).

4.3. Molecular analysis

The complete SI-MRV07 viral genome was assembled and compiled

Table 1
Neutralizing titers for the virus neutralization tests and indirect immunofluorescence assays for IgG for the patient's serum samples taken at the initial emergency visit (B_{t0}) and after 46 days (B_{t46}).

Serum sample	Neutralizing titer[a]							
	Virus neutralization test				Indirect immunofluorescence assay			
	T1L	T2J	T3D	SI-MRV07	T1L	T2J	T3D	SI-MRV07
B_{t0}	80	0	0	0	0	0	0	0
B_{t46}	80	> 1280	20	> 1280	256	1024	128	1024

Reference MRV strains: T1L (Lang; ATCC VR-230), T2J (Jones; ATCC VR-231), T3D (Dearing; ATCC VR-824).
[a] neutralizing titer: expressed as the reciprocal of the final serum dilution required to neutralize 90% of the inoculated cells.

after whole-genome shotgun sequencing. Nine genome segments were obtained by mapping next-generation sequencing reads to the MRV reference strain genome Lang. The SI-MRV07 S1 ORF was assembled *de novo*, following k-mer–based read-space restriction, as described above (see Section 3.5). The sequence identities between the S1 ORFs of the novel SI-MRV07 isolate and the T2J strain amounted to 69.5% in the nucleotide and 73.3% in the amino-acid sequence contexts, respectively. According to the similarity score and the phylogenetic analysis of the S1 genome segment shown in Fig. 1, the SI-MRV07 isolate belongs to the genetic group of serotype 2. Moreover, this strain could be regarded as a diverse variant of serotype 2. The maximum likelihood phylogenetic tree grouped this strain along with the reference strain T2J but away from the remainder of the currently detected serotype 2 strains deposited in GenBank. The newly detected MRV strain also contains the internal small ORF for σ1s within the S1 genome segment. The ORFs identified in the complete SI-MRV07 genome assembly are given in Table 2. The highest nucleotide and amino-acid identities of 10 genome segments were found with various MRV strains, belonging to serotypes 1, 2 and 3. As shown in Table 2, genome segments L1, M3 and S2 shared 89.0%–91.9% nucleotide identities to the most related strains in GenBank belonging to MRV serotype 1. On the other hand, L2, S1 and S3 were closely related to the MRV strains with serotype 2 specificity. Genome segment L3, M1 and M2 were closely related to serotype 3 strains and S4 segment shared the highest nucleotide identity to the MRV reference strains Lang (serotype 1) and Dearing (serotype 3). In phylogenetic analysis of all 10 genome segments, the isolate SI-MRV07 in most analysis clustered separately from the most related MRV strains (see Supplementary Fig. 3).

The nucleotide sequences obtained in this study have been deposited in GenBank under the following accession numbers: MG999576–MG999585.

5. Discussion

Reoviruses have been intensively investigated as potential human pathogens for a long time, but only a few have been proven to cause severe disease in humans. Lately, several novel reoviruses with zoonotic potential have been characterized in bats and other mammals. Recently a bat-like MRV, similar to those detected in bats in Germany and Italy (Kohl et al., 2012; Lelli et al., 2013), was also identified in Slovenia in a patient with diarrhea (Steyer et al., 2013). To the best of our knowledge, bat-specific MRVs had not been described prior to these reports. It is also worth noting that zoonotic transmission in the Slovenian case (Steyer et al., 2013) was established only indirectly, based on molecular characterization, and without further confirmation because no further samples or sample types could be obtained from the patient.

This case has indicated once more that novel MRVs have pathogenic potential in humans and can cause disease as the sole pathogen. Interestingly, the etiology of diarrheal disease in our patient was established using electron microscopy. The use of specific molecular or antigen-detecting tests alone would have resulted in an undetermined etiology of gastroenteritis. Thus, the use of electron microscopy in daily routine diagnostics is still valuable and can provide a definite advantage in cases of infections with rare and novel viral pathogens.

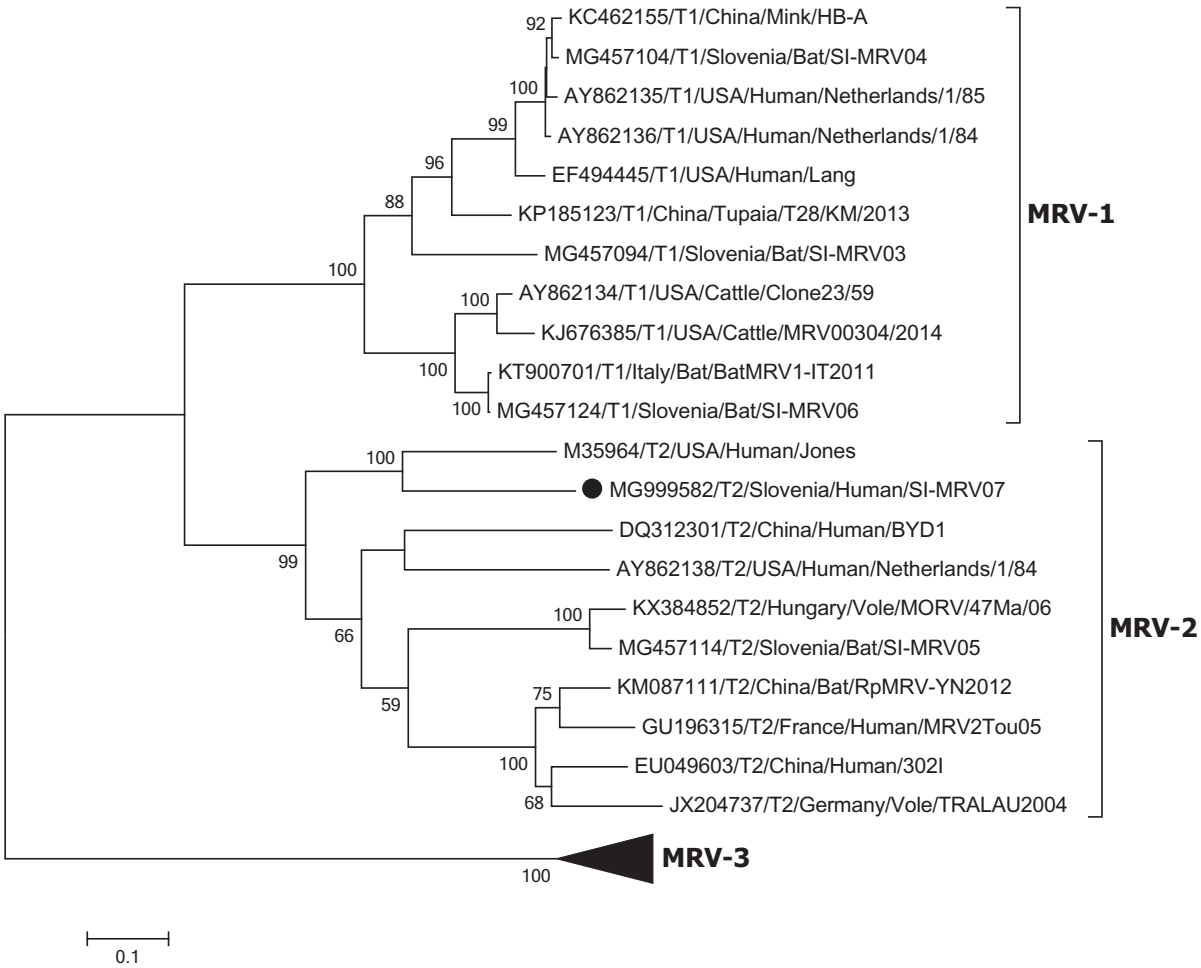

Fig. 1. Phylogenetic tree of the mammalian orthoreovirus S1 genome segments. Black dot (●) = label sequence of Slovenian MRV isolate obtained in this study (SI-MRV07). The scale bar represents the substitutions per site and is proportional to the Tamura–Nei maximum likelihood evolutionary distance.

Table 2
Genome segments with nucleotide and amino-acid identities of the reovirus SI-MRV07 isolate, compared to the most related strains in GenBank.

Serotype	BatMRV1-IT2011		MRV2Tou05		MPC/04		SI-MRV01		MRV-HLJ/2007		Lang T1L		Jones T2J		Dearing T3D	
	1		2		3		3		3		1		2		3	
	Identitiy (%)		Identitiy (%)		Identitiy (%)		Identitiy (%)		Identitiy (%)		Identitiy (%)		Identitiy (%)		Identitiy (%)	
	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa	Nucl.	Aa
L1	**90.6**	98.5	89.4	98.3	88.4	97.9	89.2	98.7	89.8	98.7	89.4	97.8	75.4	91.9	89.6	98.2
L2	76.6	93.0	**93.4**	98.5	81.4	95.5	76.0	92.7	93.1	98.3	86.2	97.0	72.8	86.6	77.0	93.1
L3	86.5	98.5	87.2	98.8	**91.0**	98.3	83.9	98.0	86.6	98.8	84.6	98.3	76.5	95.6	84.7	98.1
M1	90.1	97.1	86.4	95.3	86.5	94.2	**90.3**	97.2	86.8	95.7	88.0	96.1	71.6	81.6	87.5	95.9
M2	90.8	98.3	88.2	99.0	87.9	98.7	88.9	98.8	**95.7**	99.2	84.9	98.1	76.3	97.7	89.9	98.5
M3	84.7	96.2	84.3	96.2	83.8	95.9	84.1	96.3	83.7	95.1	**89.0**	96.3	71.9	83.4	86.0	96.5
S1	53.3	50.6	61.3	60.2	36.8	24.4	39.3	24.1	38.2	24.4	56.5	52.4	**69.5**	73.2	38.9	25.0
S2	85.8	98.3	84.3	97.8	86.1	98.5	86.1	97.1	85.6	97.6	**91.9**	98.8	75.8	94.0	84.7	98.0
S3	89.8	98.0	**95.7**	98.9	85.0	96.9	89.5	99.1	94.2	98.6	90.4	98.3	73.0	86.3	85.8	97.5
S4	86.6	97.2	86.7	97.5	86.2	96.4	86.0	97.8	86.4	95.8	**89.2**	95.6	77.5	90.6	**89.2**	95.8

GenBank designations of MRV isolates are indicated. The highest identities are indicated in bold.
Nucl. = nucleotide, Aa. = amino-acid.

Seroprevalence studies can provide some hints to reovirus infection rates because specific antibody responses with neutralization capacity were clearly observed in this study. Seroprevalence studies of MRV infections in humans were mainly performed in early studies of reovirus infections and already indicated a high seroprevalence (< 50%) among 5-year-old children (Lerner et al., 1962; Leers and Rozee, 1966). However, limited data are available regarding the more recent situation within any particular population.

The combination of the various laboratory diagnostic approaches used herein revealed a clear reovirus etiology of diarrhea in our patient. Other common causes of diarrhea were excluded using a wide syndromic diagnostics molecular panel, and reovirus infection was further confirmed with serology. In the present case, the results of the VNT were consistent with the results of IIF assays: whereas nearly no sera reactivity could be detected at the time of initial sampling, the follow-up samples clearly revealed seroconversion. Although VNT showed clear serotype 2 reactivity, the IIF assays indicated the possibility of serotype antigen cross-reactivity. Molecular analysis of the S1 genome segment encoding a serotype-specific outer capsid protein σ1 also indicated serotype 2 phylogenetic clustering.

Recently described MRVs have been shown not only to cause local respiratory and gastrointestinal infections, but also to have the potential to cause severe disseminated infections (Ouattara et al., 2011; Thimmasandra Narayanappa et al., 2015; Feher et al., 2017). It has been previously noted that MRV dissemination and pathogenesis is associated with the presence of non-structural protein σ1s (Boehme et al., 2009; Boehme et al., 2011). Due to the presence of the σ1s ORF in the SI-MRV07 genome, this MRV isolate may also have the potential to disseminate to other organs, and thus cause other clinical symptoms in addition to gastroenteritis. Because viral pathogenesis is not only dependent on the viral characteristics, but also on the host characteristics, further pathogenic studies in laboratory models need to be performed to elucidate the pathogenic potential of the SI-MRV07 isolate.

The SI-MRV07 genome assembly was challenging due to the high heterogeneity of the novel MRV isolate. We initially failed to identify the S1 genome segment among the *de novo* assembled contigs. Subsequent analysis, including a wider database of complete MRV genome assemblies used in the read-space restriction database, revealed that this was due to relatively lower nucleotide sequence similarity between S1 genome segments of the novel SI-MRV07 isolate and the reference MRV used in the initial screen. The SI-MRV07 S1 genome segment exhibited a very low level of sequence identity (in both the nucleic and amino-acid contexts) to any other known MRV isolates deposited in GenBank. This was also evident at the sequence identity matrix of SI-MRV07 genome segments, aligned to the most identical strains found in GenBank (Table 2). It was noticed, that our novel SI-MRV07 strain is most divergent in S1 genome segment. The other observation is, that SI-MRV07 probably originate through multiple reassortments, including MRV serotypes 1–3. The exact parental strain could not be determined from the current strains, deposited in GenBank or detected in our samples in previous studies. However, looking at the phylogenetics and the nucleotide identity scores, the multiple reassortment is one of the most probable explanation of SI-MRV07 genome composition.

In line with recent characterization of bat-like MRVs (Kohl et al., 2012; Lelli et al., 2013; Naglic et al., 2018), further studies are needed to establish the prevalence of MRVs in human and animal hosts. Defining natural MRV hosts would likely reveal their transmission pathways and zoonotic potential. In order to provide a realistic estimate of the burden of MRV infections in human populations, further prevalence studies are required. Indeed, such occasional MRV cases found in the human population might indicate that the frequency of MRV infections is underestimated.

In conclusion, although MRVs are important viral pathogens with significant reassortant and zoonotic potential, they can easily be overlooked using standard gastrointestinal syndromic-based molecular panels. In the case of diarrhea of unclear etiology, MRVs should also be considered and stool tested using broader-range diagnostic methods, including electron microscopy.

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