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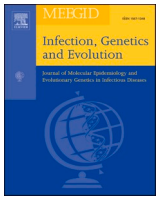

Genome sequence analysis of a novel rotavirus strain indicates a broad genetic diversity of rotavirus A in shrews

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ABSTRACT

Rotavirus A (RVA) is an etiologic agent of diarrhea in humans and animals. It shows a high degree of genetic heterogeneity. Although distinct associations of RVA genotypes with certain host species are common, interspecies-transmission has also been described. Recently, RVA strains, which are genetically distinct and cluster basally to all other RVA strains in phylogenetic trees, have been identified in common shrews (*Sorex araneus*). Here, the genome sequence analysis of another RVA strain (RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58]) from a common shrew from Germany is described. Generally, the strain shows low sequence identities to established strains, which is reflected by the assessment of the novel genotypes G42-P[58]-I32-R28-C24-M24-A39-N28-T28-E32-H28 to its genome segments. Specifically, the strain is phylogenetically distant from previously described RVA strains of common shrews, whereas it is more closely related to other avian and mammalian RVA strains including those from Asian house shrews (*Suncus murinus*). The results indicate that a broad variety of diverse RVA strains can be found in shrews suggesting a significant role of these animals in rotavirus evolution.

1. Introduction

Rotaviruses are a leading cause of gastroenteritis in humans and animals. Young children are especially affected, leading to estimated 128,500 deaths worldwide in 2016 (Troeger et al., 2018). Rotavirus gastroenteritis is also an important disease in domestic mammals and poultry (Otto et al., 2012; Otto et al., 2015). In addition, rotaviruses have been identified in wild animals, such as bats, rodents, shrews, wild boars and red foxes (Simsek et al., 2021; Sachsenröder et al., 2014; Niendorf et al., 2021; Johne et al., 2019; Moutelíková et al., 2016; Ćolić et al., 2021).

The family *Sedoreoviridae* comprises the genus *Rotavirus*, in which the species rotavirus A to rotavirus D as well as rotavirus F to rotavirus J are currently defined (Matthijnssens et al., 2022). In addition, the putative additional species rotavirus K and rotavirus L have been described recently (Johne et al., 2019; Johne et al., 2022). Rotaviruses are non-enveloped and contain a genome of 11 segments of double-stranded RNA (Crawford et al., 2017). Each genome segment codes for one of the structural proteins VP1 to VP4, VP6, VP7, or the non-structural proteins NSP1 to NSP5. The exchange of genome segments by reassortment is generally possible between strains from the same rotavirus species (Matthijnssens et al., 2022).

Among the rotavirus species, rotavirus A (RVA) has the highest clinical importance in humans and animals (Crawford et al., 2017). Generally, RVA strains show a high degree of genetic variability. Based on nucleotide sequence identities, a classification system into several genome segment-specific genotypes has been established for RVA (Matthijnssens et al., 2008). Although specific combinations of RVA genotypes are commonly found in distinct host species, interspecies-transmission has also been described and reassortment events are frequently observed (Martella et al., 2010; Dóró et al., 2015; Luchs and Timenetsky, 2016; Geletu et al., 2021).

Recently, RVA strains have also been detected in insectivorous shrews of the order Eulipotyphla (Li et al., 2016; Johne et al., 2019). First, RVA strains closely related with other strains from mammals were identified in Asian house shrews (*Suncus murinus)* from China (Li et al., 2016). Later, RVA strains were detected in common shrews (*Sorex araneus*) from Germany (Johne et al., 2019). Genome sequence analyses

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showed that the latter strains were only distantly related to all other known RVA strains; they clustered basally to the other RVA strains in phylogenetic trees leading to the hypothesis of a distinct evolutionary trajectory for the common shrew RVA strains (Falkenhagen et al., 2022).

Here, the genome sequence analysis of a novel RVA strain detected in a common shrew from Germany is presented. Sequence comparisons and phylogenetic analyses indicate a closer relationship to mammalian and avian RVA strains than to the formerly characterized common shrew RVA strains. The results indicate a broader genetic diversity of RVA strains in shrews than previously assumed. The role of shrews in rotavirus evolution and the zoonotic potential of shrew-derived RVA strains need to be investigated in future.

2. Methods

2.1. Sample

Common shrews from Germany were sampled based on snap trapping, or the shrews were found dead in live traps, as described previously (Johne et al., 2019). The animals were initially stored frozen, the intestinal contents (~0.2 g) were collected later from thawed animals and again frozen at −20 °C until further analysis. The samples were analyzed by a common shrew RVA-specific RT-PCR (Johne et al., 2019), in which sample KS11–0893 showed a faint band. It originated from a male common shrew with a body weight of 8 g, which was collected in 2010 in the area of Regensburg, Bavaria, Germany.

2.2. Next generation sequencing (NGS)

Sample KS11–0893 was diluted 1:5 in phosphate-buffered saline (PBS), and 100 µl of the solution were subjected to nucleic acid extraction using the NucliSENS® easyMAG system (BioMerieux, Marcy I'Etoile, France) according to the manufacturer's instructions. Sequence libraries were generated from nucleic acid extract as previously described (Johne et al., 2022). Briefly, the KAPA RNA HyperPrep Kit (Roche Diagnostics, Mannheim, Germany) and the KAPA Unique Dual-Indexed Adapter Kit (Roche Diagnostics) were used for library preparation following instruction of the manufacturer. Adapters were diluted to a concentration of 0.15 µM and the library was amplified in 23 PCR cycles. All purification steps during library preparation were completed using KAPA Pure Beads (Roche Diagnostics). The final library was sequenced with other additional 118 libraries using the Next Seq 500/550 Mid Output Kit v 2.5 (Illumina, San Diego, CA, USA). The generated raw sequences were subjected to assembly using metaSPAdes (Nurk et al., 2017). All resulting contigs were screened for sequence similarities with rotavirus sequences using BLASTx (Altschul et al., 1997) against all rotavirus sequences available from the National Center for Biotechnology Information (NCBI) Protein database (NCBI Resource Coordinators, 2016; https://www.ncbi.nlm.nih.gov/protein/, accessed on 20 October 2021).

2.3. Sequence analysis and phylogenetic reconstruction

The sequences of the selected contigs were analyzed for identification and translation of open reading frames (ORFs) and sequence annotation using the SeqBuilder 17 module of the DNASTAR software package (Lasergene, Madison, WI, USA). BLASTn search (https://blast.ncbi.nlm.nih.gov, accessed on 23 June 2022) was used for identification of the closest relatives present in the GenBank database. Sequence comparisons were further performed with the RVA genotype reference strains as listed by the Rotavirus Classification Working Group (RCWG, 2022). Nucleotide and amino acid sequence distances and identities were determined after alignment with ClustalW using the MegAlign Pro 17 module of DNASTAR (Lasergene). Phylogenetic analyses were performed using MEGA X version 10.1.7 (Kumar et al., 2018) by alignment of the nucleotide sequences by the ClustalW method followed by phylogenetic reconstructions by the Neighbor-Joining method (parameters: 1000 bootstrap replications, Maximum Composite Likelihood method as the optimal nucleotide substitution model, uniform rates among sites, pairwise deletion of gaps or missing sequences), or by the Maximum Likelihood method (parameters: 1000 bootstrap replications, Tamura-Nei model as the optimal nucleotide substitution model, uniform rates among sites, all sites used). Trees were thereafter manually labeled and formatted using Microsoft Powerpoint.

3. Results

3.1. Sequencing results

A total of 5,612,026 paired reads were generated by NGS, from which 59,267 contigs were assembled. Eleven of these contigs showed significant similarities to RVA strains, which had mean read coverages between 28 and 64. The contigs represented the eleven genome segments of an RVA strain, which included all complete coding regions, whereas most non-coding sequences at the 5′- and 3′-termini were missing. The sequences of the complete ORFs were submitted to the GenBank database with the accession numbers ON988153 – ON988163.

3.2. Genotyping and sequence comparison

Comparison of the nucleotide sequences from sample KS11–0893 with all RVA genotype reference strain sequences indicated identities between 42% (NSP1- and NSP4-encoding segments) and 73% (NSP5-encoding segment) (Table 1). As these identities were lower than the cut-off values for genotype definition (Matthijnssens et al., 2008), the sequences were submitted to the Rotavirus Classification Working Group (RCWG), which subsequently assigned new genotypes to all genome segments. The genotype constellation of the strain is therefore G42-P[58]-I32-R28-C24-M24-A39-N28-T28-E32-H28 and the complete strain designation RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58].

A BLASTn search of the sequences revealed the highest nucleotide sequence identities with various RVA strains from humans, mammals and birds (Table 1). Comparably high nucleotide sequence identities between 44% and 72% were also evident with the Asian house shrew RVA strain RVA/Asian House Shrew-wt/CHN/LW9/2013/G32P[46] from China, and BLASTn search identified this strain as the closest relative based on the VP2-encoding segment (Table 1). However, considerably lower nucleotide sequence identities between 42% and 65% were evident when the strain was compared to the sequences previously determined for RVA strains from common shrews collected in the Baden-Wuerttemberg region of Germany (Table 1).

3.3. Phylogenetic analysis of nucleotide sequences

To investigate the phylogenetic relationship of strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] and all RVA genotype reference strains, phylogenetic trees using the Neighbor Joining (NJ) method were generated based on the complete coding nucleotide sequences for each individual genome segment (Supplementary Data S1 and Fig. 1). Fig. 1 shows the tree for the segment encoding the major structural protein VP6. Here, the strain branches between the clades of mammalian and avian RVA strains, whereas the previously determined common shrew RVA strain RVA/Common Shrew-wt/GER/KS-11–2281/2011/GxP[x] branches basally to all RVA strains and the Asian house shrew strain RVA/Asian House Shrew-wt/CHN/LW9/2013/G32P[46] branches within the mammalian clade. A similar branching is found for the VP2-, NSP4- and NSP5-encoding segments (Supplementary Data S1). For the VP3-, VP7- and NSP1-encoding segments, the new strain branches out most closely to the previously determined common shrew RVA strain RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55]; however, with long branch lengths indicating high phylogenetic

Table 1
Nucleotide sequence identities of common shrew strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] with other RVA strains.

Genome segment-encoded protein	Closest relative by BLASTn search, GenBank acc.-no. (genotype, % nucleotide sequence identity)	Nucleotide sequence identity (%) with			Nucleotide sequence identity cutoff (%) for genotype definition*	Assigned novel genotype
		Common shrew genotype(s)	Asian house shrew genotype	All RVA genotype reference strains		
VP1	RVA/Human-wt/KEN/KLF1038/2012/G1P[8], MZ096992 (R1, 71% identity)	63–64	70	63–70	83	R28
VP2	RVA/Shrew/CHN/LW9/2013/G32P[46], KU243571 (C17, 71% identity)	65	71	65–71	82	C24
VP3	RVA/Avian/BRA/strain 1/09/2009/G19P[x], KX185136 (M7, 66% identity, partial sequence)	55	61	55–62	81	M24
VP4	RVA/Human-wt/HUN/BP271/2000/G4P[6], KF835913 (P6, 68% identity, partial sequence)	53	57	53–62	80	P[58]
VP6	RVA/Human-wt/KOR/RN-010/2013/G4P[6], MK953581 (I1, 69% identity)	62	65	62–68	85	I32
VP7	RVA/Pig/IND/TR/RV/SW49/XXXX/G26P[13], KT277525 (G26, 69% Identity)	57	64	57–68	80	G42
NSP1	RVA/Raccoon/China/ SD-MO5/2021/G3P[3], OM450984 (A9, 74% identity, short partial sequence)	44	44	42–51	79	A39
NSP2	RVA/Human-wt/KOR/RN-014/2014/G4P[6], MK953579 (N1, 70% identity)	57	67	57–69	85	N28
NSP3	RVA/Pig/BRA/ROTA5/2012/G5P[13], KJ482301 (T1, 71% identity, partial sequence)	57	67	54–67	85	T28
NSP4	RVA/Rabbit/ITA/ 160/01/XXXX/G3P[22], AF533535 (E5, 88% identity, short partial sequence)	42	52	42–55	85	E32
NSP5	RVA/Human-wt/PAK/3085/2010/G2P[4], KY497538 (H2, 80% identity, partial sequence)	56–59	72	56–73	91	H28

* according to Matthijnssens et al. (2008).

distance (Supplementary Data S1). For the remaining VP1-, VP4-, NSP2- and NSP3-encoding segments, the branching is more variable, but strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] generally branches closer to the other mammalian strains than the previously determined common shrew strains RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55] and/or RVA/Common Shrew-wt/GER/KS-11–2281/2011/GxP[x] (Supplementary Data S1). Additional trees were calculated for each genome segment using the Maximum Likelihood (ML) method, which mainly resulted in similar topologies as compared to the presented NJ trees. As an example, the ML tree for the VP6-encoding segment is shown in Supplementary Data S2.

3.4. Analysis of deduced amino acid sequences

The deduced amino acid sequences of the encoded proteins of strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] were compared to that of the RVA genotype reference strains. The comparison of the amino acid sequences generally revealed low percentages of identity. These were specifically lower compared to the previously determined common shrew strains RVA/Common Shrew-wt/GER/KS-11–2281/2011/GxP[x] and RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55] (14% - 67%) than to the Asian house shrew strain RVA/Asian House Shrew-wt/CHN/LW9/2013/G32P[46] (29% -73%) and to many other mammalian and avian strains (Supplementary Data S3). An alignment of the sequences identified several regions with deletions or insertions in the proteins of several strains. In most of these positions, the common shrew strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] was more similar to mammalian strains than to avian strains or the previously determined common shrew strains RVA/Common Shrew-wt/GER/KS-11–2281/2011/GxP[x] and RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55]; examples are shown for VP1 and NSP5 (Supplementary Data S4A and S4B). In contrast, strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] showed an insertion in the NSP3 at the same position like the previously determined common shrew strain RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55] together with a bat strain (RVA/Bat-wt/CHN/GLRL1/2005/G33P[48]), which was not present in the other strains (Supplementary Data S4C). In addition, strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] showed in the NGS-based dataset a unique insertion within NSP4 containing a stretch of five glutamine residues (Supplementary Data S4D), which was confirmed by Sanger sequencing.

4. Discussion and conclusions

RVA strains are genetically highly diverse, which is reflected by the definition of a large number of different RVA genotypes. The number of reported novel genotypes increases continuously, with the latest descriptions of new genotypes from bats, shrews and cattle (Simsek et al., 2021; Falkenhagen et al., 2022; Singh et al., 2022). Here, we describe a novel RVA strain, which again shows only low sequence identities to established genotypes and further increases the number of genotypes, e.g. to 42 G-types and 58 P-types. The high genetic diversity might only reflect the genetic variation generated during a long evolutionary history, but it may also influence biological characteristics of the viruses, such as host tropism, virulence and antigenicity. Therefore, continued surveillance of RVA strains in humans and animals remains important.

Although the new RVA strain described here originated from a sample of a common shrew in Germany, it was not closely related to previously described strains from other common shrew samples also collected in Germany. The new strain RVA/Common Shrew-wt/GER/

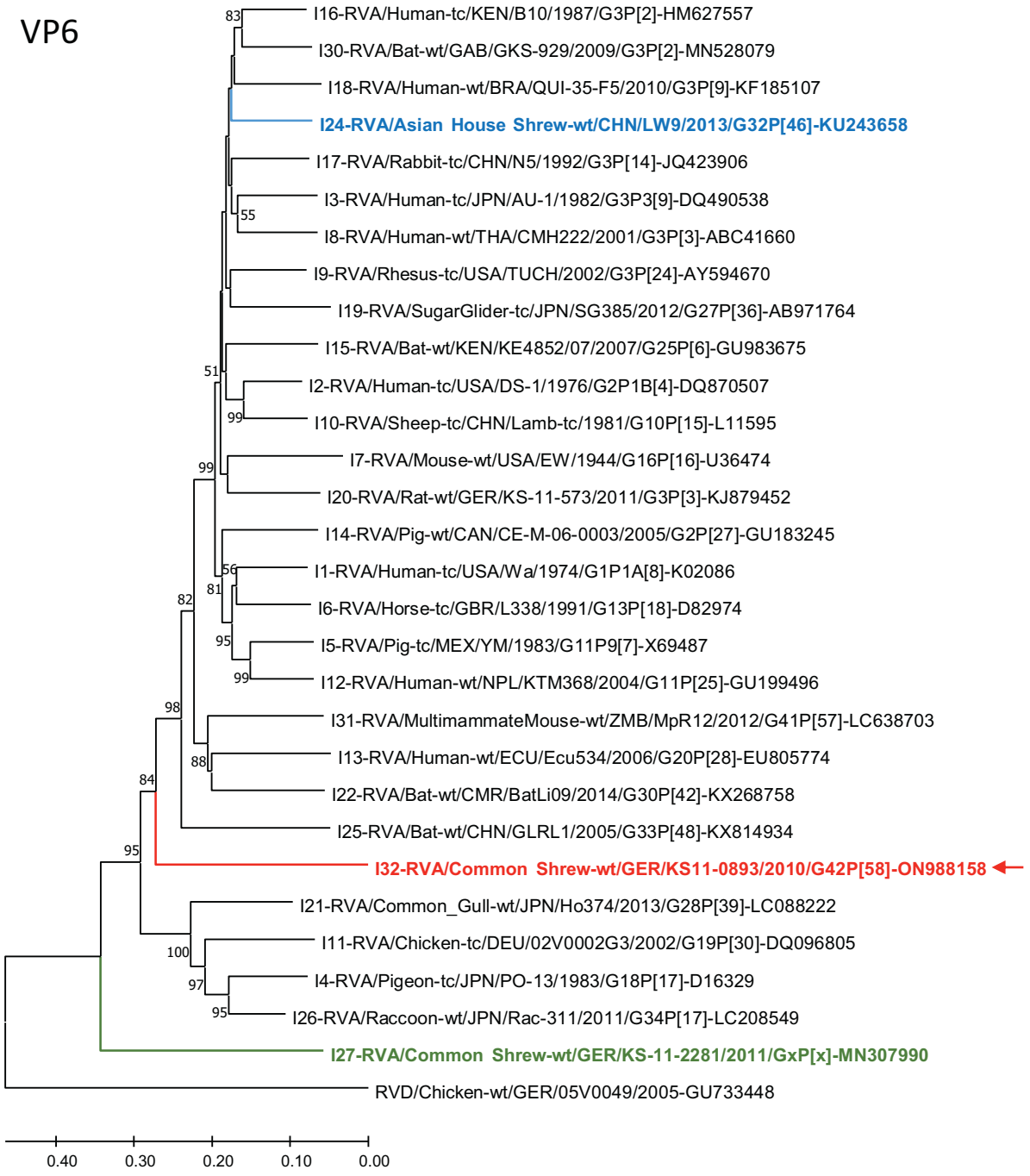

Fig. 1. Phylogenetic relationship of common shrew strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] with RVA genotype reference strains. The nucleotide sequence of the complete open reading frame of the major structural protein VP6 was compared with those of the RVA genotype reference strains by the Neighbor-joining method using MEGA X. Bootstrap values >50% are indicated. Scale bars indicate nucleotide substitutions per site. The genotypes, strain designations and GenBank accession numbers are indicated at the branches of the trees. RVD strain RVD/Chicken-wt/GER/05V0049/2005 is used as an outgroup strain. Strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] is marked in red and with an arrow. The previously identified strain from common shrew is marked in green and the strain from Asian house shrew in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

KS11–0893/2010/G42P[58] originated from a shrew in Bavaria, whereas the older strains originated from shrews from Baden-Wuerttemberg, with an approximate trapping site distance of 200 km. The strains from Baden-Wuerttemberg generally clustered basal to all other RVA strains, which led to the suggestion of an evolutionary trajectory distinct from the other RVA strains (Falkenhagen et al., 2022). In contrast, strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] showed a closer relationship to other mammalian and avian RVA strains for most of the genome segments. Interestingly, for the VP2-encoding segment, the highest sequence identities – although as low as 71% - were found with a strain from Asian house shrews from China, which branched within the mammalian RVA clade. Generally, the phylogenetic trees presented in Fig. 1 and Supplementary Data S1 and S2 show that RVA strains from shrews are scattered throughout the trees, indicating that these animal species contain a large genetic diversity of RVA strains.

Comparison of the sequence identities of the encoded amino acid sequences confirmed the closer relationship of strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] to RVA strains from other mammals and birds compared to the previously described strains from common shrews. This is also reflected by distinct deletions, which are found in the previously determined common shrew strains RVA/Common Shrew-wt/GER/KS-11–2281/2011/GxP[x] and RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55], but not in strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58], as exemplarily shown for VP1 and NSP5 (Supplementary Data S4A and S4B). However, a common insertion of all common shrew strains in the NSP3 might indicate common properties of all common shrew strains, whereas an insertion only present in the NSP4 gene of strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] underlines its unique characteristics. Further research is necessary to elucidate the functional importance of the insertions or deletions found in the distinct proteins.

The strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] with the described new genotypes was detected only in one sample of a fecal content of a common shrew. Therefore, it cannot be decided if it is a typical shrew-adapted strain, or if it represents a strain from a single spillover event from a yet unknown other host. However, the common branching of this strain with the previously determined RVA strain from a common shrew for the VP3-, VP7- and NSP1-encoding segments as well as the common insertion in NSP3 may indicate some shared characteristics of the common shrew RVA strains. Investigation of more samples from shrews and other animal species may help to clarify the origin of the strain in the future.

For some RVA strains, zoonotic transmission between animal hosts and humans have been suggested (Martella et al., 2010; Geletu et al., 2021). Moreover, typical genome segments of animal RVA strains have been repeatedly identified in some human RVA strains, which are suggested to be the result of reassortment events (Dóró et al., 2015; Luchs and Timenetsky, 2016). Attempts to generate reassortants, which contain a genome segment of the previously identified common shrew RVA strain RVA/Common Shrew-wt/GER/KS14–269/2014/G39P[55] in the backbone of a simian RVA strain using a reverse genetics approach, did not result in the generation of viable viruses (Falkenhagen et al., 2022). It was suggested that the differences between the common shrew strain and the simian strain were too high, thus preventing interactions of their viral proteins or RNAs (Falkenhagen et al., 2022). As the new RVA strain RVA/Common Shrew-wt/GER/KS11–0893/2010/G42P[58] is more closely related to other mammalian strains, it might have a higher potential for reassortment with simian or human strains, and its reassortment and zoonotic potential should be investigated in future.

In conclusion, a novel RVA strain has been identified in a common shrew, which is only distantly related to those detected previously in this animal species. The results indicate that a broad spectrum of genetically distinct RVA strains can be present in shrews. Further research on RVA strains in shrews may elucidate their role in rotavirus evolution and reveal the zoonotic potential of the strains to be transmitted to other hosts including humans.

Access to data and data analysis

Data are available as Supplementary Data S1–S4 and additional data can be retrieved upon request from R.J. The GenBank accession numbers of the nucleotide sequences of the shrew RVA strain identified in this study are presented in Section 3.1. of the manuscript.

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CRediT authorship contribution statement

Reimar Johne: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Project administration, Funding acquisition. **Simon H. Tausch:** Methodology, Software, Data curation, Writing – review & editing. **Katja Schilling-Loeffler:** Methodology, Investigation, Data curation, Writing – review & editing. **Rainer G. Ulrich:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary Data

Supplementary Data to this article can be found online at https://doi.org/10.1016/j.meegid.2022.105392.

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