Isolation and Characterization of Three Mammalian Orthoreoviruses from European Bats

Claudia Kohl1*, René Lesnik1, Annika Brinkmann1, Arnt Ebinger1, Aleksandar Radonić1, Andreas Nitsche1, Kristin Mühldorfer2, Gudrun Wibbelt2, Andreas Kurth1

1 Robert Koch Institute, Centre for Biological Security 1, Berlin, Germany, 2 Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

Abstract

In recent years novel human respiratory disease agents have been described in South East Asia and Australia. The causative pathogens were classified as pterpine orthoreoviruses with strong phylogenetic relationship to orthoreoviruses of flying foxes inhabiting these regions. Subsequently, a zoonotic bat-to-human transmission has been assumed. We report the isolation of three novel mammalian orthoreoviruses (MRVs) from European bats, comprising bat-borne orthoreovirus outside of South East Asia and Australia and moreover detected in insectivorous bats (Microchiroptera). MRVs are well known to infect a broad range of mammals including man. Although they are associated with rather mild and clinically unapparent infections in their hosts, there is growing evidence of their ability to also induce more severe illness in dogs and man. In this study, eight out of 12 vespertilionid bats proved to be infected with one out of three novel MRV isolates, with a distinct organ tropism for the intestine. One isolate was analyzed by 454 genome sequencing. The obtained strain T3/Bat/Germany/342/08 had closest phylogenetic relationship to MRV strain T3D/04, isolated from a dog. These novel reoviruses provide a rare chance of gaining insight into possible transmission events and of tracing the evolution of bat viruses.

Citation: Kohl C, Lesnik R, Brinkmann A, Ebinger A, Radonić A, et al. (2012) Isolation and Characterization of Three Mammalian Orthoreoviruses from European Bats. PLoS ONE 7(8): e43106. doi:10.1371/journal.pone.0043106

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: KohlC@rki.de

Introduction

Since most of the recent disease outbreaks have been associated with zoonotic transmission events of newly emerging viruses, it has become evident that surveillance and evaluation of viruses prevalent in wildlife are of particular importance [1–2]. Bats are increasingly known to be reservoir hosts of various viruses [3]. Since Gard et al. reported the first isolation of a novel orthoreovirus from a fruit bat in Australia (Nelson Bay) in 1973, bats are likewise known to be possible carriers of reoviruses [4]. Later, while searching for the natural reservoir host of Nipah virus, another bat-borne orthoreovirus called Pulau virus was isolated from a fruit bat’s urine on Tioman Island, Malaysia [5]. In the past five years bat-borne orthoreoviruses again received attention after another bat-borne orthoreovirus termed Melaka virus was isolated from human patients in Malaysia in 2007 [6]. A possible zoonotic bat-borne transmission was assumed for Melaka virus, a close relative of Pulau virus, and further evidence was obtained by phylogenetic calculations. Since then five additional orthoreoviruses (Xi-River, Kampar, Sikamat, HK25629/07 and Broome virus) have been isolated from fruit bats [7–8] or from humans with assumed contact to bats [9–11]. Remarkably, all these viruses, except Broome virus, appear in one distinct phylogenetic cluster [8]. This phylogenetic species (formerly known as Nelson Bay species) was recently proposed to be renamed as Pteropine orthoreoviruses within the genus Orthoreovirus [11], while Broome virus seems to represent the first member of a novel, slightly remote cluster [8].

In general, the family Reoviridae can be divided genetically into two subfamilies: the Sendavirinae with six and the Spinareovirinae with nine comprised genera. Orthoreovirus is one genus within the Spinareovirinae and consists of five virus species: Pteropine orthoreovirus, Asian orthoreovirus, Reptilian orthoreovirus, Baboon orthoreovirus and the type species Mammalian orthoreovirus (MRV) which includes the orthoreoviruses of man and the majority of mammals. Orthoreoviruses are non-enveloped viruses with a segmented double-stranded RNA genome [12]. Each orthoreovirus particle contains ten genome segments, named after their migration in gel electrophoresis separation dependent on size: L1, L2, L3 (large), M1, M2, M3 (medium) and S1, S2, S3, S4 (small). The virions have an average size of 70–80 nm with a typical icosahedral, double-layered protein capsid structure.

The species MRV includes four prototype strains: type 1 Lang (T1L), type 2 Jones (T2J), type 3 Dearing (T3D) and type 4 Ndelle (T4N) [12–13]. These prototype strains show no immunogenic cross-reactivity with serologic differentiation. Most MRV strains can be assigned to one of the serogroups T1L, T2J and T3D. An alternative typing of novel strains is common via genetic comparison of the S1 segment which most probably encodes proteins that are responsible for serotype and tissue tropism [14].

In this study we report the isolation of three novel MRV strains from organ tissue of European bats. These bat-borne MRVs occur beyond the species Pteropine orthoreovirus, in insectivorous bats and
outside of South East Asia and Australia. By phylogenetic analysis of the S1 segment a close, monophyletic relationship was observed to strain T3D/04 isolated from a dog suffering from hemorrhagic diarrhea and a simultaneous concurrent canine parvovirus type 2 infection. Although MRVs were assumed to cause rather mild respiratory or gastrointestinal diseases, recent findings indicate the occurrence of higher virulent MRV strains in man and other mammals [15–17]. The recent isolation of a novel bat virus, bat Adenovirus 2 [18], which closest phylogenetic relative is able to cause severe disease like hemorrhagic enteritis in canids, prompted us to further investigate these novel orthoreovirus isolates. This included the generation, annotation and phylogenetic analysis of the complete genome of one isolate.

Methods
Study
Since all German bats are covered by species protection through the European Commission (http://ec.europa.eu/environment/nature/legislation/habitatsdirective) and through the Agreement on the Conservation of Populations of European Bats (www.eurobats.org), investigative research requires special permission by local governmental bodies [19]. As part of a study on diseases in native bats [19], 120 bats of fourteen vespertilionid bat species were examined. (Myotis mystacinus [n = 21], Pipistrellus pipistrellus [n = 21], P. nathusii [n = 16], Vespertilio mysticus [n = 11], M. daubentoni [n = 10], Eptiuscus nilssonii [n = 10], Plecotus auritus [n = 8], Nyctalus noctula [n = 7], P. kuhl [n = 7], E. serotinus [n = 5], M. bechsteinii [n = 1], M. brandtii [n = 1], M. myotis [n = 1], M. nattereri [n = 1]). Animals were found dead, injured or moribund near roosting sites or human habitations [19] in urban and suburban areas of different regions in Germany (Bavaria [n = 85], Lower Saxony [n = 18] and Berlin greater metropolitan area [n = 17]). All bat carcasses were kindly provided by bat researchers and bat rehabilitation centres from the different geographic regions. Permits to investigate carcasses of deceased bats were granted by the respective local governmental authorities (district government of Upper Bavaria, Munich; district government of Bavarian Swabia, Augsburg; Lower Saxony water management, coastal defence and nature conservation, Hannover; senate department for urban development and the environment, Berlin). If bats died in care or had to be euthanized for medical reasons, the carcasses were immediately stored at −20°C and transferred to the Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, for histopathological and bacteriological examination [19]. Subsequently, aliquots of the individual organs were sent to the Robert Koch Institute for virological examination.

Pathological Investigation
A full necropsy was performed on each bat, followed by histopathological examination. Small slices of multiple organ tissues were fixed in 4% buffered formalin, processed using standard methods and embedded in liquid paraffin. Sections were cut at 2–5 μm and stained with hematoxylin-eosin.

Virological Investigation
Vero E6 and Vero B4 cell lines were used for cell-culture screening. Cells were seeded into 24-well cell-culture plates and incubated overnight at cell-culture conditions (5% CO₂, 37°C) to 80–90% confluence of cell monolayer. For infection, homogenates of all particular organs available per bat were pooled and incubated on either Vero E6 or Vero B4 cells for one hour at cell-culture conditions. Subsequently, the cells were sub-cultured three times, once a week and observed daily for the occurrence of cytopathogenic effects (CPE). To exclude and avoid bacterial contamination, the supernatant was passed through a 0.22 μm filter and cells were treated with a 10-fold dose of antibiotics (penicillin, streptomycin). By CPE occurrence in the third sub-cultivation, the supernatant was passed to an 80–90% confluent 175 cm² flask of fresh Vero cells and incubated at cultivation conditions for one week. To harvest virus particles, cells were homogenized by three freeze-thaw cycles and the resulting suspension was purified from cell debris by low-speed centrifugation. Aliquots were stored at −80°C. One aliquot was titrated on Vero E6 cells to estimate a titre.

For live cell imaging, Vero E6 cells were seeded into a cell culture dish. Cells were infected with a MOI (multiplicity of infection) of 0.1 with strain T3/Bat/Germany/342/08. An additional uninfected dish was prepared similarly and used as a negative control. To improve visualization Opti-MEM media without phenol red (Invitrogen Life Technologies, Germany) was used during live cell imaging. Both cell culture dishes were incubated at cultivation conditions on a NIKON Eclipse TE2000-E live cell microscope and images of both cells were captured every 15 minutes for a period of 72 hours.

Molecular Biological Investigation
DNA/RNA extraction was performed using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, Darmstadt, Germany) on all homogenized and pooled bat organs available. For cDNA synthesis the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA) was utilized with an additional denaturing step of 95°C for 5 min. Bat species were determined by amplification and sequencing of mitochondrial DNA as described by Sonntag et al. [20]. For detection of reoviral RNA in internal organs a real-time PCR assay was developed, using the following primer and probe sequences: BatReoF (5’-CACCATgTCAaGCTgTCCCGC-3’), BatReoR (5’-AG-CGGCAGTgTATgTCCGGAg-3’) and BatReoProbe (5’-6FAM-CCGAgTGgTGATgTCCACg-3’). The PCR mix contained 1x Platinum® Taq Buffer (Invitrogen), 2.5 mM of MgCl₂ (Invitrogen), 0.3 μM of each primer, 0.1 μM of probe, 0.2 mM Mix of dNTPs (Invitrogen), 1 U Platinum® Taq DNA polymerase (Invitrogen). Cycler conditions were as follows: pre-denaturation (95°C for 15 min), 40 amplification cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30 s) and final extension (72°C for 10 min). Real-time amplification was run on a Stratagene MX3000 (Agilent Technologies, Waldbronn, Germany). For sequencing the PCR products were run on a 1.5% agarose gel containing ethidium bromide. Images were captured on the E.A.S.Y. RH-3 (Herolab, Wiesloch, Germany) gel documentation system.

Amplicons were purified through the MSB® Spin PCRapace kit (invitek, Berlin, Germany) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI Genetic Analyzer 3500 XL. Ds automated sequencer (Applied Biosystems, Darmstadt, Germany). The corresponding PCR primers and reaction conditions were used in accordance with the manufacturer’s protocol.

Detection of virus nucleic acid fragments in the pooled organ tissue was followed by separate RNA extraction and cDNA synthesis of the respective bat’s particular organs. Subsequently, viral copy numbers for every organ were determined by real-time PCR and retrieved amplification products were sequenced as described above. For the phylogenetic analysis of the partial RNA-dependent RNA polymerase (RdRP) gene (L1 segment), the generic nested PCR assay previously published by Welhelmann et al.
was used [21]. Amplified products were visualized and sequenced as described.

Random PCR amplification of reoviral RNA from purified virus particles was performed as described by Victoria et al. [22]. Additionally, the retrieved PCR products were cloned into a TOPO TA Cloning Kit® 2.1 Vector System (Invitrogen) to reveal single sequences. The products from the cloning colony PCR were run on a 1.5% agarose gel containing ethidium bromide. Images were captured on the E.A.S.Y. RH-3 (Herolab) gel documentation system and sequenced with the corresponding colony PCR primers.

Sequence analysis and annotation were used to characterize the S1 segment and determine the serotypes of the three novel MRV strains isolated in this study [14,17,23].

**Virus Propagation and Purification**

For random PCR, electron microscopy and whole genome sequencing, as well as propagation and purification of the isolate T3/Bat/Germany/342/08, Vero E6 cells were infected at a Multiplicity of Infection of 0.5 and incubated at cultivation conditions (5% CO₂, 37°C). Seven days after the infection of cells, viruses were harvested and purified by low speed centrifugation (1,000 g; 10 min) after three freeze-and-thaw cycles. Afterwards products were purified by ultracentrifugation (Beckman Ultrafuge, 45 h, room temperature, 100,000 g through a CsCl gradient (37% CsCl in 0.05 M Tris- HCl, ηp = 1.3671)). A white band containing virus particles became visible, which was separated and subsequently used.

**Whole Genome Sequencing**

Purified RNA was transcribed and amplified utilizing the TransPlex Whole Transcriptome Amplification 2 Kit (Sigma-Aldrich). The resulting double-stranded DNA was then measured with the Qubit (Life Technol., Darmstadt, Germany) instrument, and 500 ng were used to generate a Titanium Rapid-Library (Roche, Mannheim, Germany). The library was prepared and sequenced on a 454 FLX instrument (Roche) according to the manufacturer’s instructions. Sequencing resulted in 35,036 reads and 6,032,630 bases. The sequencing data was used to perform a hybrid assembly using Newbler 2.5 (Roche) and Geneious Pro program package 5.4 software (Biomatters Ltd., Auckland, New Zealand).

**Sequence Analysis**

The nucleotide (nt) sequence contigs were analyzed with the Mega 4 (http://www.megasoftware.net) and the Geneious Pro program package 5.4 software (Biomatters Ltd., Auckland, New Zealand). The identity of the nucleotide fragments was confirmed by BLASTx homology search (http://www.ncbi.nlm.nih.gov/BLAST/) against the non-redundant protein database of NCBI. For genome annotation the nt sequence was translated to amino acids (aa) in six frames with the Geneious Pro package. The sequence was also analyzed by the FGENESV Trained Pattern/ Markov chain-based viral gene prediction program (http://www.softberry.com).

**Phylogenetic Tree Reconstruction**

Multiple alignments (ClustalW) of nucleotide sequences were calculated with the Mega5 program (http://www.megasoftware.net). Phylogenetic tree reconstructions were based on the complete four S segments and the complete L1 segment, respectively. A complementary calculation for the partial L1 segment (197 nt, assay by Wellehan et al.) was performed that included all available, but shorter MRV sequence data from GenBank. For all calculations MrBayes v3.1.2 [24] was used, following a first model selection by using ModelTest [25], and model GTR+G (gamma distribution) was selected for all alignments. The calculation parameters were as follows: number of runs: four, number of generations: 10,000,000 (partial L1 segment: 20,000,000), sample frequency: 100 and burn in: 25%. The results were finally visualized by the FigTree v1.2.1 program (http://tree.bio.ed.ac.uk/), a graphical viewer of phylogenetic trees.

**Results and Discussion**

**Virus Isolation and Visualization**

After inoculation and passaging of two different Vero cell lines (E6 and B4) with pooled homogenized organ tissues from 120 bats, a CPE was clearly visible on Vero E6 cells (bat 342/08) and Vero B4 cells (bats 019/09 and 021/09), respectively (Fig. 1 A and B). Cryosolates of infected cells and supernatant were purified by CsCl gradient ultracentrifugation, resulting in a distinct white band containing virus particles between ηp =1.365 and ηp = 1.373. Virus particles were visualized by negative staining electron microscopy, revealing typical inner and outer icosahedral, non-envoloped capsids of approximately 70 nm in diameter which is characteristic for reoviruses (Fig. 1C). Ultra-thin sections of infected Vero E6 cells displayed typical electron-dense virus particles organized in a paracrystalline pattern within the cytoplasm (Fig. 1D). Life cell imaging shows clearly the developing CPE caused by T3/Bat/Germany/342/08 in Vero E6 cells compared to the negative control (Fig S1).

**Molecular Biology**

The identification of the detected virus isolates of the family Reoviridae was verified by a generic nested PCR assay [21]. The obtained sequences of the partial RdRP gene (197 bp) indicated that these viruses represented novel strains within the species MRV of the genus Orthoreovirus and were tentatively named Bat MRV342/08 (JQ412755), Bat MRV019/09 (JQ412765) and Bat MRV021/09 (JQ412766) (Fig. 2). Sequence homologies between the three novel strains isolated in this study, based on the partial RdRP fragments, are given in table 1. Subsequently, isolate Bat MRV342/08 was chosen for further sequence acquisition due to the interesting pathological findings of the bat host (moderate hemorrhagic enteritis; table 2). The following random amplification, cloning and sequencing resulted in four different sequence contigs based on 20 single overlapping sequences. In parallel, extracted RNA was applied to next generation sequencing technology. Following 454 sequencing, the reads were mapped to MRV-3 Dearing as reference sequences, resulting in 10 contigs of 3,131 reads with a total of 1,054,029 bases. Only 636 bases (2.8%) had a log quality value lower than Q39. The missing terminal sequences of incomplete segments were determined by rapid amplification of cDNA ends (RACE) PCR (table 3) and revealed consensuses sequences of all segments of 5′-GCCUA...yU-GCAUG-3′ (h = A, U or C; y = C or U). The complete genome sequences of all segments (L1–L3, M1–M3, S1–S4) were submitted to GenBank (JQ412753-JQ412764). The terminal sequences of the orthoreovirus genome segments are fairly conserved among each species and clearly differ from other orthoreovirus genera [26]. These sequences are used in positive-sense orientation as a phylogenetic marker for virus type separation [10]. All terminal sequences for strain Bat MRV342/08 proved to be similar to the MRV species within the genus Orthoreovirus. These conserved segment ends are typical of MRV, confirming the MRV character of Bat MRV342/08. They
represent the end or start of the 5'- and 3'-UTR. The variable length at the 5'-UTR is reported to vary in all MRVs from 13 bp to 32 bp, and to range on the 3'-UTR between 35 bp and 83 bp [27]. The same range was observed for Bat MRV342/08: ranges between 12 bp in L2 to 32 bp on the S4 segment and the 3'UTR from 31 bp in the L1 segment to 80 bp in the M1 segment (table 3).

Agarose gel electrophoresis was used to examine the migration patterns of the ten segments of Bat MRV342/08. The S1 segment of Bat MRV342/08 was found to migrate with delay, a typical property of serotype 3 MRV strains [28].

Phylogeny and Characterization

After revealing the full genome sequence of one of the novel orthoreovirus strains (Bat MRV342/08), a more in-depth molecular characterization was performed, highlighting some special features in comparison to other strains.

Phylogenetic tree reconstructions by Bayesian and maximum likelihood analyses were based on the nucleotide sequence of the complete L1, S2, S3, and S4 segments (Fig. 3A; Fig. 3C–E). Commonly, orthoreoviruses are taxonomically classified on the basis of the S1 segment [14,23]. We based the phylogenetic reconstruction of the S1 segment on the complete ORF (nt) encoding for the σ1 protein (Fig. 3B). The topology of this phylogenetic tree assigned strain Bat MRV342/08 to serogroup 3 within the species MRV and the strain was renamed as T3/Bat/Germany/342/08. Strain T3/Bat/Germany/342/08 appears to be monophyletic and forms a distinct lineage together with the canine strain T3D/04 (Fig. 3B). In every calculation the segments S2, S3 and S4 of T3/Bat/Germany/342/08 were shown to cluster within the species MRV, without a distinct relation to a single serotype or –group (Fig. 3C–E). However, based on the L1 segment, the reconstructed tree assorted strain T3/Bat/Germany/342/08 to serogroup T2/Jones within the species MRV (Fig. 3A).

Alignments and calculations for the remaining reoviral segments (M1–3, L2–3) are based on an insufficient number of sequences, due to the unavailability of comparative sequence data from GenBank (data not shown).

Pairwise nucleotide and deduced amino acid comparison between strain T3/Bat/Germany/342/08 and the prototype strains T1 (MRV-1 Lang), T2 (MRV-2 Jones), T3 (MRV-3 Dearing) and T4 (MRV-4 Ndelle) was performed for all ten segments (table 4). The predicted protein-coding ORFs were mostly similar in length and had a similar start and stop position compared to the previously reported ORFs of the prototype strains T1/Lang, T2/Jones, T3/Dearing and T4/Ndelle (table 4).

The segments L1 and L3 of strain T3/Bat/Germany/342/08 showed high similarity to all MRV prototypes, with T2J being the most distant (supported by Fig. 3A). The L2 segment of strain T3/Bat/Germany/342/08 had the highest consistence with prototype T3/Dearing on both the nucleotide and deduced amino acid level.

The length of this segment was the same as the one reported for strains T1 and T3, while the protein described for strain T2 was
found to be reduced by one amino acid. Comparing the M segments with prototype strains resulted in different parities on nucleotide and amino acid levels. Segments M1 and M3 showed the highest identity with strains T1/Lang and T3/Dearing on the amino acid level, whereas the segment M2 appeared to be uniform with all four prototypes. On the nucleotide level M3 was similar to M2 and was uniform to all three prototype sequences available for this segment. The length of the M1 segment was identical for all strains compared. For the M2 segment only strain T4/Nèdle was found to encode for a protein longer by one amino acid. In the M3 segment the size of the protein predicted was identical to that of the other prototype strains.

The largest segments (L-segments) are encoding monocistronically for the proteins \( \lambda_1 \), \( \lambda_2 \) and \( \lambda_3 \) and the medium sized segments (M-segments) encoding the \( \mu_1 \), \( \mu_2 \) and \( \mu_3 \) proteins, respectively [12,29]. The \( \lambda \) and \( \mu \) class proteins are conserved among the orthoreovirus species. Within these segments a lesser degree of diversity was observed. The M1 and M3 segments appeared to be closely related to the prototype strains T1/Lang and T3/Dearing, while M2 showed the same relationship to all prototypes.

The highest diversity among all segments was observed for S1, which revealed the highest nucleotide and size similarity to the prototype strain T3/Dearing. Nine out of ten segments of orthoreoviruses are monocistronic; a single ORF can be identified on each segment. The S1 segment is bi- or tricistronic and overlapping ORFs are expected [30]. Among all sequences compared, the highest identity was observed for the S2 segment which agreed to 99% with T3/Dearing at the deduced amino acid level. Detailed numbers of nucleotides and deduced amino acid comparisons of all proteins are given in table 4. The four small orthoreovirus segments (S1, S2, S3 and S4) encode for the \( \sigma \) class proteins which play a crucial role in cell attachment, dsRNA binding, hemagglutination ability and antigenicity and are associated with the assortment of the different genome segments during virus assembly [31]. Genome analysis and phylogenetic

| Table 1. Sequence homology in percent between three novel orthoreovirus strains. |
|------------------------|------------------|------------------|
| Virus strain            | T3/342/08        | 019/08           | 021/09           |
| T3/Bat/Germany/342/08   | 100%             |                  |                  |
| Bat MRV019/09           | 93%              | 100%             |                  |
| Bat MRV021/09           | 88%              | 89%              | 100%             |

Based on sequences (197 nt) revealed by nested PCR amplification, targeting the RNA-dependent RNA polymerase gene. Accession numbers: JQ412755, JQ412765 and JQ412766. doi:10.1371/journal.pone.0043106.t001
### Table 2. Summary of the molecular biological and histopathologic results of vespertilionid bats found positive for orthoreoviruses.

| Bat     | Bat species/age and sex | Virus strain | Origin of bat | Positive organs | Virus load* | Pathological changes                                                                 | Auto-lysis |
|---------|-------------------------|--------------|---------------|-----------------|-------------|-------------------------------------------------------------------------------------|------------|
| E342/08 | *P. auritus* Subadult/m | T3/Bat/Germany/342/08 | Bavaria       | Intestine       | 2.4*10⁴     | Lung: non-suppurative interstitial pneumonia (++); intestine: hemorrhagic enteritis (+++) | −          |
| E019/09 | *M. mystacinus* Juvenile/f | Bat MRV 019/09 | Bavaria       | Intestine       | 1.0*10³     | Lung: non-suppurative interstitial pneumonia (+)                                    | −          |
| E021/09 | *M. mystacinus* Juvenile/f | Bat MRV 021/09 | Bavaria       | Intestine       | 1.3*10⁴     | Lung: non-suppurative interstitial pneumonia (+)                                    | −          |
| E022/09 | *M. mystacinus* Juvenile/f | Bat MRV 021/09 | Bavaria       | Lung            | 1.0*10⁴ 8.7*10⁴ | Lung: non-suppurative interstitial pneumonia (+++); spleen: follicular hyperplasia (+); intestine: coccidiosis (+++) | +          |
| E099/09 | *P. pipistrellus* Adult/m | Bat MRV 021/09 | Bavaria       | Liver Heart Brain | 1.3*10⁵ 3.5*10⁵ 2.4*10⁶ | Lung: alveolar edema (++)                                                            | −          |
| E121/09 | *P. nathusii* Subadult/m | Bat MRV 021/09 | Bavaria       | Intestine Liver | 1.0*10⁵ 2.4*10⁴ | Lung: alveolar edema (+++), non-suppurative interstitial pneumonia (+); leucocytosis of blood vessels (+++); spleen: follicular hyperplasia (+++); kidney: glomerulopathy (+++) | −          |
| E126/09 | *P. kuhlii* Adult/m   | Bat MRV 021/09 | Bavaria       | Intestine       | 1.9*10³     | Lung: non-suppurative interstitial pneumonia (+)                                    | +          |
| E131/09 | *N. noctula* Adult/f   | Bat MRV 021/09 | Bavaria       | Intestine       | 1.0*10³     | Spleen: follicular hyperplasia (+); intestine: catarrhal-hemorrhagic enteritis (+); enteritis (+) | ++         |

m, male; f, female; −, none; +, mild; ++, moderate; ++++, severe (degree of severity);

*per ml of homogenized organ tissue (average size of tissue piece 8 mm³).*

doi:10.1371/journal.pone.0043106.t002
| Segment | Segment size [nt] | UTR [nt] | Terminal sequence | ORF/Protein region [nt] | size [aa] | class | Predicted function | 3' end |
|---------|------------------|---------|-------------------|-------------------------|-----------|-------|-------------------|--------|
| L1      | 3,853 bp         | 18 bp   | GCUA CA           | 19…3,822               | 1,267     | λ.3   | RNA-dependent RNApolymerase | 31 bp  |
| L2      | 3,915 bp         | 12 bp   | GCUA AU           | 13…3,882               | 1,289     | λ.2   | Guanyltransferase, methyltransferase | 74 bp  |
| L3      | 3,901 bp         | 13 bp   | GCUA AU           | 14…3,841               | 1,275     | λ.1   | RNA binding, NTPase, helicase, RNA triphosphatase | 60 bp  |
| M1      | 2,303 bp         | 13 bp   | GCUA AU           | 14…2,224               | 736       | μ.2   | Binds RNA NTPase | 80 bp  |
| M2      | 2,197 bp         | 30 bp   | GCUA AU*          | 31…2,157               | 708       | μ.1   | Cell penetration, transcriptase activation | 40 bp  |
| M3*     | 2,241 bp         | 18 bp   | GCUA AA*          | 19…2,184               | 721       | μNS   | Unknown NS | 57 bp  |
| S1      | 1,416 bp         | 12 bp   | GCUA AU           | 13…1,380 71…433       | 455 120   | σ1 σ1s | Cell attachment Unknown NS | 36 bp  |
| S2      | 1,331 bp         | 18 bp   | GCUA AU*          | 19…1,275               | 418       | σ2    | Binds dsRNA | 56 bp  |
| S3      | 1,198 bp         | 27 bp   | GCUA AA*          | 28…1,128               | 366       | σNS   | Inclusion formation, binds ssRNA | 70 bp  |
| S4      | 1,196 bp         | 32 bp   | GCUA AU           | 33…1,130 785…567β     | 365 73 68 | σ3 σ3a σ3b | Binds dsRNA Unknown function Unknown function | 66 bp  |

Functions of proteins encoded by open reading frames in between the non-coding sequences are predicted analog to known members of the genus Orthoreovirus. bp, base pair;  
*Terminal sequence determined by RACE PCR;  
**Predicted protein functions reported by Coombs, 2006;  
*reversely directed ORF with unknown function.

doi:10.1371/journal.pone.0043106.t003
tree reconstructions have proved that strain T3/Bat/Germany/324/08 was clearly related to the species MRV type T3. The gene constellation observed in the S1 segment (σ1 and σ1s genes) was characteristic of mammalian orthoreoviruses [23]. Together with the high homology on the nucleotide (79.6%) and the amino acid (88.2%) level and the identical length of the S1 segment compared to that of strain T3, this justified the assignment of strain T3/Bat/Germany/324/08 to serogroup 3/Dearing within the species MRV. The S1 is the most divergent segment within the orthoreovirus genome, and the encoding genes are used to assign novel viruses to different species and strains [14,23,32]. The S1 segments of all known bat- and human-borne reoviruses of the species Pteropus orthoreovirus are tricistronic [12]. In contrast, MRVs are reported to have a bicistronic genome organization [23]. The S1 segment of strain T3/Bat/Germany/324/08 appeared to be bicistronic with one larger ORF encoding for the σ1 protein and a second smaller one within encoding for the σ1s protein. Orthoreoviruses can be divided into a fusogenic and a non-fusogenic cluster, depending on their ability to cause syncytial cell fusion in mammalian cell cultures [26]. The underlying gene function for building syncytial fusion is the FAST (fusion-associated transmembrane) protein located on the S1 segment of all orthoreoviruses (S4 segment in Broome). T3/Bat/Germany/324/08 and all other members of the species MRV do not code for the FAST protein and therefore were not fusogenic.

Segments S2 and S3, encoding for the σ2 (σA in fusogenic MRV species) and σNS proteins, were found to be similar in size and length to the corresponding proteins of MRV prototype strains T1/Lang, T2/Jones and T3/Dearing. The highest identity of all segments (99% aa) was found by sequence comparison of the S2 segment to strain T3/Dearing. The σ3 protein, located on the S4 segment, plays a crucial role in dsRNA binding, assortment and replication [31]. Interestingly, we detected three ORFs including two novel, reversely directed ORFs in the S4 segment. The protein structure (α-helix, β-strand, coil and turn) was predicted for all strains, and rectangles (Fig. 4) indicate higher structure similarities between strains T3/Bat/Germany/324/08 and T3/Dearing but not with T3/Dearing.

Infected Bats and Organ Tropism

To determine the viral load in individual organs of infected bats, a real-time PCR assay targeting the RNA-dependent RNA polymerase gene was developed. Reoviral RNA was confirmed in internal organs of the three bats from which the concurrent strains were isolated (summarized in Table 5). Subsequently, all 120 bats investigated in this study were screened via real-time PCR for the presence of reoviral RNA. Five additional bats were found to...
carry strain Bat MRV021/09 with a distinct organ tropism (intestine). Detailed results including the viral load are given in table 2. No distinct correlation between infection status and age or gender of the infected bats was observed. All bats positive for reoviral RNA originated from Bavaria (8/85). The number of samples from other locations in Germany (n = 35) was not sufficient to statistically correlate reovirus infections to the bat origin. Interestingly, reovirus infections were observed in six different bat species (table 2) belonging to four different genera of vespertilionid bats: *Myotis* [n = 3], *Pipistrellus* [n = 3], *Nyctalus* [n = 1] and *Plecotus* [n = 1]. Whereas strains T3/Bat/Germany/342/08 and Bat MRV 019/09 were only identified in its initial host, strain Bat MRV21/09 was detected in five different bat species. These findings indicate a broad host range and an opportunistic character of bat orthoreoviruses, as well as a lacking of host adaptation in European bats [33–34].

Pathological Findings

During necropsy, five out of eight bats positive for reoviruses had mild to severe traumatic injuries. Two bats revealed moderate to severe hemorrhagic or catarrhal-hemorrhagic enteritis, respectively (table 2). Five carcasses had early signs of decomposition. Based on histological examination, single- or multi-organ inflammatory lesions were observed in seven of the eight bats. The lung was predominantly affected. Three bats revealed additional activation of the lymphoreticular tissue of the spleen. Although two of eight bats revealed signs of hemorrhagic enteritis and six bats had mild to severe interstitial pneumonia the impact of reovirus infection on bat hosts remains unclear.

Figure 4. Free-end protein alignment of the partial e1 protein. Its abridged length is due to the availability of only a shorter T3D/04 sequence. The protein structure (α-helix, β-strand, coil and turn) was predicted by Geneious Pro for all strains, and rectangles indicate structure similarity between strains T3/Bat/Germany/342/08 and T3D/04 compared to prototype strain T3/Dearing. doi:10.1371/journal.pone.0043106.g004


### Relationship to T3D/04

Strain T3/Bat/Germany/342/08 shared a monophyletic relationship (95% nt/96% aa) with strain T3D/04 isolated from a dog with a parvovirus type 2 co-infection, showing hemorrhagic enteritis (95% nt/96% aa) on the S1 segment and on the partial L1 segment. Moreover, they form a clearly separate lineage within the T3 class of mammals including man [12]. The distinct tropism of the novel viruses to the bats’ intestines indicates a fecal-oral transmission route. Although MRVs are known to cause rather mild and unapparent infections, recent studies also reported severe disease outcomes in a dog and man [15–17,35]. Considering that reovirus-infected bats were found in close proximity to humans, a possible spill-over and zoonotic potential of bat-borne MRV needs to be discussed.

### Supporting Information

**Figure S1** Life-cell video of Vero E6 cells infected with strain Bat/342/08. Left, Vero E6 cells control. Right, Vero E6 cells infected with T3/Bat/Germany/342/08 (MOI 0.1). Images were captured every 15 minutes for a period of 72 hours. (AVI)

### Acknowledgments

The authors are grateful to Berliner Artenschutz Team - BAT-e.V., F. Brandes, I. Frey-Mann, J. Harder, M. Kistler, M. Kredler, S. Morgenroth, E. Mühlbach, R. Pfeiffer, S. Rosenau, and W. and H. Zoels for providing the bat carcasses. The authors like to thank Jule Hinmann, Jule Tesch, Silvia Muschler and Doris Krumnow for excellent technical assistance and Ursula Eriki and Amy Burroughs for copy-editing. The authors are grateful to Ute Kramer and Kazimierz Madeira for their technical assistance and scientific discussion regarding the life cell imaging.

### Author Contributions

Conceived and designed the experiments: CK RL AB AE AR AN KM GW AK. Performed the experiments: CK RL AB AE AK. Analyzed the data: CK RL AB AE AK. Contributed reagents/materials/analysis tools: CK RL AB AE AR AN KM GW AK. Wrote the paper: CK AK GW.

### References

1. Cutler SJ, Foote AR, van der Poel WH (2010) Public health threat of new, reemerging, and neglected zoonoses in the industrialized world. Emerg Infect. Dis. 16: 1–7.
2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, et al (2008) Global trends in emerging infectious diseases. Nature 451: 930–933.
3. Kuzmin IV, Bozick B, Guagliardo SA, Kunkel R, Shuk JR, et al (2011) Bats, emerging infectious diseases, and the rabies paradigm revisited. Emerg. Health Threats J. 4: 7159.
4. Gard GP, Marshall ID (1973) Nelson Bay virus. A novel reovirus. Arch. Gesamte Virusforsch. 13: 34–42.

---

Table 5. Overview: Orthoreovirus strains isolated from or associated with bats.

| Strain          | Year | Host                   | Origin          | Data type | Sequences available | Cluster | Reference          | #  |
|-----------------|------|------------------------|-----------------|-----------|---------------------|---------|--------------------|----|
| Nelson Bay      | 1970 | Pteropus (Megachiroptera) | Australia     | Isolate   | Genome              | PRV     | Gard et al. (1973)  | [4]|
| Pulau           | 1999 | Pteropus (Megachiroptera) | Malaysia      | Isolate   | S1,S2,S3,S4         | MRV     | Pritchard et al. (2006) | [5]|
| Broome          | 2002 | Pteropus (Megachiroptera) | Australia     | Isolate   | Genome              | Own cluster | Thalman et al. (2010) | [8]|
| Melaka          | 2006 | Human (bat associated)  | Malaysia       | Isolate   | S1,S2,S3,S4         | MRV     | Chua et al. (2007)  | [6]|
| Kamper          | 2006 | Human (bat associated)  | Malaysia       | Isolate   | S1,S2,S3,S4         | MRV     | Chua et al. (2008)  | [10]|
| HK23629/07      | 2007 | Human (bat associated)  | China          | Isolate   | S1,S2,S3,S4         | MRV     | Cheng et al. (2009) | [9]|
| Xi River        | 2010 | Rousettus (Megachiroptera) | China       | Isolate   | S1,S2,S3,S4         | MRV     | Du et al. (2010)    | [7]|
| Sikamat         | 2010 | Human (bat associated)  | Malaysia       | Isolate   | S1,S2,S3,S4         | MRV     | Chua et al. (2011)  | [11]|
| Bat den Cave    | 2010 | Bat spp.               | USA            | Sequence  | Partial L1          | Mammalian ORV | Acc.No. EU871040 |    |
| T3/Bat/Germany/342/08 | 2010 | Vespertilionid (Microchiroptera) | Germany | Isolate   | Genome              | Mammalian ORV | This study |    |
| Bat MRV019/09   | 2011 | Vespertilionid (Microchiroptera) | Germany | Isolate   | Partial L1          | Mammalian ORV | This study |    |
| Bat MRV021/09   | 2011 | Vespertilionid (Microchiroptera) | Germany | Isolate   | Partial L1          | Mammalian ORV | This study |    |

doi:10.1371/journal.pone.0043106.t005
Three Novel Reoviruses in European Bats

5. Pritchard L, Chua K, Cummins D, Hyatt A, Crameri G, et al. (2006) Palau virus, a new member of the Nelson Bay orthoreovirus species isolated from fruit bats in Malaysia. Arch. Virol. 151: 229–239.

6. Chua KB, Crameri G, Hyatt A, Yu M, Tompang MR, et al. (2007) A previously unknown reovirus of bat origin is associated with an acute respiratory disease in humans. Proc. Nat. Acad. Sci. U. S. A. 104: 11424–11429.

7. Du L, Liu Z, Fan Y, Meng K, Jiang Y, et al. (2010) Xi River virus, a new bat reovirus isolated in southern China. Arch. Virol. 155: 1293–1299.

8. Thalmann CM, Cummins DM, Yu M, Lunt R, Pritchard L, et al. (2010) An unknown reovirus of bat origin is associated with an acute respiratory disease in humans. Arch. Virol. 155: 1295–1299.

9. Cheng P, Lau CS, Liu A, Ho E, Leung P, et al. (2009) A novel reovirus isolated from an adult with acute respiratory disease. J. Clin. Virol. 45: 79–80.

10. Chua KB, Voon K, Chen C, Tan HS, Rosli J, et al. (2008) Identification and characterization of a new orthoreovirus from patients with acute respiratory infections. PLoS ONE 3: e3003.

11. Chua KB, Voon K, Yu M, Keniscope C, Abdul Rasid K, et al. (2011) Investigation of a potential zoonotic transmission of orthoreovirus associated with acute influenza-like illness in an adult patient. PLoS ONE 6: e25434.

12. Day JM (2009) The diversity of the orthoreoviruses: Molecular taxonomy and phylogenetic divides. Infect. Genet. Evol. 9: 390–400.

13. Attoui H, Biagini P, Stirling J, Mertens PPC, Cantaloube J, et al. (2001) "Brome virus, a new fusogenic Orthoreovirus species isolated from an Australian fruit bat. Virology 402: 26–40.

14. Song L, Zhou Y, He J, Zhu H, Huang R, et al. (2008) Comparative sequence characterization of a new orthoreovirus from patients with acute respiratory disease. J. Clin. Virol. 45: 79–80.

15. Decaro N, Campolo M, Desario C, Ricci D, Camero M, et al. (2005) Virological evidence for reassignment to the genus Orthoreovirus, family Reoviridae. Biochem. Biophys. Res. Commun. 327: 353–358.

16. Song L, Zhou Y, He J, Zhu H, Huang R, et al. (2008) Comparative sequence analyses of a novel mammalian reovirus genome and the mammalian reovirus S1 gene from six new serotype 2 human isolates. Virus Genes 37: 392–399.

17. Decaro N, Campolo M, Desario C, Ricci D, Camero M, et al. (2005) Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. Vet. Microbiol. 109: 19–27.

18. Tyler KL, Barton ES, Bäch ML, Robinson C, Campbell JA, et al. (2004) Isolation and molecular characterization of a novel type 3 reovirus from a child with meningitis. J. Infect. Dis. 189: 1664–1666.

19. Kohl C, Vedeckis MJ, Mulldorfer K, Dabrowski PW, Radonjic A, et al. (2012) Genome analysis of a bat adenovirus 2: Indications of interspecies transmission. J. Virol. 86: 1888–1892.

20. Sonntag M, Mulldorfer K, Speck S, Wibbelt G, Kurth A. (2009) New adenovirus in bats, Germany. Emerg. Infect. Dis. 15: 2052–2053.

21. Wellehan JF Jr, Childress AL, Marschang RE, Johnson AJ, Lamirande EW, et al. (2009) Consensus nested PCR amplification and sequencing of diverse reptilian, avian, and mammalian orthoreoviruses. Vet. Microbiol. 133: 34–42.

22. Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. (2008) Rapid identification of known and new RNA viruses from animal tissues. PLoS Pathogens 4: e1000165.

23. Dermody TS, Nibert ML, Basels-Duby R, Fields BN. (1990) Sequence diversity in S1 genes and S1 translation products of 11 serotype 3 reovirus strains. J. Virol. 64: 4842–4850.

24. Huenikenbeck JP, Ronquist F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 17: 754–755.

25. Posada D. (2008) jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25: 1253–1256.

26. Duncan R. (1999) Extensive sequence divergence and phylogenetic relationships between the fusogenic and nonfusogenic orthoreoviruses: a species proposal. Virology 260: 316–328.

27. Dermody TS, Deselberger U. (2010) Reoviruses, Orbiviruses, and CoViruses. Topley and Wilson’s Microbiology and Microbial Infections. John Wiley & Sons, Ltd., Hoboken, NY/Bognor Regis, UK.

28. Holy DR, Rosen L, Fields BN. (1979) Polymorphism of the migration of double-stranded RNA genome segments of reovirus isolates from humans, cattle, and mice. J. Virol. 31: 104–111.

29. Breun LA, Broering TJ, McCutcheon AM, Harrison SJ, Luongo CL, et al. (2001) Mammalian reovirus L2 gene and lambda2 core spike protein sequences and whole-genome comparisons of reoviruses type 1 Lang, type 2 Jones, and type 3 Shope. Virology 287: 333–348.

30. Urbano P, Urbano FG. (1994) The Reoviridae family. Comp. Immunol. Microbiol. Infect. Dis. 17: 151–161.

31. Coombs KM (2006) Reovirus structure and morphogenesis. Curr. Top. Microbiol. Immunol. 309: 117–167.

32. Day JM, Pantin-Jackwood MJ, Spackman E. (2007) Sequence and phylogenetic analysis of the S1 genome segment of turkey-origin reoviruses. Virus Genes 35: 235–242.

33. Woolhouse ME. (2001) Population biology of emerging and re-emerging pathogens. Trends Microbiol. 10: S3–7.

34. Woolhouse ME, Taylor LH. (2001) Population biology of multivirus pathogens. Science 292: 1109–1112.

35. Hermann L, Embree J, Hazeldon P, Wells B, Coombs R. (2004) Reovirus type 2 isolated from cerebrospinal fluid. Pediatr. Infect. Dis. J. 23: 373.