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Characterization of mammalian orthoreoviruses isolated from feces of pigs in Zambia

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Abbreviations: dsRNA, double-stranded RNA; MRV, Mammalian orthoreovirus; T1L, type 1 Lang; T2J, type 2 Jones; T3D, type 3 Dearing; T4N, type 4 Ndelle; PBS, phosphate-buffered saline; Anti-Anti, antibiotic–antimycotic solution; RT-PCR, reverse transcription-polymerase chain reaction; Vero E6, African green monkey kidney cells; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; MEM, Eagle's minimum essential medium; BSA, bovine serum albumin; CPE, cytopathic effect; RACE, rapid amplification of cDNA ends; UTR, untranslated region; DDBJ, DNA Data Bank of Japan; ISVP, infectious subviral particle.

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The GenBank/EMBL/DDBJ accession numbers for the viral sequences are LC533904–LC533933. Supplementary tables and figures are available with the online version of this article.

Footnote: † Professor Aaron S. Mweene has passed away on April 27th, 2019.

Abstract

Mammalian orthoreovirus (MRV) has been identified in humans, livestock, and wild animals; this wide host range allows individual MRV to transmit into multiple species. Although several interspecies transmission and genetic reassortment events of MRVs among humans, livestock, and wildlife have been reported, the genetic diversity and geographic distribution of MRVs in Africa are poorly understood. In this study, we report the first isolation and characterization of MRVs circulating in a pig population in Zambia. In our screening, MRV genomes were detected in 19.7% (29/147) of fecal samples collected from pigs by reverse transcription-polymerase chain reaction. Three infectious MRV strains (MRV-85, MRV-96 and MRV-117) were successfully isolated, and their complete genomes were sequenced. Recombination analyses based on the complete genome sequences of the isolated MRVs demonstrated that MRV-96 shared the S3 segment with different MRVs isolated from bats, and that the L1 and M3 segments of MRV-117 originated from bat and human MRVs, respectively. Our results suggest that the isolated MRVs emerged through genetic reassortment events with interspecies transmission. Given the lack of information regarding MRVs in Africa, further surveillance of MRVs circulating among humans, domestic animals, and wildlife is required to assess potential risk for humans and animals.

Introduction

Orthoreoviruses, belonging to the genus *Orthoreovirus* in the family *Reoviridae*, are non-enveloped, icosahedral, segmented double-stranded RNA (dsRNA) viruses that infect vertebrates and invertebrates [1]. The orthoreovirus genome consists of 10 dsRNA segments divided into three size classes based on the characteristic mobility by gel electrophoresis, including three large segments (L1–L3), three medium segments (M1–M3), and four small segments (S1–S4) [1]. The genome is encased in two concentric capsid protein shells [1]. This structural feature contributes to the thermostability and protease resistance of its virion and play a role in the entry process of orthoreoviruses [2-8]. The genus *Orthoreovirus* is divided into two phenotypic groups, namely fusogenic and nonfusogenic groups, based on the ability to induce cell–cell fusion and syncytium formation [9]. Mammalian orthoreovirus (MRV) is nonfusogenic and includes four major serotypes (MRV1–4) according to the capacity for neutralization and hemagglutination inhibition using type-specific reovirus antisera [1]. The representative prototypes of each serotype are type 1 Lang (T1L), type 2 Jones (T2J), type 3 Dearing (T3D), and type 4 Ndelle (T4N) [10, 11]. The serotype specificity is determined by σ1 protein encoded in the S1 segment, which has the largest sequence divergence [12-14]. The genome segments of MRV have been exchanged between evolutionarily distinct strains, resulting in unpredicted reassortant viruses containing various combinations of the genome segments [15-19].

MRVs have been identified in humans, livestock, and wild animals with a wide host range [2, 19-26]. Individual MRVs can infect multiple species; several interspecies transmission and genetic reassortment events involving both humans and animals have been reported [2, 15-19, 26-28]. An MRV strain isolated from a child with acute gastroenteritis had high similarity with MRVs detected in bats, indicating interspecies transmission between bats and humans [26]. Another MRV isolated from a child with encephalitis possessed a reassorted genome between human and porcine MRVs [28]. Other MRVs isolated from pigs with diarrhea in USA emerged via genetic reassortment among different evolutionarily strains from humans, bats, and pigs [2]. These previous reports suggest that livestock and wildlife play an important role in the occurrence of interspecies transmission and genetic reassortment events, and it is therefore better to consider their genomic variations in several hosts to understand their evolution.

MRV has a wide geographic distribution, and it has spread to North and South America, Asia, Europe, and Africa [2, 16, 26, 27, 29]. Among African countries, only two MRVs have been reported in Cameroon: a T4N strain, a prototype of serotype 4, isolated from a rodent [11, 30, 31]; and the CMR-HP55 strain detected from children with diarrhea [27]. Although MRVs isolated from pigs have been reported in Japan, China, South Korea, Italy, and the USA [2, 3, 18, 32-35], they have never been detected from pigs in Africa. Understanding the distribution of MRV in Africa is important for veterinary and public health perspectives. In this study, we investigated the prevalence of MRV infection in pigs in Zambia and identified novel MRV strains with evidence of reassortment events between human and animal MRVs.

Results

Detection of MRV infection in pigs in Zambia

We screened 147 fecal samples collected from pigs in Zambia for the detection of MRV via reverse transcription-polymerase chain reaction (RT-PCR). The MRV genome was detected in 19.7% of the samples (29/147; Table 1). With respect to specific subsets, the MRV genome was detected in 14.3% (1/7) and 15.0% (6/40) of non-diarrheal and diarrheal samples, respectively, collected from suckling pigs and in 29.3% (12/41) and 16.9% (10/59) of healthy and diarrheal fattening pigs, respectively. The MRV genomes were detected in specimens from farms located in the Lusaka, Chilanga, Kafue, and Chibombo districts, suggesting a widespread MRV infection across all examined farms in Zambia.

Isolation and characterization of MRV

The supernatants of fecal suspensions were inoculated onto African green monkey kidney (Vero E6) cells for viral isolation. Obvious cytopathic effects (CPEs) were observed in Vero E6 cells inoculated with fecal samples within a few days. As described in the Methods section, we attempted to isolate MRVs using Vero E6 cells cultivated in Eagle's minimum essential medium (MEM) with either 5 μg/ml trypsin and 0.3% bovine serum albumin (BSA) or 2% fetal bovine serum (FBS). In total, 18 of 147 MRV strains (12.2%) were isolated from Vero E6 cells cultivated in MEM supplemented with trypsin and BSA, whereas

five MRVs (3.4%) were obtained from cells grown in MEM supplemented with FBS. The isolation efficiency of these two methods was significantly different ($p < 0.01$), indicating that MEM supplemented with trypsin and BSA was more suitable for isolating MRVs from Vero E6 cells than MEM supplemented with FBS. MRV-85, MRV-96, and MRV-117 were isolated from fecal samples collected from Farms B, E, and C, respectively. These representative strains were used for further analyses.

Vero E6 cells infected with MRV-85, MRV-96, and MRV-117 exhibited CPEs, as indicated by the rounding of individual cells and characteristic eosinophilic inclusion bodies (Fig. S1). The viral segmented dsRNA was separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the migration patterns of the viral M and S segments of the three MRV strains differed from each other; this finding corresponds with the different genetic backgrounds of these strains (Fig. 1).

Bioinformatics analyses of nucleotide sequences obtained via next-generation sequencing and the rapid amplification of cDNA ends (RACE) method revealed that the genomes of isolated MRV strains (MRV-85, MRV-96, and MRV-117) consisted of 10 segments (3915, 3901, 3854, 2304, 2241, 2203, 1433, 1331, 1198, and 1196 bp in length) encoding 10 open-reading frames (λ1, λ2, λ3, μ1, μ2, μNS, σ1, σ2, σ3, and σNS, Table S3). Although different migration patterns of dsRNA genomes from these strains were observed via SDS-PAGE (Fig. 1F), there was no difference in the length of the respective viral segments among obtained MRV strains based on their nucleotide sequences, suggesting that this property is attributable to differences of the sequence composition as previously observed [36]. The 5′- and 3′-untranslated regions (UTRs) ranged 12–32 and 32–80 bp in length, respectively. Isolated Zambian MRV strains possessed consensus genome sequences of the 5′- and 3′-termini (5′-GCUA and UCAUC-3′, respectively) similarly as other reported MRVs [1, 37]. The determined genomic sequences of the MRVs were deposited in the DNA Data Bank of Japan (DDBJ) under the following accession numbers: MRV-85, LC533904–LC533913; MRV-96, LC533914–LC533923; and MRV-117, LC533924–LC533933.

Sequence comparison of each viral segment and encoding protein of MRVs

Homology comparison of the complete genomic sequences among the Zambian MRV strains (MRV-

85, MRV-96, and MRV-117) demonstrated that the highest nucleotide identities of the 10 segments ranged from 82.8 to 98.7% (Table 2). Based on eight MRV segments (L1, L2, L3, M1, M2, S1, S2, and S4), Zambian MRV strains shared more than 96.0% identities with each other. Of note, the M3 segment of MRV-117 was divergent from those of the other two strains (82.8–83.0%), and the S3 segment of MRV-96 also had low nucleotide identities with those of the other two strains (89.6–90.0%). Similar results were obtained in comparative analysis focused on the amino acid sequences of nine MRV viral proteins (λ1, λ2, λ3, μ1, μ2, μNS, σ1, σ2, and σ3); however, only the σNS protein of MRV-96 had high sequence identities with those of the other two strains (97.2–97.8%). These results indicate that while the viral protein encoded in the S3 segment is highly conserved among the Zambian MRV strains, the S3 segment of MRV-96 has undergone divergence.

To identify MRV strains genetically related to Zambian MRV strains, we conducted BLAST analyses using the sequence of each viral segment (Table 3). Half of the segments (L2, L3, M1, M2, and S4) of Zambian MRV strains were closely related to those of SHR-A, GD-1, and BM-100, which were isolated from pigs in China or the USA [2, 38]. In contrast, the L1 segments of Zambian MRV strains shared the highest nucleotide and amino acid sequence identities (97.1–97.2% and 99.0–99.6%, respectively, Table 3) with that of the MRV WIV8 strain identified in wild bats. Moreover, the nucleotide sequences of the S1 and S2 segments of Zambian MRV strains diverged from those of other MRV strains (85.2–88.1% nucleotide identities, Table 3). These results suggest that these segments arise from other uncharacterized MRV strain(s). Despite large divergence among the S2 segments, the amino acid identities of the σ2 protein of Zambian strains were remarkably high (98.0–98.5%) as compared with those of the bat strain (RpMRV-YN2012). These results suggested that there is little antigenic variation among the σ2 proteins of these viruses. While the M3 segments of MRV-85 and MRV-96 shared the highest nucleotide and amino acid identities with those of the porcine strain (BM-100) (90.1–90.3% and 95.5–95.7%, respectively) [2], the pairwise nucleotide and amino acid sequence identities of the M3 segment of MRV-117 with that of SI-MRV07 identified in patients with diarrhea who traveled to Southeast Asia was 89.8% and 95.7%, respectively [39]. The S3 segment of MRV-96 shared the highest identity with the HB-B strain isolated

from minks in China [21], whereas the S3 segments of the MRV-85 and MRV-117 strains shared the highest sequence identities with the T1/Human/Wash.D.C./clone62/1957 and T3/Bovine/Maryland/clone18/1961 strains [40].

The MRV S1 segment has been demonstrated to encode the cell attachment protein "σ1" which determines the virus serotype [12]. Based on the identity comparison of S1 segments, Zambian MRV strains had the closest genetic relationship with MORV/47Ma/06 belonging to the MRV2 group, which was identified in a common vole in Hungary [22] (Table 3). According to the comparative analyses of the S1 segment and σ1 protein among the Zambian MRV strains, MRV prototypes, and other representative strains, we found that the Zambian MRVs have the highest nucleotide and amino acid sequence identities with type 2 serotype MRV strains (61.0–85.2% and 58.0–87.3%, respectively; Table S4). Some of the lower-level identities among Zambian MRV strains and type 2 serotype MRV strains (around 60%) may be due to the large divergence of the domains unrelated to the serotype-specific features of σ1 protein; similar results were obtained among other type 2 serotype MRVs.

Phylogenetic characterization of Zambian MRV

Phylogenetic trees of each viral segment were constructed using the sequences of Zambian and other representative MRVs. Phylogenetic analysis based on the sequences of S1 segments revealed that Zambian MRVs formed a distinct clade near the branches of bat (SI-MRV05) and rodent (MORV/47Ma) virus strains within the larger clade encompassing the MRV2 strain (Fig. 2A); these results suggest that the isolated Zambian strains might be classified into MRV2. Based on the L2, L3, M1, M2, and S4 phylogenies, Zambian MRV strains clustered with various porcine strains (Fig. S2). The phylogenetic analysis of the L1 segment indicated that the Zambian MRV strains were monophyletic with a bat strain (WIV8). MRV-117 formed a lineage with the bat strain (WIV8) out of a different lineage consisting of other two Zambian MRVs (Fig. S2). The topologies of the phylogenetic tree based on the S2 segment revealed that a separate unique lineage was formed by Zambian MRV strains. Notably, the Zambian MRV strains were divided into two clusters on the basis of each M3 and S3 phylogeny (Fig. 2B and 2C). Whereas the M3 segments

of MRV-85 and MRV-96 were closely related to those of porcine strains, MRV-117 clustered with human strains (SI-MRV07, T1L, and CMR-HP55). Based on the S3 segment phylogeny, MRV-96 was located out of the lineage, containing MRV-85 and MRV-117 and formed the cluster with various MRV strains, such as mink, bat, human, and porcine strains (Fig. 2C). These phylogenetic incongruities suggested the occurrence of reassortment events between the MRV strains of various hosts.

To date, only two MRV strains, namely Ndelle (MRV4) and CMR-HP55 (MRV2), have been identified from rodents and humans in Africa, respectively [11, 27, 30, 31]. These strains were detected in Cameroon, and thus prompting an investigation into the evolutionary relationship between the isolated Zambian MRVs and the Cameroonian MRVs in the phylogenetic trees. The phylogenetic analyses of MRVs indicated that the genome segments of Zambian MRVs were distantly related to those of Cameroonian MRVs, excluding for M3 segment. Based on the M3 segment phylogeny, MRV-117 clustered with human strains, including CMR-HP55 as mentioned previously (Fig. 2B).

Detection of reassortment events

To further investigate the evidence of genetic recombination in Zambian MRVs, recombination analyses were conducted using SimPlot software. Because MRV-96 and MRV-117 were not grouped with other Zambian MRV strains in the phylogenetic trees based on the sequences of the S3 and M3 segments, respectively (Figs. 2B and 2C), we performed standard similarity plot and bootscan analyses based on individual MRV segments. These analyses revealed that MRV-96 displayed high degrees of sequence similarity and bootstrap support with MRV-85 and/or MRV-117 in the L1–L3, M1–M3, S1, S2, and S4 segments, but not in the other S3 segment, which exhibited similarity with the sequences of a bat virus strain (WIV8; Figs. 3A–3C). The analyses for MRV-117 demonstrated high degrees of sequence similarity and bootstrap supports for the L2, L3, M1, M2, and S1–S4 segments among the three Zambian MRVs and for the M3 segment between MRV-117 and a human strain (SI-MRV07) as expected (Fig. 3C–3E). The L1 segment exhibited higher similarity and bootstrap support between MRV-117 and a bat strain (WIV8) than those among the Zambian MRVs. These results suggest the occurrence of genetic

reassortment events among different MRV strains.

Discussion

Although MRVs are widely distributed in humans, livestock, and wild animals in Asia, Europe, and North, and South America [2, 16, 19-27, 29], they have never been detected from pigs in Africa and their epidemiological information in Africa is limited. In this study, we report the isolation and characterization of MRVs circulating in a pig population in Zambia. In our screening, 19.7% of pigs in Zambia were positive for MRVs, which were detected in all investigated areas, including Lusaka, Chilanga, Chibombo, and Kafue. A similar prevalence of MRV infection was observed in pig populations in the USA, South Korea, and China [2, 18, 32], suggesting that MRV has spread across pig populations globally.

Phylogenetic analysis based on the S1 segments of MRV demonstrated that Zambian MRV strains formed a monophyletic group with various MRV2 strains. The isolation of MRV2 in pigs has been reported in Austria and Japan (GenBank accession number JN799419) [35]. A Japanese MRV2 strain was isolated from fecal specimens collected from both diarrheal and apparently healthy pigs, and this result was similar to our findings in Zambia. Further epidemiological studies are required to estimate the impact of MRV2 infection in the pig population worldwide.

During the cell entry of MRV, the outer capsid proteins μ1 and σ3 are degraded by luminal or intracellular proteases to generate metastable intermediate particles called infectious subviral particles (ISVPs) for membrane penetration [5-8]. Protease treatment of MRV virions, resulting in the digestion of viral outer capsids and formation of ISVPs, enhances viral entry into cells [2, 41, 42]. The addition of chymotrypsin to cell culture medium promotes the viral growth of MRV1, MRV2, and MRV3 strains in several cell lines [43]. Conversely, some MRV3 strains featuring threonine at position 249 in the cell attachment protein σ1 are susceptible to cleavage by trypsin, and treatment with trypsin *in vitro* leads to the cleavage of the viral protein and a resultant 90% reduction of virus infectivity [44]. We demonstrated that MRV was efficiently isolated from fecal samples in Vero E6 cells using trypsin and BSA, suggesting

that this procedure is more useful for MRV2 isolation than the virus propagation in Vero E6 cells with medium supplemented with FBS. Despite the clear effect of protease on virus propagation *in vitro*, little information about the evaluation of protease treatment for MRV isolation using clinical and field samples is available. The present study provides an effective protocol for the isolation of MRVs excluding MRV3 strains, the σ1 protein of which is susceptible to proteolysis.

The phylogenetic analyses and recombination analyses based on whole-genome sequences of MRV clearly demonstrated some genetic reassortment events between Zambian MRVs and different MRV strains. Although the S3 segment of MRV-96 had the highest identity to that of the mink HB-B strain, the bat WIV8 strain were predicted as the genetically close strain to MRV-96 via recombination analyses as demonstrated in our dataset. Taken together, these results suggest that the number and diversity of MRV genomic sequences currently maintained in the databank are not sufficient to infer the precise origin of the S3 segment of the MRV-96 strain. Contrarily, the M3 segment of MRV-117 most likely originated from human strains, such as SI-MRV07 and CMR-HP55, but there is no information about human MRV strains in Zambia. Interestingly, these strains were recently detected in patients with gastroenteritis in South Asia and Africa [27, 39], thus suggesting that MRV causing gastroenteritis in humans might present in Zambia. Understanding the consequences of viral reassortment is important to prepare for newly emerging high pathogenic viruses, and further surveillance of MRVs circulating in human and other animals in Zambia is required to enhance veterinary and public health.

Methods

Sample collection

We selected five farms, each housing more than 500 pigs; farms A and E were located in Lusaka, farm B in Chilanga, farm C in Kafue, and farm D in the Chibombo district. Fecal samples were collected with the cooperation of local veterinary personnel from January to December 2018 [45]. For the collection of fecal samples, some pens were chosen at random, and fresh stools on the floor were collected into tubes. Collected samples were then transported at 4°C to the laboratory and stored at −80°C until analysis. In total,

47 and 100 fecal samples were obtained from suckling (0–3 weeks old) and fattening pigs (4–12 weeks old), including those with or without diarrhea, respectively (Table 1).

Detection of MRV by RT-PCR

Fecal samples were suspended in phosphate-buffered saline (PBS) (10% [w/v]) containing 5% antibiotic–antimycotic solution (Anti-Anti; Gibco, Waltham, MA, USA) and briefly centrifuged. Total RNAs were extracted from the supernatants using TRIzol-LS (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. RNA samples were examined to detect MRVs by RT-PCR using two specific primer sets based on the conserved sequence within the L1 and L3 segments as previously described [46-48] (Table S1). The protocol of RT-PCR for detecting the L3 segment was modified from that of the PrimeScript One Step RT-PCR Kit v2 (Takara, Shiga, Japan). The RT-PCR conditions were as follows: an initial reverse transcription step at 50°C for 30 min; a PCR activation step at 94°C for 2 min; 43 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. PCR was performed in a thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were subjected to direct sequencing using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Virus isolation

Vero E6 cells were maintained in Dulbecco's Modified Eagle's Medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 3.5 mg/ml D-glucose, and 1.0 mg/ml $NaHCO_3$ at 37°C in an atmosphere of 5% CO_2. The supernatants of fecal suspensions were inoculated onto Vero E6 cell cultures, followed by 1 h of incubation at 37°C in 5% CO_2 for virus adsorption. After the inocula were removed, the cells were washed twice with PBS and maintained in MEM (Nissui Pharmaceutical Co.) containing 5 µg/ml trypsin, 0.3% BSA, 2 mM L-glutamine, 4% Anti-Anti, and 1.0 mg/ml $NaHCO_3$ at 37°C in 5% CO_2. To evaluate the trypsin sensitivity of MRV isolation, confluent monolayers of Vero E6 cells inoculated with each fecal sample were prepared

and maintained in MEM containing 2% FBS instead of trypsin and BSA. The supernatants of Vero E6 cells were passaged three times to observe CPEs. The culture medium collected from cells displaying CPEs were filtered through 0.45-μm membrane filters (Iwaki, Tokyo, Japan) to remove bacterial cells and re-inoculated into the cells. Viral isolation was confirmed by RT-PCR. The morphology and staining features of MRV-infected cells were assessed via hematoxylin and eosin staining.

The electrophoretic patterns of the viral dsRNA were observed by SDS-PAGE as previously described [36]. Vero E6 cell monolayers grown in T-175 flasks were infected with the isolated MRVs. The infected cells exhibiting CPEs were subjected to three freeze-thaw cycles, and the debris was removed via centrifugation at 1750 × *g* at 4°C for 10 min. For virus concentration, the supernatants were ultracentrifuged at 28,000 rpm and 4°C for 2 h using an SW-28 rotor and an Optima L-90K (Beckman Coulter, Brea, CA), and the pellets were resuspended in PBS. Total RNAs were extracted from the re-suspensions using QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Viral dsRNAs were purified as previously described [49]. Briefly, extracted total RNAs were mixed with equal volumes of 4 M LiCl and incubated at 4°C overnight. The dsRNAs were precipitated by adding isopropanol and ammonium acetate at −30°C and pelleted via centrifugation. Purified dsRNA was separated by SDS-PAGE using 4–15% gradient gel. MRV genome segments were visualized via ethidium bromide staining.

Whole-genome sequencing

Total RNAs were extracted from the supernatants of MRV-infected cells using QIAamp Viral RNA Mini Kits (Qiagen), and cDNAs were synthesized using a PrimeScript Double Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. The cDNA libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instruction and subjected to whole-genome sequencing on the MiSeq instrument with a MiSeq Reagent Kit v3 (600 cycles) (Illumina). Sequencing data was analyzed using CLC Genomics Workbench software (CLC bio, Hilden, Germany). After trimming low-quality reads, remaining reads were *de novo* assembled with

the default settings. The obtained contigs were analyzed via local BLAST against the virus reference downloaded from the NCBI database. Consensus sequences obtained by mapping to the reference sequences of other known MRVs were aligned with MRV contigs obtained via *de novo* assembly. Finally, all trimmed reads were re-mapped to the aligned sequences and consensus sequences. Additionally, the 5′- and the 3′-terminal regions of each MRV segment were amplified by RACE (Table S1). The RACE method was performed using a SMARTer RACE cDNA Amplification Kit (Takara) according to the manufacturer's protocol. PCR products were subjected to direct sequencing using a Big Dye Terminator v3.1 Cycle Sequencing kit.

Genetic comparison and phylogenetic analysis

The open-reading frame positions and encoded proteins were predicted via comparisons with other known MRV strains. Bioinformatic analyses were performed using various MRV sequences deposited in the DDBJ/EMBL-Bank/GenBank databases (Table S2). Global homology analyses were conducted among Zambian MRV strains using GENETYX version 12 (GENETYX Corporation, Tokyo, Japan). The highest nucleotide identities for each viral segment of Zambian MRVs were identified via BLAST analyses. Phylogenetic analyses based on the nucleotide sequence of each viral segment were performed using MEGA7 [50]. The MUSCLE protocol was used to align the sequences, and phylogenetic trees were constructed using the maximum likelihood method based on the Tamura–Nei model with 1000 bootstrap replicates.

Recombination analyses

Homologous recombination within each genome segment among Zambian MRVs and other MRVs was analyzed. Putative reassortant MRVs were preliminary identified by topological incongruity among phylogenies of different segments. Such incongruities were further investigated using a dataset of MRVs composed of the sequences of Zambian MRVs (n = 3) and other MRVs (n = 27) with complete genomic sequences available in the reference list (Table S2). Alignments for each viral segment were analyzed

using the similarity and Bootscan methods as implemented in SimPlot software version 3.5.1 [51].

Statistical analysis

Differences in the rates of MRV isolation in the presence and absence of trypsin were analyzed using the chi-squared test. $p < 0.05$ denoted statistical significance.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethics approval and consent to participate

Not applicable

Consent for publication

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498 **Tables**

499 **Table 1.** Summary of sampling areas, profiles of pigs, and results of reverse transcription-polymerase chain

500 reaction for mammalian orthoreoviruses

			No. of positive/No. of tested fecal sample from pigs (%)			
			Suckling pigs (0-3 weeks)		Fattening pigs (4-12 weeks)	
Farm	District	Sampling date	No diarrhea	Diarrhea	No diarrhea	Diarrhea
A	Lusaka	7/January/2018	1/5 (20)	0/12 (0)	-	1/7 (14)
		14/June/2018	-	0/4 (0)	0/2 (0)	0/9 (0)
B	Chilanga	25/January/2018	-	-	-	0/4 (0)
		10/July/2018	-	-	2/9 (22)	1/6 (17)
		4/December/2018	0/1 (0)	1/2 (50)	0/1 (0)	1/7 (14)
C	Kafue	13/February/2018	-	1/5 (25)	-	2/10 (20)
		8/June/2018	-	1/7 (14)	2/8 (25)	0/1 (0)
		16/August/2018	-	3/10 (30)	5/13 (38)	0/4 (0)
D	Chibombo	2/March/2018	-	-	-	2/4 (50)
E	Lusaka	17/July/2018	-	-	2/4 (50)	2/6 (33)
		20/December/2018	0/1 (0)	-	1/4 (25)	1/1 (100)
Total			1/7 (14)	6/40 (15)	12/41 (29)	10/59 (16)

501

502 **Table 2.** Identity comparison of each genome segment among Zambian strains (MRV-85, MRV-96, and

503 MRV-117)

Segment	Nucleotide sequence identity (%) between			Amino acid sequence identity (%) between		
(Protein)	85 and 96	85 and 117	96 and 117	85 and 96	85 and 117	96 and 117
L1 (λ3)	98.7	96.0	96.0	99.9	99.1	99.0
L2 (λ2)	98.7	98.7	98.5	99.3	99.3	99.0
L3 (λ1)	98.3	98.5	98.6	99.2	99.1	99.6
M1 (μ2)	98.6	98.5	98.7	99.1	98.9	99.1
M2 (μ1)	98.3	98.1	98.2	99.0	99.2	99.4
M3 (μNS)	98.4	83.0	82.8	98.7	91.1	91.2
S1 (σ1)	97.6	97.6	97.3	95.6	96.0	96.5
S2 (σ2)	98.2	98.5	98.3	98.8	99.2	99.0
S3 (σNS)	90.0	98.4	89.6	97.8	98.9	97.2
S4 (σ3)	98.4	98.7	98.5	98.6	99.1	98.9

504

505 **Table 3.** Highest nucleotide and amino acid identities for each genome segment of strains isolated in Zambia

Segment (Protein)	Strain	% identity		Strain	Serotype	Host	Country	year	Acession No.
		nt	aa						
L1 (λ3)	85, 96, 117	97.1-97.2	99.0-99.6	WIV8	3	bat	China	2011	KT444562
L2 (λ2)	85, 96, 117	91.2-91.3	95.8-96.1	SHR-A	1	pig	China	2011	JX415467
L3 (λ1)	85, 96, 117	95.8-96.0	98.6-99.0	SHR-A	1	pig	China	2011	JX415472
M1 (μ2)	85, 96, 117	95.0-95.4	97.5-98.2	GD-1	3	pig	China	2012	JX486060
		94.9-95.4	97.1-97.6	Netherlands 84	1	human	Netherlands	1984	AY428872
M2 (μ1)	85, 96, 117	93.2-93.5	97.7-98.1	BM-100	3	pig	USA	2014	KM820748
M3 (μNS)	85, 96	90.1-90.3	95.5-95.7	BM-100	3	pig	USA	2014	KM820749
	117	89.8	95.7	SI-MRV07	2	human	Slovenia	2017	MG999581
S1 (σ1)	85, 96, 117	85.2-85.5	85.8-87.3	MORV/47Ma/06	2	rodent	Hungary	2006	KX384852
S2 (σ2)	85, 96, 117	87.8-88.1	98.0-98.5	RpMRV-YN2012	2	bat	China	2012	KM087112
S3 (σNS)	85, 117	95.8-96.3	97.8-98.3	T3/Bovine/Maryland/clone18/1961	3	cattle	USA	1961	U35358
		96.0-96.3	97.8-98.3	T1/Human/Wash.D.C/clone62/1957	1	human	USA	1957	U35356
	96	97.8	99.7	HB-B	1	mink	China	2013	KF013856
S4 (σ3)	85, 96, 117	96.4-96.9	97.5-98.0	GD-1	3	pig	China	2012	JX486066

506 Abbreviation: nt, nucleotide sequence; aa, amino acid sequence

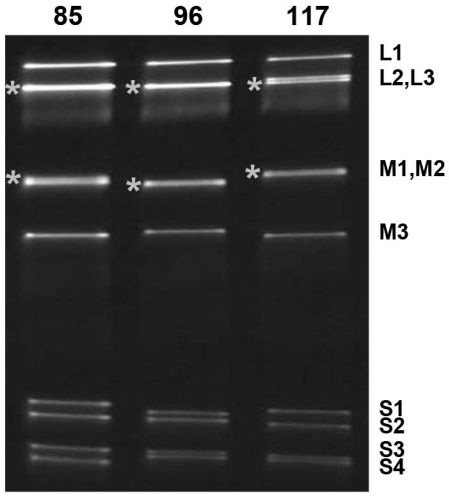
507

Figure legends

Fig. 1. Electrophoresis analysis of the genome of mammalian orthoreoviruses (MRVs) isolated from fecal samples of pigs. Electropherotypes of viral double-stranded RNAs isolated from MRV strains (MRV-85, MRV-96, and MRV-117) were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4–15% gradient gel. The locations of three large segments (L1–L3), three medium segments (M1–M3), and four small segments (S1–S4) are indicated. The L2 and L3 segments and the M1 and M2 are considered to have co-migrated, forming single bands (*).

Fig. 2. Phylogenetic analysis of the S1, M3, and S3 segments. Phylogenetic trees based on the nucleotide sequence of the complete S1 (A), M3 (B), and S3 (C) segments were constructed using the maximum likelihood method with 1000 bootstrap replicates. Bootstrap values greater than 70% are shown on the interior branch nodes, and the scale bar indicates the number of substitutions per site. Group names (MRV1, MRV2, MRV3, and MRV4) and cluster names are indicated on the tree. Black circles represent isolated MRVs in this study. MRV, mammalian orthoreovirus.

Fig. 3. Recombination analysis of mammalian orthoreovirus (MRV). Similarity plot (A and D) and Bootscan analyses (B and E) based on the sequences of each viral segment were conducted using SimPlot software v3.5.1. Two putative reassortant viruses, namely MRV-96 (A and B) and MRV-117 (D and E) were analyzed. (C) Schematic diagram of the each MRV genome segments. Window and step sizes of 800 and 200 bp, respectively, were used for the similarity plot and Bootscan analyses. Compared sequences and color coding are specified on the top of panels A and D.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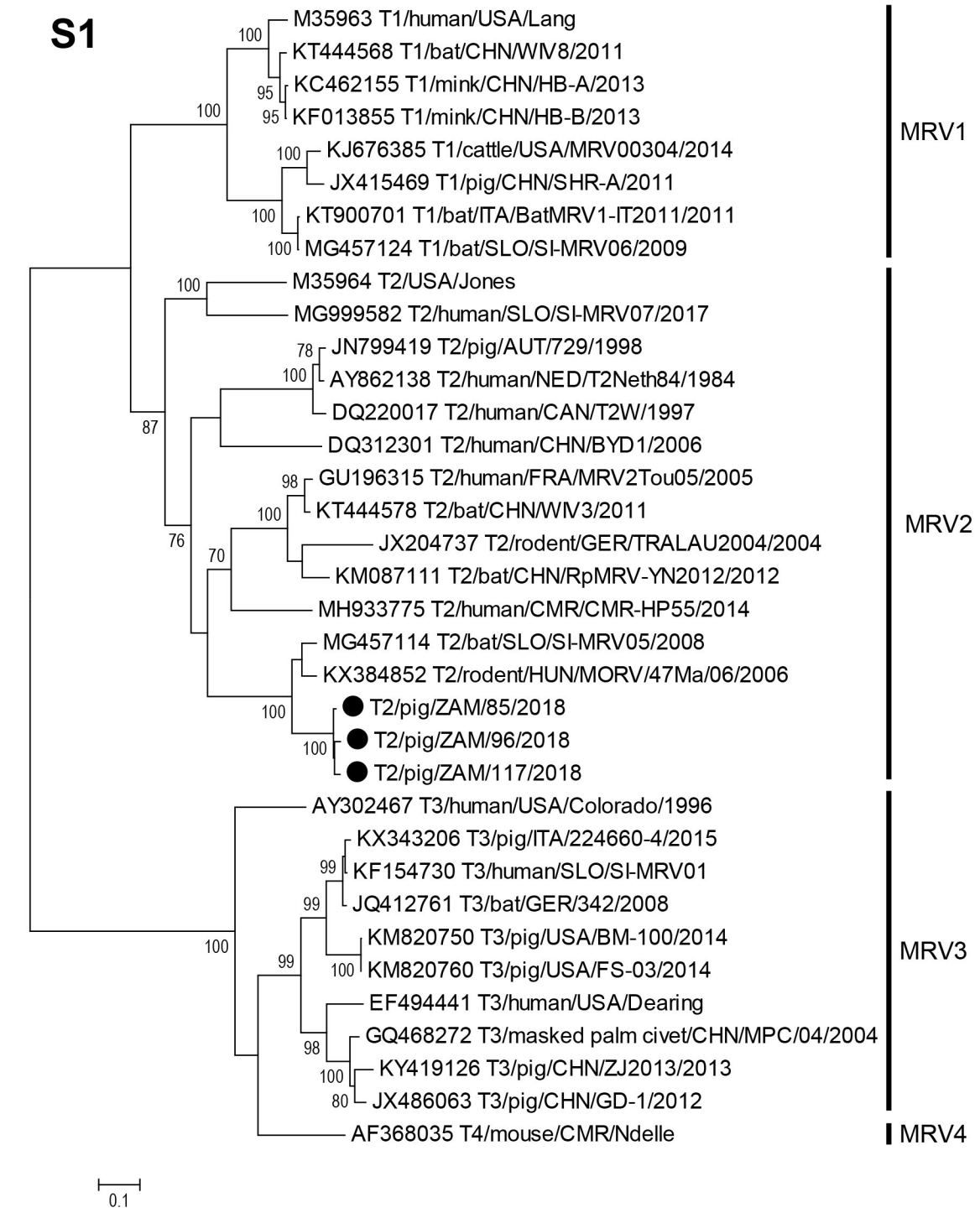
96
117
L1
L2,L3
M1,M2
M3
S1
S2
S3
S4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KJ676385 T1/cattle/USA/MRV00304/2014
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MG457124 T1/bat/SLO/SI-MRV06/2009
M35964 T2/USA/Jones
MG999582 T2/human/SLO/SI-MRV07/2017
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DQ220017 T2/human/CAN/T2W/1997
DQ312301 T2/human/CHN/BYD1/2006
GU196315 T2/human/FRA/MRV2Tou05/2005
KT444578 T2/bat/CHN/WIV3/2011
JX204737 T2/rodent/GER/TRALAU2004/2004
KM087111 T2/bat/CHN/RpMRV-YN2012/2012
MH933775 T2/human/CMR/CMR-HP55/2014
MG457114 T2/bat/SLO/SI-MRV05/2008
KX384852 T2/rodent/HUN/MORV/47Ma/06/2006
T2/pig/ZAM/85/2018
T2/pig/ZAM/96/2018
T2/pig/ZAM/117/2018
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KF154730 T3/human/SLO/SI-MRV01
JQ412761 T3/bat/GER/342/2008
KM820750 T3/pig/USA/BM-100/2014
KM820760 T3/pig/USA/FS-03/2014
EF494441 T3/human/USA/Dearing
GQ468272 T3/masked palm civet/CHN/MPC/04/2004
KY419126 T3/pig/CHN/ZJ2013/2013
JX486063 T3/pig/CHN/GD-1/2012
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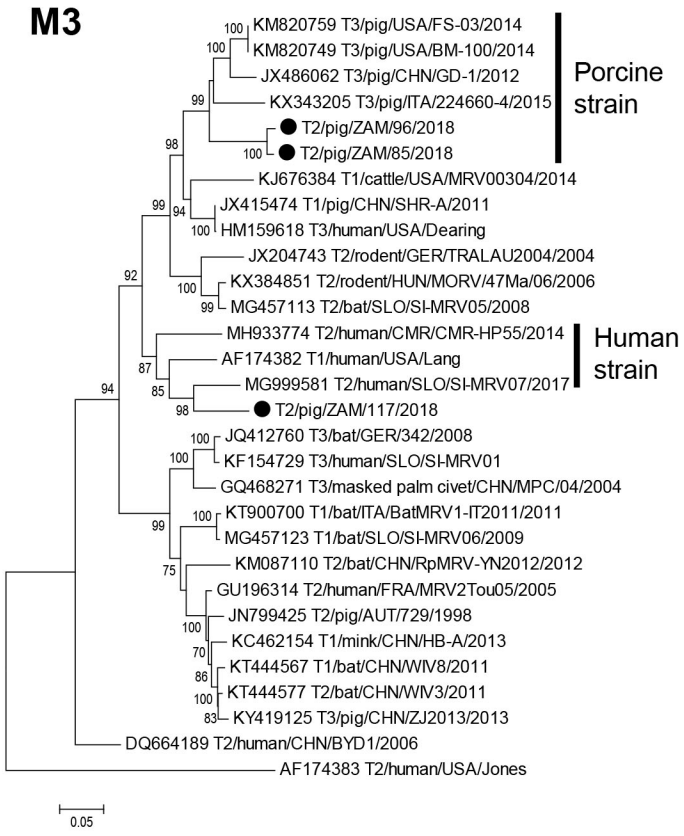
MRV1
MRV2
MRV3
MRV4
0.1

B)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T2/pig/ZAM/96/2018
T2/pig/ZAM/85/2018
Porcine strain
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JX415474 T1/pig/CHN/SHR-A/2011
HM159618 T3/human/USA/Dearing
JX204743 T2/rodent/GER/TRALAU2004/2004
KX384851 T2/rodent/HUN/MORV/47Ma/06/2006
MG457113 T2/bat/SLO/SI-MRV05/2008
MH933774 T2/human/CMR/CMR-HP55/2014
AF174382 T1/human/USA/Lang
MG999581 T2/human/SLO/SI-MRV07/2017
Human strain
T2/pig/ZAM/117/2018
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KF154729 T3/human/SLO/SI-MRV01
GQ468271 T3/masked palm civet/CHN/MPC/04/2004
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MG457123 T1/bat/SLO/SI-MRV06/2009
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KT444577 T2/bat/CHN/WIV3/2011
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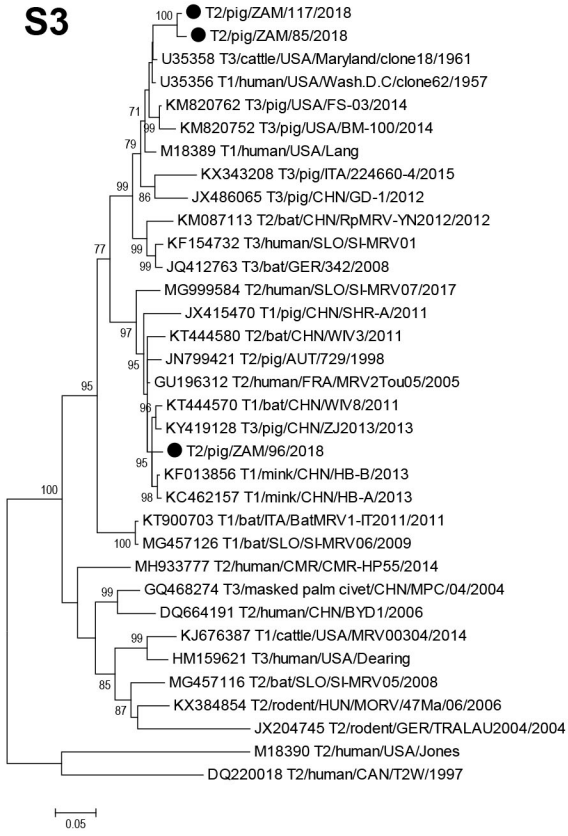
0.05

C)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KM820762 T3/pig/USA/FS-03/2014
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M18389 T1/human/USA/Lang
KX343208 T3/pig/ITA/224660-4/2015
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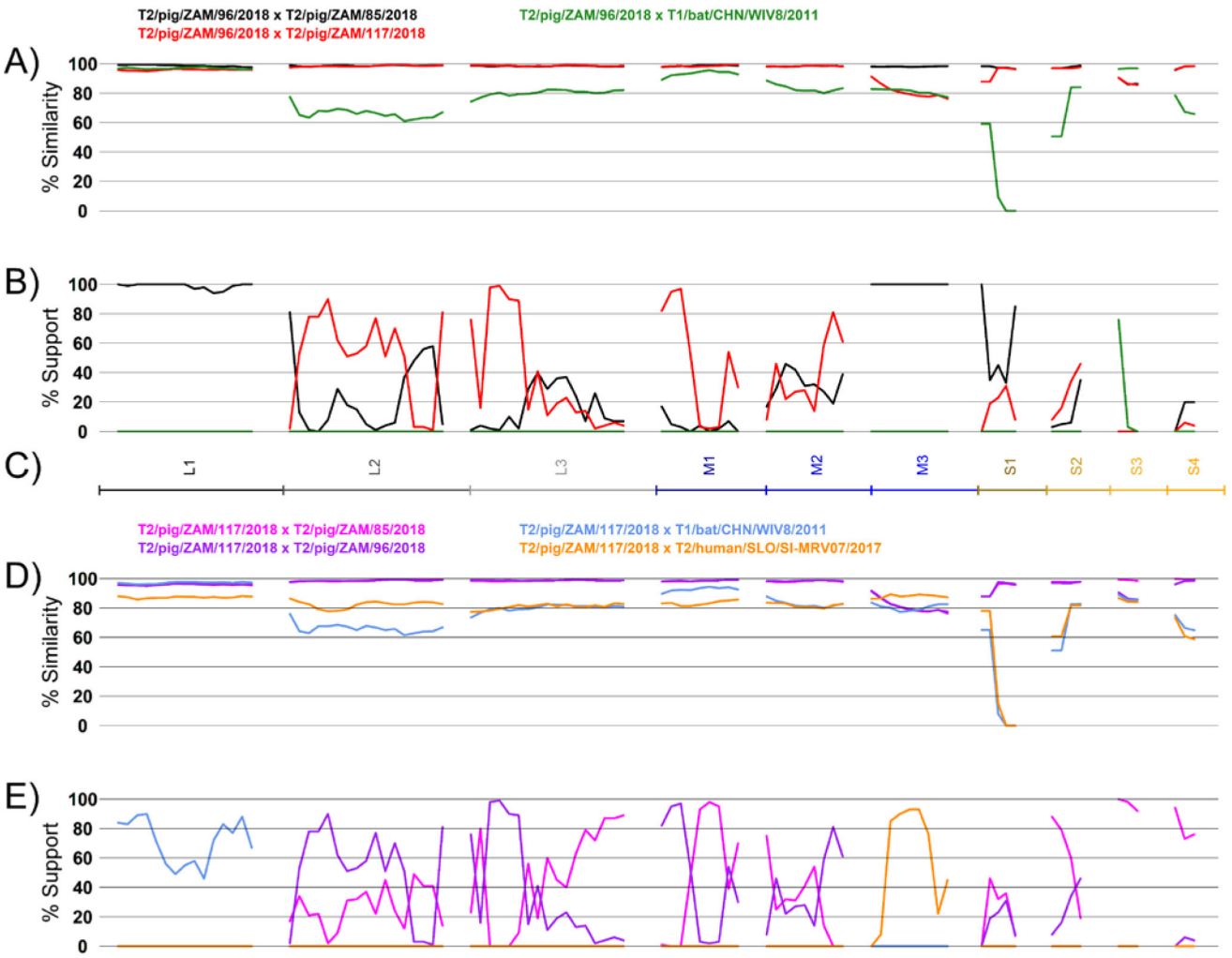
KT444570 T1/bat/CHN/WIV8/2011
KY419128 T3/pig/CHN/ZJ2013/2013
T2/pig/ZAM/96/2018
KF013856 T1/mink/CHN/HB-B/2013
KC462157 T1/mink/CHN/HB-A/2013
KT900703 T1/bat/ITA/BatMRV1-IT2011/2011
MG457126 T1/bat/SLO/SI-MRV06/2009
MH933777 T2/human/CMR/CMR-HP55/2014
GQ468274 T3/masked palm civet/CHN/MPC/04/2004
DQ664191 T2/human/CHN/BYD1/2006
KJ676387 T1/cattle/USA/MRV00304/2014
HM159621 T3/human/USA/Dearing
MG457116 T2/bat/SLO/SI-MRV05/2008
KX384854 T2/rodent/HUN/MORV/47Ma/06/2006
JX204745 T2/rodent/GER/TRALAU2004/2004
M18390 T2/human/USA/Jones
DQ220018 T2/human/CAN/T2W/1997
0.05


T2/pig/ZAM/96/2018 x T2/pig/ZAM/85/2018
T2/pig/ZAM/96/2018 x T2/pig/ZAM/117/2018
T2/pig/ZAM/96/2018 x T1/bat/CHN/WIV8/2011
A)
% Similarity
100
80
60
40
20
0
B)
% Support
100
80
60
40
20
0
C)
L1
L2
L3
M1
M2
M3
S1
S2
S3
S4
T2/pig/ZAM/117/2018 x T2/pig/ZAM/85/2018
T2/pig/ZAM/117/2018 x T2/pig/ZAM/96/2018
T2/pig/ZAM/117/2018 x T1/bat/CHN/WIV8/2011
T2/pig/ZAM/117/2018 x T2/human/SLO/SI-MRV07/2017
D)
% Similarity
100
80
60
40
20
0
E)
% Support
100
80
60
40
20
0