Isolation and characterization of a mammalian orthoreovirus type 3 in a fecal sample of wild boar in Japan

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ABSTRACT

Mammalian orthoreoviruses (MRVs) have been identified in various mammalian species, including humans, bats, and pigs. However, MRV isolation and complete genome sequences from wild boars have not yet been reported. In this study, we isolated, sequenced, and analyzed an MRV from a free-living wild boar in Japan using a porcine sapelovirus-resistant cell line N1380. The complete and empty virus particles were obtained from the N1380 cell culture supernatants, and complete genome sequences were obtained from complete virus particles. Sequence analyses revealed that the isolated MRV, named TY-14, was classified as MRV3 and had a close genetic relationship with lion in a Japanese zoo MRV2 (L2, L3, and M3 genes) and human MRV2 from Japan (S2 gene). Phylogenetic analyses showed that TY-14 clustered only with bat MRVs in the M1 gene, while TY-14 formed a cluster with several animal MRVs in the M2 and S3 genes, and independently branched in the L1, S1, and S4 genes, suggesting a genetic relationship with other unknown origins. Recombination events were identified in the M2 gene. These results suggest that TY-14 was generated by reassortment and recombination events involving MRVs circulating in Japan, bats, and other unknown origins.

Keywords: mammalian orthoreovirus (MRV), reassortment, recombination, wild boar, N1380 cells

1. INTRODUCTION

The genus *Orthoreovirus* includes viruses that infect mammalians, birds, and reptiles, and belongs to the family *Reoviridae*. *Mammalian orthoreovirus* (MRV) is a species of the genus *Orthoreovirus* that also includes the species *Avian orthoreovirus*, *Baboon orthoreovirus*, *Nelson Bay orthoreovirus*, and *Reptilian orthoreovirus* (Day, 2009). The virus particles of MRV have an icosahedral symmetry, with a diameter of 60-80 nm, and contains 10 segments of linear double-stranded RNA (dsRNA), with a total genome size of approximately 23,500 base pairs (bp). Based on the mobility of gel electrophoresis, the genome segments of MRV divide into three large (L1, L2, and L3), three medium (M1, M2, and M3), and four small (S1, S2, S3, and S4) sized-class segments that encode three λ, three μ, and four σ proteins, respectively (Day, 2009; Dermody et al., 2013). The σ1 protein that is encoded by the S1 gene is the serotype-specific antigen of the MRV, which is recognized by neutralization antibodies. Based on the antigenic recognition of σ1 protein, three major serotypes (MRV1, MRV2, and MRV3) have been identified for MRV. In addition to the three serotypes of MRV, Ndelle virus, which was isolated from a mouse and has been classified as an orbivirus, has been reclassified as MRV4 due to its sequence similarity to MRVs and its antigenic difference to three MRV serotypes (https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsrna-viruses-2011/w/dsrna_viruses/188/reoviridae as of November 2020).

MRVs are mainly spread by fecal-oral or respiratory routes, with no arthropod vectors are involved, and are distributed worldwide. MRVs infect a variety of mammals, including swine (Cao et al., 2018; Cavicchio et al., 2020; Dai et al., 2012; Fukutomi et al., 1996; Harima et al., 2020; Hirahara et al., 1988; Kwon et al., 2012; Lelli et al., 2016; Luo et al., 2020; Qin et al., 2017; Steyer et al., 2013; Thimmasandra Narayanappa et al., 2015; Ye et al., 2020; Zhang et al., 2011; Zhang et al., 2020), humans (Mikuletič et al., 2019;

Rosa et al., 2019; Tyler et al., 2004; Yamamoto et al., 2020), bats (Kohl et al., 2012; Lelli et al., 2013, 2015; Li et al., 2016; Naglič et al., 2018; Wang et al., 2015; Yang et al., 2015), cattle (Anbalagan et al., 2014), horses (Conner et al., 1984), chamois (Besozzi et al., 2019), deer (Ahasan et al., 2019), minks (Lian et al., 2013; Zhang et al., 2016), masked palm civet (Li et al., 2015), dogs (Decaro et al., 2005; Kokubu et al., 1993), cats (Mochizuki et al., 1992; Muir et al., 1992), shrews (Li et al., 2020), and rodents (Fehér et al., 2017; Gauvin et al., 2013). MRVs have been associated with both asymptomatic and symptomatic infections. Symptomatic cases, including diarrhea, respiratory disease, and encephalopathy in children and young animals, associated with MRV are indicative of its zoonotic potential.

In Japan, MRV1 and MRV2 were isolated from the respiratory tract of pigs with respiratory disease and the diarrheic feces of pigs, respectively (Fukutomi et al., 1996; Hirahara et al., 1988). MRV2 viruses have also been found in children with meningitis and gastroenteritis, and cats with or without diarrhea (Mochizuki et al., 1992; Yamamoto et al., 2020). Although MRV3 was isolated from fecal samples of diarrheic dogs in Japan (Kokubu et al., 1993), with the greatest prevalence among cats in Japan (Mochizuki et al., 1992), genome sequence data for MRV3 is currently lacking. Moreover, no MRV genome sequence has been reported from wild boars, not only in Japan, but also in other areas. In the present study, we report the first isolation and characterization of MRV3 from wild boar in Japan.

2 MATERIALS AND METHODS

2.1 Fecal specimens of wild boar

Eighty-two fecal samples (rectal contents) of hunted wild boars were collected during 2018-2019 from the Toyama and Ishikawa prefectures of the central region of

mainland Japan. Fecal samples were diluted with 10 mM phosphate-buffered saline (PBS) to prepare a 10% (w/v) suspension. The suspension was shaken at 4°C for 1 h, clarified by centrifugation at 10,000 × *g* for 30 min, passed through a 0.45-μm membrane filter (Millipore, Bedford, MA), and stored at –80°C used for genome analyses and virus isolation.

2.2 Viral RNA extraction and genome sequencing

Total RNA was extracted from the supernatants of 10% wild boar fecal suspension using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA), followed by treatment with DNase I (Takara Bio, Shiga, Japan). RNA was extracted from cell culture supernatants using a MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science). To determine the nucleotide (nt) sequence of the entire genome of isolated MRV, next generation sequencing (NGS) was performed. A library was constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina version 2.0 (New England Biolabs) according to the manufacturer's protocol. Library purification was performed using Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). A 151-cycle paired-end read sequencing run was carried out on a MiSeq desktop sequencer (Illumina) using a MiSeq Reagent Kit (version 2) (300 cycles). Sequence reads were assembled into contigs by *de novo* assembly using the CLC Genomics Workbench 7.5.5 (CLC bio).

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) for MRV detection

Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems, Foster City, CA). PCR was performed to amplify a portion of the L1 genome, with a forward primer (Reo-L1-F, 5'-

TCCATCGTAAATGATGAGTCTG-3') and a reverse primer (Reo-L1-R, 5'-GAAATCAGTTCTAACATCCTCTG-3'), based on the sequence of L1 of sR1590 (Zhang et al., 2020). The amplification was carried out for 35 cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s) after denaturation at 95°C for 60 s, followed by a final extension at 72°C for 7 min. The PCR products with 410 bp nucleotides were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Nucleotide sequencing was carried out using an ABI 3130 Genetic Analyzer Automated Sequencer (Applied Biosystems, Foster City, CA) and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

2.4 Cell culture and inoculation

A porcine sapelovirus-resistant cell line, N1380, was previously established by a genome-wide genetic screening script (Zhang et al., 2020). The N1380 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Nichirei, Biosciences, Tokyo) at 37°C in a humidified 5% CO_2 atmosphere, and passaged every 3 days. One milliliter of the 10% wild boar fecal suspension was inoculated onto N1380 cells, and adsorption was performed at 37°C for 1 h. The cells were washed three times with PBS, and then 10 mL of maintenance medium consisting of medium DMEM (Invitrogen, Carlsbad, CA) containing 2% (v/v) heat-inactivated FBS was added. Further incubation was performed at 36°C. The culture medium was replaced with fresh medium every 4 days. If no cytopathic effect (CPE) was observed until day 8 post-inoculation (p.i.), the cells were monodispersed by trypsinization, diluted twice with the maintenance medium, and used for the second passage.

2.5 Purification of virus particles

The virus-infected N1380 cells were harvested on day 4 p.i. after extensive CPE was observed, followed by centrifugation at 10,000 × *g* for 60 min. The resulting supernatant was collected and centrifuged at 32,000 rpm for 3 h in a Beckman SW32Ti rotor. The resulting pellet was re-suspended in PBS buffer at 4°C overnight, and then purified by an equilibrium cesium chloride (CsCl) gradient centrifugation at an initial density of 0.42 g/ml. Centrifugation was performed at 35,000 rpm for 24 h at 10°C in a Beckman SW55Ti rotor. The gradient was fractionated into 250-μl aliquots, and each fraction was weighed to estimate the buoyant density and isopycnic point. Finally, each fraction was diluted with PBS and centrifuged for 2 h at 50,000 rpm in a Beckman TLA55 rotor to remove the CsCl and concentrate the samples (Bai et al., 2018; Zhang et al., 2019).

2.6 SDS-PAGE

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5-20% e-Pagel (ATTO, Tokyo), followed by staining with GelCode blue stain reagent (Thermo Fisher Scientific, Rockford IL, USA).

2.7 Electron microscopy (EM)

The purified virus particles were placed on glow-discharged 300-mesh carbon-coated Cu grids (Veco grids; Nisshin EM, Tokyo) for 2 min, rinsed with distilled water, and negatively stained with 2% phosphotungstic acid solution. The grids were observed under a transmission electron microscope (HT7700; Hitachi High Technologies, Tokyo) at 80 kV.

2.8 Phylogenetic analyses

The nt sequences of the complete ten segments of the MRV genome with sequences obtained from GenBank/EMBL/DDBJ database were aligned using ClustalW (Thompson et al., 1997). Phylogenetic analyses were performed based on nt sequences using the maximum likelihood method with the best-fit model in MEGA7 (Kumar et al., 2016). The trees were evaluated using bootstrap analysis with 1000 replicates (Felsenstein, 1985). Pairwise nt and amino acid (aa) sequence identities were calculated using the CLC Genomics Workbench. The aligned concatenated whole genomes were analyzed and visualized using SimPlot software (version 3.5.1) (Lole et al., 1999) and the mVISTA online platform (Frazer et al., 2004). Recombination analysis was performed using SimPlot software (version 3.5.1) and Recombination Detection Program (RDP) (version 4.80) (Martin et al., 2015).

3 RESULTS

3.1 Metagenomics identification and isolation of MRV from wild boar fecal specimens

MRV sequence read searches using the BLAST program and the read mapper tool in CLC Genomics Workbench revealed that the MRV sequence reads were identified in one specimen collected from a healthy wild boar (approximately five months old) in 2018 in Toyama Prefecture (Toyama 14) among 82 (1.2%) wild boar specimens. This sample also contained the viral nt sequences of the picobirnavirus. To isolate the MRV by cell culture, the stool suspension was inoculated onto N1380 cells. Since no CPE was observed until day 8 p.i., the adherent cells were monodispersed by trypsinization and diluted twice with the maintenance medium. Then, extensive CPE appeared in the cells 3 days after the passage. The culture supernatants were collected and inoculated again into N1380 cells. CPE was clearly observed at day 4 p.i, and the MRV RNA in the cell culture

supernatants was detected by RT-PCR (data not shown). These findings indicate that the MRV was replicated in N1380 cells.

To purify the MRV particles, a large-scale cell culture was performed using 20 bottles (75 cm^2) of N1380 cells. A total of 400 mL of the virus-infected cell culture supernatants was concentrated and purified by CsCl gradient centrifugation, as described in the Materials and Methods. The gradient was separated into 20 fractions, and the proteins contained in each fraction were examined by SDS-PAGE. The protein bands were primarily distributed in fractions 4–6 and 12–13 (Fig. 1A). We observed six protein bands, with molecular masses of ~140 kDa, ~83 kDa, ~72 kDa, ~42 kDa, ~37 kDa, and ~25 kDa in fractions 4 to 6 and 12 to 13 (Fig. 1A). Using electron microscopy (EM) to visualize fraction 5, many spherical virus particles with a diameter of approximately 90 nm were observed (Fig. 1B). In contrast, empty particles with identical diameters were observed in fractions 13 (Fig. 1C). The density of the virus particles in fractions 4 to 6 was 1.360 g/cm^3, while that of the virus particles in fractions 13 and 14 was 1.300 g/cm^3. The morphological properties of the virus particles are similar to those of reoviruses previously isolated from pigs (Zhang et al., 2020).

3.2 Analysis of the entire genome sequences of MRV

To analyze the entire genome sequence of RMV, the RNAs were extracted from the purified particles with a density of 1.360 g/cm^3 and subjected to NGS. The complete genome of the isolated MRV (GenBank/DDBJ/EMBL accession no. LC579751-LC579760), named Wild_boar_TY-14_2018_JPN (TY-14), was comprised of 3,854 nt (L1), 3,915 nt (L2), 3,902 nt (L3), 2,304 nt (M1), 2,203 nt (M2), 2,241 nt (M3), 1,416 nt (S1), 1,332 nt (S2), 1,198 nt (S3), and 1,196 nt (S4) that encompassed 1,268 aa (L1), 1,290 aa (L2), 1,276 aa (L3), 737 aa (M1), 709 aa (M2), 722 aa (M3), 456 aa (S1), 419 aa

(S2), 367 aa (S3), and 366 aa (S4), respectively (Fig. 2). The S1 gene encodes the σ1 protein, which is the serotype-specific antigen of the MRV. Phylogenetic analysis based on the complete nt sequences of the S1 gene revealed that TY-14 was classified as MRV3 (Supplementary Fig. 1G). The pairwise nt and aa sequence comparison of the S1 gene showed that TY-14 was most related to porcine MRV GD-1 from China, exhibiting 92.51% (nt) and 94.95% (aa) sequence identities (Table 1). However, TY-14 independently branched and did not cluster with GD-1 in the S1 phylogenetic tree (Fig. 2). In the L2, L3, and M3 phylogenetic trees, TY-14 clustered with the MRV2 strain isolated from a lion (lion Yamaguchi) in 2011 at the zoo in Yamaguchi Prefecture, located on the western part of mainland Japan, with high nt sequence identities (98.57%, 98.77%, and 98.26% for L2, L3, and M3 genes, respectively) (Fig. 2 and Table 1). Furthermore, SimPlot and mVISTA analyses showed that lion Yamaguchi exhibited a high sequence similarity to TY-14 throughout the L2, L3, and M3 genes (Fig. 3). The S2 gene of TY-14 shared a high sequence similarity (98.57% and 100% for nt and aa, respectively) to human MRV Osaka_2014, which was isolated in 2014 in a central region of mainland Japan (Table 1, Fig. 3), and TY-14 clustered with Osaka_2014 in the S2 tree (Fig. 2). In the M1 phylogenetic tree, TY-14 and bat MRVs from China formed a cluster (Fig. 2), while the M1 nt sequence of bat MRV WIV2 had the highest nt similarity (97.57%) with TY-14 (Table 1). TY-14 clustered with human, chamois, and porcine MRVs, and porcine, human, mink, deer, and bat MRVs in the M2 and S3 phylogenetic trees, respectively, while TY-14 monophyletically branched in the L1 and S4 trees (Fig. 2).

Similarity plot analysis of the M2 gene using SimPlot with the TY-14 sequence as a separate query indicated that TY-14 had an nt sequence similarity with lion Yamaguchi in the 5’ half and 3’-end of the M1 gene, whereas the middle of the M2 gene

was similar to that of bat MRV WIV2 (Fig. 4A). Recombination analysis using RDP4 revealed a cross-over event in the M2 gene (Fig. 4B).

4 DISCUSSION

We successfully isolated MRV from fecal samples of wild boars using N1380 cells, which are resistant to porcine sapelovirus infection (Zhang et al., 2020). Sapelovirus is prevalent in the Japanese wild boar population (Abe et al., 2011); therefore, N1380 is a powerful cell line for the isolation of MRV from the fecal samples of wild boar. We also successfully determined the complete genome sequence of MRV from isolated complete viral particles using an Illumina MiSeq platform. To the best of our knowledge, this study is the first to describe the complete genome sequence of MRV from wild boar.

Phylogenetic analysis using the complete S1 gene nt sequences indicated that TY-14 was classified as MRV3, which has been found in pigs from the USA, China, Korea, and Italy (Kwon et al., 2012; Lelli et al., 2016; Thimmasandra Narayanappa et al., 2015). Similarly, MRV1 and MRV2 have been isolated from pigs, pigs, humans, and cats, respectively, in Japan (Fukutomi et al., 1996; Hirahara et al., 1988; Mochizuki et al., 1992; Yamamoto et al., 2020). However, only one study has described MRV3 from the diarrheic fecal samples of dogs (Kokubu et al., 1993). Thus, TY-14 represents the second MRV3 strain to be found in Japan. In a previous study using MRV2 empty particle-based ELISA, a total of 95 wild boar serum samples were collected in Japan, among which 52.6% (50/95) were found to be positive for MRV2, indicating that this infection occurs frequently in free-living wild boar in Japan (Zhang et al., 2020). On the other hand, although a MRV3 seroepidemiological survey of wild boar in Japan has not been previously conducted, MRV3 is known to be the most prevalent of the MRV serotypes in cats in Japan (Mochizuki et al., 1992). However, between 1981 and 2018 in Japan, the

yearly distribution of MRV cases was 57%, 13%, and 0.7% for MRV-2, MRV-1, and MRV-3, respectively (Yamamoto et al., 2020). In the present study, only one MRV3 genome sequence was identified from 82 wild boar fecal samples using a metagenomics approach. Additional genetic and serological studies on the prevalence of MRV in wild boars will be needed to gain further insights.

Reoviruses have discrete segmented genes; therefore, their genomes have a tendency to undergo genetic reassortment derived from intra- and interspecies transmission, as well as gain genetic diversity among circulating viruses (Cowley et al., 2016; Lian et al., 2013; McDonald et al., 2016). Our results suggest that the genome of TY-14 is likely to be a reassortment segment derived from several mammalian species. The M1 gene of TY-14 formed a cluster with bat MRVs. Bats are reservoirs of various viruses, including MRV, and play an important role in the interspecies transmission of MRVs to other mammalian species (Chua et al., 2007; Lelli et al., 2015; Li et al., 2016; Uehara et al., 2019). The M1 gene of TY-14 may be derived from that of bats. However, a report describing MRV from Japanese bats has yet to be published. Therefore, further studies are needed. In the present study, TY-14 was found to share a high sequence similarity with lion Yamaguchi (isolated in 2011 in a Japanese zoo) across the L2, L3, and M3 genes. Furthermore, the S2 gene of TY-14 showed a sequence similarity with that of Japanese human MRV Osaka_2014. In Japan, free-living wild boar populations are expanding, and their area of distribution is increasing (Ohdachi et al., 2009; Yamazaki et al., 2016). Occasionally, wild boars migrate close to human habitats, increasing the potential of natural pathogen transmission between humans and animals under the control of human and wild boars (Meier & Ryser-Degiorgis, 2018; Meng et al., 2009). Our analyses suggest that TY-14 appears to be a naturally occurring reassortment in which the

ancestral virus of TY-14 acquired the L2, L3, M3, and S2 gene segments from animals under the control of human and human origin in Japan.

Recombination events contribute to the acquisition of genetic diversity and the emergence of novel viruses, resulting in their adaptation to new host species (Nora et al., 2007; Oberste et al., 2004; Pérez-Losada et al., 2015; Simmonds, 2006). Recombination in the L3, M2, and S2 genes have been reported in porcine MRV and avian orthoreovirus, respectively (Noh et al., 2018; Ye et al., 2020). Our analysis showed the possibility of intra-segment recombination of the M2 gene of TY-14 with MRV2 lion Yamaguchi and MRV2 bat WIV5. RNA recombination events require co-infection with more than one MRV strain, suggesting that mixed infection with multiple MRVs occurred in wild boars or other animals, indicating that MRV was transmitted to wild boars in the past.

In summary, our results suggest that MRV3 TY-14 is a reassortant possessing MRV gene segments found in the lions of a Japanese zoo, as well as humans and bats. These findings imply that the evolution of MRVs is geographically driven, and that several MRV strains may circulate in mammalian species in Japan. Furthermore, recombination events in the intra-segment of the M2 gene were identified. These findings underline the importance of the whole-genome characterization of MRV strains; however, the origin of the five gene segments of TY-14 was not elucidated by our analyses. The broad host range of MRVs and the scarcity of data deposited in the DDBJ/EMBL/GenBank databases makes it difficult to characterize their origin. Considering that the pathogenic capacity of pigs, humans, and other animals has been reported (Cao et al., 2018; Ouattara et al., 2011; Qin et al., 2017; Thimmasandra Narayanappa et al., 2015; Yamamoto et al., 2020), there is a need for the large-scale surveillance and characterization of the whole genome of circulating MRVs, not only wild boars, but also in other animals.

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CONFLICT OF INTREST

The authors have no conflicts of interest to declare.

ETHICAL APPLOVAL

Since the rectal content samples were collected from a free-living wild boar population controlled by the authority under the Ishikawa Prefectural Wildlife Management Plan, which encourages the use of captured animals for scientific research to manage wildlife populations, no specific approval was needed. We contacted hunters and asked for their assistance prior to the commencement of this study.

DATA AVA I L A B I LIT Y S TATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Fig. 1. Purification of MRV. The supernatants of Toyama14-inoculated N1380 cells were concentrated by ultracentrifugation, and virus particles were purified by CsCl gradient centrifugation. Aliquots from each fraction were analyzed using 5–20% SDS-PAGE, and the virion proteins were visualized by GelCode blue stain reagent (**A**). Virus particles in fractions 5 (**B**) and 13 (**C**) were observed by EM. Scale bar, 200 nm.

Fig. 2. Genome structure of TY-14 and phylogenetic analyses based on complete L1-L3, M1-M3, and S1-S4 gene nucleotide sequences of TY-14 and selected MRVs, which had a sequence similarity with TY-14, obtained from the DDBJ/EMBL/GenBank databases. The phylogenetic trees were constructed using the maximum-likelihood method in MEGA7 with best fit models (T92+G for L1, M1, M3, and S1 genes, GTR+G for L2, K2+G for L3, M2, and S4, K2+I for S2 and S3). Bootstrap values above 70 (1,000 replicates) are indicated. The bars indicate the corrected genetic distances. TY-14, T2_Lion_Yamaguchi_2011_JPN, human MRVs, bat MRVs, porcine MRVs, and another origin MRVs are indicated in red, pink, blue, green, orange, and black, respectively.

Fig. 3. (A) Similarity plots of the concatenated ten segments of human MRVs (blue curves), T2_Lion_Yamaguchi_2011_JPN (pink curve), porcine MRVs (orange curves), bat MRVs (green curves), and mink MRV (black curve), and T3_Wild_boar_TY-14_2018_JPN TY-14 as query sequence, with a sliding window of 200 nucleotides and a moving step size of 20 nucleotides. (B) mVISTA concatenated ten segments nucleotide alignment TY-14 with T2_Lion_Yamaguchi_2011_JPN, T2_Human_Osaka_2014_JPN, and T1_Bat_WIV2_2007_CHN. Percentile values on the right indicate sequence-based

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identities between TY-14 and another strains. Shading in pink indicates the level of conservation.

Fig. 4. (A) Similarity plots of the M2 gene of T2_Lion_Yamaguchi_2011_JPN (blue curve), and T2_Bat_WIV5_2011_CHN (blue curve), and TY-14 as query sequence, with a sliding window of 200 nucleotides and a moving step size of 20 nucleotides. (B) Recombination analysis of TY-14 vs. T2_Bat_WIV5_2011_CHN (yellow curve), TY-14 vs. T2_Lion_Yamaguchi_2011_JPN (blue curve), and TY-14 vs. T2_Bat_WIV5_2011_CHN (purple curve).

Supplementary Fig. 1. The phylogenetic analyses based on complete L1-L3, M1-M3, and S1-S4 gene nucleotide sequences of TY-14 and MRVs obtained from the DDBJ/EMBL/GenBank databases. The phylogenetic trees were constructed using the maximum-likelihood method in MEGA7 with best fit models (GTR+G+I for L1-L3, M1-M3, S2 and S3 genes, GTR+G for S1 and S4). Bootstrap values above 70 (1,000 replicates) are indicated. The bars represent the corrected genetic distances. TY-14, T2_Lion_Yamaguchi_2011_JPN, human MRVs, bat MRVs, porcine MRVs, and another origin MRVs are indicated in red, pink, blue, green, orange, and black, respectively.

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