First Report on Co-isolation and Whole-genomic Characterization of Mammalian Orthorubulavirus 5 and Mammalian Orthoreovirus Type-3 From Domestic Pigs in India

Fateh Singh (fateh.ars07@gmail.com)
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases https://orcid.org/0000-0001-6653-7570

Katherukamem Rajukumar
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Dhanapal Senthilkumar
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Govindarajulu Venkatesh
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Deepali Srivastava
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Subbiah Kombiah
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Sandeep Kumar Jhade
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Vijendra Pal Singh
National Institute of High Security Animal Diseases of Indian Council of Agricultural Research: National Institute of High Security Animal Diseases

Research Article

Keywords: Genomic characterization, India, co-isolation, mammalian orthoreovirus type-3, mammalian orthorubulavirus 5, pig

Posted Date: January 6th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1219383/v1

Abstract

During a surveillance study to monitor porcine epidemic diarrohoea virus and transmissible gastroenteritis virus in India, a total of 1043 swine samples including faeces (n=264) and clotted blood (n=779) were collected and tested. Five samples (four faecal and one serum) showed cytopathic effects in Vero cells. Transmission electron microscopy of infective cell supernatant revealed the presence of two types of virions. Next generation sequencing (*de novo*) enabled complete genome assembly of Mammalian orthorubulavirus 5 (MRuV5; 15246 bp) and all 10 gene segments of Mammalian orthoreovirus (MRV; 22219 bp and 20512 bp). Genetic analysis of the MRuV5 revealed grouping of the Indian MRuV5 with those isolated from various mammalian species in South Korea and China, sharing more than 99% nucleotide identity. Deduced amino acid sequences of the HN, NP and F genes of MRuV5 isolates showed three (92L, 111R, 447H), two (86S, 121S) and two (139T, 246T) amino acid substitutions, respectively, compared to previously reported virus strains. The Indian MRV isolates were identified as MRV type-3 based on genetic analysis of S1 gene, showing the highest nucleotide identity (97.73%) with the MRV3 strain ZJ2013 isolated from pigs in China. Deduced amino acid sequences of MRV3 S1 gene revealed amino acid residues 198-204NLAIRLP, 249I, 340D, 419E known for sialic acid binding and neurotropism. We report the co-isolation and whole-genomic characterization of MRuV5 and MRV3 recorded incidentally for the first time from domestic pigs in India. It attracts attention to perform detailed surveillance studies and continuous monitoring of evolution and spread of emerging viruses, which may have pathogenic potential in animal and human hosts.

Introduction

Continuously increasing threats of emerging pathogens causing several infectious diseases in human and animal population are a big challenge to the global health care system. Due to high dynamics of microbial evolution in nature, especially in the current scenario of climate change, the viruses are continuously evolving in a diverse way due to genetic changes. A number of novel viruses have emerged in the last few decades, and some of them have shown to infect multiple host species including human [1]. The RNA viruses, which undergo frequent genetic changes, contribute substantially to the occurrence of emerging infectious diseases, sometimes overcoming species barriers to adapt in a new host or niche.

Mammalian orthorubulavirus 5 (MRuV5), previously called as parainfluenza virus 5 (PIV5), belongs to the genus *Orthorubulavirus* under the family *Paramyxoviridae*. It has a genome of 15246 bp single-stranded RNA assembled in a circular or polymorphous-shaped virion of 50–200 nm diameter [2]. The MRuV5 genome is comprised of a 3' leader region, a 5' trailer region, and seven non-overlapping genes (NP, V/P, M, F, SH, HN and L) [3]. Due to a specific RNA editing mechanism, the V/P gene encodes two distinct structural proteins, V and P, and thus seven genes in MRuV5 genome encode a total of eight proteins [1]. MRuV5 has been isolated from various host species such as humans, pigs, dogs, cattle, cats, hamsters, guinea pigs, and lesser pandas [1, 4-6]. It has been often reported to cause asymptomatic respiratory infections in many mammalian species [5]. Moreover, it has been reported to cause enteric disease in pigs [7].

The Mammalian orthoreovirus (MRV), classified under the genus *Orthoreovirus* and family *Reoviridae,* is a non-enveloped double-stranded RNA virus having double-capsid structure around a core of ten genomic RNA-segments [8]. These viral RNA segments include three large (L), three medium (M) and four small (S) based on electrophoretic mobility [9]. Inner capsid is formed by proteins λ-1 and σ-2, while the outer capsid of the virion is made up of proteins λ-2, μ-1, σ-3, and σ-1 [10]. Due to segmented genome, genetic recombination among segments of the reoviruses is possible and their reassortant strains are frequently detected. There are four known serotypes of MRV based on the sequence and the antigenic behaviour of hemagglutination protein σ-1. These serotypes include prototype strain type-1 (Lang, T1L), type-2 (Jones, T2J), type-3 (Dearing, T3D), and type-4 (Ndelle, T4N) [11].

Like MRuV5, MRV also have a wide host range and infect multiple mammalian species including pigs, dogs, cattle, sheep, horses, cats, mice, and human and non-human primates [12]. Previously, the MRVs were known to develop asymptomatic or mild respiratory and enteric infections [12]. However, in recent past, MRVs have been found producing several clinical infections both in human and other mammalian species. Clinical cases of enteritis, acute respiratory infections and encephalitis in humans due to reassortant MRV strains have increased worldwide, and the causative MRVs have animal reservoirs [13-14]. MRV-2 and 3 are known to cause respiratory infections, encephalitis and diarrhoea in human [13-14]. While, MRV-3, alone or with other pathogens, has been reported in association with enteric disease in pigs from Asia, North America and Europe [15-19].

Owing to the presence of large biodiversity, dense population of humans and animals and porous international borders, tropical regions like India, are vulnerable 'hotspots' for the emergence of exotic and emerging diseases. Due to lack of species barriers, the prime concern is zoonotic transmission and spread of these viruses in humans and animal species. Monitoring the pigs might be an important strategy to detect emerging pathogens as they are reservoirs of a diverse range of viruses. Identification of emerging or exotic viruses provides valuable information on upcoming potential threats and can aid in providing disease alerts for necessary preparedness. In this paper we present the first report on co-isolation and whole-genomic characterization of MRuV5 and MRV type-3 from domestic pigs in India.

Materials And Methods

Samples

During a surveillance study to monitor emerging and exotic swine enteric viruses such as porcine epidemic diarrohea virus (PEDV) and transmissible gastroenteritis virus (TGEV), a total of 1043 swine samples including faeces (n=264) and clotted blood (n=779) were collected from different states in India. The faecal samples were from the states of Mizoram (153), Assam (76), Chhattisgarh (24) and Madhya Pradesh (11), and the blood samples were from the states of Mizoram (399), West Bengal (150), Madhya Pradesh (113), Assam (73), Chhattisgarh (24), and Maharashtra (20). Faecal samples in the form of rectal swabs were collected in 1 ml of sterile phosphate buffered saline (PBS) containing 2× antibiotics and anti-mycotic solution (Sigma-Aldrich, USA). All the samples were transported under chilled condition to the biosafety level-3 (BSL-3) laboratory at ICAR-National Institute of High Security Animal Diseases, Bhopal for testing.

Sample processing

Serum was separated from the clotted blood by centrifugation at 1200 g for 15 min. Faecal swab samples were suspended in sterilized phosphate buffered saline to make 20% (w/v) homogenate. Supernatants of faecal homogenates were collected after centrifugation at 8000 g for 15 min. The serum and faecal supernatants were kept in two sets; one set to extract the viral RNA and the other for virus isolation. For virus isolation, the faecal supernatants were filtered through 0.22 µm pore-sized syringe filters for decontamination.

Screening of samples by RT-PCR

RNA was extracted from faecal supernatants and serum samples using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany). One-step reverse transcription-PCR was carried out for the detection of genome of PEDV and TGEV as per the method described previously using virus specific primers (Kim et al., 2000) (Table 1) and Access RT-PCR System (Promega Corporation, Madison, USA). RT-PCR products were analysed by agarose gel electrophoresis.

Virus isolation

For virus isolation, dilutions (1:5 and 1:10) of the faecal supernatants and clarified serum samples were inoculated in duplicate into sub-confluent monolayers of Vero cells grown in Eagles minimum essential medium (EMEM) in 96-well cell culture plates. Plain EMEM containing trypsin at a concentration of 10 µg/ml was used for virus infection. Inoculated plates were incubated at 37ºC for 5-6 days in an incubator at 5% CO_2 concentration. The cells were observed daily under inverted microscope for the development of any cytopathic effect (CPE). Subsequently, two more serial passages were given using 1:5 diluted frozen and thawed cell culture supernatants of the previous passage. At the end of third passage, RNA extracted from the cell supernatents were tested for the genome of PEDV and TGEV by RT-PCR.

Screening of cell culture supernatants by reverse transcription-PCR/ PCR

The CPE producing cells which tested negative for PEDV and TGEV were further investigated for the presence of other swine enteric viruses such as porcine rotavirus (PRV), porcine enterovirus (PEV), porcine sapelovirus (PSV) and porcine parvovirus (PPV).

Viral RNA and DNA were extracted from the CPE-positive as well as uninfected control cell culture supernatants using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) and QIAamp viral DNA extraction kit (Qiagen, Hilden, Germany), respectively. The

nucleic acid was used as template for RT-PCR or PCR, as the case may be, using virus specific primers and methods described previously (Table 1).

Haemagglutination test

Cell culture supernatants from the CPE-positive wells were also tested for the haemagglutination (HA) using red blood cells (RBCs) of pig, guinea pig and chicken as per the standard protocol (Decaro et al., 2005).

Ultracentrifugation

The CPE-positive cell culture supernatants were processed for ultracentrifugation to concentrate the virus particles. The cell suspensions after three freeze-thaw cycles were clarified by centrifugation at 8000 g for 15 min at 4ºC. The supernatants were overlaid on one-third volume of 20% sucrose (w/v, prepared in TNE buffer) in ultracentrifuge tubes. Ultracentrifugation was carried out at 150,000 g and 4ºC for 2 h in Sorvall™ WX 90 ultracentrifuge with swinging bucket rotor (Thermo Fisher Scientific, USA). After ultracentrifugation, the supernatants were discarded and the sediment at the tube bottom was resuspended in 200 µl of autoclaved water.

Transmission electron microscopy

For electron microscopy, a drop of the ultracentrifuged suspension was allowed to get adsorbed over formvar-carbon–coated copper grids for 3-5 min. The adsorbed grids were negatively stained with 2% phosphotungstic acid for 45 sec. The stained grids were air-dried and viewed in a transmission electron microscope (Talos, FEI, Hillsboro, OR, USA).

Next generation sequencing

RNA was extracted from ultracentrifuged cell culture supernatant of one faecal (3013814) and one serum (3013789) sample and subjected to next generation sequencing (*de novo*) using NextSeq500 platform (Illumina) commercially at Eurofins Genomics India Pvt. Ltd., Bengaluru. Sequenced raw data was processed to obtain high quality clean reads using Trimmomatic v0.38. The resulting reads were aligned to hg 19 human reference genome using BWA MEM (0.7.17). The unmapped reads were fetched from the mapping using Samtools. *De novo* assembly of unmapped reads was performed using CLC Workbench9. Blastn was performed on the longest scaffolds to identify the top-hit virus species. Two top-hit virus species were subjected to gene prediction using GeneMarkS tool and the genes identified were annotated using Diamond tool (BlastX).

Phylogenetic analysis

The sequence data was assembled and analysed with respect to the reference sequences available in NCBI GenBank database. The whole genome sequence of MRuV5 and segment sequences of MRV were used to perform phylogenetic analysis. MRV serotype was identified based on genetic analysis of S1 segment sequence. The evolutionary history was inferred by using the neighbour-joining/ maximum likelihood method and Tamura-Nei model available in MEGA X software (*https://www.megasoftware.net/*).

Detection of specific genes of MRuV5 and MRV

MRuV5 and MRV were also identified by one-step RT-PCR and sequencing. Previously published primers specific to nucleoprotein (NP), fusion (F) protein, phosphoprotein (P) and membrane (M) protein gene were used to verify the presence of MRuV5, while the primers specific to L1 and L3 segments were used to detect the MRV in Vero cell supernatants. The RT-PCR amplification was performed by adding 4 µl of AMV/ *Tfl* 5× reaction buffer, 0.2 mM of dNTPs, 0.75 mM of MgSO4, AMV reverse transcriptase (2 U), *Tfl* DNA polymerase (2 U) using Access RT-PCR System (Promega Corporation, Madison, USA), 10 picomoles of each primer and 2 µl of RNA template for 20 µl amplification reactions. One-step RT-PCR conditions include reverse transcription of RNA at 45ºC for 30 min followed by initial denaturization at 95ºC for 3 min and 35 cycles comprised of denaturization at 95ºC for 20 sec, annealing at 50ºC for 45 sec, extension at 72ºC for 30 sec to 1.5 min (30 sec for L1 and L3 segments of MRV, 1 min for M and P gene, 1.5 min for NP and F gene of MRuV5) and the final extension at 72ºC for 5 min. PCR products were analysed by agarose gel electrophoresis. The primer details are provided in Table 1.

The PCR products in excised gels were purified by PCR purification kit (Qiagen, Hilden, Germany) following the Manufacturer's instructions and subjected to nucleotide sequencing using Sanger's sequencing method for confirmation.

Results

All the samples were negative for the genome of PEDV and TGEV by RT-PCR. Four faecal samples (Lab accession no. 3013772, 3013814, 3013820, 3013823) and one serum sample (Lab accession no. 3013789) collected from Mizoram state showed CPE in Vero cells by third passage. The CPE, characterized by rounding and detachment of cells, started at 48 h post infection (hpi) and by 72 hpi more than 50% cells were found detached (Figure 1). About 80% cells were found detached by 96-120 hpi after which the cells were frozen.

The CPE-positive cell culture supernatants were found to be negative for the genome of PEDV, TGEV, PRV, PEV, PSV, and PPV when tested by RT-PCR/ PCR. The cell culture supernatants of CPE producing samples showed haemagglutination (HA) of RBCs of pig and guinea pig. The HA activity was not observed with chicken RBCs.

TEM analysis showed the presence of two types of viruses. A pleomorphic enveloped virion with a diameter ranging from 99 nm to 261 nm (common diameter range of 150-200 nm) resembling a paramyxovirus was seen (Figure 2). Characteristic 'Herringbone-like' nucleocapsid of about 17 nm diameter could be seen outside the disrupted virus particles. The other type was seen as icosahedral, non-enveloped virus particles ranging from 68 to 89 nm in diameter (common diameter range of 78-85 nm), resembling a reovirus (Figure 2).

Next generation sequencing generated 828 mb and 476 mb data for the sample number 3013789 and 3013814, respectively. Blastn analysis of the longest scaffolds showed the top-hit species as mammalian orthorubulavirus 5 (MRuV5) and the mammalian orthoreovirus (MRV) in both the samples. Nucleotide assembly and gene prediction showed the presence of 7 genes encoding nucleocapsid protein (NP) (1530 nucleotides, 509 amino acids), V protein (669 nucleotides, 222 amino acids), membrane (M) protein (1134 nucleotides, 377 amino acids), fusion (F) protein (1656 nucleotides, 551 amino acids), small hydrophobic (SH) protein (135 nucleotides, 44 amino acids), haemagglutinin-neuraminidase (HN) (1698 nucleotides, 565 amino acids) and large (L) protein (6735 nucleotides, 2244 amino acids) in MRuV5. The whole genome size of MRuV5 was estimated to be 15246 bp. The whole genome sequences of MRuV5 obtained in this study have been submitted to the NCBI GenBank database [accession numbers: MW273368 (IND/MZ/3013789) and MW273369 (IND/MZ/3013814)]. The gene prediction analysis also showed the presence of MRV with segmented genome consisting of three large (L1, L2, L3), three medium (M1, M2, M3) and 4 small (S1, S2, S3, S4) segments, and genome length of 22219 bp and 20512 bp could be assembled for the IND/MZ/3013789/reo and IND/MZ/3013814/reo isolates, respectively. The segment-wise nucleotide sequences of MRV (IND/MZ/3013789/reo and IND/MZ/3013814/reo) isolated in this study have been submitted to the NCBI GenBank database (accession numbers MZ516371 to MZ516381, and MZ541850 to MZ541858).

Whole genome based sequence and phylogenetic analysis of the MRuV5 isolated from pigs in the present study showed grouping of the Indian MRuV5 with those isolated from various mammalian species in South Korea and China, with more than 99% nucleotide identity (Figure 3). Nucleotide and deduced amino acid sequence analysis of all seven individual ORFs (NP, V/P, M, F, SH, HN and L) showed >99% identity with several strains of MRuV5 reported from China, South Korea and Thailand (Table 2).

Deduced amino acid sequences of the HN, NP and F gene coding regions showed the presence of three (92L, 111R, 447H), two (86S, 121S) and two (139T, 246T) unique amino acid substitutions, respectively, as compared to previously reported strains of the virus. Predicted amino acid residues in HN protein revealed QDHVS (186–190) at receptor binding site, and E390 and Y523 at the cleavage site. Predicted amino acid residues for the HN stalk region included S60, Y77, L90, E91 and Q102, similar to the other strains of MRuV5 reported previously. Amino acid residues responsible for the development of neutralizing antibodies were recorded as E37, Q342, T437, and F457. Amino acid residues predicted for host preference were observed as I22, A49, R57, T254, N318 and K460 in the HN protein and as T3, S19, T438, L498, S530 and R536 in the Fusion protein. Membrane protein showed the presence of one unique amino acid (184L) substitution compared to reported strains of the virus.

S1 gene segment based genetic analysis of the MRV isolated in this study showed the highest nucleotide identity (97.73%) and deduced amino acid identity (96.7%) with the MRV3 isolate ZJ2013 (KY419126.1) of porcine origin reported from China, and the

virus was grouped into MRV type-3 serotype (Dearing, T3D) (Figure 4). The nucleotide and deduced amino acid sequence identity of all gene segments of the MRV with respect to some reported strains are shown in Table 3. The phylogenetic trees of the segments L1, L2, L3, M1, M2, M3, S2, S3, and S4 of MRV are shown in Figure 5.

Deduced amino acid sequence of full coding region (codes for sigma-1 protein) of the S1 segment showed changes in 15 amino acids compared to the MRV3 strain ZJ2013. The MRV strains reported in the present study showed three unique amino acids changes (V49, H65 and I186) which are distinct from most of the strains reported so far. Further analysis of the deduced amino acid sequence of S1 gene revealed the amino acid residues NLAIRLP(198-204) associated with sialic acid binding. The present strains have amino acid isoleucine (I) at position 249 in σ1 protein which affects the susceptibility of type-3 σ1 protein to cleavage by proteases in host intestine. The present strains also have amino acid residues 340D and 419E that have been linked with the affinity of MRV to the central nervous system (Figure 6).

The MRuV5 and MRV were also detected in the CPE-positive Vero cell supernatants by one-step RT-PCR using specific primers targeting NP, F, M and P protein gene of MRuV5, and L1 and L3 gene of MRV. The PCR products were confirmed by nucleotide sequencing using Sanger's sequencing method. The uninfected cell culture supernatants were negative for MRuV5 and MRV by RT-PCR.

Discussion

In the recent past, the pig has been recognised as an important reservoir host for numerous emerging viruses that can cross species barriers to infect multiple mammalian species including humans. In this study, we have co-isolated the MRuV5 and MRV3 form domestic pigs incidentally during a surveillance study for PED and TGE. MRuV5 and MRV3 were characterized based on genetic analysis of their whole genome. Concurrently, the MRuV5 and MRV3 were remarkably cytopathogenic in Vero cells, showing CPE within 48 hpi. However, their individual cytopathogenicity could not be assessed due to the presence of both the viruses in same samples. Similarly, the haemagglutination activity of MRuV5 and MRV3 infected Vero cell supernatants with swine RBCs could not be evaluated individually.

Previous reports have shown isolation of MRuV5 strains from the respiratory system of several mammalian species [1, 5-6, 28] and there are only a few reports on MRuV5 isolation from intestinal tissues or faecal samples [7]. Isolation of a cytopathogenic MRuV5 strain (SER) from a pig concurrently infected with porcine reproductive and respiratory syndrome virus in Germany has been reported [29]. The MRuV5 strains IND/MZ/3013789 and IND/MZ/3013814 isolated in the current study showed high genetic homology with several MRuV5 strains reported from China and South Korea. They revealed no change in predicted amino acid residues at the receptor binding site in HN protein, cleavage site or HN stalk region. Host specificity related amino acid residues I22, A49, R57, T254, N318, K460 and M536 observed in the HN protein of Indian isolates were unlike to the amino acid residues (L22, S49, G57, A254, S318, T460 and T536) of MRuV5 strains of human. Similarly, variation was also recorded in the fusion protein, which had amino acid residues T3, S19, T438, L498, S530 and R536 as against I3, G19, S438, F498, Q530 and Q536 reported in human strains of MRuV5. Thus, the results show that the present MRuV5 strains of porcine origin are different from MRuV5 strains of human [30].

Besides this, MRV strains have been isolated from cases of enteric infections and diarrhoea in pigs [15, 17, 19]. Moreover, MRV3 strains have been described to be mildly pathogenic to piglets, without causing diarrhoea and vomiting [19]. Furthermore, MRV3 has also been associated with respiratory signs in pigs, indicating its extraintestinal pathogenic potential [15, 19].

Establishment of reovirus infection in intestine depends on proteolysis of outer-capsid proteins to yield infectious subvirion particles (ISVPs). Amino acid residue Threonine at position 249 determines susceptibility of σ1 protein to proteolytic cleavage [31]. The presence of isoleucine (I) at position 249 in σ1 protein of the Indian MRV3 isolates indicates its resistance to cleavage by trypsin. S1 gene amino acid sequence NLAIRLP (positions 198-204), which attribute to the sialic acid binding site, was present in both the MRV3 isolates in the present study [32]. Moreover, two amino acids residues (340D and 419E) corresponding to σ1 protein of MRV3 in the present study have been reported to be associated with the neurotropism of orthoreoviruses [33]. MRV type-3 strains with protease resistant σ1 proteins are able to migrate and replicate in other organ systems including the central nervous system [31]. Thus, the presence of isoleucine (I) instead of Threonine at position 249 along with 340D and 419E in σ1 protein indicates that the Indian MRV strains may have potentially neurotropic features similar to other strains of the virus reported earlier [32, 34].

All the segments of MRV3 strains IND/MZ/3013789/reo and IND/MZ/3013814/reo have the highest nucleotide homology with the strains/ serotypes of the virus isolated from pigs and bats in China, human in Japan indicating circulation of MRVs of common genetic signature in Asian countries. Nucleotide sequences of all the segments except S1 showed identity with the corresponding segment sequences of any of the three MRV serotypes, which reveals that these segments are more conserved in MRVs across serotypes. Reoviruses possess segmented genome that can undergo genetic reassortment derived from intra- and interspecies transmission, and attain genetic diversity [35]. Genome segments recombination (reassortment) among different MRV serotypes play an important role in the emergence of new variants of MRV, and such genomic recombination between MRV strains have been reported earlier [18-19].

The present study represents co-isolation and whole-genomic characterization of MRuV5 and MRV3 from domestic pigs for the first time in India, substantiating the pigs as animal reservoir host. However, the origin and prevalence of these viruses, or their pathogenic role in swine, was not elucidated. Nevertheless, mild subclinical symptoms or sporadic severe infections caused by these viruses cannot be ruled out, because the data on thorough and comprehensive observations of backyard pigs was not available to correlate. Faecal carriage of MRuV5 and MRV3 reiterates the role of environmental contamination in the spread of these viruses possibly through fomites. In addition to this, close human-animal interactions are known to be the risk factors for emerging viruses. Since there are no species barriers in transmission of MRuV5 and MRV3; they may spread from animals to humans and vice versa, facilitating emergence of novel virus strains across different geographical areas.

In conclusion, a brief knowledge on the circulation of MRuV5 and MRV3 in pigs has been gained for the first time in India. The present study attracts attention for the widespread scope of systematic studies on epidemiology, genetic evolution, host adaptability and pathogenic potential of these viruses in mammalian reservoirs, or their possible spread or transmission to domestic animals and humans.

Declarations

Statements and Declarations

Funding

The present research work was carried out under the institutional project entitled “Diagnostic preparedness for porcine epidemic diarrhoea and transmissible gastroenteritis in pigs” (Project code IXX 13779) funded by Indian Council of Agricultural Research, New Delhi through institutional research contingency.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Samples were collected by Fateh Singh, Katherukamem Rajukumar, Dhanapal Senthilkumar and Govindarajulu Venkatesh. Material preparation, laboratory tests and analysis were performed by Fateh Singh, Katherukamem Rajukumar, Dhanapal Senthilkumar, Deepali Srivastava, Subbiah Kombiah and Sandeep Kumar Jhade. The first draft of the manuscript was written by Fateh Singh and Katherukamem Rajukumar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Research involving human participants and/or animals: This study did not involve any human participants or animals.

Acknowledgements

We thank the staff of the Department of Animal Husbandry and Veterinary of the government of Mizoram state who helped in collection of blood and faecal samples from pigs.

References

[1] Lee YN, Lee C (2013) Complete genome sequence of a novel porcine parainfluenza virus 5 isolate in Korea. Ach Virol 158:1765–1772. https:// doi: 10.1007/s00705-013-1770-z

[2] Terrier O, Rolland JP, Rosa-Calatrava M, Lina B, Thomas D, Moules V (2009) Parainfluenza virus type 5 (PIV-5) morphology revealed by cryo-electron microscopy. Virus Res 142:200–203. https:// doi: 10.1016/j.virusres.2008.12.017.

[3] Henrickson KJ (2003) Parainfluenza viruses. Clin Microbiol Rev 16:242–264. https:// doi: 10.1128/CMR.16.2.242-264.2003

[4] Chatziandreou N, Stock N, Young D, Andrejeva J, Hagmaier K, McGeoch DJ, Randall RE (2004) Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5). J Gen Virol 85:3007–3016. https:// doi: 10.1099/vir.0.80200-0

[5] Liu Y, Li N, Zhang S, Zhang F, Lian H, Hu R (2015a) Parainfluenza virus 5 as possible cause of severe respiratory disease in calves, China. Emerg Infect Dis 21:2242–2244. https:// doi: 10.3201/eid2112.141111

[6] Zhai JQ, Zhai SL, Lin T, Liu JK, Wang HX, Li B, Zhang H, Zou SZ, Zhou X, Wu MF, Chen W, Luo ML (2017) First complete genome sequence of parainfluenza virus 5 isolated from lesser panda. Arch Virol 162:1413–1418.https://doi:10.1007/s00705-017-3245-0

[7] Jiang N, Wang E, Guo D, Wang X, Su M, Kong F, Yuan D, Zhai J, Sun D (2018) Isolation and molecular characterization of parainfluenza virus 5 in diarrhea-affected piglets in China. J Vet Med Sci 80:590–593. https://doi: 10.1292/jvms.17-0581

[8] Shatkin AJ, Sipe JD, Loh P (1968) Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J Virol 2:986–991. https://doi.org/10.1128/JVI.2.10.986-991.1968

[9] Nibert ML, Schiff LA (2001) Reoviruses and their replication. In: Knipe DM, Howley PM (Eds.) Fields Virology. 4th Ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1679–1728.

[10] Fields BN (1982) Molecular basis of Reovirus virulence. Arch Virol 71:95–107. https://doi: 10.1007/BF01314880

[11] Day JM (2009) The diversity of the orthoreoviruses: molecular taxonomy and phylogentic divides. Infect Genet Evol 9:390–400. https://doi: 10.1016/j.meegid.2009.01.011

[12] Tyler KL (2001) Mammalian reoviruses. In: Knipe DM, Howley PM (Eds), Fields Virology. 4th Ed. Lippincott Williams and Wilkins, Philadelphia. pp. 1729–45.

[13] Tyler KL, Barton ES, Ibach ML, Robinson C, Campbell JA, O'Donnell SM, Valyi-Nagy T, Clarke P, Wetzel JD, Dermody TS (2004) Isolation and molecular characterization of a novel type 3 reovirus from a child with meningitis. J Infect Dis 189:1664-1675. https://doi: 10.1086/383129

[14] Steyer A, Gutiérrez-Aguire I, Kolenc M, Koren S, Kutnjak D, Pokorn M, Poljšak-Prijatelj M, Racki N, Ravnikar M, Sagadin M, Fratnik Steyer A, Toplak N (2013) High similarity of novel orthoreovirus detected in a child hospitalized with acute gastroenteritis to mammalian orthoreoviruses found in bats in Europe. J Clin Microbiol 51:3818–3825. https://doi.org/10.1128/JCM.01531-13

[15] Zhang C, Liu L, Wang P, Liu S, Lin W, Hu F, Wu W, Chen W, Cui S (2011) A potentially novel reovirus isolated from swine in northeastern China in 2007. Virus Genes 43:342-349.https://doi:10.1007/s11262-011-0642-4

[16] Kwon HJ, Kim HH, Kim HJ, Park JG, Son KY, Jung J, Lee WS, Cho KO, Park SJ, Kang MI (2012) Detection and molecular characterization of porcine type 3 orthoreoviruses circulating in South Korea. Vet Microbiol 157:456-463. https://doi: 10.1016/j.vetmic.2011.12.032

[17] Thimmasandra Narayanappa A, Sooryanarain H, Deventhiran J, Cao D, Ammayappan Venkatachalam B, Kambiranda DM, LeRoith T, Heffron CL, Lindstrom NM, Hall K, Jobst PM, Sexton C, Meng X, Elankumaran S (2015) A Novel Pathogenic Mammalian Orthoreovirus from Diarrheic Pigs and Swine Blood Meal in the United States. *mBio 6:593*. https://doi:10.1128/mBio.00593-15

[18] Lelli D, Beato MS, Cavicchio L, Lavazza A, Chiapponi C, Leopardi S, Baioni L, Benedictis PD, Moreno A (2016) First Identification of Mammalian Orthoreovirus Type 3 in Diarrheic Pigs in Europe. Virol J 13:139. https://doi.org/10.1186/s12985-016-0593-4

[19] Qin P, Li H, Wang JW, Wang B, Xie RH, Xu H, Zhao LY, Li L, Pan Y, Song Y, Huang YW (2017) Genetic and pathogenic characterization of a novel reassortant mammalian orthoreovirus 3 (MRV3) from a diarrheic piglet and seroepidemiological survey of MRV3 in diarrheic pigs from east China. Vet Microbiol 208:126-136. https://doi: 10.1016/j.vetmic.2017.07.021

[20] Decaro N, Campolo M, Desario C, Ricci D, Camero M, Lorusso E, Elia G, Lavazza A, Martella V, Buonavoglia C (2005) Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. Vet Microbiol 109:19–27. https://doi.org/10.1016/j.vetmic.2005.05.014

[21] Kim O, Choi C, Kim B, Chae C (2000) Detection and differentiation of porcine epidemic diarrhoea virus and transmissible gastroenteritis virus in clinical samples by multiplex RT-PCR. Vet Rec 146:637-640. https://doi.org/10.1128/jcm.30.6.1365-1373.1992

[22] Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, Das BK, Bhan MK (1992) Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol 30:1365–1373. https://doi: 10.1128/jcm.30.6.1365-1373.1992.

[23] Boros A, Pankovics, P, Reuter G (2011) Characterization of a novel porcine enterovirus in domestic pig in Hungary. Infect Genet Evol 11:1096–1102. https://doi: 10.1016/j.meegid.2011.04.003

[24] Krumbholz A, Wurm R, Scheck, O, Birch-Hirschfeld E, Egerer R, Henke A, Wutzler P, Zell R (2003) Detection of porcine teschoviruses and enteroviruses by LightCycler real-time PCR. *J Virol Methods 113*:51-63. https://doi: 10.1016/s0166-0934(03)00227-1

[25] Liu JK, Wei CH, Yang XY, Dai AL, Li XH (2015b) Simultaneous detection and differentiation of porcine circovirus type 2, type 2 porcine reproductive and respiratory syndrome virus, porcine parvovirus and pseudorabies virus in pigs with postweaning multisystemic wasting syndrome (PMWS) by multiplex PCR. Vet Arh 85:511-521.

[26] Leary TP, Erker JC, Chalmers ML, Cruz AT, Wetzel JD, Desai SM, Mushahwar IK, Dermody TS (2002) Detection of mammalian reovirus RNA by using reverse transcription-PCR: sequence diversity within the lambda3-encoding L1 gene. J Clin Microbiol 40 1368-1375. https://doi: 10.1128/JCM.40.4.1368-1375.2002

[27] Ouattara LA, Barin F, Barthez MA, Bonnaud B, Roingeard P, Goudeau A, Castelnau P, Vernet G, Paranhos-Baccalà G, Komurian-Pradel F (2011) Novel human reovirus isolated from children with acute necrotizing encephalopathy. Emerg Infect Dis 17:1436-1444. https://doi: 10.3201/eid1708.101528

[28] Liu C, Li X, Zhang J, Yang L, Li F, Deng J, Tan F, Sun M, Liu Y, Tian K (2017) Isolation and genomic characterization of a canine parainfluenza virus type 5 strain in China. Arch Virol 162:2337-2344. https://doi: 10.1007/s00705-017-3387-0

[29] Heinen E, Herbst W, Schmeer N (1998) Isolation of a cytopathogenic virus from a case of porcine reproductive and respiratory syndrome (PRRS) and its characterization as parainfluenza virus 2. Arch Virol 143:2233–2239. https://doi: 10.1007/s007050050454

[30] Charoenkul K, Nasamran C, Janetanakit T, Chaiyawong S, Bunpapong N, Boonyapisitsopa S, Tangwangvivat R, Amonsin A (2021) Molecular detection and whole genome characterization of Canine Parainfluenza type 5 in Thailand. Sci Rep 11:3866. https://doi:10.1038/s41598-021-83323-9

[31] Chappell JD, Barton ES, Smith TH, Baer GS, Duong DT, Nibert ML, Dermody TS (1998) Cleavage susceptibility of reovirus attachment protein sigma1 during proteolytic disassembly of virions is determined by a sequence polymorphism in the sigma1 neck. J Virol 72:8205–8213. https://doi.org/10.1128/JVI.72.10.8205-8213.1998

[32] Lelli D, Moreno A, Lavazza A, Bresaola M, Canelli E, Boniotti MB, Cordioli P (2013) Identification of Mammalian orthoreovirus type 3 in Italian bats. Zoonoses Public Health. 60:84-92. https://doi: 10.1111/zph.12001

[33] Bassel-Duby R, Spriggs DR, Tyler KL, Fields BN (1986) Identification of attenuating mutations on the reovirus type 3 S1 double-stranded RNA segment with a rapid sequencing technique. J Virol 60:64-67. https://doi: 10.1128/JVI.60.1.64-67.1986

[34] Besozzi M, Lauzi S, Lelli D, Lavazza A, Chiapponi C, Pisoni G, Viganò R, Lanfranchi P, Luzzago C (2019) Host range of mammalian orthoreovirus type 3 widening to alpine chamois. Vet Microbiol 230:72-77. https://doi.org/10.1016/j.vetmic.2019.01.012

[35] McDonald SM, Nelson MI, Turner PE, Patton JT (2016) Reassortment in segmented RNA viruses: mechanisms and outcomes. Nat Rev Microbiol 14:448-460. https://doi: 10.1038/nrmicro.2016.46

Tables

Table 1. Primers used for screening and specific detection of swine viruses

PEDV-porcine epidemic diarrhoea virus, TGEV- transmissible gastroenteritis virus, PRV- porcine rotavirus, PEV- porcine enterovirus, PPV- porcine parvovirus, MRuV5- mammalian orthorubulavirus 5, MRV- mammalian orthoreovirus

Table 2. Comparative analysis of MRuV5 strains IND/MZ/3013789 and IND/MZ/3013814 with reported strains

Virus/ gene	Primers	Amplicon size (bp)	Annealing temp (°C)	Reference
Primers used for screening				
PEDV M gene	5′-GGGCGCCTGTATAGAGTTTA-3′	412	55	[21]
	5′-AGACCACCAAGAATGTGTCC-3′			
TGEV N gene	5′-GATGGCGACCAGATAGAAGT-3′	612	55	[21]
	5′-GCAATAGGGTTGCTTGTACC-3′			
PRV VP4	F-5′-TGGCTTCGCCATTTTATAGACA-3′	876	50	[22]
	R-5′-ATTTCGGACCATTTATAACC-3′			
Enterovirus	F-5′-GTACCYTTGTRCGCCTGTT-3′	536	52	[23]
	R-5′-ATTGTCACCATAAGCAGCCA-3′			
Sapelovirus (PEV8)	F-5′-CTTAAGGTGGTTGTATCCCCTAC-3′	413	52	[24]
	F-5′-TTCAACTGACTATACTAGTTACAGG-3′			
PPV	F-5′-ACACGCATCAAGACTCATAC-3′	531	56	[25]
	R-5′-TCACTGTGTAGTCCTGTTTG-3′			
Primers used for specific detection of MRuV5 and MRV				
MRuV5-NP	F- 5′-ATGTCATCCGTGCTTAAAGC-3′	1530	50	[1]
	R- 5′-CTAGATGTCAAGATCACCCA-3′			
MRuV5-P	F- 5′-ATGGATCCCACTGATCTGAG-3′	1177	50	[1]
	R- 5′-TCAAATTGCACTGCGGATGA-3′			
MRuV5-FP	F1- 5′-AAATCATATTAAGACTAT-3′	1749	50	[7]
	R1- 5′-GTCCTATCGTTTTTTTCTTA-3′			
MRuV5-M	F- 5′-ATGCCATCCATCAGCATCCC-3′	1134	50	[1]
	R- 5′-TCATTCCAGCTCCGTCAGGT-3′			
MRV L1	F1-5′-GCATCCATTGTAAATGACGAGTCTG-3′	416	50	[26]
	R1-5′-CTTGAGATTAGCTCTAGCATCTTCTG-3′			
MRV L1	F2 5′-GCTAGGCCGATATCGGGAATGCAG-3′	344	50	[26]
	R2 5′-GTCTCACTATTCACCTTACCAGCAG-3′			
MRV L3	F1 5′-CAGGATGAAGCGGATTCCAA-3′	696	50	[27]
	R1 5′-GGATGATTCTGCCATGAGCT-3′			

Gene	MRuV5 strain	GenBank accession number	Nucleotide identity	Amino acid identity	Host	Country
NP	PIV5-GD18	MG921602.1	99.74	99.61	Pangolin	China
	SH/2015/1202	MK028670.1	99.67	99.41	Pig	China
	M32	MK423238.1	98.37	97.45	Pig	South Korea
	T263	MK423233.1	97.71	97.25	Pig	South Korea
	T434	MK423237.1	96.41	97.45	Pig	South Korea
	RUS/Moskva/2015	MK593539.1	87.52	93.91	Vero cells*	Russia
V	PIV5-GD18	MG921602.1	99.85	99.55	Pangolin	China
	CU-D399	MT604025.1	99.7	99.55	Dog	Thailand
	Rigel	MF170889.1	98.51	97.75	Pig	South Korea
	CH19-MMH	MN735204.1	97.91	98.65	Cattle	Switzerland
	T434	MK423237.1	96.86	96.4	Pig	South Korea
	RUS/Moskva/2015	MK593539.1	87.89	96.4	Vero cells*	Russia
M	1168-1	KC237064.1	99.82	99.73	Canine	South Korea
	ZJQ-221	KX100034.1	99.65	99.47	Lesser Panda	China
	SH/2015/1202	MK028670.1	99.56	99.2	Pig	China
	XJ033	MN604146.1	97.27	97.35	Horse	China
	T434	MK423237.1	96.12	97.08	Pig	South Korea

	RUS/Moskva/2015	MK593539.1	86.95	95.38	Vero cells*	Russia
F	1168-1	KC237064.1	99.76	99.46	Canine	South Korea
	CU-D399	MT604022.1	99.7	99.64	Dog	Thailand
	SH/2015/1202	MK028670.1	99.64	99.09	Pig	China
	T263	MK423233.1	97.52	97.28	Pig	South Korea
	T434	MK423237.1	95.89	95.28	Pig	South Korea
	RUS/Moskva/2015	MK593539.1	85.27	94.01	Vero cells*	Russia
SH	SH/2015/1202	MK028670.1	100	100	Pig	China
	PIV5-GD18	MG921602.1	100	100	Pangolin	China
	T263	MK423233.1	97.04	95.45	Pig	South Korea
	CH19-MMH	MN735204.1	92.42	88.64	Cattle	Switzerland
	Rigel	MF170889.1	85.38	72.09	Pig	South Korea
	M32	MK423238.1	84.62	72.09	Pig	South Korea
HN	CU-D399	MT604023.1	99.59	99.29	Dog	Thailand
	PIV5-GD18	MG921602.1	99.29	98.58	Pangolin	China
	M32	MK423238.1	98.65	97.35	Pig	South Korea
	CH19-MMH	MN735204.1	97.59	97.35	Cattle	Switzerland
	T434	MK423237.1	97.35	97.17	Pig	South Korea

	RUS/Moskva/2015	MK593539.1	85.51	92.57	Vero cells*	Russia
L	1168-1	KC237064.1	99.87	99.82	Canine	South Korea
	PIV5-GD18	MG921602.1	99.85	99.82	Pangolin	China
	HMZ	MH370862.1	99.82	99.73	Tiger	China
	XJ033	MN604146.1	98.07	98.4	Horse	China
	T434	MK423237.1	97.83	98.4	Pig	South Korea
	RUS/Moskva/2015	MK593539.1	88.15	97.64	Vero cells*	Russia

*Vero cells exhibited cytopathic effect and hypothesized to be contaminated by a scientist with respiratory symptoms

Table 3. Comparison of MRV strains IND/MZ/3013789/reo and IND/MZ/3013814/reo with reported strains

Gene	MRV strain	Serotype	GenBank accession number	Nucleotide identity	Amino acid identity	Host	Country
L1	WIV8	1	KT444562.1	98.29	99.17	Bats	China
	ZJ2013	3	KY419120.1	95.87	98.82	Pig	China
	SI-MRV02	3	MG457078.1	90.9	98.23	Bats	Slovenia
	BYD1	2	DQ664184.1	88.94	98.1	Human	China
	SI-MRV03	1	MG457088.1	81.52	95.53	Bats	Slovenia
L2	WIV7	3	KT444553.1	97.38	99.32	Bats	China
	HB-A	1	KC462150.1	93.88	99.1	Mink	China
	BYD1	2	DQ664185.1	81.12	96.2	Human	China
	SI-MRV02	3	MG457079.1	76.7	92.84	Bats	Slovenia
	SI-MRV03	1	MG457089.1	74.77	91.4	Bats	Slovenia
L3	Osaka1994	2	LC476897.1	97.13	99.29	Human	Japan
	THK0617	1	LC613221.1	96.55	99.41	Wastewater	Japan
	WIV7	3	KT444554.1	94.91	99.17	Bats	China
	BYD1	2	DQ664186.1	83.58	97.3	Human	China
	SI-MRV03	1	MG457090.1	79.85	96.13	Bats	Slovenia
M1	ZJ2013	3	KY419123.1	96.92	97.33	Pig	China
	HB-A	1	KC462152.1	95.21	95.3	Mink	China
	BYD1	2	DQ664187.1	89.15	92.06	Human	China

	SI-MRV02	3	MG457081.1	86.89	93.48	Bats	Slovenia
	SI-MRV03	1	MG457091.1	79.82	87.88	Bats	Slovenia
M2	Osaka1994	2	LC476899.1	97.6	99.16	Human	Japan
	HB-A	1	KC462153.1	95.02	99.17	Mink	China
	ZJ2013	3	KY419124.1	94.69	99.16	Pig	China
	BYD1	2	DQ664188.1	89.05	98.12	Human	China
	96	2	LC533918.1	83.38	96.67	Pig	Zambia
M3	B/03	1	KX263312.1	96.35	98.09	Fruit bat	China
	ZJ2013	3	KY419125.1	95.61	97.88	Pig	China
	SI-MRV02	3	MG457083.1	90.35	96.62	Bats	Slovenia
	BYD1	2	DQ664189.1	83.94	91.39	Human	China
	MRV2/Swine/Italy/90178-3/2018_M3	2	MT151669.1	82.2	90.4	Pig	Italy
S1	ZJ2013	3	KY419126.1	97.73	95.16	Pig	China
	KPR146	3	JF829216.1	94.89	93.27	Pig	South Korea
	WIV7	3	KT444558.1	92.99	89.1	Bats	China
	SI-MRV02	3	MG457084.1	80.22	83.55	Bats	Slovenia
	BM-100	3	KM820750.1	78.41	78.34	Pig	USA
S2	WIV7	3	KT444559.1	98.73	97.84	Bats	China
	Osaka2005	2	LC476912.1	98.57	98.2	Human	Japan
	BM-100	3	KM820751.1	91.96	96.43	Pig	USA
	BYD1	2	DQ664190.1	84.73	96.11	Human	China
	SI-MRV03	1	MG457095.1	80.40	93.62	Bats	Slovenia

S3	Osaka2014	2	LC476923.1	97.09	98.81	Human	Japan
	HB-A	1	KC462157.1	95.73	96.44	Mink	China
	SI-MRV02	3	MG457086.1	89.46	97.24	Bats	Slovenia
	BYD1	2	DQ664191.1	82.53	92.58	Human	China
	SI-MRV08	2	MT518192.1	74.67	79.15	Human	Slovenia
S4	ZJ2013	3	KY419129.1	97.63	98.84	Pig	China
	HB-A	1	KC462158.1	96.81	98.08	Mink	China
	Osaka1994	2	LC476904.1	93.99	97.68	Human	Japan
	BYD1	2	DQ318037.2	85.06	94.59	Human	China
	MRV3/Pig/4476/USA/2014	3	MN233067.1	76.47	83.72	Pig	USA

Figures

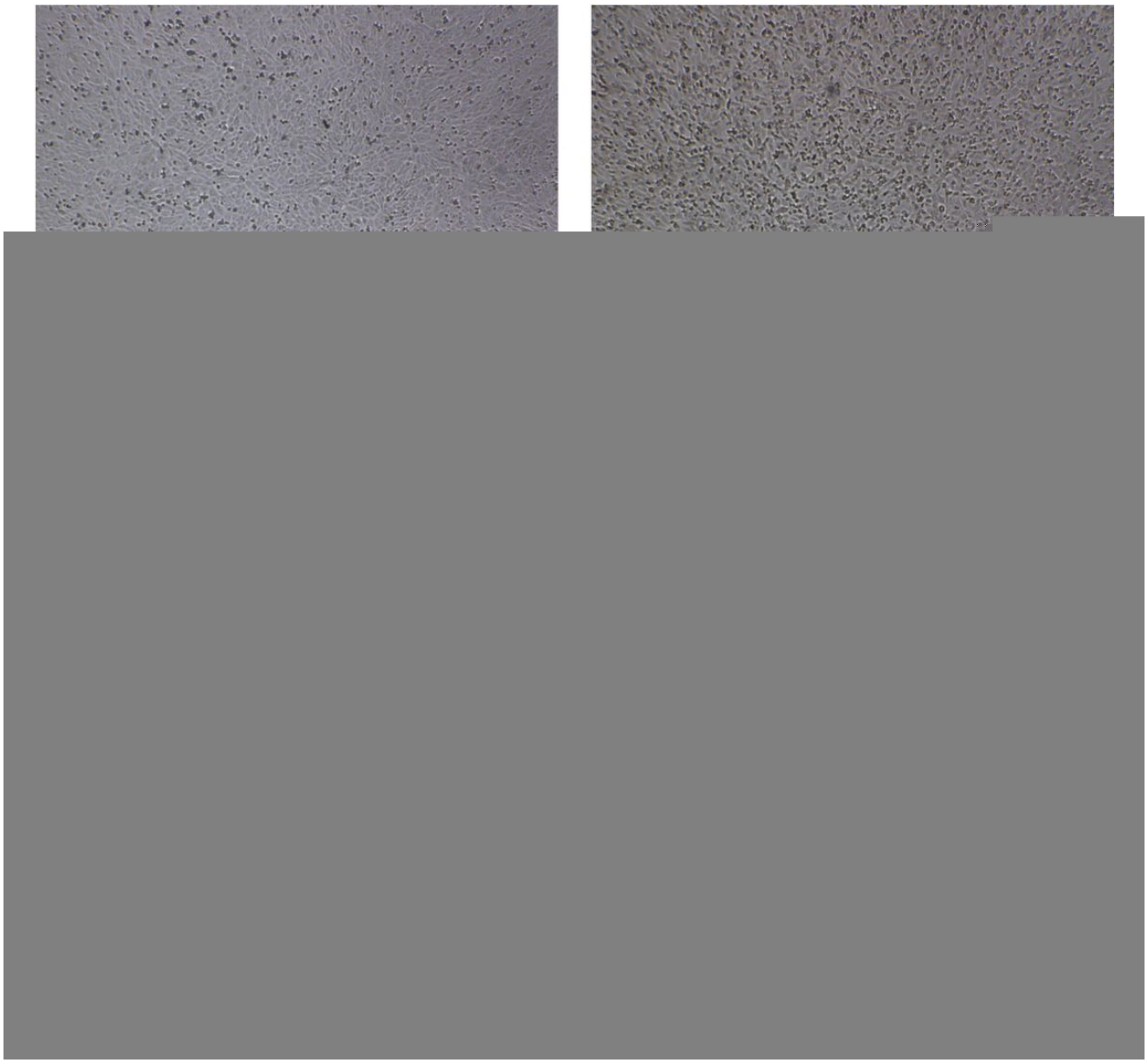

Figure 1

MRuV5 and MRV3 strains showing cytopathic effect (CPE) in Vero cells. a- Uninoculated cells, b- rounding and clumping of cells (48 hpi), c- moderate CPE with cell detachment (72 hpi), d-severe CPE with more cell detachment (96 hpi).

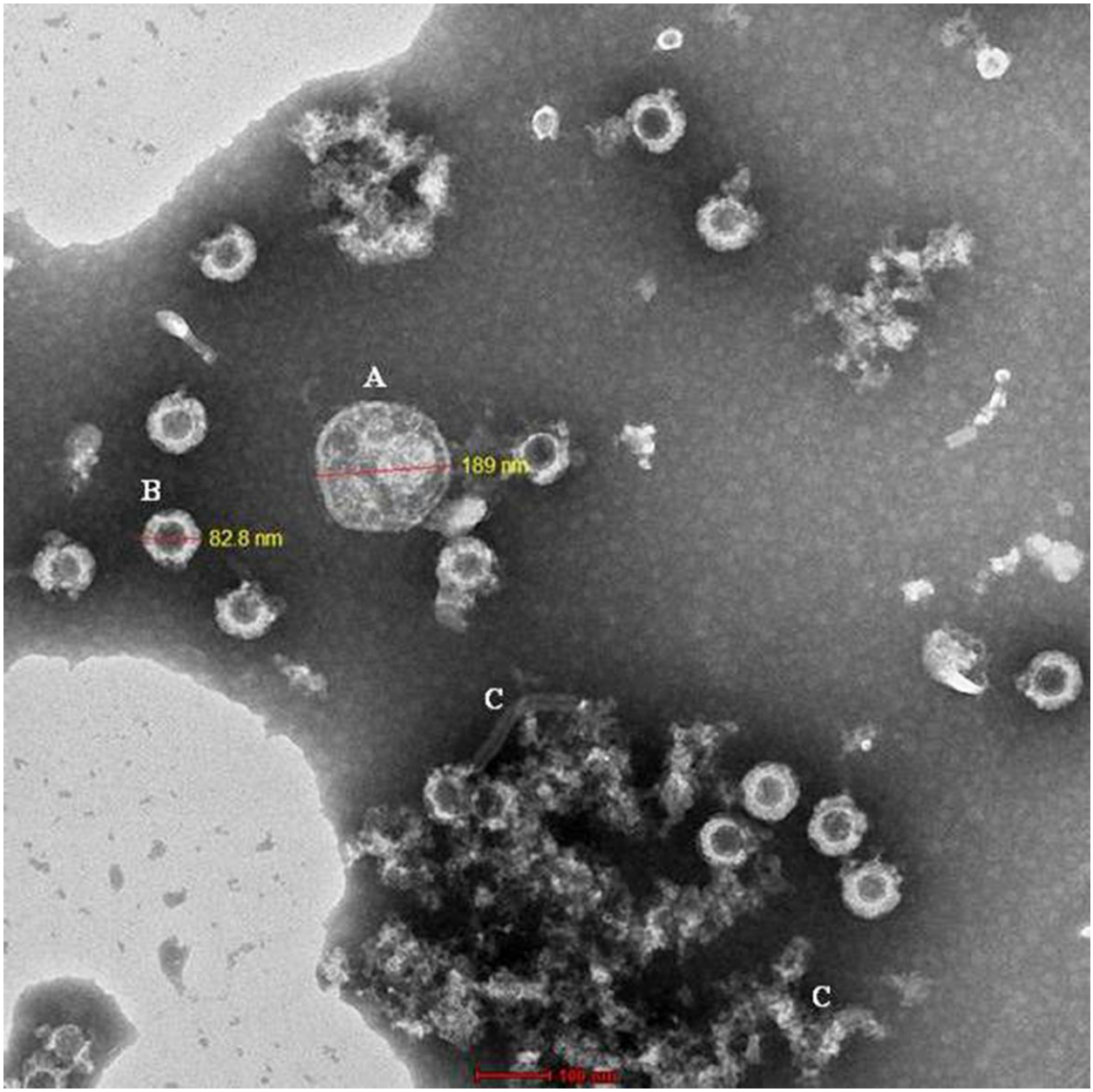

Figure 2

Transmission electron microcopy of the supernatants of the infected Vero cells using negative staining with 2% sodium phosphotungstate. The micrograph is showing round enveloped paramyxovirus (MRuV5) virion (A), icosahedral, nonenveloped reovirus virion (MRV3) (B) and ultrastructure (nucleocapsid core) of MRuV5 (C). (Scale bar=100 nm).

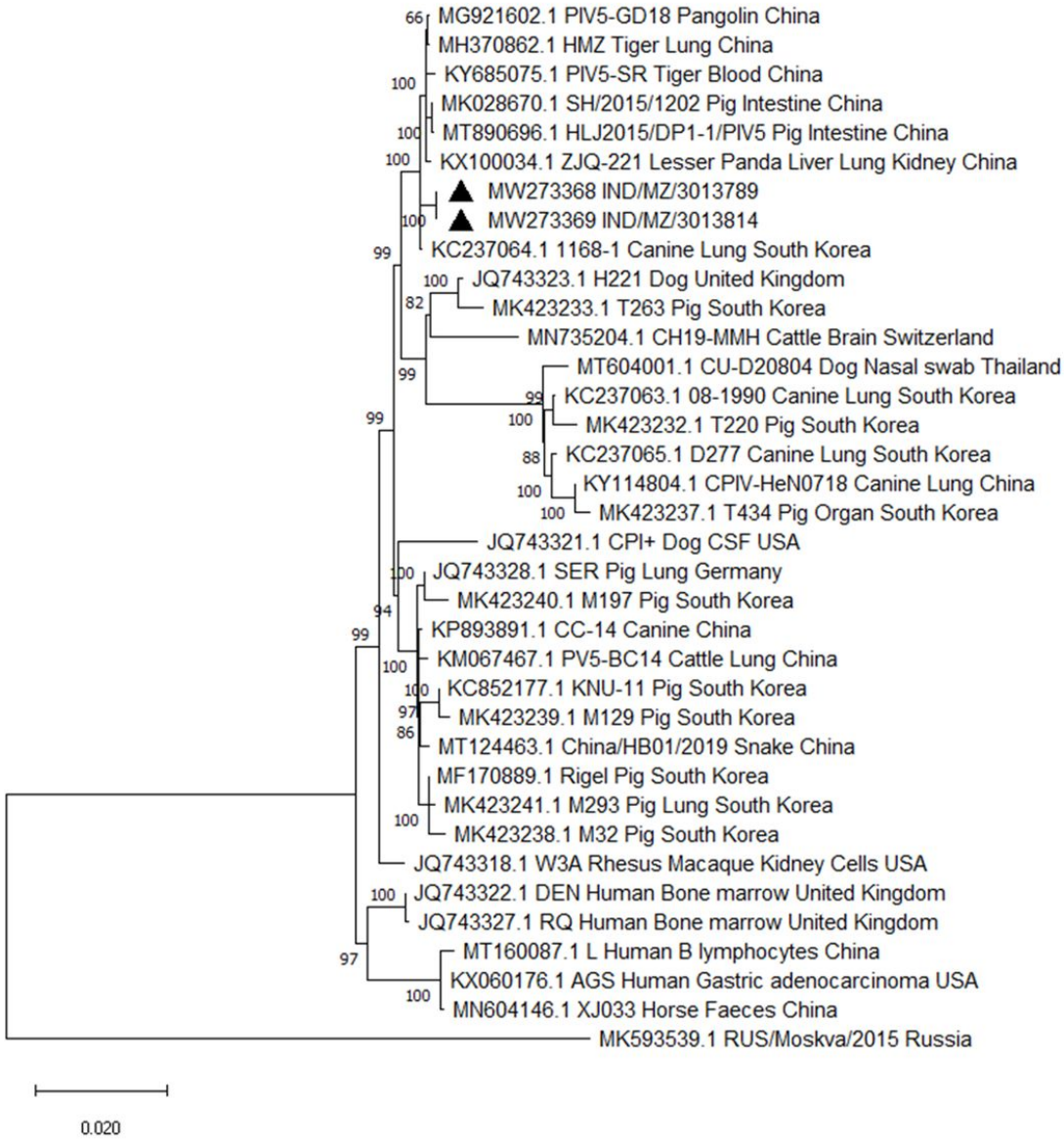

Figure 3

Phylogenetic analysis of MRuV5 based on the whole genome sequence. Phylogenetic tree was constructed by MEGA X software, using the Construct/Test Neighbor-Joining method, Tamura-Nei model and bootstrap with 1000 replications. Bootstrap values >60% are shown. The dark triangles represent isolates of the present study.

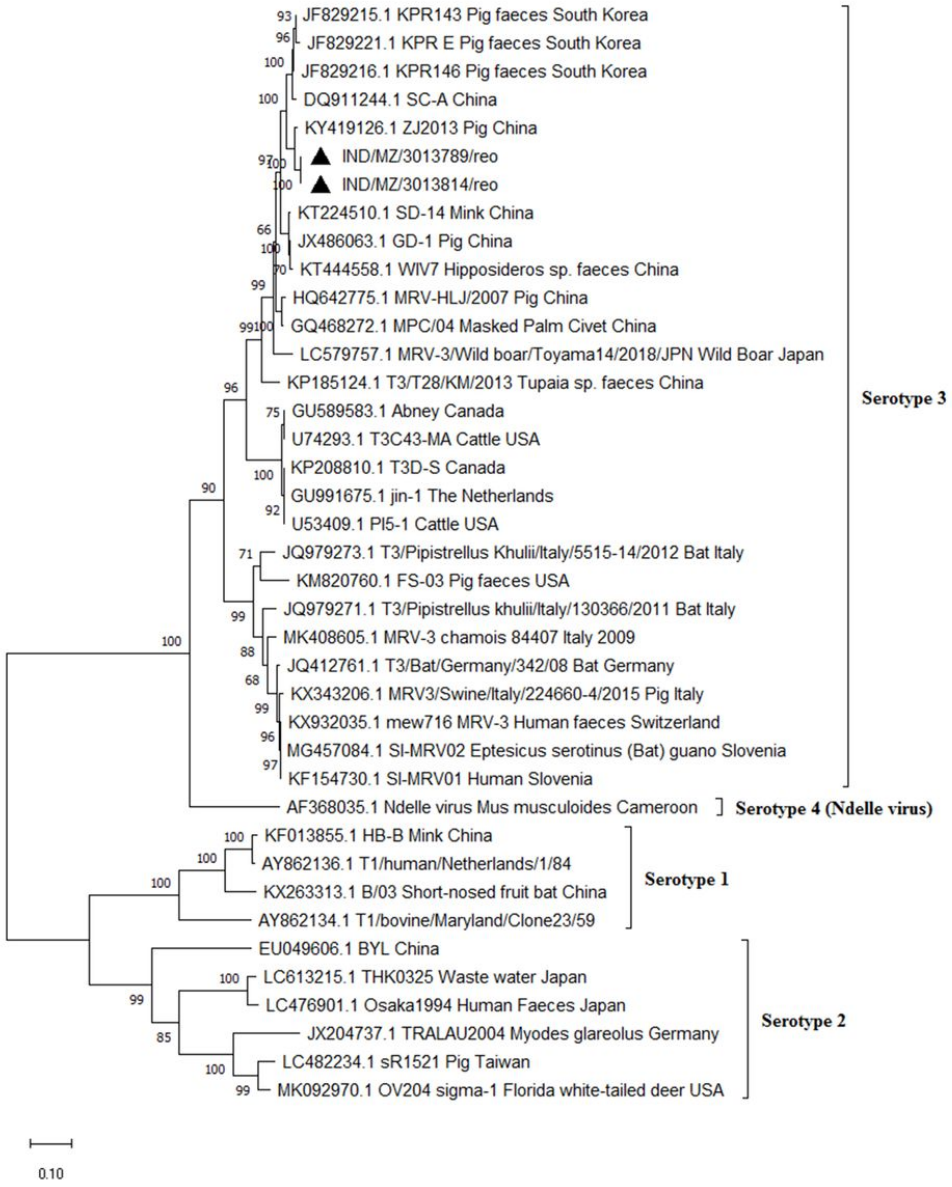

Figure 4

Phylogenetic analysis of MRV3 S1 gene. Phylogenetic tree was constructed by MEGA X software, using the Construct/Test Maximum-Likelihood method, Tamura-Nei model and bootstrap with 1000 replications. Bootstrap values >60% are shown. The dark triangles represent isolates of the present study.

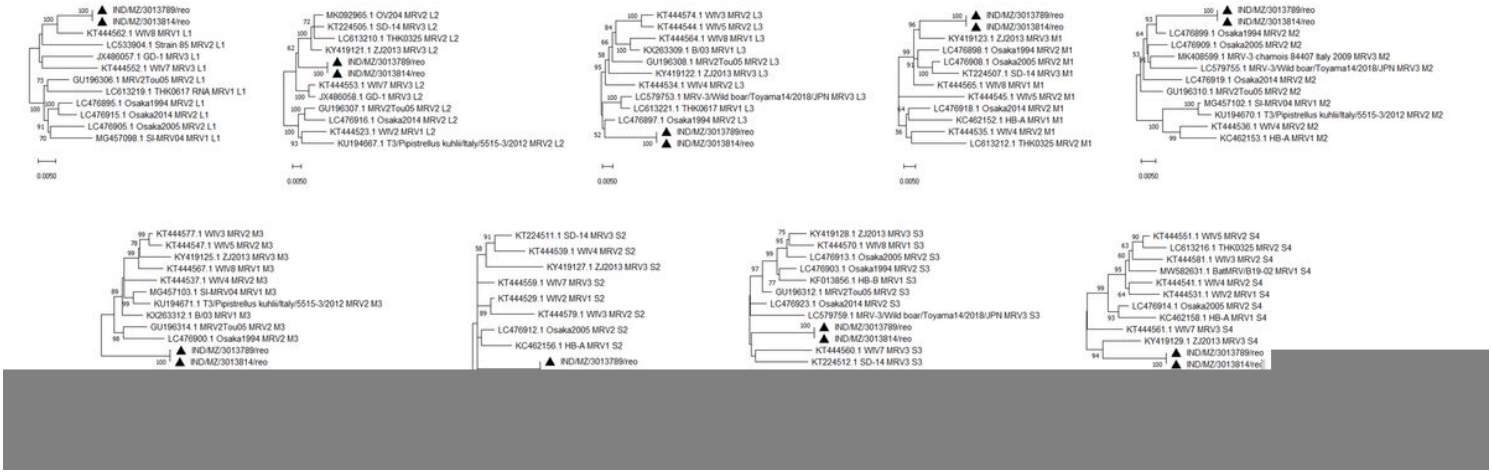

Figure 5

Phylogenetic analyses of different genes of MRV3. a (L1), b (L2), c (L3), d (M1), e (M2), f (M3), g (S2), h (S3) and i (S4). Phylogenetic trees were constructed by MEGA X software, using the Construct/Test Maximum-Likelihood method, Tamura-Nei model and bootstrap with 1000 replications. The dark triangles represent isolates of the present study.

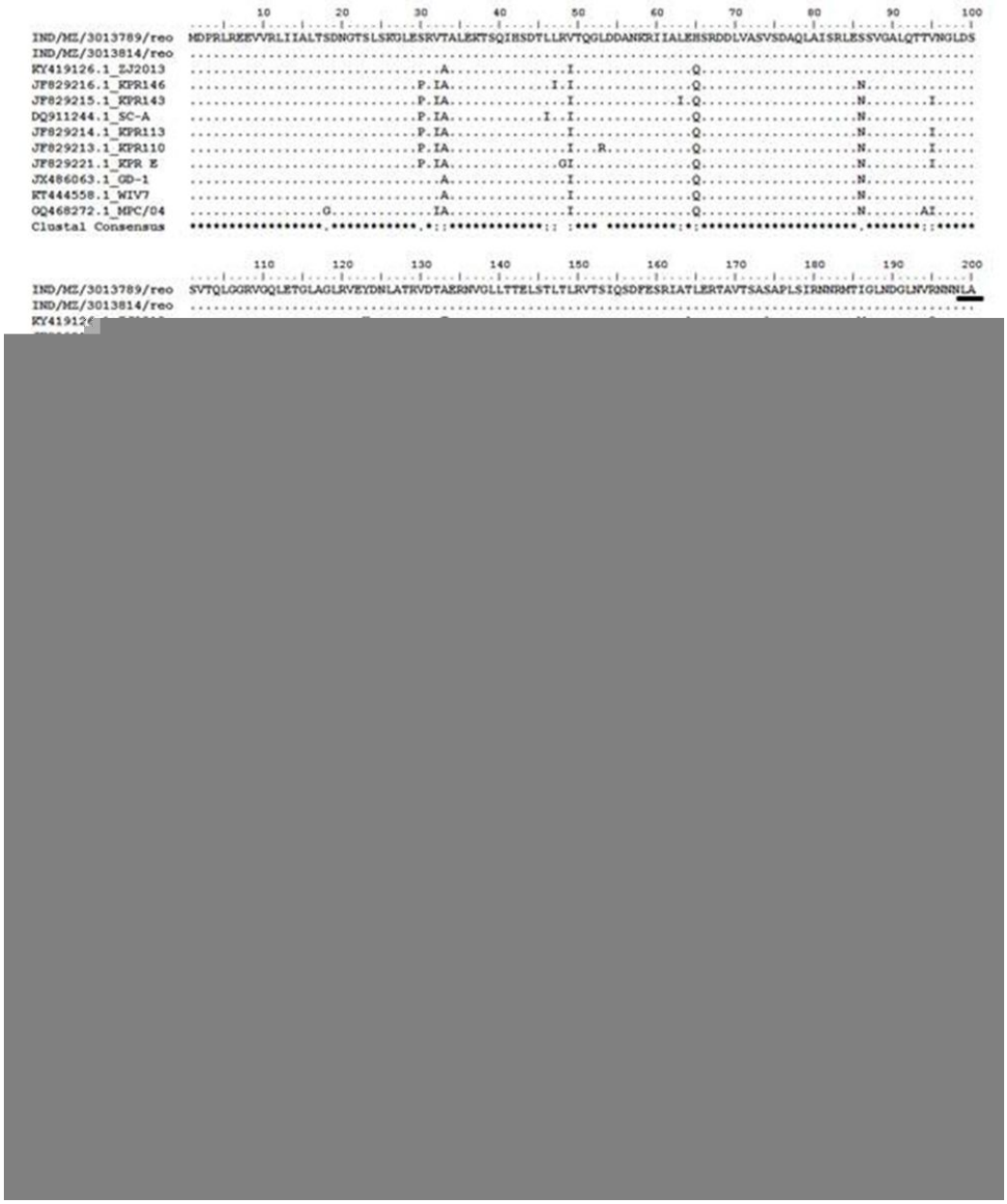

Figure 6

Alignment and comparison of deduced amino acid sequences of the σ 1 protein of the IND/MZ/3013789/reo and IND/MZ/3013814/reo strains of MRV3 with Asian MRV-3 strains. The sequences predicted for sialic acid-binding site are underlined; the sequence responsible for resistance to cleavage by intestinal proteases is arrowed downward; the sequences predicted for neurotropism are arrowed upward.