# Epidemiology and genetic characteristics of murine kobuvirus from fecal samples of Rattus losea, Rattus tanezumi and Rattus norvegicus in Southern China

Minyi Zhang[1], Fang-Fei You[1], Fei Wu[1], Huan He[1], Qiushuang Li[1], and Qing Chen[1]

[1]Southern Medical University

March 30, 2022

**Abstract**

Background: Most recently, murine kobuvirus (MuKV), a novel recognized member of the family Picornaviridae, has been initially identified from fecal samples of Rattus norvegicus in China. The circulation of MuKV from other murine rodent species is not fully understood, prompted us to investigate the prevalence and genetic characterization of MuKV in Rattus losea, Rattus tanezumi and Rattus norvegicus in Southern China. Results: Between 2015 and 2017, a total of 243 fecal samples from three murine rodent species were screened for the presence of murine kobuvirus (MuKV) from three cities in Southern China, with an overall prevalence of 23.0% (56/243). Phylogenetic analysis based on the complete VP1 gene suggested our sequences were genetically closely related to each other and the Chinese strains. Three complete polyprotein nucleotide sequences of MuKV and the genome organization were acquired in the present study. Phylogenetic analyses also showed our sequences belong to the members of genus Kobuvirus within the genotype of Achivirus A. Conclusion: The present study indicated that MuKV is very common in murine rodent populations.

## Introduction

Picornaviruses (family *Picornaviridae* ) are small, non-enveloped RNA viruses with single-stranded and positive-sense polarity. As of March 2020, 147 species divided into 63 genera have been known currently in the family *Picornaviridae*(*https://www.picornaviridae.com/*), many of which had been found to result in significant diseases in humans and a wide variety of animals. The genus *Kobuvirus* is a novel recognized member of the family *Picornaviridae* , which contains a genomic organization similar to that of other picornaviruses and has a linear genome ranging from 8.2 to 8.4 kb in length. The genome structure of *Kobuvirus* is composed of VPg, 5' $UTR^{IRES\text{-}V}$, a large open reading frame (ORF) encoding a leader protein (L) and other three functional regions (P1-P2-P3), 3' UTR and poly (A) tail. The P1 gene region encodes the structural proteins (VP0-VP3-VP1), whereas P2 ($2A^{H\text{-}Box/NC}$-2B-2C) and P3 (3A-$3B^{VPg}$-$3C^{pro}$-$3D^{pol}$) gene regions encode non-structural proteins (Khamrin, Maneekarn, Okitsu, & Ushijima, 2014). Of these, the 3D region as the most conserved region plays an essential role in viral replication (Lescar & Canard, 2009), whereas the VP1 gene is the highly variable motif encoding the most important viral capsid protein that determines the pathogenicity and antigenicity for kobuvirus (Reuter, Boros, & Pankovics, 2011).

Since the determination of the first human kobuvirus (Achi virus, AiV) in a patient with nonbacterial diarrhea in March 1989, Japan (Yamashita, Sakae, Ishihara, Isomura, & Utagawa, 1993), the novel kobuvirus strains have been determined as members of the genus *Kobuvirus* from a variety of animal species. In 2003, bovine kobuvirus (BKV) as a cytopathic agent was first recognized in the culture medium of HeLa cells that had been used over 30 years in a laboratory in Japan (Yamashita et al., 2003). Porcine kobuvirus (PKoV) was initially discovered in 2008 from the fecal samples of domestic pigs in Hungary (Reuter, Boldizsár, Kiss, & Pankovics, 2008). Subsequently, ovine kobuvirus (OKV) that was found to be related to BKV was initially

isolated from young, healthy and domestic sheep in Hungary in 2009 (Reuter, Boros, Pankovics, & Egyed, 2010). In the last ten years,*Kobuviruses* have been described worldwide from a wide range of hosts, including black goats in South Korean (Lee et al., 2012), rabbits in Hungary (Pankovics et al., 2016), dogs in South Korean and Africa (Oem, Choi, Lee, Lee, & Choi, 2014; Olarte-Castillo et al., 2015), cats in South Korean and China (Cho et al., 2014; Niu et al., 2019), ferrets in the Netherlands and Sweden (Smits et al., 2013), rats (You et al., 2020) and bats (Wu et al., 2016) in China, wolves (Melegari et al., 2018), red foxes (Di Martino et al., 2014) and roe deer (Di Martino et al., 2015) in Italy.

According to the International Committee on Taxonomy of Viruses (ICTV),*Kobuviruses* comprise six officially recognized species to date. The species *Aichivirus A* contains Achi virus (found in humans), canine kobuvirus, feline kobuvirus, murine kobuvirus and a kobuvirus found in sewage (Ng et al., 2012), whereas *Aichivirus B* consists of bovine kobuvirus (Yamashita et al., 2003), ferret kobuvirus (Smits et al., 2013) and ovine kobuvirus (Reuter et al., 2010). Regarding*Aichivirus C* , two distinct types have been described, including porcine kobuvirus and caprine kobuvirus (Reuter, Boldizsár, & Pankovics, 2009), while cattle kobuvirus (Otomaru et al., 2016), rabbit (Pankovics et al., 2016) and bat kobu-like viruses represent*Aichivirus D, E* and *F* , respectively.

Murine kobuvirus (MuKV) belongs to a member of the species*Aichivirus A* that initially detected from the stools of a canyon mouse (*Peromyscus crinitus* ) in the USA in 2010 (Phan et al., 2011). Subsequently, MuKVs were identified in Hungary (Reuter et al., 2011), Vietnam (Lu et al., 2018) and USA (Williams et al., 2018) from fecal samples of several rodent species, including striped field mouse (*Apodemus agrarius* ), Norway rat (*Rattus norvegicus* ),*Rattus losea* , *Rattus argentiventer* and *Mus musculus* . We have recently initially conducted an epidemiological study for rat kobuvirus in *Rattus norvegicus* from Guangdong, China (You et al., 2020). However, lack of information on kobuvirus in other murine rodent species in China; here, we investigate the prevalence and genetic characterization of MuKV in *Rattus losea* , *Rattus tanezumi*and *Rattus norvegicus* among several regions in Southern China.

## Materials and methods

### Sample collection

A total of 243 fresh fecal samples were collected between October 2015 and September 2017 from the rats with unknown health status captured close to human residences in three different regions in China, including Xiamen city in Fujian province, Yiyang city in Hunan province and Malipo county in Yunnan province. Individual fresh stool samples were immediately placed in RNase-free tubes with 700μl phosphate-buffered saline (PBS) (0.3% homogenate) and stored at -80 until further use. These stool specimens have also been examined for rat bocavirus (Xiong, Zhou, et al., 2018), porcine bocaviruses (Xiong et al., 2019) and torque teno virus (Xiong, Mo, et al., 2018).

### Nucleic acid extraction and cDNA synthesis

Viral nucleic acid was extracted from 200μl of each stool supernatant using the MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa, Japan), and the obtained nucleic acid was then reverse-transcribed to synthesize cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) in accordance with the manufacturer's instructions. The cDNA was used as the template directly for polymerase chain reaction (PCR) or kept frozen at -20.

### Detection for kobuvirus and other emerging diarrhea viruses

The presence of MuKV was detected by PCR using universal primers according to the previous description, UNIV-kobu-F/UNIV-kobu-R, targeting a 217-bp fragment of partial 3D region for all kobuvirus strains (Reuter et al., 2009). Two sets of primers for a longer 3D gene (461bp) and complete VP1 gene (831bp) of MuKV were also used in order to examine the nucleotide sequences among the 217-bp 3D gene-positive samples, in line with the previous research (You et al., 2020). Each PCR mixture was conducted in a total volume of 25μl, including 12.5μl Green Master Mix (Promega, USA), 8.5μl sterilized $H_2O$, 2μl cDNA template and 1.0μM each of the primers. The mixtures were amplified by 95 for 2min, 40 cycles of 95 for 30s, 56 for

1min, 72 for 1min, with a final elongation step at 72 for 5min. The amplified products were analyzed by 1.0% agarose gels electrophoresis and further sequenced using ABI Prism 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Moreover, kobuviruses have found to be related to gastroenteritis and considered as one of the emerging diarrhea viruses in recent years, containing *Bocavirus, Salivirus, Cosavirus, Bufavirus, Parechovirus* and *Tusavirus* (Finkbeiner et al., 2008); we thereby screened for other emerging diarrhea viruses among these samples according to PCR assays previously reported (Kapoor et al., 2010; Kapoor et al., 2008; Phan et al., 2014; Shan et al., 2010; Yang et al., 2016).

### Genome acquisition of complete polyprotein gene

Three MuKV-positive samples were randomly selected to amplify the full-length polyprotein gene sequences using ten pairs of primers designed in this study on the Benchling website (*https://benchling.com*), referred to the unpublished sequences in the Genbank database (accession number: MF352432.1). The primer sequences were shown in Table 1. After sequencing, there full-length polyprotein genomes were assembled using Lasergene SeqMan software (DNASTAR, Inc. USA, Wisconsin, Madison), and available in Genbank under the accession numbers MW292480-MW292482.

### Genetic and phylogenetic analysis

The nucleotide sequences of MuKV identified in the present study were compared to the corresponding sequences of other kobuvirus strains previously reported in the Genbank database using Basic Local Alignment Search Tool (BLAST;*https://www.ncbi.nlm.nih.gov*). Multiple sequences were aligned with the corresponding regions of kobuvirus reference strains retrieved from Genbank database using the CLUSTAL W program in Molecular Evolutionary Genetics Analysis (MEGA version 7.0, Oxford Molecular Ltd., UK). The pairwise nucleotide (nt) and amino acid (aa) identities among all sequences were calculated using MegAlign program (DNASTAR, Inc. USA, Wisconsin, Madison). The open reading frame (ORF) was predicted for the obtained genome sequences by ORF finder (*https://www.ncbi.nlm.nih.gov/orffinder*). Similarity plot analysis of the full-length polyprotein genome was performed using SimPlot 3.5.1 software. The RNA secondary structure prediction for 5' and 3' UTR was conducted by Mfold program (Zuker, 2003). Phylogenetic analyses were generated by maximum-likelihood method with 1000 bootstrap replicates using MEGA v7.0 and further visualized in FigTree v1.4.0 software. The model selections were based on the results of 'Find best DNA/Protein models' in MEGA v7.0.

## Results

### Detection of MuKV in stool samples

Two hundred and forty three fecal samples of murine rodents from Xiamen (n=96), Malipo (n=48) and Yiyang (n=99) in Southern China were screened for the presence of MuKV by PCR utilizing a universal primer pair targeted to their corresponding 3D regions, with an overall prevalence of 23.0% (56/243). The MuKV was examined at the highest positive rate of 28.1% (41/146) in *Rattus norvegicus* , following by 23.7% (9/38) in *Rattus tanezumi* and 10.2% (6/59) in *Rattus losea* . Regarding other emerging diarrhea viruses, human*Bocavirus* , *Parechovirus A* and *Parechovirus B* were found in the same stool samples, while the co-infection of MuKV with other emerging diarrhea viruses was not observed in the present study.

### Phylogenetic analysis of 3D gene and VP1 gene sequences

Twenty-two representative sequences of partial 3D gene (461nt) were selected randomly for further PCR amplification and sequencing within these positive samples. Sequences isolated from different areas and different species were highly conserved with each other, at the nucleotide level of 92.7%-99.5% and deduced amino acid level of 95%-100%. Maximum-likelihood phylogenetic analysis based on partial 3D regions was generated with representative kobuvirus strains from multiple species, indicating that our sequences belonged to*Achivirus A* and displayed the closet relatedness with MuKVs. Interestingly, only one strain (YY101) determined during this study showed the closest relationship to the Chinese MuKV strain, GZ85, isolated from *Rattus norvegicus* (You et al., 2020), while the other 21 sequences formed a major group with MuKV reference strains identified from USA, China and Hungary (Figure 1).

Meanwhile, seven complete VP1 regions (917nt) were successfully determined in the present study, which share 88.7%-99.3% nucleotide identities and 95.1%-100% amino acid identities with one another. In contrast to other references kobuvirus previously reported in Genbank database, they have the highest sequence similarity with the Chinese MuKV strain, MM33 (accession number MN648600.1), at the nucleotide level of 91.3%-93.9%. A phylogenetic tree has been conducted based on the seven VP1 sequences identified in the current study and the available complete nucleotide sequences for MuKV VP1 from three different countries, indicating that our VP1 sequences clustered along with two MuKV strains, GZ80 and GZ84, previously described in Guangdong, China, while other kobuvirus reference strains from China, Hungary and Vietnam formed another groups (Figure 2). The partial 3D and complete VP1 nucleotide sequences of MuKV determined in the present study have been lodged within the Genbank database under the accession numbers MW292451-MW292472 and MW292473-MW292479 , respectively.

**Genomic and phylogenetic analyses of complete polyprotein genome**

Three complete MuKV polyprotein genome sequences (except the 5' and 3'UTR) were successfully obtained from three MuKV-positive fecal samples using ten primer pairs in the current study, designating MuKV/XM34/CHN (accession no. MW292482), MuKV/XM86/CHN (accession no. MW292481) and MuKV/YN27/CHN (accession no. MW292480). The obtained genome sequence of MuKV/XM34/CHN was 7714nt long and possessed one complete ORF (7293nt) encoding a potential polyprotein of 2430 amino acids, whereas the acquired 7714-nt-long genome strain of MuKV/XM86/CHN and 7711-nt-long genome strain of MuKV/YN27/CHN contained the complete ORF of 7293nt (2430aa) and 7290nt (2429aa) in length, respectively. Interestingly, the lack of one-amino-acid was found in 3A region of the strain MuKV/YN27/CHN when multiple aligned with the genome of MuKV/XM34/CHN and MuKV/XM86/CHN, and they shared 92.5-97.1% nucleotide identities and 97.1-98.1% amino acid identities with each other. The predicted genomes of our study strains have the same genome organization as identified for other kobuviruses, and the detailed information was shown in Figure 3A.

The genomes identified in the present study showed the highest similarity to the currently published MuKV strain GZ85 (MN648601.1) as the closest match determined by BLASTn search, with 93.2-94.5% nucleotide identities. Moreover, the comparison results indicated our study strains were also closely related to the previously described MuKVs. Consequently, we conducted a comparative sequence analysis for each functional region between our study strains and representative kobuviruses available in the Genbank database. The kobuviruses identified in the present study showed amino acid identities with those of rat (96.0-98.3%), human (80.1-80.7%), canine (80.1-80.5%), and feline kobuvirus reference strains (80.1-80.3%) based upon the complete polyprotein gene. For MuKV/XM34/CHN and MuKV/XM86/CHN, the highest nucleotide identities were presented in P2 region of rat kobuvirus GZ85 strain, with values of 94.6% and 94.7%, respectively, while they also shared the highest amino acid identities (98.7%) to the P2 region of rat08/rAiA/HUN. Regarding MuKV/YN27/CHN, the highest nucleotide (95.4%) and amino acid identities (99.8%) were found in P2 and P1 regions of GZ85, respectively (Table 2). Besides, the similarity plot analysis was generated to further analyze the genetic characteristics, contrasting the complete polyprotein nucleotide gene of our study strains and one feline kobuvirus strain WHJ-1/MF598159.1 (used as an out-group sequence) to the rat kobuvirus reference strain GZ85/MN648601.1 (used as a query sequence). The findings suggested the sequences displayed relatively high similarities to the query sequence in the VP0, VP3, 2A-2C, 3C and 3D regions, whereas different similarities were exhibited in the L, VP1, 3A and 3B regions. Additionally, MuKV/YN27/CHN presented considerably higher similarities in the L, 3A, 3B regions and the sequence connecting 2A and 2B than MuKV/XM34/CHN and MuKV/XM86/CHN (Figure 3B).

Table 3 shows the predicted protease-cleavage sites of our study strains and other reference kobuviruses, including Q/G, P/Q, Q/T, Q/S, Y/V and Q/A. The predicted protease-cleavage sites between L and VP0 (Q/G), 2A and 2B (Q/G), 2B and 2C (Q/G), 2C and 3A (Q/G), and 3C and 3D (Q/S) are conserved among kobuviruses from different species. Besides, the protease-cleavage sites between 3A and 3B is Q/A for the kobuvirus strains, except for porcine kobuvirus (Q/G).

Maximum-likelihood phylogenetic analysis was conducted based on the complete polyprotein nucleotide

sequences between our study strains and other representative members of family *Picornaviridae* , suggesting the strains identified in the present study were located at the same branch as the members of genus *Kobuvirus* , where the sequences of MuKV are the closest relatives (Figure 4).

**Discussion**

The circulation of kobuvirus in rats has been reported in Guangdong, China, but only from *Rattus norvegicus* . To our best knowledge, the present study represents the first study to investigate the prevalence of murine kobuvirus isolated from three murine rodent species in southern China, including *Rattus losea* , *Rattus tanezumi*and *Rattus norvegicus* .

The detection results indicated that fifty-six strains of MuKV were identified in fecal samples from these murine rodents, with an approximate detection rate of 23.0% (56/243). Of these, the prevalence of MuKV in *Rattus losea* , *Rattus tanezumi* and *Rattus norvegicus* was 10.2% (6/59), 23.7% (9/38) and 28.1% (41/146), respectively, confirming these three murine rodent species in China are infected with MuKV, as many studies reported that the kobuvirus infection is widely distributed in the world (Milićević et al., 2020). The MuKVs were examined in 50% of*Rattus norvegicus* fecal samples in USA in 2014 and later the MuKV strains were presented in approximately 50% of *Rattus norvegicus*in Hungary (Boros et al., 2019; Firth et al., 2014), while the detection rate of MuKV was 17% in Vietnam (Lu et al., 2018). Thus, our present finding confirmed murine rodent species are hosts of kuboviruses. The detection of kobuvirus is common in murine rodents, even if the different prevalence.

Several previous studies have detected kobuviruses isolated from different animals with diarrhea, suggesting kobuviruses were the causative agent for gastroenteritis (Niu et al., 2019; Wang, Fredrickson, Duncan, Samuelson, & Hsiao, 2020). Simultaneously, it has been described that kobuviruses have a serious impact on systemic infections (Ribeiro et al., 2017). Although the health status of the analyzed live-trapped murine rodents in the present study is currently unknown due to none of observable clinical manifestation, the frequent presence of kobuviruses identified in fecal samples implied a viral affinity to the gastrointestinal tract (Milićević et al., 2020). Furthermore, a previous study suggested that kobuviruses are frequently mixed infected with other pathogens (Jackova et al., 2017). Hence, we simultaneously screened for those as emerging diarrhea viruses, including *Bocavirus, Salivirus, Cosavirus, Bufavirus, Parechovirus* and *Tusavirus,* while no co-infection was found in this investigation. Considering that murine rodents are in close contact with human life, there is still a high risk of human transmissibility of MuKVs increasing human health concerns. Taken together, these give valuable insight into future experimental studies focused on its pathogenesis and the real associations with infectious diseases, especially for gastroenteritis.

Phylogenetic analysis based upon partial 3D gene confirmed our sequences to be members of genus *Kobuvirus* of species *Achivirus A* , clearly branched together with murine, canine and feline kobuviruses and human Achi viruses. The 21 MuKV sequences identified in the current study formed a large group with the American, Chinese and Hungarian MuKV strains, indicating no geographic clustering based on 3D region in line with previously published results (Milićević et al., 2020), whereas only one strain (YY101) showed the closest relationship to the Chinese MuKV strain, GZ85. Moreover, our sequences were more likely to phylogenetically cluster at the same branch according to their host species.

The VP1 capsid protein of picornaviruses is the most immunodominant portion of kobuvirus determining the antigenicity and pathogenicity; it is the most variable structural protein for kobuviruses as well (Chen et al., 2013; Reuter et al., 2011). The phylogenetic analysis based on the complete VP1 nucleotide sequences of the study strains with the species of murine rodent (in italics) shows our study strains clustered closely together with the Chinese strains and separated from other published VP1 sequences of MuKVs from Hungary and Vietnam, indicating the MuKV strains from China may possess a similar evolution background and could be circulating among the different murine rodent populations in China (Y. Wang et al., 2020). This finding presents the geographical location-specific clustering of the selected VP1 strains, while geographic clustering has not been observed in the 3D region. Whether these MuKV sequences from different geography impact protein function still warrants research attention in the future.

The acquirement of complete polyprotein genomes in the present study allowed us to obtain information on the genetic characteristics of MuKV strains circulating in murine rodents in Southern China, which was named MuKV/XM34/CHN (7296nt), MuKV/XM86/CHN (7296nt) and MuKV/YN27/CHN (7293nt). Genomic analysis revealed that the polyprotein of our study strains have a similar genome architecture to other kobuviruses, including L , VP0, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D. Interestingly, one-amino-acid deletion was presented in the deduced 3A viral protein region of MuKV/YN27/CHN, similar to the canine strain, CH-1, and feline strain, WHJ-1. According to the previous studies, one-amino-acid deletion was also found in VP0 region of feline kobuvirus from a diarrhoeic cat (Niu et al., 2019). In contrast, thirty-amino-acid deletion was presented in 2B region of porcine kobuvirus from health piglets that might be associated with the pathogenicity of porcine kobuvirus (Jin et al., 2015). Nevertheless, natural mutation and recombination exist in viruses of the family *Picornaviridae* , playing an important role in genetic diversities (Lukashev, 2010). Whether this amino acid deletion has an impact on the pathogenicity of kobuviruese from different species requires further investigation via structure prediction and genomic analysis. A phylogenetic analysis of*Kobuvirus* and other members of the family *Picornaviridae*demonstrated that our study strains belong to the members of genus*Kobuvirus* .

Furthermore, a phylogenetic analysis based on the complete polyprotein sequences of our study strains and different kobuvirus species (*Achivirus A-F* ) revealed our MuKV strains were more closely related to canine kobuvirus and human Achi virus than to bovine kobuvirus. This result suggests that cross-species transmission of kobuviruses can occur due to frequent contact between rats, dogs, and humans in the natural environment, in accordance with a prior study donmenstrating multiple cross-species transmissions have a possibility to exist within and among mammalian species (Lu et al., 2018). These findings increase further understanding of evolution and genomic characteristics for *Kobuvirus* in murine rodent populations.

Besides, the 49-nt-long partial 3'UTR of MuKVs identified in our study showed the highest sequence identities (98.0%-100%) to the Chinese strain Wencheng-Rt386-2 (accession no. MF352432.1). The predicted RNA secondary structure of the partial 3'UTR also contain the characteristic "barbell-like" structure with conserved motifs of AGGGAAC (Figure 5), which is identical in other partial 3'UTR of MuKV references currently available in the Genbank database. The presence of barbell-like structures was recognizable in the genus of *Kobuvirus* among different nucleotide positions of the 3'UTR gene (Boros et al., 2019; Choi, Lee, Lee, & Oem, 2015). It is important to note that intense studies should figure out the question about the functions of this structure.

In conclusion, we first identify kobuviruses in *Rattus losea* and*Rattus tanezumi* , expanding the host range of kobuviruses. The combined findings of this study provide molecular characteristics of MuKV and show widespread circulation in different murine rodent species. The limitation of the present study is small sample size. Therefore, more detailed analyses including epidemiological and experimental investigations are needed to emphasize the pathogenicity, genetic diversity and potential risk to the public health of murine kobuvirus.

**Ethics Statement**

The research protocol has been approved by the Animal Ethics and Welfare Committee of the School of Public Health, Southern Medical University and met the guidelines for the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China.

**Data available statement**

All data generated or analyzed during this study are included in this published article. Access to raw data can be acquired by connecting to the corresponding author via email.

**Acknowledgments**

We are grateful for the generous support of our teammates in the sample collection and technical assistance. This work was supported by the National Natural Science Foundation of China (Grant No.81973107).

**Conflict of interest statement**

No conflict of interest exits in the submission of this manuscript.

**References**

Boros, Á., Orlovácz, K., Pankovics, P., Szekeres, S., Földvári, G., Fahsbender, E., . . . Reuter, G. (2019). Diverse picornaviruses are prevalent among free-living and laboratory rats (Rattus norvegicus) in Hungary and can cause disseminated infections. *Infect Genet Evol, 75* , 103988. doi:10.1016/j.meegid.2019.103988

Chen, L., Zhu, L., Zhou, Y. C., Xu, Z. W., Guo, W. Z., & Yang, W. Y. (2013). Molecular and phylogenetic analysis of the porcine kobuvirus VP1 region using infected pigs from Sichuan Province, China. *Virol J, 10* , 281. doi:10.1186/1743-422x-10-281

Cho, Y. Y., Lim, S. I., Kim, Y. K., Song, J. Y., Lee, J. B., & An, D. J. (2014). Molecular characterization of the full kobuvirus genome in a cat. *Genome Announc, 2* (2). doi:10.1128/genomeA.00420-14

Choi, J. W., Lee, M. H., Lee, K. K., & Oem, J. K. (2015). Genetic characteristics of the complete feline kobuvirus genome. *Virus Genes, 50* (1), 52-57. doi:10.1007/s11262-014-1144-y

Di Martino, B., Di Profio, F., Melegari, I., Di Felice, E., Robetto, S., Guidetti, C., . . . Marsilio, F. (2015). Molecular detection of kobuviruses in European roe deer (Capreolus capreolus) in Italy.*Arch Virol, 160* (8), 2083-2086. doi:10.1007/s00705-015-2464-5

Di Martino, B., Di Profio, F., Melegari, I., Robetto, S., Di Felice, E., Orusa, R., & Marsilio, F. (2014). Molecular evidence of kobuviruses in free-ranging red foxes (Vulpes vulpes). *Arch Virol, 159* (7), 1803-1806. doi:10.1007/s00705-014-1975-9

Finkbeiner, S. R., Allred, A. F., Tarr, P. I., Klein, E. J., Kirkwood, C. D., & Wang, D. (2008). Metagenomic analysis of human diarrhea: viral detection and discovery. *PLoS Pathog, 4* (2), e1000011. doi:10.1371/journal.ppat.1000011

Firth, C., Bhat, M., Firth, M. A., Williams, S. H., Frye, M. J., Simmonds, P., . . . Lipkin, W. I. (2014). Detection of zoonotic pathogens and characterization of novel viruses carried by commensal Rattus norvegicus in New York City. *mBio, 5* (5), e01933-01914. doi:10.1128/mBio.01933-14

Jackova, A., Sliz, I., Mandelik, R., Salamunova, S., Novotny, J., Kolesarova, M., . . . Vilcek, S. (2017). Porcine kobuvirus 1 in healthy and diarrheic pigs: Genetic detection and characterization of virus and co-infection with rotavirus A. *Infect Genet Evol, 49* , 73-77. doi:10.1016/j.meegid.2017.01.011

Jin, W. J., Yang, Z., Zhao, Z. P., Wang, W. Y., Yang, J., Qin, A. J., & Yang, H. C. (2015). Genetic characterization of porcine kobuvirus variants identified from healthy piglets in China. *Infect Genet Evol, 35* , 89-95. doi:10.1016/j.meegid.2015.07.035

Kapoor, A., Mehta, N., Esper, F., Poljsak-Prijatelj, M., Quan, P. L., Qaisar, N., . . . Lipkin, W. I. (2010). Identification and characterization of a new bocavirus species in gorillas. *PLoS One, 5* (7), e11948. doi:10.1371/journal.pone.0011948

Kapoor, A., Victoria, J., Simmonds, P., Slikas, E., Chieochansin, T., Naeem, A., . . . Delwart, E. (2008). A highly prevalent and genetically diversified Picornaviridae genus in South Asian children. *Proc Natl Acad Sci U S A, 105* (51), 20482-20487. doi:10.1073/pnas.0807979105

Khamrin, P., Maneekarn, N., Okitsu, S., & Ushijima, H. (2014). Epidemiology of human and animal kobuviruses. *Virusdisease, 25* (2), 195-200. doi:10.1007/s13337-014-0200-5

Lee, M. H., Jeoung, H. Y., Lim, J. A., Song, J. Y., Song, D. S., & An, D. J. (2012). Kobuvirus in South Korean black goats. *Virus Genes, 45* (1), 186-189. doi:10.1007/s11262-012-0745-6

Lescar, J., & Canard, B. (2009). RNA-dependent RNA polymerases from flaviviruses and Picornaviridae. *Curr Opin Struct Biol, 19* (6), 759-767. doi:10.1016/j.sbi.2009.10.011

Lu, L., Van Dung, N., Ivens, A., Bogaardt, C., O'Toole, A., Bryant, J. E., . . . Woolhouse, M. E. (2018). Genetic diversity and cross-species transmission of kobuviruses in Vietnam. *Virus Evol, 4* (1), vey002. doi:10.1093/ve/vey002

Lukashev, A. N. (2010). Recombination among picornaviruses. *Rev Med Virol, 20* (5), 327-337. doi:10.1002/rmv.660

Melegari, I., Sarchese, V., Di Profio, F., Robetto, S., Carella, E., Bermudez Sanchez, S., . . . Di Martino, B. (2018). First molecular identification of kobuviruses in wolves (Canis lupus) in Italy.*Arch Virol, 163* (2), 509-513. doi:10.1007/s00705-017-3637-1

Milićević, V., Kureljušić, B., Maksimović-Zorić, J., Savić, B., Spalević, L., & Žutić, J. (2020). Molecular detection and characterization of Porcine Kobuvirus in domestic pigs and wild boars in Serbia. *Res Vet Sci, 132* , 404-406. doi:10.1016/j.rvsc.2020.07.028

Ng, T. F., Marine, R., Wang, C., Simmonds, P., Kapusinszky, B., Bodhidatta, L., . . . Delwart, E. (2012). High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. *J Virol, 86* (22), 12161-12175. doi:10.1128/jvi.00869-12

Niu, T. J., Yi, S. S., Wang, X., Wang, L. H., Guo, B. Y., Zhao, L. Y., . . . Hu, X. G. (2019). Detection and genetic characterization of kobuvirus in cats: The first molecular evidence from Northeast China.*Infect Genet Evol, 68* , 58-67. doi:10.1016/j.meegid.2018.12.010

Oem, J. K., Choi, J. W., Lee, M. H., Lee, K. K., & Choi, K. S. (2014). Canine kobuvirus infections in Korean dogs. *Arch Virol, 159* (10), 2751-2755. doi:10.1007/s00705-014-2136-x

Olarte-Castillo, X. A., Heeger, F., Mazzoni, C. J., Greenwood, A. D., Fyumagwa, R., Moehlman, P. D., . . . East, M. L. (2015). Molecular characterization of canine kobuvirus in wild carnivores and the domestic dog in Africa. *Virology, 477* , 89-97. doi:10.1016/j.virol.2015.01.010

Otomaru, K., Naoi, Y., Haga, K., Omatsu, T., Uto, T., Koizumi, M., . . . Nagai, M. (2016). Detection of novel kobu-like viruses in Japanese black cattle in Japan. *J Vet Med Sci, 78* (2), 321-324. doi:10.1292/jvms.15-0447

Pankovics, P., Boros, Á., Bíró, H., Horváth, K. B., Phan, T. G., Delwart, E., & Reuter, G. (2016). Novel picornavirus in domestic rabbits (Oryctolagus cuniculus var. domestica). *Infect Genet Evol, 37* , 117-122. doi:10.1016/j.meegid.2015.11.012

Phan, T. G., Kapusinszky, B., Wang, C., Rose, R. K., Lipton, H. L., & Delwart, E. L. (2011). The fecal viral flora of wild rodents. *PLoS Pathog, 7* (9), e1002218. doi:10.1371/journal.ppat.1002218

Phan, T. G., Sdiri-Loulizi, K., Aouni, M., Ambert-Balay, K., Pothier, P., Deng, X., & Delwart, E. (2014). New parvovirus in child with unexplained diarrhea, Tunisia. *Emerg Infect Dis, 20* (11), 1911-1913. doi:10.3201/eid2011.140428

Reuter, G., Boldizsár, A., Kiss, I., & Pankovics, P. (2008). Candidate new species of Kobuvirus in porcine hosts. *Emerg Infect Dis, 14* (12), 1968-1970. doi:10.3201/eid1412.080797

Reuter, G., Boldizsár, A., & Pankovics, P. (2009). Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in the genus Kobuvirus, family Picornaviridae.*Arch Virol, 154* (1), 101-108. doi:10.1007/s00705-008-0288-2

Reuter, G., Boros, A., & Pankovics, P. (2011). Kobuviruses - a comprehensive review. *Rev Med Virol, 21* (1), 32-41. doi:10.1002/rmv.677

Reuter, G., Boros, A., Pankovics, P., & Egyed, L. (2010). Kobuvirus in domestic sheep, Hungary. *Emerg Infect Dis, 16* (5), 869-870. doi:10.3201/eid1605.091934

Ribeiro, J., Headley, S. A., Diniz, J. A., Pereira, A. H., Lorenzetti, E., Alfieri, A. A., & Alfieri, A. F. (2017). Extra-intestinal detection of canine kobuvirus in a puppy from Southern Brazil. *Arch Virol, 162* (3), 867-872. doi:10.1007/s00705-016-3164-5

Shan, T., Wang, C., Cui, L., Yu, Y., Delwart, E., Zhao, W., . . . Hua, X. (2010). Picornavirus salivirus/klassevirus in children with diarrhea, China. *Emerg Infect Dis, 16* (8), 1303-1305. doi:10.3201/eid1608.100087

Smits, S. L., Raj, V. S., Oduber, M. D., Schapendonk, C. M., Bodewes, R., Provacia, L., . . . Haagmans, B. L. (2013). Metagenomic analysis of the ferret fecal viral flora. *PLoS One, 8* (8), e71595. doi:10.1371/journal.pone.0071595

Wang, L., Fredrickson, R., Duncan, M., Samuelson, J., & Hsiao, S. H. (2020). Bovine Kobuvirus in Calves with Diarrhea, United States.*Emerg Infect Dis, 26* (1), 176-178. doi:10.3201/eid2601.191227

Wang, Y., Cui, Y., Li, Y., Wang, X., Yang, K., Zhang, D., . . . Li, Y. (2020). Identification and full-genome sequencing of canine kobuvirus in canine fecal samples collected from Anhui Province, eastern China.*Arch Virol, 165* (11), 2495-2501. doi:10.1007/s00705-020-04773-6

Williams, S. H., Che, X., Garcia, J. A., Klena, J. D., Lee, B., Muller, D., . . . Lipkin, W. I. (2018). Viral Diversity of House Mice in New York City. *mBio, 9* (2). doi:10.1128/mBio.01354-17

Wu, Z., Yang, L., Ren, X., He, G., Zhang, J., Yang, J., . . . Jin, Q. (2016). Deciphering the bat virome catalog to better understand the ecological diversity of bat viruses and the bat origin of emerging infectious diseases. *Isme j, 10* (3), 609-620. doi:10.1038/ismej.2015.138

Xiong, Y. Q., Mo, Y., Chen, M. J., Cai, W., He, W. Q., & Chen, Q. (2018). Detection and phylogenetic analysis of torque teno virus (TTV) carried by murine rodents and house shrews in China. *Virology, 516* , 189-195. doi:10.1016/j.virol.2018.01.017

Xiong, Y. Q., You, F. F., Chen, X. J., Chen, Y. X., Wen, Y. Q., & Chen, Q. (2019). Detection and phylogenetic analysis of porcine bocaviruses carried by murine rodents and house shrews in China. *Transbound Emerg Dis, 66* (1), 259-267. doi:10.1111/tbed.13011

Xiong, Y. Q., Zhou, J. H., Zhang, M. Y., You, F. F., Li, D. L., & Chen, Q. (2018). Presence of rat bocavirus in oropharyngeal and fecal samples from murine rodents in China. *Arch Virol, 163* (11), 3099-3103. doi:10.1007/s00705-018-3943-2

Yamashita, T., Ito, M., Kabashima, Y., Tsuzuki, H., Fujiura, A., & Sakae, K. (2003). Isolation and characterization of a new species of kobuvirus associated with cattle. *J Gen Virol, 84* (Pt 11), 3069-3077. doi:10.1099/vir.0.19266-0

Yamashita, T., Sakae, K., Ishihara, Y., Isomura, S., & Utagawa, E. (1993). Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *J Clin Microbiol, 31* (11), 2938-2943. doi:10.1128/jcm.31.11.2938-2943.1993

Yang, S., Liu, D., Wang, Y., Qu, F., He, Y., Sun, Z., . . . Delwart, E. (2016). Bufavirus Protoparvovirus in feces of wild rats in China.*Virus Genes, 52* (1), 130-133. doi:10.1007/s11262-015-1262-1

You, F. F., Zhang, M. Y., He, H., He, W. Q., Li, Y. Z., & Chen, Q. (2020). Kobuviruses carried by Rattus norvegicus in Guangdong, China.*BMC Microbiol, 20* (1), 94. doi:10.1186/s12866-020-01767-x

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res, 31* (13), 3406-3415. doi:10.1093/nar/gkg595

**Hosted file**

`Table 1.docx` available at https://authorea.com/users/468115/articles/561933-epidemiology-and-genetic-characteristics-of-murine-kobuvirus-from-fecal-samples-of-rattus-losea-

rattus-tanezumi-and-rattus-norvegicus-in-southern-china

**Hosted file**

Table 2.docx available at https://authorea.com/users/468115/articles/561933-epidemiology-and-genetic-characteristics-of-murine-kobuvirus-from-fecal-samples-of-rattus-losea-rattus-tanezumi-and-rattus-norvegicus-in-southern-china

**Hosted file**

Table 3.docx available at https://authorea.com/users/468115/articles/561933-epidemiology-and-genetic-characteristics-of-murine-kobuvirus-from-fecal-samples-of-rattus-losea-rattus-tanezumi-and-rattus-norvegicus-in-southern-china

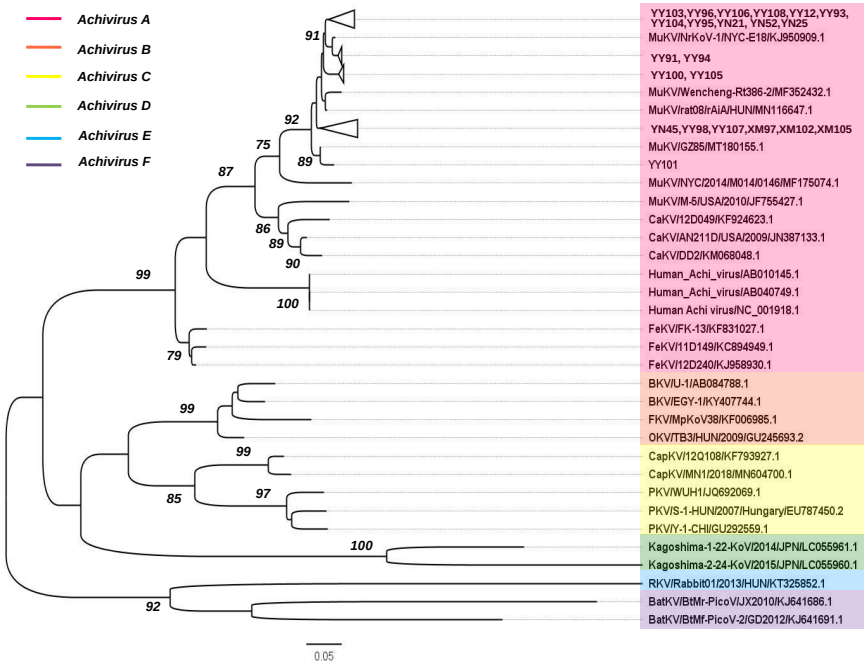


**Figure 1 Phylogenetic analysis of the MuKVs based on the nucleotide sequences of partial 3D regions.** The tree is generated by maximum-likelihood method based on Kimura 2-parameter model (Gamma distributed and partial deletion) with 1000 bootstrap replicates, and the statistics values ＞70% are displayed above the tree branches. YY101 (MW292457) and each of various lateral blank triangles indicate the MuKVs identified in the present study. The strains YY12 (MW292465), YY91 (MW292464), YY93 (MW292463), YY95 (MW292461), YY96 (MW292460), YY98 (MW292459), YY100 (MW292458), YY101, YY103 (MW292456), YY104 (MW292455), YY106 (MW292453), YY108 (MW292451), YN21 (MW292469), YN25 (accession no.MW292468), YN45 (MW292467) and YN52 (MW292466) were isolated from *Rattus norvegicus*. The strains YY94 (MW292462), YY105 (MW292454), YY107 (MW292452) and XM105 (MW292470) were isolated from *Rattus tanezumi*. The strains XM97 (MW292472) and XM102 (MW292471) were isolated from *Rattus losea*. BKV, bovine kobuvirus; BatKV, bat kobuvirus; CaKV, canine kobuvirus; CapKV, caprine kobuvirus; FKV, ferret kobuvirus; FeKV, feline kobuvirus; PKV, porcine kobuvirus; RKV, rabbit kobuvirus. (For better view of the sequences covered by different colors, the reader is referred to the web version of this article.)

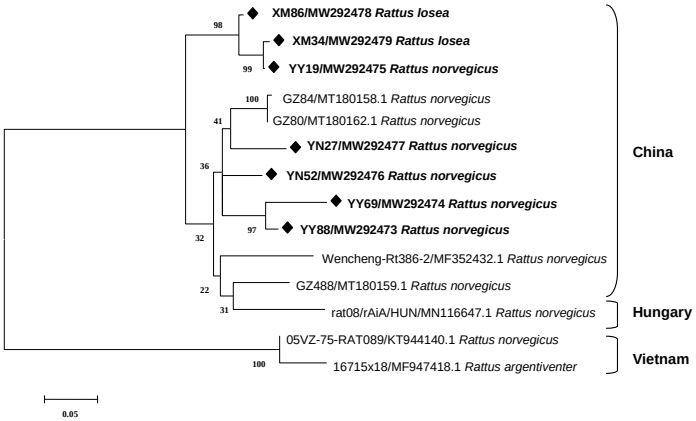


**Figure 2 Phylogenetic tree of the MuKVs based on the complete VP1 sequences.** The tree is generated on the basis of nucleotide sequences by the maximum-likelihood method based on General Time Reversible (GTR) model (Gamma distributed and partial deletion) with 1000 bootstrap replicates. Black diamond symbol refers to the sequences of MuKV presented in the current study.

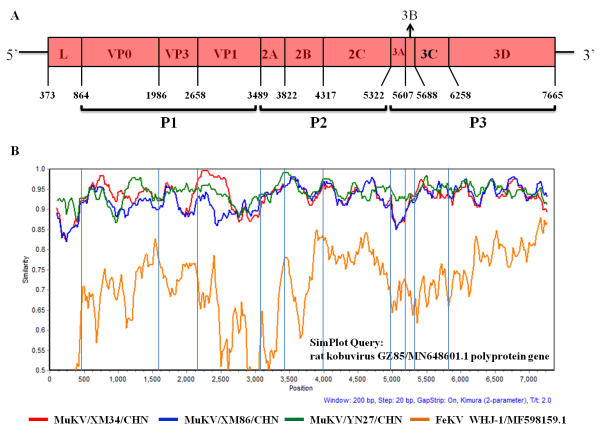


**Figure 3 Genome organization and genetic characterization of the MuKV strains identified in the present study.** A) Genome organization of MuKV/XM34/CHN with initial nucleotide positions labeled for each functional region. The partial 5'UTR is located in positions 1-372 and the partial 3'UTR is located in positions 7666-7714. P1 represents viral structural proteins, and P2 and P3 represent nonstructural proteins. B) Similarity plot analysis of the complete polyprotein genome of MuKV/XM34/CHN (*red line*), MuKV/XM86/CHN (*blue line*) and MuKV/YN27/CHN (*green line*), feline kobuvirus strain WHJ-1/MF598159.1 (*orange line*) as an out-group sequence and rat kobuvirus strain GZ85/MN648601.1 as a query sequence using Kimura (2-parameter) model in Simplot 3.5.1 software.

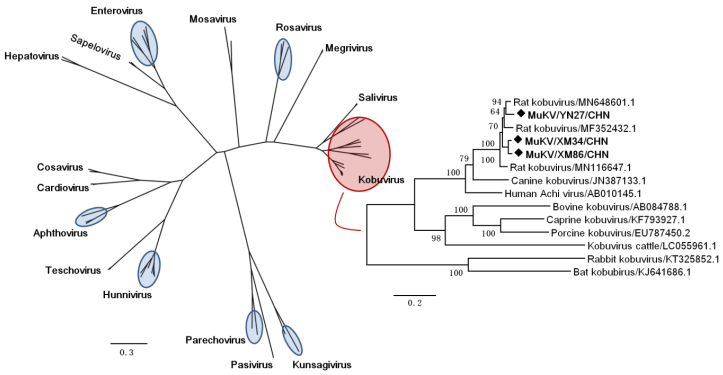


**Figure 4 Phylogenetic relationships between kobuviruses and other picornaviruses based on nucleotide sequences of complete polyprotein gene.** The tree is generated by the maximum-likelihood method based on the General Time Reversible (GTR) model (Gamma distributed with Invariant sites [G+I]) and partial deletion) with 1000 bootstrap replicates. Representative members of family *Picornaviridae* are marked with letters and some with blue circles). Black diamond symbol indicates the sequences of MuKVs identified in the present study.

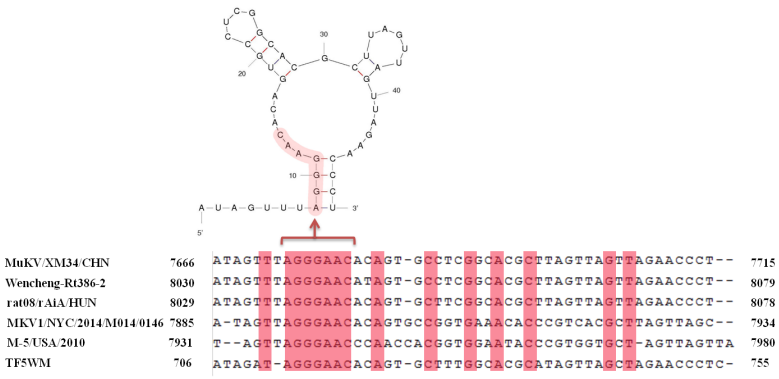


**Figure 5 Predicted barbell-like structure of partial 3'UTR of MuKV/XM34/CHN and sequence alignment of partial 3'UTRs of murine kobuvirus strains.** The sequence alignment in the 49-nt-long partial 3'UTRs begins after the stop codon, and the numbers on both sides of the sequences indicate the nucleotide position. The reference *Kobuvirus* strains include Wencheng-Rt386-2 (accession no.MF352432.1), rat08/rAiA/HUN (accession no.MN116647.1), MKV1/NYC/2014/M014/0146 (accession no.MF175074.1), M-5/USA/2010 (accession no.JF755427.1) and TF5WM (accession no.JQ408726.1).