

# Epidemiology and genetic characteristics of murine kobuvirus from faecal samples of *Rattus losea*, *Rattus tanezumi* and *Rattus norvegicus* in southern China

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## Abstract

Recently, murine kobuvirus (MuKV), a novel member of the family *Picornaviridae*, was identified in faecal samples of *Rattus norvegicus* in China. The limited information on the circulation of MuKV in other murine rodent species prompted us to investigate its prevalence and conduct a genetic characterization of MuKV in *Rattus losea*, *Rattus tanezumi* and *Rattus norvegicus* in China. Between 2015 and 2017, 243 faecal samples of these three murine rodent species from three regions in southern China were screened for the presence of MuKV. The overall prevalence was 23.0% (56/243). Three complete MuKV polyprotein sequences were acquired, and the genome organization was determined. Phylogenetic analyses suggested that our sequences were closely related to Chinese strains and belong to the species *Aichivirus A* in the genus *Kobuvirus*. Additional studies are required to understand the true prevalence of MuKV in murine rodent populations in China.

## DATA SUMMARY

The new sequences in this study have been deposited in the GenBank database under accession numbers MW292451 to MW292482.

## INTRODUCTION

Picornaviruses (family *Picornaviridae*) are small, non-enveloped viruses with positive-sense single-stranded RNA genomes. As of March 2020, there were 147 known species in 63 genera in the family *Picornaviridae* (<https://www.picornaviridae.com/>), many of which result in significant diseases in humans and a wide variety of animals. The genus *Kobuvirus* is a member of the family *Picornaviridae*. It has a genomic organization similar to that of other picornaviruses and has a linear genome ranging from 8.2 to 8.4 kb. *Kobuvirus* genomes are composed of VPg, 5'UTR<sup>IRIS-V</sup>, a large ORF encoding a leader protein (L) and three other functional regions (P1-P2-P3), a 3'UTR, and a poly(A) tail. The P1 region encodes structural proteins (VP0-VP3-VP1), whereas the P2 (2A<sup>H-Box/NC</sup>-2B-2C) and P3 (3A-3B<sup>VPg</sup>-3C<sup>pro</sup>-3D<sup>pol</sup>) regions encode

nonstructural proteins [1]. Of these, the three-dimensional (3D) region is the most conserved region and plays an essential role in viral replication [2]. The VP1 region is highly variable, encoding the most important viral capsid protein that determines viral pathogenicity and antigenicity [3].

Since the determination of the first human kobuvirus (Aichi virus) in a patient with nonbacterial diarrhoea in Japan in March 1989 [4], several additional strains isolated from a variety of animal species have been determined to be members of the genus *Kobuvirus*. Bovine kobuvirus was first recognized as a cytopathic agent in 2003 in the culture medium of HeLa cells that had been used for over 30 years in a laboratory in Japan [5]. Porcine kobuvirus was initially discovered in 2008 in faecal samples from domestic pigs in Hungary [6]. Subsequently, ovine kobuvirus related to bovine kobuvirus was isolated from young, healthy domestic sheep in Hungary in 2009 [7]. In the last 10 years, kobuviruses have been described worldwide in a wide range of hosts, including black goats in South Korea [8], rabbits in Hungary [9], dogs in South Korea and Africa [10, 11], cats in South Korea and China [12, 13], ferrets in the Netherlands and Sweden [14], rats [15] and bats [16] in China, and wolves [17], red foxes [18] and roe deer [19] in Italy.

Received 15 December 2020; Accepted 09 July 2021; Published 06 September 2021

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**Keywords:** detection; genetic characterization; MuKV; murine rodent.

**Abbreviations:** aa, amino acid; BLAST, Basic Local Alignment Search Tool; 3D, three-dimensional; ICTV, International Committee on Taxonomy of Viruses; MuKV, murine kobuvirus; nt, nucleotide; PBoV, porcine bocavirus; RBoV, rat bocavirus; RoTTV, rodent torque teno virus.

Two supplementary tables and one supplementary figures are available with the online version of this article.

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According to the International Committee on Taxonomy of Viruses (ICTV), there are currently six officially recognized *Kobuvirus* species (*Aichivirus A–F*). *Aichivirus A* consists of Aichi virus (found in humans), murine kobuvirus (MuKV), canine kobuvirus, feline kobuvirus and a kobuvirus found in sewage [20]. *Aichivirus B* consists of bovine kobuvirus [5], ferret kobuvirus [14] and ovine kobuvirus [7]. Regarding *Aichivirus C*, two distinct types have been described, porcine and caprine kobuvirus [21]. Cattle kobuvirus [22], rabbit kobuvirus [9] and bat kobu-like viruses represent *Aichivirus D, E* and *F*, respectively.

MuKV was initially detected in the stool of a canyon mouse (*Peromyscus crinitus*) in the USA in 2010 [23]. Subsequently, MuKVs were identified in Hungary [3], Vietnam [24] and the USA [25] in faecal samples from several rodent species, including striped field mouse (*Apodemus agrarius*), Norway rat (*Rattus norvegicus*), Rice-field rat (*Rattus losea* and *Rattus argentiventer*) and house mouse (*Mus musculus*). We recently conducted an initial epidemiological study on MuKV in *Rattus norvegicus* in Guangdong, China [15]. However, there is a lack of information on kobuvirus strains in other murine rodent species in China. This study investigated the prevalence and genetic characterization of MuKV in *Rattus losea*, *Rattus tanezumi* and *Rattus norvegicus* in several regions in southern China.

## METHODS

### Sample collection

A total of 243 fresh faecal samples were collected between October 2015 and September 2017 from rats with unknown health status (they appeared healthy at capture and dissection) captured close to human residences in three regions in China, comprising 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 700 µl PBS (0.3% homogenate) and stored at –80 °C until further use. These stool specimens have been previously examined for rat bocavirus (RBoV) [26], porcine bocaviruses (PBoV) [27], and rodent torque teno virus (RoTTV) [28].

### Nucleic acid extraction and cDNA synthesis

The thawed faecal samples were fully resuspended in PBS and centrifuged at 10000 *g* for 10 min. Viral nucleic acid was extracted from 200 µl of each supernatant using a MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa, Japan). The obtained viral RNA was then reverse transcribed to synthesize cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) and random hexamer primers. The cDNA was directly used as the template for PCR or kept frozen at –20 °C.

### Detection of MuKV

The presence of MuKV was detected by PCR using universal primers (UNIV-kobu-F/UNIV-kobu-R) targeting a 217 bp fragment of the partial 3D region found in all kobuvirus strains, in line with previous research [21]. Thereafter, to determine other nucleotide (nt) sequences of the 217 bp 3D region-positive samples, two sets of primers for a longer 3D region (461 bp)

and the complete VP1 region (831 bp) of MuKV were used [15]. Each PCR mixture had a total volume of 25 µl, including 12.5 µl Green Master Mix (Promega, USA), 8.5 µl sterilized H<sub>2</sub>O, 2 µl cDNA template, and 1.0 µM of each primer. The mixtures were amplified using the following programme: 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. The amplified products were subjected to 1.0% agarose gel electrophoresis and sequenced using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

### Acquisition of complete polyprotein sequences

Three MuKV-positive samples were randomly selected to undergo amplification of the full-length polyprotein sequences using ten pairs of primers designed in this study using the Benchling website (<https://benchling.com>), based on the Chinese sequence Wencheng-Rt386-2 (accession no. MF352432.1). The primer sequences are shown in Table S1 (available in the online version of this article). After sequencing, the full-length polyprotein sequences were assembled using Lasergene SeqMan software (DNASTAR, Madison, WI, USA) and are available in GenBank under accession no. MW292480–MW292482.

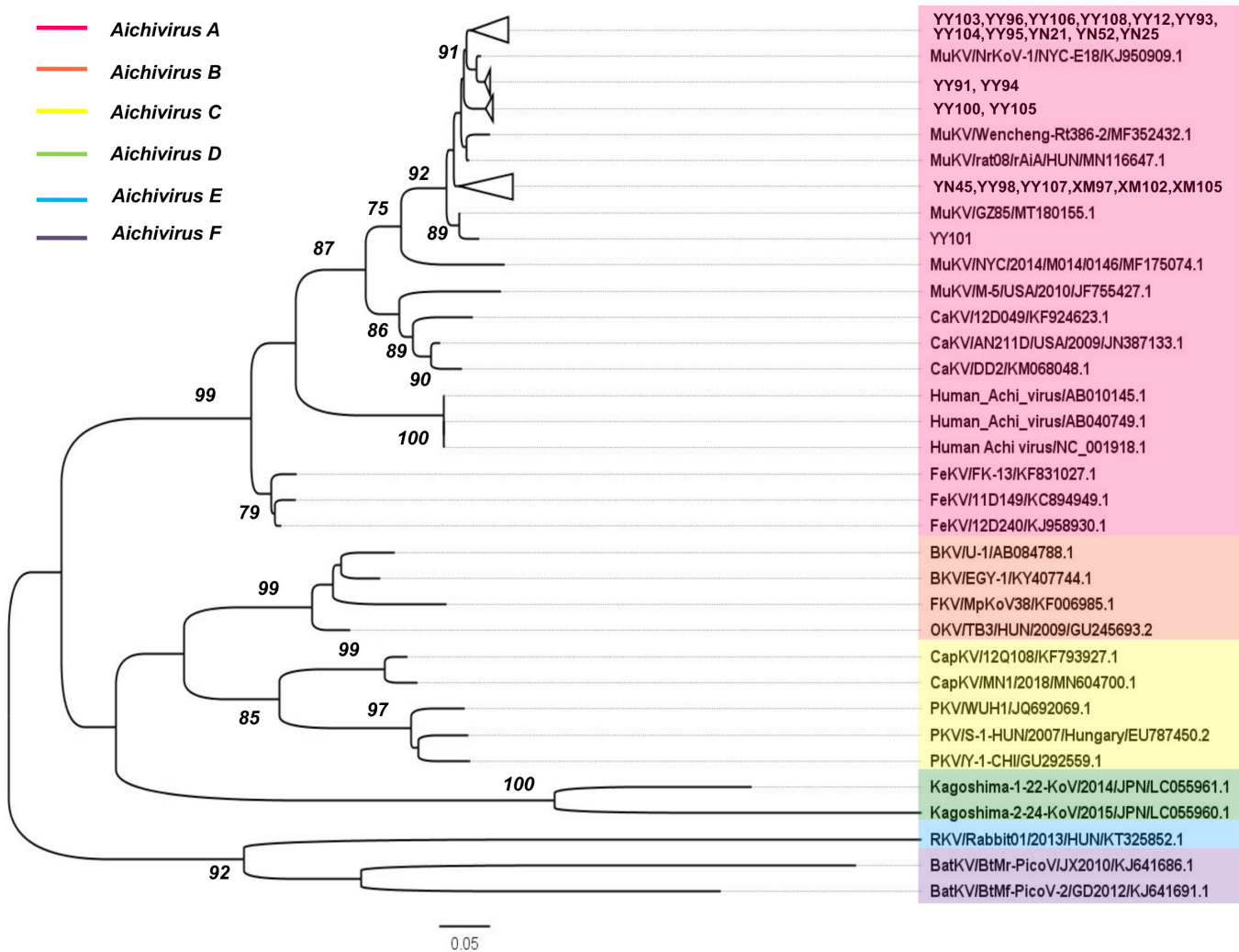
### Genetic and phylogenetic analyses

The MuKV nt sequences identified in this study were compared to the corresponding sequences of other kobuvirus strains in GenBank using Basic Local Alignment Search Tool (BLAST; <https://www.ncbi.nlm.nih.gov>). Multiple sequence alignment was conducted using ClustalW MEGA v7.0 (Oxford Molecular, UK) [29], involving corresponding regions of kobuvirus reference sequences in GenBank. The pairwise nt and amino acid (aa) identities among all sequences were calculated using MegAlign (DNASTAR, Madison, WI, USA). The ORFs were predicted for the identified sequences using ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder>). A similarity plot analysis of the full-length polyprotein sequences was performed using SimPlot v3.5.1 [30, 31]. The 5' and 3'UTR RNA secondary structures were predicted using Mfold [32]. Phylogenetic analyses were conducted using the maximum-likelihood method with 1000 bootstrap replicates in MEGA v7.0 and further visualized in FigTree v1.4.0. 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

Using PCR with a universal primer pair targeting a partial 3D region found in all kobuvirus strains, 243 faecal 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. The overall prevalence was 23.0% (56/243). The prevalence was 28.1%(41/146) in *Rattus norvegicus*, followed by 23.7 %(9/38) in *Rattus tanezumi* and 10.2% (6/59) in *Rattus losea* (Table S2).



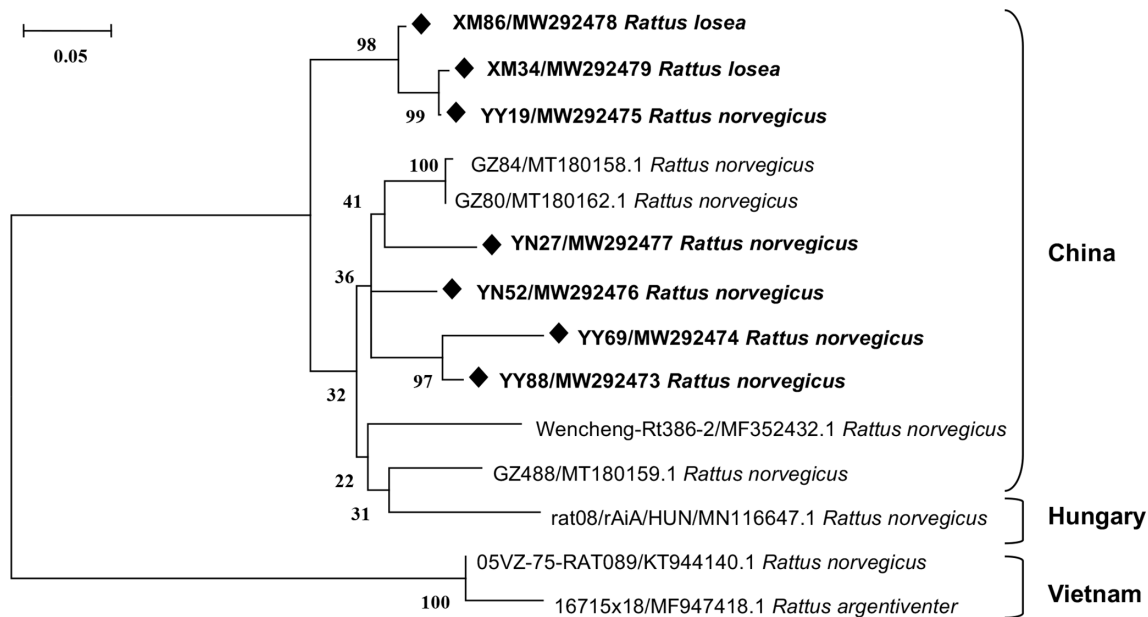
**Fig. 1.** Phylogenetic analysis of the MuKVs based on the nucleotide sequences of partial 3D regions. The tree was generated by the maximum-likelihood method based on the Kimura 2-parameter model (gamma distributed and partial deletion) with 1000 bootstrap replicates. Statistics values >70% are displayed above the tree branches. Lateral blank triangles indicate the MuKVs identified in this study, and YY101 (MW292457) is the other MuKV identified in this study. The sequences 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 (MW292468), YN45 (MW292467) and YN52 (MW292466) were isolated from *Rattus norvegicus*. The sequences YY94 (MW292462), YY105 (MW292454), YY107 (MW292452) and XM105 (MW292470) were isolated from *Rattus tanezumii*. The sequences 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 a better view of the sequences with different colours, refer to the web version of this article.).

### Phylogenetic analyses of 3D and VP1 region sequences

Twenty-two sequences of the partial 3D region (461 bp) were randomly selected for further PCR amplification and sequencing within the 56 of positive samples. Our MuKV sequences that were isolated from three different rodent species captured in different geographic locations were found to be highly conserved in relation to each other (92.7–99.5% at the nt level and 95–100% at the aa level). Maximum-likelihood phylogenetic analysis based on the partial 3D

regions was generated using representative kobuvirus strains from multiple species. The analysis showed that our sequences belonged to *Aichivirus A* and clustered with MuKVs. Interestingly, only one of our sequences, YY101, was closely related to the Chinese MuKV sequence (GZ85) isolated from *Rattus norvegicus* [15]. The other 21 sequences formed a major group with MuKVs from USA, China and Hungary (Fig. 1).

Seven complete VP1 regions (917 nt) were successfully determined, which had 88.7–99.3% nt and 95.1–100% aa



**Fig. 2.** Phylogenetic tree of the MuKVs based on the complete nucleotide sequences of VP1. The tree was generated by the maximum-likelihood method based on the general time Reversible (GTR) model (gamma distributed and partial deletion) with 1000 bootstrap replicates. Black diamonds indicate the MuKV sequences identified in this study.

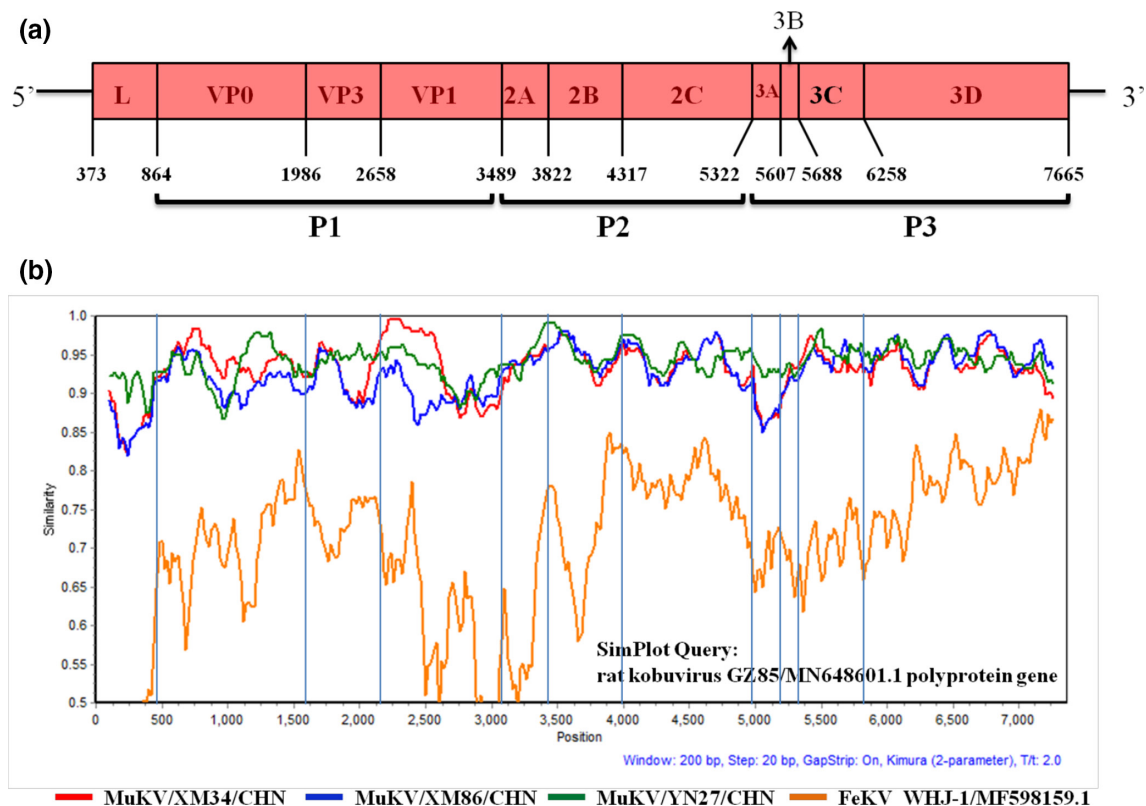
identities with one another. Our sequences shared the highest nt sequence similarity (91.3–93.9%) with the Chinese MuKV MM33 (accession no. MN648600.1). A phylogenetic tree was constructed based on our seven VP1 sequences and the available complete MuKV VP1 nt sequences from three countries, indicating that our VP1 sequences clustered with two MuKV sequences, GZ80 and GZ84, previously described in Guangdong, China. The other kobuvirus reference sequences from China, Hungary and Vietnam formed another group (Fig. 2). The partial 3D and complete VP1 nt sequences of MuKV identified in this study have been submitted to GenBank (accession no. MW292451–MW292472 and MW292473–MW292479, respectively).

### Genomic and phylogenetic analyses of complete polyprotein sequences

Three complete MuKV polyprotein sequences (except the 5' and 3'UTR) were successfully obtained using ten primer pairs. They were designated MuKV/XM34/CHN (accession no. MW292482), MuKV/XM86/CHN (accession no. MW292481) and MuKV/YN27/CHN (accession no. MW292480). These sequences were 7714, 7714 and 7711 nt in length and each had a single complete ORF (7293, 7293 and 7290 nt) encoding a potential polyprotein of 2430, 2430 and 2429 aa, respectively. We performed additional multiple sequence alignments with these three sequences, and they shared 92.5–97.1% nt and 97.1–98.1% aa identities with each other. Interestingly, there was a single amino acid deletion in the 3A region of MuKV/YN27/CHN. The predicted genome organization of our sequences was the same as in other previously identified kobuviruses, and the detailed information is

shown in Fig. 3(a). Further, the predicted RNA secondary structure of the partial 3'UTR displayed a characteristic 'barbell-like' structure (Fig. S1).

According to BLASTn analysis, our sequences showed the highest similarity to the previously published MuKV strain GZ85 (MN648601.1), with identities of 93.2–94.5% nt. Moreover, our sequences were also closely related to other previously described MuKVs. Consequently, we conducted a comparative sequence analysis for each functional region between our sequences and representative kobuvirus sequences in GenBank. Our complete polyprotein sequences showed aa identities with those of rat (96.0–98.3%), human (80.1–80.7%), canine (80.1–80.5%) and feline (80.1–80.3%) kobuvirus reference sequences. Regarding MuKV/XM34/CHN and MuKV/XM86/CHN, the highest nt identities (94.6 and 94.7%, respectively) were found for the P2 region of MuKV sequence GZ85, and the highest aa identities (both 98.7%) were found for the P2 region of rat08/rAiA/HUN. Regarding MuKV/YN27/CHN, the highest nt (95.4%) and aa (99.8%) identities were found for the P2 and P1 regions of GZ85, respectively (Table 1). In addition, a similarity plot analysis was conducted to further analyse the genetic characteristics of the complete polyprotein nt sequences, with the feline kobuvirus sequence WHJ-1 (MF598159.1) used as the out-group sequence and the MuKV reference sequence GZ85 (MN648601.1) used as the query sequence. The findings showed that the VP0, VP3, 2A–2C, 3C and 3D regions displayed relatively high similarities to the query sequence, whereas low similarities were exhibited in the L,



**Fig. 3.** Genome organization and genetic characterization of the MuKV sequences identified in this study. (a) Genome organization of MuKV/XM34/CHN, with initial nucleotide positions labelled for each functional region. The partial 5'UTR is located at positions 1–372 and the partial 3'UTR is located at positions 7666–7714. P1 represents the structural proteins, and P2 and P3 represent the nonstructural proteins. (b) Similarity plot analysis of the complete polyprotein sequences of MuKV/XM34/CHN (red line), MuKV/XM86/CHN (blue line) and MuKV/YN27/CHN (green line), with feline kobuvirus sequence WHJ-1/MF598159.1 (orange line) as the out-group sequence and MuKV sequence GZ85/MN648601.1 as the query sequence, using the Kimura 2-parameter model in Simplot v3.5.1.

VP1, 3A and 3B regions. Additionally, MuKV/YN27/CHN had considerably higher similarities in the L, 3A and 3B regions and the 2A-2B junction than MuKV/XM34/CHN and MuKV/XM86/CHN (Fig. 3b).

Table 2 shows the predicted protease-cleavage sites of our sequences and the reference kobuviruses, including Q/G, Q/H, Q/T, Q/S, Q/A, A/Q and L/T. The predicted protease-cleavage sites between L and VP0 (Q/G), VP0 and VP3 (Q/H), 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 isolated from different species. In comparison, the protease-cleavage site between 3A and 3B is Q/A for all the kobuvirus sequences except for porcine kobuvirus (Q/G). The protease cleavage sites in our sequences are highly conserved with those typically found across the genus [3].

Maximum-likelihood phylogenetic analysis was conducted based on our complete polyprotein nt sequences and those from representative picornaviruses. The results indicated that our sequences were located at the same branch as the members of the genus *Kobuvirus*, and other MuKV sequences represented the closest relatives (Fig. 4).

## DISCUSSION

Kobuvirus circulation in rats has been reported in Guangdong, China, but only in *Rattus norvegicus* [15]. To the best of our knowledge, this study represents the first study to investigate the prevalence of MuKV in the other two murine rodent species (*Rattus losea* and *Rattus tanezumi*) in southern China.

The detection results indicated that 56 MuKV sequences were identified in faecal samples from these murine rodents, with a detection rate of 23.0% (56/243). 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, highlighting the circulation of MuKV within the three murine rodent populations. In comparison, MuKVs were detected in 50 and 55.5% of *Rattus norvegicus* faecal samples in the USA and Hungary, respectively [33, 34]. Likewise, a previous study in Vietnam reported that the occurrence of rodent-derived kobuvirus sequences was 10.9% in six murine species [24]. Even though our findings confirmed that murine rodent species are hosts of MuKV, additional studies are required to fully understand the true prevalence of MuKV in murine rodents in China.

**Table 1.** Nucleotide and putative amino acid sequence identity of the complete polyprotein sequences and L<sub>1</sub>-P<sub>3</sub> regions between MuKV/XM34/XM86/YN27/CHN and other *Kobuvirus* reference sequences\*

Gene region	Rat			Human		Canine		Feline	
	MuKV/XM86/CHN	MuKV/YN27/CHN	GZ85	Wencheng-Rt386-2	rat08/rAIA/HUN	A846/88	CH-1	WHJ-1	WHJ-1
MuKV/XM34/CHN									
L	99.0/98.8	89.4/94.2	89.9/93.0	88.4/90.7	84.5/87.2	64.7/60.5	57.3/51.2	57.3/48.8	
P1	96.0/97.5	92.2/98.3	94.0/98.3	91.4/97.9	92.0/98.1	74.7/77.4	74.4/77.4	75.0/79.5	
P2	98.3/99.3	93.8/97.9	94.6/98.2	94.2/98.2	94.5/98.7	77.9/82.5	79.8/85.3	78.6/84.9	
P3	97.4/97.8	93.5/97.1	90.4/93.5	92.9/97.1	93.1/97.1	79.3/86.3	81.7/86.2	78.9/83.8	
Polyprotein	97.2/98.2	92.8/97.5	92.7/96.4	92.4/97.2	92.4/97.1	76.3/80.4	76.9/80.3	75.9/80.1	
MuKV/XM86/CHN									
L	100/100	88.8/94.2	89.4/93.0	88.2/90.7	84.3/87.2	64.5/61.0	56.9/50.6	57.4/49.4	
P1	100/100	91.0/96.7	91.9/96.5	90.6/96.3	90.9/96.4	74.0/76.3	73.9/76.5	74.6/78.6	
P2	100/100	93.9/97.9	94.7/98.2	93.9/98.2	94.5/98.7	78.1/82.5	79.9/85.4	78.8/85.4	
P3	100/100	93.3/97.7	91.0/94.5	93.6/97.8	93.5/97.9	79.7/86.7	82.0/86.4	79.4/84.4	
Polyprotein	100/100	92.3/97.1	92.1/96.0	92.2/96.9	92.2/96.8	76.3/80.1	76.8/80.1	76.0/80.1	
MuKV/YN27/CHN									
L	88.8/94.2	100/100	92.3/96.5	90.5/94.2	86.5/90.7	63.5/62.2	59.2/51.2	56.3/48.3	
P1	91.0/96.7	100/100	93.9/99.8	92.4/99.6	91.3/99.2	74.3/77.5	74.2/77.8	75.3/79.9	
P2	93.9/97.9	100/100	95.4/98.9	93.6/98.7	93.5/98.5	78.0/82.8	79.9/85.1	78.8/84.8	
P3	93.3/97.7	100/100	91.4/95.0	93.0/97.4	93.0/97.6	79.1/86.5	81.3/86.4	78.4/84.4	
Polyprotein	92.3/97.1	100/100	93.3/98.0	92.8/98.3	92.0/97.9	76.1/80.7	76.8/80.5	75.8/80.3	

Values were described as nucleotide identity (%) / amino acid identity (%).

\*Reference *Kobuvirus* sequences include: GZ85 (accession no. MN648601.1), Wencheng-Rt386-2 (accession no. MF352432.1), rat08/rAIA/HUN (accession no. MN116647.1), A846/88 (accession no. AB010145.1), CH-1 (accession no. JQ911763.1) and WHJ-1 (accession no. MF598159.1).

Table 2. Protease-cleavage sites of MuKV sequences identified in this study and other Kobuvirus reference sequences from several species

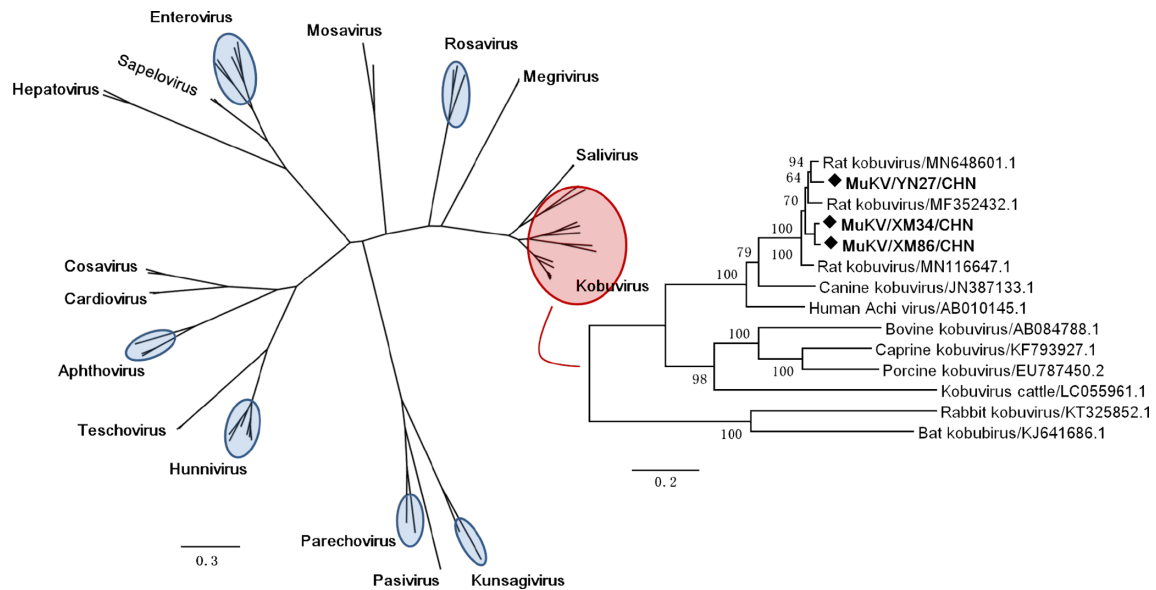
Cleavage between	Rat		Human	Bovine	Canine	Feline	Porcine
	MuKV/XM34/CHN	MuKV/XM86/CHN					
L/VP0	LQRQ/GNST	LQRQ/GNST	A846/88 AB010145.1	U-1 AB084788.1	CH-1 JQ911763.1	WHJ-1 MF598159.1	S-1-HUN EU787450.2
VP0/VP3	VAPQ/HWKT	VAPQ/HWKT	LAPQ/HWKT	VSKQ/HWKT	VAPQ/HWKT	VAPQ/HWKT	VQIQ/HWKI
VP3/VP1	LSSQ/TLSE	LSSQ/TLSE	LTSQ/TLSE	LALQ/ADGD	LTSQ/ANSE	LSSQ/GNSE	FQIQ/AADD
VP1/2A	VVRQ/GANY	VVRQ/GANY	VIKQ/GAAS	RQCA/GESL	VVKQ/GAHT	VKRQ/GAPA	RQCL/TNDV
2A/2B	IRRQ/GLLT	IRRQ/GLLT	IRRQ/GLLT	ITRQ/GLLT	IKRQ/GLLT	IKRQ/GLLT	IRQ/GLLS
2B/2C	LEHQ/GLKD	LEHQ/GLKD	LEPQ/GLKD	LEPQ/GVRD	LEPQ/GLKD	LEPQ/GLKD	VEHQ/GVRD
2C/3A	IKRQ/GNRV	IKRQ/GNRV	IRRO/GNRV	IKRQ/GAHS	IKRQ/GRTI	IKRQ/GRTI	VKRQ/GLTI
3A/3B	SSAQ/AAYS	SSAQ/AAYS	QEPQ/AAYS	DEPQ/AAYS	SETQ/AAYS	SAAQ/AAYS	DKSQ/GAYS
3B/3C	IQRQ/GISP	IQRQ/GISP	IQRQ/GISP	VVRQ/AGPS	IQRQ/GLSP	IQRQ/GLSP	VVRQ/SLSP
3C/3D	TTHQ/SQII	TTHQ/SQII	TTQ/SQII	TECQ/SLII	TTHQ/SLIV	TTQ/SQII	IDQQ/SIII

As mentioned above, the faecal samples in this study had been previously used for RBoV, PBoV and RoTTV detection, with the respective prevalence of 43.6, 44.4 and 25.5%. Notably, coinfections of MuKV and RBoV were found in 10.3% of the 243 specimens, coinfections of MuKV and PBoV in 10.3%, and coinfections of MuKV and RoTTV in 8.6%. Several studies have reported that coinfections of porcine kobuvirus and other viruses were very common [35, 36], whereas limited data are available on the coinfections of MuKV and other pathogens. Although we reported that MuKV co-infected with RBoV, PBoV and RoTTV, many other pathogens have not been ruled out in these specimens. Additional studies are needed to consider the coinfections of MuKV and other pathogens in rats.

Previous studies have detected kobuviruses in humans and animals with diarrhoea and other clinical manifestations, suggesting that kobuviruses are a causative agent of gastroenteritis [13, 37–43]. However, as in this study, others have also identified kobuviruses in asymptomatic animals or individuals co-infected with other pathogens [4, 13, 19, 37–39, 41, 44, 45]. Consequently, additional studies are warranted to determine the actual influence of MuKV and other kobuviruses on animal health. The presence of MuKV in rodent populations living near humans poses a potential threat to public health. Susceptible animal models should be established to verify the clinical significance of kobuviruses in gastrointestinal disease. Taken together, our epidemiological evidence will help to promote experimental studies on MuKV pathogenesis and clinical manifestations, especially regarding gastroenteritis.

Phylogenetic analysis based on a partial 3D region confirmed our sequences to be members of the species *Aichivirus A* in the genus *Kobuvirus*. Furthermore, the sequences clustered with MuKV, canine and feline kobuviruses, and human Aichi viruses. The 21 MuKV 3D region sequences identified in this study formed a large group with the US, Chinese and Hungarian MuKV sequences. This is in accordance with previous research indicating no geographic clustering [46]. Besides those 21, the other, one sequence (YY101) exhibited the closest relationship to the Chinese MuKV sequence GZ85. Moreover, our sequences tended to phylogenetically cluster at a branch associated with their host species (Fig. 1).

The VP1 protein is the most immunodominant portion of the kobuvirus capsid, determining the viral antigenicity and pathogenicity; it is also the most variable kobuvirus structural protein [3, 47]. The phylogenetic analysis based on our complete VP1 nt sequences (with the murine rodent host species shown in italics) indicates that our VP1 sequences clustered closely with those from China but were separated from MuKV VP1 sequences from Hungary and Vietnam (Fig. 2). This demonstrates that the MuKV sequences from China may share a similar evolutionary background and circulate among the various murine rodent populations in China [48]. This provides evidence of geographic clustering of the analysed VP1 sequences, though geographic clustering was not observed regarding the 3D region. Whether the MuKV sequences from different geographic regions impact



**Fig. 4.** Phylogenetic relationships between kobuviruses and other picornaviruses based on complete polyprotein nucleotide sequences. The tree was generated by the maximum-likelihood method based on the GTR model (gamma distributed with Invariant sites [G+I]) and partial deletion) with 1000 bootstrap replicates. Representative members of the family *Picornaviridae* are marked with letters and some are marked with blue circles. Black diamonds indicate the MuKV sequences identified in this study.

pathogenicity and antigenicity warrants attention in the future.

Acquiring complete polyprotein sequences in this study allowed us to obtain comprehensive information on the genetic characteristics of MuKVs circulating in murine rodents in southern China. The three sequences were designated MuKV/XM34/CHN (7296 nt), MuKV/XM86/CHN (7296 nt) and MuKV/YN27/CHN (7293 nt). Genomic analysis revealed that our polyprotein sequences exhibited a similar genome organization to those of other kobuviruses, including L, VP0, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D (Fig. 3a). Interestingly, a single amino acid deletion was found in the deduced 3A region of MuKV/YN27/CHN, similar to the canine strain CH-1 and the feline strain WHJ-1. According to previous research, a single amino acid deletion was also found in the VP0 region of feline kobuvirus from a cat with diarrhoea [13]. In contrast, there was a thirty-amino-acid deletion in the 2B region of porcine kobuvirus from healthy piglets, which might be associated with the pathogenicity of porcine kobuvirus [49]. Natural mutation and recombination occurs in picornaviruses, playing an important role in genetic diversity [50]. Whether the amino acid deletions influence the pathogenicity of kobuviruses from different species requires further investigation via structural prediction and genomic analysis.

The 49-nt partial 3'UTR of the MuKVs identified in this study showed the highest sequence identities (98.0–100 %) to the Chinese sequences Wencheng-Rt386-2 (accession no. MF352432.1). The predicted partial 3'UTR RNA secondary structure had a characteristic 'barbell-like' structure with a conserved AGGGAAC motif, as in the MuKV reference

sequences in GenBank. Barbell-like structures have been identified in members of the genus *Kobuvirus* at different nt positions in the 3'UTR [33, 51]. Future studies should determine the functions of this structure.

The predicted cleavage sites generate a typical picornavirus gene order of VP0–VP3–VP1–2A1–2A2–2B–2C–3A–3B–3C–3D [52]. Prior studies have reported the hypothetical protease cleavage sites in feline kobuvirus [51, 53], canine kobuvirus [54] and even different rodent picornavirus lineages [55]. This study predicted the cleavage site positions in the MuKV polyprotein by sequence alignment with those putative or demonstrated cleavage sites in kobuviruses. Predicted cleavage sites of our MuKV sequences mainly comprised Q/G, Q/H, Q/T, Q/S and Q/A amino acid sequences, except for A/G and L/T between VP1 and 2A. We further compared the protease cleavage sites among kobuvirus sequences isolated from humans and various animal hosts. The findings supported a previous review indicating predicted cleavage sites are similar to those in other picornaviruses [3].

A phylogenetic analysis of *Kobuvirus* and other picornaviruses demonstrated that our sequences belong to the genus *Kobuvirus* (Fig. 4). The phylogenetic relationships between our complete polyprotein sequences and other kobuvirus sequences (*Aichivirus A–F* species) were also shown in Fig. 4, illustrating that our MuKV sequences shared a common root with the kobuviruses identified in canine and humans but related more distantly to bovine kobuvirus, as indicated by the nucleotide and amino acid homology analyses. In addition, this result suggests that cross-species kobuvirus transmission might occur due to frequent contact between rats, dogs and humans in the



natural environment, in line with a study demonstrating that cross-species transmission may occur within and among mammalian species [24, 56]. These findings increase understanding of *Kobuvirus* evolution and genomic characteristics in murine rodent populations.

## CONCLUSIONS

For the first time, we identified MuKV in *Rattus losea* and *Rattus tanezumi* in China, demonstrating the expanded host range of kobuviruses. We identified the molecular characteristics of MuKV and provided evidence of its circulation in three murine rodent species. A limitation of the study is the small sample size. Therefore, more detailed analyses, including epidemiological and experimental investigations, are needed to investigate the pathogenicity, genetic diversity and potential risk to human health of MuKV.

### Funding information

This work was supported by the National Natural Science Foundation of China (grant no. 81973107).

### Acknowledgements

We are grateful for the generous support of our colleagues regarding sample collection and technical assistance.

### Author contributions

M.Z.: conceptualization, investigation, writing-original draft preparation; F.Y.: methodology, investigation; F.W.: investigation; H.H.: visualization; Q.L.: investigation; Q.C.: conceptualization, writing-review and editing, supervision.

### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Ethical statement

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

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