First detection and genetic characterization of donkey-like kirkovirus in diarrhoeal piglets in Xinjiang, China

Panpan Tong
Xinjiang Agricultural University

Zunbao Wang
Jilin University

Yueyi Dang
Xinjiang Agricultural University

Lei Zhang
Aksu Regional Animal Disease Control and Diagnostic Center

Guangwei Song
Tecon Bio-technology Co, LTD

Xiaozhen Song
People's Government of Ziniquanzi Town

Juanjuan Pan
Xinjiang Agricultural University

Ling Kuang
Xinjiang Agricultural University

Junhui Li
Tecon Bio-technology Co, LTD

Gang Lu
South China Agricultural University

Jinxin Xie (xiejinxin198683@163.com)
Xinjiang Agricultural University

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Abstract

Kirkovirus (kirV), a seemingly novel virus family, has been found in horses and donkey. The objectives of the study intend to investigate the presence of the virus in swine. In this study, donkey-like kirkovirus (kirV) was detected in anal swabs of piglets with diarrhoea and the positive rate was found to be 100% (149/149), but this virus was detected in only one of 261 clinically healthy piglets, which suggested a strong relationship between the kirV and the diarrhoeal disease. We obtained the whole-genome sequences of three kirVs (named Cj-D5, Cj-D32 and Cj-D43), with length of 3750 nt and sharing 99.9% nucleotide (nt)-identity with donkey kirVs. Furthermore, three viruses shared 88.5%–100%, and 23%–51% of the Rep protein sequence identity with available reference strains of the families Kirkoviridae, Circoviridae, respectively. Moreover, like horse and donkey kirVs, RCR domain and P-loop NTPase domains of Rep protein and nonanucleotide motif (CAATATTAC), of the three viruses, were similar to those of Circoviruses and Cycloviruses. Phylogenetic analysis showed that these viruses could be potentially grouped together with members in the proposed family Kirkoviridae. This is the first report to describe that kirV can circulate in piglets with diarrhoea and future studies are needed to determine the pathogenesis of this virus.

Introduction

Kirkovirus (kirV), belonging to the family “Kirkoviridae”, has been identified in the liver, spleen of a thoroughbred horse with fatal idiopathic hepatopathy (Li et al. 2015), fecal sample of a dead thoroughbred mare (Xie et al. 2020), and the fecal samples of healthy milk donkey (Tong et al. 2022). It has been established that similar to the members of the family Circoviridae (Meehan et al. 1998; Morozov et al. 1998; Palinski et al. 2016), kirV is characterized by a circular genome, but they possess relatively larger genome (3,732 to 3,800 nt) (GenBank nos. KR902498, MK520880, MW147105, MW147107, MW147108) and a more complex genomic structure (six ORFs, ORF> 300 nt) in comparison to circovirus and cyclovirus (Li et al. 2015; Xie et al. 2020; Tong et al. 2022). So far, kirV has been only reported in horse and donkey, but the positive rate for kirV in horse and donkey fecal samples was found to range from 11% to 60% (Xie et al. 2020; Tong et al. 2022).

In this study, we have identified a donkey-like kirV in diarrhoea piglets’ anal swabs of a farm in Changji City, Xinjiang, China. Further investigations revealed that the virus may be a potential swine diarrhoea virus.

Materials And Methods

Sample collection

In January 2020, a fatal outbreak of diarrhoeal disease among piglets (< 14 days) was observed in a pig farm in Changji, Xinjiang province, China. The morbidity rate ranged from 50% to 70%, however, the mortality rate was as high as 70%. RT-PCR results for major diarrhoea viruses (porcine epidemic diarrhea
virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine rotavirus A (PRV-A), and porcine deltacoronavirus (PDCoV)) demonstrated that 80% diarrhoea anal swabs were positive for PEDV and PRV, and these two viruses were found to cause co-infection in about 10% diarrhoea samples, by using primers reported in a previous study (Ding et al. 2020).

In the current study, 410 anal samples of piglet were collected from the farm and were observed to be negative for four viruses above (Ding et al. 2020). Among these, 149 samples were obtained from piglets with severe clinical signs, including diarrhoea, loss of appetite, haemorrhagic enteritis and eventually death, whereas 261 samples were derived from the clinically healthy piglets.

**PCR**

The viral DNA was extracted from anal swabs with the Virus DNA Isolation Kit (Geneaid Biotech Co.) based on the manufacturer’s instructions. One universal primer pair targeting a 413-nt fragment of kirV hypothetical protein 4 (Hp4) gene (Table S1) was used as per the prior published report (Xie et al. 2020; Tong et al. 2022). The 2× TransStart® FastPfu Fly PCR SuperMix was used to amplify the target gene as follows: 94 °C for 2 min, denaturation (35 cycles) at 94 °C for 20 s, annealing at 60 °C for 20 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The positive PCR amplicons were ligated into pEASY®-Blunt T vector to transform Trans1-T1 phage resistant chemically competent cells. Thereafter, Sanger sequencing was performed on ten randomly selected clones of each amplicon

**Sequences analysis**

DNAMAN 6.0 software and ORF Finder were used for sequence assembly and prediction of the genome organization, respectively. Sequences similarity analysis was conducted by using MegAlign in Lasergene.v7.1. The maximum likelihood (ML) method (Tamura–Nei model) was employed for phylogenetic analysis and evaluated with 1,000 bootstrap replicates (Kumar et al. 2016).

**Results**

**Detection of KirV in samples obtained from diarrheal piglets**

A number of previous studies have showed that kirV can exhibit genetic diversity in both horse and donkey (Xie et al. 2020; Tong et al. 2022), hence to investigate the presence of kirV in swine, DNA was extracted from the collected anal swab samples. After performing PCR with the primer pairs, Hp4-F and Hp4-R, as well as agarose gel electrophoresis, 150 PCR products were identified that presented bright bands of 413 bp. The subsequent sequencing and BLAST hits indicated that 150 anal swab samples were kirV-positive, with positive rate of 36.6% (150/410). Among these kirV-positive samples, 149 diarrhea samples were all found to be positive for kirV, however, one out of 261 (0.4%) healthy samples was observed to be positive for the virus. These results indicated that the kirV was circulating in piglets and exhibited strong association with piglet diarrhoea. In addition, all porcine kirV Hp4 genes displayed high nt sequence identities of 99.6%–100% with each other, nt sequence identity of 86.5-94.7% with horse
KirV (GenBank no. KR902498 and MK520880), and 99.2-99.5% with donkey KirV respectively (GenBank nos. MW147105, MW147107, and MW147108), thereby indicating porcine KirV was a donkey-like KirV.

**Genetic characterization of porcine KirV**

Thereafter, to facilitate understanding of the genetic characterization of porcine KirV, all porcine KirV *Hp4* genes sequences were aligned and one primer pairs, Com-F and Com-R, were designed based on the *Hp4* gene conservative region (Table S1). After agarose gel electrophoresis, the PCR products amplified by the primer pairs displayed bands of 3,410 bp. After sequencing and assembly with *Hp4* gene identified as belonging to donkey KirVs, three 3,750-nt complete genome sequences were obtained (named Cj-D5, Cj-D32, and Cj-D43) (GenBank nos. MW504209–MW504211), with 12 nt longer than KirV Cj-7-7 and 50 nt shorter than that of KirV Equ1. It shared 86.5%–89.0% homology with horse KirV Cj-7-7 as well as Equ1, and shared 99.9% homology with donkey KirV Hetian-46, Hetian-48 and Hetian-58, thus indicating that both porcine and donkey KirV might originate from the same one. Similar to the complete genomic map of horse and donkey KirVs, porcine KirV possessed two forward reading frame (nt 1–918, and nt 1605–1991) and four reverse reading frames (nt 3629–3156, nt 3133–2567, nt 2544–1973, and nt 1940–1350) (Fig. 1). Among various identified reading frames, forward reading frame (nt 1–918) and reverse reading frames (nt 3133–2567) encoded the replicase protein (Rep, 305 amino acid (aa)) and the putative capsid protein (Cap, 188 aa), respectively. The other four ORFs encoded four hypothetical proteins (Hp) of relatively unknown function with sequences of 196, 190, 157 and 128 aa, respectively (Fig. 1).

Like horse and donkey KirV Rep, and porcine KirV Rep in this study contained RCR and SF3 helicase motifs, including RCR motif I [FTIN], motif II [PHIQG] and motif III [YCSK] and dNTP-binding or P-loop NTPase domains which are characteristic of superfamily 3 (SF3) helicases motifs Walker-A [GPAGVGKS], Walker-B [RVVLDD], and motif C [VTSN]. Moreover, similar to horse and donkey KirV, porcine KirV ori was characterized by a conserved nonanucleotide motif “CAATATTAC” (Figure 1).

**Analysis of porcine KirV Rep genes**

A comparison of the *Rep* genes revealed that three distinct KirV identified in our study shared 99.9%–100% nt identity, and 99.7% aa similarity, thus indicating that the porcine KirV possessed a high genetic identity. Further multiple sequence alignment of three porcine KirV identified in the present study revealed a sequence similarity of 98.7%–100%, 36.7%–40% 40.5%–42.7%, for the *Rep* gene sequence and a sequence similarity of 88.5%–100%, and 23%–51%, 22.8%–27.1%, 22.2%–25.7% for the *Rep* protein sequence after taking into account the sequences of available reference strains of the proposed family *Kirkoviridae*, genus *Circovirus* and genus *Cyclovirus*, respectively.

A phylogenetic tree was then reconstructed using the maximum likelihood method based on the *Rep* protein sequences to understand the potential genetic relationships between porcine KirV and available reference strains. The results indicated that porcine, donkey, and horse KirVs formed an independent branch, and displayed most close relationship to donkey KirV Hetian-58, Hetian-48, and Hetian-47, as indicated by both nt and amino acid homology analyses. These KirV and human, rodent, horse, porcine,
bovine and macaca mulatta-origin CRESS DNA viruses were clustered into the proposed family \textit{Kirkoviridae} but was divergent from the lineages of \textit{Circovirus}, and \textit{Cyclovirus} (Figure 2). Unfortunately, similar to previous studies (Xie et al. 2020; Tong et al. 2022), we also attempted to isolate the kirV using PK15, ST and Vero cells, but were not successful. Next study will continue to try to isolate viruses using other types of cells.

\textbf{Discussion}

A number of viruses have been discovered that can cause acute diarrhea in China's piglets, which has resulted in massive losses to the pig husbandry (Ding et al. 2020; Li et al. 2023). KirV has a larger circular genome (3,732 to 3,800 nt), and been reported in horses, and donkey till date (Li et al. 2015; Xie et al. 2020; Tong et al. 2022). In the present study, kirV was detected in 150 of 410 anal swabs of piglets negative for the major diarrhoea viruses (PEDV, TGEV, PRV-A, and PDCoV), thus indicating that this virus can infect porcine. Our results suggested that kirV can infect a wide range of host as the virus was also detected in sheep and calf (data not shown). However, further studies are needed to investigate the infection of kirV in others livestock and to confirm host range of this virus.

Interestingly, among 150 kirV-positive anal swabs, 149 kirVs were detected in all diarrhoeic samples, however, one of 261 healthy samples was positive for this virus. Therefore, based on the results, it can be concluded that the kirV infection was strongly association with diarrhoea of piglet, Xinjiang, China. Until now, several viruses, like PEDV, etc., have been reported to cause severe damage to the development of the pig industry, thus causing major economic losses to global pig farms (Ding et al. 2020; Li et al. 2023).

In the current study, we have established that kirV could be a potential novel diarrhoea virus, but this aspect needs to be investigated on porcine population of worldwide. Thus, the future studies should aim to isolate the kirV, then perform challenge assay and confirm the association between the virus and piglet diarrhoea.

In addition, as reported for horse and donkey kirVs in two previous studies, some variations in \textit{Hp4} genes of porcine kirVs were also observed (Xie et al. 2020; Tong et al. 2022). However, the HP4 sequences from all porcine kirVs in this study shared 99.6-100\% nt identity, thereby indicating the genetic conservation. Indeed, porcine and donkey kirV \textit{Hp4} genes displayed high 99.2-99.5\% nt identity, which suggested that the kirVs of porcine and donkey could have common evolutionary origins.

Interestingly, several prior studies have showed that kirV had relatively larger genomes (Li et al. 2015; Xie et al. 2020; Tong et al. 2022). Among these viruses, the complete genome sequences of horse kirV are 3,732, and 3,800 nt, and donkey kirV is composed of 3,750 nt. It was found in the present study that similar to donkey kirV genome, the complete genome sequence of porcine kirV was 3,750 nt.

Moreover, the complete genome sequence of porcine and donkey kirV shared 99.9\% nt identity. Therefore, based on these findings, it could be inferred that porcine, and donkey kirV had common evolutionary origins.
In addition, a previously published article has already revealed that circovirus and cyclovirus Rep have six well-conserved RCR and SF3 helicase motifs, including motif I [FT(L/I)N], motif II [PHLQG], motif III [YC(S/x)K], Walker-A [G(P/x)(P/x)GxGK(S/t)], Walker-B [uuDDF], and motif C [uTSN] where "x" represents any residue, and "u" represents a hydrophobic amino acid (F, I, L, V, M) (Karyna et al. 2017; Rosario et al. 2012; Shulman et al. 2017).

It was noted that similar but different to Rep of the members of the family Circoviridae, Rep of porcine kirVs identified in this study, as well as that of donkey and horse kirVs reported in in previous three studies (Li et al. 2015; Xie et al. 2020; Tong et al. 2022) all had conserved motifs RCR and SF3 helicase motifs, including RCR motif I [FTIN], motif II [PHIQG] and motif III [YCSK] and dNTP-binding or P-loop NTPase domains characteristic of the superfamily 3 (SF3) helicases motifs Walker-A [GPAGVGS], Walker-B [RVVLDD], and motif C [VTSN]. But kirV had more complex genomic structure (six ORFs, ORF> 300 nt) in comparison to circovirus and cyclovirus (Figure 1). Thus, based on our findings kirV could be potentially classified into a new family: Kirkoviridae.

Moreover, identical to circovirus and cyclovirus conserved origin of replication (ori) “TAGTATTAC” (Karyna et al. 2017; Rosario et al. 2012; Shulman et al. 2017), kirV ori of different host was characterized by a conserved nonanucleotide motif “CAATATTAC” (Figure 1). These results suggested that the kirV could have a similar replication strategy as that of the members of the family Circoviridae.

Overall, this study provides the first molecular evidence for existence of porcine kirV, and our observations indicate that the virus may function as a novel porcine diarrhoeal virus. however, further research is required to develop innovative methods for virus isolation and to explore pathogenesis mechanisms associated with this virus.

Declarations

Author contributions P.T., J.X. and G.L. performed the research, analyzed the data, and drafted the manuscript. Y.D., L.Z., X.S., J.P., Z.W., G.S., J.L. and L.K. contributed to the collection of samples and detection of PCR. P.T., G.L. and J.X. revised the manuscript. J.X. conceived the study, carried out additional analyses and finalized the manuscript. All authors have contributed to the editing of the manuscript. The authors have also read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its additional files. Sequences of three porcine kirV strains generated in this study have been submitted to GenBank under accession nos. MW504209-MW504211.
Conflict of interest The authors declare no conflict of interest to be disclosed for the present study.

Ethics approval All experimental procedures involving animals were approved by the Animal Care and Use Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China under animal protocol number: 2020012, and performed according to the Animal Ethics Procedures and Guidelines of the Ministry of Agriculture of China. Anal samples of piglet were collected by the farm's veterinarian according to the approved procedures.

References


Figures

Figure 1

Predicted genome organization of the porcine kirV and the stem loop of the virus.
Figure 2

Phylogenetic reconstruction of the amino acid sequences of the Rep protein. Fixed circles indicate the porcine kirV detected in this study.

Supplementary Files
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- TableS1.docx