Detection of pigeon circoviruses in ticks of sheep and camels in Inner Mongolia, China

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Abstract: Circovirus is one of the smallest known DNA viruses infecting animals. In this report, circovirus was detected in two tick species- *Hyalomma asiaticum* and *Dermacentor nuttalli* collected from sheep and camels in Inner Mongolia, China by high-throughput sequencing. The reverse semi-nested PCR assay revealed a complete genomic sequence of 2042 base pairs of cyclic single-stranded DNA (ssDNA). Evolutionary analysis further revealed that it belonged to circovirus with two major open read frames (ORFs) that putatively encode replicase and capsid proteins, respectively. Sequence and phylogenetic analysis suggested that the circovirus belonged to pigeon circovirus. The results demonstrated the presence of pigeon circovirus in ticks in Inner Mongolia, which provide the basis for future exploration of the prevalence of pigeon circovirus in Inner Mongolia and the pathogens carried by ticks in Inner Mongolia.

Key words: Circovirus; pigeon circovirus; ticks; phylogenetic analysis

Introduction

Taxonomically, circovirus is a small, nonenveloped, single-stranded DNA virus with a circular genome, which is categorized into the genus *Circovirus* of the *Circoviridae* family (Rosario *et al.* 2012). Circoviral genomes range from 1.8 to 2.1 kb in size and contain two open reading frames that encode the replication (Rep) and capsid proteins (Cap) (Breitbart *et al.* 2017). Vertebrates, including birds, poultry and mammals, are natural hosts of circovirus. Circovirus infection may cause diseases in animals (Delwart and Li 2012). For example, porcine circovirus type 2 (PCV2) has been associated with various disease syndromes in pigs, primarily postweaning multisystemic wasting syndrome (PMWS) (Allan and Ellis 2000). Other symptoms in sows include abortion, dermatitis, and nephropathy syndrome (de Castro *et al.* 2012; Zhai *et al.* 2014; Kekarainen and Segalés 2015). Canine

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circovirus infection has also been reported to cause hemorrhagic enteritis that was associated with a sudden onset of weakened appetite, vomiting, and bloody diarrhea in dogs(Hsu *et al.* 2016; Dowgier *et al.* 2017). Similarly, avian circoviruses are related to symptoms of immunosuppression and feather abnormalities in birds or poultry(Todd 2000; Shearer *et al.* 2008; Zhang *et al.* 2009; Stenzel and Koncicki 2017).

Although most circoviruses currently cannot readily be cultured in vitro, with the exception that porcine circovirus (PCV) is able to grow in PK-15 cells(Stevenson *et al.* 1999), an increasing number of novel circovirus-like genomes have been discovered from wild mammals and insects using high-throughput sequencing techniques(Ge *et al.* 2011; Marton *et al.* 2015). Among them, however, there are few reports of circovirus in ticks, the second most common vectors of pathogens, despite porcine circovirus and raven-related circovirus being discovered in ticks(Wang *et al.* 2018; Franzo *et al.* 2019). To explore whether the ticks in Inner Mongolia autonomous region were the vectors of circovirus, we tried to detect circular ssDNA genomic sequences from ticks collected from sheep and camels in this location by high-throughput sequencing technology and the reverse semi-nested PCR.

Materials and methods

A total of 2,662 ticks were collected from sheep and camels in the Alxa Left Banner, Alxa Right Banner and Siziwang Banners of Inner Mongolia, China, from April to May 2016. For each sampling site, three flocks of sheep or camels were selected, and more than 50 animals in each flock were randomly selected for sampling. Ticks on the skin surface of animals were caught and collected in a container. All samples were frozen and stored at -80 °C. The collected ticks were pooled according to the hosts they were collected from and the species of ticks. Approximately 15 ticks were pooled in one sample for processing and analysis. To prepare samples, ticks of each pool were put into a 2-mL centrifuge tube containing 1 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) prior to being homogenized in a frozen grinder (Jingxin, Shanghai, China). The homogenized samples were then centrifuged at 4 °C and 12,000× g for 10 min. The supernatants were collected, and total RNA was extracted from tick pools with an RNeasy Mini Kit (QIAGEN, Hiden, Germany), followed by cDNA synthesis using M-MLV reverse transcriptase (TaKaRa Biotechnology, Dalian, China). The qualified cDNAs were subjected to high-throughput sequencing for viral metagenomic analysis by the Beijing Genome Institute (BGI, Shenzhen, China).

After removing host-derived sequences, 3,568,561 reads with an average length of 145 nt were obtained, of which 12,931 (0.004%) were annotated to viruses of 20 families and some unclassified viruses, including dsDNA, ssDNA, and ssRNA viruses (Supplemental Fig. 1). According to the sequencing results, the DNA of each tick pool was extracted using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China), and the fractional genes of circovirus were determined by PCR using the primers TiCV-F1, TiCV-F2 and TiCV-R (Supplemental Table 1). The viral metagenomic results were also corroborated by a PCR assay. To obtain the complete sequence of circovirus, primers for amplifying the complete genome were designed according to the results of high-throughput sequencing (Supplemental Table 2). The PCR was performed in a 50 µL reaction system containing 1 µL of genomic DNA as a template from each pool sample, 25 µL of 2× Phabta Max Master Mix (Vazyme, Nanjing, China), 20 µL of ddH₂O, and 2 µL each of the forward and reverse primers (10 µM). The PCRs were conducted in a thermal cycler (Analytik Jena, Thuringia, Germany) using the following

thermocycling conditions: predenaturation at 95°C for 3 min, followed by 35 cycles (denaturation for 15 s at 95°C, annealing for 15 s at 57°C, and extension for 15-47 s at 72°C), and a final incubation for 5 min at 72°C. The PCR products were analyzed by electrophoresis using a 1.0% agarose gel stained with ethidium bromide.

All amplified products were analyzed by Sanger sequencing (ComateBio, Changchun, China). Nucleotide sequence homology was performed by BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>). All the sequencing results were assembled and aligned using CLUSTAL W in MEGA 7.0 (<http://www.megasoftware.net/>) and compared with the sequences of other circovirus strains in GenBank. A phylogenetic tree was constructed with the maximum-likelihood method by MEGA 7.0 software. The reliability of the branches of the tree was assessed using a bootstrap analysis with 1,000 replicates.

Results

All samples were screened for the presence of circovirus by half-nested PCR. The distribution of circovirus in these three regions was assessed, and circoviral DNA was detected in tick samples from Alxa Left Banner and Siziwang Banner but not in samples from Alxa Right Banner. The positive percentages of circoviral DNA were 0.093% in ticks from sheep and 1.268% in ticks from camels in Alxa Left Banner, Inner Mongolia and 3.734% in ticks from sheep in Siziwang Banner, Inner Mongolia. The positive rate was determined by calculating the minimum infection rate (MIR), which was the ratio of the number of positive tick pools to the total number of analyzed ticks (Gu *et al.* 2003). The analysis data are listed in Table 1.

The complete sequences of circoviruses recovered by half-nested PCR using the specific primer sets based on Sanger sequencing revealed a circular viral genome of 2,042 nucleotides (nt) in ticks. The genomic sequence obtained in this study was submitted to GenBank with accession number MN920392.

To explore the evolutionary relationship between this circovirus and other circoviruses, the complete genomic sequences and ORFs of circoviruses were used for phylogenetic tree mapping and homology analysis. In this report, genome sequences of 19 members of this family were analyzed. The phylogenetic analysis of the complete viral genome showed that this virus was close to pigeon circovirus (PiCV) (Fig. 1 A). In addition, the phylogeny also suggested that tick circovirus (TiCV) has a common ancestor with the evolutionary branches of starling circovirus (StCV), raven circovirus (RaCV), finch circovirus (FiCV) and zebra finch circovirus. Except for chimpanzee feces-associated circovirus (accession no. GQ404851), mammalian and avian circoviruses were relatively independent of the evolutionary tree. The phylogenetic trees of Rep and Cap were consistent with the whole genome (Fig 2).

Through comparison of the TiCV genome with those of other circoviruses, a high identity of 93.5% to 95.5% with the genome of PiCV and a low identity (42.7.0% to 59.8%) with duck circovirus (DuCV), goose circovirus (GoCV), gull circovirus (GuCV), RaCV, FiCV and StCV were found (Supplemental Table 2). There were two large ORFs for this circovirus, which encode the replication-associated proteins (Rep) and the viral capsid protein (Cap). The intergenic region between the two ORFs could

form a potential stem-loop structure, according to the mfold web server (Fig. 1 B). The putative Rep protein of TiCV was 315 amino acids (aa), and it had a high identity with the Rep proteins of FiCV (75.2%), RaCV (74.5%), StCV (73.3%), and PiCVs that were isolated from China (96.1%) and Brazil (97.1%). It had a low identity with those of GuCV (61.3%), human stool-associated circovirus (55.9%), DuCV (50.9%), and GoCV (51.9%) (Table 2). The putative Cap protein of TiCV was 273 aa and shared a higher identity with that of PiCVs from China (98.9%) and Brazil (98.6%) than that of with other circoviruses, including FiCV (44.9%), Tick circovirus (41.0%), and StCV (60.1%) (Table 2).

Discussion

Ticks are efficient vectors for the transmission of pathogens, including many arboviruses. To our knowledge, the identified tick-borne viruses include members of two orders, nine families and at least twelve genera (Shi *et al.* 2018). The infection of most of these tick-borne viruses may cause diseases in humans or animals (Vandegrift and Kapoor 2019). With a vast territory and well-balanced ecological environment in Inner Mongolia, it relies heavily on the animal husbandry industry. In this report, we attempted to detect circular ssDNA genomic sequences from ticks collected from sheep and camels in Inner Mongolia, China, by high-throughput sequencing technology.

Notably, genomic DNA of PiCV was detected in ticks parasitized on sheep and camels at two remote sampling sites in Inner Mongolia, suggesting that PiCV exists in ticks in Inner Mongolia. Interestingly, however, the ticks were collected from sheep and camels herding on the desert steppe or meadow grassland in Inner Mongolia, where no pigeons live.

In addition, circoviruses may be able to transmit between different species. It has been reported that a PiCV was detected in chicken and that a PCV was detected in cow or calf samples (Li *et al.* 2010; Li *et al.* 2011). However, there is no direct evidence that this virus can infect ruminants, such as sheep or camels. These findings suggest that we can establish a serological detection method using antibodies against circovirus in further research, combined with nucleic acid detection methods, which may solve this problem. Notably, the role played by ticks in the transmission route of circovirus is currently uncertain. Whether ticks are used as a source of transmission to spread the virus to the host or as an intermediate host from an infected animal to a tick is worthy of additional experiments, such as a serological detection of circovirus, for further validation (Estrada-Pena and de la Fuente 2014). Nevertheless, the existence of genomic DNA of PiCV was detected in ticks of sheep and camels in Inner Mongolia, China.

Conclusions

In this study, we detected for the first time that the PiCV was found in ticks of sheep and camels in Inner Mongolia, China. Sequence comparison and phylogenetic analysis further identified that the new circovirus sequence belongs to PiCV and was detected in *Hyalomma asiaticum* and *Dermacentor nuttalli*. This study thus made a preliminary report on the genome structure and epidemic situation of circovirus in ticks of sheep and camels in Inner Mongolia, China, for the first time.

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Author contributions YL designed the research. YYK, CY, GZ, YRC and BH helped with the experiments, data analysis, and discussion. YYK and YL wrote the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest with respect to the data, authorship, or publication of this article.

Ethics approval and consent to participate

The experiments involving sheep and camels were performed according to protocols approved by the Institutional Animal Care and Use Committee of Ningxia University (NXU-2019-007). The need for consent is deemed unnecessary according to national regulations, but an informed verbal consent was obtained from the sheep and camel owners. The ethics committee for the use of animals of Ningxia University approved this study.

Informed consent

Informed consent was obtained from all individual participants in the study.

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Supplemental Table 1. Oligonucleotide primers for amplifying the complete genome of circovirus in ticks

Primers	Sequence (5'→3')	Location	Interest fragment (bp)
TiCV-F1	ACAGCTGAGTCTGGGTCTATA	1132-1152	465
TiCV-F2	GTCACATACATGCATGAGTTC	1185-1205	
TiCV-R	AGACCACTCGGAACAGAAATC	1629-1649	
TiCV-F3	GTCACATACATGCATGAGTTC	1185-1205	465
TiCV-R1	CAGAAAGTGGGATCTGAGAAA	1501-1521	
TiCV-R2	AGACCACTCGGAACAGAAATC	1629-1649	
TiCV-F4	TTTCTCAGATCCCACTTTCTG	1501-1521	1,577
TiCV-F5	GATTTCTGTTCCGAGTGGTCT	1629-1649	
TiCV-R3	GAACTCATGCATGTATGTGAC	1185-1205	

Supplemental Table 2. Full-length sequence comparison of tick circoviruses with others

Viral name	GenBank accession number	TiCV
		nt (%)
Porcine circovirus 1	AF071879	42.7
Goose circovirus	AJ304456	43.3
Porcine circovirus 2	AY424401	42.5
Duck circovirus	DQ100076	42.7
Raven circovirus	DQ146997	57.4
Finch circovirus	DQ845075	57.3
Starling circovirus	DQ172906	59.8
Gull circovirus	DQ845074	52.1
Swan circovirus	EU056309	42.7
Chimpanzee feces-associated circovirus	GQ404851	65.3
Human stool-associated circular virus	GQ404856	39.9
Barbel circovirus	GU799606	42.1
Bat associated circovirus 1	JX863737	42.5
Canine circovirus	KC241983	43.2
Bat associated circovirus 2	KC339249	42.6
Mink circovirus	KJ020099	42.2
Zebra finch circovirus	KP793918	57.1
Tick circovirus	KX987147	56.5
Pigeon circovirus-PL44B	KF738858	94.7
Pigeon circovirus-zjl	DQ090945	93.5
Pigeon circovirus- SLMA4	MF664482	95.5

Supplementary Fig. 1 Numbers of reads annotated to viruses classified by viral genus, family and nucleic acid group.

Table 1 Molecular detection of circovirus from ticks isolated from different regions in Inner Mongolia, China

Areas of Sampling	Coordinates	Host species	Tick species	Number of ticks	Number of pools	Number of positive pools	Positive rate (%)
Alxa Left Banner	N39°02'36.68"	Sheep	<i>Hyalomma asiaticum</i>	1,075	72	1	0.093
	E104°38'36.04"	Camels	<i>Hyalomma asiaticum</i>	946	63	12	1.268
	E105°41'25.04"		<i>Hyalomma asiaticum</i>	367	25	0	0
Alxa Right Banner	N40°20'46.14"	Camels	<i>Dermacentor nuttalli</i>	33	2	0	0
	E104°23'3.74"		<i>Dermacentor nuttalli</i>	241	16	9	3.734
Siziwang Banner	N41°37'36.69"	Sheep	<i>Dermacentor nuttalli</i>				
	E112°01'25.72"						

Table 2 Comparison of amino acid and nucleotide sequence identity between tick circovirus and other circoviruses

Viral name	GenBank accession number	Rep segment of TiCV		Cap segment of TiCV	
		nt (%)	aa (%)	nt (%)	aa (%)
Porcine circovirus 1	AF071879	49.4	43.5	37.7	39.7
Goose circovirus	AJ304456	54.2	51.9	40.5	35.4
Porcine circovirus 2	AY424401	48.8	43.8	37.7	39.3
Tick circovirus	KX987147	56.6	71.8	49.4	41.0
Duck circovirus	DQ100076	56.2	50.7	36.4	31.9
Raven circovirus	DQ146997	72.8	74.5	54.6	48.3
Starling circovirus	DQ172906	72.2	73.3	62.5	60.1
Gull circovirus	DQ845074	64.9	61.3	52.2	56.4
Finch circovirus	DQ845075	74.2	75.2	54.5	44.9
Swan circovirus	EU056309	53.4	51.2	40.3	37.7
Chimpanzee feces-associated circovirus	GQ404851	72.4	74.8	63.2	66.5
Human stool-associated circular virus	GQ404856	53.1	55.9	32.9	30.4
Barbel circovirus	GU799606	51.6	51.2	37.3	34.2
Bat associated circovirus 1	JX863737	51.3	47.8	36.6	36.6
Canine circovirus	KC241983	53.7	45.7	29.4	23.3
Bat associated circovirus 2	KC339249	48.6	44.1	38.4	42.4
Mink circovirus	KJ020099	52.9	47.8	34.5	38.1
Zebra finch circovirus	KP793918	71.6	75.3	52.5	54.9
Pigeon circovirus-zj1	DQ090945	94.9	96.0	95.4	98.9
Pigeon circovirus-PL44B	KF738858	94.7	96.6	95.6	98.0
Pigeon circovirus- SLMA4	MF664482	94.6	97.1	95.8	98.6

Fig. 1 Phylogenetic tree and schematic representation of circovirus in ticks. A. Phylogenetic tree of the tick circovirus (TiCV) complete sequence. The tree was constructed using the maximum-likelihood method with MEGA 7.0 software. Bootstrap testing (1,000 replicates) was performed, and values lower than 60% are not shown. The bar at the bottom of the figure denotes the distance. B. Genome map of TiCV showing the ORFs encoding Rep and Cap.

Fig. 2 Phylogenetic tree of Tick circovirus (TiCV) based on Rep and Cap sequences