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Detection of co-infection and phylogenetic analysis of Mink circovirus and Aleutian mink disease virus in minks, foxes and raccoon dogs in northern China

Xue Leng[1], Chenyan Sheng[2], Tingting Wang[2], Jian Liang[2], Yu Song[2], Xiaolin Wang[2], Siddique Sehrish[2], Jianming Li[1], Kun Shi[1], Qinglong Gong[2] and Rui Du[1*]

[1]College of Chinese Medicine Materials, Jilin Agricultural University, Changchun, China
[2]College of Animal Science and Technology, Jilin Agricultural University, Changchun, China

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ABSTRACT

Mink circovirus (MiCV) and Aleutian mink disease virus (AMDV) have severely affected the reproductive performance of mink and caused significant economic losses to fur-bearing animal farms. To date, there are no effective vaccines or drugs to prevent and control these two diseases. To understand the current co-infection and evolution of MiCV and AMDV in minks, foxes, and raccoon dogs, a TaqMan-based real-time duplex fluorescent quantitative PCR assay for MiCV and AMDV was developed. In addition, a total of 18 samples from minks, fox- es and raccoon dogs with mixed MiCV and AMDV infection were selected for genetic evolution analysis of the MiCV Cap gene and the AMDV VP2 gene. The detection limits of the real-time duplex fluorescent quantitative PCR assay for both MiCV and AMDV were 1.0×101 copies/μL, with higher sensitivity than conventional PCR, and no specific fluorescence signals of other non-target pathogens were detected. The intra- and inter-batch coef- ficients of variation were less than 2%. The total detection rates of 213 samples from minks, foxes and raccoon dogs were 46.0% (98/213) and 55.4% (118/213) for MiCV and AMDV, respectively, and 31.9% (68/213) for mixed infection. The MiCV Cap gene was relatively conserved, while the AMDV VP2 gene had more variants, which mainly occurred in the VP2 virulence-related loci and hypervariable region. Phylogenetic analysis of the MiCV Cap gene and the AMDV VP2 gene showed that the phylogenetic clusters were not formed on the basis of species or isolation time, but had a clear relationship with geographic origin. This study enriches the epidemio- logical data on MiCV and AMDV. It provides an important reference for the prevention and control of MiCV and AMDV in China.

Key words: Mink circovirus (MiCV); Aleutian mink disease virus (AMDV); real-time duplex quantitative PCR; co-infection; phylogenetic analysis

* Corresponding author:
Rui Du, College of Animal Science and Technology, Jilin Agricultural University, Changchun, 130118, China, e-mail: durui197101@sina.com

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Introduction

Mink circovirus (MiCV) is a circovirus discovered in 2013, associated with intestinal dis- ease (LIAN et al., 2014), and it is now generally accepted that consequent viral infections are predominantly lymphatic depletion and immunosuppression, even with the risk of secondary infection (TODD, 2000). Mink circovirus invades mink and causes intractable diarrhea, which mainly manifests as red and white diarrhea in mink, with lethargy, anorexia, dehydration, and poor fur quality, and even leads to death. Mink circovirus has been found to be prevalent in most mink-rearing areas in China, and the trend is increasing yearly. The prevalence of mink circovirus has seriously affected the reproductive performance of mink and caused great economic losses to the mink farming industry (GE et al., 2018a). Some studies have shown that mink circovirus can also infect foxes and raccoon dogs with a high infection rate (YANG et al., 2018).

Aleutian mink disease virus (AMDV) was reported in the United States in the 1990s. The virus causes high levels of non-neutralizing antibodies to form immune complexes and be deposited in the body tissues of the blue-gray Aleutian mink species, with enlargement of the kidneys, spleen, and lymph nodes in infected minks (MARKARIAN and ABRAHAMYAN, 2021). The early clinical signs of AMDV infection in farmed minks are loss of appetite and weight loss, followed by reduced activity and coat thinning, which can even lead to sterility and abortion (ALEXANDERSEN et al., 1988). Mink with AMDV may appear clinically healthy, but are usually less fertile (REICHERT and KOSTRO, 2014), and clinical signs of advanced disease are weakness, irritability, anemia, and diarrhea (EKLUND et al., 1968). Currently, AMDV is widely prevalent in most mink breeding countries around the world, including Sweden, Denmark, Ireland, the Netherlands, Finland, Canada and China. Furthermore, AMDV can also infect different species such as raccoons, otters, foxes, raccoon dogs, skunks, dogs, and cats, which may act as hosts to facilitate the spread of AMDV (JEPSEN et al., 2009; LEIMANN et al., 2015; ZALESKA-WAWRO et al., 2021).

Unfortunately, there are no approved vaccines or specific drugs for the control of MiCV and AMDV. To prevent mink circovirus, farmers have used a homemade inactivated vaccine, prepared by formalin-inactivating the tissue suspension supernatant from infected mink. While this approach initially reduced the incidence of the disease, immunized mink eventually developed MiCV symptoms after a period of application (AASTED et al., 1998; WANG et al., 2021).

At present, no method for simultaneous AMDV and MiCV detection has been established, and there are only a few molecular epidemiological reports on these two viruses (GE et al., 2018b; TONG et al., 2020). In China, the provinces of Shandong, Jilin, and Heilongjiang are recognized as the primary regions for breeding fur-bearing animals. To better understand MiCV and AMDV infections, as well as the genetic diversity and relationships between different lineages of MiCV and AMDV in minks, foxes, and raccoon dogs in China, a real-time dual quantitative PCR assay was developed for detecting MiCV and AMDV. Additionally, the genetic evolution of MiCV and AMDV was analyzed separately.

Materials and methods

Viruses and samples. The MiCV HB3 strain (GenBank accession No. MK561562), AMDV LN1 strain (GenBank accession No.MF362625), Pseudorabies virus (PRV) JL-3 strain and Porcine circovirus 2 (PCV-2) were stored in the economic animal infectious diseases laboratory of Jilin Agricultural University. The vaccine strains of mink enteritis virus (MEV), canine distemper virus (CDV) and canine adenovirus type 2 (CAdV-2) were purchased from Teyan Biotechnology Ltd., Jilin, China and QiLu Animal Health Products Ltd., Shandong, China. A total of 213 tissue samples (including spleen, liver and kidney) from 91 minks, 65 foxes and 57 raccoon dogs were collected from 6 fur-bearing animal farms in Jilin, Heilongjiang and Shandong Provinces, during the pelting season in 2022. Precautions were taken during sample collection to avoid cross-contamination of samples. All samples were stored at -80°C immediately after being transported back to the laboratory at low temperature.

Primer design and synthesis. All MiCV Cap gene sequences and AMDV VP2 gene sequences were retrieved from GenBank. Multiple sequence alignments were performed using DNAMAN (LynnonBiosoft, USA) to identify highly conserved regions within the MiCV Cap gene and the AMDV VP2 gene. Primer premier 5.0 (PREMIER Bio- soft International, Palo Alto, CA, USA) was used to design the required primers (duplex real-time quantitative PCR primers and common PCR primers) and probes (Table 1). The primers and probes were synthesized by Comate Bioscience Co. Ltd., (Changchun, China).

DNA extraction. All samples were repeated- ly frozen and thawed three times and centrifuged according to the manufacturer's protocol. A viral DNA Kit (Omega Biotek, USA) was used to extract DNA from 200 μL supernatant. After DNA was eluted with 100 μL elution buffer, it was stored at -20°C.

Establishment of duplex real-time quantita- tive PCR. The 684 bp fragment of the MiCV Cap gene was amplified from the DNA of the MiCV HB3 strain by PCR using MiCV-Cap-F and MiCV- Cap-R, the 530 bp fragment of the AMDV VP2 gene was amplified from the DNA of the AMDV LN1 strain by PCR using AMDV-VP2-F and AMDV-VP2-R. The 25 μl amplification system for the MiCV Cap gene and the AMDV VP2 gene were all as follows: 12.5 μl of Ex Taq DNA polymerase (Takara Biomedical Technology, Dalian, China), 10.5 μl of ddH_2O, 0.5 μl of MiCV-Cap-F/AMDV-VP2-F (10 mM), 0.5 μl of MiCV-Cap-R/AMDV-VP2-R (10 mM), and 1 μl of the template. The PCR procedure consisted of 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. A Midi Purification Kit (Tiangen Biotech, Beijing, China) was used to purify the amplification products from agarose gel, and they were then ligated into the vector pMD18-T (Takara Biotechnology, Dalian, China) to construct recombinant plasmids of pMD-MiCV and pMD-AMDV, respectively, following the manufacturer's instructions. pMD-MiCV and pMD-AMDV were purified using a Plasma Miniprep Kit (Axygen A Corning Brand, Suzhou, China), and verified by a sequencing service (Comate Bioscience, Changchun, China), respectively. The original concentrations of the target plasmids pMD-MiCV and pMD-AMDV were 210.47 ng/μL and 247.07 ng/μL which were

Table 1. Primers and probes used in this study

Virus	Name	Sequence (5' to 3')	Length (bp)	Position[a]	Purpose
MiCV	MiCV-F	AGGGCCTTTGGGCATCATTG	107	1473-1579	qPCR
	MiCV-R	CCCGCCTGCAAACTGAAGAA			
	MiCV-Cap-F	TTAAGTTTGCTTTGGGAAATTGACT	684	1020-1703	Ordinary PCR
	MiCV-Cap-R	ATGCCCGTAAGATCGCGATACTCGC			
	MiCV-P	FAM-ACGGAGTTGCTGCAGATGCCACGGT-TAMR	-	1529-1553	Probe
AMDV	AMDV-F	GGAAGAAGAAGGTTGGC	166	3685-3850	qPCR
	AMDV-R	AACTGTTGGTTGGTATGAG			
	AMDV-VP2-F	GGACCAAGACAGTGGAGTGACACAA	530	3440-3969	Ordinary PCR
	AMDV-VP2-R	GGAGGGTTGTTTTTACATACAAATG			
	AMDV-P	ROX-TGCTGCTTCAGGCACACA-BHQ2	-	3702-3720	Probe

[a]Position: The positions of primers and probes represent the start and end positions of nucleotides in MiCV and AMDV (refer to the sequence positions of MK561562 and KU513985 in GenBank)

measured using a Nanodrop One spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

According to the formula: the copy number of plasmid (copies/μL) = [concentration of plasmid (ng/μL) × (6.02×10^{23})]/ [(plasmid length ×660×10^{9})], the copy numbers of pMD-MiCV and pMD-AMDV were 5.68×10^{10} copies/μL and 7.00×10^{10} copies/μL. The recombinant positive plasmids of pMD-MiCV and pMD-AMDV were respectively diluted with elution buffer to a concentration of 1.0×10^{10} copies/μL, then stored at -20°C.

The standard plasmid mixtures of pMD-MiCV and pMD-AMDV were serially diluted 10-fold using an elution buffer, and the plasmids with dilution concentrations ranging from 1.0×10^{1} copies/μL to1.0×10^{6} copies/μL were used as templates to establish standard curves in triplicate for duplex qPCR detection. The logarithm of the plasmid copy number was plotted against the measured cycle threshold (Ct). The standard curve, the cor- relation coefficient of the standard curve and ef- ficiency were calculated using GraphPad Prism 7 software. DNA quantification for each sample was expressed as copy number/reaction number. The fi- nal reaction volume was 20 μL including 10 μL of TB Green® Premix Ex Taq™ II (Takara Biomed- ical Technology, Beijing, China), 0.8 μL each of MiCV-F and MiCV-R, 0.4 μL MiCV-P, 0.8 μL each of AMDV-F and AMDV -R, 0.8 μL AMDV-P, 2 μL of template and 3.6 μL of ddH_2O. qPCR reactions were performed in 8 tubes with fluorescent qPCR qTOWER3 G (Analytik Jena AG, Germany). The procedure was as follows: incubation at 95°C for 3 minutes, followed by 45 cycles of amplification at 95°C for 15 s and 57°C for 45 s.

To determine the absence of cross-reactivity of the established method with other pathogens, DNA or cDNA of MiCV, AMDV, PCV-2, MEV, CDV, CAdV-2 and PRV were used as templates for duplex real-time PCR reactions for specificity evaluation. Sterile water was used as the negative control.

To determine the detection limit and efficiency of the assay, the standard plasmid DNA of pMD-MiCV and pMD-AMDV were subjected to 10-fold serial dilutions (from 1.0×10^{0} copies/μL - 1.0×10^{5} copies/μL) as templates for duplex qPCR reactions and conventional PCR reactions, respectively. The conventional PCR reaction was performed using the protocol described above with the primer pair of MiCV-Cap-F and MiCV-Cap-R, AMDV-VP2-F and AMDV-VP2-R. In addition, the diagnostic sensitivity of the duplex qPCR was tested by the positive clinical samples verified by sequencing.

Using pMD-MiCV and pMD-AMDV mixed plasmids at concentrations of 1.0×10^{2} copies/μL, 1.0×10^{4} copies/μL and 1.0×10^{6} copies/μL as templates, three replicates of each sample were made and the test was repeated three times under the same conditions to evaluate the intra-and inter-assay repeatability and stability in the qPCR method. The coefficient of variation (CV) was calculated according to the formula CV = (SD [Ct value]/ overall average [Ct value]) × 100.

Detection of MiCV and AMDV in clinical samples. Nucleic acids were extracted from 213 tissue samples collected from fur-bearing animal farms in northern China, where minks, foxes, and raccoon dogs were being raised together. The extraction was performed using magnetic bead grinding and lysis with a Viral DNA Kit (Omega Biotek, USA). The positive rates of MiCV and AMDV in the samples were then analyzed using the duplex real-time PCR method established in this study.

Sequencing and phylogenetic analyses. Following duplex real-time PCR, a total of 18 samples from minks, foxes, and raccoon dogs from different farms with mixed infections and small Ct values in the assay were selected. The Cap gene of MiCV was amplified using the primers MiCV-Cap-F and MiCV-Cap-R (Table 1). The VP2 gene of AMDV was amplified following a previously established method (LIU et al., 2018). The amplified PCR products were purified using an Agarose Gel DNA Purification Kit (Axygen, USA) according to the manufacturer's instructions, and cloned into the pMD-18T vector (Takara) for sequencing (Comate Bioscience Co., Ltd., Changchun, China). For each fragment, three clones were sequenced in both directions. The sequencing results were compared for homology using the ClustalW Method in the MegAlign program of DNAStar software (DNAstar, Inc., Madison, Wisconsin, USA). The phylogenetic tree was constructed using the Neighbor Joining

method in the program MEGA7 (SUDHIR et al., 2016), and the confidence levels of the branches were assessed using bootstrap analysis with 1000 replications (SAITOU and NEI, 1987). The reference information of the Cap genes of MiCV and VP2 genes of AMDV are shown in Table 2 and Table 3, respectively.

Results

Standard curve of the duplex qPCR. The mixed plasmids of pMD-MiCV and pMD-AMDV were diluted 10-fold (1.0×10^1 copies/μL-1.0×10^6 copies/μL), and the MiCV and AMDV duplex qPCR reactions were performed to establish a standard curve (Fig.1). The MiCV standard equation was y=-3.438x+37.501, the correlation coefficient (R^2) was 0.9984, and the amplification efficiency was 95%. The AMDV standard equation was y=-

Table 2. Details of the MiCV strains included in the phylogenetic analysis reported in this study

Strain	Reference	Country	Isolation year
DL16-6	KY362745	China	2016
DL-16	KY320498	China	2016
HB3	MK561562	China	2015
DL-13	NC_023885	China	2013
JL28	MG001457	China	2015
LN19	MG001456	China	2016
SD16	MG001455	China	2017
HL24	MG001454	China	2016
HB18	MG001453	China	2016
HL6	MG001452	China	2015
HEB15	KX268345	China	2015
Hebei13	KM586846	China	2013

Table 3. Details of the AMDV strains included in the phylogenetic analysis reported in this study

Strain	Reference	Country	Isolation year
G	M20036	USA	Late 1970s
Utah	Z18276	USA	Late 1970s
TR	U39013	USA	1995
Utah1-Kit	U39015	USA	1995
Pullman	U39014	USA	1995
SL3	X97629	Germany	Early 1980s
Far East	DQ371395	Russia	2006
FIN	GQ336866	Finland	2009
LN2	GU183265	China	2009
LN3	GU269892	China	2009
Beijing	KT329428	China	2015
LN1	MF362625	China	2017
HLJ1	MF362627	China	2017
G#1	KU513985	Denmark	2012
M173	KT878958	Canada	2014
FMN	MT770839	Canada	2020

3.5797x+38.074, the correlation coefficient (R^2) was 0.9974, the amplification efficiency was 90%.

Specificity, sensitivity, and reproducibility of the duplex qPCR. The specificity of the established duplex qPCR method was evaluated using DNA or cDNA of MiCV, AMDV, PCV-2, MEV, CDV, CAdV-2, and PRV. The results indicated that only the detected MiCV and AMDV gave amplification products. Under the same conditions, PCV-2, MEV, CDV, CAdV-2 and PRV did not provide positive reactions.

The mixed standard plasmids pMD-MiCV and pMD-AMDV, at concentrations of 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL, were selected as templates for sensitivity evaluation. The minimum detection limits (LODs) of duplex qPCR for both MiCV and AMDV were 1.0×10^1 copies/μL (Fig. 2), and the sensitivity of LOD was 100 times higher than that of conventional PCR. The minimum detection limits of conventional PCR for both were 1.0×10^3 copies/μL. For the positive clinical samples, 23 out of 23 positive samples were verified as positive by duplex qPCR assay.

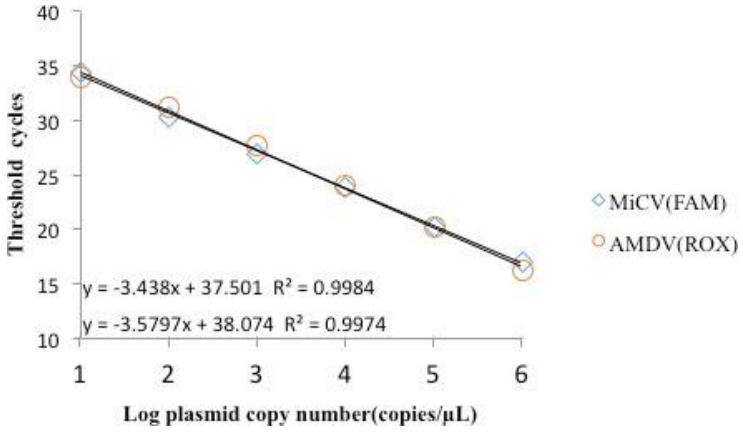

Fig. 1. Standard curves of the duplex qPCR assay for MiCV and AMDV

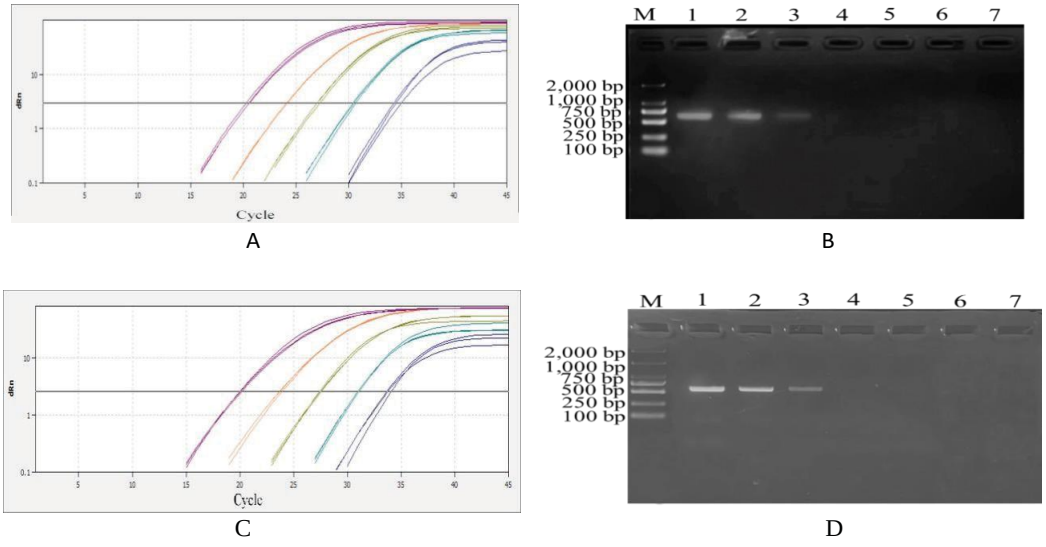

Fig. 2. Sensitivity of the duplex qPCR assay for MiCV and AMDV

(A) Sensitivity of the duplex qPCR assay for MiCV (FAM channel); (B) Sensitivity of the conventional PCR assay for MiCV; (C) Sensitivity of the duplex qPCR assay for AMDV (ROX channel); (D) Sensitivity of the conventional PCR assay for AMDV. The template concentrations were 1.0×105 copies/μL, 1.0×104 copies/μL, 1.0×103 copies/μL, 1.0×102 copies/μL, 1.0×101 copies/μL and 1.0×100 copies/μL, and the negative control

Three dilutions of the mixed standard plasmids (10^2 copies/μL, 10^4 copies/μL and 10^6 copies/μL) were tested under the same conditions, and amplified in three parallel assays. The intra-assay coefficient of variation (CV) ranged from 0.34% to 0.75%, and the inter-assay CV ranged from 0.98% to 1.23%, indicating that the duplex qPCR method was highly reproducible.

Detection of MiCV and AMDV in minks, foxes, and raccoon dogs. When all 213 clinical samples were tested by duplex qPCR, the positive rates of MiCV were 51.6% (47/91), 43.1% (28/65) and 40.4% (23/57) in minks, foxes and raccoon dogs, respectively. The total positive rate was 46.0% (98/213). The positive rates of AMDV were 59.3% (54/91), 55.4% (36/65) and 49.1% (28/57) in minks, foxes, and raccoon dogs, respectively. The total positive rate was 55.4% (118/213). The co-infection rates for both viruses were 35.2% (32/91), 27.7% (18/65) and 31.6% (18/57) in minks, foxes and raccoon dogs, respectively, and the total positive rate was 31.9% (68/213) (Table 4).

Gene homology and phylogenetic analysis of the MiCV Cap gene. The nucleotide sequence length of the 18 newly sequenced MiCV Cap genes were all 685 bp, which was the same as the reference strains. The nucleotide homology of the MiCV Cap genes ranged from 97.8% to 100.0% among the isolated strains from minks, foxes, and raccoon dog s. Compared with other reference strains, the sequence homology of the circovirus Cap genes was 97.8%-99.9%, 98.2%-100.0%, and 98.5%-100% in minks, foxes, and raccoon dogs, respectively.

The alignment was built using the deduced amino acid sequences predicted from the Cap gene

Table 4. Detection of MiCV and AMDV in minks, foxes, and raccoon dogs

Infection	Mink	Fox	Raccoon dog	Total
MiCV	51.6% (47/91)	43.1% (28/65)	40.4% (23/57)	46.0% (98/213)
AMDV	59.3% (54/91)	55.4% (36/65)	49.1% (28/57)	55.4% (118/213)
MiCV+ AMDV	35.2% (32/91)	27.7% (18/65)	31.6% (18/57)	31.9% (68/213)

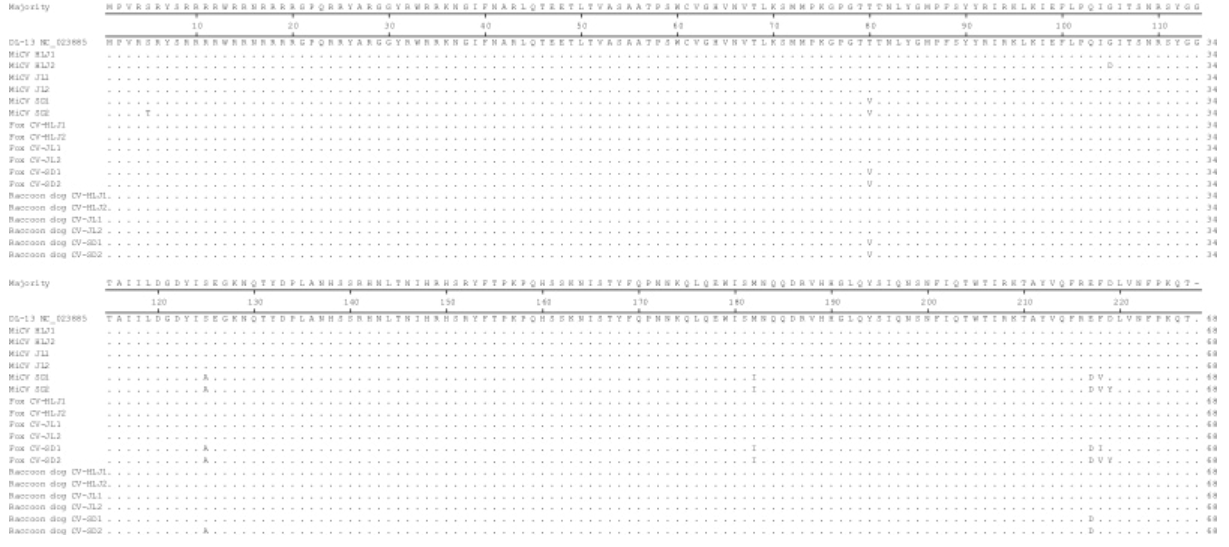

Fig. 3. Results of amino acid sequence alignment of MiCV Cap gene

Comparison of the amino acid sequences of the Cap proteins from different MiCV strain isolates. Amino acids with identical sequences are indicated by dots. The numbers at the top represent the amino acid positions of the MiCV Cap proteins

sequences from 18 newly sequenced MiCV strains and the compared DL-13 strain (Fig. 3). No deletion or insertion events were identified. No amino acid mutations were observed in the MiCV strains from minks, foxes, and raccoon dogs in Jilin and Heilongjiang provinces, except for MiCV HLJ2, which exhibited a single amino acid mutation (105G to 105D). However, there were 2 to 7 amino acid mutations that occurred in the isolated circovirus from Shandong province.

To understand the relationship between circovirus sequences detected in major fur-bearing animal breeding regions in China, the 18 sequenced circovirus Cap genes were subjected to genetic evolutionary analysis with reference strains (Table 2) using MEGA 7 software to draw a circovirus Cap gene phylogenetic tree (Fig. 4). All the MiCV strains were divided into three groups, designated as A, B, and C. The phylogenetic clusters were not formed on the basis of species or isolation time, but there was a clear relationship with geographic origin. The MiCV strains from Heilongjiang province were all in group A, the MiCV strains from Shandong province were all in group C but Raccoon dog CV-SD1. The MiCV strains from Jilin province were divided into groups A and B.

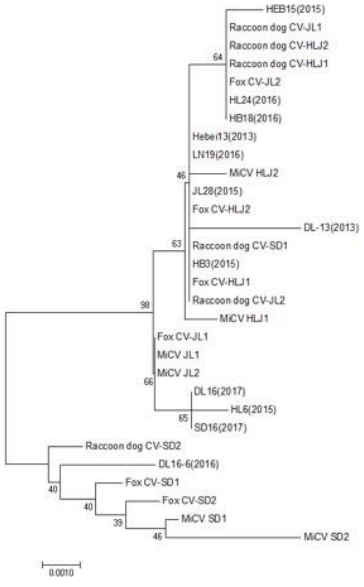

Fig. 4. Phylogenetic tree of MiCV isolates based on full-length fragment alignment of the Cap gene

The tree was constructed using the neighbor joining method with MEGA 7, and the virulent strains were divided into three groups

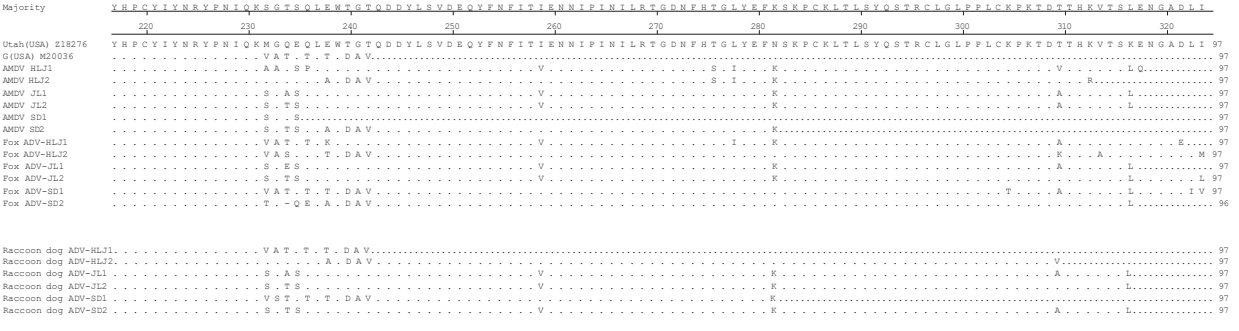

Fig. 5. Amino acid sequence comparison results of hypervariable region of the AMDV VP2 gene

In the comparison of amino acid sequences of the VP2 proteins isolated from different AMDV strains, amino acids with the same sequence are indicated by dots, the top number represents the amino acid position of the AMDV VP2 protein, and - represents the deletion of acid residues

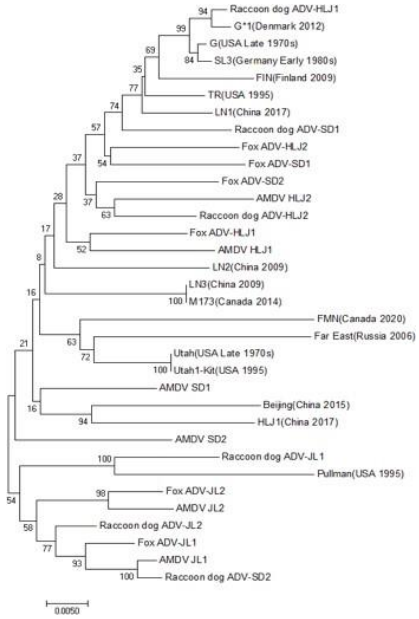

Fig. 6. Phylogenetic tree of ADV isolates based on full-length fragment alignment of the VP2 gene
The tree was constructed using the neighbor joining method with MEGA 7, and the virulent strains were divided into four groups

Gene homology and phylogenetic analysis of the AMDV VP2 gene. The nucleotide and amino acid sequences of the AMDV VP2 gene were obtained by sequencing. The full length of the open read- ing frame of the gene was about 1.945 bp, which was the same as that of the reference strains. The sequencing results of 18 samples were compared with the homology analysis of 16 domestic and foreign reference strains retrieved from GenBank. The AMDV VP2 nucleotide homology among the three species was 93.9%-99.5%. The AMDV VP2 gene nucleotide sequences of the strains isolated from minks, foxes, and raccoon dogs were 93.8%-97.1%, 94.2%-97.8%, and 93.3%-98.2% homologous to the reference strains, respectively.

The results of amino acid sequence homology showed that the AMDV VP2 gene variation was relatively obvious compared with AMDV Utah (Fig. 5). Approximately 15 to 31 amino acid variations were observed, including one single amino acid deletion (Gln) at position 234aa in Fox ADV-SD2. Of these, 8 different residues were found in an 11-amino-acid region (aa232–242), corresponding to the hypervariable region of the VP2 gene. Among them, the AMDV strains from Jilin province were different from other strains.

Phylogenetic analysis was performed using the full-length VP2 sequences from 18 strains from this study, and 16 reference strains from GenBank (Table 3). On the basis of the evolutionary tree, the AMDV sequences fell into four gene groups, designated A, B, C and D (Fig. 6). Phylogenetic clusters were not formed on the basis of species or isolation time, but had a clear relationship with geographic origin. The AMDV strains from Heilongjiang province were all in group A. The AMDV strains from Jilin province were all in group D and similar to the reference strain AMDV-Pullman. The AMDV strains from Shandong province were divided into groups A, B and D. There were no isolated strains or Chinese strains in group C.

Discussion

Currently, there are a few methods to detect MiCV or AMDV, mainly including polymerase chain reaction, recombinant enzyme polymerase amplification, indirect enzyme-linked immunosorbent method and the SYBR Green I real-time fluorescence quantitative PCR method (WANG et al., 2015; CUI et al., 2018; GE et al., 2018a). Although convective immunoelectrophoresis is the

"gold standard" for the detection of Aleutian virus in mink, it is prone to false positives or false negatives (CHRISTENSEN et al., 2011). MiCV and AMDV are able to co-infect, indicating that the infection may be exacerbated and disease control more difficult. However, there is no method to detect both viruses simultaneously. Therefore, in this study, a duplex qPCR assay was developed for the simultaneous detection of MiCV and AMDV.

Duplex fluorescent quantitative PCR is derived from fluorescent quantitative PCR and is able to detect two different target genes simultaneously in one reaction tube, with higher efficiency and lower cost (MACKAY et al., 2002). In this study, the TaqMan-based real-time dual fluorescence quantitative PCR assay was developed by optimizing the reaction system and conditions. For MiCV, the LOD of the duplex qPCR assay was 10 copies/ uL, which was the same as that of the nested PCR and RPA assays for MiCV (GE et al., 2018c). The method detected AMDV with a LOD of 10 copies/ ul, which was higher than the LOD of AMDV detection by TaqMan-based qPCR established by Wu (WU et al., 2020). The specificity of the method for the detection of MiCV and AMDV showed that there was no fluorescent signal detected for PCV-2, MEV, CDV, CAdV-2 or PRV. In addition, the response efficiencies of 95% and 90% for MiCV and AMDV, respectively, lie in the adoptable range between 90% and 105% (ZHENG et al., 2020).

To further understand the co-infection phenomenon of MiCV and AMDV, the 213 clinical samples were tested by the duplex qPCR established in this study. The positive rates of MiCV were 51.6% (47/91), 43.1% (28/65) and 40.4% (23/57) in minks, foxes and raccoon dogs, respectively. The positive rates of AMDV were 59.3% (54/91), 55.4% (36/65) and 49.1% (28/57) in minks, foxes and raccoon dogs, respectively. The total co-infection rate for both viruses was 31.9% (68/213). The results showed that the co-infection rate of MiCV and AMDV was high in minks, foxes and raccoon dogs in northern China. So, adequate attention should be paid to disease prevention and detection, controlling virus transmission, and culling positive animals at the time of retaining breeding animals.

Currently, no effective vaccines are availa- ble for MiCV and AMDV. To prevent the further spread and transmission of these diseases, it is essential to conduct studies on their genetic diversity and perform analytical epidemiological investigations (MARKARIAN and ABRAHAMYAN, 2021; WANG et al., 2021). Sequence analysis of MiCV with other circoviruses (CVS) revealed a higher homology with mammalian CVS compared to avian CVS (GE et al., 2018b). It has been shown that the recently identified porcine circovirus type 4 (PCV4) has high amino acid homology with the Cap protein of MiCV, and it is speculated that PCV4 may have a common origin with MiCV or involve mink in the virus transmission process (LI et al., 2022). For AMDV, although it poses a serious threat to free-ranging and farmed mink worldwide, molecular epidemiological studies of AMDV are scarce and have mainly analyzed partial fragments of NS1 or VP2 (RYT-HANSEN et al., 2017; TONG et al., 2020).

The sequences of MiCV strains from different regions were analyzed to help assess their level of genetic diversity. The Cap had the highest nucleotide and amino acid homology with its reference strains, and the MiCV Cap protein was relatively conserved. Conserved amino acid motifs of unknown function such as WWDGY (aa 195-199), DDFYGW (aa 208-213) and DRYP (aa 224 -227) can be used to design specific DNA primers for amplification of CVs and used to discover new CVs in other hosts (TODD et al., 2001; STEWART et al., 2006). The Cap protein of MiCV is required for viral genome packaging, capsid assembly and virus-host interactions, and plays an important role in the induction of protective immunity against viral infection as the only structural protein (JIANG et al., 2020; ZHAN et al., 2020). In this study, phylogenetic analysis of 18 sequenced MiCV Cap genes and 12 reference MiCV Cap genes showed that MiCV Cap genes from minks, foxes, and raccoon dogs were divided into three groups, designated as A, B, and C. The phylogenetic clusters were not formed on the basis of species or isolation time, but they had a clear relationship with geographic origin. The strains from Shandong province exhibited more amino acid mutations and

were grouped into a separate cluster, except for Raccoon dog CV-SD1.

The VP2 protein of AMDV plays an important role in viral particle assembly and nuclear transport (MARKARIAN and ABRAHAMYAN, 2021), and contains the major immunogenic epitope of AMDV on its surface. AMDV isolates can be distinguished by detecting the highly variable region in the VP2 gene (SANG et al., 2012). Phylogenetic analysis of 34 AMDV VP2 genes showed that VP2 genes were present in groups A, B, C and D. The phylogenetic clusters were not formed on the basis of species, but had a clear relationship with geographic or- igin. The MiCV strains from Jilin province were all in group D and similar to the reference strain AMDV-Pullman. The AMDV strains from Shan- dong province were divided into groups A, B and D. There were no isolated strains or Chinese strains in group C. The AMDV strains from Heilongjiang province were all in group A, together with most of the foreign reference strains.

Comparison of the nucleotide and amino acid sequences of the AMDV VP2 gene fragments showed that the AMDV isolates from minks, foxes, and raccoon dogs were highly homologous and closely related. Comparative amino acid sequence analysis revealed that the variants in the VP2 gene were primarily located in the virulence-associated sites and high-variation regions. No amino acid deletions were observed in the isolated samples, except for Fox ADV-SD2. All isolates from Jilin had the same amino acid mutation at position 394. Mutation of amino acid residues at position 92 or 94 reduces viral transcription and viral infectivity levels (XI et al., 2017). The isolated samples showed similar changes in the VP2 hypervariable region, suggesting that the domestic AMDV strains are somewhat different from those of other countries. It has been suggested that mutations in residue 434 may lead to a change in the host range of AMDV (MCKENNA et al., 1999). Interestingly, Fox ADV-SD1 and Raccoon dog ADV-HLJ1 had amino acid mutations in the high-variable region that were identical to those of AMDV-G. The high conservation at locus 420 in all strains suggests that this feature may be associated with persistent AMDV infection in vivo (CHENG et al., 2010).

MiCV and AMDV are commonly found in fur-bearing animal farms, with high detection rates and co-infection rates, therefore, further research on prevalence, virulence levels and detection methods must be given more attention. Due to the fact that this study mainly focuses on fur-bearing animals raised in northern China, including minks, foxes, and raccoons, the generalizability of the research results to other regions with different environmental and epidemiological conditions is limited.

Conclusions

In this study, a highly sensitive, specific, and repeatable duplex fluorescent quantitative PCR assay for MiCV and AMDV was developed. The results showed that the co-infection positive detection rate of MiCV and AMDV in minks was higher than that in foxes and raccoon dogs. Phylogenet- ic analysis of the MiCV Cap gene and the AMDV VP2 gene showed that the phylogenetic clusters were not formed on the basis of species or isolation time, but had a clear relationship with geographic origin. This study could contribute to controlling the spread of MiCV and AMDV.

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Authorship contribution statement

Xue Leng and Chenyan Sheng contributed equally to this paper.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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SAŽETAK

Cirkovirus kuna (Mink circovirus, MiCV) i virus aleutske bolesti kuna (Aleutian mink disease virus, AMDV) u znatnoj su mjeri utjecali na reproduktivnu sposobnost kuna, uzrokujući goleme ekonomske gubitke na farmama životinja koje se uzgajaju za proizvodnju krzna. Još uvijek nisu pronađena učinkovita cjepiva ni lijekovi kojima bi se ti gubici mogli spriječiti i kontrolirati. Kako bi se razumjela koinfekcija i evolucija MiCV-a i AMDV-a u kuna, lisica i rakunopasa, razvijen je dupleks fluorescencijski kvantitativni PCR u stvarnom vremenu, zasnovan na polim- erazi TaqMan za viruse MiCV i AMDV. Osim toga, prikupljeno je ukupno 18 uzoraka od kuna, lisica i rakunopasa s mješovitom infekcijom virusima MiCV i AMDV za analizu genske evolucije gena *Cap* u virusu MiCV i gena *VP2* u virusu AMDV. Granice detekcije dupleks fluorescencijskog kvantitativnog PCR testa u stvarnom vremenu za oba su virusa bile 1,0 $\times 10^1$ kopija/μL, s većom osjetljivošću od konvencionalnog PCR-a, te nije bilo znakova specifične fluorescencije drugih, neciljnih patogena. Koeficijenti varijacije unutar i između serija bili su manji od 2%. Ukupne stope detekcije u 213 uzoraka od kuna, lisica i rakunopasa bile su 46,0% (98/213) za MiCV i 55,4% (118/213) za AMDV, te 31,9% (68/213) za koinfekciju obama virusima. Gen *Cap* virusa MiCV bio je evolucijski relativno očuvan, dok je gen *VP2* virusa AMDV imao više varijanti, s najčešćom pojavom u lokusima i hipervarijabilnoj regiji povezanoj s VP2 virulencijom. Analiza gena *Cap* u virusu MiCV i gena *VP2* u virusu AMDV pokazala je da filogenetske skupine nisu formirane na temelju vrste ili vremena izolacije, već su povezane s geografskim podrijetlom. Rezultati istraživanja doprinos su epidemiološkim podacima o virusima MiCV i AMDV te su važna referencija u prevenciji bolesti i kontroli virusa MiCV i AMDV u Kini.

Ključne riječi: cirkovirus kuna (MiCV); virus aleutske bolesti kuna (AMDV); dupleks kvantitativni PCR u stvarnom vremenu; koinfekcija; filogenetska analiza