Japanese Journal of Veterinary Research 73(2): 27–35, 2025

JJVR

REGULAR PAPER Experimental Research

Establishment and application of fluorescent RT-PCR assay for detecting Aichivirus D

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Received for publication, November 10, 2024; accepted, April 23, 2025

Abstract

Aichivirus D (AiV-D) is a newly emerging diarrhea-associated pathogen in cattle and sheep, and comprised two officially recognized genotypes (AiV-D1 and AiV-D2) and two potential novel genotypes (AiV-D3 and AiV-D4). This study aimed to establish a fluorescent RT-PCR assay for detecting AiV-D and to investigate its prevalence in yak diarrheic samples. A SYBR Green fluorescent RT-PCR assay was successfully established by targeting the conserved region of viral 3D genes, enabling detection of all known AiV-D genotypes. The established assay was specific for AiV-D, exhibiting no cross-reactivity with other common diarrhea-causing pathogens in ruminants, with coefficients of variation for reproducibility ranging from 0.23% to 2.16% and detection limits for the positive plasmids of AiV-D1 to AiV-D4 at 2.21, 4.78, 4.10, and 5.94 copies/μL, respectively. Of the 166 diarrheic samples collected from calf yaks across nine farms in Sichuan province, China, between May and August 2023, 22.3% (37/166) tested positive for AiV-D, suggesting its substantial prevalence within the sampled region. Furthermore, seven complete 3D gene sequences were cloned from positive samples, and subsequent phylogenetic analysis revealed distinct evolutionary patterns among these isolates. In conclusion, this newly established assay represents a reliable diagnostic tool for AiV-D detection, while our findings provide significant insights into the epidemiological distribution and evolutionary dynamics of AiV-D in the yak population.

Key Words: Aichivirus D, fluorescent RT-PCR, 3D gene, yak

Introduction

Kobuvirus is classified in the genus *Kobuvirus* of the family *Picornaviridae* (https://ictv.global/). To date, kobuviruses has been classified into six species: *Kobuvirus aichi*, *Kobuvirus bejaponia*, *Kobuvirus cebes*, *Kobuvirus dekago*, *Kobuvirus ecuni* and *Kobuvirus femyomini* by International Committee on Taxonomy of Viruses, and can be further divided into 20 genotypes by phylogenetic analysis[9)]. Kobuvirus has a wide host range, and multiple species having been identified as diarrhea-associated pathogens in humans and animals[1,2,4,17-21)].

Kobuvirus dekago, also called Aichivirus D (AiV-D), has been confirmed to infect various ruminant species, including cattle, sheep, goats, and yaks[2,15,18,19)]. Currently, AiV-D comprises two officially recognized genotypes (AiV-D1 and AiV-D2) and two potential novel genotypes

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doi: 10.57494/jjvr.73.2_27

(AiV-D3 and AiV-D4)[2,15,18,19]. AiV-D1 and AiV-D2 were detected in diarrhea of Japanese black cattle[15]. AiV-D3 was identified in fecal samples from sheep and goats in China, with significantly higher positivity in diarrhea samples from goats compared to healthy samples, suggesting a potential link to goat diarrhea[2,19]. AiV-D4 has been identified as diarrhea-causing pathogens in calf yaks, and has been circulating among yaks in the Qinghai-Tibet Plateau of China[18]. To date, ten AiV-D genomes are available in the GenBank database, including one nearly complete genome of the AiV-D1 strain, two complete genomes of the AiV-D2 strains[15], two complete genomes of the AiV-D3 strains[2,19], three complete genomes of the AiV-D4 strains[18], and two unverified AiV-D strains (GenBank accession number: OR148642 and OR148643).

The genomic architecture of kobuvirus comprises a 5' untranslated region (5'UTR), a large open reading frame (ORF), and a 3'UTR. The ORF encodes the structural proteins P1 (VP0, VP3 and VP1), the non-structural Leader protein (L), and non-structural proteins P2 (2A, 2B, and 2C) and P3 (3A, 3B, 3C, and 3D)[16]. The 3D gene represents the most highly conserved genomic region within kobuvirus and consequently serves as the principal target for molecular detection strategies[3,5,14,18,19]. Currently, the GenBank database contains 23 complete AiV-D 3D gene sequences. Intra-genotypic analysis reveals relatively high conservation of 3D genes within individual AiV-D genotypes, whereas genetic heterogeneity exists among 3D genes across different genotypes.

Given the emergent nature of AiV-D, current diagnostic methodologies warrant further optimization and standardization. Several studies have reported PCR assays for AiV-D, but all were designed to target specific AiV-D genotypes[2,15,18,19]. Comprehensive sequence analysis revealed that existing primer sets exhibit incomplete complementarity across all known AiV-D genotypes. Considering the potential co-circulation of multiple AiV-D genotypes within the same host species[2,15,18,19], this study aimed to establish a broad-spectrum fluorescent RT-PCR assay for comprehensive AiV-D detection and subsequently employ it to evaluate the prevalence of AiV-D in yak diarrhea samples from China.

Materials and Methods

Nucleic acids and clinical samples

The nucleic acids of AiV-D2, AiV-D3, and AiV-D4 were extracted from PCR-validated fecal samples that were confirmed negative for other common diarrhea-causing pathogens through comprehensive molecular screening[2,18,19], while the nucleic acid of AiV-D1 was a full-length synthetic 3D gene (Sangon Bio, Shanghai, China). For specificity validation, nucleic acids of the common diarrhea-causing pathogens in ruminants were used, including bovine coronavirus (BCoV), bovine rotavirus (BRVA), bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis virus (IBRV), bovine nebovirus (NeV), bovine norovirus (BNoV), bovine torovirus (BToV), bovine astrovirus (BAoV), caprine astrovirus (CAoV), bovine enterovirus (BEV), enterotoxigenic *Escherichia coli* (ETEC), and *Salmonella enterica*. All nucleic acids were preserved at -80 °C in the Veterinary Medicine Laboratory of Southwest Minzu University.

For assay validation, 120 clinical fecal samples were collected from Sichuan province, China, between May 2022 and May 2024, comprising equal numbers (n=30) of samples from diarrheic cattle, sheep, goats, and yaks. To investigate AiV-D prevalence, 166 additional clinical fecal samples were collected during diarrheal outbreaks from nine yak farms across three districts (Ruoergai, Hongyuan, and Daofu) of Sichuan province, China, between May and August 2023. The samples were obtained from calf yaks (1–3 months of age) in herds situated along the southeastern margin of the Qinghai-Tibet Plateau, at locations with mean elevations exceeding 3,400 meters above sea level. All samples were preserved at -80 °C in our laboratory, until further analysis.

Detection primers design and synthesis

All of 23 complete 3D gene sequences of different AiV-D genotypes in GenBank (GenBank accession number: LC055960, LC055961, OP376931, OQ267712, MW296158, MZ558247–MZ558259, OP776109–OP776111) were aligned to identify the conserved region using the MEGA7.0 software[10]. The primers were designed targeting the identified conserved regions using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA), with reference to the BKV/Yak/XZNQX7/2021/CHN strain sequence (GenBank accession number: OP776111). The primers were synthesized by Sangon Biotech (Shanghai, China).

RNA extraction and cDNA synthesis

Clinical fecal samples were homogenized with PBS at a 1:5 volume ratio, and the freeze-thaw process was repeated three times at -80 °C. The homogenates underwent initial centrifugation at 3,000 rpm for 10 min at 4 °C, followed by a secondary centrifugation of the supernatant at 12,000 rpm for 15 min at 4 °C. Total RNA was extracted from the supernatant using Trizol reagent (TaKaRa Bio, Kusatsu, Japan) following the manufacturer's protocol. cDNA was synthesized using PrimeScript RT Reagent Kit (TaKaRa Bio) according to the manufacturer's instructions using a random hexamer. The synthesized cDNA was stored at -20 °C.

Preparation of standards

The amplified PCR products of AiV-D1 to AiV-D4 were purified using the Universal DNA Purification kit (Tiangen Bio, Beijing, China). The purified products were ligated into the pClone007 vector (Tsingke Bio, Beijing, China), and subsequently, transformed into E.coli DH5α competent cells (Tsingke Bio). Recombinant plasmids were extracted using the TIANprep Mini Plasmid kit (Tiangen Bio), their concentrations were measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations were then converted to copy numbers using the formulary copies/μL = (6.02 × 10^{23} copies/mol) × (concentration ng/μL) × 10^{-9} / (molecular mass g/mol).

Optimization of the fluorescent RT-PCR assay

All fluorescent RT-PCR assays were conducted on a QuantStudio thermal cycler system (Thermo Fisher Scientific). Each reaction consisted of qPCR SYBR Green Master Mix (High Rox Plus) (Yeasen Bio, Shanghai, China) 12.5 μL, 2 μL of AiV-D positive control, in addition to different volumes of 10 μmol/μL primers (0.6, 0.8, 1.0, 1.2, 1.4 μL), which were used to optimize the assay and made up to 25 μL with the RNase-Free ddH_2O. Thermal cycling parameters were optimized under the following reaction conditions: 50 ˚C for 2 min and 95 ˚C for 2 min, followed by 40 cycles of 95 ˚C for 15 s and 55–65 ˚C for 20 s, which were used to confirm the optimal amplification conditions.

Establishment of standard and melting curves

The standard curves of the developed fluorescent RT-PCR assay were generated by detecting 10-fold serial dilutions (from 10^{-1} to 10^{-10}) of the standards of AiV-D1 to AiV-D4 using the QuantStudio system (Thermo Fisher Scientific). The analysis was repeated three times, and the PCR amplification efficiency (E), standard curve slope, and correlation coefficient (R^2) were calculated.

Evaluation of the fluorescent RT-PCR assay

To evaluate analytical specificity, the assay was tested against a panel of twelve common ruminant diarrheic pathogens: BCoV, BRVA, BVDV, IBRV, NeV, BNoV, BToV, BAoV, CAoV, BEV, ETEC, and Salmonella enterica. The nucleic acids of AiV-D1 to AiV-D4 were used as positive controls, nucleic acid extracted from AiV-D-negative fecal was used as negative control, and ddH_2O was used as non-template control. Assay reproducibility was evaluated by determining the coefficient of variation (CV) for both intra-assay and inter-assay variations. CV values were calculated from threshold cycle (Ct) measurements obtained from three independent experiments (inter-assay) and triplicate reactions (intra-assay), using AiV-D1 to AiV-D4 standards at concentrations of 1.0 × 10^6, 1.0 × 10^5, and 1.0 × 10^4 copies per reaction. Analytical sensitivity was determined using serial dilutions of AiV-D1 to AiV-D4 standards ranging from 1.0 ×

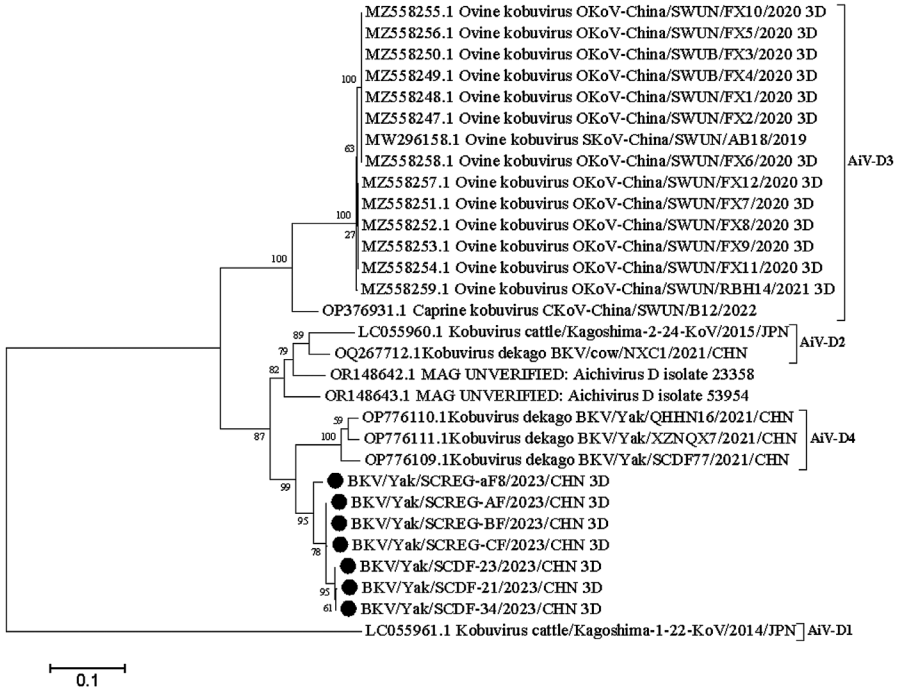

Fig. 1. Phylogenetic tree of complete 3D gene sequences of AiV-D.
Maximum-likelihood analysis in combination with 1,000 bootstrap replicates was used to derive a phylogenetic tree based on the complete 3D gene of AiV-D. Black circles indicate the 3D genes detected in this study.

10^9 to 1.0×10^0 copies/μL, with all measurements performed in triplicate.

Additionally, a total of 120 clinical fecal samples from different animal species were used to validate the developed assay in this study and previously reported RT-PCR assays for detecting AiV-D1 to AiV-D4[2,15,18,19].

Detection of AiV-D in yak diarrhea samples

A total of 166 clinical samples were detected for AiV-D using the optimized fluorescent RT-PCR assay, with 2 μL of synthesized cDNA serving as template for each reaction. Samples yielding Ct value <35 were considered positive. For samples with Ct values between 35 and 40, nucleic acid extraction and amplification were repeated. Samples were confirmed positive if they exhibited characteristic amplification curves and Ct values <40; otherwise, they were classified as negative. Additionally, to validate the accuracy of the assay results, 20% of the positive samples were randomly selected for confirmatory Sanger sequencing.

Cloning and analysis of the complete 3D gene

Two pairs of primers were designed to amplify the complete 3D gene from AiV-D-positive fecal samples, and the primers were synthesized by Sangon Biotech. The primers were: F1 (5'-CAGTCGCTGATCTTCTCCAAAG-3') and R1 (5'-GCAGAGAGAAGAACAGAAGGGAT-3'), which amplified a 764 bp fragment (positions 6872-7635 nt); and F2 (5'-CTTGACTACAAATGCTTTGATGC-3') and R2 (5'-GCAAGAGTGACCAACACCATTAC-3'), which amplified a 797 bp fragment (positions 7586-8382 nt) of the BKV/Yak/XZNQX7/2021/CHN strain (GenBank accession number: OP776111). The amplified products were purified using the Universal DNA Purification kit, ligated into pClone007 vector, and subsequently transformed into *E.coli* DH5α competent cells. Recombinant plasmids were isolated and subjected to Sanger sequencing at Sangon Biotech. The 3D genes were assembled using SeqMan software (DNASTAR, Madison, WI, USA), and nucleotide and deduced

Table 1. Primer information for RT-PCR assays

Test method	Primer name	Target gene	Fragment length, bp	Primer sequence (5'-3')	Source of primers
SYBR Green fluorescent RT-PCR	AiV-D-F/R	3D	118	F: TCCACCATCTATGACGTCACGT R: TCATCCACATAACTGATTGCTC	This study
TB Green real-time RT-PCR	AiV-D4-F/R	3D	157	F: CGCTGTCTGGAGAACCCTGAGTA R: GTTCGATGATACCACCAAGGAGC	8)
RT-PCR	AiV-D1 F/R	5′UTR	197	F: TCTGCAAACAACTACCAGAT R: GTGGGAAGCGGTTTGTGACAGC	10)
RT-PCR	AiV-D2-F/R	5′UTR	466	F: GCTTTCATGGACCATTGCGCG R: GGAGAGTCGAGAGCACCGCCGAA	10)
RT-PCR	AiV-D3-sheep-F/R	3D	306	F: AAGCCCTTGCTCTTTACACTTCTACCC R: CACGGGTCAGCCCCTTCTTCAC	11)
RT-PCR	AiV-D3-goat-F/R	3D	362	F: TAAACACGATAAGGGCGACCA R: ACTCAACAGCACGGGTCAGG	9)

amino acid sequence similarities were determined using the MegAlign program in DNASTAR 7.0 software (DNASTAR). The complete 3D gene sequences of AiV-D, including sequences in this study and that available from GenBank, were used to constructed a phylogenetic tree by MEGA7.0 software[10].

Results

Primers design and evaluation

Multiple sequence alignment of the 23 known complete 3D genes was performed using MEGA7.0 software[10]. Based on the aligned sequences, a pair of primers targeting a highly conserved region was designed using Primer Premier 5.0 software (Premier Biosoft). The designed primer pair consisted of F (5'-TCCACCATCTATGACGTCACGT-3') and R (5'-TCATCCACATAACTGATTGCTC-3'), which specifically amplified a 118 bp fragment (position 8000-8117 nt of the BKV/Yak/XZNQX7/2021/CHN genomic sequence, GenBank accession number: OP776111), shown in Table 1. The primer demonstrated successful amplification of target fragments from AiV-D1 to AiV-D4, as illustrated in Supplementary Material Figures S1 and S2.

Fluorescent RT-PCR assay for detecting AiV-D

The optimal volumes of primers and the annealing temperature were determined based on the presence of a plateau period and the minimum Ct value. The optimal reaction conditions were as follows: 1 μL of each primer (10 μmol/μL) and an annealing temperature of 65 °C for 20 s.

The generated standard curves of AiV-D1, AiV-D2, AiV-D3, and AiV-D4 showed a strong linear relationship within the range of 10^3 to 10^9 copies per reaction and the parameters revealed slopes of -3.276, -3.254, -3.112, and -3.51; efficiencies of 101.94%, 102.93%, 109.59%, and 103.04%; and R^2 values of 0.998, 0.998, 0.997, and 0.999, respectively. Melting curve analysis revealed consistent melting temperatures (Tm) of 82.5 °C ± 0.5 °C across all AiV-D (D1–D4). The details of the copy number calculation process and results are shown on page 3 in the Supplementary Material, while the standard curves and melting curves are presented in Figure S3.

The established fluorescent RT-PCR assay exhibited high specificity for known AiV-D (D1–D4), demonstrating no cross-reactivity with common diarrhea-causing pathogens in ruminants, including BCoV, BRVA, BVDV, IBRV, NeV, BNoV, BToV, BAoV, CAoV, BEV, ETEC, and *Salmonella enterica* (Fig. S4). The intra-assay variability (reproducibility) and inter-assay variability (repeatability) for AiV-D1 to AiV-D4 ranged from 0.23% to 2.16% (Table S1 and S2). The detection limits for the standards of AiV-D1 to AiV-D4 were 2.21, 4.78, 4.10, and 5.94 copies/μL, respectively (Fig. S5).

Table 2. The results of 6 kinds of AiV-D RT-PCR assay

Test method	No. of AiV-D positive/No. of tested (%)					Source of primers
	Diarrhea samples from cattle	Diarrhea samples from sheep	Diarrhea samples from goat	Diarrhea samples from yak	Total	
RT-PCR	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	0/120 (0)	15)
RT-PCR	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	0/120 (0)	15)
RT-PCR	0/30 (0)	3/30 (10.0)	0/30 (0)	0/30 (0)	3/120 (2.5)	2)
RT-PCR	0/30 (0)	0/30 (0)	16/30 (53.3)	0/30 (0)	16/120 (13.3)	19)
TB Green real-time RT-PCR	5/30 (16.7)	0/30 (0)	0/30 (0)	9/30 (30.0)	12/120 (10.0)	18)
Fluorescent RT-PCR	5/30 (16.7)	3/30 (10.0)	16/30 (53.3)	9/30 (30.0)	33/120 (27.5)	This study

Table 3. Yak fecal sample collection and AiV-D detection

Characteristic	Region in Sichuan province, China and farm no.									Total
	Control				Hongyuan county				Daofu county	
	1	2	3	4	5	6	7	8	9	
Sample number	23	21	21	11	13	10	16	11	40	166
No. of AiV-D positive/ No. of tested (%)	1/23 (4.3)	4/21 (19.0)	7/21 (33.3)	3/11 (27.3)	0/13 (0)	1/10 (10.0)	7/16 (43.8)	2/11 (18.2)	12/40 (30.0)	37/166 (22.3)

Evaluation of the fluorescent RT-PCR assay using clinical samples

Comparative analysis of AiV-D detection rates between our newly developed assay and five previously established RT-PCR methods was conducted using 120 clinical samples, with results summarized in Table 2. The newly developed assay demonstrated robust detection capabilities across diverse ruminant species (cattle, sheep, goats, and yaks), achieving detection rates exceeding those of previously established methods. Notably, this assay successfully identified all AiV-D-positive samples that were detected by existing RT-PCR assays.

Detection of AiV-D in yak diarrhea samples

The results of the developed assay revealed AiV-D presence in 22.3% (37/166) of clinical fecal samples obtained from diarrheic yak calves, with a high farm-level prevalence of 89% (8/9 farms), as detailed in Table 3. These findings indicate that AiV-D had been widely distributed among the sampled region.

Cloning and analysis of the complete 3D gene

Seven full-length 3D genes, each 1422 bp in length, were successfully amplified and sequenced from independent AiV-D-positive samples (GenBank accession numbers: PQ469009–PQ469015). Sequence analysis revealed nucleotide identity levels of 96.2–100% among the seven newly sequenced 3D genes, with maximum sequence similarity (91.3–91.9%) to three previously characterized yak-derived AiV-D strains in GenBank. Comparative sequence analysis demonstrated that the ten yak-derived 3D genes exhibited nucleotide sequence identities ranging from 73.1% to 90.8% when compared with other documented AiV-D strains in GenBank.

Phylogenetic analysis was performed using MEGA7.0 software10), employing the Maximum-likelihood method with the Tamura-Nei substitution model to construct a phylogenetic tree based on AiV-D 3D gene sequences. Phylogenetic analysis demonstrated that all seven newly identified 3D genes, together with the three previously reported yak-derived sequences, formed a distinct monophyletic clade (Fig. 1). Compared to 3D genes from other animal hosts in GenBank, the ten 3D genes from yaks shared 10 identical nucleotide mutations: (G/C)10T, (C/G/A)13T, C16A, G17A, (T/C)184G, (A/G) 213T, T831G, C864G, G930T, and (G/A)1209T; and six amino acid mutations: (D/N/P)5S, R6(N/K), (S/L)62A, (Q/

K)71(H/D), (Y/F)277W, and D288E, among which four amino acid mutations ((S/L)62A, (Q/K)71(H/D), (Y/F)277W, and D288E) were located in the RNA-dependent RNA polymerase motif (Fig. S6).

Discussion

Diarrhea represents the predominant disease affecting ruminants, with viral infections serving as a primary etiological factor, leading to considerable economic losses through elevated mortality rates and diminished livestock productivity[6,12]. AiV-D, an emerging member of the *Kobuvirus* genus, has been confirmed as a diarrhea-associated viruses in ruminant species[2,15,18,19]. Consequently, establishing effective detection methods and investigating the epidemiological prevalence of AiV-D are crucial for the diagnosis and prophylaxis of ruminant diarrheal diseases. The AiV-D classification currently encompasses two officially recognized genotypes (AiV-D1 and AiV-D2) and two potential novel genotypes (AiV-D3 and AiV-D4)[2,15,18,19], along with two recently documented but unvalidated AiV-D strains from South Korea (GenBank accession numbers: OR148642 and OR148643), and substantial genetic heterogeneity has been documented within the 3D gene regions across different genotypes. While various studies have reported RT-PCR assays for genotype-specific AiV-D detection, the capacity for multiple genotypes to co-infect individual host species necessitates a more comprehensive approach[2,15,18,19]. Furthermore, an extensive sequence analysis revealed that existing primer sets show incomplete complementarity across all known AiV-D genotypes (Fig. S2). Consequently, the development of a universal PCR assay capable of detecting diverse AiV-D strains is imperative to circumvent the limitations inherent in existing genotype-specific PCR assays[2,15,18,19].

Among kobuviruses, the 3D gene exhibits the highest degree of sequence conservation, rendering it an optimal target for molecular diagnostic applications[13,16-18]. Through comprehensive analysis of all available AiV-D 3D gene sequences in GenBank, we designed primers targeting highly conserved regions and subsequently established a SYBR Green fluorescent RT-PCR assay for AiV-D detection. The developed assay demonstrates high specificity for AiV-D detection across known genotypes (AiV-D1 to AiV-D4), while exhibiting no cross-reactivity with other common diarrhea-causing pathogens in ruminant, and maintains excellent analytical sensitivity and reproducibility. Therefore, the assay developed in this study overcomes the limitations of previously reported genotype-specific RT-PCR assays[2,15,18,19], providing a valuable tool for the detection and investigation of the prevalence of AiV-D.

Endemic to the Tibetan Plateau, yaks represent a significant economic resource in China, with an estimated population of 14 million animals contributing substantially to the regional agricultural economy[7,8]. Our research team recently confirmed that AiV-D4 is a diarrhea-causing pathogen in calf yaks, detected with a positivity rate of 24.8% in yak diarrhea samples collected from May to August 2021, and all sampled farms were positive for AiV-D4[18]. These epidemiological data indicate the extensive distribution of AiV-D throughout the Qinghai-Tibet Plateau, underscoring the imperative for systematic viral surveillance programs. In the present study, an AiV-D positivity rate of 22.3% (37/166) among the samples and 89% (8/9) at the farm level were detected using the developed assay, which are similar to those of our previous research, suggesting that AiV-D is prevalent among yaks in China. The results demonstrated the necessity of including AiV-D in routine pathogen monitoring for yak diarrhea.

The 3D gene encodes the critical RNA-dependent RNA polymerase motif, a fundamental component essential for viral genome replication11). Prior to this study, only three complete 3D gene sequences from yak-derived AiV-D were available in GenBank. Our investigation successfully characterized seven additional complete 3D gene sequences of yak-derived AiV-D. Compared to 3D genes of AiV-D from other host species in GenBank, the ten AiV-D 3D genes from yaks share ten identical

nucleotide mutations and four identical amino acid mutations, making these 3D genes from yaks to cluster into an independent branch; the result indicated that AiV-D has a unique evolutionary pattern within the yak species.

In conclusion, we have successfully established a SYBR Green fluorescent RT-PCR detection system for AiV-D detection. This diagnostic platform demonstrates robust specificity for all known AiV-D genotypes (AiV-D1 to AiV-D4) while maintaining complete analytical specificity against common diarrhea-causing pathogens in ruminant, thereby offering a reliable tool for both diagnostic applications and epidemiological investigations. Furthermore, 22.3% (37/166) of calf yak diarrhea samples from nine farms were detected as AiV-D positive, indicating that AiV-D was widespread in the sampled area. Additionally, seven complete 3D genes of AiV-D originating from yaks were obtained, and phylogenetic analysis revealed that these genes exhibit a distinct evolutionary trend.

Conflicts of Interest and Ethical Statement

The authors declare that there are no conflicts of interest. This study did not involve animal experiments besides the fecal sampling of diarrheic sheep, goats, and yaks.

Acknowledgments

This work was funded by the National Key Research and Development Program of China during the 14th Five-Year Plan (Grant number 2023YFD180250402) and Southwest Minzu University Double World-Class Project (Grant number XM2023014).

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