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Multiplex one-step RT–qPCR assays for simultaneous detection of AMDV, MEV and CDV

Zhi Cao[1,2], Hang Xu[1], Xinru Zhao[1], Ke Zhang[1,2], Dehua Yin[1,3], Shuai Ma[4], Wenling Li[1], Siyu Li[1], Jianwei Ren[1] and Jianxin Wen[1*]

Abstract

Background Aleutian mink disease, mink viral enteritis and canine distemper are known as the three most serious diseases that cause great economic loss in the mink industry. In clinical practice, aleutian mink disease virus (AMDV), mink enteritis virus (MEV) and canine distemper virus (CDV) are common mixed infections, and they have similar clinical clinical signs, such as diarrhoea. Therefore, a rapid and accurate differential diagnosis method for use on mink ranches is essential for the control of these three pathogens. Here, we developed multiplex one-step real-time quantitative PCR (RT–qPCR) assays for the simultaneous detection and quantification of AMDV, MEV and CDV by using three primers and probes based on the conserved NS1, VP2 and N genes, respectively.

Results The results showed that the established method can not cross-react with other mink pathogens, with a detection sensitivity of 25 copies/μL and a coefficient of variation less than 3.51%. Moreover, the interference experiment showed that the presence of AMDV, MEV and CDV templates at different concentrations would not interfere with the detection results. Furthermore, two hundred clinical samples of mink with diarrhoea were simultaneously analysed using multiplex RT–qPCR and single RT–qPCR, the Kappa values were all greater than 0.921, indicating that there was a high degree of coincidence between the two detection methods.

Conclusions In conclusion, multiplex RT–qPCR exhibited high specificity, sensitivity, and reproducibility, indicating that this method can be used as a reliable and specific tool for the differential detection and quantification of AMDV, MEV and CDV.

Keywords AMDV, MEV, CDV, Multiplex RT–qPCR, Differential detection

*Correspondence:
Jianxin Wen
wenjianxin@126.com
[1]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao 266109, China
[2]Shandong Collaborative Innovation Center for Development of Veterinary Pharmaceuticals, Qingdao 266109, China
[3]Innovus Solarex Biotech Co., Ltd, Qingdao 266109, China
[4]Qingdao Animal Disease Prevention and Control Center, Qingdao 266100, China

Introduction

Aleutian mink disease (AMD), caused by Aleutian mink disease virus (AMDV), is reported in many mink-producing countries [1]. AMD can significantly increase the empty rate of female minks and the mortality of young minks, and decrease the mating ability of male minks. AMDV belongs to the genus *Amdoparvovirus* within the family *Parvoviridae*, and its single-stranded DNA genome encodes two structural proteins (VP1 and VP2) and three nonstructural proteins (NS1, NS2 and NS3) [2, 3]. Because of the special pathogenic infection mechanism of AMD, there is currently no commercial vaccine or treatment for AMDV [4]. Thus, a relatively successful control strategy is to screen infected animals through testing and cull them. Serology and qPCR are common screening methods for AMDV, and a cheaper serological test is often used clinically for mass screening and confirmation by qPCR.

Mink viral enteritis, caused by Mink enteritis virus (MEV), is an acute and highly infectious disease whose symptoms include violent diarrhoea. MEV belongs to the genus *Parvovirus* within the family *Parvoviridae*. Its single-stranded DNA genome encodes two nonstructural proteins (NS1 and NS2) and the capsid proteins VP1 and VP2. The molecular diagnosis of MEV is an important measure for disease control [5–7]. Among them, nanoparticle-assisted PCR and loop-mediated isothermal amplification (LAMP) have been widely used for the detection of MEV through amplification of the highly conserved NS1 and VP2 genes [8]. Additionally, real-time PCR has not been used to detect MEV, but it has been used to detect other parvoviruses [9–11].

Canine distemper (CD) is caused by canine distemper virus (CDV), which can cause high mortality on mink farms with clinical symptoms, including conjunctivitis, diarrhoea, encephalitis, and so on [12]. CDV belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* and is an enveloped negative-strand RNA genome that encodes six structural and two nonstructural proteins. There is no specific therapeutic drug for mink CD, and the best prevention method is vaccination [8]. However, immunization failure leads to an increase in the incidence of infection in immune mink. Therefore, highly sensitive and rapid detection methods are highly important for targeted control of the epidemic and spread of CD.

The multiplex real-time quantitative PCR (RT-qPCR) method has the characteristics of high specificity, high sensitivity, and high throughput and has been widely used in the diagnosis of clinical mixed infection diseases, the differentiation of different serotypes of bacteria, the identification of different virus strains, etc [13–16]. At present, there is no relevant literature on multiple RT-qPCR methods for simultaneous differential detection of AMDV, MEV and CDV. Herein, specific primers and fluorescent probes were designed for the specific genes of AMDV, MEV and CDV, and multiple RT-qPCR methods for the simultaneous identification and detection of AMDV, MEV and CDV were established; these methods provide specific, efficient, and sensitive technical means for the detection and epidemiological investigation of three important diseases in mink breeding.

Results

The best reaction sets

The optimal reaction conditions for the different concentrations of primers and probes are shown in Fig. 1. The primer concentration and probe concentration (CDV and AMDV: 0.5 μM and 0.4 μM, MEV: 0.2 μM and 0.4 μM, respectively) were determined to be the optimal RT-qPCR concentrations according to the low Ct value. Therefore, RT-qPCR was carried out in a 25 μL reaction mixture consisting of 12.5 μL of RT-qPCR 5G Premix (TOROIVD, QST-200P), 0.5 μL of primer pair and 0.4 μL of probe containing the corresponding target fragment, 1.0 μL of the target recombinant plasmid or sample nucleic acid, and ddH_2O.

Establishment of the standard curve for multiple RT–qPCR

Tenfold-diluted standard plasmids were prepared from 2.5×10^7 to 2.5×10^1 copies/μL and mixed with equal volumes to carry out multiple RT-qPCRs and establish standard curves following the best reaction sets. The results showed that the Ct values and copy numbers of the standard recombinant plasmids (over the range of 2.5×10^7 copies/μL ~ 2.5×10^1 copies/μL) exhibited good linear

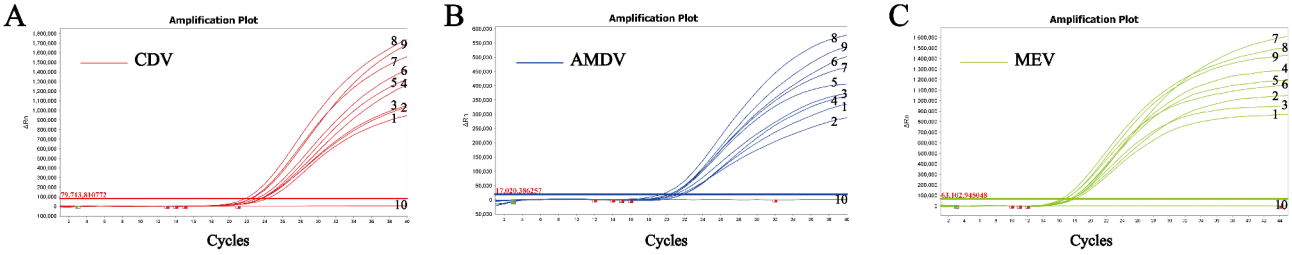

Fig. 1 Optimization results of primer probes. **A**: CDV, **B**: AMDV, **C**: MEV. The primer and probe concentrations from 1 ~ 9 were as follows: 0.2 μM, 0.2 μM; 0.5 μM, 0.2 μM; 0.8 μL, 0.2 μM, 0.2 μM, 0.25 μM; 0.5 μM, 0.25 μM; 0.8 μM, 0.25 μM; 0.2 μM, 0.4 μM; 0.5 μM, 0.4 μM; and 0.8 μM, 0.4 μM. 10: Negative control

relationships with correlation coefficients (R^2s) of 0.9980 and Y = -3.0832X + 39.886 for AMDV; R^2s of 0.9987 and Y = -3.0742X + 37.426 for MEV; and R^2s of 0.9982 and Y = -3.2080X + 40.352 for CDV (Fig. 2).

Specificity of the multiple RT–qPCR method

We tested the specificity of the multiple RT-qPCR assays using three positive standard plasmids. Meanwhile, CCoV and CPIV cDNAs, PRV and CPV DNAs were regarded as control. All three assays amplified only AMDV, MEV and CDV without cross-reaction with any of the other viruses, indicating satisfactory specificity of the established multiple RT-qPCR methods (Fig. 3).

Sensitivity of the multiple RT–qPCR method

The limit of detection (LOD) of the multiple RT-qPCR assay was determined using equal volume mixtures of tenfold-diluted serially diluted standard plasmids of AMDV, MEV and CDV. The results showed that the LODs of the multiple RT-qPCR assays were 25 copies/μL, 25 copies/μL and 25 copies/μL for AMDV, MEV and CDV, respectively (Fig. 4). Hence, the multiple RT-qPCR assay was found to be sensitive.

Reproducibility of the multiple RT–qPCR method

The intrabatch repeatability test and the interbatch repeatability test were performed using a mixture of three positive standard plasmids at final concentrations of 7.5×10^7 copies/μL, 7.5×10^5 copies/μL and 7.5×10^3 copies/μL by multiple RT-qPCR. The results showed that the reproducibility was excellent, and the intrabatch coefficient of variation (CV, 0.1%~3.37%) and interbatch coefficient of variation (CV, 1.3%~3.51%) were less than 4% (Table 1).

Anti-interference experiment

The anti-interference assays showed that all amplification curves and Ct values could be obtained by using any testing random concentration combination of three standard plasmids in any single qPCR test for detection of AMDV, MEV or CDV, thus indicating that the multiple RT-qPCRs could not contribute to identify the single viral copies (Fig. 5).

Performance of multiple RT-qPCR methods for clinical samples compared with the single highly sensitive detection methods for AMDV, MEV and CDV.

To evaluate the practical performance of the established multiple RT-qPCR methods, 200 faecal samples were analysed, and the results were compared with those of single highly sensitive detection methods for AMDV, MEV and CDV.

Using multiple RT-qPCR, the detection rates of single pathogens AMDV, MEV and CDV were 9%, 11.5% and 3%, respectively. Similarly, the detection rates of mixed infections of AMDV + CDV, AMDV + MEV, CDV + MEV, and CDV + MEV + AMDV were 1.5%, 6%, 3.5% and 2%, respectively. When using the single high-sensitivity detection method, the detection rates of the single pathogens AMDV, MEV and CDV in the sample were 8.5%, 10% and 3%, respectively. Similarly, the detection rates of mixed infections of AMDV + CDV, AMDV + MEV,

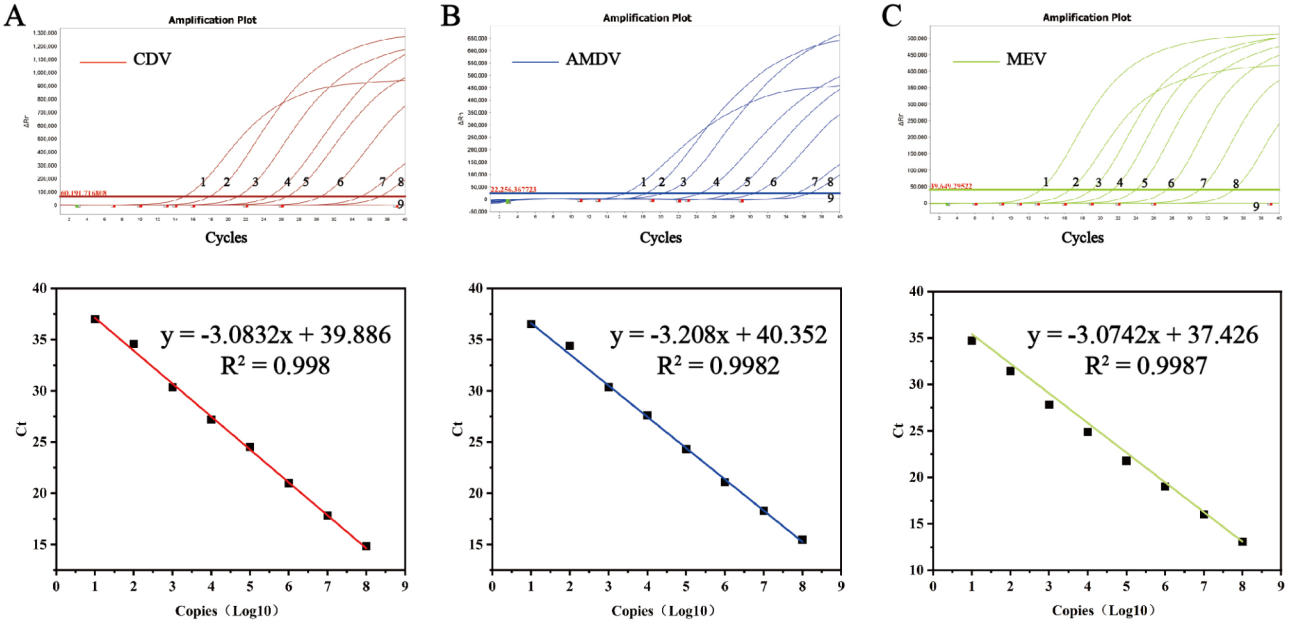

Fig. 2 Establishment of the standard curve for multiple RT–qPCR. **A**: CDV, **B**: AMDV, **C**: MEV. 1 ~ 8: The concentrations of the mixed plasmids were 2.5×10^8 ~ 2.5×10^1 copies/μL. 9: Negative control

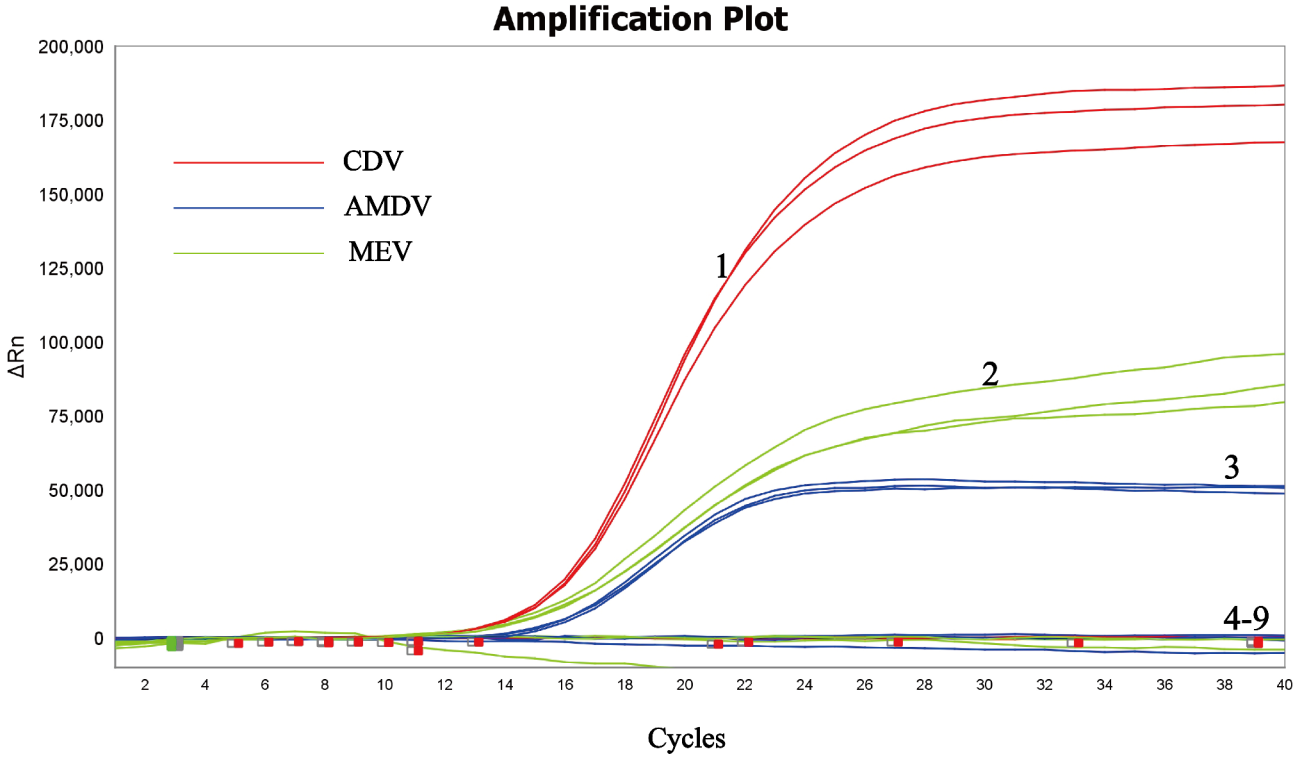

Fig. 3 Specificity of the multiple RT–qPCR method. 1 ~ 8 were CDV, MEV, AMDV, PRV, CPV, CCoV, and the CPIV standard positive template, and 9 was the standard negative control template

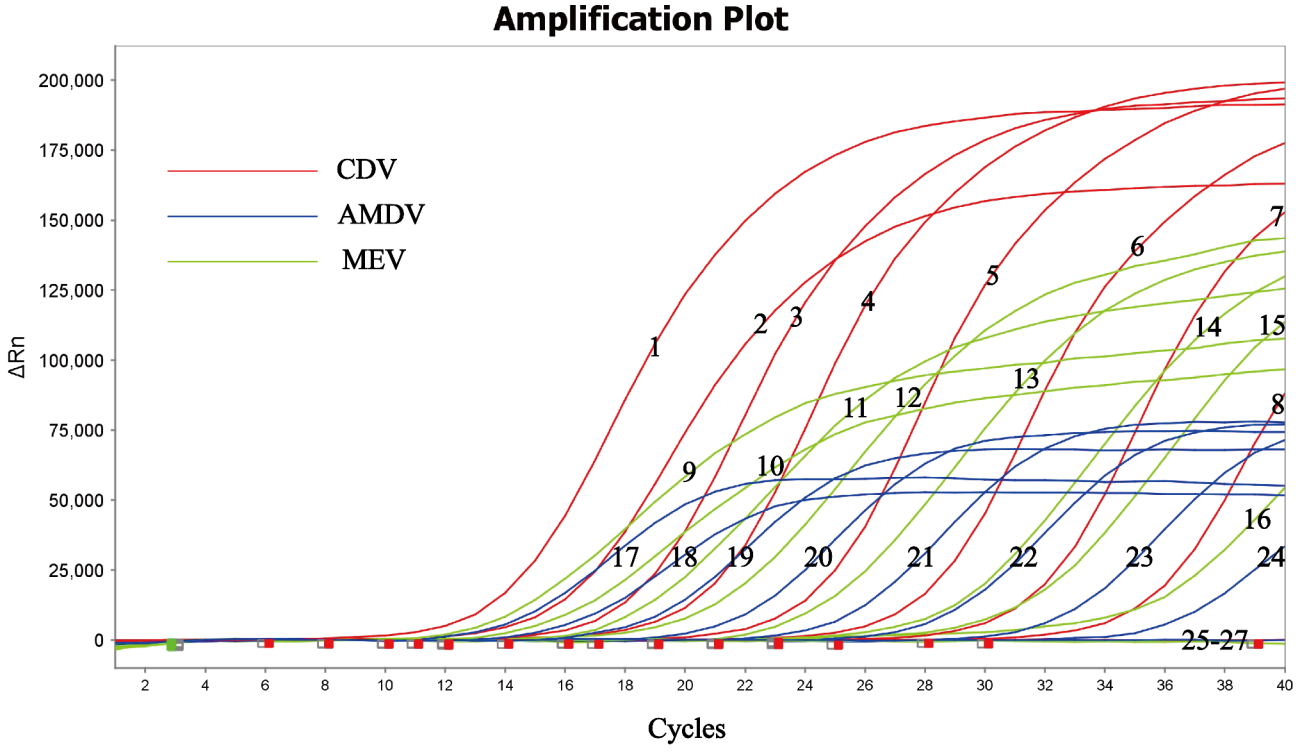

Fig. 4 Sensitivity of multiplex RT–qPCR. 1 ~ 8: The concentrations were $2.5 \times 10^8 \sim 2.5 \times 10^1$ copies/μL pCDV-N, respectively. 9 ~ 16: The concentration was $2.5 \times 10^8 \sim 2.5 \times 10^1$ copies/μL of pMEV-VP2. 17 ~ 24: The concentrations were $2.5 \times 10^8 \sim 2.5 \times 10^1$ copies/μL of pAMDV-NS1; 25 ~ 27: Negative control

Table 1 Multiplex *Taq*Man PCR repeatability tests for RT–PCR

Plasmid	(copies/μL) Concentration	Intra-assay Ct value			Interassay Ct value		
		average value $\bar{x}$	standard deviation SD	Coefficient CV%	average value $\bar{x}$	standard deviation SD	Coefficient CV%
pMEV-VP2	2.5×10^7	14.73	0.01	0.10	14.81	0.31	2.00
	2.5×10^5	22.53	0.10	0.43	22.81	0.49	2.16
	2.5×10^3	29.30	0.29	0.99	29.67	0.70	2.37
pCDV-N	2.5×10^7	15.44	0.47	3.06	15.21	0.39	2.5
	2.5×10^5	22.50	0.23	1.03	22.12	0.50	2.3
	2.5×10^3	29.20	0.57	1.95	29.04	0.38	1.3
pAMDV-NS1	2.5×10^7	16.04	0.54	3.37	16.09	0.56	3.51
	2.5×10^5	23.30	0.69	2.94	23.06	0.60	2.60
	2.5×10^3	29.63	0.80	2.70	29.80	0.69	2.31

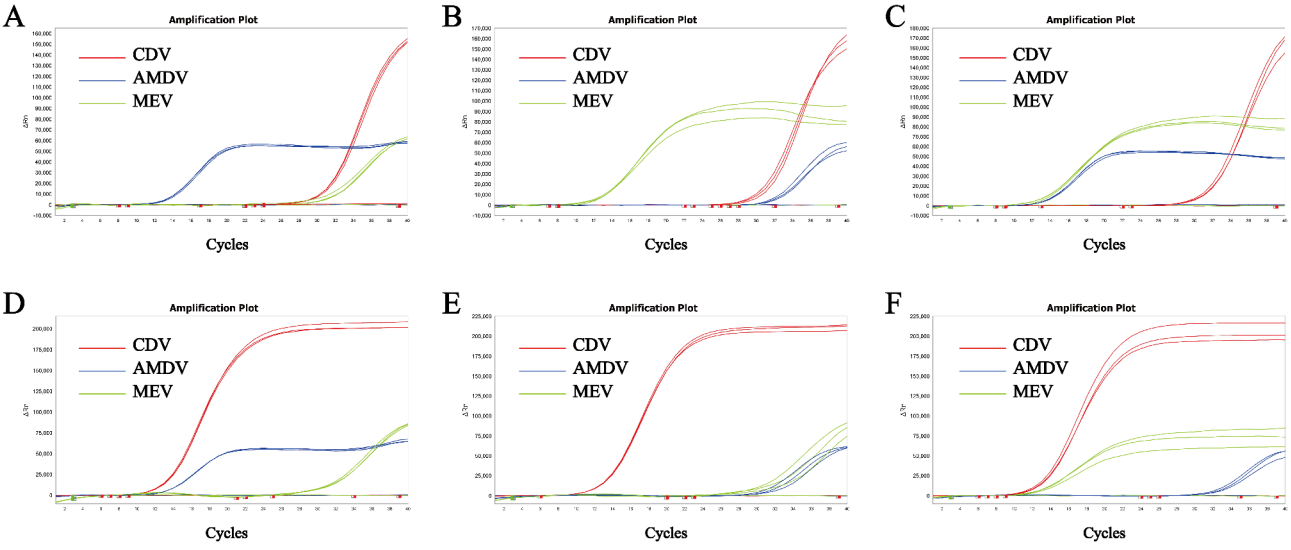

Fig. 5 Anti-interference experiment. **A**: The concentrations of pAMDV-NS1, pMEV-VP2, and pCDV-N were 10^8 copies/μL, 10^3 copies/μL, and 10^3 copies/μL, respectively. **B**: The concentrations of pAMDV-NS1, pMEV-VP2, and pCDV-N were 10^3 copies/μL, 10^8 copies/μL, and 10^3 copies/μL, respectively. **C**: The concentrations of pAMDV-NS1, pMEV-VP2 and pCDV-N were 10^8 copies/μL, 10^8 copies/μL and 10^3 copies/μL, respectively. **D**: The concentrations of pAMDV-NS1, pMEV-VP2 and pCDV-N were 10^8 copies/μL, 10^3 copies/μL and 10^8 copies/μL, respectively. **E**: The concentrations of pAMDV-NS1, pMEV-VP2, and pCDV-N were 10^3 copies/μL, 10^3 copies/μL, and 10^8 copies/μL, respectively. **F**: The concentrations of pAMDV-NS1, pMEV-VP2, and pCDV-N were 10^3 copies/μL, 10^8 copies/μL, and 10^8 copies/μL, respectively

CDV + MEV, and CDV + MEV + AMDV were 1.5%, 5.5%, 3% and 2%, respectively.

Overall, the DSe values determined via multiple RT–qPCR for the single or mixed pathogens tested 100%, the DSp values from 97% to 100%, and the kappa values varied from 0.921 to 1.00 (Table 2). The results indicated that multiple RT–qPCRs were more effective than single highly sensitive detection methods for AMDV, MEV and CDV.

Discussion

The infectious diseases AD and CD and Mink viral enteritis lead to high morbidity and mortality, causing enormous economic losses in the mink farming industry [4, 17, 18]. According to the results of clinical sample detection, an increasing tendency towards mixed infection caused by AMDV, MEV and CDV was found, suggesting that a detection method capable of detecting and distinguishing AMDV, MEV and CDV simultaneously is a prerequisite for the epidemiological investigation of mink infections. The multiplex real-time PCR (qPCR) method decreases detection costs, enhances efficiency and enhances accuracy, and these advantages give qPCR a primary position in disease screening and clinical settings [19–21].

The practical use of *Taq*Man qPCR has predominated for AMDV- and CDV-infected clinical samples. The limits of detection (LODs) of *Taq*Man qPCR for the AMDV-NS1 gene and CDV-N gene are 20 copies/μL and 100 copies/μL, respectively, and both are regarded as reference methods in this paper [22, 23]. *Taq*Man qPCR targeting the MEV-VP2 gene has not been reported previously; therefore, the use of the nanoPCR method, which has an LOD of 87.5 copies/μL and was described by

Table 2 Comparison of the samples tested by multiple RT–qPCR and the single highly sensitive detection methods for AMDV, MEV and CDV

Assays	Pathogen	Results	The single highly sensitive detection method			Performance Characteristics(%)		Kappa
			P	*N*	Total	DSe	DSp	
Multiple RT–qPCR	AMDV	*P*	99	3	102	100	97	0.970
		N	0	98	98			
	MEV	*P*	20	3	23	100	98.3	0.922
		N	0	177	177			
	CDV	*P*	6	0	6	100	100	1
		N	0	194	194			
	AMDV + CDV	*P*	3	0	3	100	100	1
		N	0	197	197			
	AMDV + MEV	*P*	11	1	12	100	99.5	0.954
		N	0	188	188			
	MEV + CDV	*P*	6	1	7	100	99.5	0.921
		N	0	193	193			
	AMDV + CDV + MEV	*P*	4	0	4	100	100	1
		N	0	196	196			

Wang, is the reference method [24]. However, multiplex *Taq*Man qPCR methods capable of detecting AMDV, CDV and MEV simultaneously are rare. Considering the description above, we established a one-step multiplex *Taq*Man qPCR with an LOD of 2.5×10^1 copies/μL to detect AMDV, CDV and MEV in a single reaction system. Lower sensitivity was not achieved, which may be due to the reduced sensitivity of the multiplex fluorescence quantification method itself. Our results showed that the standard curves of AMDV, CDV and MEV had slopes of -3.208, -3.082 and – 3.1852, respectively, and R2 values of 0.9982, 0.9980 and 0.9987, respectively. No cross-reaction signals were observed for other usual pathogens of mink, with 100% specificity. In addition, stability is also a considerable index for evaluating an emerging detection assay. In our research, repeatability was measured with a CV of less than 4%, indicating that ideal repeatability was achieved. These results suggested that the one-step *Taq*Man qPCR assay could be a potential and reliable platform in clinical mixed infection settings.

To further assess the performance of this multiplex *Taq*Man qPCR method, we collected 200 faecal samples and analysed the infection status of the samples; 4 mixed infection samples were detected. Similarly, single-time qPCR also detected 4 samples infected by AMDV, CDV and MEV, which is in perfect agreement with the results of single-time *Taq*Man qPCR. Judging from the test results, the detection rate of AMD is more than 50%, which is also being verified with clinical practice, indicating that the actual clinical infection rate of ADMV has remained high. Additionally, some "negative" samples identified by PCR were "positive" according to established one-step *Taq*Man qPCR, which indicated that one-step *Taq*Man qPCR showed better sensitivity than conventional PCR and effectively avoided misdiagnosis, especially for mixed infection samples. One potential reason underlying this observation is that one-step *Taq*Man qPCR can detect copies of viruses in samples via digitized results to detect false-negative negatives.

Moreover, we propose a protocol for point-of-care tests on farms that includes qPCR equipment and a kit consisting of collection tubes with lysis buffer, reaction tubes with premixed solution, primers and probes for AMDV, CDV and MEV, reverse transcription enzymes and sampling swabs. In the protocol, a pipettor and centrifuge are not needed, and personnel who are poor in professionally performing the tests are also needed. DNA extraction was unnecessary, the collected samples were added to tubes using swabs, and the results were one-click derivated or printed. The test procedure is as follows: (1) The anal swabs were collected and soaked in collecting tubes for 3 min at room temperature; (2) A drop of sample solution and reaction solution were added to the reaction tubes; (3) qPCR was conducted for approximately 1 h (Fig. 6B). Compared to the POCT kit for CDV developed by Brown, our protocol is more convenient and easier to use [3].

Conclusions

In conclusion, we established a one-step multiplex qPCR assay to detect and differentiate AMDV, CDV and MEV in a single system. The advantages of the newly developed one-step multiplex qPCR assay, that is, in terms of analytical sensitivity and specificity, support the attractive applications of this method as a reliable tool for the rapid detection of common viruses and diagnosis of this disease in mink.

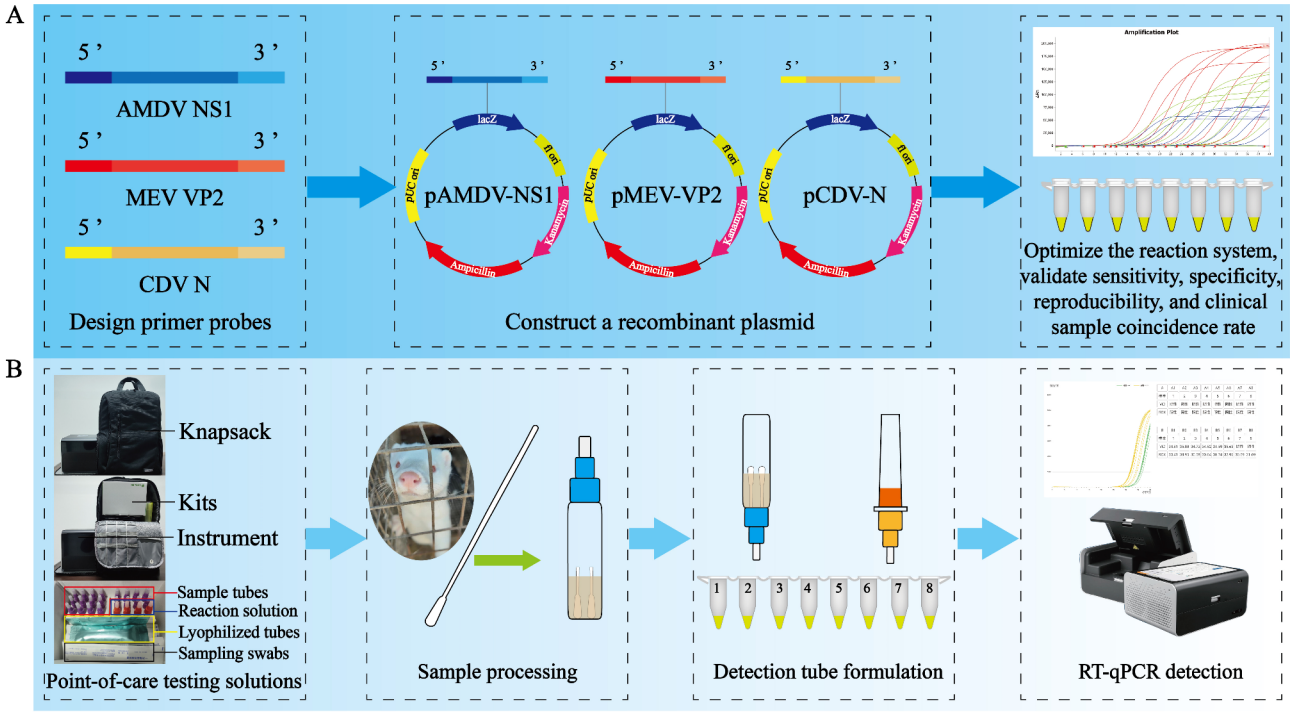

Fig. 6 Experimental flowchart. **A**: A multiplex, one-step RT–qPCR assay was used to detect CDV, AMDV, and MEV simultaneously. **B**: A protocol for point-of-care testing of CDV, AMDV, and MEV

Table 3 Primers and probes

Target virus	Name	Gene	Length (bp)	Sequence (5′-3′)
MEV	QF	VP2	190	AACACCTATTGCAGCAGGACG
	QR			GTTTCTCCTGTTGTGGTAGTTTTTT
	Probe			FAM-ATCCAAGATATGCATTTGGTAG ACA-BHQ1
CDV	QF	N	202	AAATCAACGGACCTAAATTAACTGG
	QR			TCATCTGCCTCAGAATCCAAAC
	Probe			ROX-ACTCTGTTTGTGGTCTTACAT TTGC-BHQ2
AMDV	QF	NS1	119	CTTACAAATACCATCACAAACAA ACC
	QR			ACCATCCATACCTTCCTCAGTTATC
	Probe			HEX-GCAGTAGACATGCGTGATTAT ACAT-BHQ1

Materials and methods

Samples, primers and probes

MEV and CDV were purchased from Jilin Teyan Biotechnology Co., Ltd. The nucleic acids AMDV, canine parvovirus (CPV), pseudorabies virus (PRV), canine coronavirus (CCoV) and canine parainfluenza virus (CPIV) were preserved by the Preventive Veterinary Laboratory of Qingdao Agricultural University. Two hundred suspected samples were collected from mink herds in Shandong Province.

The conserved region sequences of the NS1 gene in AMDV (KU513985.1), the VP2 gene in MEV (KT899745.1) and the N gene in CDV (HM063009.1) were retrieved from GenBank. The primers and probes used were designed with Primer Explorer V5 to amplify fragments of approximately 119 bp, 190 bp and 202 bp, and the probe characteristics of AMDV, MEV and CDV included the following reporter dyes: HEX, FAM, and ROX; and the BHQ1, BHQ1, and BHQ2 quenchers. The primers and probes used were synthesized by Shanghai Personal Biotechnology Co., Ltd. (China), and the oligonucleotide sequences of the primers and probes are shown in Table 3.

Viral DNA/RNA extraction

Total DNA and RNA were extracted from faecal samples and from MEV and CDV vaccines using a FastPure® Viral DNA/RNA Mini Kit (Vazyme, China) according to the manufacturer’s instructions. The extracted DNA/RNA samples were used as templates in the RT−qPCR assays.

Construction of recombinant plasmids

The DNA of AMDV and MEV and the RNA of CDV were used as templates. Using primers for AMDV-AF/AR, MEV-MF/MR, and CDV-CF/CR, the genes of the corresponding viruses were amplified via PCR and subsequently cloned and inserted into the pEASY-T1 vector (TransGen Biotech, Beijing, China) (Fig. 6A). Sequencing was carried out by Shanghai Personal Biotechnology Co., Ltd., to support correct construction, and the

recombinant plasmids used were named pAMDV-NS1, pMEV-VP2 and pCDV-N. The concentrations of the recombinant plasmids were measured according to the following formula. Then, tenfold-diluted recombinant plasmids were prepared from 2.5×10^7 copies/μL to 2.5×10^1 copies/μL for RT–qPCR. Standard curves were drawn to determine the reliability of the diluted recombinant plasmids.

Optimization of the RT–qPCR system

The recombinant plasmids were subjected to RT–qPCR to determine the optimal concentrations of the primers (0.2 μM, 0.5 μM, and 0.8 μM) and probes (0.2 μM, 0.25 μM, and 0.4 μM), which were determined via orthogonal tests according to Ct values and amplification efficacy. The amplification reagent was TOROIVD® 5G qPCR Premix BB with UNG (TOROIVD, China). Amplification was carried out with a QuantStudio 5 (Thermo Fisher Scientific, United States) instrument with the following reaction conditions: 37 °C for 30 s for initial denaturation, 40 cycles of denaturation at 95 °C for 120 s and 95 °C for 10 s, and annealing at 60 °C for 30 s.

Establishment of standard curves

Three recombinant plasmids were tenfold-diluted ($10^{-1} \sim 10^{-8}$ copies/μL) for RT–qPCR, in which ddH_2O was used as a negative control. Standard curves were established according to the Ct values and dilutions.

Specificity assay

The specificity for multiple RT–qPCR assays was evaluated using three mixed recombinant plasmids and other DNAs (PRV and CPV) and cDNAs (CCoV and CPIV) as negative control. Three independent experiments were performed for each sample, and ddH_2O served as the negative control.

Sensitivity assay

The sensitivity of the multiple RT–qPCR assays was evaluated. The recombinant plasmids of AMDV, MEV and CDV were diluted from 2.5×10^8 copies/μL to 2.5×10^1 copies/μL and mixed with an equal volume. Three independent experiments were performed for each sample, and ddH_2O served as the negative control.

Reproducibility assay

The three recombinant plasmids (pAMDV-NS1, pMEV-VP2 and pCDV-N) were diluted, and three different concentrations were selected for use in reproducibility assays (2.5×10^7, 2.5×10^5, and 2.5×10^3 copies/μL). Each concentration was tested 3 times, and a one-time qPCR assay was conducted to determine the intrabatch repeatability by comparing the standard deviation (SD) and the coefficient of variation (CV). Three qPCR assays using three diluted standard plasmids were conducted to test interbatch repeatability by comparing the SD and the CV.

Anti-interference assay and clinical sample detection

To confirm whether the original concentrations of standard plasmids can affect the performance of multiple RT–qPCRs, an anti-interference assay was designed. Briefly, three standard plasmids at different concentrations (10^8 and 10^3 copies/μL) were combined randomly, and multiple RT–qPCR and single RT–qPCR were conducted for AMDV, MEV and CDV.

The diagnostic performance of multiple RT–qPCRs was assessed by collecting and testing 200 facial samples suspected to suffer from diarrhoea from mink herds in Shandong Province, China. The positive Ct value is between 15 and 35, between 35 and 40 is suspicious, more than 40 is negative, suspicious samples need to be tested again, if the Ct value is less than 40 is positive, greater than 40 is negative. Moreover, retesting was performed using the highly sensitive detection method for AMDV, MEV and CDV, as previously reported [22–24]. The feasibility of multiple RT–qPCR methods was evaluated by measuring the diagnostic specificity (DSp), diagnostic sensitivity (DSe) and degree of agreement with the highly sensitive detection methods used for AMDV, MEV and CDV.

Statistical analysis

The calculation of DSe and DSp between the two methods was based on the following formula. $DSe = TP/(TP + FN)$ and $DSp = TN/(TN + FP)$, where TP means true-positive cases, FN means false-negative cases, TN means true-negative cases, and FP means false-positive cases. Precision was evaluated by obtaining the mean time-to-detection values and standard deviations (SDs) of each set of replicates at a given concentration and calculating the coefficients of variation (CVs) (SD/mean).

Author contributions

Z.C. and J.W. designed the experiments; Z.C., H.X. and X.Z. performed the experiments; Z.C., H.X., X.Z., K.Z., D.Y., S.M., W.L., S.L., J.R., and J.W. contributed reagents/materials/analysis tools; Z.C., H.X., and J.W. analyzed the data; Z.C. and J.W. wrote the paper. All authors read and approved the final manuscript.

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Data availability

Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate

All experiments received approval and were supervised by the Research Ethics Committee of Qingdao Agricultural University. Informed consent has been obtained from owners for fecal samples collection. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Informed consent
Not applicable.

Consent to participate
Not applicable.

Conflict of interest
The authors declare no conflict of interest.

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