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Development of a triplex TaqMan probe-based real-time PCR assay for simultaneous detection of swine influenza virus, porcine reproductive and respiratory syndrome virus, and porcine circovirus type 2

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Abstract

Background Porcine respiratory disease complex poses a substantial economic challenge for the swine farms due to its prevalence in pig farming environments. The disease is primarily caused by viral pathogens such as swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus type 2 (PCV2). Given the high incidence and morbidity associated with these viruses, effective control strategies rely on rapid and accurate diagnosis.

Results To this end, we developed and validated a triplex TaqMan probe-based real-time PCR assay for the simultaneous detection and differentiation of SIV, PRRSV, and PCV2. This assay demonstrated high specificity, with no observed cross-reactivity between the target viruses. The assay's sensitivity was determined to be 100 copies/μL for SIV and 10 copies/μL for PRRSV and PCV2. Comparison with a national standard detection method using 110 clinical samples revealed 100% agreement, confirming the reliability of the newly developed assay for clinical diagnostic applications.

Conclusions Given the zoonotic potential of SIV, this assay not only provides a valuable diagnostic tool for veterinary medicine but also contributes to enhanced public health surveillance efforts.

Keywords Real-time PCR, Detection, SIV, PRRSV, PCV2

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Background

The intensification of livestock farming in recent years has led to the widespread occurrence of porcine respiratory disease complex (PRDC), posing a significant challenging to its control. PRDC is primarily driven by viral pathogens that damage respiratory epithelial cells and lung tissues, predisposing animals to secondary bacterial pathogens [1]. SIV, PRRSV, and PCV2 are recognized as the main viral pathogens associated with PRDC [2]. Co-infections with these viral pathogens can negatively impact growth performance and reduce the efficacy of vaccines [3, 4].

SIV is globally distributed and represents a substantial threat to the swine industry due to its detrimental impact on animal health and productivity [5]. The zoonotic potential of SIV, including the transmission of swine-origin H1N1 influenza subtypes to humans, has resulted in human fatalities [6]. Subtypes such as H1N1, H1N2, and H3N2 are prevalent in swine populations worldwide, often leading to high morbidity (up to 100%) but relatively low mortality in pigs [7]. The conserved nature of matrix protein (M) gene makes it a common target for diagnostic primer and probe design [7].

PRRSV, a positive single-stranded RNA virus belonging to *Arteriviridae* family, can cause immunosuppression and significant economic losses in the swine industry [8, 9]. The high mutation rate and recombination frequency of PRRSV strains make it a persistent and challenging pathogen in global animal husbandry [10]. The matrix (M) and nucleocapsid (N) genes of PRRSV are relatively conserved compared to other genomic regions.

PCV2 is a small, non-enveloped icosahedral virus belonging to the genus *Circovirus* within the family *Circoviridae* [11]. The capsid (Cap) protein, the sole structural protein and principal immunogen of PCV2, is crucial for vaccine development and diagnostic assays [12, 13]. PCV2 infections can lead to immunosuppression and a spectrum of subclinical symptoms, increasing susceptibility to secondary infections and further compromising animal health [1, 14].

While single real-time fluorescent quantitative PCR (qPCR) assays exist for detecting SIV, PRRSV, and PCV2 [15–17], single-plex assays require separate reaction, limiting efficiency in terms of time and resources. Furthermore, a readily available, single quantitative assay for the direct simultaneous detection of these three pathogens is lacking. In this study, we designed specific primers and probes targeting M gene of SIV, the M and N genes of PRRSV, and the Cap gene of PCV2 to develop a triplex qPCR assay. This novel assay offers a robust and efficient approach for the diagnosis and surveillance of these important swine pathogens.

Materials and methods

Primers and probes design

Based on the relatively conserved regions of the M gene of SIV, the M and N genes of PRRSV, and the Cap gene of PCV2, gene sequences of these pathogens were retrieved from NCBI and aligned using MegAlign software to facilitate primers and probes design (Figure S1). Primers were designed using Premier 5 and Primer Express 3.0.1 software, targeting amplification lengths between 100 and 200 bp and incorporating distinct fluorophore wavelengths (Table 1). To confirm specificity, the designed primers and probes were validated using NCBI Primer-BLAST before synthesis.

Viral nucleic acid extraction and reverse transcription

Viral DNA or RNA was extracted from clinical samples exhibiting respiratory symptoms using the TIANGEN Virus DNA/RNA Kit (Beijing, China). Briefly, nasal swabs and tissue samples were resuspended in 2 mL PBS, homogenized, and centrifuged at 5,000 rpm for 10 min. The resulting supernatant was used for viral nucleic acid extraction. Complementary DNA (cDNA) was then synthesized utilizing the 5×Prime Script RT Master Mix (for Real Time) from Takara (Beijing, China). Our laboratory's nucleic acid collection, including classical swine fever virus (CSFV), pseudorabies virus (PRV), SIV, PRRSV, PCV2, *Streptococcus suis* (SS), and *Haemophilus parasuis*

Table 1 The primers/probes for detection in this study

Pathogens	Primer/probes	Sequence (5'-3')	Length	Gene	Position
SIV	SIV-F	TTCACGCTCACCGTGCCC	199bp[a]	M	11,503–11,701
	SIV-R	GGCCCAWACAACTGGCAAGT			
	SIV-Pro	NED-AGCGAGGACTGCAGCGTAG-MGB			
PRRSV	PRRSV-F	CACCTCCAGRTGCCGKTTG	125bp[b]	M	14,570–14,694
	PRRSV-R	GGACGACAAATGCGTGGTTAT			
	PRRSV-Pro	VIC-TAGGCCGCAAGTACATTCTGGCCC-BHQ1			
PCV2	PCV2-F	ATGGTGAAGAAGTGGTTGTTATTG	147bp[c]	Cap	670–816
	PCV2-R	TGCTGGTAATCAGAATACTGCGG			
	PCV2-Pro	FAM-TGACTTTTATGGCTGGCTGCCGTGG-MGB			

Note: The lengths of the amplified genes were determined by GenBank No. [a] MN418820.1, [b] EF635006.1, and [c] MH465471.1

(HPS) was also used in this study. All nucleic acid samples were stored at -80 °C until use.

Standard plasmids construction

The M gene of SIV, the M and N genes of PRRSV, and Rep and Cap gene fragment of PCV2 were amplified using the primers listed in Table 2 and subsequently cloned into the pMD18-T Vector. The resulting plasmids were sequenced to verify the correct insertion of the target fragments. The concentrations of the plasmids were then quantified using a Thermo Scientific NanoDrop2000 spectrophotometers and calculated using the following formula: Plasmid copies/μL= (A260 ng/μL×10^{-9} × 6.02 × 10^{23}) / (plasmid length ×660). Three standard plasmids, serially diluted tenfold from 10^7copies/μL to 10^2 copies/μL, were used to generate standard curves.

Optimization of the triplex qPCR

The triplex qPCR was conducted on the Applied Biosystems QuantStudio™ 5 instrumentation (Thermo Fisher Scientific) utilizing Premix Ex Taq™ enzyme (Takara Biotechnology, Dalian, China). The reaction mixture contained 10 uL of enzyme, 0.2 uL of ROX reference dye for normalization, and 1 uL of mixed plasmid templates containing the target genes. To optimize the assay conditions, we evaluated primer concentrations ranging from 0.1 to 0.4 μM and probe concentrations ranging from 0.2 μM to 0.6 μM. The cycling conditions consisted of an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at a temperature between 52 °C and 62 °C for 30 s. The optimal reaction conditions were determined based on a combination of achieving the lowest Ct values (cycle threshold) and the highest fluorescence intensity, while also considering cost-effectiveness. Standard plasmids with a known concentration of 1 × 10^7 copies/μL were used to optimize the reaction conditions.

Specificity, sensitivity, and repeatability assessment

To assess the specificity of the primers and probes used in this experiment, negative controls were selected to include viruses such as CSFV and PRV, as well as bacteria like *HPS* and *SS*, which are known to cause PRDC. Additionally, the genome of porcine alveolar macrophage (PAM) was used as a negative control, given that the nucleic acids extracted from clinical samples were derived from the cells of pigs exhibiting respiratory symptoms.

The sensitivity of the triplex qPCR was assessed under optimized reaction conditions by performing 10-fold serial dilutions of a standard plasmid, spanning a concentration range from 10^8 copies/μL to 1 copy/μL. To ascertain the limit of detection (LOD) for this assay, we conducted 23 replicate experiments using three specific dilutions of the standard plasmid: 100 copies/μL, 10 copies/μL, and 1 copy/μL. The LOD was defined as the concentration at which the standard plasmid consistently yielded a positive detection rate of 95%.

To evaluate the repeatability of the method, both intra-assay and inter-assay variability were evaluated by conducting experiments with four different concentrations of the standard plasmid: 10^6, 10^5, 10^4, and 10^3 copies/μL. Each concentration was tested in triplicate, and the entire assay was repeated three times using plasmids from different batches. The coefficient of variation (CV) was calculated for both in-batch and out-of-batch cycle threshold (Ct) values across all concentrations to quantitatively assess the reproducibility of the assay.

Clinical sample detection and phylogenetic analysis

A total of 110 clinical samples showing signs of respiratory tract disease were collected from the southwestern region of China. The nucleic acids extracted from these samples were tested using the triplex qPCR assay developed in this study. Subsequently, the extracted viral nucleic acids were also tested for various pathogens using the National Standard Detection Methods, as recommended. The primers and probes used in these assays are listed in Table S1.

The clinical samples consisted of both diseased tissues and nasal swabs. One portion of the samples was used for viral nucleic acid extraction and analysis via the triplex

Table 2 The primes for standard plasmids construction and phylogenetic analysis

Purpose	Primer	Sequence (5′-3′)	Length	Gene
Standard plasmids constructions	SIV-F	AGTCTTCTAACCGAGGTCGAAACGT	978 bp	M
	SIV-R	TTACTCCAGCTCTATGTTGACAAAA		
	PRRSV-F	ATACCAACTTCCTTCTGGACACTAA	1191 bp	M, N
	PRRSV-R	CATGGTTCTCGCCAATTAAA		
	PCV2-F	CAGCACCTCGGCAGCACCTCAGCAG	1725 bp	Rep, Cap
	PCV2-R	CGGTTTCAGCGATGACGTATCCAAG		
Phylogenetic analysis	PRRSV-F	GTTTTAGCCTGTCTTTTTGCC	704 bp	GP5
	PRRSV-R	AAGGTGCTTTTGGCGTTTTC		
	PCV2-F	CAGCACCTCGGCAGCACCTCAGCAG	1700 bp	Rep, Cap
	PCV2-R	CGGTTTCAGCGATGACGTATCCAAG		

Table 3 Triple fluorescence quantitative PCR reaction system

Reagent	Volume
Premix Ex Taq(Probe qPCR)(2X)	10 μL
ROX Reference Dye II	0.2 μL
SIV-F and SIV-R (10μM)	0.8 μL (0.4μM)
SIV-Pro (10μM)	0.4 μL (0.2μM)
PRRSV-F and PRRSV-R (10μM)	0.8 μL (0.4μM)
PRRSV- Pro (10μM)	0.6 μL (0.3μM)
PCV2-F and PCV2-R (10μM)	0.8 μL (0.4μM)
PCV2- Pro (10μM)	0.4 μL (0.2μM)
Template	2 μL
Enzyme-free water	Up to 20 μL

Table 4 Specificity of the multiplex real-time PCR assay

Sample Type	Controls	Multiplex Real-Time PCR in the Study (Ct Value)		
		PRRSV	PCV2	SIV
Lung	PRRSV	26.214	-	-
Serum	PCV2	-	26.950	-
Nasal swab	SIV	-	-	30.110
Lung	CSFV	-	-	-
Lung	PRV	-	-	-
Bacterial fluid	HPS	-	-	-
Bacterial fluid	SS	-	-	-
Porcine alveolar macrophages	PAM	-	-	-

qPCR assay, while the other portion was sent for testing according to standard diagnostic methods. Since the experimental animals were either deceased or euthanized for other research purposes, no additional interventions or injuries were inflicted upon them. According to the *Regulations on the Administration of Laboratory Animals in China*, this study did not require review or approval by an ethics committee.

Some of the positive samples identified by the triplex qPCR assay were further analyzed by sequencing after amplification with a single sequencing primer. Representative strain sequences were retrieved from NCBI and used to construct a phylogenetic tree using MEGA6.4 software. The tree was generated using the neighbor-joining method and bootstrap sampling with 1000 replicates. To enhance the clarity of the phylogenetic tree, iTOL software was used for sequence visualization and analysis.

Result

Optimization of the triplex qPCR reaction system and conditions

To enhance the efficiency and cost-effectiveness of the triplex qPCR assay, we focused on optimizing key parameters, including primer and probe concentrations and the annealing temperature. Our primary objective was to minimize non-specific amplification while achieving lower Ct values. Among comparable conditions, those that exhibited higher fluorescence intensity and required lower amounts of primers and probes were preferred.

After conducting a series of experiments involving varying temperature gradients, an optimal annealing temperature of 60 °C was determined. To further refine the assay conditions, a matrix cross-test was performed, varying the primer concentrations between 0.1 and 0.4 μM and the probe concentrations between 0.2 and 0.6 μM. The optimized reaction system is summarized in Table 3.

Validation of the specificity of primers and probes used in the triplex qPCR assay

To evaluate the specificity of the primers and probes selected for the triplex qPCR assay, templates were extracted from positive controls, including PRRSV, PCV2, and SIV, as well as from negative controls, such as PRV, CSFV, HPS, SS, and PAM. The results demonstrated that the designed primers and probes effectively recognized and amplified the target gene fragments of the corresponding viruses. No amplification or fluorescent signal was observed for any unrelated pathogens (Table 4). These findings confirm that each primer-probe pair exhibits high specificity, making them well-suited for use in the triplex qPCR assay (Fig. 1).

Establishment of standard curves for the triplex qPCR assay

Using the optimized reaction conditions, we established standard curves by selecting six different concentrations of plasmids, ranging from 10^7 to 10^2 copies/μL, diluted in 10-fold increments. The results showed the following coefficients of determination (R^2) and amplification efficiencies (Eff%) for each pathogen: SIV ($R^2 = 0.997$, Eff% = 91.562), PRRSV ($R^2 = 0.999$, Eff% = 100.530), and PCV2 ($R^2 = 0.999$, Eff% = 108.767). With R^2 values close to 1 and amplification efficiencies within the optimal range of 90-110%, these results indicated a strong linear correlation between the template plasmid concentrations and the corresponding Ct values (Fig. 2). This demonstrates the reliability of the assay for quantitative analysis of nucleic acids in amplified samples.

Evaluation of the triplex qPCR sensitivity

The sensitivity of the triplex qPCR experiments was evaluated using an optimized reaction system with varying concentrations of standard plasmids. The results of 23 replicates conducted at low concentrations are summarized in Table 5. At concentration of 100 copies/μL, the positive detection rate of the three pathogens was 100%, the assay achieved a positive detection rate of 100% for all three pathogens. When the concentration was reduced

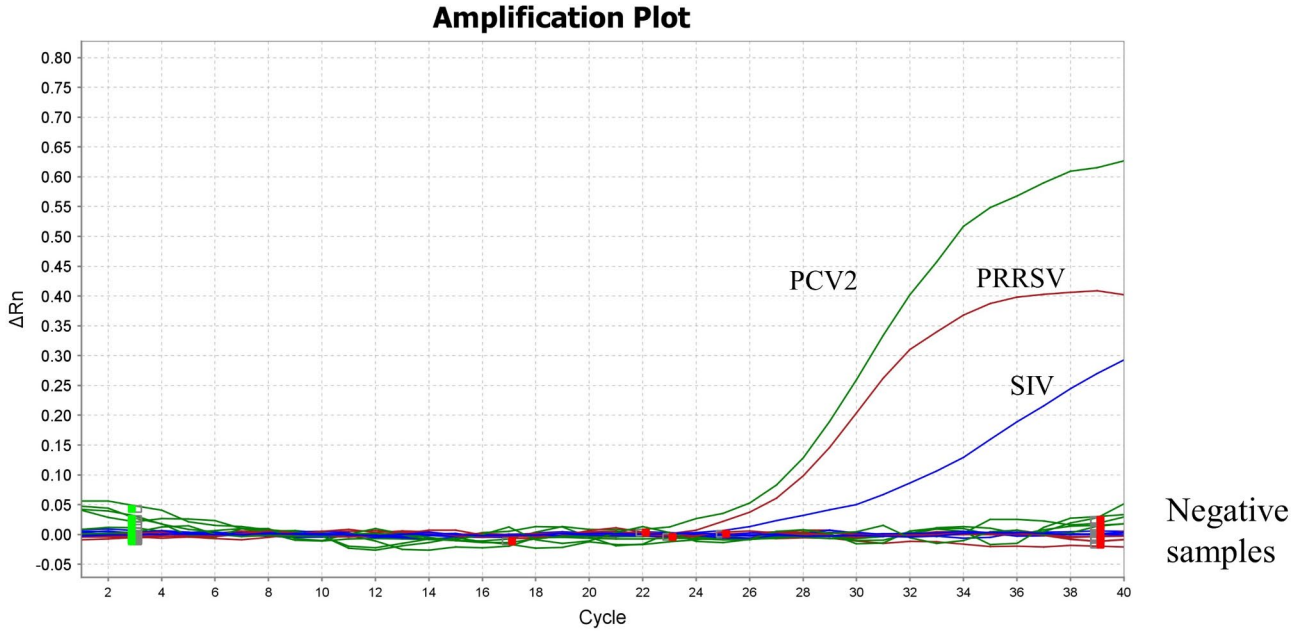

Fig. 1 Specificity test of the triple qPCR assay. Amplification curves represent samples positive for PRRSV, SIV and PCV2 detected by the triple qPCR assay. Negative samples included CSFV, PRV, HPS, SS, PAM

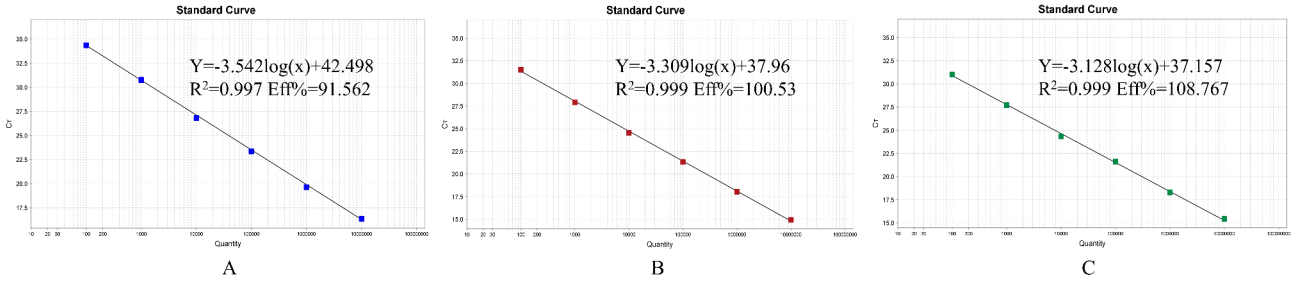

Fig. 2 Standard curve test of the triple triplex qPCR assay. **A** standard curve test of SIV; **B** standard curve test of PRRSV; **C** standard curve test of PCV2

Table 5 Sensitivity of triplex qPCR assay

Pathogen	Concentrations (Copies/μL)	Repeat times	Positive number	Positive rate	95% positive rate
SIV	100	23	23	100%	>95%
	10	23	12	52%	<95%
	1	23	0	0%	<95%
PRRSV	100	23	23	100%	>95%
	10	23	22	96%	>95%
	1	23	0	0%	<95%
PCV2	100	23	23	100%	>95%
	10	23	23	100%	>95%
	1	23	0	0%	<95%

to 10 copies/μL, the positive detection rates were 52% for SIV, 96% for PRRSV, and 100% for PCV2. At the lowest concentration tested, 1 copy/μL, none of the pathogens were detected. The LOD for this experiment was defined as the lowest concentration at which the positive detection rate exceeded 95%. Accordingly, the sensitivity thresholds for the triple qPCR were determined to be 100 copies/μL for SIV, 10 copies/μL for both PRRSV and PCV2 (Fig. 3). At LOD, the Ct values ranged from approximately 34 to 36. A cutoff value of 36 was established, which indicates that samples with a Ct value of 36 or less are considered positive, while those with a Ct value above 36 are classified as negative.

Assessment of repeatability in triplex qPCR assay

A series of repetitive tests were conducted using standard plasmids diluted from 10^6 to 10^3 copies/μL to assess the repeatability of the triplex qPCR assay. The evaluation was performed by calculating the mean values and standard deviations of the results. As shown in Table 6, the coefficient of variation (CV) for intra assay repeatability was ≤1%, while the inter CV was ≤5%. These results indicate that the triplex qPCR detection method demonstrates high stability and reliability.

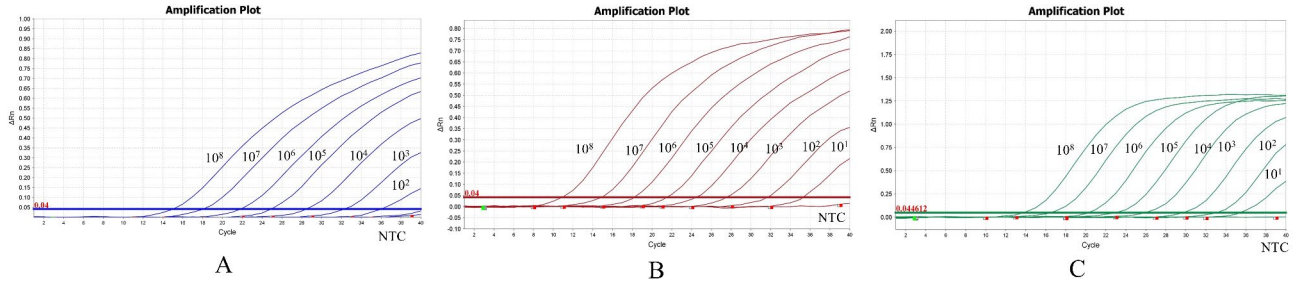

Fig. 3 Sensitivity test of the triple fluorescence quantitative PCR assay. **A** sensitivity test of SIV; **B** sensitivity test of PRRSV; **C** sensitivity test of PCV2

Table 6 Repeatability test of the multiplex real-time PCR assay

Plasmid	Concentration(copies/μL)	Inter-assay Ct value		Intra-assay Ct value	
		X ± SD	CV(%)	X ± SD	CV(%)
PRRSV	10^6	21.392 ± 0.090	0.400	21.984 ± 0.717	3.300
	10^5	24.470 ± 0.246	1.000	25.446 ± 0.547	2.100
	10^4	27.712 ± 0.184	0.700	28.909 ± 0.909	3.100
	10^3	31.478 ± 0.09	0.300	32.515 ± 0.791	2.400
SIV	10^6	21.881 ± 0.106	0.500	22.181 ± 0.416	1.900
	10^5	24.787 ± 0.085	0.300	25.928 ± 0.148	2.600
	10^4	28.374 ± 0.049	0.200	29.882 ± 0.527	1.800
	10^3	32.340 ± 0.103	0.300	33.847 ± 0.851	2.500
PCV2	10^6	20.173 ± 0.121	0.600	20.652 ± 0.677	3.300
	10^5	23.075 ± 0.073	0.300	23.774 ± 0.989	4.200
	10^4	26.048 ± 0.043	0.200	26.948 ± 1.272	4.700
	10^3	29.749 ± 0.118	0.400	30.664 ± 1.293	4.200

Assessment of co-infection in triplex qPCR assay

In co-infection analysis, standard plasmids were used to simulate mixed infections commonly observed in clinical samples (Fig. 4). The results demonstrated that the triplex qPCR was capable of detecting two or three pathogens simultaneously.

Detection of clinical samples

To evaluate the clinical applicability of the established method, we tested 110 clinical samples. The results revealed that 2.7% of the samples were positive for SIV, 10.9% for PRRSV, and 7.2% for PCV2 (Table 7). To confirm the accuracy of our detection method, all samples were subsequently analyzed using national standard detection method of China. The results showed a 100% concordance with our method, indicating that the assay is reliable for detecting pathogens in clinical samples.

Phylogenetic analysis

Some of the clinically positive samples tested were subjected to genetic analysis using primers of Table 2 to assess their evolutionary relationships. Using MEGA6.4, phylogenetic trees for PRRSV and PCV2 were constructed employing the neighbor-joining method and bootstrap analysis with 1000 repetitions. For PRRSV, a phylogenetic tree was generated that included the ORF5 sequences of 12 positive strains from this study, alongside 28 reference strains obtained from the GenBank database. The result showed that the sequences of the 12 positive samples were classified into two major lineages: lineage 1 (NADC30/34-like PRRSV) and lineage 8 (highly-pathogenic PRRSV). Notably, four out of seven samples in lineage 1 were closely related to sublinage 1.5 (NADC30-like PRRSV), and three clustered into sublinage 1.8 (NADC34-like PRRSV). For PCV2, a genetic evolution tree was constructed based on full-length sequences, incorporating 23 reference sequences from the GenBank database along with seven positive sequences identified in this study. The analysis indicated that the positive sequences belonged to subtypes PCV2b, PCV2c, PCV2d, and PCV2e. Among these, subtype PCV2d was predominant across all tested samples, suggesting that it may be the most prevalent subtype in this context (Fig. 5).

Discussion

Co-infection is a prevalent challenge in livestock farming, particularly affecting pig production through the interaction of bacterial and viral pathogens. A notable example of this phenomenon is PRDC, which is significantly influenced by viral pathogens, including SIV, PRRSV, and PCV2 [18]. Studies have shown higher detection rates of PRRSV and PCV2 in clinical samples related to PRDC, indicating their crucial roles in disease manifestation [1,

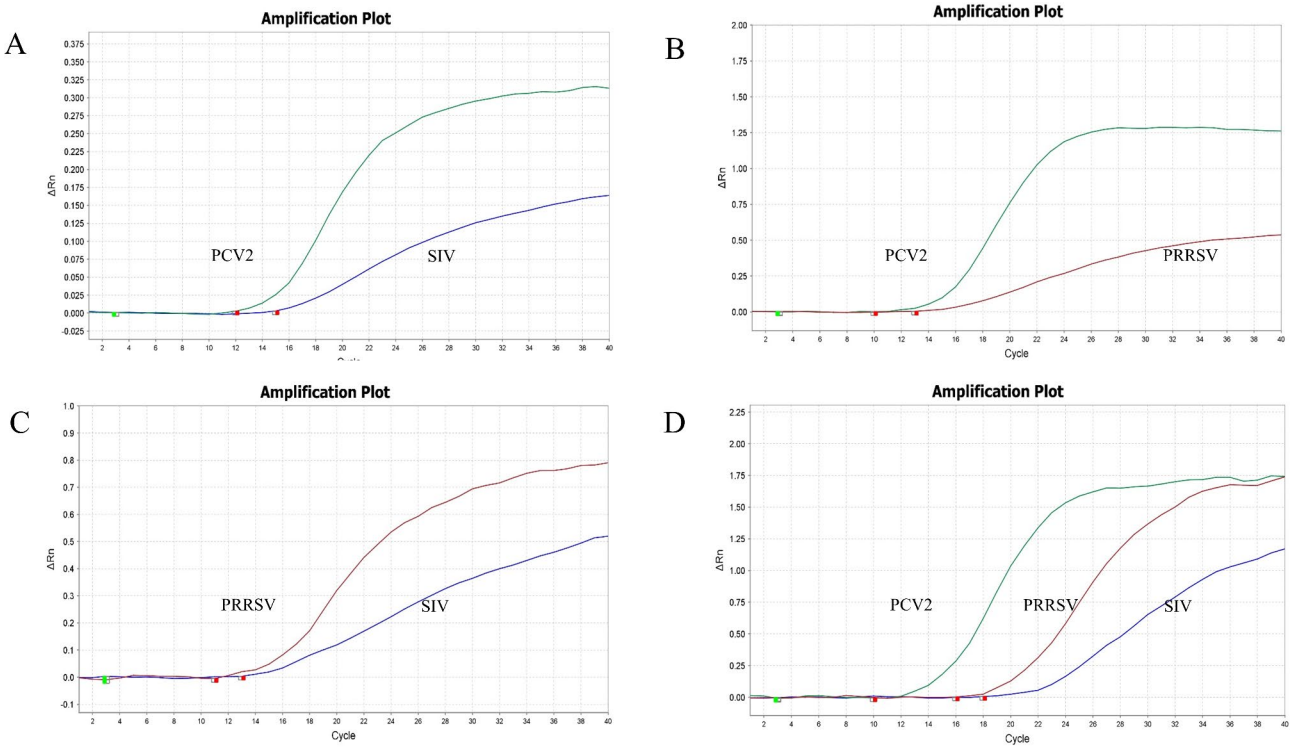

Fig. 4 Co-infection simulation experiments with two or three pathogens. **A** PCV2 + SIV; **B** PCV2 + PRRSV; **C** PRRSV + SIV; **D** PCV2 + PRRSSV + SIV with the same concentration

Table 7 The detection of clinical samples

Pathogens	Triple qPCR assay results	National standard test results	Coincidence rate
PRRSV	10.9%(12/110)	10.9%(12/110)	100%
PCV2	7.2%(8/110)	7.2%(8/110)	100%
SIV	2.7%(3/110)	2.7%(3/110)	100%
PRRSV + PCV2	2.7%(3/110)	2.7%(3/110)	100%
PRRSV + SIV + PCV2	1.8%(2/110)	1.8%(2/110)	100%

19]. Moreover, co-infections involving PRRSV and SIV can impair the efficacy of PRRSV vaccines, while the combination of PCV2 and SIV exacerbates clinical symptoms [20, 21]. Consequently, establishing accurate and efficient detection methods is imperative for the timely identification and control of these pathogens.

Current detection strategies are primarily categorized into pathogen-based and serology-based methods. Among pathogen-based approaches, nucleic acid detection technologies, such as PCR, are commonly employed to amplify pathogen-specific nucleic acid sequences. Multiplex fluorescent qPCR offers notable advantages over single PCR methods, including reduced reaction time and resource consumption by enabling the simultaneous detection of multiple pathogens in a single assay. This approach minimizes sample handling steps and lowers the risk of cross-contamination. For instance, a multiplex PCR and microarray system have been developed to differentiate four viruses and four bacteria associated with PRDC [22].While this method demonstrates high specificity, its sensitivity is comparatively lower than the method introduced in this study. In addition to multiplex techniques, single and dual PCR methods are also utilized for detecting pathogens linked to porcine respiratory diseases [23, 24].Dual nested PCR for simultaneous detection of both PCV2 and PRRSV pathogens have been developed [25]. However, this method increases the risk of cross-contamination due to the need to perform two PCR reactions, and the double-stranded nested PCR involves more steps and requires the design of two pairs of primers compared to a single PCR. Although the Point-of-Care and Label-Free assays applied to the field detection of PRRSV and SIV viruses show good sensitivity and specificity, they are limited to two respiratory pathogens [26]. Additionally, existing detection methods require updates to address the rapid emergence of new subtypes, such as NADC30-like and NADC34-like PRRSV strains, PCV2f subtype, and G4EA H1N1 subtype [27, 28]. Thus,

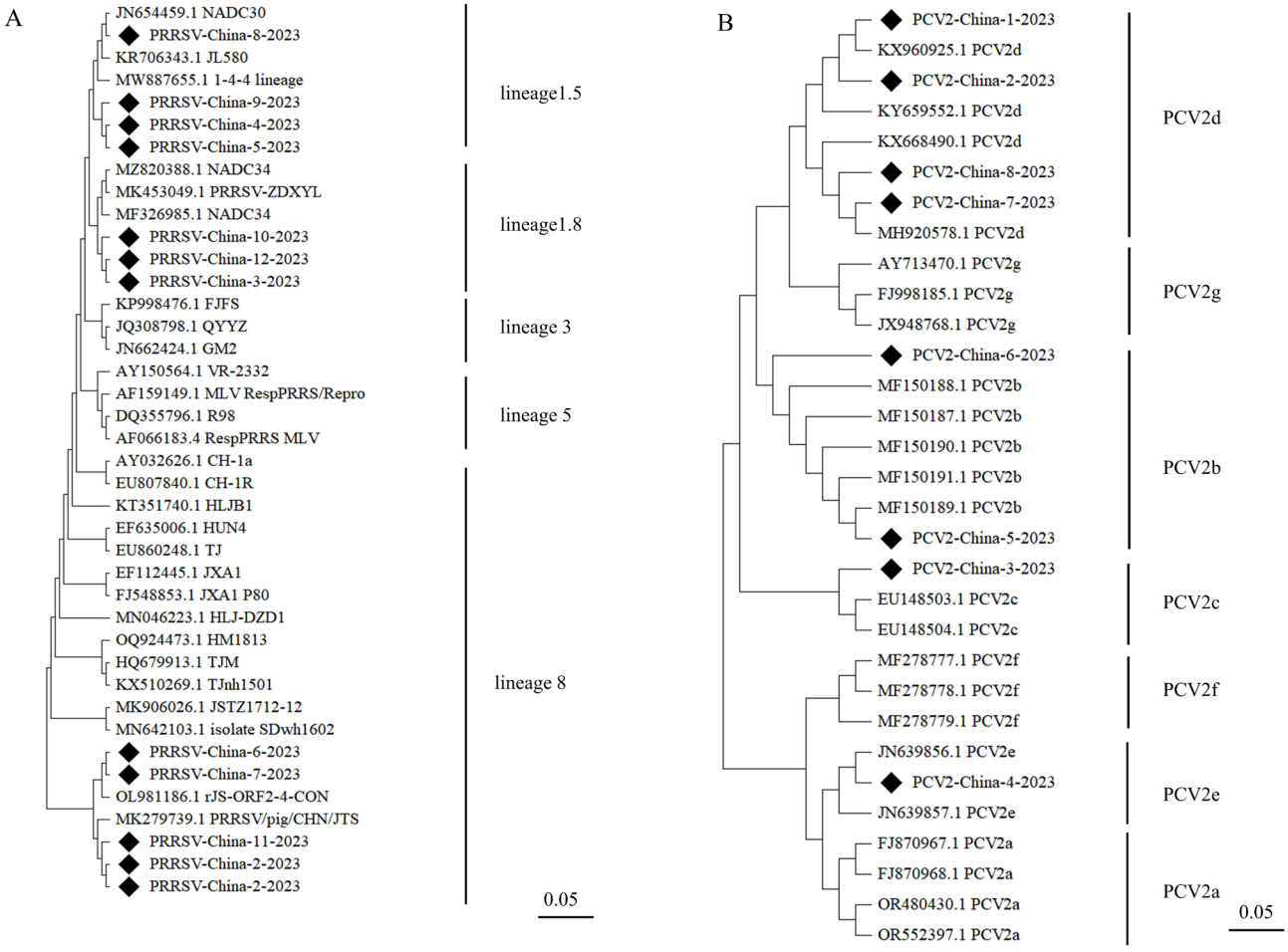

Fig. 5 Phylogenetic analysis of PRRSV and PCV2. The GenBank accession number of the reference sequence are shown on each branch of the phylogenetic tree. Strains detected in this study are labeled with). (**A**) Phylogenetic tree of PRRSV strains. (**B**) Phylogenetic tree of PCV2 strains

this study aimed to develop a method capable of detecting three respiratory disease-related pathogens while also accommodating recently prevalent strains. Primers and probes were designed to target conservative regions of new subtypes, shown in supplementary material. The reaction conditions, including temperature and concentrations of primers and probes, were optimized to enhance amplification efficiency and reduce fluorescence interference. Notably, the sensitivity of SIV detection was lower within the optimized system, likely due to interference from other fluorescence signals. Sensitivity testing established minimum detection limits of 100 copies/μL for SIV, and 10 copies/μL for both PRRSV and PCV2, demonstrating good sensitivity compared to established methods [29]. For sensitivity test, the selected analogue items are constructed standard plasmids, viral nucleic acids extracted directly from the virus and the addition of standard plasmids and viruses to negative material for sensitivity test. However, all of these have certain drawbacks, nasal swabs or even more tissue homogenates are more complex than standard plasmids and viral nucleic acids; the reference standard of negative patient material and the actual clinical negative and positive samples have certain differences, and can not completely simulate the actual clinical samples. Therefore, sensitivity test results may differ slightly from the sensitivity of clinical samples.Specificity testing confirmed that this triplex real-time PCR assay accurately detects SIV, PRRSV, and PCV2 without cross-reactivity to other pathogens in the analyzed clinical samples. Additionally, intra- and inter-batch stability tests yielded coefficients of variation below 5%, indicating the method's reliability for clinical application [30].

The triplex qPCR detection method was evaluated using clinical samples collected from pig farms in China in 2023, where signs of respiratory tract disease were present. The results indicated positivity rates of 10.9% for PRRSV, 7.2% for PCV2, and 2.7% for SIV, with co-infections observed in 2.7% of cases involving PRRSV and PCV2, and 1.8% with all three pathogens. The high prevalence of PRRSV aligns with previous findings [19]. In contrast, a study of Italian pig farms reported significantly

higher positivity rates for PCV2 [31]. The variation in pathogen prevalence affecting swine respiratory diseases necessitates continuous monitoring and timely intervention strategies. Furthermore, clinical samples were verified against national standards, achieving a compliance rate of 100%, affirming the applicability of triplex qPCR for clinical diagnostics. To further characterize positive samples, a genetic evolution analysis was performed, revealing that PRRSV positive samples in this study were classified as PRRSV-2 type 1 and 8 lineages. This aligns with the prevailing PRRSV-2 genotype in China, particularly lineages 1, 3, 5, and 8 [32]. The phylogenetic analysis of PCV2 identified subtypes PCV2b, PCV2c, PCV2d, and PCV2e, highlighting the virus's genetic variability and propensity for recombination [31, 33]. The detection of multiple virus subtypes underscores the potential risks to pig productivity, emphasizing the necessity for timely and accurate testing to mitigate such threats.

Conclusions

In summary, our study developed a method to detect respiratory diseases in pigs early. The triplex qPCR assay, developed in this study, can detect three main pathogens including PRRSV, PCV2 and SIV without interference from other pathogens. This method is quick, flexible, and sensitive, making it a reliable tool for early diseases detection. Using this test helps us catch these diseases early, which helps manage them better. Moreover, it keeps pork safe by ensuring it's free from these pathogens, which is good for consumers and the pork industry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04688-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Not applicable.

Author contributions
MW: Investigations, data curation, data analysis, original draft preparation. YP: Investigations, data curation, review and editing. WM: Data curation, review and editing. XW: Data curation, review and editing. HC: Supervision and funds acquisition. CX: Conceptualization, review and editing, funds acquisition. YW: Conceptualization, supervision, review and editing, funds acquisition.

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Data availability
Data is provided within supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All participants consent to publications of the data.

Competing interests
The authors declare no competing interests.

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References

1. Qin S, Ruan W, Yue H, Tang C, Zhou KZhang B. Viral communities associated with Porcine respiratory disease complex in intensive commercial farms in Sichuan Province. China Sci Rep. 2018;8(1):13341. https://doi.org/10.1038/s41598-018-31554-8.
2. Opriessnig T, Giménez-Lirola L, GHalbur PG. Polymicrobial respiratory disease in pigs. Anim Health Res Rev. 2011;12(2):133–48. https://doi.org/10.1017/s1466252311000120.
3. Niederwerder MC, Jaing CJ, Thissen JB, Cino-Ozuna AG, Mcloughlin K, SRowland. R R. Microbiome associations in pigs with the best and worst clinical outcomes following co-infection with Porcine reproductive and respiratory syndrome virus (PRRSV) and Porcine circovirus type 2 (PCV2). Vet Microbiol. 2016;188:1–11. https://doi.org/10.1016/j.vetmic.2016.03.008.
4. Kitikoon P, Vincent AL, Jones KR, Nilubol D, Yu S, Janke BH et al. Vaccine efficacy and immune response to swine influenza virus challenge in pigs infected with porcine reproductive and respiratory syndrome virus at the time of SIV vaccination. Vet Microbiol, 2009, 139(3–4): 235–44.https://doi.org/10.1016/j.vetmic.2009.06.003
5. Baudon E, Peyre M, Peiris MCowling BJ. Epidemiological features of influenza circulation in swine populations: A systematic review and meta-analysis. PLoS ONE. 2017;12(6). https://doi.org/10.1371/journal.pone.0179044.
6. Ma W. Swine influenza virus: current status and challenge. Virus Res. 2020;288:198118. https://doi.org/10.1016/j.virusres.2020.198118.
7. Anderson TK, Chang J, Arendsee ZW, Venkatesh D, Souza CK, Kimble JB, et al. Swine influenza A viruses and the tangled relationship with humans. Cold Spring Harb Perspect Med. 2021;11(3). https://doi.org/10.1101/cshperspect.a038737.
8. Dokland T. The structural biology of PRRSV. Virus Res. 2010;154(1–2):86. https://doi.org/10.1016/j.virusres.2010.07.029.
9. Wang S, Zhang M, Liu XC, Lin T, Yang HC, Yuan SS, et al. Investigation on the co-infections of Toxoplasma gondii with PRRSV, CSFV or PCV-2 in swine in part of China. J Integr Agric. 2015;14(9):1838. https://doi.org/10.1016/s2095-3119(15)61044-9.
10. Lv C, Zheng Y, Liu K, Li G, Luo Q, Zhang H, et al. Genetic variation and recombination analysis of PRRSV-2 GP3 gene in China from 1996 to 2023. Front Microbiol. 2024;15:1435373. https://doi.org/10.3389/fmicb.2024.1435373.
11. Yang Y, Xu T, Wen J, Yang L, Lai S, Sun X, et al. Prevalence and phylogenetic analysis of Porcine circovirus type 2 (PCV2) and type 3 (PCV3) in the Southwest of China during 2020–2022. Front Vet Sci. 2022;9:1042792. https://doi.org/10.3389/fvets.2022.1042792.
12. Qiu H, Sun M, Wang N, Zhang S, Deng Z, Xu H, et al. Efficacy comparison in cap VLPs of PCV2 and PCV3 as swine vaccine vehicle. Int J Biol Macromol. 2024;278(Pt 3):134955. https://doi.org/10.1016/j.ijbiomac.2024.134955.
13. Wang Y, Li R, Zhang Y, Zhang W, Hu SLi Z. Visual and label-free ASFV and PCV2 detection by CRISPR-Cas12a combined with G-quadruplex. Front Vet Sci. 2022;9:1036744. https://doi.org/10.3389/fvets.2022.1036744.
14. Xu Y, Zheng J, Sun P, Guo J, Zheng X, Sun Y, et al. Cepharanthine and Curcumin inhibited mitochondrial apoptosis induced by PCV2. BMC Vet Res. 2020;16(1):345. https://doi.org/10.1186/s12917-020-02568-0.
15. Ma Y, Shi K, Chen Z, Shi Y, Zhou Q, Mo S, et al. Simultaneous detection of Porcine respiratory coronavirus, Porcine reproductive and respiratory syndrome virus, swine influenza virus, and pseudorabies virus via quadruplex One-Step RT-qPCR. Pathogens. 2024;13(4). https://doi.org/10.3390/pathogens13040341.
16. Liu H, Zou J, Liu R, Chen J, Li X, Zheng H, et al. Development of a TaqMan-Probe-Based multiplex Real-Time PCR for the simultaneous detection of

African swine fever virus, Porcine circovirus 2, and pseudorabies virus in East China from 2020 to 2022. Vet Sci. 2023;10(2). https://doi.org/10.3390/vetsci10020106.
17. Osemeke OH, Cezar GA, Paiva RC, Moraes DCA, Machado IF, Magalhaes ES, et al. A cross-sectional assessment of PRRSV nucleic acid detection by RT-qPCR in serum, ear-vein blood swabs, nasal swabs, and oral swabs from weaning-age pigs under field conditions. Front Vet Sci. 2023;10:1200376. https://doi.org/10.3389/fvets.2023.1200376.
18. Eddicks M, Eddicks L, Stadler J, Hermanns WRitzmann M. [The Porcine respiratory disease complex (PRDC) - a clinical review]. Tierarztl Prax Ausg G Grosstiere Nutztiere. 2021;49(2):120–32. https://doi.org/10.1055/a-1403-1976.
19. Sun Q, Yu X, He D, Ku X, Hong B, Zeng W, et al. Investigation and analysis of etiology associated with Porcine respiratory disease complex in China from 2017 to 2021. Front Vet Sci. 2022;9:960033. https://doi.org/10.3389/fvets.2022.960033.
20. Chrun T, Maze EA, Vatzia E, Martini V, Paudyal B, Edmans MD, et al. Simultaneous infection with Porcine reproductive and respiratory syndrome and influenza viruses abrogates clinical protection induced by live attenuated Porcine reproductive and respiratory syndrome vaccination. Front Immunol. 2021;12:758368. https://doi.org/10.3389/fimmu.2021.758368.
21. Ouyang T, Zhang X, Liu XRen L. Co-Infection of swine with Porcine circovirus type 2 and other swine viruses. Viruses. 2019;11(2). https://doi.org/10.3390/v11020185.
22. Lung O, Ohene-Adjei S, Buchanan C, Joseph T, King R, Erickson A, et al. Multiplex PCR and microarray for detection of swine respiratory pathogens. Transbound Emerg Dis. 2017;64(3):834–48. https://doi.org/10.1111/tbed.12449.
23. Chang CY, Deng MC, Wang FI, Tsai HJ, Yang CH, Chang C, et al. The application of a duplex reverse transcription real-time PCR for the surveillance of Porcine reproductive and respiratory syndrome virus and Porcine circovirus type 2. J Virol Methods. 2014;201:13–9. https://doi.org/10.1016/j.jviromet.2014.01.016.
24. Shi X, Liu X, Wang Q, Das A, Ma G, Xu L, et al. A multiplex real-time PCR panel assay for simultaneous detection and differentiation of 12 common swine viruses. J Virol Methods. 2016;236:258–65. https://doi.org/10.1016/j.jviromet.2016.08.005.
25. Kim HK, Lyoo KS, Huynh TML, Moon HJ, Nguyen V, GPark BK. Duplex nested reverse transcriptase polymerase chain reaction for simultaneous detection of type 2 Porcine reproductive and respiratory syndrome virus and Porcine circovirus type 2 from tissue samples. J Vet Sci. 2017;18(2):253. https://doi.org/10.4142/jvs.2017.18.2.253.
26. Manessis G, Frant M, Wozniakowski G, Nannucci L, Benedetti M, Denes L, et al. Point-of-Care and Label-Free detection of Porcine reproductive and respiratory syndrome and swine influenza viruses using a microfluidic device with. Photonic Integr Circuits Viruses. 2022;14(5). https://doi.org/10.3390/v14050988.
27. Sun H, Xiao Y, Liu J, Wang D, Li F, Wang C, et al. Prevalent Eurasian avian-like H1N1 swine influenza virus with 2009 pandemic viral genes facilitating human infection. Proc Natl Acad Sci U S A. 2020;117(29):17204. https://doi.org/10.1073/pnas.1921186117.
28. Bao F, Mi S, Luo Q, Guo H, Tu C, Zhu G, et al. Retrospective study of Porcine circovirus type 2 infection reveals a novel genotype PCV2f. Transbound Emerg Dis. 2018;65(2):432–40. https://doi.org/10.1111/tbed.12721.
29. Zheng LL, Chai LY, Tian RB, Zhao Y, Chen H, YWang Z Y. Simultaneous detection of Porcine reproductive and respiratory syndrome virus and Porcine circovirus 3 by SYBR green I-based duplex real-time PCR. Mol Cell Probes. 2020;49:101474. https://doi.org/10.1016/j.mcp.2019.101474.
30. Pan Z, Lu J, Wang N, He WT, Zhang L, Zhao W, et al. Development of a TaqMan-probe-based multiplex real-time PCR for the simultaneous detection of emerging and reemerging swine coronaviruses. Virulence. 2020;11(1):707. https://doi.org/10.1080/21505594.2020.1771980.
31. D'annunzio G, Ostanello F, Muscatello LV, Orioles M, Jacumin N, Tommasini N, et al. Porcine circovirus type 2 and Porcine reproductive and respiratory syndrome virus alone or associated are frequent intralesional detected viruses in Porcine respiratory disease complex cases in Northern Italy. Front Vet Sci. 2023;10:1234779. https://doi.org/10.3389/fvets.2023.1234779.
32. Zhao J, Xu L, Xu Z, Deng H, Li F, Sun X, et al. Emergence and spread of NADC34-like PRRSV in Southwest China. Transbound Emerg Dis. 2022;69(5):e3416–e. https://doi.org/10.1111/tbed.14463.
33. Chen S, Li X, Zhang L, Zheng J, Yang L, Niu G, et al. Phylogenetic and structural analysis of Porcine circovirus type 2 from 2016 to 2021 in Jilin Province, China. Microorganisms. 2023;11(4). https://doi.org/10.3390/microorganisms11040983.

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