Establishment and Application of Dual TaqMan Fluorescence Quantitative Polymerase Chain Reaction (PCR) for the Detection of Porcine Circovirus Types 2 and 3

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Title: Establishment and application of dual TaqMan fluorescence quantitative polymerase chain reaction (PCR) for the detection of porcine circovirus types 2 and 3

Running Title: Dual TaqMan real-time PCR assay for PCV2 and PCV3

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Declarations

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The authors declare they have no conflicts of interest in this study.

Availability of data and material (data transparency)

All the data and materials are available by authors if necessary.

Code availability (software application or custom code)

Not applicable.

Authors' contributions

Wang Yixin and Li Jianliang contributed to the conception of the study; Cao Fengyang, Luan Xiaoning, Li Yuyan and Guo Longzong performed the experiment; Wang Wenwen contributed significantly to analysis and manuscript preparation; Cao Fengyang and Wang Yixin performed the data analyses and wrote the manuscript; Li Jianliang and Guo Longzong helped perform the analysis with constructive discussions.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

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Not applicable.

Abstract: To establish a rapid method for the simultaneous differential detection of porcine circovirus types 2 (PCV2) and 3 (PCV3), two pairs of primers and two TaqMan probes were designed according to the gene sequences of PCV2 and PCV3, and a dual TaqMan fluorescence quantitative polymerase chain reaction (PCR) method for the simultaneous detection of two virus nucleic acids was established. The results showed that the correlation coefficients (R^2) of the standard curves drawn using the recombinant plasmids of PCV2 and PCV3 were greater than 0.99 and had a good linear relationship. The specific detection results of PCV type 1, Porcine parvovirus, and Porcine pseudorabies virus were negative. The detection limits of this experimental method for PCV2 and PCV3 were 10 and 1 copies/μL, respectively, which were more sensitive than those of the common PCR detection methods. The established method was used to detect 76 samples from some pig farms in Shandong Province. 11 of the 76 samples were PCV3 positive (positive rate of 14.47%), 24 were PCV2 positive (positive rate of 34.58%), and PCV3 and PCV2 were mixed in six samples (positive rate of 7.89%). The whole-genome sequence of PCV3 was amplified and sequenced to further understand the molecular biological characteristics and genetic variation of PCV3 in Shandong Province. The genomes of 11 PCV3 strains were all 2000 bp long, and the whole-genome sequence homology between them ranged from 98.4% to 99.9%. There were mutation sites in the amino acid sequences of Cap and Rep proteins, and the strains isolated in this experiment were concentrated in the PCV3a and PCV3c subgroups. This study provides technical support and molecular biological basis for nucleic acid detection, epidemiological characteristics, genetic variation, and control of PCV3 in Shandong Province.

Keywords: dual fluorescence quantitative PCR; PCV2; PCV3; Sequence analysis

Porcine circovirus (PCV) disease is an infectious disease caused by PCV, which is prevalent worldwide and has caused great harm to the pig breeding industry. There are four subtypes of PCV, namely, PCV1, PCV2, PCV3, and the recently discovered PCV4, among which PCV1 is less pathogenic (Opriessnig et al. 2020; Zhang et al. 2020). The main symptoms of PCV infection in pigs are reproductive dysfunction, respiratory diseases, porcine dermatitis nephrotic syndrome (PDNS), and postweaning multisystem wasting syndrome (PMWS). PCV can also induce immunosuppression, leading to secondary infection of other pathogens.

PCV2 was first isolated from pigs that demonstrated PMWS symptoms (Allan et al. 1998). PCV2 is the main pathogenic microorganism causing "PCV-related diseases," including piglet multiple system failure syndrome, porcine respiratory disease syndrome, PDNS, proliferative and necrotizing interstitial pneumonia, and reproductive disorders. PCV3 is a new type of circovirus discovered in recent years. It was first identified by Kansas State University researchers from sows with reproductive disorders and PDNS and their aborted fetuses (Palinski et al. 2017; Phan et al. 2016). According to related reports, PCV3 may be related to PMWS, reproductive disorders, PDNS, myocarditis, and multisystem inflammation (Palinski et al. 2017). In 2019, researchers discovered a new type of PCV (PCV4) in China, which is 66.9% similar to the circovirus found in minks, but its clinical pathogenic characteristics, pathogenic mechanism, and prevention and control measures need further study (Zhang et al. 2020).

PCV2 and PCV3 have similar genomic structures and mainly encode two large open reading frames (ORFs). ORF1 encodes a viral replication-associated protein (Rep) on the left

side of the genome, and ORF2 encodes a viral antigen-related protein (Cap) on the right side

of the genome (Palinski et al. 2017; Phan et al. 2016). During single or mixed infection, the

virus will cause great losses to the pig industry in China. At present, there is no available

vaccine or drug for PCV3, so a fast and effective detection method has become an important

measure to prevent the disease. Researchers have established nucleic acid detection methods

for PCV2 and PCV3 (Wang et al. 2019b; Yuan et al. 2020; Zhao et al. 2019). This study

intends to establish a dual TaqMan fluorescence quantitative polymerase chain reaction (PCR)

method for PCV2 and PCV3 to improve the sensitivity and efficiency of detection, realize the

rapid differential detection of PCV2 and PCV3, and provide technical support for the

epidemiological investigation and pathogenic mechanism research of PCV2 and PCV3.

1. Materials and methods

1.1. Virus strains

PCV1, PCV2, Porcine parvovirus (PPV), and Porcine pseudorabies virus (PRV) were

isolated, identified, and preserved at the Animal Quarantine Laboratory of Shandong

Agricultural University. PCV3-positive samples were identified and preserved in the same

laboratory.

1.2. Construction of PCV2 and PCV3 standard plasmids

PCV2 and PCV3 DNA were extracted using a viral genomic DNA extraction kit (Omega

Bio-Tek, Norcross, GA, USA). PCV2 *rep* and PCV3 *cap* genes were amplified using the

primers listed in Table 1. The amplification system comprised 18.2 μL water, 2.5 μL 10×

buffer, 2 μL dNTP, 0.3 μL rTaq DNA polymerase (Takara, Dalian, China), 0.5 μL upstream

and downstream primers, and 1 μL template DNA. The amplification procedure was as

follows: 94°C for 5 min; 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s (34 cycles); 72°C for 10 min (35 cycles); and 4°C. The PCR products were identified using 1% agarose gel electrophoresis. The target fragments were collected and cloned into a pMD18-T cloning vector to construct PCV2 and PCV3 standard plasmids. The standard plasmid was quantified by a nucleic acid quantitative analyzer, and the copy number was calculated and continuously diluted by 10 times.

1.3. Establishment and optimization of the dual TaqMan fluorescence quantitative PCR method

The sequences of the PCV2 *rep* and PCV3 *cap* genes were analyzed and compared with the reference sequences registered in GenBank. The conserved regions were selected as the design regions of specific primers and probes listed in Table 1. Among them, the 5′-end and the 3′-end of the detection probe of PCV2 are modified with HEX and BHQ1, respectively, and those of the detection probe of PCV3 are modified with FAM and BHQ1, respectively. The primers and probes were synthesized by Suzhou Hongxun Biotechnology Company. The reaction system comprised 2× premix EX Taq (probe qPCR), template DNA, primers, and probes. The annealing temperature (54°C–64°C), different primer concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μM), and different probe concentrations (0.05, 0.1, 0.25, and 0.50 μM) were optimized using the highest fluorescence value (ΔRN) and the minimum cyclic threshold (CT) value (template control). The amplification cycle conditions were as follows: 95°C for 5 min, 95°C for 30 s, and 56°C to 62°C for 34 s for 40 cycles.

1.4. Drawing of the standard curve

The standard plasmids PCV2-Rep and PCV3-Cap were serially diluted (10^1–10^8 copies

/μL) 10 times. The optimized dual TaqMan fluorescence quantitative PCR method was used to amplify the recombinant plasmids, and the amplification kinetic curve was obtained. Three repeats were set for each gradient, and the standard curve was drawn to calculate the correlation coefficient. The standard curve of the dual TaqMan fluorescence quantitative PCR method was calculated with the logarithm of the standard recombinant plasmid copy number (log) as the abscissa and the CT value as the ordinate.

1.5. Specificity experiment

The genomic DNA of PCV1, PCV2, PPV, and PRV was extracted by a virus genomic DNA extraction kit, and quantified by a nucleic acid quantitative analyzer. The method established in this study was used for detection, and the specificity of the dual TaqMan fluorescence quantitative PCR method was evaluated. During detection, the standard was used as the positive control, and the negative control was set at the same time, which was repeated in three groups.

1.6. Sensitivity experiment

With the plasmid standard mixture diluted 10 times (10^0–10^4 copies/μL) as the template, amplification was conducted according to the dual TaqMan fluorescence quantitative PCR method of PCV2 and PCV3 established in this study, and each dilution was repeated in three groups to determine the sensitivity of the dual TaqMan fluorescence quantitative PCR method.

1.7. Repetitive experiment

Using plasmids with 10^4 to 10^8 copies/μL dilutions as templates, the method established in this study was used to detect and calculate the coefficient of variation within the group

(intragroup). The standard positive standard plasmid was stored in a refrigerator at −20℃ and detected in batches for three consecutive times to calculate its intergroup variation coefficient.

1.8. Clinical sample detection

The 76 samples of pig clinical tissues obtained from Shandong Province from 2017 to 2019 were collected in our laboratory. After the corresponding nucleic acid DNA was extracted by the viral genomic DNA extraction kit, PCV3 was detected using the dual TaqMan fluorescence quantitative PCR method of PCV2 and PCV3. To ensure the accuracy of detection, the common PCR method was used for parallel detection, and the agreement between the two was compared.

1.9. Amplification and sequencing of the PCV3 complete genome sequence

The DNA of the positive samples was extracted using a DNA extraction kit. Primers were synthesized according to the literature (Fan et al. 2017), and the whole-genome sequence of PCV3 was amplified via PCR. The amplified products were cloned into a pMD-18T vector and sequenced by Qingdao Ruibo Xingke Biological Company.

1.10. Sequence analysis and genetic evolution tree construction analysis

The sequencing results were edited and spliced with DNASTAR software for the whole genome, and compared with the representative PCV3 strains published in GenBank at home and abroad. To evaluate the diversity and evolution of PCV3 in Chinese swine herds in Shandong province, multiple sequence alignments were performed by ClustalX 2.0 and phylogenetic trees were constructed using MEGA 6.06 based on the aligned sequences using the neighbor-joining method and the maximum composite likelihood model. The robustness of the phylogenetic trees was evaluated by bootstrapping using 1000 replicates. The reference

sequence was listed in Table 2.

2. Results

2.1. Establishment of the dual TaqMan fluorescence quantitative PCR assay for PCV2 and PCV3

The PCV2 *rep* and PCV3 *cap* genes were amplified and cloned into a pMD-18T vector to construct the recombinant plasmids PCV2-Rep and PCV3-Cap, respectively. The dual TaqMan fluorescence quantitative PCR assay for PCV2 and PCV3 was established, and the conditions were optimized by adapting the concentrations of the primers and probes in different proportions. The final optimal system was: 2× Premix Ex Taq (Probe qPCR) 10 μL, PCV3 F/R (10 μM) 0.1 μL, PCV3 probe (10 μM) 0.2 μL, PCV2 F/R (10 μM) 0.1 μL, PCV2 probe (10 μM) 0.2 μL, and template DNA 2 μL; water was added to make up to 20 μL. The TaqMan real-time fluorescent quantitative PCR detection conditions were as follows: 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s, for 40 cycles. The prepared recombinant plasmid standards PCV2-Rep and PCV3-Cap were mixed, and the linear relationship of eight gradients with gradient concentrations of 10^3- to 10^{10} copies/μL was detected by this method. As shown in Fig. 1, the regression equations of PCV2 and PCV3 are y = −2.6625x + 34.416 (R^2 = 0.9984) and y = −2.7335x + 35.22 (R^2 = 0.9976). The results showed that the concentration of this detection method has a good linear relationship with the CT value.

2.2. Sensitivity, specificity, and repeatability tests

2.2.1. Sensitivity test

The sensitivity of the test was detected by ordinary PCR and dual TaqMan fluorescence quantitative PCR. As shown in Fig.2 and Fig.3, the minimum number of plasmid copies

detected by PCR for PCV3 and PCV2 was 10 and 10^2 copies/µL, respectively, while the minimum number of plasmid copies detected by dual TaqMan fluorescence quantitative PCR for PCV3 and PCV2 was 1 and 10 copies/µL. The sensitivity of the method established in this experiment was more than 10 times that of ordinary PCR. These results showed that the dual TaqMan fluorescence quantitative PCR method established in this experiment has good sensitivity.

2.2.2. Specificity test

Using the nucleic acid samples of PCV1, PCV2, PCV3, PPV, and PRV as templates, the established dual TaqMan fluorescence quantitative PCR amplification method was used for detection. At the same time, a negative control was set up, and three groups of repeats were performed. The result showed that only the PCV2 and PCV3 tests have an amplification curve, the CT value is less than 30, and the result is positive; other viruses and negative control tests have no amplification curve, and no CT value, and the result is negative, which indicates that the test method has good specificity.

2.2.3. Repeatability test

The established dual TaqMan fluorescence quantitative PCR method was used to test the intragroup and intergroup repeatabilities with standard quality plasmids (10^4–10^8 copies/µL) with five dilutions as the template (Table 3). The intragroup coefficient of variation was between 0.75% and 2.46%, and the intergroup coefficient of variation was between 0.14% and 1.90%. Both intergroup and intragroup coefficients of variation were less than 3%, which indicates that the method established in this experiment has good repeatability.

2.3. Clinical test

The dual TaqMan fluorescence quantitative PCR method established in this experiment was used to detect disease feeds from some pig farms in Shandong Province (Table 4). Among the 76 samples, 11 were PCV3 -positive samples with a positive rate of 14.47%, 24 were PCV2-positive samples with a positive rate of 34.58%, and six were PCV3 and PCV2 mixed-infection samples with a positive rate of 7.89%. The detection results of the method established in this experiment were consistent with the PCR detection results, and the sensitivity was higher than PCR.

2.4. Amplification and sequence determination of the complete genome of PCV3

PCR was used to conduct the segmental amplification of the complete genome of 11 PCV3-positive samples from Shandong Province, and the target fragments of the same size as expected were amplified. The gene fragment was sequenced and spliced with DNASTAR software to obtain the PCV3 whole-genome sequence. The results showed that the entire genomes of these 11 PCV3 strains were all 2000 bp long. The full gene sequences of these 11 PCV3 strains were named as SDJNI-2018, SDJN2-2018, SDLYI-2018, SDLY2-2018, SDTA1-2018, SDTA2-2018, SDWF1-2018, SDWF2-2018, SDWF3-2018, SDWF4-2018, and SDWF5-2018 respectively.

2.5. Sequencing analysis of the PCV3 complete genome sequence

The complete genome sequences of 11 PCV3 strains in Shandong Province were analyzed and compared to those published in GenBank. The results showed that the nucleic acid homology of the complete genome of the 11 strains obtained in this study was between 98.4% and 99.9%. As shown in Fig.4, the genetic evolutionary tree of the PCV3 genomic sequence was divided into PCV3a, PCV3b, and PCV3c. SDTA2-2018, SDTA1-2018,

SDLYI-2018, SDLY2-2018, SDWF1-2018, SDWF3-2018, SDWF4-2018, and SDWF5-2018 had high similarity with certain American strains and belonged to PCV3c. SDJN1-2018, SDJN2-2018, and SDWF2-2018 had high similarity with Chinese strains in Shandong, Henan, and Fujian and belonged to PCV3a.

2.6. Genetic evolution analysis of *cap* gene

The homology of cap gene within strains isolated in this study was higher than 97.8%, and the homology of *cap* gene between those strains and the reference strains was higher than 97.7%. The homology of the Cap protein between the isolated strains and reference strains was higher than 97.8%. SDTA1-2018 had the highest homology with CO2017-2017-Italy and KU1605-2016-SouthKorea, and this was also similar to SDLY1-2018, SDLY2-2018, SDTA2-2018, SDWF1-2018, SDWF3-2018, SDWF4-2018, and SDWF5-2018. SDJN1-2018 and SDJN2-2018 were quite different from other strains, but still belonged to the same PCV3 genus in this genetic evolution tree (Fig. 5). When compared with the cap protein sequence, several mutations were found in strains isolated in this study. The results revealed that strains SDJN1-2018, and SDJN2-2018 had mutations in A24V, R27K, and V199D; SDLYI-2018 and SDLY2-2018 had a mutation in N204K; and SDWF2-2018 had mutations in K9R, A24V, R27K, T75A, T77S, and L150I. SDWF3-2018 and SDWF5-2018 had mutations in Q149P, V150I, and G152A.

2.7. Genetic evolution analysis of the *rep* gene

The homology of the *rep* gene within the strain isolated in this study was 98.8% to 99.8%, and the sequence homology of the *rep* gene between the isolated and reference strains was higher than 98.5%. The homology of the rep protein within strains isolated in this study

was higher than 98.7%, and the amino acid homology of the *rep* gene between the isolated and reference strains was higher than 98.3%. The *rep* gene of SDTA1-2018, SDTA2-2018, SDLY2-2018, SDWF2-2018, SDWF3-2018, SDWF4-2018, and SDWF5-2018 had the highest amino acid sequence homology with KU1602-2016-SouthKorea, PB01-2017-Thailand, and Guangdong-HY1-2016-China (Fig. 6). According to the amino acid sequence analysis, SDJN1-2018 had a mutation in E282G, SDJN2-2018 had a mutation in E282G, SDLY1-2018 had a mutation in A122S, SDLY2-2018 had a mutation in E225D, SDWF2-2018 had a mutation in R44K, and SDWF4-2018 had a mutation in F212Y.

3. Discussion

PCV disease is one of the important pig diseases that harm the pig industry caused by PCV (Opriessnig et al. 2020), a single-stranded circular DNA virus with a particle size of only 17 to 20 nm and belongs to the Circovirus genus, Circoviridae family. Clinically, PCV infection is mainly manifested as PMWS, porcine dermatitis and nephropathy syndrome, pig reproductive disorders, and acute pulmonary edema. PCV can be divided into four genotypes, namely, PCV1, PCV2, PCV3, and the newly discovered PCV4. PCV1 was first found in cultured cells, and it is widely present in pigs but not pathogenic (Tischer et al. 1986), and PCV2 has always been considered to be the main factor of PCV. PCV3 was first isolated from pigs with PDNS symptoms in the United States in 2016, and became popular in pig herds all over the world, such as Poland, Italy, South Korea, and China, causing huge economic losses to the pig industry (Bera et al. 2020; Hayashi et al. 2018; Kim et al. 2018; Wang et al. 2020b; Xia et al. 2019; Yuzhakov et al. 2018). Retrospective research data demonstrated that PCV3 possessed a high sequence homology to bat circovirus, suggesting that PCV3 might have been

derived early from bats and gradually adapted to pigs (Li et al. 2018). In China, the infection

rate of PCV3 is increasing in pig farms (Chen et al. 2019; Geng et al. 2019; Liu et al. 2019;

Xu et al. 2018). Seriously, the positive rate of PCV2 and PCV3 co-infection in China is

getting higher and higher (Guo et al. 2020; Wang et al. 2020a; Xia et al. 2019; Zhang et al.

2020).

Because of the difficulty of virus isolation and the lack of a commercial serological test

kit, the detection of viral nucleic acids is presently the most effective diagnostic method for

PCV detection. The main detection methods for PCV3 include conventional PCR, nested PCR,

multiplex PCR, fluorescence quantitative PCR, and loop-mediated isothermal amplification

(Ha et al. 2018; Kim et al. 2020; Wang et al. 2019a; Zheng et al. 2020). Several scholars have

designed various primers for PCR detection according to the PCV3 gene, and established a

rapid, specific, and sensitive PCR detection method and applied it in practice. However,

conventional PCR is not sensitive and quantitative. In addition, because of the increasing

infection rate of PCV2 and PCV3, it is urgent to establish a method for the simultaneous

detection of these two pathogens. In this study, the complete gene sequences of PCV3 and

PCV2 were analyzed, and specific primers and TaqMan probes labeled with FAM and HEX

fluorescent groups, respectively, were designed. The annealing temperature, primer and probe

concentrations, and reaction system and conditions were optimized. The established dual

TaqMan fluorescence quantitative PCR method can detect PCV2 and PCV3 simultaneously,

and the detection limits arewere 10 and 1 copies/μL, respectively. PCV2 and PCV3

recombinant plasmids were used as templates, the standard curves were drawn, and the

correlation coefficient (R^2) was greater than 0.99, demonstrating a good linear relationship.

The coefficient of variation of the intergroup and intragroup repeated tests was less than 3%, demonstrating that this method had good repeatability. This method iswas only positive for PCV2 and PCV3, but negative for other viruses, indicating that the method established in this experiment has good specificity. The detection results of the positive clinical samples were consistent with those of ordinary PCR, and the time consumed was shorter than that of ordinary PCR, which could reduce aerosol pollution better.

To understand the infection of PCV2 and PCV3 in pig herds in some areas of Shandong Province, 76 samples of suspected PCV were collected in 2018, and the dual TaqMan fluorescence quantitative PCR method established in this study was used to detect PCV3 in the diseased samples. The results showed 11 positive cases of PCV3, with a positive rate of 14.47%, indicating that PCV3 is prevalent in Shandong, Province and should be given attention and action. Notably, six of the tested samples were positive for both PCV2 and PCV3, with a positive rate of 7.89%, suggesting a need to further research the pathogenic mechanism of PCV3 and PCV2 mixed infection and coordinated infection.

The genetic evolution of the complete gene sequences of 11 PCV3 strains was analyzed to further understand the characteristics of the PCV3 genome sequence in some areas of Shandong Province. The sequencing results of 11 swine virus strains isolated in the experiment showed that the length of the virus genome sequence was 2000 bp, and the homology between each other was between 98.4% and 99.9%, indicating that PCV3 is relatively conservative in genetic evolution. PCV3 mainly has two coding proteins, namely, ORF1 (Rep) and ORF2 (Cap), both with similar functions to PCV2 (Palinski et al. 2017). Rep protein is related to virus replication, and the Cap protein is the main immunogenic protein.

PCV3 gene subtypes are classified according to the phylogenetic relationship between Cap protein mutations (A24V and R27K) and the Cap gene. A comparison of the amino acid sequence of the Cap protein of PCV3 isolated in this study showed that the strains obtained in this study had high amino acid homology. The amino acid mutation sites mainly existed at the 9, 24, 27, 75, 77, 149, 150, 152, 199, and 204 amino acid positions. The amino acid sequence of the Cap protein of SDJN1-2018 and SDJN2-2018 isolated in this experiment was quite different from other strains. By contrast, in the amino acid sequence of the Rep protein of PCV3, the amino acid mutation sites were mainly concentrated at the 3, 44, 122, 225, and 282 amino acid positions. The relationship between these mutations and pathogenicity and immunogenicity remains unclear and needs further research.

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Tables and Figure legends

Table 1 Primer sequence used in this study

Primers/Probes	Sequences (5'-3')
PCV2 Rep-F	ATGCCCAGCAAGAAGAATGGAAGAAG
PCV2 Rep-R	TCAGTAATTTATTTCATATGGAAAT
PCV3 Cap-F	TTAGAGAACGGACTTGTAACGAATCCA
PCV3 Cap-R	ATGAGACACAGAGCTATATTCAGAAGA
PCV2-F	TTGGCCCGCAGTATTCTGAT
PCV2-R	CTGGGACAGCAGTTGAGGAGTA
PCV2 Probe	HEX-ACCAGCAATCAGACCCCGTTGGAA-BHQ1
PCV3-F	TCACCCCCAAACCACTTCTG
PCV3-R	CTGGAGAAAAGAAGAGGCTTTGT
PCV3 Probe	FAM-CGGGAACTACCAGCGCTCACCCA-BHQ1

Table 2 Reference strain of PCV3 used in this study

Name	Genbank accession NO.	Country	Year
Chongqing-155	KY075993	China	2016
DE18.2	MG014366	Germany	2015
29160	KT869077	USA	2015
BRRS6	MF079253	Brazil	2016
Fujian-5	KY075986	China	2016
Guangdong-HY1	MF589102	China	2016
Henan13	KY075988	China	2016
Hubei-618	KY354039	China	2016
KU1602	KY996338	SouthKorea	2016
MO2015	KX778720	USA	2015
PCK3-1703	MF611878	SouthKorea	2017
Shandong-1	KY778776	China	2017
CN-FJ-1	KY753912	China	2016
GD2016	KY421347	China	2016
Jiangxi-B1	MF589107	China	2017
KU-1608	KY996344	SouthKorea	2016
1621	MF805719	Italy	2017
2164	KX458235	USA	2015
BRRS8	MF079254	Brazil	2016
CC2016	KY421348	China	2016
CO2017	MF162298	Italy	2017
DE13.20	MG014365	Germany	2015
GD1	KY753911	China	2016
GDBL1	MF405272	China	2017
Guangdong-MX	MF589104	China	2015
GXHJ2	MF405277	China	2017
Jiangxi3	MF589106	China	2016
KU1607	KY996343	SouthKorea	2016
NWHEB21	MG564174	China	2016
NWHUN2	MG564175	China	2016
SD2016	KX966193	USA	2016
Shanghai0708	KY865243	China	2016

Table 3 Repetitive experimental results

Concentration (copies/μl)	N	Intra-assay variability (CT)		Inter-assay variability (CT)	
		X ±SD	CV（%）	X ±SD	CV（%）
PCV3					
10^8	3	13.30±0.18	1.35	13.33±0.08	0.60
10^7	3	16.07±0.23	1.43	16.28±0.31	1.90
10^6	3	19.04±0.45	2.36	18..65±0.15	0.80
10^5	3	21.39±0.46	2.15	21.43±0.23	1.07
10^4	3	24.81±0.61	2.46	24.20±0.34	1.40
PCV2					
10^8	3	13.36±0.10	0.75	13.25±0.10	0.75
10^7	3	16.24±0.32	1.97	16.00±0.24	1.50
10^6	3	18.33±0.33	1.80	18.11±0.23	1.27
10^5	3	20.89±0.48	2.29	20.87±0.03	0.14
10^4	3	23.68±0.56	2.36	23.74±0.06	0.25

Table 4 Clinical test using TaqMan quantitative PCR

Method	PCV2		PCV3		PCV2+PCV3	
	Positive number	positive rate	Positive number	Positive rate	Positive number	Positive rate
TaqMan quantitative PCR	24/76	31.58%	11/76	14.47%	6/76	7.89%
Traditional PCR	22/76	28.94%	10/76	13.15%	6/76	7.89%

Fig. 1 Amplification curve and standard curve of dual TaqMan fluorescence quantitative PCR

A. The TaqMan fluorescence quantitative PCR amplification curve of PCV3. B. The standard curve of PCV3 drawn with LC96 software. C. The TaqMan fluorescence quantitative PCR amplification curve of PCV2. D. The standard curve of PCV2 drawn with LC96 software.

Fig. 2 Sensitivity detection of PCV3 using dual TaqMan fluorescence quantitative PCR

A. Amplification results of PCV3 using ordinary PCR assay. B. Results of PCV3 using dual TaqMan fluorescence quantitative PCR established in this study.

Fig. 3 Sensitivity detection of PCV2 using dual TaqMan fluorescence quantitative PCR

A. Amplification results of PCV2 using ordinary PCR assay. B. Results of PCV2 using dual TaqMan real-time PCR established in this study.

Fig. 4 Phylogenetic tree of PCV3 full-length genomic sequence

Phylogenetic tree of PCV3 full-length genomic sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the PCV3 strains identified in this study.

Fig. 5 Phylogenetic tree of PCV3 *cap* gene sequence

Phylogenetic tree of PCV3 *cap* gene sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the PCV3 strains identified in this study.

Fig. 6 Phylogenetic tree of PCV3 *rep* gene sequence

Phylogenetic tree of PCV3 *rep* gene sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the

PCV3 strains identified in this study.

Fig.1
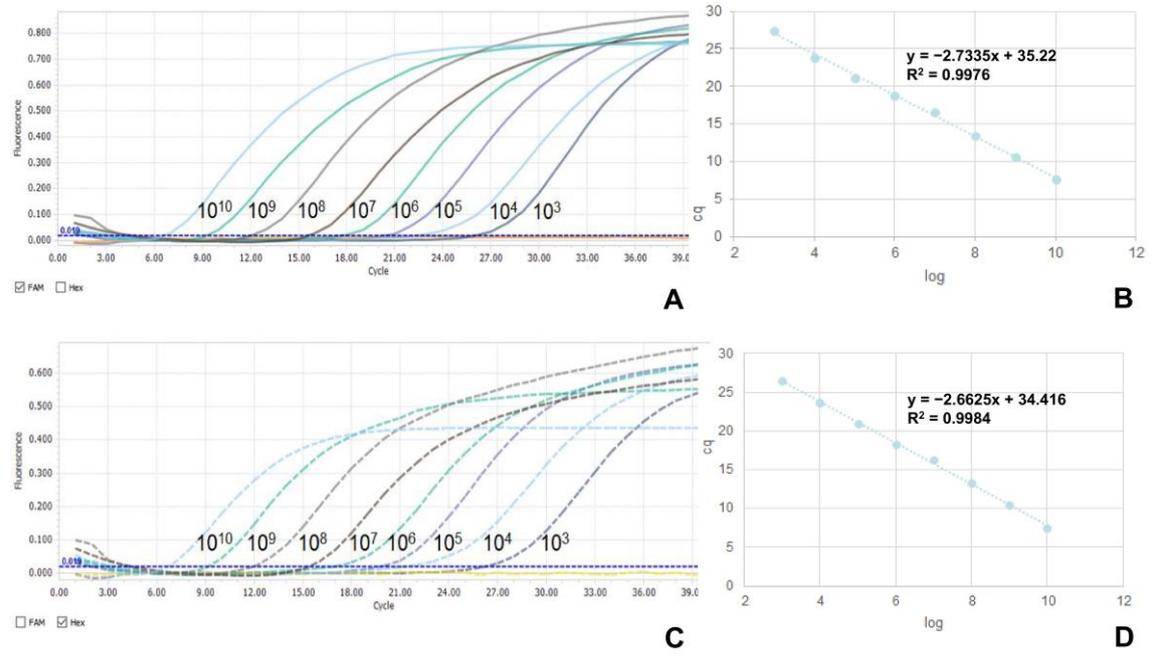
Fig.2
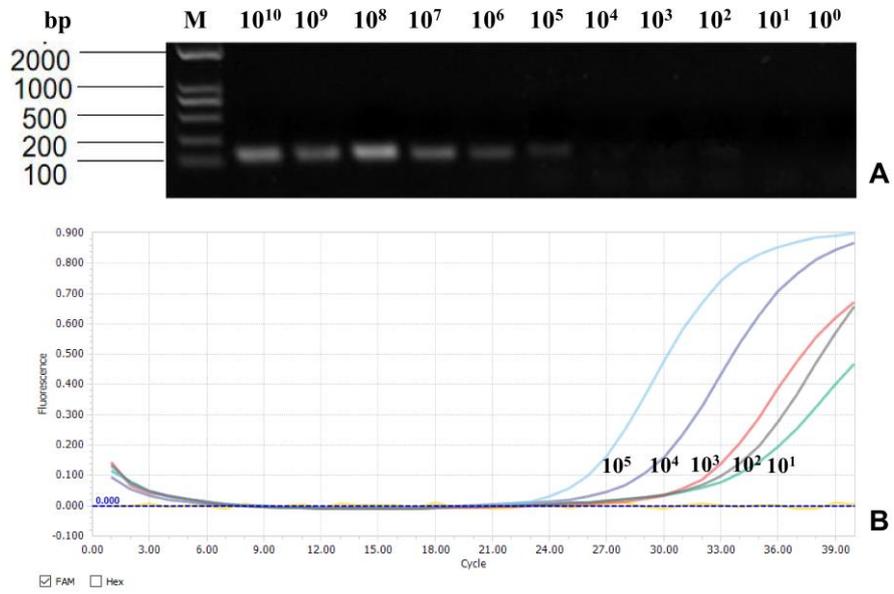
Fig.3
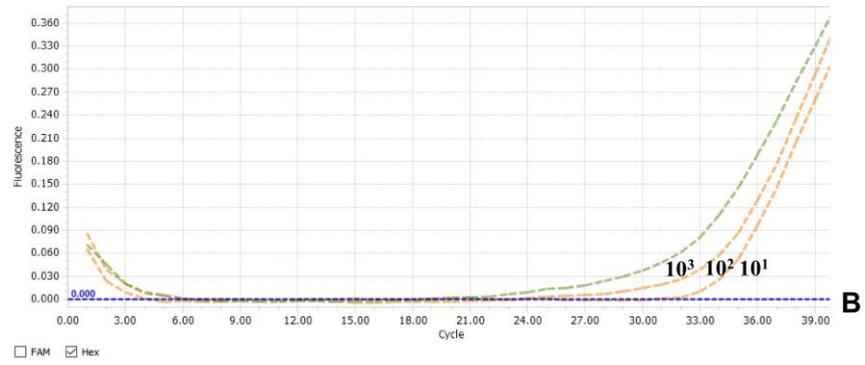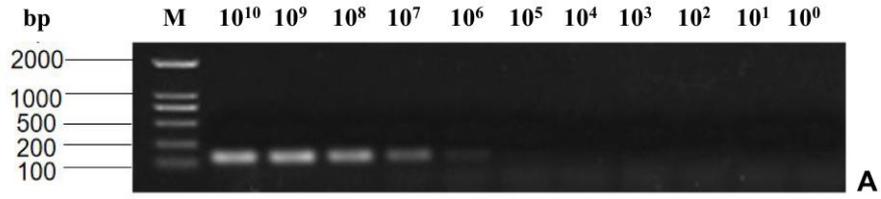
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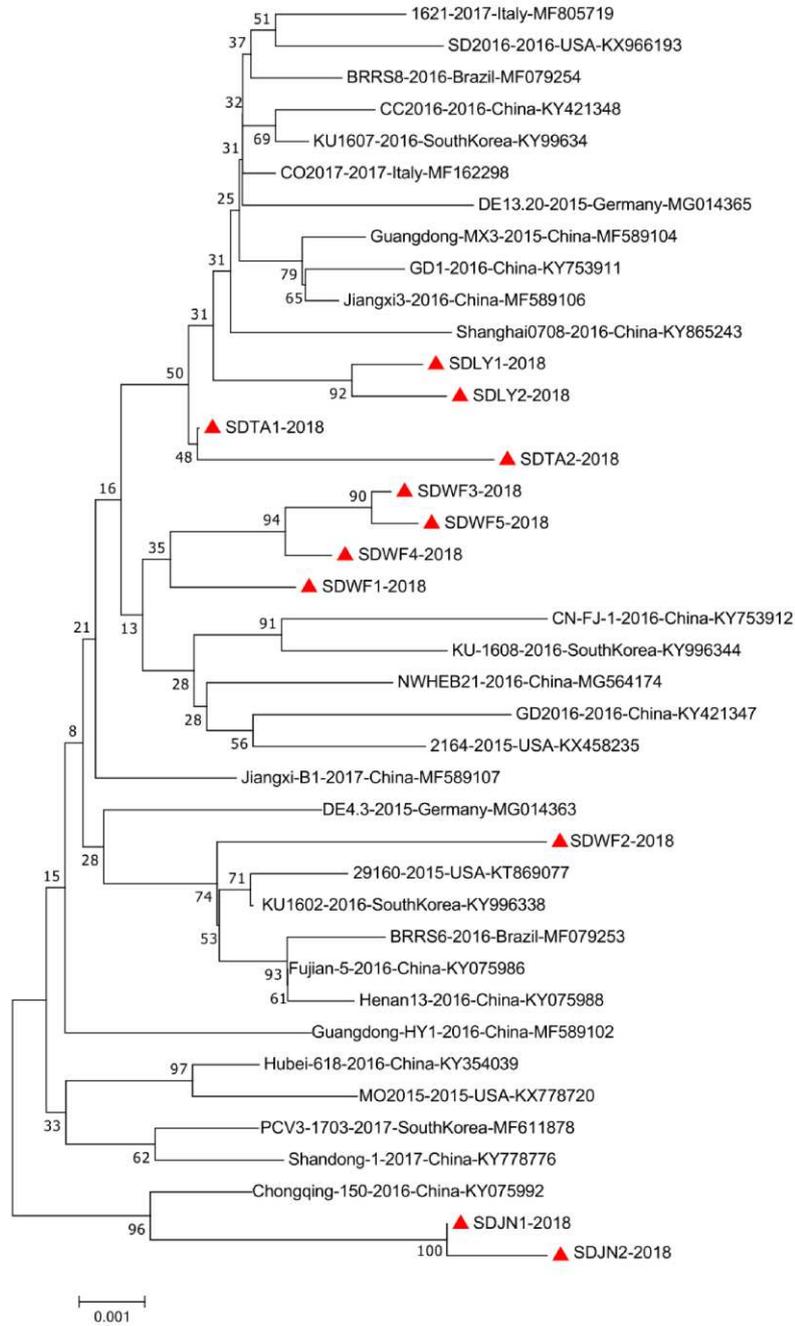
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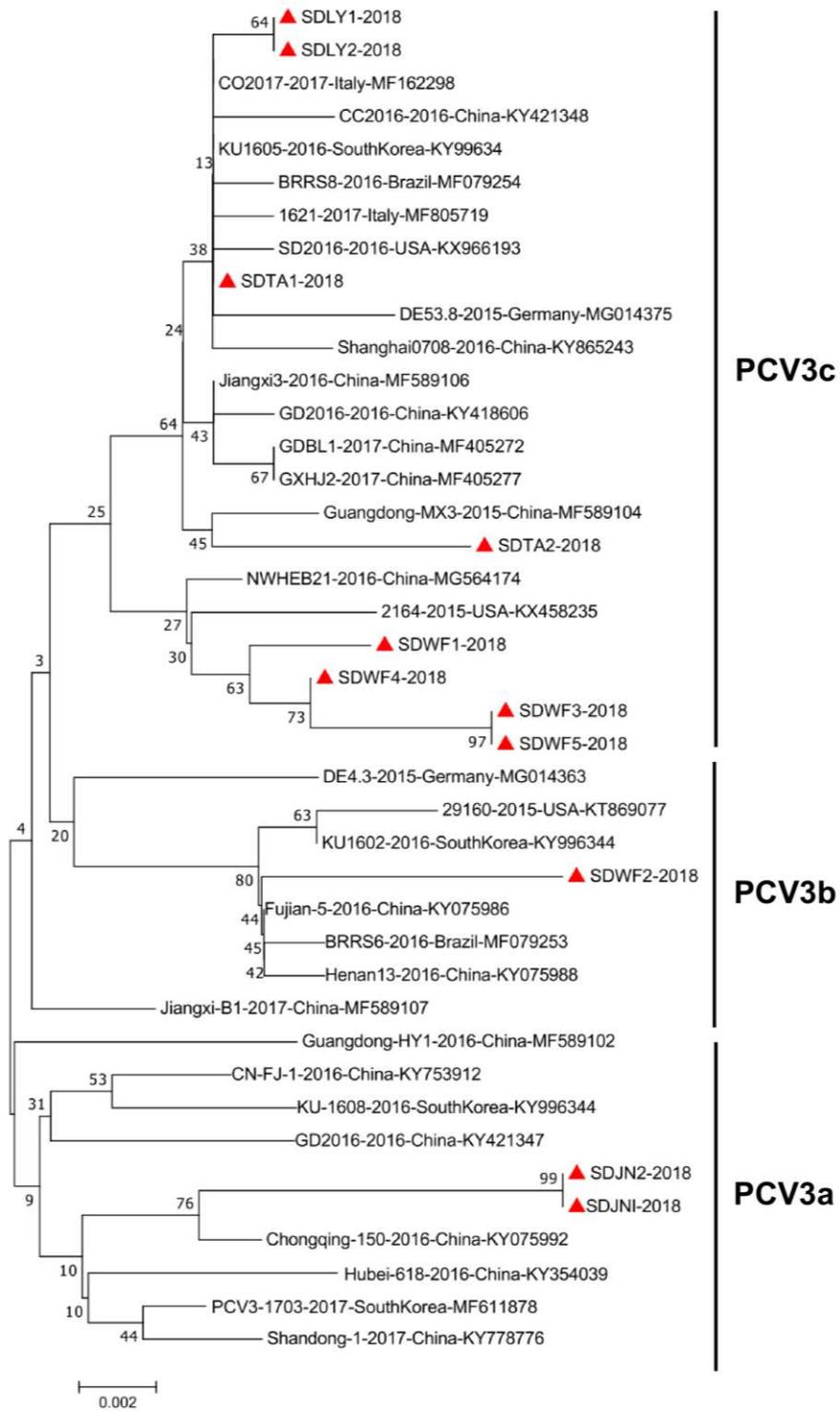
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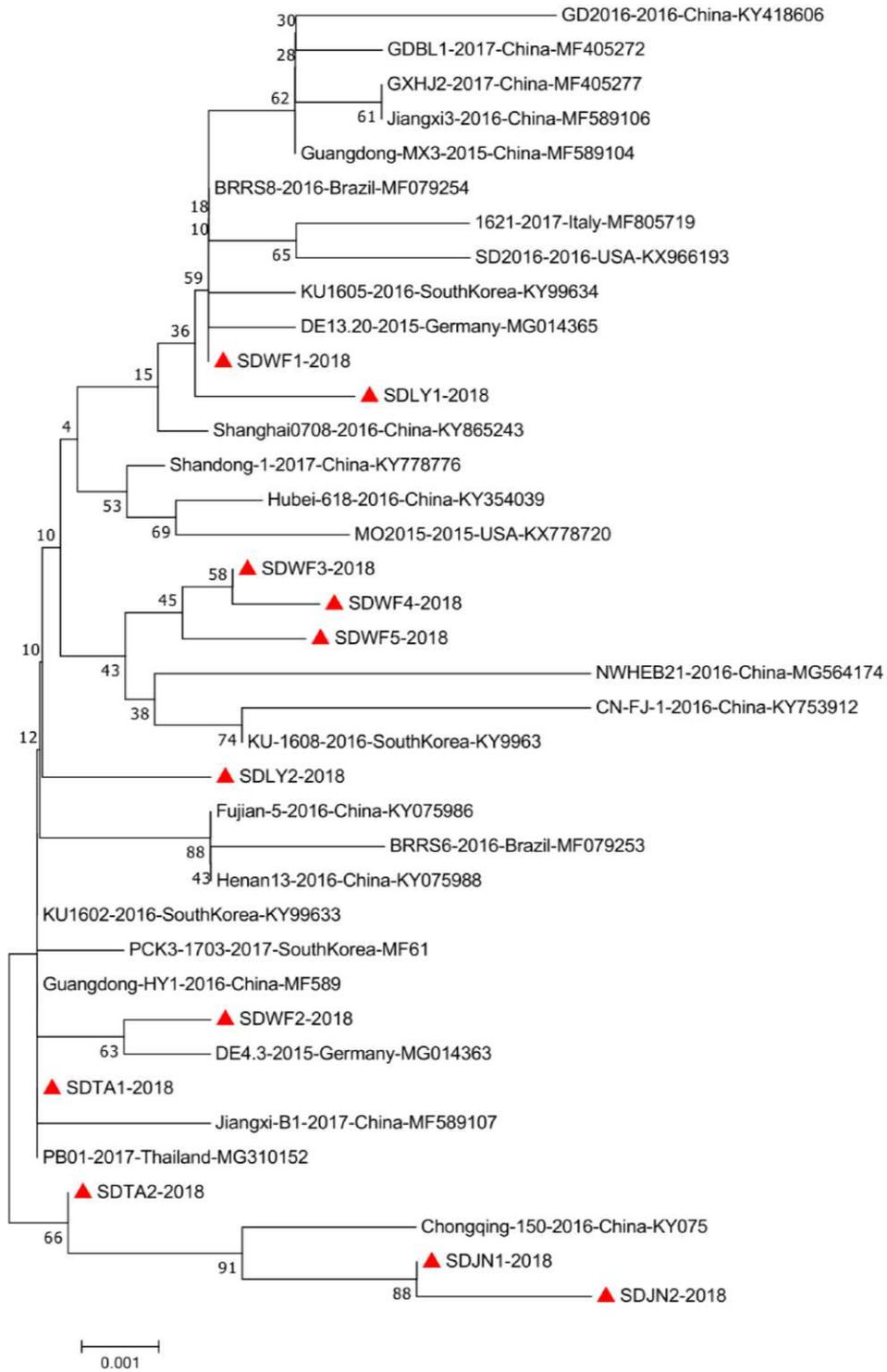
Figures
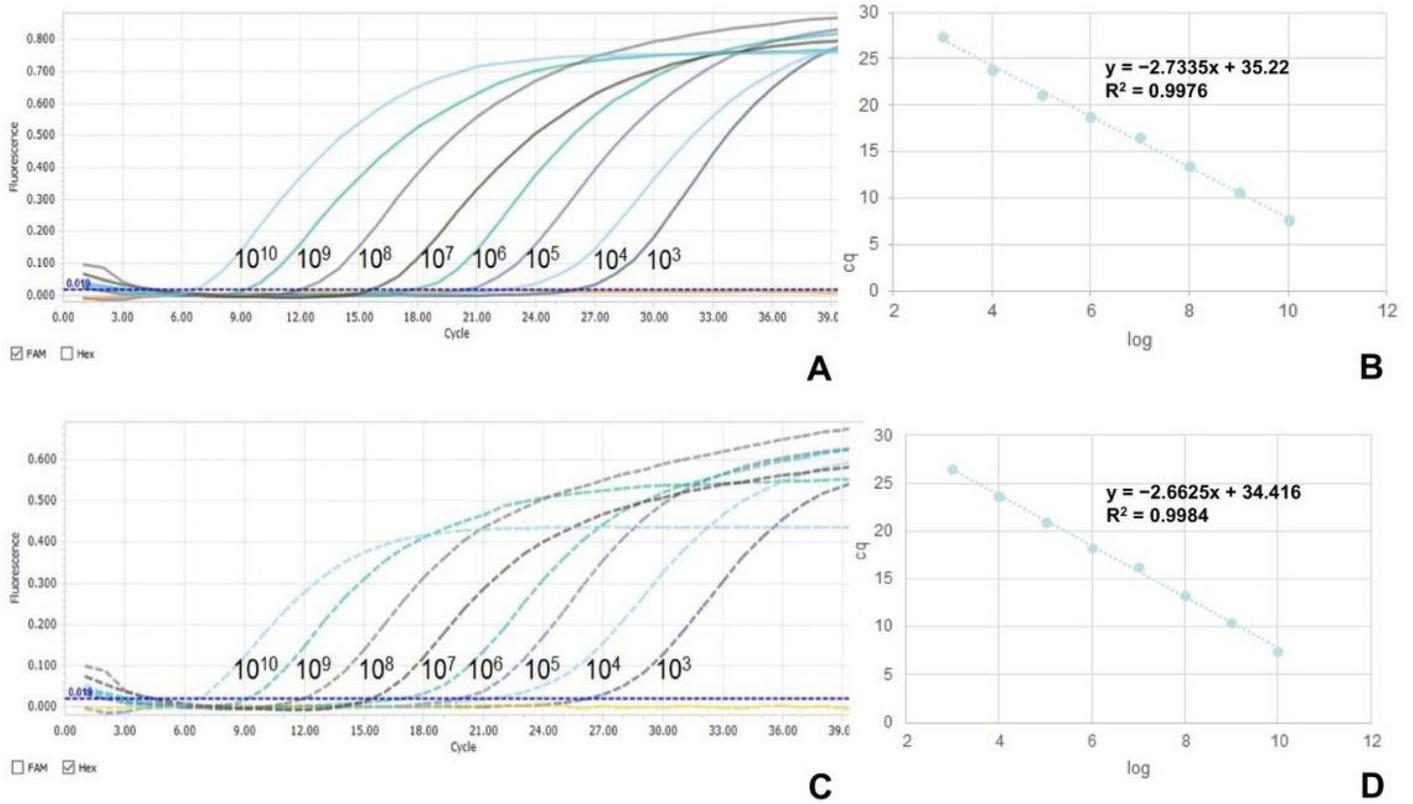
Figure 1

Amplification curve and standard curve of dual TaqMan fluorescence quantitative PCR A. The TaqMan fluorescence quantitative PCR amplification curve of PCV3. B. The standard curve of PCV3 drawn with LC96 software. C. The TaqMan fluorescence quantitative PCR amplification curve of PCV2. D. The standard curve of PCV2 drawn with LC96 software.
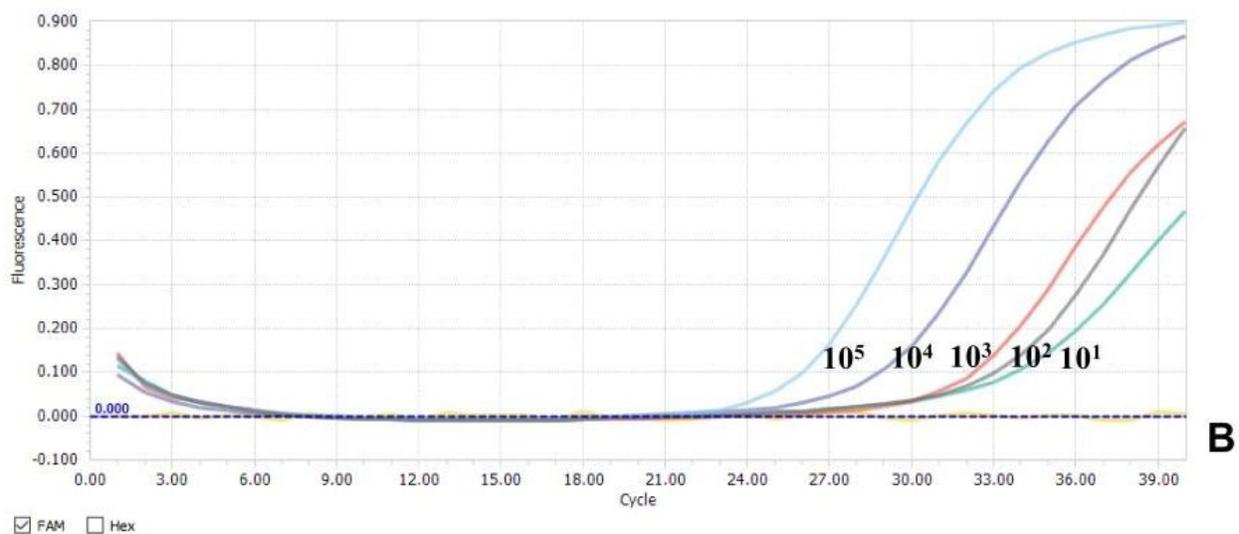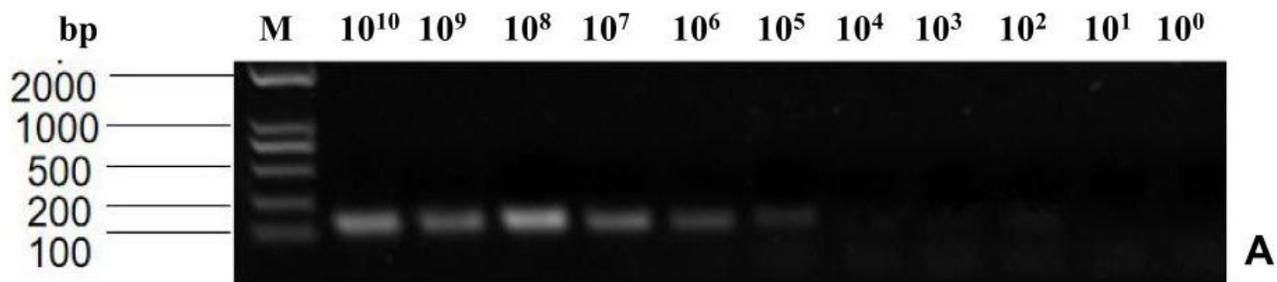
Figure 2

Sensitivity detection of PCV3 using dual TaqMan fluorescence quantitative PCR A. Amplification results of PCV3 using ordinary PCR assay. B. Results of PCV3 using dual TaqMan fluorescence quantitative PCR established in this study.
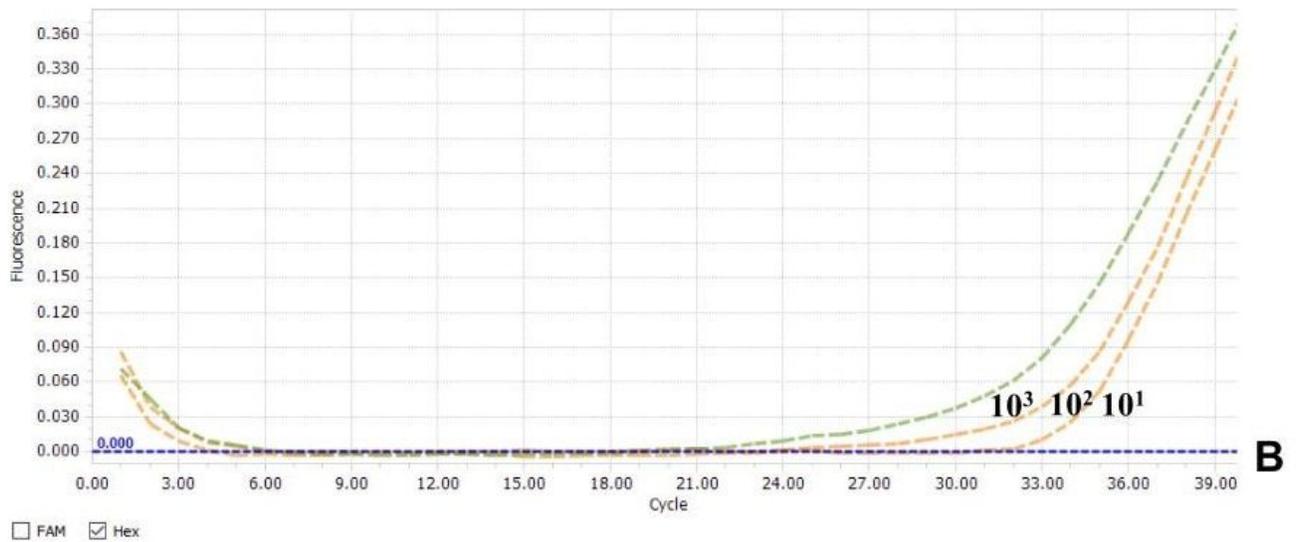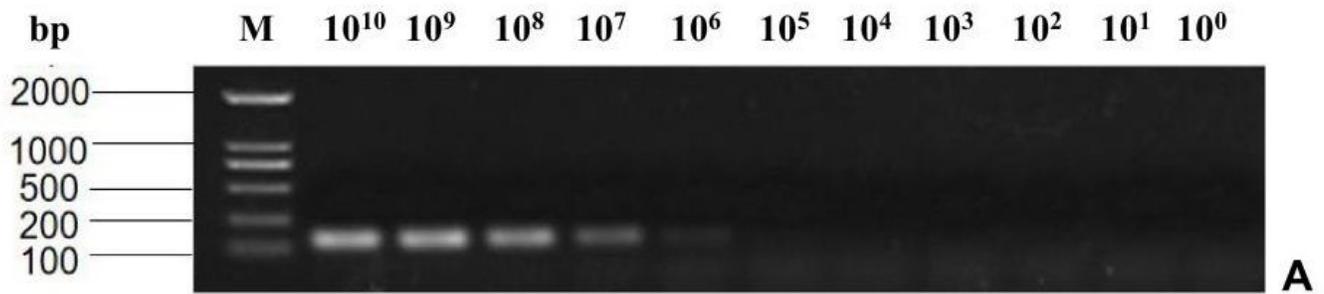
Figure 3

Sensitivity detection of PCV2 using dual TaqMan fluorescence quantitative PCR A. Amplification results of PCV2 using ordinary PCR assay. B. Results of PCV2 using dual TaqMan real-time PCR established in this study.
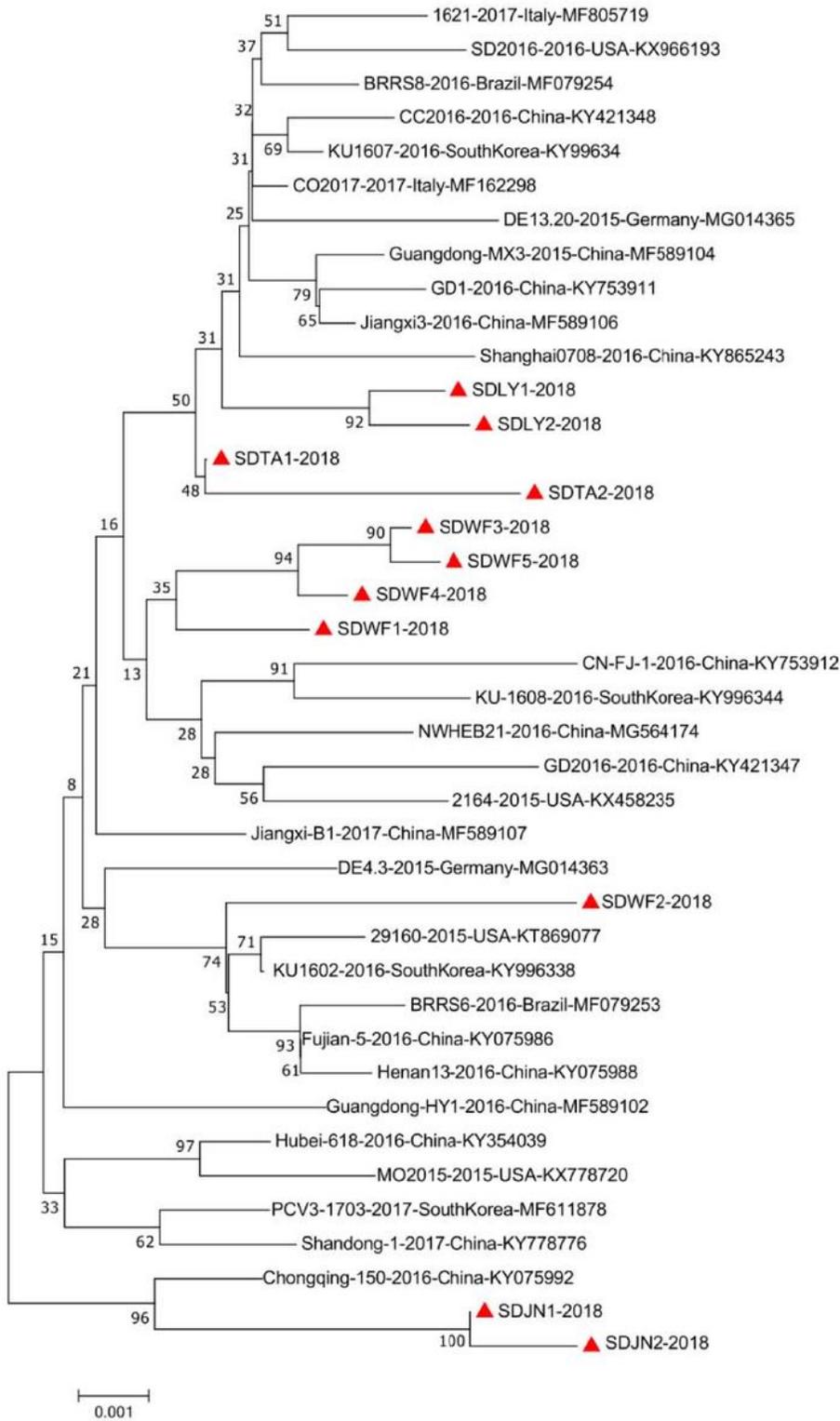
Figure 4

Phylogenetic tree of PCV3 full-length genomic sequence. Phylogenetic tree of PCV3 full-length genomic sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the PCV3 strains identified in this study.
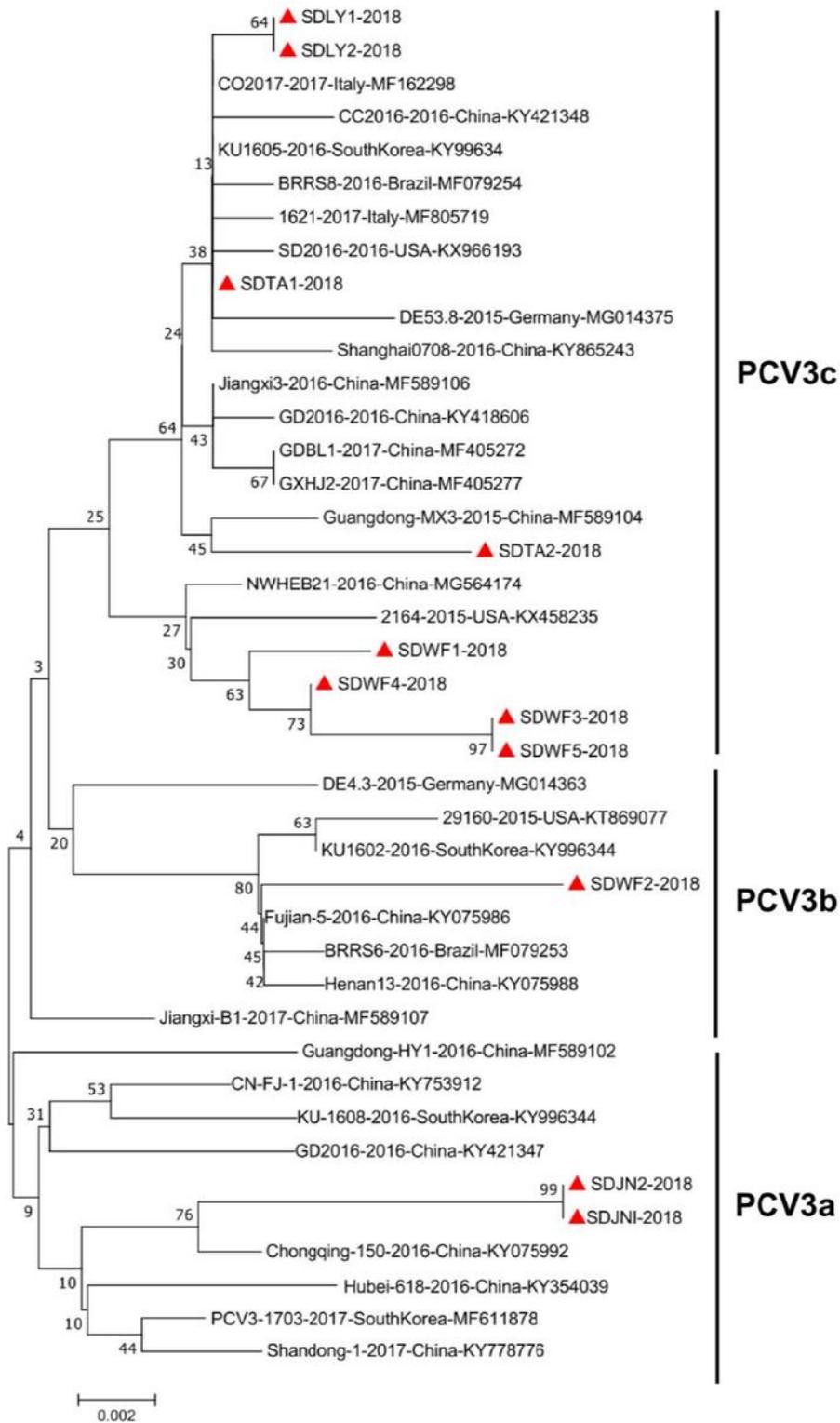
Figure 5

Phylogenetic tree of PCV3 cap gene sequence. Phylogenetic tree of PCV3 cap gene sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the PCV3 strains identified in this study.
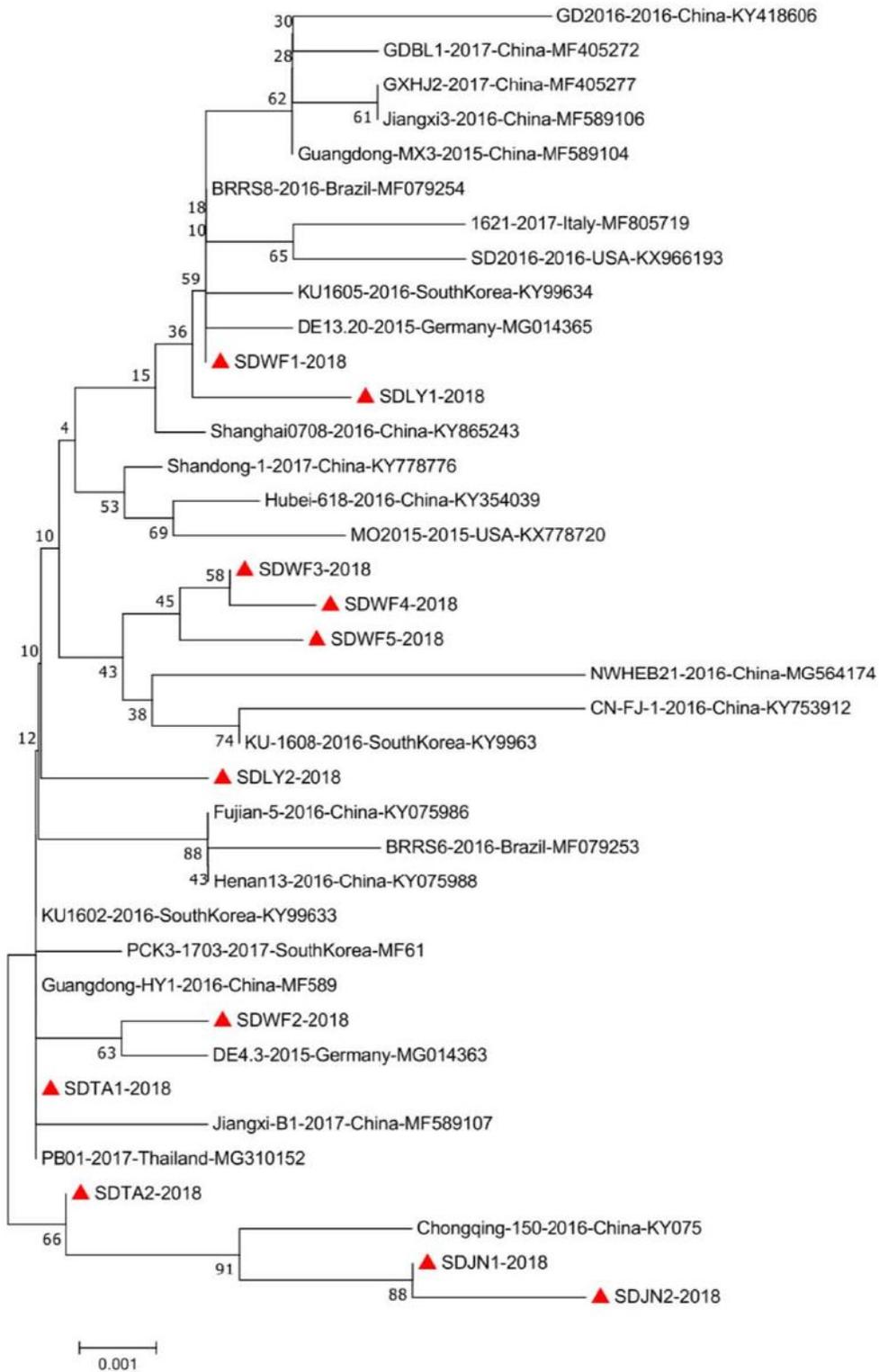
Figure 6

Phylogenetic tree of PCV3 rep gene sequence. Phylogenetic tree of PCV3 rep gene sequence was constructed using the neighbor-joining method. Bootstrap values were calculated with 1000 replicates. Filled triangles indicated the PCV3 strains identified in this study.