Identification of Porcine circovirus type (PCV2) and type 3 (PCV3), Porcine 2 parvovirus (PPV) in swine by multiplex PCR test

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Thoai Kim Tran  
Nong Lam University

Trang Thi Thanh Nguyen  
Nong Lam University

Hiep Lai Xuan Vu  
University of Nebraska Lincoln

Phat Xuan Dinh  
Nong Lam University

dinhxuanphat@hcmuaf.edu.vn  
Corresponding Author  
ORCiD: https://orcid.org/0000-0003-3589-7252

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Abstract
Background: Aiming to simultaneously detect three important viruses known to be involved in reproductive problems of sows, a multiplex PCR (mPCR) test was developed to provide rapid diagnosis of porcine circovirus type 2 and 3 (PCV2, PCV3) and to illustrate parvovirus (PPV) prevalence in sow herds.

Methods: Three pairs of specific primers were designed to target PCV2 Cap gene, PCV3 Cap gene and PPV NS1 gene, with predicted mPCR products of 702 bp, 267 bp and 380 bp, respectively.

Results: The detection limit of mPCR was 100 copies/reaction per target gene. Sequencing of mPCR products performed with clinical serum samples accurately confirmed results. The mPCR was run against a panel of 94 swine serum samples whose infection status had been pre-determined by commercial real-time PCR kits. Overall, the mPCR results matched 100% with the real-time PCRs.

Conclusions: The developed mPCR test functions successfully and can be used in routine rapid diagnosis of PCV2, PCV3 and PPV.

Introduction
Reproductive disorders are among the most concerned problems in sow herds, causing huge economic losses to swine producers. Many infectious pathogens are involved which include: Porcine parvovirus (PPV), porcine circovirus type 2 (PCV2) and porcine circovirus type 3 (PCV3), a newly discovered viral pathogen [1].

PCV2 and PCV3 belong to the family Circoviridae. They are non-enveloped and single-circular DNA viruses [1, 2]. PCV2 has been proved to play significant roles in different swine syndromes such as Postweaning multisystemic wasting syndrome (PMWS), Porcine dermatitis and nephropathy syndrome (PDNS), or Porcine Circovirus associated diseases (PCVAD) [3, 4, 5, 6]. The virus was first identified in western Canada in a case of wasting disease using electron microscopy, immunohistochemical, and in situ hybridization methods [5]. Later, multiple other publications confirmed the circulation and the pathogenesis of PCV2 in swine abortions, and births of weak piglets [7, 8, 9, 10]. PCV3 was recently described in a case of PDNS syndrome in a sow farm in North Carolina (USA) [1]. The virus was found in samples of skin, kidney, lung, and lymph node without the existence of PCV2. From this
observation, PCV3 is also suspected to play a pathogenic role in swine, especially in the sow herds although the evidence of its pathogenesis remains to be elusive. In addition to the causative lists of reproductive disorders in swine is PPV, a member of the Paroviridae family, that was identified at the end of the 1960s [11, 12, 13]. Classical manifestations associated with a PPV infection of pregnant sows include stillbirths, mummification, embryonic death, and infertility, a condition that is collectively identified as SMEDI syndrome.

These three viruses are endemic in Vietnam and cause significant losses [14, 15, 16]. PPV was reported to be circulating in the Vietnamese swine herds since 1998 [17], while PCV2 and PCV3 were reported in 2005 and 2017, respectively [18, 19].

Several conventional and real-time PCRs have been developed for molecular diagnosis of these three viruses [20, 21, 22, 23, 24, 25]. However, those PCRs are designed for detection of a single viral agent at a time. Recently, duplex PCRs were developed for simultaneous detection of both PCV2 and PCV3 [26, 27, 28]. In the present study, we report the development and validation of a mPCR that allows simultaneous detection of these three important viruses in clinical samples.

Materials And Methods

Controls and clinical samples

Positive controls: DNA fragments of PCV3 Cap gene and PPV NS1 gene were synthesized by Integrated DNA Technologies (IDT - USA). DNA template of PCV2 was originally obtained from a field isolate followed by sequencing confirmation. The resulting sequence exhibited 99.72% identity to the previously reported PCV2 (Gene ID LC383449.1).

Unrelated pathogens for specificity evaluation: bacteria and viruses that are commonly present in the swine farms and potentially contaminated into the samples were used to confirm the specificity of the PCR. Staphylococcus aureus (ATCC 6338); Escherichia coli (E. coli (ATCC 25922)) were offered by Sanphar Vietnam laboratory (belonging to Erber group, Austria); Streptococcus suis and Clostridium perfringens were obtained from previous study [29]. Pseudorabies virus (gE gene were synthesized by Integrated DNA Technologies (IDT - USA). The bacterial DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution (Cat#P1037, Sigma; Cat#25666, Merck).
Clinical samples: Ninety-four serum samples from sows were collected and used to evaluate this mPCR procedure. Each serum sample was pooled from five individual sows. All samples were submitted to the laboratory and the senders signed agreement for the laboratory to use result data for the purposes of teaching and publication. The infectious status of these 94 samples were predetermined by using commercial Realtime PCR kits or master mix, as per the manufacturers’ instructions. The Realtime kit for PCV2 was PowerChek™ PCV2 Real-time PCR Kit (Cat#R0809, Primerdesign), for PPV was PowerChek™ ADV/PPV Realtime PCR Kit (Cat#R0832, Primerdesign) and Luna® Universal qPCR Master Mix (Cat#M3003S, NEB) was for PCV3.

**DNA preparation from clinical samples**

Serum samples were extracted by advanced phenol-chloroform method [30]. Two hundred microliters of a serum sample was mixed with 500 µl extraction buffer (200 mM Tris-HCl, pH 7.5; 25 mM EDTA; 250 mM NaCl and 0.5% SDS) and vortexed vigorously, followed by incubation on ice for 15 min. The mixture was centrifuged for 8 min at 4000 rpm and the upper aqueous layer was collected and transferred to pre-chilled 1.5 mL tube, followed by the addition of 0.25 mL of phenol and 0.25 mL of chloroform/isoamyl-alcohol (24:1, v/v). After the tube was inverted several times and centrifuged at 14000 rpm for 6 min. The supernatant was then collected and transferred to a new tube, followed by an addition of 0.5 mL of chloroform/isoamyl-alcohol mixture (24:1, v/v) and centrifugation at 14000 rpm for 3 min. Finally, the upper aqueous layer was transferred to a clean 1.5 ml tube, followed by the addition of 1 mL absolute ethanol and incubation at -20 °C overnight to facilitate DNA precipitation. The DNA was then pelleted by centrifugation at 14000 rpm for 30 min, followed by a wash with 1 mL of 70% cold-ethanol. The pellet was allowed to air-dry at 40 °C, dissolved in 30 µl 1X tris-EDTA buffer, pH 8.0 and was stored at -20 °C for subsequent assays.

**Primers**
Table 1  Primer sequences and estimated product sizes

| Virus | Primers | Gene | Primer sequences (5' – 3') | Product size (bp) | Reference |
|-------|---------|------|---------------------------|-------------------|-----------|
| PCV2  | PCV2    | Cap  | F:ATGACGTATCCAA GGAGGCG  | 702               | Present study |
|       |         |      | R:TTAAGGGTTAAGTG GGGGGTC |                   |           |
| PCV3  | PCV3    | Cap  | F:TTCCGGGACATAAT GCT    | 267               | Lan Tian et al., (2017) [31] |
|       |         |      | R:GGGCACACAGCCAT AGAT  |                   |           |
| PPV   | PPV     | NS1  | F:GCTTTAGCCTTGGA GCCGTGGA | 380               | Present study |
|       |         |      | R:CGTGTTCTTTTGCT CGGCGTC |                   |           |

The primers used in this study are listed in Table 1. The primers for amplification of PCV2 were designed based on the alignment of 65 PCV2 cap gene sequences recorded in the NCBI GenBank. This primer pair will result in a PCR product of 702 bp. The primers for amplification of PPV was designed based on the alignment of 67 PPV NS1 sequences recorded in the NCBI GenBank. The expected product size for the PPV PCR is 380 bp. Finally, the primers for amplification of PCV3 were obtained from Lan Tian et al. (2017) [30]. This pair of primers was designed based on the PCV3 cap gene with the expected product size of 267 bp.

**Multiplex PCR (mPCR)**

After multiple rounds of optimization, a primer mix containing three primer pairs was created at a mixing ratio of 1 PPV: 2 PCV2 : 4 PCV3. Accordingly, the final concentrations of the PPV, PCV2 and PCV3 primer pairs in the mixture were 0.2 µM, 0.4 µM, 0.8 µM, respectively. The mPCR was performed in a 20 µl reaction volume containing 10 µl of DreamTaq 2X (Cat#K1072, No MAN0012702, Thermo Scientific), 1.4 µl of the primer mix, 3 µl DNA template, and 5.6 µl of nuclease free water (ThermoFisher). After initial denaturation at 94 °C for 5 min, 35 cycles were conducted at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s, followed by a final extension at 72 °C for 5 min. Ten microliters of amplified products were then analyzed by electrophoresis in a 1.5% (w/v) agarose gel in 1X Tris-Boric-EDTA (TBE) containing Midori Green Advance DNA stain. A 1 Kb Plus DNA ladder (Invitrogen) was used as the molecular weight marker to indicate the sizes of the PCR products.
Evaluation of the specificity of the mPCR

Genomic DNA of *Staphylococcus aureus* (ATCC 6338); *Escherichia coli* (*E. coli* (ATCC 25922)); *Streptococcus suis*, *Clostridium perfringens* and Pseudorabies virus (PRV) *gE* gene were used as templates in the mPCR to determine the specificity of the test. The concentration of DNA templates used in the reaction was 30 ng per reaction in the cases of bacteria genomic template and and $10^8$ copy per reaction in the case of Pseudorabies *gE* gene.

Evaluation of the sensitivity of the mPCR

Ten-fold serial dilutions of each positive control DNA templates ranging from $10^7$ to $10^0$ copies were used as the DNA template in the mPCR to estimate its limit of detection. The minimum number of template copies that enable successful amplification of each product was considered as its limit of detection.

DNA sequencing

Sequencing of each PCR product was performed by using both forward and reverse primers used in corresponding PCR (Table 1). The sequencing results were aligned with the reference PCV2 (accession no. MH470234.1), PCV3 (accession no. MK058529.1) and PPV (accession no. MK378155.1) using Clustal Omega program.

Results

Optimal condition for the multiplex PCR

Before combining the primers to form a mPCR, each primer pair was tested for its performance in individual PCRs at different annealing temperatures. Gel electrophoresis analysis indicated that only one single DNA product was generated for each individual PCR, with the product sizes of 267 bp for PCV3, 380 bp for PPV and 702 bp for PCV2 (Fig 1). It appeared that the three primer pairs worked well at the annealing temperatures between 52 °C and 58 °C. Subsequently, the annealing temperature of 56 °C was chosen for mPCR. As shown in Fig. 2, the mPCR produced three amplicons that were clearly visible and easily distinguishable from each other. Importantly, the sizes of the amplicons were as expected and were similar to the ones observed in single PCRs (Fig. 2). Although the templates were
the three positive control DNAs, the three products in this mPCR were recovered from agarose gel and subjected to sequencing. The sequencing results confirmed that the three pairs of primers were compatible, functioning accurately and without interfering with each other when combined in one reaction.

**Specificity, sensitivity and functioning under interfering conditions**

Next, we evaluated the specificity of the mPCR by running with unrelated DNA templates isolated from bacteria and/or viruses commonly found in pig farms which included *Staphylococcus aureus, Escherichia coli, Streptococcus suis, Clostridium perfringens* and Pseudorabies virus. Results showed that the three primer pairs did not cross-react with any of these DNA templates (Fig. 3A), indicating the established mPCR has expected specificity.

We then evaluated the sensitivity of the mPCR by running the assay against a set of 10-fold serially diluted positive control DNA templates with the copy numbers ranging from $10^7$ to $10^0$ copies per reaction. As shown in Fig. 3B, three distinct DNA bands at expected molecular weights were observed at the concentration of $10^2$ copies per reaction. Thus the sensitivity of this mPCR ranks at the level of 100 copies/gene/reaction.

**Performance of the mPCR with clinical samples**

The ultimate aim of this experiment was to evaluate the diagnostic performance of the mPCR using real life clinical samples. Ninety-four sow serum samples whose infectious statuses were predetermined by using commercial real-time PCR kits were used to evaluate the mPCR. The DNA electrophoresis results of the first 18 samples were shown in Fig. 4 for demonstration purposes.

Detailed information of the mPCR results is listed in Tables 2 and 3. The single infection rates for PCV2, PCV3 and PPV in these 94 samples were 43.6%, 39.4% and 55.3% respectively (Table 2). More importantly, the mPCR results matched 100% with the commercial real-time PCR results. The co-infection of three tested viruses (PCV2, PCV3 and PPV) were identified in 17.0% samples while the dual infection between PCV2 and PCV3; PCV3 and PPV, or PCV2 and PPV were detected in 7.4%, 5.3%, and 11.7% sample, respectively (Table 3).
Sequence analysis of the PCR amplicons

Table 2 Detection of clinical specimens by multiplex PCR and real-time PCR

| Pig Farm | No. of specimens | Multiplex PCR | Real-time PCR |
|----------|------------------|---------------|---------------|
|          |                  | PCV2 positive (%) | PCV3 positive (%) | PCV2 positive (%) | PCV3 positive (%) |
| 1        | 5                | 1 (20.0)       | 2 (40.0)       | 1 (20.0)         | 3 (60.0)         |
| 2        | 5                | 3 (60.0)       | 1 (20.0)       | 3 (60.0)         | 1 (20.0)         |
| 3        | 5                | 2 (40.0)       | 0 (0.0)        | 2 (40.0)         | 0 (0.0)          |
| 4        | 5                | 4 (80.0)       | 2 (40.0)       | 4 (80.0)         | 2 (40.0)         |
| 5        | 5                | 2 (40.0)       | 3 (60.0)       | 2 (40.0)         | 3 (60.0)         |
| 6        | 5                | 4 (80.0)       | 3 (60.0)       | 4 (80.0)         | 3 (60.0)         |
| 7        | 5                | 4 (80.0)       | 4 (80.0)       | 4 (80.0)         | 4 (80.0)         |
| 8        | 5                | 3 (60.0)       | 3 (60.0)       | 5 (100)          | 3 (60.0)         |
| 9        | 5                | 3 (60.0)       | 3 (60.0)       | 5 (100)          | 3 (60.0)         |
| 10       | 5                | 4 (80.0)       | 4 (80.0)       | 2 (40.0)         | 4 (80.0)         |
| 11       | 5                | 2 (40.0)       | 2 (40.0)       | 2 (40.0)         | 2 (40.0)         |
| 12       | 5                | 2 (40.0)       | 2 (40.0)       | 2 (40.0)         | 2 (40.0)         |
| 13       | 5                | 0 (0.0)        | 2 (40.0)       | 0 (0.0)          | 2 (40.0)         |
| 14       | 5                | 2 (40.0)       | 2 (40.0)       | 1 (20.0)         | 1 (20.0)         |
| 15       | 5                | 1 (20.0)       | 2 (40.0)       | 1 (20.0)         | 1 (20.0)         |
| 16       | 5                | 1 (20.0)       | 0 (0.0)        | 1 (20.0)         | 0 (0.0)          |
| 17       | 5                | 1 (20.0)       | 1 (20.0)       | 1 (20.0)         | 1 (20.0)         |
| 18       | 5                | 1 (20.0)       | 1 (20.0)       | 1 (20.0)         | 1 (20.0)         |
| 19       | 4                | 1 (20.0)       | 1 (20.0)       | 1 (20.0)         | 1 (20.0)         |
| Total    | 94               | 41 (43.6)      | 37 (39.4)      | 52 (35.3)        | 41 (43.6)        |

Table 3 Detection of the co-infection of clinical specimens by multiplex PCR

| Pig Farm | No. of specimens | PCV2 and PCV3 positive (%) | PCV3 and PPV positive (%) | PCV2 and PPV positive (%) | PCV2 and PPV positive (%) |
|----------|------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 1        | 5                | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  |
| 2        | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 1 (20.0)                  |
| 3        | 5                | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   |
| 4        | 5                | 1 (20.0)                  | 1 (20.0)                  | 2 (40.0)                  | 2 (40.0)                  |
| 5        | 5                | 1 (20.0)                  | 1 (20.0)                  | 0 (0.0)                   | 0 (0.0)                   |
| 6        | 5                | 1 (20.0)                  | 1 (20.0)                  | 1 (20.0)                  | 1 (20.0)                  |
| 7        | 5                | 0 (0.0)                   | 1 (20.0)                  | 1 (20.0)                  | 1 (20.0)                  |
| 8        | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 2 (40.0)                  |
| 9        | 5                | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   | 3 (60.0)                  |
| 10       | 5                | 2 (40.0)                  | 0 (0.0)                   | 0 (0.0)                   | 3 (60.0)                  |
| 11       | 5                | 1 (20.0)                  | 0 (0.0)                   | 2 (40.0)                  | 2 (40.0)                  |
| 12       | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   |
| 13       | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   |
| 14       | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   |
| 15       | 5                | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   | 0 (0.0)                   |
| 16       | 5                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   |
| 17       | 5                | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   |
| 18       | 5                | 1 (20.0)                  | 0 (0.0)                   | 0 (0.0)                   | 0 (0.0)                   |
| 19       | 4                | 0 (0.0)                   | 0 (0.0)                   | 1 (20.0)                  | 0 (0.0)                   |
| Total    | 94               | 7 (7.4)                   | 5 (5.3)                   | 11 (11.7)                 | 16 (17.0)                 |

To further confirm the specificity of the mPCR assay, PCR products of PPV, PCV3 and PCV2 amplified from the field samples were excised from the agarose gel and subjected to DNA sequencing. The resulting sequences were 97%, 98.88%, 98.42% identity to the reference sequences for PCV2; PCV3 and PPV, respectively. The results clearly demonstrate that the mPCR specifically amplified the targeted viral DNA templates.

Discussion
Since its initial identification in the US [1], PCV3 has been subsequently, but almost simultaneously, found in various pig-producing countries. The 39.4% detection rate for PCV3 in our study was also comparable to other reports elsewhere, such as 44.2% in Korea [32]; 31.18% in Central China [33]; 50% in Germany [34]; 56.4%, 37.4%, 14.8% in Denmark, Italy, Spain respectively [35]; 61.1% in United States [36]; and 47.8% in Brazil [37]. It is therefore possible that this virus had already emerged and spread through the global swine population before it was discovered in the US.

Co-infection between PCV2 and other swine pathogens are frequently detected in swine herds [38]. One of the plausible reasons for this is that PCV2 can suppress the host immune system, therefore, rendering the host more susceptible to infection with other pathogens. PPV is one of the common swine pathogens that are detected in PCV2 infected pigs [38]. Previous studies showed that co-infection of pigs with PCV2 and PPV is more effective in inducing PMWS disease than coinfection between PCV2 and other pathogens [39, 1, 41]. Consequently, PPV is often used in combination with PCV2 to induce PMWS in experimental studies [1]. Coinfection between PCV2 and PCV3 is also commonly observed. Several studies from China have reported the PCV2/PCV3 coinfection rates varying from 27.6–39.39% [38]. While it has been demonstrated that PCV2, PCV3 and PPV are endemic in Vietnam, the co-infection rates of these 3 viruses in Vietnamese sow herds have not been studied. Currently, singleplex realtime PCRs are used for molecular detection of these viruses. The use of single-target real-time PCRs used to detect multiple pathogens within one sample significantly increases the diagnostic costs, thus, deterring swine producers from using this service. Therefore, we are interested in developing a quick and reliable mPCR that allow simultaneous detection of these three pathogens. The results indicate that the diagnostic performance of our mPCR is similar to that of commercial real-time PCRs that are currently used in Vietnam (Table 2). The detection limit of our mPCR is 100 copies of each target gene per reaction. Liu et al. (2015) established a mPCR to detect and differentiate of PCV2, Porcine reproductive and respiratory syndrome virus 2 (PRRSV-2), PPV and Pseudorabies virus in pigs with PMWS [42]. These authors reported that the sensitivity of their mPCR was $4.0 \times 10^3$ and $3.0 \times 10^2$ copies to PCV2 and PPV, respectively. Recently, Yang et al. (2018) developed a mPCR for simultaneous detection of PCV1, PCV2 and PCV3 with the detection limit of 50
copies of each target gene per reaction [43]. Thus, our mPCR herein reported has similar levels of sensitivity as compared to those reported in recent publications.

Conclusions
In summary, we successfully developed and validated a mPCR to simultaneously detect three common swine DNA viruses in clinical samples. This mPCR should provide quick and reliable molecular diagnosis of reproductive failure in sow herds.

Declarations

Ethics approval and consent to participate
Not applicable (written in the part of Clinical Samples)

Consent for publication
Not applicable (written in the part of Clinical Samples)

Competeing interests
The authors declare that they have no competing interests.

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Availability of data and materials
Not applicable (all necessary data have been shown in the manuscript).

Author’s contributions

TKT organized experiment, analysed data and established initial manuscript. TTTN collected samples and performed PCR assay. HLXV provided suggestion in research and substantially revised the manuscript. PXD initiated the project, supervised the study, and prepared the final manuscript.

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Figures
Figure 1. Optimization of annealing temperature in s-PCR. (A) PCV3, (B) PPV, (C) PCV2. M: DNA ladder 1 Kb plus, (1): 50°C; (2): 52°C, (3): 54°C, (4): 56°C, (5): 58°C, (6): negative control with pure water. The thermal cycling conditions were: 94°C/5 min; 35 Cycles of 94°C/30 s, 56°C/30 s and 72°C/40 s, a final extension at 72°C/5 min; Gel electrophoresis was at 90 Vol/25 minutes.

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Figure 2. Products of sPCRs and mPCR. (1-3) sPCR: PCV3 276bp (1), (2) PPV 380bp, (3) PCV2 702bp; (4) mPCR of all three targets. (-) negative control with pure water. The thermal cycling conditions were: 94°C/5 min; 35 Cycles of 94 °C/30 s, 56 °C/30 s and 72 °C/40 s and a final extension at 72 °C/5 min. Gel electrophoresis was at 90 Vol/25 minutes.

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Products of sPCRs and mPCR. (1-3) sPCR: PCV3 276bp (1), (2) PPV 380bp, (3) PCV2 702bp; (4) mPCR of all three targets, (-) negative control with pure water. The thermal cycling conditions were: 94°C/5 min; 35 Cycles of 94 °C/30 s, 56 °C/30 s and 72 °C/40 s and a final extension at 72 °C/5 min; Gel electrophoresis was at 90 Vol/25 minutes.
Specificity and sensitivity of the mRCR. (A) Specificity test. M: ladder 1 Kb plus, (1): positive control, (2): E. coli, (3): Streptococcus suis, (4): Staphylococcus aureus, (5): C. perfringens, (6): Pseudorabies virus, (7): negative control with pure water. B) Detection limit of the mPCR. DNA template of PCV2, PCV3 and PPV were serially diluted and used at $10^2$-$10^8$ copy/reaction as indicated. M: ladder 1 Kb plus, (-) negative control with pure water. The thermal cycling conditions were: 94 °C/5 min; 35 Cycles of 94 °C/30 s, 56 °C/30 s and 72 °C/40 s, a final extension at 72 °C/5 min; Gel electrophoresis was at 90 Vol/25 minutes.

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Specificity and sensitivity of the mRCR. (A) Specificity test. M: ladder 1 Kb plus, (1): positive control, (2): E. coli, (3): Streptococcus suis, (4): Staphylococcus aureus, (5): C. perfringens, (6): Pseudorabies virus, (7): negative control with pure water. B) Detection limit of the mPCR. DNA template of PCV2, PCV3 and PPV were serially diluted and used at $10^2$-$10^8$ copy/reaction as indicated. M: ladder 1 Kb plus, (-) negative control with pure water. The thermal cycling conditions were: 94 °C/5 min; 35 Cycles of 94 °C/30 s, 56 °C/30 s and 72 °C/40 s, a final extension at 72 °C/5 min; Gel electrophoresis was at 90 Vol/25 minutes.
Figure 4. Multiplex PCR test using clinical samples. (1) - (18) Clinical samples. M: DNA ladder 1 Kb plus, (+) positive control, (-) negative control with pure water. The thermal cycling conditions were: 94 °C/5 min; 35 Cycles of 94 °C/30 s, 56 °C/30 s and 72°C/40 s, a final extension at 72 °C/5 min; Gel electrophoresis was at 90 Vol/25 minutes.

Multiplex PCR test using clinical samples. (1) - (18) Clinical samples. M: DNA ladder 1 Kb plus, (+) positive control; (-) negative control with pure water. The thermal cycling conditions were: 94 oC/5 min; 35 Cycles of 94 oC/30 s, 56 oC/30 s and 72oC/40 s, a final extension at 72 oC/5 min; Gel electrophoresis was at 90 Vol/25 minutes.