

Development and optimization of Multiplex-PCR for simultaneous detection of Porcine Pseudorabies Virus, Porcine Parvovirus, and Porcine Circovirus Type 2

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KEY WORDS: Multiplex-PCR; Porcine pseudorabies virus; Porcine parvovirus; Porcine circovirus type 2; Detection pathogen

ABSTRACT

Multiple infections by pathogens are currently the most serious problems in pig herds. Clinically, accurate diagnosis is difficult due to similarity of the symptoms of porcine pseudorabies virus (PRV), porcine parvovirus (PPV), and porcine circovirus type 2 (PCV2). A multiplex polymerase chain reaction (multiplex-PCR) was developed and optimized for the simultaneous detection of the three DNA viral infections in pigs. Four pairs of specific primers were designed for each of the three viruses. Each of the four target fragments produced a specific amplicon 657 bp (PPV, NS1), 490 bp (PCV2, ORF2), 372 bp (PRV, gB), and 298 bp (PRV, gE) in a single PCR. The optimal parameters, individual reaction component concentrations (the concentrations of prim-

ers, MgCl₂, dNTP, and Taq DNA polymerase), and annealing temperature, of the multiplex PCR were defined based on single PCR conditions. The sensitivity of the multiplex PCR for NS1, ORF2, gB, and gE in a 20 µl mixture using purified recombinant plasmids containing the viral target genes was 10⁻⁵ (1.375 × 10⁻⁴ ng). The specificity of primer pairs for the classical swine fever virus, as well as porcine reproductive and respiratory syndrome virus was analyzed by multiplex PCR. The PCR products tested negative. The multiplex-PCR method is a convenient diagnostic tool for the routine surveillance of viral co-infections for the simultaneous detection of PCV2, PRV, and PPV.

INTRODUCTION

Multiple infections with pathogens are currently the most serious problems in the hog industry worldwide¹, especially in intensive swine production. Swine simultaneously infected with two or more viral pathogens

is becoming prevalent. Clinically, this often leads to difficulty in definitively diagnosing pig viral infections because of the similarity in the presentation of clinical symptoms, such as reproductive failure, diarrhea, fever, abortion, and/or stillbirth. Determining whether the causative agent is porcine pseudorabies virus (PRV), porcine parvovirus (PPV), porcine circovirus type 2 (PCV2), or others is often difficult if based only on clinical signs. However, the diagnostic standard for identifying viral infections based on pathogen isolation from cell cultures of suspected samples is time consuming, costly for individuals, and is not favorable for controlling the diseases.

Multiplex-PCR and multiplex RT-PCR for multiple viral infectious have been reported. However, these methods are specific for simultaneous differential diagnostic tests related to particular symptoms based on amplicon size in a single sample²⁻⁴. In the present report, we describe the development of multiplex PCR methods for the simultaneous detection of three swine DNA viruses, namely, PCV2, PRV, and PPV.

MATERIAL AND METHODS

Viruses and clinical samples

The PPV NADL-2 and PRV Bathar strains were from the China Institute of Veterinary Drug Control. The PCV2 strain was stored in the laboratory. All of the viruses were propagated in PCV1-free PK-15 cells. Up to 30 samples from suspected clinical cases, including lymph nodes, tonsils, lungs and

spleens, were collected from piglets and weaned piglet with respiratory and/or reproductive problems accompanied by progressive weight loss, as well as from aborted pig fetuses.

Primer design

Four pairs of primers for simultaneous amplification for the three target viruses, PCV2 (ORF2), PPV (NS1), and PRV (gB and gE), were designed by Sangon Biotech (Shanghai) Co., Ltd. (Table 1).

Viral genomic DNA extraction

Viral genomic DNA was extracted from cell cultures infected with each virus or frozen clinical samples using the Universal Genomic DNA Mini Isolation Kit (Sangon) following the manufacturer’s protocol.

The optimum concentration of primers in a single PCR

The concentration of primers for the amplification of NS1, ORF2, gB, and gE was optimized for single PCRs under standard PCR conditions. The basic concentration of the primers ranged from 0.1 μM to 0.5 μM in a 20 μl mixture. The annealing temperature gradient was at 58 °C with 18 cycles and at 57 °C with 15 cycles. Amplicons were observed by electrophoresis of 8 μl aliquots through 2.5% agarose gel in 1× TAE buffer (40 mM Tris–acetate, pH 8.0, 1 mM EDTA). Four target fragments from the cell viruses were cloned into the plasmid pMD18-T (TaKaRa), and all the DNA fragments were further sequenced and analyzed by Sangon to determine their specificity.

Table 1. Primers used for multiplex PCR and the length of the corresponding target genes

Primer	Sequence	Length of target gene
PPC675f	5?-catgggccagcatctacagg-3?	657 bp
PPV657r	5?-tgttggctcgctccacggct	
PCV490f	5?-gagaagggctgggttatggtatgg-3?	490bp
PCV490r	5?-acagcgcacttcttctgtttcag-3?	
PrVgB372f	5?-agtactcgcagggcgcaact-3?	372bp
PrVgB372r	5?-cgccgatcttgggttaggtgt-3?	
PrVgE298f	5?-gccacgcacgaggactactacga-3?	298bp
PrVgE298r	5?-ttaagcggggcgggacatcaacag-3?	

Table 2. Detection of PRV, PPV and PCV2 viruses in clinical samples by multiplex-PCR and single

Single PCR	Multiplex-PCR	Single PCR	Positives (%)
PRV	4	4	13.33
PPV	7	7	23.33
PCV2	18	18	60
Co-infection			
PRV+PCV2	2	2	6.67
PPV+PCV2	1	1	3.33
PPV+PCV2	3	3	10
PPV+PRV+PCV2	1	1	3.33

Optimization the multiplex-PCR

Further research was performed to obtain the concentration of each component, such as MgCl₂, dNTP, and Taq DNA polymerase, under standard single PCR or multiplex PCR to ensure outcomes are appropriate. Different MgCl₂ concentrations ranging from 1.3 to 1.7 mM in a 20 µl mixture were tested, and the optimal concentration selected in the single PCR. The concentrations of the dNTP and Taq DNA polymerase, also determined experimentally, ranged from 0.07 to 0.42 mM and 0.025 to 0.225 U in a 20 µl mixture, respectively, under the multiplex-PCR reaction.

Sensitivity and specificity of single and multiplex-PCR assays

The sensitivity of the multiplex-PCR was determined using the plasmid containing NS1, ORF2, gB, and gE as template after a 10-fold serial dilution, from 137.5 to 1.375 pg plasmid per reaction in the 20 µl reaction system. The specificity of the multiplex PCR was further evaluated depending on the virus-infected cell culture and the suspected clinical samples, and the amplified fragments were

identified by DNA sequencing and analysis. Two common RNA viruses in pigs and no template-reaction system, including classical swine fever virus (CSFV) and reproductive and respiratory syndrome virus (PRRSV), were the negative controls.

RESULTS

Optimization and standardization of the multiplex-PCR

Four primer sets, corresponding to each virus, were designed to optimize amplification, especially annealing temperature, and to amplify the length gradient of the target fragments to distinguish them on electrophoresis gel. The nonspecific products were minimized whenever possible and the specificity of the primers was also evaluated in

Figure 1: Optimum concentration of primers of a single PCR for PPV-NS1 (A), PCV2-ORF2 (B), PRV-gB (C), and PRV-gE (D) Lanes 1–5: Concentrations of primers form 0.1 µM to 0.5 µM in a 20 µl mixture, respectively; lane M: DL2000 marker

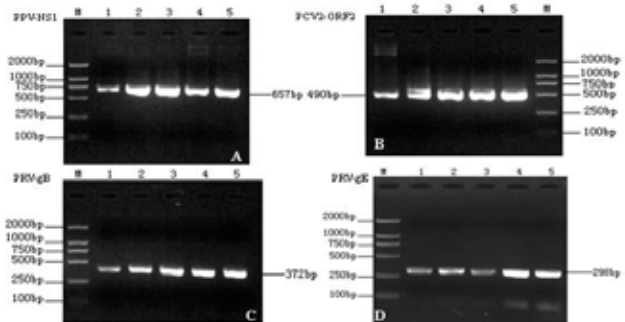


Figure 1

Figure 2: Results of simultaneous detection of PRV, PPV, and PCV2 by multiplex-PCR. PPV+PCV2+PRV mixture M. DL2000

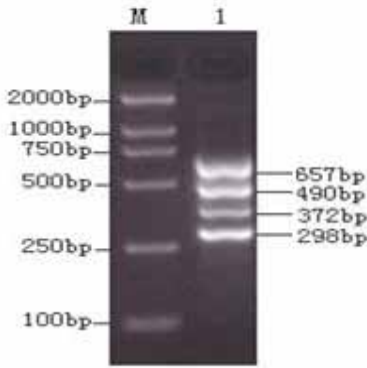


Figure 2

the single PCR of ORF2, NS1, gB, and gE (Fig. 1).

The multiplex PCR simultaneously amplified all four target genes by testing combinations of the four viral strains prepared from the infected cell culture. Four distinct bands of the expected sizes, 657 bp (PPV, NS1), 490 bp (PCV2, ORF2), 372 bp (PRV, gB), and 298 bp (PRV, gE), were observed clearly under the same gradient annealing temperature (58 °C and 57 °C) in the sample multiplex PCR (Fig. 2). The optimal concentrations of MgCl₂ were assessed in the single PCR. The best working concentration is 1.54 mM in a 20 µl mixture. The appropriate concentrations of dNTP and Taq polymerase were also obtained at 0.35 mM and 0.2 U in the 20 µl mixture, respectively (Figs. 4 and 5).

Sensitivity and specificity of the multiplex-PCR assay

The specificity of the primers for each virus was analyzed via single PCR (Fig. 3). The minimum concentration of the target genes was 1.375×10^{-2} ng in the 20 µl mixture. The multiplex PCR was specific for the four DNA viruses because the nonspecific bands were amplified with CSFV, PRRSV, and

dH₂O in lanes 8–10 (Fig. 6).

Evaluation of the multiplex-PCR using clinical samples

The multiplex and single PCR were assessed using 30 suspected clinical samples collected from different places between 2006 and 2010 within China. The results matched both PCR methods well (Table 2) and they were further confirmed by two positive sequencing results for each virus.

DISCUSSION

Diseases that cause reproductive failure seriously affect the efficiency and cost of swine production. The etiology of reproductive diseases is complex. PRV, PPV, PCV2, CSFV, and PRRSV are the common viral agents implicated in these diseases in pigs. Under typical conditions of intensive swine production, pigs are commonly infected simultaneously by two or more viral pathogens, which can induce similar clinical syndromes and lesions [5]. Furthermore, an accurate diagnosis of mixed infections often requires more effort, especially for some clinical signs in swine.

PRV, an alpha herpes virus, is the etiologic agent of Aujeszky's disease in swine [6]. This disease is responsible for causing severe economic losses in infected herds and is often fatal due to the central nervous system disorders in young piglets. Additionally, older pigs generally develop respiratory disease, including encephalitis and pneumonia, whereas those who survive the acute infection carry the virus form and exhibits persistent viral infection for their entire life⁷. In pregnant sows, PRV infection normally causes reproductive failure⁸. The gE-deleted marker vaccine is used for eradication of PRV worldwide. Therefore, most eradication or control programs are performed using the marker vaccines, an accompanying differentiating infected from vaccinated animal serologic tests that detect serum antibodies against the gE protein, and etiological detection that detects the gE gene^{9,10}. Compared with other methods, such as virus isolation, fluorescent antibody tissue section test, serum virus neutralization, latex agglutination

Figure 3: Optimal MgCl₂ concentration of single PCR for PPV-NS1 (A), PCV2-ORF2 (B), PRV-gB (C), and PRV-gE (D). Lanes 1–5: Varying MgCl₂ concentrations from 1.3 mM to 1.7 mM in a 20 µl mixture, respectively; lane M: DL2000 marker

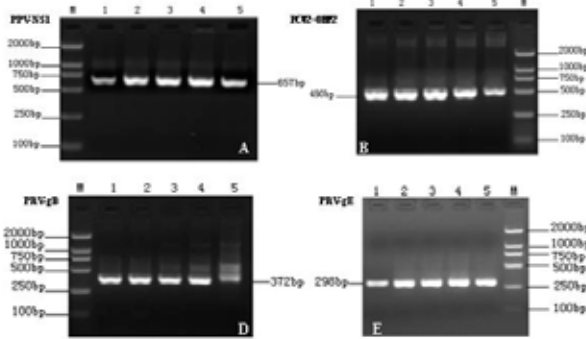


Figure 3

Figure 4: Optimum concentration (µM) of dNTP of mPCR for PPV-NS1, PCV2-ORF2, PRV-gB, and PRV-gE. Lanes 1–5: Concentrations of dNTP: 0.07, 0.14, 0.21, 0.28, 0.35, and 0.42 in a 20µl mixture respectively; Lane M: DL2000 marker.

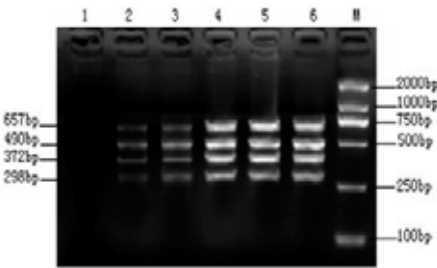


Figure 4

Figure 5: Optimum concentration (U) of mPCR of Taq polymerase. Lanes 1–9: Concentrations of Taq polymerase: 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, 0.2, and 0.225 in a 20 µl mixture, respectively; lane M: DL2000 marker.

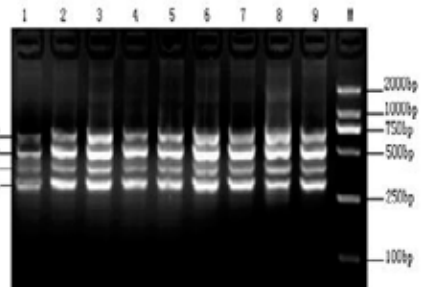


Figure 5

Figure 6: Sensitivity of mPCR for PPV-NS1, PCV2-ORF2, PRV-gB, and PRV-gE. Lanes 1–10: Reactions performed in 10-fold serial dilution from 10⁻¹–10⁻¹⁰ (from 137.5 ng to 1.375 pg)

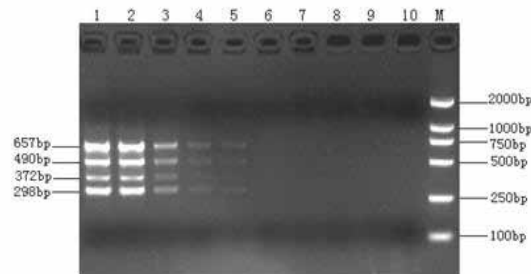


Figure 6

test, and ELISA¹¹, PCR is a time-saving, sensitive, and accurate assay in which the results may also support the utility and value of molecular assays for diagnostic investigation and surveillance because it can detect both infectious and noninfectious viral materials by investigating the gB-positive/gE-negative cases¹⁰.

PPV is an extremely durable and highly infectious virus and causes severe reproductive failure in pregnant sows. The disease caused by this pathogen is characterized by embryonic and fetal death, mummification, stillbirths, and delayed return to estrus¹². PPV is widespread among swine throughout the world, and is even endemic in most herd infections. Hence, the economic effects in a susceptible herd can be serious^{12,13}. Thus, continuous vaccination is the most effective method for avoiding large-scale economic losses. Recently, PPV has gained importance as a syndicated agent to enhance the effects of PCV2 infection in the clinical course of postweaning multisystemic wasting syndrome^{14,15}. PCV2-associated reproductive failure can be reproduced experimentally showing that porcine embryos and fetuses are susceptible to PCV2 infection^{16,17}, and that intrauterine spread¹⁸ and vertical transmission can occur¹⁹. Moreover, naturally occurring PCV2-associated reproductive failure cases have been reported²⁰. Rapid and reliable detection of the three DNA viruses is essential for epidemiological surveillance and disease prevention.

Conventional usage of single PCR to detect several viruses individually is labor intensive and expensive. These limitations can be overcome by establishing a multiplex-PCR assay that incorporates multiple specific primers that amplify several RNA or DNA viruses simultaneously in a single PCR [21]. A recent study reported the development of multiplex RT-PCR to detect major viruses in pigs with multiple infections [22]. The multiplex-PCR method was developed in the present study to specifically detect and differentiate the three DNA viruses in swine. The development of the multiplex-PCR

method is usually confronted with the aforementioned problems. Under optimization of the single PCR reaction condition, the multiple primers require concordant annealing temperatures and reduction of any possible formation of primer dimers. Therefore, correctly designed primers should conform to the above principles. Experimentation was performed repeatedly in a single PCR for individual viral target gene (Fig. 3). The multiplex-PCR reaction system was carefully optimized to obtain maximal sensitivity and specificity, including the concentrations of dNTP and Taq polymerase (Figs. 4 and 5). The sensitivity of the developed multiplex-PCR was evaluated comparatively using serial ten-fold dilutions of each virus. The lowest detection limit for the target genes was 13.75 pg (Fig. 6). Pathogen detection results show that PPV, gB, and gE were detected only once, whereas PCV2 was the most frequently detected agent among the clinical samples (Table 2).

CONCLUSIONS

In the present work, the multiplex-PCR method has been developed for simultaneous detection of PRV, PPV, and PCV2, providing for a more convenient and reliable method for the rapid diagnosis of major pathogenic viruses in swine.

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REFERENCES

1. Opriessnig T, Meng XJ, Halbur PG: Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* 2007, 19(6): 591–615.
2. Kim SY, Song DS, Park BK: Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. *J Vet Diagn Invest* 2001, 13(6): 516–520.
3. Song DS, Kang BK, Oh JS, Ha GW, Yang JS, Moon HJ, Jang YS, Park BK: Multiplex reverse transcription-PCR for rapid differential detection of porcine epidemic diarrhea virus, transmissible gastroenteritis virus, and porcine group A rotavirus. *J Vet Diagn Invest* 2006, 18(3): 278–281.
4. Bellau-Pujol S, Vabret A, Legrand L, Dina J,

- Gouarin S, Petitjean-Lecherbonnier J, Pozzetto B, Ginevra C, Freymuth F: Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. *J Virol Methods* 2005, 126(1-2): 53–63.
5. Pabst R: The respiratory immune system of pigs. *Vet Immunol Immunopathol* 1996, 54 (1-4): 191–195.
 6. Mettenleiter TC: Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis—state of the art. *Vet Res* 2000, 31(1): 99–115.
 7. Nauwynck HJ: Functional aspects of Aujeszky's disease (pseudorabies) viral proteins with relation to invasion, virulence and immunogenicity. *Vet Microbiol* 1997, 55 (1-4): 3–11.
 8. Zuckermann FA: Aujeszky's disease virus: opportunities and challenges. *Vet Res* 2000, 31(1): 121–131.
 9. Ao JQ, Wang JW, Chen XH, Wang XZ, Long QX: Expression of pseudorabies virus gE epitopes in *Pichia pastoris* and its utilization in an indirect PRVgE-ELISA. *J Virol Methods* 2003, 114(2): 145–150.
 10. Ma W, Lager KM, Richt JA, Stoffregen WC, Zhou F, Yoon KJ: Development of real-time polymerase chain reaction assays for rapid detection and differentiation of wild-type pseudorabies and gene-deleted vaccine virus. *J Vet Diagn Invest* 2008, 20 (4): 440–447.
 11. Yong T, Huan-chun C, Shao-bo X, Ya-li Q, Qi-gai H, Yu-qi R: Development of a latex agglutination test using the major epitope domain of glycoprotein E of pseudorabies virus expressed in *E. coli* to differentiate between immune responses in pigs naturally infected or vaccinated with pseudorabies virus. *Vet Res Commun* 2005, 29 (6): 487–497.
 12. Mengeling WL, Lager KM, Zimmerman JK, Samarikermani N, Beran GW: A current assessment of the role of porcine parvovirus as a cause of fetal porcine death. *J Vet Diagn Invest* 1991, 3(1): 33–35.
 13. Parke CR, Burgess GW: An economic assessment of porcine parvovirus vaccination. *Aust Vet J* 1993, 70(5): 177–80.
 14. Krakowka S, Ellis JA, Meehan B, Kennedy S, McNeilly F, Allan G: Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. *Vet Pathol* 2000, 37(3), 254–263.
 15. Segale's J, Allan GM, Domingo M: Porcine circovirus diseases. *Anim Health Res Rev* 2005, 6 (2): 119–142.
 16. Mateusen B, Maes DG, Van SA, Lefebvre D, Nauwynck HJ: Effect of a porcine circovirus type 2 infection on embryos during early pregnancy. *Theriogenology* 2007, 68 (6): 896–901.
 17. Mateusen B, Sanchez RE, Van SA, Meerts P, Maes DG, Nauwynck HJ: Susceptibility of pig embryos to porcine circovirus type 2 infection. *Theriogenology* 2004, 61(1): 91–101.
 18. Pensaert MB, Sanchez Jr RE, Ladekjaer-Mikkelsen AS, Allan GM, Nauwynck HJ: Viremia and effect of fetal infection with porcine viruses with special reference to porcine circovirus 2 infection. *Vet Microbiol* 2004, 98(2): 175–183.
 19. Park JS, Kim J, Ha Y, Jung K, Choi C, Lim JK, Kim SH, Chae C: Birth abnormalities in pregnant sows infected intranasally with porcine circovirus 2. *J Comp Pathol* 2005, 132(2-3): 139–144.
 20. Hansen MS, Hjulsgaard CK, Bille-Hansen V, Haugegaard S, Dupont K, Høgedal P, Kunstmann L, Larsen LE: Selection of method is crucial for the diagnosis of porcine circovirus type 2 associated reproductive failures. *Vet Microbiol* 2010, 144 (1-2): 203–209.
 21. Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE: Multiplex PCR: optimization and application in diagnostic virology. *Clin Microbiol Rev* 2000, 13(4): 559–570.
 22. Ogawa H, Taira O, Hirai T, Takeuchi H, Nagao A, Ishikawa Y, Tuchiya K, Nunoya T, Ueda S: Multiplex PCR and multiplex RT-PCR for inclusive detection of major swine DNA and RNA viruses in pigs with multiple infections. *J Virol Methods* 2009, 160(1-2): 210–214.