VETERINARSKI ARHIV 85 (5), 511-521, 2015

Simultaneous detection and differentiation of porcine circovirus type 2, type 2 porcine reproductive and respiratory syndrome virus, porcine parvovirus and pseudorabies virus in pigs with postweaning multisystemic wasting syndrome (PMWS) by multiplex PCR

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LIU, J. K., C. H. WEI, X. Y. YANG, A. L. DAI, X. H. LI: Simultaneous detection and differentiation of porcine circovirus type 2, type 2 porcine reproductive and respiratory syndrome virus, porcine parvovirus and pseudorabies virus in pigs with PMWS by multiplex PCR. Vet. arhiv 85, 511-521, 2015.

ABSTRACT

A multiplex polymerase chain reaction (PCR) was designed for the simultaneous detection of four viruses involved in postweaning multisystemic wasting syndrome (PMWS) in pigs: porcine circovirus type 2 (PCV-2), type 2 porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV) and pseudorabies virus (PRV). Each of the four pairs of primers exclusively amplified the targeted fragment of the specific viruses. The sensitivity of the multiplex PCR, using purified plasmid constructs containing the specific viral target fragments, was 4.0×10^3, 4.5×10^3, 3.0×10^2 and 5.0×10^2 copies for PCV-2, type 2 PRRSV, PPV, PRV. Among 82 clinical samples, coinfection by PCV-2 and type 2 PRRSV was the most common. When compared with the virus isolation method commonly used to detect viruses, the multiplex PCR assay was found to be more sensitive and rapid and, as such, may prove to be a good alternative method for the detection of and differentiation in PMWS in pigs.

Key words: postweaning multisystemic wasting syndrome, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, porcine parvovirus, pseudorabies virus, PCR

Introduction

Postweaning multisystemic wasting syndrome (PMWS) was first described in Canada (HARDING and CLARK, 1997) and was a relatively new disease of swine associated with

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ISSN 0372-5480
Printed in Croatia

important mortality rates in nursery and fattening pigs (MAGAR et al., 2000). PMWS has since been recognized as one of the economically most important swine diseases worldwide (SEGALES et al., 1997; ALLAN et al., 1998; KIUPEL et al., 1998; HINRICHS et al., 1999; GRABAREVIĆ et al., 2004; LIPEJ et al., 2006), in acute outbreaks overall mortality can reach up to 50 % (CHEUNG et al., 2007). PMWS is an acute or chronic disease affecting animals at the age of 5 to 16 weeks (HARDING and CLARK, 1997; ALLAN et al., 2000b; SEGALES et al., 2004). The characteristic clinical symptoms of PMWS include progressive mass loss, dyspnea, enlargement of lymph nodes, diarrhea, pallor, and jaundice (ALLAN and ELLIS, 2000; SEGALES et al., 2004).

Porcine circovirus 2 (PCV2) is a single-stranded circular DNA virus in the *Circoviridae* family that has been demonstrated to be the cause of PMWS (KENNEDY et al., 2000; KRAKOWKA et al., 2001). In previous studies, susceptible pigs inoculated with PCV2 develop the typical microscopic lesions of PMWS, but only a mild form of the clinical disease. Experimental studies of coinfection with PRRSV and PCV2 have reproduced microscopic lesions of PMWS and/or PMWS (ALLAN et al., 2000a; HARMS et al., 2001; ROVIRA et al., 2002). Porcine parvovirus (PPV) has also been confirmed to potentiate the progression of PCV2 infection to clinical PMWS (ALLAN et al., 1998; KENNEDY et al., 2000; KIM et al., 2003; KRAKOWKA et al., 2000; OPRIESSNIG et al., 2004). These experimental results have been further supported by field epidemiological data. PRRSV was detected in 51.9 % of the PMWS cases in a retrospective investigation (PALLARES et al., 2002) and was present on farms during outbreaks of PMWS (QUINTANA et al., 2001; SEGALÉS et al., 2002). The clinical PMWS on a farm increased when nursery pigs were concurrently infected with PPV and PRRSV (POGRANICHNIY et al., 2002; ROSE et al., 2003). Three clinically relevant viral infections (PCV-2, PRRSV and PRV) occurred simultaneously on a farm during an outbreak of PMWS (QUINTANA et al., 2001). These results further confirmed that PCV2 coinfections with PRRSV or PPV or PRV appear to be important cofactors in the pathogenesis of PMWS.

The objective of the present study was to develop a multiplex PCR for the simultaneous detection and differentiation of PCV2, type 2 PRRSV, PPV and PRV in clinical specimens from pigs with PMWS. This will provide a method for pathogen investigation of PMWS.

Materials and methods

Primer design. A multiplex PCR assay was designed with primers based on the sequences of the ORF1 of PCV-2, ORF5 of type 2 PRRSV, VP2 of PPV, gE of PRV (LIN et al., 2009; ZHANG et al., 2009; JIANG et al., 2010; LIU et al., 2013). The specificities of these primers were checked against the GenBank. The sequences and locations of the primers are given in Table 1.

Extraction of viral genomic DNA and RNA. Viral genomic DNA and RNA were extracted from cell cultures infected with each virus or fresh or frozen clinical specimens using the Roche TriPure Isolation Reagent Viral RNA/DNA Extraction Kit Ver.3.0 (TaKaRa) following the manufacturer's protocol.

Table 1. Specific primer pairs used to amplify each target gene.

Virus	Target gene	Primer sequence (5′-3′)	Expected product (bp)
PRRSV	*ORF5*	catttcatgacacctgagaccaa	718
		agagcatatatcatcaactggcgt	
PCV-2	*ORF1*	cgagaaagcgaaaggaacaga	371
		ggtaaccatcccaccactt	
PPV	*VP2*	acacgcatcaagactcatac	531
		tcactgtgtagtcctgtttg	
PRV	*gE*	gcccacgcacgaggactactacga	298
		ttaagcggggcgggacatcaacag	

PRRSV: porcine reproductive and respiratory syndrome virus. PPV: porcine parvovirus; PCV-2: porcine circovirus type 2; PRV: pseudorabies virus

Single PCR and mPCR. Single PCR was performed in 50- μL reactions in 0.2 mL thin-walled PCR tubes. The reaction mixtures contained 5 μL 10× PCR buffer, 5 μL 2.5 mM $MgCl_2$, 5 μL 2 mM dNTP mix, 2 μL Taq DNA polymerase (TaKaRa), 1 μL of each 10 pmol primer, 2 μL template DNA/CDNA and sterile double distilled water to bring the final volume to 50 μL. The negative controls included the reagents without the cDNA template. The PCR was performed at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 7 min and holding at 4 °C. The PCR products were detected by electrophoresing 5 μL aliquots through 2 % agarose gels in 1×TAE (40 mM Tris - aceate [pH 8.0], 1 mM EDTA).

The multiplex PCR contained a mixture of all four primer pairs and was performed similarly to the single PCRs with some optimization.

Sensitivity and specificity assays. For optimization of multiplex PCR and to measure the limit of detection for each virus, we constructed Plasmid containing specific viral target fragments which were used as templates for single and multiplex PCR. The constructs were verified by PCR and DNA sequencing (Sangon). The DNA concentrations of each construct were determined spectrophotometrically. To evaluate the detection limit of the multiplex PCR assay, tenfold dilutions in the plasmid constructs of the target sequence were used. The specificity of multiplex PCR assays using primers for PCV-2, type 2 PRRSV, PPV and PRV was determined by subjecting the following purified viruses (and bacterium) to independent assays: PCV-2, type 2 PRRSV, PPV, PRV, CSFV, TGEV, PEDV, ddH_2O and *Escherichia coli*.

Field samples. Eight-two clinical specimens of PMWS, including lymph nodes, kidney, lungs and spleens, were collected from commercial herds during 2011 to 2013.

Results

Optimization of multiplex PCR In this study the primers were selected carefully for amplification of the *ORF1, ORF5, VP2*, and *gE* gene for PCV-2, type 2 PRRSV, PPV, and PRV, respectively. The primers met the optimal PCR primers, and they could be used together in a multiplex reaction under similar conditions for amplification and to produce products which could be clearly differentiated on the basis of their sizes. The optimal annealing temperature was found to be approximately 56 °C, primer concentration 0.4 mM, dNTP concentrations 0.04 mM and the number of cycles was 30.

Sensitivity of multiplex PCR. The multiplex PCR is highly sensitive, detecting the limits of PCV-2, type 2 PRRSV, PPV, PRV was 4.0×10^3, 4.5×10^3, 3.0×10^2 and 5.0×10^2 copies, from the viral mixture DNAs (Fig 1A). The results were similar, except the sensitivity of PPV of the multiplex PCR is 10-fold lower than that of the single PCRs (Fig 1B). These primers produce PCR products of 718 bp for type 2 PRRSV, 371bp for PCV-2, 531bp for PPV, and 298 bp for PRV. Thus, the amplicons can be visualized and easily differentiated by agarose gel electrophoresis (Fig. 2).

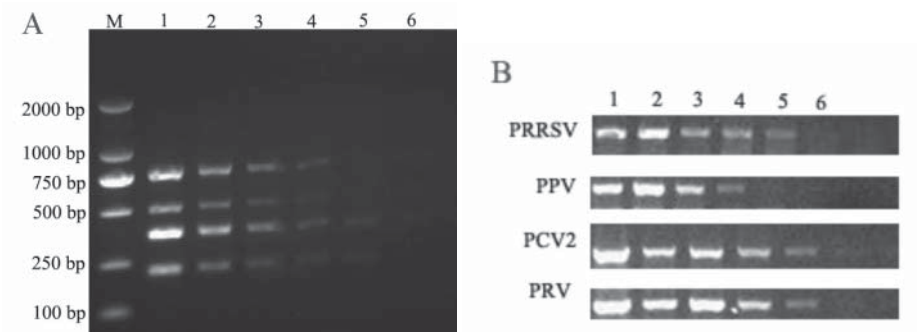

Fig. 1. Sensitivity of the multiplex PCR for simultaneous amplification of all viral target DNAs (A) and single PCR/RT-PCR for each viral target (B). M: DL2000 DNA Marker. Lanes 1-6 are 1: 10^{-1}; 2: 10^{-2}; 3: 10^{-3}; 4: 10^{-4}; 5: 10^{-5}; 6: 10^{-6}.

Specificity of multiplex PCR. When different combinations of the four viruses (one or more viruses) were used in the multiplex PCR, the respective virus amplicons were produced clearly. However, no amplicons were produced in the negative controls including CSFV, TGEV, PEDV, ddH_2O and *Escherichia coli* (data not shown).

Application of multiplex PCR to clinical specimens. To test the multiplex PCR for diagnosis of porcine viruses, 82 clinical specimens were tested for PCV-2, type 2 PRRSV,

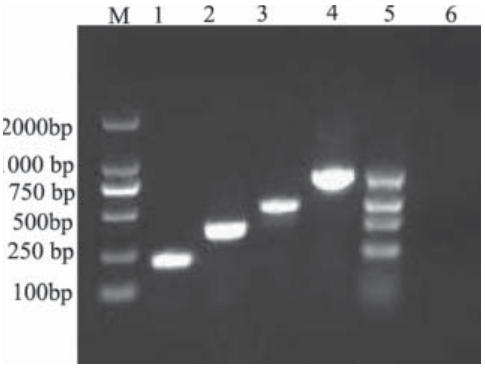

Fig. 2. Multiplex PCR products from four viruses. Lanes (M) DL2000 DNA Marker; (1) 298-bp band from PRV; (2) 371-bp band from PCV2; (3) 531-bp band from PPV; (4) 718-bp bands from PRRSV (5) 298-, 371-, 531-, 718-bp bands from a combination of PRV, PCV2, PPV and PRRSV (6) negative control.

PPV, and PRV, using the multiplex PCR and confirmed by routine PCR/RT-PCR, using the same four sets of specific primers and DNA sequencing of PCR products. The results were similar when compared with single PCRs.

The number of cases with each of the different combinations of pathogens is illustrated in Table 2. Coinfection of PCV-2+type 2 PRRSV was the most common combination, with 28 cases (34.15 %). In decreasing order, the other coinfections were: PCV-2+PPV (18 cases, 21.95 %), PCV-2+type 2 PRRSV+PPV (13 cases, 15.85 %), PCV-2+PRV+PPV (13 cases, 15.85 %), PCV-2+PRV (6 cases, 7.32 %), and PCV-2+PPV+type 2 PRRSV+PRV (2 cases, 2.44 %), there were only 2 cases in which singular PCV-2 infection was confirmed without any co-infecting pathogens.

Table 2. Frequency of viruses alone or in combination in 82 sick piglets from 2011 to 2013

Viruses	Number of cases positive	Positive (%)
PCV-2+PRRSV	28	34.15
PCV-2+PPV	18	21.95
PRRSV+PCV-2+PPV	13	15.85
PCV-2+PRV	6	7.32
PRRSV+PPV+PCV-2+PRV	2	2.44
PCV-2+PPV+PRV	13	15.85
PCV-2 only	2	2.44

PRRSV: porcine reproductive and respiratory syndrome virus. PPV: porcine parvovirus; PCV-2: porcine circovirus type 2; PRV: pseudorabies virus

In the present study, by sequencing confirmation, 100 % specificity was accomplished compared with the results of virus isolation and single PCR. Therefore, the multiplex PCR assay in this study can also be used to directly diagnose PCV2, type 2 PRRSV, PPV and PRV infection from pig tissues without virus isolation.

Discussion

PCV2 is the primary causative agent of PMWS, characterized by severe progressive mass loss, dyspnea, lymph node enlargement, diarrhea, pallor, and jaundice in pigs of 7-15 weeks of age. The hallmark lesion of PMWS and PCV2 infection is lymphoid depletion (KRAKOWKA et al., 2005; RAMAMOORTHY and MENG, 2009; SEGALÉs et al., 2008). Although PCV2 is a necessary pathogen in the development of PMWS, experimental infection with PCV2 alone only produced minimal symptoms, and mild bronchiolitis and interstitial pneumonia (ALLAN et al., 2000b; BOLIN et al., 2001; ELLIS et al., 1999; MAGAR et al., 2000). Piglets coinfected with PCV2 and PPV, or PCV2 and PRRSV, or PCV2 and PRV had a more severe clinical disease and PCV2-associated lesions than piglets infected with PCV2 alone (ALLAN et al., 2000a; ELLIS et al., 2000; KRAKOWKA et al., 2000; QUINTANA et al., 2001; ROVIRA et al., 2002; OPRIESSNIG et al., 2004).These results confirm that coinfections with PRRSV or PPV or PRV appear to be important cofactors in the pathogenesis of PMWS. Therefore a specific and sensitive multiplex PCR is described and applied for simultaneously and differential detection of four important swine viruses with PMWS in one reaction. Multiplex PCR would produce considerable savings of time and effort within the laboratory, without compromising the robustness and sensitivity of the virus detection assays (BELÁK, 2007). An additional advantage of multiplex PCR is the reduced sample requirement, which is particularly important when sample material is limited (PERSSON et al., 2005). In general, optimization of a multiplex PCR compromises the concentrations of reagents, the annealing temperature and the number of cycles, in order to obtain the best strategy for amplification of more than one target sequence. Furthermore, the most common problem of multiplex PCR is that several primer pairs used in the same tube may interact with each other, thus blocking the reaction (ELNIFRO et al., 2000). In this study, the reaction conditions were optimized to obtain maximum sensitivity and specificity, and, despite four primer sets in one reaction, the multiplex PCR was able to detect four viruses at a high level of sensitivity and yield amplified products that could clearly be differentiated by agarose-gel lectrophoresis.

In this study, the multiplex PCR assay was developed for the simultaneous detection of PCV-2, type 2 PRRSV, PPV, and PRV in PMWS in pigs. This multiplex PCR is highly sensitive, detecting PCV-2, type 2 PRRSV, PPV, PRV was 4.0×10^3, 4.5×10^3, 3.0×10^2 and 5.0×10^2 copies from a viral mixture. Previous studies showed that sensitivity was lower with multiplex PCR than with routine PCR (JACQUES et al., 2004; YUE et al., 2009; LIU

et al., 2011). In the current study, the results of multiplex PCR were similar, except the sensitivity of PPV of the multiplex PCR is 10-fold lower than that of the single PCRs in detecting the field samples. In addition, primer selection for the four viruses was based on the sizes of the generated amplicons. These primers produce PCR products of 718 bp for type 2 PRRSV, 531bp for PPV, 371bp for PCV-2, and 298 bp for PRV. Thus, the amplicons can be visualized and easily differentiated by agarose gel electrophoresis. By sequencing confirmation, 100 percent specificity was accomplished compared with the results of virus isolation and single PCR. Therefore, the multiplex PCR assay described here can be used to diagnose PCV-2, type 2 PRRSV, PPV and PRV infection directly from pig tissues without virus isolation, situ hybridization and immune-ohistochemistry, especially in detection of several viruses in swine.

Experimental studies of coinfection with PRRSV and PCV2 reproduced microscopic lesions of PMWS and/or PMWS (ALLAN et al., 2000a; HARMS et al., 2001). PPV has also been found to potentiate the progression of PCV2 infection to clinical PMWS. Since PRRSV also replicates in macrophages, it has been suggested that this virus can produce an effect similar to that observed with PPV (ALLAN et al., 2000a). Our study strongly supports the idea that a variety of pathogens may share a common mechanism in stimulating the immune system (ALLAN et al., 2000b; KRAKOWKA et al., 2001), with the subsequent progression of PCV-2 infection to PMWS, that is, PCV-2 makes pigs more susceptible to other pathogens. In this study, type 2 PRRSV, and PPV were the most common agents that coexisted with PCV-2 in cases of PMWS, and type 2 PRRSV+PCV-2 coinfection was found in 34 % of the cases of PMWS, which is considerably higher than the 29.3 % reported in South Korea (KIM et al., 2002) and considerably lower than the 67 % and 48 % reported in Canada and Spain (LAROCHELLE et al., 1999; SEGALÉS et al., 2002). Because type 2 PRRSV+PCV-2 coinfection was found in 34 % of the cases of PMWS in our study, effective PRRSV control or an eradication program may be important in reducing the incidence of PMWS in Chinese herds.

In summary, a sensitive and reproducible method was established for the simultaneous detection and differentiation among PCV-2, type 2 PRRSV, PPV and PRV from pig tissues. This method could be a good alternative for diagnostic laboratories in detecting these four viruses in PMWS.

Acknowledgements

This work was supported by the Climbing Program of Longyan University (LQ2013023), a major project of Science and Technology Program of Fujian Province, China (2014NZ0002-3) and the Climbing Program of Longyan University (LQ2013022).

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Received: 26 October 2013
Accepted: 9 June 2015

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SAŽETAK

Višestruka lančana reakcija polimerazom (PCR) razvijena je za istodobni dokaz četiriju virusa koji se nalaze kod sindroma kržljavosti odbijene prasadi: svinjskog cirkovirusa tip 2, virusa reprodukcijskog i respiratornog sindroma tip 2, parvovirusa i virusa bolesti Aujeszkoga. Svaki od četiri para početnica bio je specifičan isključivo za umnožavanje specifičnog fragmenta određenog virusa. Osjetljivost višestrukog PCR-a upotrebom pročišćenih konstrukata plazmida koji sadrže specifične virusne fragmente bila je na razini $4{,}0\times10^3$ kopija za svinjski cirkovirus tip 2, $4{,}5\times10^3$ kopija za virus reprodukcijskog i respiratornog sindroma, $3{,}0\times10^2$ za svinjski parvovirus i $5{,}0\times10^2$ kopija za virus bolesti Aujeszkoga. Među 82 klinička uzorka često je dokazana koinfekcija svinjskim cirkovirusom tip 2 i virusom respiratornog i reprodukcijskog sindroma. U usporedbi s izolacijom virusa, višestruka lančana reakcija polimerazom pokazala se osjetljivijom i bržom te se kao takva može rabiti kao alternativna metoda za dokaz i razlikovanje uzročnika sindroma kržljavosti prasadi.

Ključne riječi: sindrom kržljavosti odbijene prasadi, svinjski cirkovirus 2, virus reprodukcijskog i respiratornog sindroma, svinjski parvovirus, virus bolesti Aujeszkoga, lančana reakcija polimerazom