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LETTER TO THE EDITOR

A touchdown multiplex PCR for porcine circovirus type 2 and pseudorabies virus

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Multiplex polymerase chain reaction (mPCR) is a variant of PCR in which more than one target sequence can be amplified by including more than one pair of primers in the same reaction (*8, 12*). In comparison with single PCR (sPCR), it uses less DNA template, reduces the extensive costs, time requirements, and allows for rapid identification. It is practically a reasonable choice for simultaneous identification and differentiation of multiple viruses or bacteria in a single sample on the basis of amplicon size (*4, 7, 11*). However, if the degree of complementarity between primers and template is uncertain the mPCR is not recommended. Touchdown PCR (TD-PCR) is another modified conventional PCR that may result in circumventing spurious priming during gene amplification (*2*). In this technique the first hybridization occurs between primers and genomic DNA sequences with the greatest complementarity, so it minimizes the number of spurious bands due to mispriming (*5*). Therefore it offers a simple and rapid means to optimize PCR, increases specificity, sensitivity, and yield, without need for lengthy optimizations and/or the redesigning of primers (*6*).

Under typical conditions of intensive pork meat production, it is common for swine to be simultaneously infected with two or more viral pathogens (*1*). Clinical diagnosis of swine viral diseases remains based on virus isolation, identification, and serological diagnostic methods, which are time-consuming and tedious. In addition, because it is customarily polyinfection, a definitive diagnosis of multiple infections is often difficult because of variable clinical signs. Thus, it often fails to provide a clear diagnosis and the most appropriate diagnostic test. Yue and Liu used mPCR and mRT-PCR in rapid and simultaneous detection of porcine viruses and both proved to be feasible methods for polyinfection (*9, 13*). Porcine circovirus type 2 (PCV2) and pseudorabies virus (PRV) are two of the primary pathogens causing reproductive and/or respiratory failure in pigs. They both cause common polyinfection in swine. In this study, we developed a novel technique of mPCR coupled with TD-PCR (TD-mPCR) to identify combination of two common swine DNA viruses, PCV2 and PRV.

PCV2 and PRV were isolated and identified from swine liver samples. We used PCV1-free PK-15 cells for the propagation and titration of PCV2 and PRV. Detailed procedure was carried out according to Ellis (*3*). In order to evaluate the efficiency and sensitivity of the TD-mPCR method, a total of 41 liver samples were collected from a local farm in Yangling, Shaanxi, China, during the year 2010. Genomic DNA was extracted from PCV2 and PRV virus strains according to the instruction of E.Z.N.A.® viral DNA kit (Omega). The extraction of genomic DNA from liver tissue samples was performed according to the method provided by manufacturer (Beyotime). Primers specific for PCV2 and PRV were designed

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Abbreviations: PCV1, PCV2 = porcine circovirus type 1 and 2; PRV = pseudorabies virus; mPCR = conventional multiplex PCR; sPCR = single PCR; TD-PCR = touchdown PCR

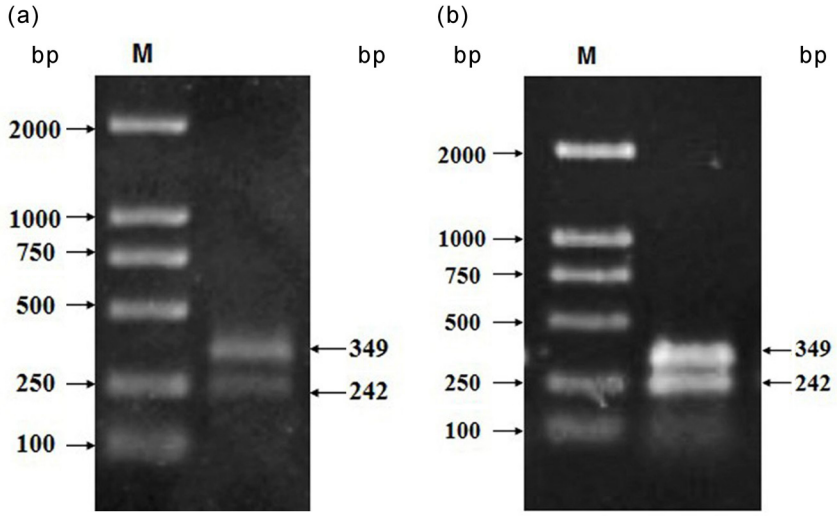

Fig. 1

mPCR and TD-mPCR of a mixture of PCV2 and PRV

Agarose gel electrophoresis of products after mPCR (a) and TD-mPCR (b) with mixture of PCV2 (242 bp) and PRV (349 bp). DNA size markers (lane M).

on the basis of ORF1 and gD region (Accession Number: FJ667596, FJ477296), respectively. The primers are as follows: PCV2: sense 5′-GCTGAACTTTTGAAAGTGAGCGGG-3′, antisense 5′-TCACACAGTCTCAGT AGATCATCCCA-3′; PRV: sense 5′-GCACCTGCTGTACTTTA TCG-3′, antisense 5′-CGTCAGGAATCGCATCAC-3′. We used 4 experimental techniques: sPCR, TD-sPCR, mPCR, and TD-mPCR. The total PCR reaction volume of 50 μl contained 25 μl 2×PCR of reaction mix (Majorbio), 2 μl of virus DNA (1 ng/μl), 2 μl of each primer, 1 μl of Taq polymerase and 18 μl of distilled water. In the sPCR and TD-sPCR, we used as template either PCV2 or PRV DNA, while in mPCR and TD-mPCR, we used equal volumes of both PCV2 and PRV DNA, respectively. In the sPCR we used following cycles: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C (PCV2) or 50°C (PRV) for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. For mPCR, the annealing temperature was fixed after optimization experiments. By contrast, following hot start in TD-sPCR and TD-mPCR, samples were subjected to 10 cycles in a touchdown program (94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec for 1 cycle, followed by a 1°C decrease of the annealing temperature every cycle). Thirty cycles were subsequently run (94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec) ending with a 10 min extension at 72°C .

To evaluate the specificity of the designed primers, the size-specific PCR products obtained from the sPCR were purified and sequenced. The sequencing results showed that the PCR products were exactly 242 bp and 349 bp in size, and had 97.7% and 100% of similarity with the sequences in GenBank respectively. After optimization in mPCR, 55°C was fixed as the optimal annealing temperature. The samples positive in sPCR could be detected perfectly by TD-sPCR, mPCR (Fig. 1a), and TD-mPCR (Fig. 1b). mPCR and TD-mPCR showed two bands in the same lane, and the target bands were brighter in TD-mPCR. The detection results of pig liver samples showed that 11 samples were positive for PCV2 (infection rate 26.83%) and non of the samples were positive for PRV (infection rate 0%) by conventional mPCR. However, by the TD-mPCR we obtained 13 positive samples for PCV2 (infection rate 31.71%) and 0 positive samples for PRV (infection rate 0%), suggesting that TD-mPCR increased the detection efficiency.

In general, mPCR provides a best tool for diagnosis of multiple infections which are often difficult to identify due to unclear clinical signs (*9, 13*). The development of an mPCR assay is not an easy task. In order to obtain the best overall strategy for amplification of more than one target sequence, optimization of a multiplex reaction requires compromises in: concentrations of reagents, annealing temperature, cycling conditions, and especially determining an agreeable annealing temperature (*10*). TD-PCR offers a simple and rapid means to optimize PCR, increasing specificity, sensitivity, and yield without the need for lengthy optimizations and/or the redesigning of primers (*6*). All samples positive in conventional mPCR could be detected perfectly by TD-mPCR using mixed primer pairs. In the clinical application, the introduction of touchdown

program significantly reduces the non-specific PCR bands, and the brightness of the target bands. The detection rate of PCV2 by TD-mPCR was higher than by conventional mPCR, which shows that the introduction of touchdown program to some extent improved the sensitivity of multiplex PCR. The establishment of TD-mPCR was of high pragmatic value and practical significance for simultaneous infection on clinical testing.

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