JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2015. Vol. **9**(Spl. Edn. 1), p. 171-176

A Simple Method for the Rapid Detection of Porcine Pseudorabies in Clinical

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(Received: 06 February 2015; accepted: 29 April 2015)

Pseudorabies (porcine pseudorabies) is an acute infectious disease, which caused by pseudorabies virus (PRV). Clinical features of infected pigs was temperature rises, newborn piglets nerve symptom and affects the digestive system. Adult pig often hidden infection, pregnant pigs can cause abortion, stillbirth and respiratory clinical symptom, no itching. The clinical symptom of boar was reproductive failure and respiratory. This disease may also occur in other domestic and wild animal. The virulence of PRV was coordinated control by several genes, mainly gE, gD, gI and TK genes. At present the main diagnosis methods of PRV are PCR, gE-ELISA, gG-ELISA, gC-ELISA, gE-LAT (latex agglutination test) and gG-LAT etc. PCR has the advantages of fast, sensitive, specificity, can simultaneously detect large quantities of samples, and can be suitable for in vivo detection, suitable for clinical diagnosis. In this study, a pair of primers were designed for specific amplificationaccording to the PRV virus genome databases.The optimal PCR reaction system was determined through optimizing concentration of primers and the template concentration and PCR amplification conditions.The best amplification conditions as follows: 10×Buffer 5 ¼l, $MgCl_2$ (15 mmol/L) 5 ¼l, dNTPs (2 mmol/L) 3 ¼l, the forword primer(2 ¼mol/L) 2 ¼l, reverse primer (2 ¼mol/L) 2 ¼l, Taq enzyme 0.3¼l (1.5U), DNA template 5 ¼l, add water to the 50 ¼l. PCR reaction conditions are as follows: 95! for 4 min, 35 cycles of 95! for 1 min, 65! for 1min and 72! for 1 min, and a final extension at 72! for 5 min. Electrophorese in 1% agarose and detect by ethidium bromide staining.This study provides a simple method for PRV clinical diagnosis, can be detected in 2 hours, can be used for clinical diagnosis and epidemiological survey of PRV.

Key words: Porcine pseudorabies, PCR, Molecular diagnosis.

Multiple animals can be infected by PRV, pigs of which are the most sensitive and most serious[1]. Sickness of pigs, carriers of PRV and mice carries of PRV are the important infection source of this disease. Health pig direct contact with the sickness of pigs or carries of PRV pigs can be infected with this disease[2]. The milk can be detected the virus after sows infected with this disease and lasted 3~5 d. Suckling pigs can be infected with this disease through the suckling[3]. Pregnant sows infected with this disease, can cause the virus invade fetal through vertical transmission. Infection of swine and piglets can be long-term carrier with virus[4,5].It is difficult to eradicate the long-term carrier with virus, which become an important cause of the disease epidemic. Piglets of the younger, the morbidity and mortality is higher, and decreased with age growth, after weaning piglets no morbidity but can be long-term carrier with virus[6]. The mortality up to 100% within 2 week suckling piglet. The mortality can be 40%~60% 3~4 week old pig. Above two monthpigscan occasionally cause death. PRV diagnosis methods are mainly PCR, gE-ELISA, gG-ELISA, gC-ELISA,

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gE-LAT (latex agglutination test), gG-LAT[7-10]. PCR has the advantages of fast, sensitive and strong specificity, can simultaneously detect large quantities of samples, can be suitable for in vivo detection, clinical diagnosis. This study conducted on the PRV-PCR reaction system and condition was optimized, which provides an effective method for molecular diagnosis of PRV in clinical diagnosis and molecular epidemiological survey.

MATERIALS AND METHODS

Reagents

Taq DNA polymerase, 10×Buffer, DL2000 bp DNA Ladder, dNTPs, $MgCl_2$, DEPC-treated ddH_2O were purchased from Dalian TaKaRa.

Sample collection and processing

Aseptic collection sick animal heart, liver, spleen, lung, kidney, brain, lymph nodes in the groin, and small intestine contents and blood, repeated freezing and thawing grinding centrifugal, the supernatant was placed at -20! saving or immediate extraction DNA.

Primers design

According to the PRV virus genome database sequences of gD gene nucleotide sequence specific amplification, a pair of primers were designed, Foword primer: 5'-CACGGAGGA CGAGCTGGGGCT-3', Reverse primer 3'-GTCCACGCCCCGCTTGAAGCT-5'. The expected PCR fragment was 220 BP, primer were by Shanghai Sangon Biotech. The primer were diluted to 25 pmol/µl.

Virus nucleic acid extraction

According to the genomic DNA Extraction Kit (LifeFeng Cat# DK622-01) extracted DNA from tissues, according to the following method:

1 Homogenize sample using a sonicator rotor-stator homogenizer, and 50 µl supernatant to clean 1.5 ml centrifuge tube.

2 Add 150µl Buffer CS, vortex the sample to mix.

3. Add 5µl Proteinase K and 300 µl Buffer CL, vortex 10 seconds or violent shaking 20 mixed evenly, for10 min at 70!.

4. Add 230µlabsolute alcohol, mixed uniformly moderate turnover, to avoid a lot of bubbles, a brief centrifugation, removal of the liquid on the cover.

5 Pipette the step 4 solution to DNA adsorption column -C30 (placed in the collection tube), at room temperature for 2 min.

6 12000 centrifugal 1min, abandoned the collection tube, DNA column -C30 put into a clean collection tube.

7 Add 500 µl buffer WAG to the DNA adsorbent column -C30, at room temperature for 2 min, 12000 centrifugal 1min, abandoned the collection tube, put the adsorption of DNA column -C30 into a clean collection tube.

8 Add 500 µl buffer WB1 to the adsorbent column -C30, 12000 centrifugal 1 min, abandoned wastewater, put the adsorption of DNA column -C30 in the recovery tube.

9 Repeat step 8.

10 12000 centrifugal 2 min.

11 Take the DNA adsorption column -B into 1.5 ml centrifuge tube, with 50 µl deionized water to the silicone rubber mould center (pH=7). At room temperature for 2 min, 12000 centrifugal for 1 min. The DNA were then resuspended in deionized water.

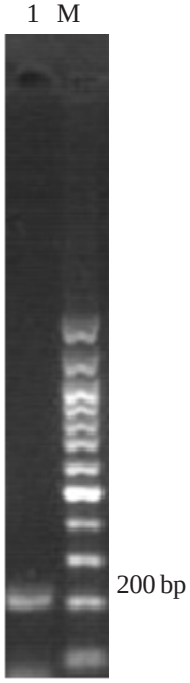

Fig. 1. PRV-PCR

PCR amplification

The extracted tissue DNA as template, the PCR reaction system was 50 µl: 10×Buffer 5.0 µl, $MgCl_2$(15 mmol/L), dNTPs (2 mmol/L) 3.0 µl, upstream primer (2 µ mol/L) 2.0 µl, downstream primer (2 µ mol/L) 2.0 µl, Taq enzyme 0.3 µl (1.5U), DNA template 5 µl, add water to the volume of 50 µl. PCR reaction conditions are as follows: 95! for 4 min, 35 cycles of 95! for 1 min, 65! for 1min and 72! for 1 min, and a final extension at 72! for 5 min. Electrophorese in 1% agarose and detect by ethidium bromide staining.

PCR specific test

Collect clinical samples disease of blood, brain, liver, kidney, lung, molecular diagnosis according to the above method.

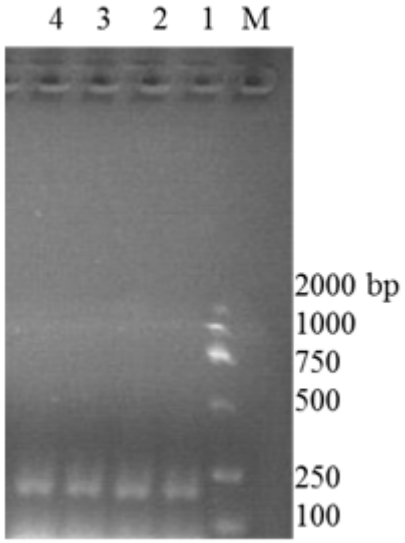

Fig. 2. PRV-PCR

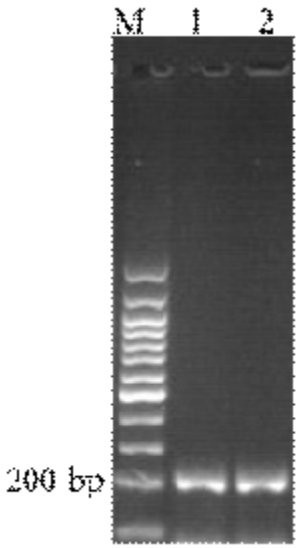

Fig. 3. PRV-PCR

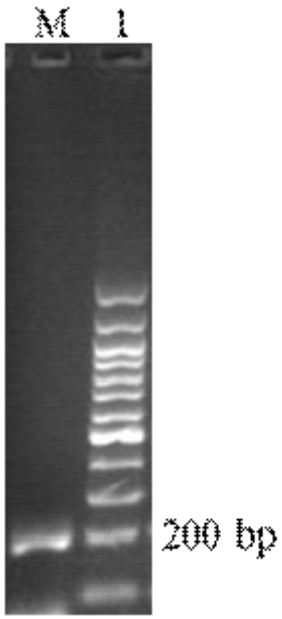

Fig. 4. PRV-PCR

Fig. 5. PRV-PCR

Sequence analysis of PCR product

RT-PCR product was gel purified, then send to TaKaRa Biological Engineering Co., Ltd for sequencing.The sequences obtained were compared with the NCBI database.

Results and analysis

Detection of PCR products

The PCR product electrophoresis results see Figure 1. The Marker was 100 bp, from top to the bottom as follows, 1500 bp, 1200 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp. 1 was the amplification products extracted from brain tissue template.

As you can see from Figure 1, the PRV template RT-PCR amplified fragment of approximately 220 bp, which prove that the PRV amplified fragment was the purpose fragment, consistent with the expected size of the design.

PCR specific test

It can be seen from the figures, samples from clinically collected, according to the above method, all the samples could amplify the fragment with the expected, which showed good specificity. Figure 2 was the blood sample, figure 3 for liver, kidney and spleen of mixed samples, figure 4 was tonsil samples, figure 5 for intestinal contents of samples.

Analysis of the PCR product sequence

According to the conventional method, the PCR product was cloned into pMD18-T vector, the recombinant plasmid was sequenced at TaKaRa Biological Engineering Co., Ltd. The sequence was 99% homology compared with the data obtained with the PRV sequence in the NCBI.

DISCUSSION

A variety of domestic and wild animal can be infected by PRV, which is an acute infectious disease[11]. Different day old pigs can be infected. Pregnancy can cause miscarriage, stillbirth, mummy after infection[12]. Gilts and barren sow can cause infertility, slow or not oestrus, return rate as high as 90% after infection. The boar with reproductive failure and respiratory manifestations of clinical symptom, mainly for testicular swelling, atrophy[13-16].Within 2 week old suckling piglets after infection, the mortality was 100%, 3~4 week old pig disease death rate up to 40%~60%. The main pathological changes was kidney, lung, gastric mucosa, brain tissue, have pinpoint bleeding point, meningeal congestion, hemorrhage and edema.Tonsil, liver and spleen have scattered white necrotic foci.

PRV is a kind of frequent disease of respiratory and nervous system in pigs, swine upon the occurrence of the disease[17]. It is difficult to eradicate and purification. Disease and infected pigs are the major source of infection, the main route of transmission is through the nose and mouth. The clinical cases often solitary or with porcine transmissible gastroenteritis virus, porcine epidemic diarrhea mixed infection, sometimes with porcine circovirus infection, classical swine fever or with bacterial infection[18]. There is no good treatment and effective drug can be use, in case of emergency available hyperimmune serum treatment, can reduce the mortality rate. PRV is difficult to purify, regularly pathogens detection and serum neutralization test on pigs.Isolation and elimination of positive pigs detected, reexamination after interval of 3~4 weeks, until two times tests are negative, establishment of disease-free pigs.

The main measures to prevent and control this disease is vaccination. At present, the main vaccine in the market was porcine pseudorabies attenuated vaccine, inactivated vaccine, attenuated wild virus inactivated vaccine and gene deletion vaccine. Gene deletion vaccine have the good effect. It was application in many endemic areas.It can effectively relieve the clinical symptom after infection pigs, greatly reduce the incidence of this disease, reduce economic losses, but cannot eliminate the disease only by vaccination. The clinical application of swine vaccine should be used carefully. The research proved that, gene deletion vaccine can be occurrence of genetic recombination phenomena. So only the use of a gene deletion vaccine in the same animal body, in order to avoid recombination between vaccine strains[19-21]. To prevent the disease is to do the work of feeding and management. Isolation and disinfection when found this disease, reduce the flow of personnel, with all in and all out system and other measures to prevent and control.

This study established PRV-PCR molecular diagnosis method, has good specificity. This study provides a simple method for the rapid detection of PRV in clinical, and laid the foundation for PRV epidemiology and molecular diagnosis.

ACKNOWLEDGMENTS

This work has been supported by Henan academy of agricultural sciences outstanding youth fund of science and technology (Project number: 2013YQ22) and the Doctoral Starting up Foundation of Henan Academy of Agricultural Sciences and.

REFERENCES

1. Mahjoub N, Dhorne-Pollet S, Fuchs W, Endale Ahanda ML, Lange E, Klupp B, Arya A, Loveland JE, Lefevre F, Mettenleiter TC et al: A 2.5-Kilobase Deletion Containing a Cluster of Nine MicroRNAs in the Latency-Associated-Transcript Locus of the Pseudorabies Virus Affects the Host Response of Porcine Trigeminal Ganglia during Established Latency. *J Virol* 2015; **89**(1):428-442.
2. Chang HT, Liu HM, Guo ZD, Du JM, Zhao J, Chen L, Yang X, Wang XW, Yao HX, Wang CQ: [Investigation of etiology of massive infection with porcine pseudorabies virus in Henan and neighboring Provinces]. *Bing Du Xue Bao* 2014; **30**(4): 441-449.
3. Caruso C, Gobbi E, Biosa T, Andra M, Cavallazzi U, Masoero L: Evaluation of viral inactivation of pseudorabies virus, encephalomyocarditis virus, bovine viral diarrhea virus and porcine parvovirus in pancreatin of porcine origin. *J Virol Methods* 2014; **208**: 79-84.
4. Hao X, Shen Z, Wang J, Zhang Q, Li B, Wang C, Cao W: In vitro inactivation of porcine reproductive and respiratory syndrome virus and pseudorabies virus by slightly acidic electrolyzed water. *Vet J* 2013; **197**(2): 297-301.
5. Yang X, Forier K, Steukers L, Van Vlierberghe S, Dubruel P, Braeckmans K, Glorieux S, Nauwynck HJ: Immobilization of pseudorabies virus in porcine tracheal respiratory mucus revealed by single particle tracking. *PLoS One* 2012; **7**(12):e51054.
6. Faurez F, Grasland B, Beven V, Cariolet R, Keranflec'h A, Henry A, Jestin A, Dory D: The protective immune response against Pseudorabies virus induced by DNA vaccination is impaired if the plasmid harbors a functional Porcine circovirus type 2 rep and origin of replication. *Antiviral Res* 2012; **96**(3): 271-279.
7. Liu XQ, Xu K, Zhang ZY: A touchdown multiplex PCR for porcine circovirus type 2 and pseudorabies virus. *Acta Virol* 2012; **56**(2): 163-165.
8. Sandfoss MR, DePerno CS, Betsill CW, Palamar MB, Erickson G, Kennedy-Stoskopf S: A serosurvey for Brucella suis, classical swine fever virus, porcine circovirus type 2, and pseudorabies virus in feral swine (Sus scrofa) of eastern North Carolina. *J Wildl Dis* 2012; **48**(2): 462-466.
9. Wu YQ, Chen DJ, He HB, Chen DS, Chen LL, Chen HC, Liu ZF: Pseudorabies virus infected porcine epithelial cell line generates a diverse set of host microRNAs and a special cluster of viral microRNAs. *PLoS One* 2012; **7**(1):e30988.
10. Perez LJ, Perera CL, Frias MT, Nunez JI, Ganges L, de Arce HD: A multiple SYBR Green I-based real-time PCR system for the simultaneous detection of porcine circovirus type 2, porcine parvovirus, pseudorabies virus and Torque teno sus virus 1 and 2 in pigs. *J Virol Methods* 2012; **179**(1): 233-241.
11. Glorieux S, Favoreel HW, Steukers L, Vandekerckhove AP, Nauwynck HJ: A trypsin-like serine protease is involved in pseudorabies virus invasion through the basement membrane barrier of porcine nasal respiratory mucosa. *Vet Res* 2011; **42**: 58.
12. Yue F, Cui S, Zhang C, Yoon KJ: A multiplex PCR for rapid and simultaneous detection of porcine circovirus type 2, porcine parvovirus, porcine pseudorabies virus, and porcine reproductive and respiratory syndrome virus in clinical specimens. *Virus Genes* 2009; **38**(3): 392-397.
13. Tomioka Y, Morimatsu M, Amagai K, Kuramochi M, Watanabe Y, Kouda S, Wada T, Kuboki N, Ono E: Fusion protein consisting of the first immunoglobulin-like domain of porcine nectin-1 and Fc portion of human IgG1 provides a marked resistance against pseudorabies virus infection to transgenic mice. *Microbiol Immunol* 2009; **53**(1): 8-15.
14. Glorieux S, Favoreel HW, Meesen G, de Vos W, Van den Broeck W, Nauwynck HJ: Different replication characteristics of historical pseudorabies virus strains in porcine respiratory nasal mucosa explants. *Vet Microbiol* 2009; **136**(3-4): 341-346.
15. Flori L, Rogel-Gaillard C, Mariani V, Lemonnier G, Cochet M, Hugot K, Chardon P, Robin S, Lefevre F: A combined transcriptomic approach to analyse the dialogue between pseudorabies virus and porcine cells. *Dev Biol* (Basel) 2008; **132**: 99-104.
16. Yao Q, Qian P, Cao Y, He Y, Si Y, Xu Z, Chen H: Synergistic inhibition of pseudorabies virus replication by porcine alpha/beta interferon and

gamma interferon in vitro. *Eur Cytokine Netw* 2007; **18**(2): 71-77.
17. Mateusen B, Van Soom A, Maes DG, Favoreel H, Nauwynck HJ: Receptor-determined susceptibility of preimplantation embryos to pseudorabies virus and porcine reproductive and respiratory syndrome virus. *Biol Reprod* 2007; **76**(3): 415-423.
18. Lee CS, Moon HJ, Yang JS, Park SJ, Song DS, Kang BK, Park BK: Multiplex PCR for the simultaneous detection of pseudorabies virus, porcine cytomegalovirus, and porcine circovirus in pigs. *J Virol Methods* 2007; **139**(1): 39-43.
19. Jiang Y, Fang L, Xiao S, Zhang H, Pan Y, Luo R, Li B, Chen H: Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. *Vaccine* 2007; **25**(3): 547-560.
20. Ono E, Tomioka Y, Watanabe Y, Amagai K, Taharaguchi S, Glenisson J, Cherel P: The first immunoglobulin-like domain of porcine nectin-1 is sufficient to confer resistance to pseudorabies virus infection in transgenic mice. *Arch Virol* 2006; **151**(9): 1827-1839.
21. Ono E, Tomioka Y, Taharaguchi S, Cherel P: Comparison of protection levels against pseudorabies virus infection of transgenic mice expressing a soluble form of porcine nectin-1/HveC and vaccinated mice. *Vet Microbiol* 2006; **114**(3-4): 327-330.