Differential diagnosis of CSFV wild strain and vaccine strain by the Fluorescent Quantitative RT-PCR Technique

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Abstract. The purpose of this study was to establish a rapid method for distinguishing the virulent strains of CSFV from vaccine strains. In GeneBank, sequence alignment was performed using DNAstar's Meglign. A specific amplification primer was designed according to the independent T rich insertion sequence in 3'-UTR of CSFV vaccine strain. The fluorescent quantitative RT-PCR technique showed a high specificity and sensitivity. The CSFV Shimen strain, CSFV live vaccine cell line origin, CSFV Thiverval strain and CSFV live vaccine spleen-lymph tissue-origin showed specific band, while the other BVDV C24V strain, PRRSV vaccine Tianjin strain, JEV vaccine 14-14-2 strain, and PEDV-TGEV-PoRV live vaccine strains showed no specific bands. The length of PCR product for CSFV wild strain and vaccine strain are 99 bp and 115-140 bp, respectively. The 10⁵ TCID50 of the vaccine was diluted into 10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶. The maximum times of dilution could be detected by the real-time RT-PCR was 10⁻⁶. So, the sensitivity reached up to 0.1 TCID50. The fluorescent quantitative RT-PCR technique was set up successfully for differential diagnosis of CSFV wild strain and vaccine strain, which is prerequisite for eradication of CSF in swine farm.

1. Introduction

Classical swine fever (CSF) is a highly contagious and lethal viral disease caused by classical swine fever virus (CSFV) [1]. CSFV infection is a great threat to pig husbandry, and the immunosuppression and immune evasion features of CSF often lead to a persistent infection of CSFV, causing leukocytopenia, thrombosis and endothelial damage. The virus can also cross the placental barrier to infect the fetus, and widespread reproductive failure in pregnant sows including mummified, stillborn and aborted fetuses. The clinical symptoms of the infected swine are classified into acute, subacute and chronic [2]. Although the application of HCLV vaccine [3] reduce the spread of CSFV [4], only means of vaccination is not enough to effectively inhibit the spread of CSFV [5]. The differential diagnosis of CSFV wild strain and vaccine strain is prerequisite for eradication of CSF in swine farm.

There are many methods of detecting wild-type strains of CSFV, such as virus isolation [6], direct immunofluorescence assay [7], ELISA [8] and RT-PCR method [9, 10]. Among them, virus isolation requires special equipment and professional technology, which takes a long time. When the CSFV proliferates in cells, there are fewer antigens exposed to the surface of infected cells, and it is difficult to detect antigens by immunological methods. In the case of fluorescence experiments, it is not easy to set standards; In addition, the attenuated vaccine strain of CSF can persist in the inoculated swines for
about 48 days [11], which will interfere with the detection of CSFV wild strains in laboratory. With the development of molecular biology, the quantitative PCR technology has the advantages of high sensitivity [12], strong specificity, accurate quantitative and convenient detection in quantitative detection [13], and the fluorescent quantitative reverse transcription polymerase chain reaction assay is one of the most popular techniques [14]. This study designed specific primer based on the highly conserved 3′ non-coding region of CSFV, to provide a method of the differential diagnosis of CSFV virulent strains and vaccine strains by using fluorescence quantitative RT-PCR technique.

2. Materials and Methods

2.1 Viruses and Materials

CSFV live vaccine cell line origin, CSFV live vaccine spleen-lymph tissue-origin, CSFV Thiverval strain, CSFV Shimen strain, Bovine Viral Diarrhea Virus (BVDV) C24V strain, Porcine Reproductive Respiratory Syndrome Virus (PRRSV) live vaccine Tianjin Strain, Japanese Encephalitis Virus (JEV) live vaccine 14-14-2 strain, Porcine Epidemic Diarrhea Virus, Transmissible Gastroenteritis of Swine Virus, Porcine Rotavirus (PEDV-TGEV-PoRV) live vaccine strains.

2.2 Main Reagents

gDNA Eraser, SYBRgreenII kit, PrimeScript™ 1st Strand cDNA Synthesis Kit, RoxII (50×), DL500 Marker, Loading buffer: Takara® (Dalian); DEPC: Sangon Biotech (Shanghai); 4S Red Plus Nucleic Acid Stain: Sangon Biotech (Dalian); Column Viral RNAOUT: TIANDZ.

2.3 Primer Design and Synthesis

Sequence alignment using DNAstar's Meglign in GeneBank, and a T-rich insert was independently generated based on the HCLV 3′-UTR, and specific amplification primers were designed. The primer sequences are as follows:

3′-UTR For: 5′- GACCCTATTGTAGATAACAC-3′
3′-UTR Rev: 5′- GAGGTAGTTTATACCAGTTC-3′

2.4 Methods

2.4.1 Extraction of RNA. The viral RNA in the sample was extracted using the Column Viral RNAOUT. The cDNA was stored at -20 °C for use.

2.4.2 Reverse transcription synthesis of cDNA. Using the total RNA extracted in the above procedure as a template, the total RNA is reverse-transcribed according to the user manual (PrimeScript™ 1st Strand cDNA Synthesis Kit). Shortly, prepare a mixed system in a microtube tube, take 5 μL of total RNA extracted in the above steps as a template, add 1 μL of gDNA Eraser, 2 μL of 5×gDNA Eraser Buffer, 2 μL of RNase Free·dH2O, total volume of reaction 10 μL, the reaction condition is 42 °C for 2 min, 4 °C for 5 min; And then, add 5×primer script Buffer 4 μL, Primer script Enzyme mix 1 μL, RT·primer mix 1 μL, RNase Free·dH2O 4 μL. A reaction system with a total volume of 10 μL was prepared, reacted at 37 °C for 15 min, reacted at 85 °C for 5 s, and incubated at 4 °C for 5 min.

2.4.3 Real-time fluorescence quantitative PCR amplification. Reaction system: The cDNA obtained by reverse transcription in the above step was used as a template, a distinguishable specific primer was added, and a TARAKA kit was used for real-time PCR amplification on a PCR machine. A PCR amplification mix was prepared for a microtone tube, including the following components: Primer F (10 μM), 1 μL, Primer R (10 μM) 1 μL, RoxII (50×) 0.5 μL, cDNA 2 μL, dH2O 8 μL, SYBRgreen II 12.5 μL. The total volume of the PCR reaction was 25 μL. The reaction conditions were: 95 °C for 3 min, 95 °C for 30 s, 53 °C for 20 s, 72 °C for 50 s, 40 cycles, the last cycle of 72 °C for 5 min, and finally 4 °C.

2.4.4 Prepare agarose gel (1.5%) for electrophoresis. Use 1× TAE as gel running buffer, use DNAgreen to visualize nucleic acids, and use a 500-bp ladder as size standard.

2.4.5 The specificity of Real-time quantitative RT-PCR for the detection of CSFV. CSFV live vaccine
cell line origin, CSFV live vaccine spleen-lymph tissue-origin, CSFV Thiverval Strain, CSFV Shimen Strain, BVDV C24V strain, PRRSV live vaccine Tianjin strain, JEV live vaccine 14-14-2 strain and PEDV-TGEV-PoRV live vaccine strains were used as a template for specific fluorescent RT-PCR reaction of CSFV, and the specificity of the primer was determined by the amplification curve.

2.4.6 The sensitivity of Real-time quantitative RT-PCR detection of CSFV. The TCID50 of the vaccine virus was determined by virus neutralization test, and the vaccine was diluted 10-fold (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) from the diluted sample. RNA was extracted and then subjected to real-time PCR, and the maximum dilution factor of the amplification curve, that is, the sensitivity of the fluorescent quantitative PCR, was detected on a fluorescent quantitative PCR instrument.

3. Results

3.1 Specificity
As shown in Fig. 1, CSFV Shimen strain, CSFV live vaccine cell origin, CSFV Thiverval strain, CSFV live vaccine spleen-lymph tissue-origin. Other BVDV C24V strain, PRRSV live vaccine TJ strain, JEV live vaccine 14-14-2 strain, PEDV-TGEV-PoRV live vaccine strain were used to verify the specificity of the real-time quantitative RT-PCR detection of CSFV. The result showed good specificity, only CSFV have amplification curves and the others strain, like BVDV C24V strain and PRRSV live vaccine TJ strain, JEV live vaccine 14-14-2 strain and PEDV-TGEV-PoRV live vaccine strains have not. So, the goal of distinguishing between CSFV and other virus, like PRRSV, JEV, BVDV, PEDV, TGEV, PoRV was reached. Furthermore, we can distinguish CSFV wild strain from the vaccine strain, as shown in Fig. 2, the fragment size of the CSFV wild strain was 99 bp, and the vaccine strains were between 115 bp and 140 bp.

Figure 1. The amplification curve of real-time quantitative RT-PCR detection of CSFV. From left to right, CSFV Shimen strain, CSFV live vaccine cell origin, CSFV Thiverval strain, CSFV live vaccine spleen-lymph tissue-origin. Other virus strain showed no amplification curve, including BVDV C24V strain, PRRSV live vaccine TJ strain, JEV live vaccine 14-14-2 strain and PEDV-TGEV-PoRV live vaccine strain.

Figure 2. The fragment size of the product of real-time quantitative RT-PCR detection of CSFV detected by electrophoresis. M:DL500 Maker, 1: PBS (Negative control), 2: DEPC (Blank control), 3-6: CSFV live vaccine, 7: CSFV Shimen strain, 8: BVDV C24V strain, 9: PRRSV live vaccine (TJ strain), 10: JEV Vaccine (14-14-2 strain), 11: PEDV-TGEV-PoRV live vaccine strain.

3.2 Sensitivity
The sensitivity of the fluorescent quantitative PCR detection was performed according to the method of 2.4.6. The 10^5 TCID50 of the vaccine was diluted into 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The maximum times of dilution could be detected by the real-time RT-PCR was 10^6. So, the sensitivity reached up to 0.1 TCID50.
4. Discussion

In recent years, the epidemic and pathogenesis of CSFV infection in China has undergone great changes, and the clinical diagnosis has become more complicated. The pathological necropsy is often different from the typical classic swine fever, which brings great difficulties to the veterinarian's clinical diagnosis. Also in laboratory diagnosis, the large-scale application of HCLV also makes it difficult to distinguish between CSFV wild-infected pigs and vaccinated pigs.

Currently, the diagnosis of CSFV infection in the laboratory mainly relies on the immunofluorescent antibody technique to detect antigen and ELISA technique to detect antibody, which cannot differentiate the CSFV wild strain infection from vaccinated pigs. If swine are also infected with BVDV [15], which exist cross antigen with HCV antigens, the false positive will interfere with the specificity of immunological detection.

In recent years, the real-time fluorescent quantitative PCR (FQ-PCR) technology has been widely used in the analysis of mRNA expression for its rapid, sensitive and specific effects. Studies have shown that the sensitivity of fluorescence quantitative RT-PCR is 100 times higher than that of RT-PCR, and the sensitivity is near to a reverse transcription-multiplex nested polymerase chain reaction (RT-nPCR). The specificity can reach 100% [16]. RT-nPCR may be considered to set up for identifying CSFV virulent and attenuated strains, but the RT-nPCR technique is time-consuming and easy to get contaminated.

In this study, according to T-rich insert in the 3'-UTR of the CSFV attenuated vaccine strain, the specific amplification primer was designed. The SYBRgreen fluorescence quantitative PCR method can specifically distinguish CSFV virulent strains and vaccine strains. Also, the specificity and sensitivity of the method were verified, the results showed no cross-amplification reactions to the BVDV C24V strain, the PRRSV live vaccine TJ strain, the JEV live vaccine 14-14-2 strain, and the PEDV-TGEV-PoRV live vaccine strain. Sensitivity detection can reach up to 0.1 TCID50. FQ-PCR production is simple and economical and can be used to monitor a large number of pigs with high sensitivity and specificity. According to the amplification curve and melting curve of CSFV real-time fluorescent quantitative PCR and the electrophoresis results of PCR products, we can identify the sample detected is from CSFV vaccinated or wild strain infected. Thus, CSFV wild-infected pigs were quickly found out from immunized ones, and to remove the infected pigs timely is critical to prevent the spread of the epidemic.

5. Conclusion

The fluorescent quantitative RT-PCR technique showed high specificity and sensitivity. The CSFV wild strain and vaccine strain showed a specific band, while the other virus showed no specific bands. The fragment size of PCR product for CSFV wild strain and vaccine strain are 99 bp and 115-140 bp, respectively. The sensitivity of the fluorescent quantitative RT-PCR technique for detection of CSFV reached up to 0.1 TCID50. The fluorescent quantitative RT-PCR technique was set up successfully for differential diagnosis of CSFV wild strain and vaccine strain, which is prerequisite for eradication of CSF in swine farm.
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References

[1] W. He, H. Xu, H. Go. CSFV Infection Up-Regulates the Unfolded Protein Response to Promote Its Replication, J. Frontiers in microbiology, 8 (2017) 21-29.
[2] G.Y. Wen, T. Zhang, J. Yang. Evaluation of a real-time RT-PCR assay using minor groove binding probe for specific detection of Chinese wild-type classical swine fever virus, J. Journal of Virological Methods, 176 (2011) 96-102.
[3] X. J. Zhang, Q. Y. Han, Y. Sun. Development of a loop-mediated isothermal amplification for visual detection of the HCLV vaccine against classical swine in China, J. Journal of virological methods, 171 (2010) 5-200.
[4] F. Hao, D. Y. Tang, Z. Y. Zeng. Study on the epidemic variation of swine fever virus gene in China, J. China Swine Industry, 8 (2013) 42-44.
[5] X. J. Zhang, Q. Y. Han, Y. Sun. Development of a triplex TaqMan real-time RT-PCR assay for differential detection of wild-type and HCLV vaccine strains of classical swine fever virus and bovine viral diarrhea virus 1, J. Research in Veterinary Science, 92 (2012) 512-518.
[6] N. Kumar, S. Barua, T. Riyesh. Complexities in Isolation and Purification of Multiple Viruses from Mixed Viral Infections_ Viral Interference, Persistence and Exclusion, J. PloS one, 11 (2016) e0156110.
[7] A.C. Caviness, L. L. Oelze, U. E. Saz. Direct immunofluorescence assay compared to cell culture for the diagnosis of mucocutaneous herpes simplex virus infections in children, J. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology, 49 (2010) 58-60.
[8] Y. Panyasing, R. Thanawongnuwech, J. Ji. Detection of classical swine fever virus (CSFV) E2 and E antibody (IgG, IgA) in oral fluid specimens from inoculated (ALD strain) or vaccinated (LOM strain) pigs, J. Elsevier B.V. 224 (2018) 70-77.
[9] B. Hoffmann, S. Blome, P. Bonilauri. Classical swine fever virus detection_results of a real-time reverse transcription polymerase chain reaction ring trial conducted in the framework of the European network of excellence for epizootic, J. Journal of veterinary diagnostic investigation: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 23 (2011) 999-1004.
[10] E. M. Wagner. Monitoring Gene Expression: Quantitative Real-time Rt-pcr, M. [S.l.]: Humana Press, 2013.
[11] W. H. Yao, X.Z. Fan, L. Xu. Detection of antigen after vaccination of piglets that were infected with classical swine fever virus (CSFV), J. Chinese Journal of Veterinary Medicine, 47 (2011) 6-8.
[12] J. P. Levesque-Sergerie, M. Duquette, C. Thibault. Detection limits of several commercial reverse transcriptase enzymes impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR, J. BMC Molecular Biology, 8 (2007) 93.
[13] J. Dewulf, F. Koenen, K. Mintsiens. Analytical performance of several classical swine fever laboratory diagnostic techniques on live animals for detection of infection, J. Journal of virological methods, 119 (2004) 43-137.
[14] Y. L. Huang, V. F. Pang, C. H. Pan. Development of a reverse transcription multiplex real-time PCR for thedetection and genotyping of Classical swine fever virus, J. Journal of Virological Methods, 160 (2009) 111-118.
[15] M. Ammari, F. M. McCarthy, B. Nanduri. Analysis of Bovine Viral Diarrhea Viruses-infected monocytes_identification of cytopathic and non-cytopathic biotype differences, J. BMC bioinformatics, 11 (2010) Suppl 6: S9.
[16] B Hoffmann, M Beer, C Schelp, et al. Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever, J. Journal of virological methods, 130 (2005) 36-44.