Development and application of real-time TaqMan RT-PCR assay for improved detection of classical swine fever virus in slaughtered pigs

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ABSTRACT

Classical swine fever (CSF) is an economically devastating disease of pigs. Instrumental to the control of CSF is a well-characterized sensitive assay that can deliver a rapid and accurate diagnosis before the onset of clinical signs. With this objective, a real-time fluorogenic-probe hydrolysis (TaqMan) reverse transcription-polymerase chain reaction (RT-PCR) assay was developed for rapid and specific detection of classical swine fever virus (CSFV) and applied on samples derived from infected slaughtered pigs. A pair of PCR primers targeting 5'-non-coding region (CSFL1 and CSFR1) in conjunction with a CSFV-specific fluorogenic probe (CSFP1) was designed and assessed in real-time PCR. During PCR, when the target of interest was present, the CSFV specific FAM-labeled TaqMan probe annealed to the amplicon between the forward and reverse primers and was subsequently cleaved via the 5'-3' exonuclease activity of the DNA polymerase resulting in the release of the fluorescent reporter dye. This assay was found to be rapid and strain-specific for CSFV detection.

Key words: Classical swine fever virus, Pig, Real-time TaqMan RT-PCR assay.

INTRODUCTION

Classical swine fever virus (CSFV), belonging to the genus Pestivirus in the Flaviviridae family, is the causative agent of ‘classical swine fever’ (CSF), or ‘hog cholera’, a deadly menace causing serious economic losses to the pig industry (Leifer et al., 2010). Clinical signs of CSF may remain undiagnosed, particularly during infections with CSFV strains of low virulence (Terpstra, 1991). Moreover, the gross lesions observed at necropsy are diverse and often not pathognomonic (Moennig and Plagemann, 1992; De Smit et al., 1999). As the disease is notifiable due to the high mortality rates associated with its acute forms, rapid and precise detection of the virus becomes very crucial for disease containment. Current diagnostic methods including demonstration of viral antigens in tonsils using fluorescent conjugated antibodies (Ressang and de Boer, 1968) or antigen capture enzyme-linked immunosorbent assay (Shannon et al., 1993; Clavijo et al., 1998) and detection of genomic RNA by reverse transcription-polymerase chain reaction (RT-PCR) (Wirtz et al., 1993; Harding et al., 1994), are relatively rapid. However, these techniques require centralized sophisticated laboratory facilities and clinical specimen submissions that might delay the disease diagnosis, thereby affecting the efficiency of emergency disease management measures. A rapid, presumptive diagnosis at the site of suspected disease outbreak would rather be very useful for controlling CSF. Real-time PCR is reportedly an accurate, rapid and reliable method that can be used for the detection and quantification of specific DNA molecules. The basic principle lies on recurring estimation of fluorescent signal that is proportional to the amount of amplification product. In this study, a fluorogenic-probe hydrolysis (TaqMan) RT-PCR assay for rapid and strain-specific detection of CSFV was developed and applied on the samples collected from infected slaughtered pigs.

MATERIALS AND METHODS

Sample collection: Tissue specimens were collected randomly from 1120 pigs during 2005 - 2006 from a local abattoir at Prem Nagar, Bareilly, Uttar Pradesh. The carcasses were examined for CSF suggestive pathological lesions and lymphoid tissues were collected from mesenteric lymph nodes, spleen and tonsil. Acute, chronic and inapparent forms of CSF were observed based on standard gross lesions as described by Van Oirschot (1999) and Moennig et al. (2003). Of 1120 cases examined, 110 (9.82%) cases showed lesions suggestive for CSFV, 110 (9.82%) cases showed lesions suggestive for CSF. Based on pathological findings, 58.18% (64/110) cases were categorized as acute CSF, 16.36% (18/110) as chronic and 25.45% (28/110) as inapparent form of CSF. As the TaqMan RT-PCR assay aids in strain-specific detection of CSFV, only two typical cases from each of the three categories of CSF (with an assumption that the three
different clinical forms of CSF might have been caused by different strains of the virus) were selected at random and tested in this assay.

RNA extraction and reverse transcription: Representative tissues of spleen from two typical cases each of acute, chronic and inapparent CSF were selected. Total RNA was extracted from spleen using TRIZOL reagent (Invitrogen, USA) as per manufacturer’s recommendation. RNA was eluted with 30 μl of RNase-free water and after spectrophotometric quantification, reverse transcription was carried out in a 20 μl reaction volume using 2 μg of total RNA, 10 pmol specific reverse primer CSFR1 and 200 units of M-MLV RT-RT (Invitrogen, USA) following the manufacturer’s instruction.

Designing of oligonucleotide primers and TaqMan probe: CSFV nucleotide sequences available in GenBank were aligned by using BioEdit sequence alignment software. Specific oligonucleotide primers and the TaqMan probe were designed to target a highly conserved region within the 5' untranslated region (UTR) of the CSFV genome. The oligonucleotide primers targeting 5'UTR (CSFL1-sense 5'-TGGTTGGTCTAAGTCCTGAGT-3'; CSFR1-antisense 5'-GTGTGATTTCACCCTAGCGA-3') and TaqMan probe (CSFP1- 5'-FAM-ACGCGACGAGAAGCCCACCT - TAMRA- 3') used in the study were designed and custom synthesized (Hysel, India Pvt. Ltd). The TaqMan probe was labeled with a reporter dye, 6-carboxyfluorescein (FAM) at the 5' end and with non-fluorescent quencher dye, 6-carboxy-N,N,N',N' -tetr methylrhodamine (TAMRA) at the 3' end.

TaqMan RT-PCR assay: The assay was carried out with real-time TaqMan RT-PCR assay kit (Eppendorf) using Stratagene Mx 3000P real-time PCR machine. The real-time RT-PCR amplification was performed in a total volume of 25 μl containing 10 μl of 2.5X mastermix, 0.25 μl of forward primer CSFL1 (10 pmol/ μl), 0.25 μl of reverse primer CSFR1 (10 pmol/ μl), 1 μl of probe CSFP1 (10 pmol/ μl), 1.25 μl of 2X probe enhancer solution, 3 μl of template c-DNA and 9.25 μl nuclease-free water to make a final volume of 25 μl for each sample. The mixture was centrifuged and dispensed into transparent strip-capped 200 μl real-time PCR tubes. Positive and negative controls consisting of viral c-DNA and a non-template reaction mixture (with nuclease-free water instead of template DNA), respectively were included in each evaluation. The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Fluorescence data were acquired during the annealing phase in the reaction using the FAM detection channel on the instrument. The detection was done by determining the cycle threshold (Ct) that is the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. The results were designated positive for Ct values <40, and negative for Ct values ≥40 or negative for samples with which threshold had not been attained before cycling completed at 40 cycles.

RESULTS AND DISCUSSION

CSF has been reported to be an important disease leading to heavy pig mortality. Hence, rapid and sensitive detection assays for CSFV are urgently needed by the pig industry and research community as well. Novel nucleic acid amplification systems designed to decrease assay time by monitoring amplification of desired sequences in real-time by fluorescence resonance energy transfer analysis are widely used for basic research and diagnostics. Real-time RT-PCR thus offers obvious advantages over more traditional RT-PCR platforms. In comparison with conventional PCR, TaqMan real-time PCR is more sensitive and less prone to contamination. The main drawback of conventional PCR is the contamination that occurs when products are visualized in gels leading to false-positive results. This is the reason why, real-time PCR is widely used, and additionally, it has heightened sensitivity requiring less time than conventional PCR. Control of CSF has remained a big challenge for the pig industry. The control measures should therefore much rely on accurate and reasonable laboratory diagnosis to identify and segregate the infected animals from the healthy ones. Rapid laboratory diagnosis of CSF at an early phase of the disease can yield information relevant to swine industry management and help facilitate biosecurity protocols.

The TaqMan RT-PCR assay used herein could detect only one sample with a Ct value of 18.40 for FAM dye (Figure 1). The assay was found to be promising for fast and specific detection of CSFV genome in samples from infected slaughtered pigs. It took no more than 3.5 hrs to complete the whole assay, which included only 1.5 hr of sample preparation and 1.5 hrs for DNA amplification, because of faster thermal cycling and real-time detection of amplicon. The 5'UTR region (as targeted in this assay) is the major conserved region in CSFV genome, and is obviously the ideal reference fragment to detect CSFV than any other region. Furthermore, the primers recognizing this region have high specificity and do not cross-amplify the sequences from other related porcine viruses. Detection of CSFV by RT-PCR with TaqMan probe was done by McGoldrick et al. (1998) and they showed that the method could be used to detect viral RNA extracted from infected pig blood with sensitivity greater than that of virus isolation. McGoldrick et al. (1999) also used closed one-tube RT-nested PCR for the detection of pestiviral RNA with fluorescent probes for border disease virus and for bovine viral diarrhoea virus type II (BVDV-II). With suitable modifications in probe designing, the test can also be adapted for simultaneous detection and genotyping of CSFV as was applied for BVDV by Letellier and Kerkhofs (2003). Hoffmann et al. (2005) found the TaqMan assay to be very
useful because of its high sensitivity and specificity. Ciglenecki et al. (2008) reported that the one-step real-time TaqMan RT-PCR assay was more appropriate for pestivirus quantification that reduces the risk of contamination and is less time consuming as compared to the conventional PCR. A TaqMan-based real-time PCR assay was also devised for the detection of porcine circovirus type 2 (Zhao et al., 2010), porcine bocavirus (Li et al., 2011), caprine arthritis-encephalitis virus (Li et al., 2013) and porcine parvovirus (Song et al., 2010; Gava et al., 2015).

In summary, the real-time TaqMan RT-PCR assay was used for rapid and strain-specific detection of CSFV. This assay has proven useful for routine diagnostic assay in clinical settings because it eliminates post-amplification product processing thereby shortening the turn-around time for reporting results, which remains to be crucial in an outbreak setting. The simplicity and portability of the assay may allow for its use as a complementary pen-side diagnostic for rapid on-site diagnosis and can be used as a routine molecular tool or a confirmative tool for diagnosis of CSF, especially in the case of samples that yield an inconclusive result by other assays. The assay should be used as a useful tool for analyzing the clinical and molecular epidemiology of CSF in swine populations in the country.

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