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SHORT COMMUNICATION

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Stability and integrity of classical swine fever virus RNA stored at room temperature

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Abstract

Worldwide cooperation between laboratories working with classical swine fever virus (CSFV) requires exchange of virus isolates. For this purpose, shipment of CSFV RNA is a safe alternative to the exchange of infectious material. New techniques using desiccation have been developed to store RNA at room temperature and are reported as effective means of preserving RNA integrity. In this study, we evaluated the stability and integrity of dried CSFV RNA stored at room temperature. First, we determined the stability of CSFV RNA covering CSFV genome regions used typically for the detection of viral RNA in diagnostic samples by reverse transcription-polymerase chain reaction (RT-PCR). To this end, different concentrations of *in vitro*-transcribed RNAs of the 5'-untranslated region and of the NS5B gene were stored as dried RNA at 4, 20, and 37ºC for two months. Aliquots were analyzed every week by CSFV-specific quantitative real-time RT-PCR. Neither the RNA concentration nor the storage temperature did affect CSFV RNA yields at any of the time evaluated until the end of the experiment. Furthermore, it was possible to recover infectious CSFV after transfection of SK-6 cells with dried viral RNA stored at room temperature for one week. The full-length E2 of CSFV was amplified from all the recovered viruses, and nucleotide sequence analysis revealed 100% identity with the corresponding sequence obtained from RNA of the original material. These results show that CSFV RNA stored as dried RNA at room temperature is stable, maintaining its integrity for downstream analyses and applications.

Additional keywords: dried RNA; degradation; virus rescue; viral diagnosis; viral characterization; RNA shipment.

Abbreviations used: CSF (classical swine fever); CSFV (classical swine fever virus); RSB (RNA safe buffer); RT-PCR (reverse transcription polymerase chain reaction); UTR (untranslated region).

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Introduction

Classical swine fever (CSF) is a contagious viral disease of swine affecting both domestic and wild *Suidae* of all breeds and ages. It is a notifiable disease to the World Organization for Animal Health (OIE) due to the highly contagious nature of the virus, the high mortality associated with the acute forms of the disease, and the huge economic losses incurred by the affected countries, along with the impact on the international trade (Haines *et al.*, 2013). CSF is caused by classical swine fever virus (CSFV), an enveloped single-stranded, positive-sense RNA virus belonging to the *Pestivirus* genus of the *Flaviviridae* family (Thiel *et al.*, 2005). The polyprotein of approximately 4000 amino acids is processed co-translationally, giving rise to 12 final cleavage products: N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Rice, 1996). The 5´ untranslated region (UTR) and the E2 and NS5B coding regions have been proposed as standard targets for the genetic characterization and diagnosis of CSFV (Díaz de Arce *et al.*, 1998; Paton *et al.*, 2000; Risatti *et al.*, 2005; Postel *et al.*, 2012).

Since the CSFV genome is an RNA molecule, it can be affected by multiple degradation mechanisms. Firstly, the RNA is very sensitive to oxidation by reactive oxygen species. Oxidation of RNA can be mediated by ozone, an atmospheric pollutant that rapidly reacts with RNA either in solution or in the solid state. Degradation can also be mediated by contaminating nucleases. However, the main mechanism of RNA degradation is the spontaneous cleavage of the phosphodiester linkage through transesterification resulting from a nucleophilic attack of the phosphorus atom by neighboring 2'OH. Water is involved, for instance, by providing hydroxyl or hydronium ions or by allowing proton transfer. Consequently, dehydration of RNA strongly inhibits its degradation (Fabre *et al.*, 2014).

Typically, the development of molecular tools for the diagnosis and characterization of CSFV involves intensive exchange of methods and material between laboratories (Haines *et al.*, 2013; Coronado *et al.*, 2017). The exchange of genetic material has been proposed as an alternative to the exchange of infectious virus representing a major concern in terms of biosafety and biosecurity. However, the main challenge when exchanging RNA is maintenance of RNA integrity during storage and shipping. Loss of RNA quality during shipment would strongly compromise experimental results and diagnostic (Fleige & Pfaffl, 2006).

Traditional processes for handling nucleic acid samples rely on an uninterrupted cold chain throughout all steps, from sample collection trough transport, processing and storage, which is costly and time-consuming. Thus, there is a need for improved methods for storing and shipping samples at ambient temperature (Liu *et al.*, 2015). In recent years, new room temperature storage technologies based on anhydrobiosis have been reported as effective alternative approaches for preservation of nucleic acid integrity (Muller *et al.*, 2016). The objective of the present study was to determine whether RNA of CSFV stored as dried RNA at room temperature would preserve its stability and integrity for common downstream analyses

Material and methods

Sample collection and RNA isolation

The study was carried out using supernatants of SK-6 cells infected with four Cuban isolates, *i.e.* the reference strain 'Margarita' and the strains 'Holguín' (CSF1056), 'Santiago de Cuba' (CSF1057), and 'Pinar del Río' (CSF1058). These latter viruses as well as spleen suspension from CSFV-free pigs were obtained from the Animal Virology laboratory of the National Center for Animal and Plant Health, La Habana, Cuba. Total RNA was isolated from 150 µL of supernatant obtained from infected cells using the QIAamp Viral RNA Mini Kit (Qiagen GmbH) following the manufacturer's instructions. The eluted RNA was stored at -80ºC until further processing for evaluating RNA stability and integrity.

In vitro transcription

In order to evaluate the CSFV RNA stability, *in vitro*-transcribed RNAs of 5´UTR and the NS5B gene were analyzed. Briefly, the 5´UTR and NS5B fragments of the 'Pinar del Río' (CSF1058) strain were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the primer pairs reported by Hoffmann *et al.* (2005) and Díaz de Arce *et al.* (1998), respectively. In both cases, the T7 promoter was coupled to the reverse primer. The PCR products were *in vitro*-transcribed by using the MEGAscript® T7 Kit (Ambion). The concentration of the RNA transcripts was determined by photometric quantification (Nanodrop, Thermo Scientific). Ten-fold serial dilutions of the *in vitro*-transcribed were used to generate standard curves for quantification of CSFV-specific RNA copies per µL of RNA.

RNA sample processing and storage

Different concentrations of RNA transcripts of the 5'UTR and the NS5B gene (high RNA concentration: 10^6 copies/µL, medium RNA concentration: 10^4 copies/µL and low RNA concentration: 10^2 copies/µL) were used to spike total RNA extracted from spleen tissue of CSFV-free pigs. Each RNA preparation was divided into several aliquots of 20 µL in sterile 0.2 mL Eppendorf tubes. The RNA was then precipitated by adding 53.6 µL of precipitation solution composed of 1.6 µL glycogen (20 mg/mL), 2.0 µL ammonium acetate (7.4 M) and 50 µL ethanol. The samples were then centrifuged at maximum speed (approx. 13,000 ×g) for 10 min. After centrifugation, the aqueous phase was removed and the RNA pellet was dried in a laminar flow cabinet prior to sealing the tube hermetically. The dried RNA samples were then stored at 4, 20, and 37ºC (t=0) and tested every week for two months. At each time point, three aliquots of each dried RNA sample were rehydrated in 20 µL nuclease-free water for 10 min and resuspended gently by pipetting prior to being analyzed by CSFV-specific real-time reverse transcription-quantitative polymerase chain reaction (rRT-qPCR).

cDNA synthesis and RT-qPCR

The cDNA was synthesized by random priming using M-MLV reverse transcriptase as previously described by Díaz de Arce *et al.* (2009). In order to quantify the number of copies/µL of RNA for each target region, two CSFV-specifics rRT-qPCR were performed using the LightCycler1.5 (Roche Applied Science, Mannheim, Germany): the NS5B RNA was amplified with the primer pair CSFV1-CCTGAGGACCAAACACATGTTG/CSFV2-TGGTGGAAGTTGGTTGTGTCTG as described by Pérez *et al.* (2011), and the 5´UTR was detected with the primer pair CSF 100-F: ATGCCCAYAGTAGGACTAGCA / CSF 192-R: CTACTGACGACTGTCCTGTAC and the FAM-TGGCGAGCTCCCTGGGTGGTCTAAGT-TAMRA probe as previously described Hoffmann *et al.* (2005). Reaction efficiencies were calculated with the LightCycler software (vers. 4.05).

Integrity and infectivity of RNA recovered after storage at room temperature

Virus recovery from dried CSFV RNA stored at room temperature was evaluated using total RNA from the three Cuban isolates CSF1056, CSF1057 and CSF1058. The reference strain 'Margarita' (CSF0705) was used as positive control. The RNA samples were stored at room temperature for one week, either as dried RNA (see above) or diluted in the RNA safe buffer (RSB) previously described by Hoffmann *et al.* (2006). Each dried RNA was rehydrated with nuclease-free water for further processing. After one week of storage, the number of RNA copies/µL was determined for each viral RNA preparation by CSFV-specific rRT-qPCR using the primers CSF 100-F, CSF-192-R and the corresponding probe as described above (Hoffmann *et al.*, 2005). For sequence analysis, the E2 gene was amplified by conventional RT-PCR and subjected to nucleotide sequencing as described previously by Postel *et al.* (2012).

Transfection of SK-6 cells with viral RNA was performed according to Meyer *et al.* (2015). The titer of the rescued virus was determined by end-point dilution 72 h after electroporation. For this purpose, the supernatant was harvested, clarified (5 min at 3,000×g), and used to inoculate SK-6 cells with 10-fold dilutions. After 72 h, the cells were fixed and CSFV protein expression was detected by a peroxidase-linked assay (PLA) (Wensvoort *et al.*, 1986). The titers were calculated according to Reed & Muench (1938). The supernatants of infected SK-6 cells were harvested and used for RNA isolation with the QiaAmp Viral RNA Kit according to the manufacturer's instructions (Qiagen).

Results and discussion

Effect of storage conditions on the stability of RNA from the 5´UTR and the NS5B gene of CSFV

Quantitative real-time RT-PCR (rRT-qPCR) has become one of the most widely used methods in CSFV diagnostics. In this context, RNA stability and integrity are key issues during sample shipment and storage. The 5'UTR region is highly conserved among CSFV isolates, and most laboratories use this genome segment as a target for diagnostic rRT-qPCR (Hoffmann *et al.*, 2005). Alternatively, we and others have used the NS5B gene as a target region for diagnostic rRT-qPCR with excellent results (Díaz de Arce *et al.*, 1998).

Here we analyzed the RNA stability of the 5´UTR and NS5B genome fragments of CSFV by rRT-qPCR. For this purpose, dried *in vitro* transcribed RNA preparations representing the 5'UTR and the NS5B gene of CSFV were stored at concentrations of 10^6, 10^4 and 10^2 copies/µL at 4, 20, and 37°C for two months. Every week, triplicate aliquots of each RNA were rehydrated and quantified by rRT-qPCR. The two-genome regions were amplified at each time point evaluated until the end of the experiment (Fig. 1). Neither the RNA concentration nor the storage temperature did affect the number of RNA copies detected. The number of RNA copies/µL determined for each RNA preparation remained constant from t=0 to the last time point evaluated. Importantly, the stability of the RNA stored at 20°C or 37°C was not significantly different from the stability observed after storage of the RNA at 4°C. This shows that CSFV RNA remains stable when stored dried, even at low concentrations, indicating that RNA shipment under these conditions may be suitable for downstream diagnostic applications by rRT-PCR. Our results are consistent with previous data reported by Fabre *et al.* (2014) who found that nearly complete removal of water strongly reduced the rate of RNA degradation. This was expected since dehydration reduces the rate of RNA phosphodiester bond cleavage mediated by water in transesterification reactions. A loss of molecular mobility related to the solid state may also contribute to reducing the rate of RNA degradation. Earlier studies had already suggested that the atmospheric water and oxygen can greatly affect the degradation rate of RNA in the solid state at room temperature (Liu *et al.*, 2015). We did not find any sign of RNA degradation when the dried CSFV RNA was stored in hermetically sealed laboratory plastic tubes at room temperature for two months. This is in line with the studies by Mathay *et al.* (2012) and Fabre *et al.* (2014) evaluating methods for the storage of dried RNA at room temperature. In these reports, welded-

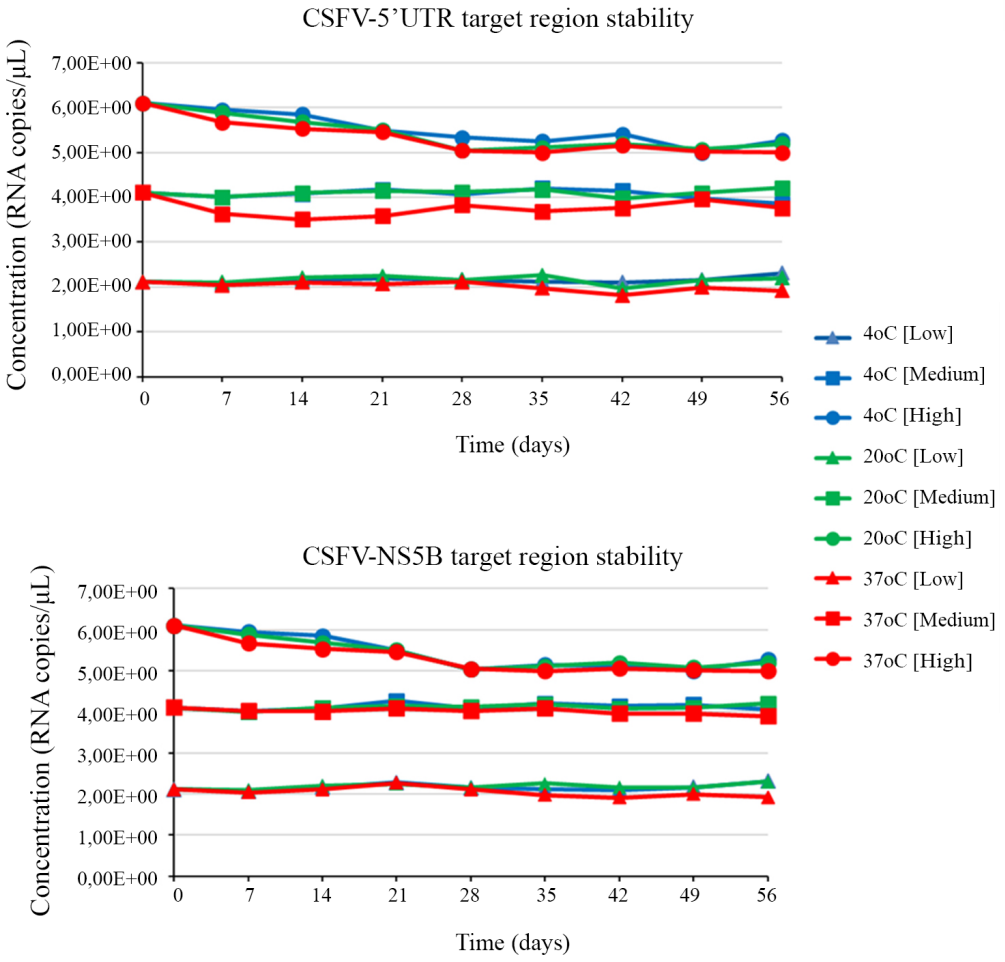

Figure 1. Stability of 5´UTR and NS5B RNA fragments of the CSFV genome stored as dry pellets at 4, 20 and 37ºC for two months. RNA samples were analyzed with 7 days intervals by specific CSFV rRT-qPCR.

sealed stainless steel minicapsules were recommended to maintain an anhydrous and anoxic atmosphere and increase RNA stability over time.

Integrity of dried CSFV RNA genomes stored at room temperature

The shipment of infectious CSFV between biocontainment facilities requires extensive safety measures to be applied. Therefore, shipment of viral RNA from which infectious virus can be recovered represents an attractive alternative (Hofmann *et al.*, 2000). This is technically possible since RNA from viruses with a single-stranded RNA genome of positive polarity is typically infectious when introduced into mammalian cells by transfection (Lemire *et al.*, 2016). However, this requires intact full-length viral RNA. Therefore, we evaluated the integrity of dried CSFV RNA stored at room temperature by determining the efficiency of virus recovery after RNA transfection. We compared total RNA extracted from four different Cuban CSFV strains (CSF1056, CSF1057, CSF1058, and Margarita) and stored at room temperature, either as dried RNA or as RNA diluted in RSB. Following one week of storage, SK-6 cells were transfected by electroporation with approximately 10^4 CSFV RNA copies/µL. According to Meyer *et al.* (2015), this amount of total RNA is sufficient for successful virus rescue after cell transfection. Three days after transfection of SK-6 cells with CSFV RNA that had been stored as dried RNA, a 1460 bp RT-PCR product representing the E2 gene of CSFV was amplified from the clarified cell culture supernatant (Fig. 2). Eventually, infectious virus was recovered with at titer of 10^5 $TCID_{50}$/mL. In contrast, following transfection with CSFV RNA stored in RSB, two passages in SK-6 cells were required before the E2 gene could be amplified by RT-PCR (Fig. 2), indicating reduced RNA stability in RSB when compared to dried RNA. Of note, for the two conditions compared, the amount of transfected RNA was adjusted to 10^4 copies/µL using rRT-qPCR covering only a short fragment of the genome, which did not provide any information on the integrity of

full-length genome. Therefore, our data suggest that fewer full-length RNA molecules were present after storage in RSB than after storage as dried RNA. Similarly, Hofmann *et al.* (2000) and Meyer *et al.* (2015) reported that RNA integrity was a limiting factor for efficient CSFV recovery from full-length CSFV RNA genome preparations. In accordance with the detection of the rescued virus by RT-PCR, only the SK-6 cells transfected with RNA that had been stored as dried RNA resulted in positive signals in the peroxidase-linked assay following transfection (Fig. 3). The RNA stored in RSB required at least one passage in SK-6 cells after transfection before viral antigen expression was detected. This confirmed that the integrity of the full-length viral RNA stored in RSB was inferior to that of the RNA stored as dried RNA. Similar results were reported by Hofmann *et al.* (2000) with SK-6 cells transfected with RNA extracted from samples stored in Trizol. Finally, the E2 gene of a rescued virus (CSF1058) was 100% identical to the corresponding sequence obtained from total RNA of the original material stored as dried RNA.

The RSB did not perform as well as dried RNA for long-term storage, although it was described as an efficient method for the short-term storage of RNA at room temperature (Hoffmann *et al.*, 2006). Consistent with the present study, our previous results showed that the RNA from avian influenza virus (AIV) had better stability when stored as dried RNA than in RSB. Chemical matrices such as RSB use various combinations of reagents that minimize or prevent oxidation, hydrolysis, and nuclease activity. Most of the matrices are proprietary, and there is only limited information available on their composition. In studies conducted by Mathay *et al.* (2012), RNA samples stored in Biomatrica RNAstable or GenTegra RNA for 2 weeks were equally amenable to RNA quality analyses and real-time PCR than RNA stored at minus 80°C. On the other hand, studies by Seelenfreund *et al.* (2014) on the long-term storage of dried versus frozen RNA indicated that dried RNA kept at room temperature for up to one year was comparable to cryopreserved RNA for downstream analysis, including real-time PCR and RNA sequencing.

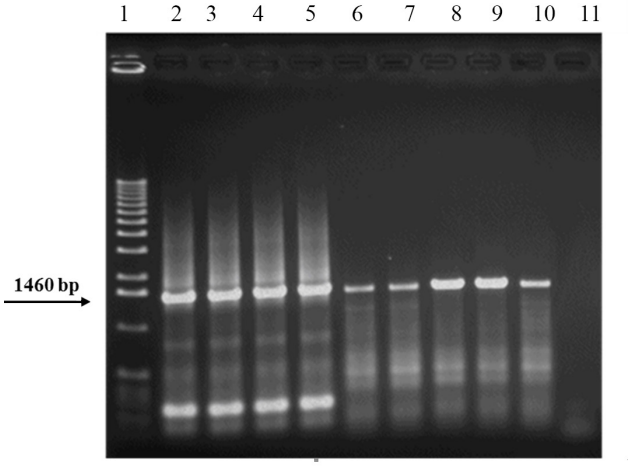

Figure 2. Evaluation of CSFV RNA integrity by amplification of the E2 gene (CSFV 2250f/CSFV 3710r) from supernatants of SK-6 cells transfected with RNA stored as dried RNA (lanes 2-5) or passaged twice after transfection of RNA kept in RSB (lines 6-9). The amplified products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. Lane 1: 1 kb DNA ladder (Promega), Lane 2: dried RNA from CSF1056, Lane 3: dried RNA from CSF1057, Lane 4: dried RNA from CSF1058, Lane 5: dried RNA from CSFV Margarita strain, Lane 6: RNA from CSF1056 diluted in RSB, Lane 7: RNA from CSF1057 diluted in RSB, Lane 8: RNA from CSF1058 diluted in RSB, Lane 9: RNA from CSFV Margarita diluted in RSB, Lane 10: PCR positive control (CSFV RNA), lane 11: PCR negative control.

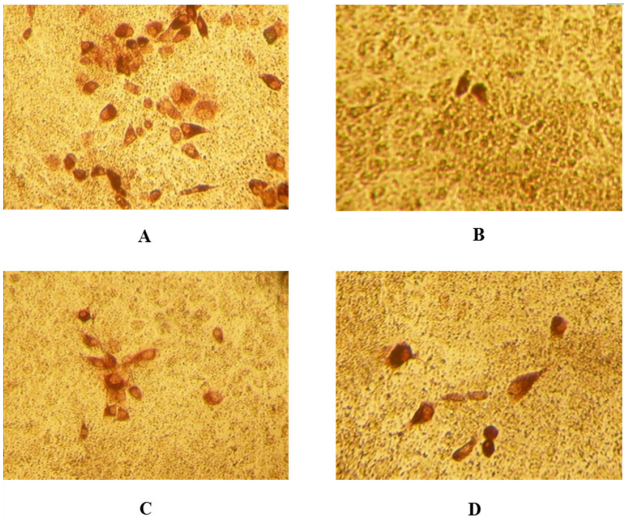

Figure 3. Recovery of infectious virus by electroporation of SK-6 cells with CSFV RNA stored at room temperature as dried RNA. Virus amplification after transfection of RNA from the Margarita strain (CSF0705) (A), and from the strains CSF1058 (B), CSF1056 (C) and CSF1057 (D) was visualized by using a peroxidase-linked assay.

In conclusion, this study demonstrates an efficient method for room temperature storage of dried CSFV RNA. Dehydration appears as a very promising technique for RNA storage. Storage of CSFV RNA in a dried state preserved its stability and integrity, and the RNA was used successfully as a template for diagnosis by rRT-PCR, full genome amplification, sequencing, and recovery of infectious virus upon transfection. The results of the present study may have useful applications in the exchange of RNA samples between laboratories for virus diagnosis and research without biosafety concerns or risks of RNA degradation during shipment.

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