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Comparison of various commercial products for phenol-guanidine-based classical swine fever virus RNA extraction

Martin A. Hofmann* and Markus Mader

Institute of Virology and Immunology IVI, Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland.

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TRIzol®, TRI Reagent®, and RNAzol® are widely used commercial reagents for the extraction of cellular or viral RNA. Several other brand name products, some of which are advertised for the processing of specific sample types such as blood, are also available. Here, we compare the efficiency of these products for classical swine fever virus RNA extraction from cell culture supernatant, serum, and tonsil tissue, assessed by quantitative RT-PCR. Furthermore, the detection of a synthetic RNA transcript used as an internal positive control for extraction and RT-qCR was compared as well. Most tested products showed a similar extraction efficiency, and none of the products recommended for specific sample types performed better than the all-purpose reagents. We also show that the homogenization method for tissue samples has a significant impact on the detection efficiency of the RNA after extraction from the homogenized tissue. Homogenization of 100 mg tissue in 5 ml cell culture medium and using an UltraTurrax® tissue grinder yielded the best results, whereas TissueLyser®-mediated homogenization in 1 ml cell culture medium or direct homogenization in RNA extraction medium proved to be less efficient.

Key words: RNA extraction, homogenization, comparison, TRIzol, TRI reagent, reverse transcription-quantitative PCR (RT-qPCR).

INTRODUCTION

Nucleic acids (NA) can be extracted by various methods. For diagnostic purposes, today most methods basically follow the same principle: samples are being lysed in a protein-denaturing agent such as guanidine and/or phenol, followed by purification of the NA from the precipitated proteins, either by centrifugation resulting in organic/-inorganic phase separation followed by NA precipitation, or by NA adsorption to silica membranes or coated magnetic beads followed by washing and subsequent elution.

One of the most widely used methods is the isolation of total cellular or viral RNA by TRIzol® which is a mono-phasic solution of phenol and guanidine isothiocyanate. The extraction protocol was initially developed by Chomczynski and Sacchi (Chomczynski, 1993; Chomczynski and Sacchi, 1987). Today, several additional

*Corresponding author. E-mail: martin.hofmann@ivi.admin.ch.

commercial products are available, many of them being advertised to be superior for specific sample types, for example, TRI Reagent® BD for processing blood derivatives. We have previously shown, that full-length viral RNA is still detectable after long-term storage of clinical samples in TRIzol® (Hofmann et al., 2000). Since the advent of reverse transcription-quantitative PCR (RT-qPCR) for the detection of cellular or viral RNA, the consistent isolation and purification of RNA has become a crucial step in any laboratory employing RT-qPCR.

Classical swine fever (CSF) is one of the most devastating pig diseases worldwide (Penrith et al., 2011). It is caused by CSF virus (CSFV) which belongs to the genus pestivirus within the family Flaviviridae. CSFV has an unsegmented, plus-oriented, single-stranded RNA genome. Whereas the virus was traditionally detected by virus isolation on susceptible cell lines, RT-qPCR has become the method of choice for CSFV detection, based on its superior sensitivity and specificity (Hoffmann et al., 2009). Furthermore RT-qPCR still allows detecting CSFV-specific RNA in samples that do not contain any infectious virus anymore. CSFV can be readily detected in serum and specific organs, in particular the tonsils of infected pigs. Since CSFV replicates in commonly used cell cultures without producing any cytopathic effect, RT-qPCR is often also used to identify CSFV upon cell culture infection.

Whereas numerous studies have been published which compare different RNA extraction procedures, both for cellular (Ruettger et al., 2010; Kong et al., 2006) and for viral (Deng et al., 2005; Guarino et al., 1997; Scheibner et al., 2000) RNA extraction, no published data are available on the comparison of various commercial products that are all based on the phenol-guanidine principle. In the present study we compare the performance of TRIzol® with several other brand name products based on the same principle for the extraction of CSFV RNA from cell culture supernatant, serum and tonsils.

MATERIALS AND METHODS

CSFV-positive samples

Clarified supernatant from SK-6 swine kidney cell cultures infected with the moderately virulent CSFV strain Alfort/187 (Greiser-Wilke et al., 1990), and serum and tonsils collected 7 days post infection from a pig infected with the highly virulent CSFV strain Koslov (Kaden et al., 2001) were used for the comparative RNA extraction. All extractions were done with the same sample materials.

Extraction media and RNA extraction

TRIzol® (Invitrogen, Carlsbad, CA, USA) was compared to the following alternative brand name products (all from Molecular Research Center, Inc., Cincinnati, OH, USA): TRI Reagent®, TRI Reagent® BD, TRI Reagent® LS, TRI Reagent® RT, TRI Reagent® RT-Blood, TRI Reagent® RT-Liquid Samples, RNAzol®.

All RNA extractions were run in triplicates and were performed according to the respective manufacturer's protocol, except that 10 µl of a 1 mg/ml solution of glycogen (type III, from rabbit liver; Sigma, Buchs, Switzerland) and 10 µl of an *in vitro*-transcribed EGFP RNA (Hoffmann et al., 2006) corresponding to 10^4 RNA copies were added immediately before sample extraction. Precipitated RNA was dissolved in 20 µl RNase-free H_2O.

Tissue homogenization

Aliquots of 100 mg of tonsil epithelium were homogenized in isolation medium [Eagles Minimal Essential Medium supplemented with 2 % horse serum and antibiotics (EMEM)], either in 5 ml in an UltraTurrax® tissue grinder, or in 1 ml in the TissueLyser® homogenizer (Qiagen, Hilden, Germany). To assess the suitability of RNA extraction media (REM) for direct one-step homogenization and RNA extraction, 100 mg slices of tonsil tissue were also homogenized in the TissueLyser® in 1 ml of TRIzol®, TRI Reagent®, TRI Reagent® RT-Blood, respectively.

RT-qPCR

All samples were tested in triplicates in a CSFV-specific RT-qPCR (Hoffmann et al., 2005) for the presence of viral RNA. The added EGFP RNA was used as an internal positive control (IPC) to monitor both extraction and RT-qPCR. Mean values and standard deviations for the cycle of threshold (Ct) value of the 9 replicates for all REM (that is, 3 independent RNA extractions that were each tested in triplicates in the RT-qPCR) were calculated and used to compare the efficiency and robustness of RNA extraction.

RESULTS AND DISCUSSION

Quantitative detection of CSFV RNA by RT-qPCR following extraction in various guanidine/phenol-based extraction media was compared by analyzing two different liquid sample types, that is, cell culture supernatant or serum. Furthermore, tissue specimens that had been homogenized either in 1 or 5 ml EMEM, or directly in 1 ml of selected REM before RNA extraction, respectively, were also included in the study. Raw Ct values were normalized in order to refer to the same original volume/weight of sample before extraction. For example when results of UltraTurrax®-and TissueLyser®-homogenized tissue were compared, 2.3 Ct were subtracted from the mean of the UltraTurrax®-derived Ct values to take in account the 5 times lower amount of homogenized tissue used for RNA extraction, due to the different volumes used for homogenization (that is, 5 ml for UltraTurrax® versus 1 ml EMEM for TissueLyser®). Differences in original sample amount due to the varying volumes recommended by the manufacturers of the REM to be used for extraction were taken into account as well.

The synthetic EGFP RNA added as IPC prior to extraction of cell culture supernatant was detected with nearly the same efficiency with all REM (Figure 1A), whereas the IPC added to serum samples was detected less consistently, illustrated as greater Ct fluctuations between the REMs. Tri Reagent® showed the lowest Ct values for IPC detection and was the only product that yielded sample type-independent, similar results. In most

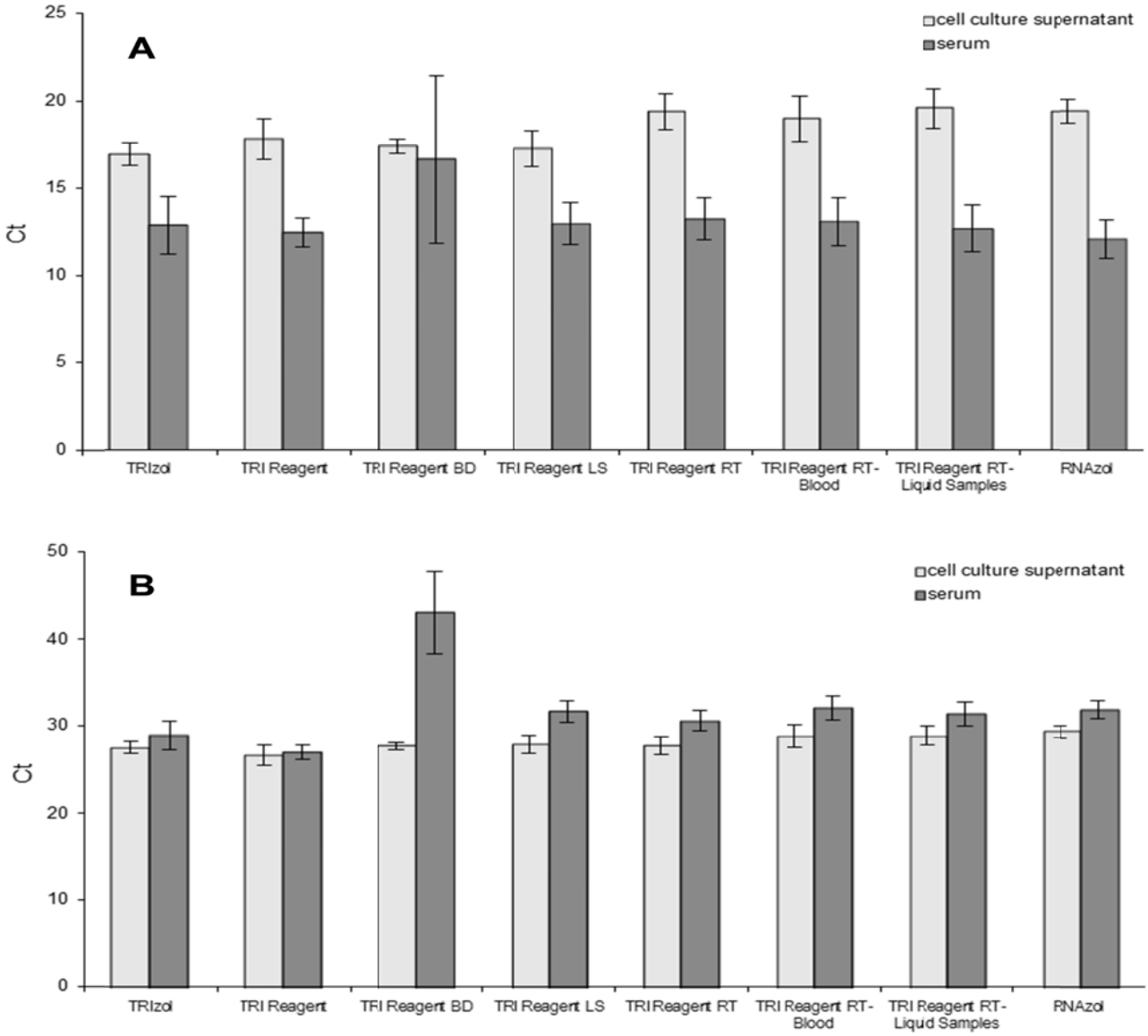

Figure 1. Comparison of RNA extraction media for EGFP IPC (A) and CSFV (B) RNA from cell culture supernatant and serum. Sample volume-normalized Ct values are shown. Error bars indicate standard deviation of the 9 individual RT-qPCR reactions (triplicates from 3 individual extractions).

extractions, IPC detection in serum was less efficient than in cell culture supernatant. In particular, a 100-fold inhibition of the IPC detection was observed after Tri Reagent® BD extraction which is advertised as advantageous for blood derivates (BD). These results indicates that none of the RNA extraction protocols led to a quantitative recovery of the spiked IPC RNA or was able to completely remove RT-qPCR inhibitors present in serum but not in cell culture supernatant.

When the efficiency of CSFV RNA extraction was compared, again only minor differences but the same tendency as for IPC detection was observed (Figure 1B). For the detection of viral RNA from CSFV-positive cell culture supernatant, TRIzol® and TRI Reagent® BD performed slightly better than the other products. However, when viral RNA was extracted from serum, all extractions showed a very similar efficiency, except TRI Reagent® BD which as in IPC detection again showed an inferior efficacy and a larger fluctuation. These results suggest that none of the REM from the TRI Reagent product line nor RNAzol® performed better than TRIzol®. In particular the TRI Reagent® products advertised for specific sample types did not lead to more efficient RNA extraction than the all-purpose products.

RNA extraction from tonsil tissue samples from a CSFV-infected pig was also compared between TRIzol®, TRI Reagent® and TRI Reagent® RT. As shown in Figure 2, the efficiency of CSFV (A) and IPC (B) RNA extraction

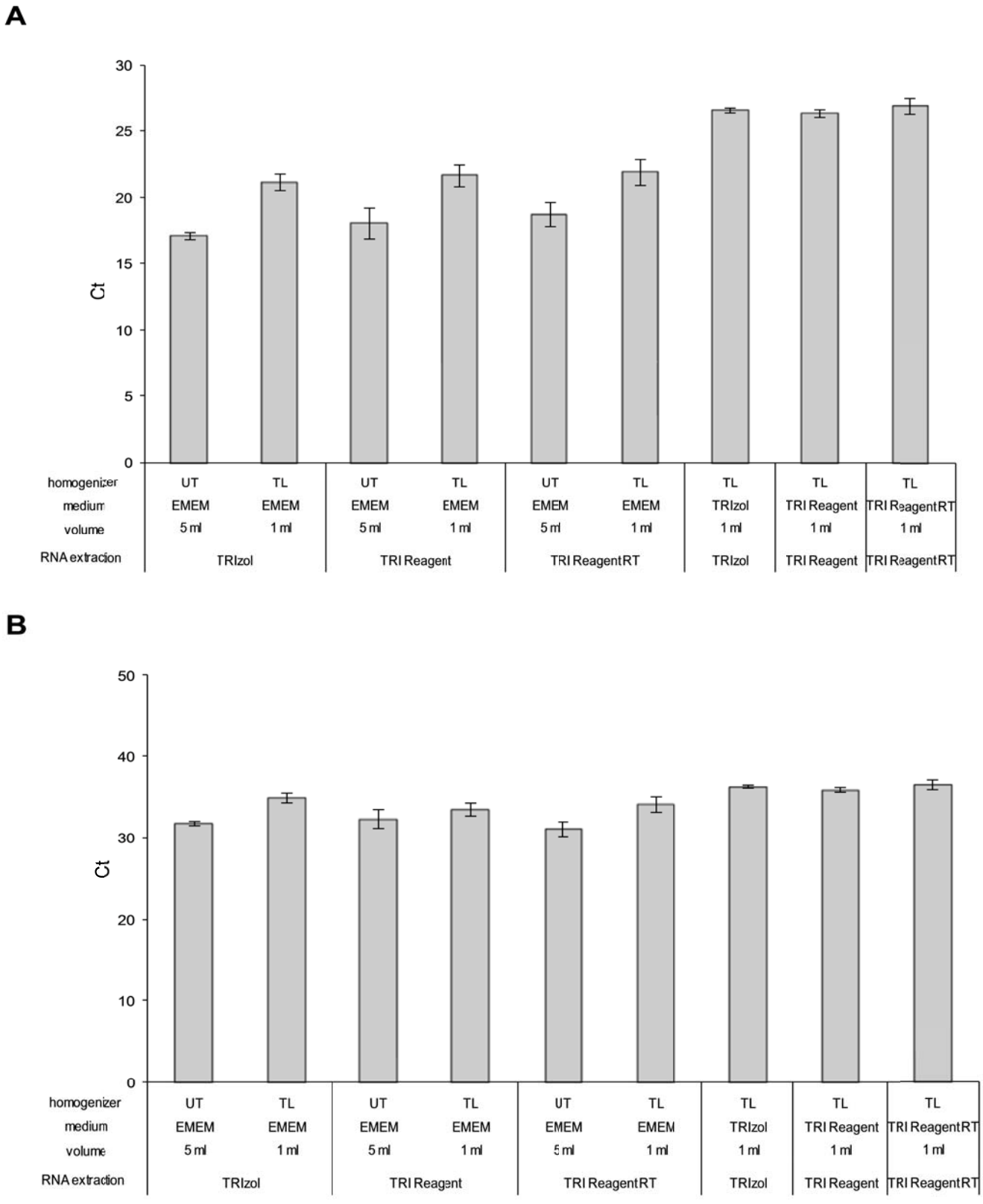

Figure 2. Comparison of homogenization and RNA extraction media for CSFV (A) and EGFP IPC (B) RNA from tonsil tissue. 100 mg tissue was homogenized with an UltraTurrax® grinder (UT) in 5 ml EMEM, or with a TissueLyser® homogenizer (TL), either in 1 ml of EMEM or directly in 1 ml of TRIzol®, TRI Reagent®, or TRI Reagent® RT, respectively. Tissue weight-normalized Ct values are shown. Error bars indicate the standard deviation of the 9 individual RT-qPCR reactions (triplicates from 3 individual homogenizations/extractions).

was mainly dependent on the method used for homogenization of the tissue. The best results were obtained when the 100 mg tissue sample was homogenized in 5 ml EMEM. All 3 REM yielded comparable Ct values that were distinctly lower than if the tissue had been extracted in a smaller volume (1 ml) and using a different homogenization device. On the other hand, direct homogenization in the REM before RNA extraction was significantly less efficient. Results for IPC detection were similar to the CSFV data. Again RNA detection was most efficient in those samples that had been extracted in EMEM in a relatively large volume. Spiked IPC RNA added to the samples homogenized directly in the REM again yielded higher Ct values for all 3 REM. This indicates that the most important factor for efficient RNA extraction is the medium and the volume used for homogenizing the tissue. Differences between the 5 ml and the 1 ml homogenization in EMEM could be due to a different efficiency of the UltraTurrax® compared with the TissueLyser®. However, since the 5 ml homogenization also showed lower Ct values for IPC detection, it is more likely that the volume itself is a critical factor, for example, by leading to a higher dilution of tissue fragments that could interfere with RNA extraction or inhibit the RT-qPCR. Direct homogenization in REM was clearly inferior, most likely due to an inefficient homogenization due to the "tanning" effect of phenol that rendered the tissue more solid. Furthermore, tissue homogenization in EMEM allows - in contrast to protein denaturing agents - infectious virus detection by inoculating susceptible cell cultures.

In conclusion, our data demonstrate that no major differences in RNA extraction/detection efficiency exist between the REM compared in this study. None of the TRI Reagent® products recommended either for blood derivates or for liquid samples performed better than the all-purpose TRIzol® or TRI Reagent®, suggesting that these 2 REM can be used for virtually any sample type. Virus-containing samples should be homogenized in a large volume (for example, 5 ml) of EMEM for efficient RNA extraction and detection by RT-qPCR, respectively, and to allow isolation and subsequent characterization of infectious virus present in the sample.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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