Effect of temperature on the detection of porcine epidemic diarrhea virus and transmissible gastroenteritis virus in fecal samples by reverse transcription–polymerase chain reaction

Kwonil Jung, Chanhee Chae

Abstract. The effect of storage temperature was determined for the detection of porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) in fecal samples from experimentally and naturally infected pigs by multiplex reverse transcription–polymerase chain reaction (RT-PCR). To examine the effect of storage temperature on the ability to detect PEDV and TGEV RNA by multiplex RT-PCR, fecal samples were stored for different temperatures (4, 21, 36, and 45 C) before extracting viral RNA. The PEDV and TGEV nucleic acids in fecal samples were stable up to 3 days at 4 C. The PEDV and TGEV nucleic acids were consistently detected in fecal samples up to 60 hours at 21 C and 24 hours only at 36 and 45 C. Thereafter, the number of positive signals declined. Positive signals were detected in fecal samples stored at 4 C by 240 hours. The PEDV and TGEV nucleic acids were consistently detected in fecal samples up to 60 hours at 21 C and 24 hours only at 36 and 45 C. The results of this study suggested that storage temperature has significant effect on the detection of PEDV and TGEV nucleic acids from fecal samples by multiplex RT-PCR.

Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) are 2 highly contagious enteric viruses affecting mainly neonatal pigs. The PEDV and TGEV infections are considered difficult to distinguish clinically and histopathologically. Rapid differential diagnosis and treatment are crucial to reduce mortality and morbidity from PEDV- and TGEV-induced enteritis in piglets.

A definitive diagnosis of porcine epidemic diarrhea and transmissible gastroenteritis requires isolation of PEDV and TGEV from intestinal contents. However, there are inherent difficulties in isolating PEDV in cell culture. In recent years, there has been much progress in the development of molecular techniques, such as reverse transcription–polymerase chain reaction (RT-PCR), for the differential diagnosis of PEDV and TGEV from fecal samples. Collection of fecal samples does not require euthanasia of sick animals for necropsy or shipping of whole-intestine specimens through courier services, as is currently done. In 2002, more than 1,200 fecal samples were submitted to the Department of Veterinary Pathology at Seoul National University in Seoul to diagnose PEDV and TGEV (C. Chae, personal communication).

Ideally, clinical fecal samples collected for the detection of PEDV and TGEV must be kept cool and transported to the diagnostic laboratory as quickly as possible. This is often a problem especially if farms are located a long distance from a specialized diagnostic laboratory. Harsh environmental conditions during shipment of fecal samples could degrade viral RNA and result in failure of its detection. The objective of this study was to evaluate the effect of various temperatures on the stability of PEDV and TGEV nucleic acids from fecal samples for multiplex RT-PCR assay.

Twenty colostrum-deprived, 3-day-old pigs were randomly divided into 4 groups of 5 pigs each. Each group was randomly assigned to 1 of 4 treatments. The 4 treatments included oral inoculation with cell culture containing 1 of 2 viruses, PEDV strain SNUVR971496 or TGEV strain SNUVR980473, with both PEDV and TGEV, or with uninfected cell culture medium. Pigs in each of the 3 groups were inoculated orally with 3 ml of tissue culture fluid containing 10⁴ 50% tissue culture infective doses (TCID₅₀)/ml of PEDV, 3 ml of tissue culture fluid containing 10⁴ TCID₅₀/ml of TGEV, or 3 ml of tissue culture fluid mixture containing 10⁴ TCID₅₀/ml of PEDV and TGEV (1:1). Five control pigs were exposed in the same manner to uninfected cell culture supernatant. All pigs from each group were euthanized and necropsied at 36 hours postinoculation. The methods were approved previously by the Seoul National University, Institutional Animal Care and Use Committee.

To examine the effect of storage temperature on the ability to detect PEDV and TGEV RNA by multiplex RT-PCR, fecal samples were stored at different temperatures before extracting viral RNA. At intervals (12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, and 240 hours), a representative fecal sample from each pig was used to extract viral RNA and then RT-PCR was performed as described previously. The multiplex RT-PCR amplified a 412-base pair (bp) region from the membrane protein gene of PEDV and a 612-bp region from the nucleocapsid protein gene of TGEV. Vero and swine testicular cells were used to isolate PEDV and TGEV from fecal samples as described previously. A total of 480 fecal samples (2 fecal samples per storage time and temperature) were randomly selected for virus isolation.

An additional 10 fecal samples from each pig naturally infected with either PEDV or TGEV were also used in this investigation. Cases were selected on the basis of virus isolation, clinical signs, histopathological lesions, and in situ hybridization, as described previously.

To determine whether prolonged storage of fecal samples at different temperatures affected the functionality of the
mulitplex RT-PCR, 300 fecal samples from 15 pigs experimentally inoculated with PEDV, TGEV, and both viruses were examined after storage for 12–240 hours at temperatures from 4 to 45 C. Each specific primer pair for PEDV and TGEV yielded an RT-PCR product of the expected size from RNA extracted from fecal samples (Fig. 1). One positive sample per storage time and temperature was randomly selected for sequence analysis. RT-PCR products of each virus were sequenced, and their identity was confirmed as PEDV and TGEV (data not shown).

PEDV nucleic acid was consistently detected in fecal samples up to 132 hours at 4 C, 72 hours at 21 C, 24 hours at 36 C, and 12 hours at 5 C. Thereafter, the number of positive signals declined. Positive signals were detected in fecal samples stored at 4 C by 216 hours, 21 C by 180 hours, 36 C by 108 hours, and 45 C by 24 hours. TGEV nucleic acid was consistently detected in fecal samples up to 96 hours at 4 C, 60 hours at 21 C, 36 hours at 36 C, and 12 hours at 45 C. Thereafter, the number of positive signals declined. Positive signals were detected in fecal samples stored at 4 C by 228 hours, 21 C by 168 hours, 36 C by 120 hours, and 45 C by 24 hours. In the pigs from coinfected group, PEDV and TGEV were detected simultaneously in fecal samples up to 72 hours at 4 C. Thereafter, the number of positive signals declined. By 240 hours, no positive signals were detected in fecal samples stored at 4 C (Table 1). PEDV and TGEV were isolated from fecal samples from pigs experimentally inoculated with PEDV and TGEV and both viruses. Neither PEDV nor TGEV was isolated from fecal samples that were found negative by multiplex RT-PCR. No positive signal for PEDV and TGEV was detected in fecal samples from control pigs. Therefore, PEDV and TGEV were not recovered from any control pig.

In the pigs naturally infected with either PEDV or TGEV, PEDV nucleic acid was consistently detected in fecal samples up to 108 hours at 4 C, 48 hours at 21 C, 12 hours at 36 C, and 12 hours at 5 C. Thereafter, the number of positive signals declined. Positive signals were detected in fecal samples stored at 4 C by 228 hours, 21 C by 168 hours, 36 C by 96 hours, and 45 C by 24 hours. TGEV nucleic acid was consistently detected in fecal samples up to 84 hours at 4 C, 48 hours at 21 C, 36 hours at 36 C, and 12 hours at 45 C.
Thereafter, the number of positive signals declined. Positive signals were detected in fecal samples stored at 4°C by 204 hours, 21°C by 132 hours, 36°C by 84 hours, and 45°C by 24 hours. PEDV and TGEV were isolated from fecal samples from pigs naturally infected with either PEDV or TGEV. Neither PEDV nor TGEV was isolated from fecal samples that were found negative by multiplex RT-PCR.

This study has important implications for the diagnosis of PEDV and TGEV in the fecal samples. An important practical point is the effect of transportation temperature of the specimens over long distances on the performance of the RT-PCR assay. Fecal sample integrity is a key factor for successful RT-PCR assays for accurate diagnosis. Storage temperature of clinical specimens is known to influence the stability of viral RNA. The degradation of viral RNA at routine storage temperature could result in the failure of its detection. Based on RT-PCR assay, PEDV and TGEV nucleic acids were stable in fecal samples up to 3 days at 21°C. Stability would be presumably even higher at 4°C. It must be emphasized that care should be taken to guarantee the temperature during transport and prevent exposure of the samples to severe temperature fluctuations.

A multiplex RT-PCR is naturally less sensitive than individual RT-PCR or nested PCR. In this study, all fecal samples that were negative by multiplex PCR were also tested by individual RT-PCR. Five samples that were negative by multiplex RT-PCR were positive by individual RT-PCR (data not shown). It is recommended to retest the negative fecal samples by individual RT-PCR if viral infection was suspected. No attempt was made to check with porcine respiratory coronavirus (PRCV). The PRCV evolved from TGEV when a spontaneous deletion occurred near the N-terminus of the spike gene. Because the target gene for TGEV in multiplex RT-PCR was N protein, the multiplex RT-PCR used in this study could detect the nucleic acid of PRCV. Therefore, some fecal samples from naturally infected pigs may be positive because of the presence of PRCV.

It is not always possible to transport fecal samples to a diagnostic laboratory in <1 day, and prolonged delays between collection of specimens and RT-PCR assay in the laboratory, as well as potential exposure to extreme environmental temperatures during shipping, may have deleterious effects on the identification of PEDV and TGEV. Because freezing is commonly recommended when an extended delay is expected, this study suggests that it would be better if samples were stored at subzero.

**Acknowledgement.** This work was supported by contract research funds of the Research Institute for Veterinary Science from the College of Veterinary Medicine, Seoul National University and BK21, Republic of Korea.

**References**