Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus

Seong-Hee Kim, In-Joong Kim, Hyun-Mi Pyo, Dong-Seob Tark, Jae-Young Song, Bang-Hun Hyun *

Department of Virology, National Veterinary Research and Quarantine Service, Ministry of Agriculture and Forestry, Republic of Korea

Received 6 February 2007; received in revised form 7 May 2007; accepted 27 June 2007
Available online 13 August 2007

Abstract

Transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) are major etiological agents of diarrhea and death in piglets. Multiplex real-time reverse transcriptase (RT)-PCR was developed for simultaneous differential quantification of each virus in a single reaction tube, using Cy5- and FAM-labeled TaqMan-probes based on sequences from the TGEV and PEDV nucleocapsid genes. The copy numbers for transcripts of TGEV and PEDV were quantified using this assay over a range from $9 \times 10^7$ to $9 \times 10^1$ copies and $7 \times 10^7$ to $7 \times 10^1$ copies, respectively. The variability of the intra-assay and inter-assay were evaluated using standard solutions of each transcript, with coefficients of variation (CV) less than 3.43 and 3.33%, respectively. Piglets were experimentally infected with virulent TGEV and PEDV, and the amounts of virus from the onset of diarrhea were measured. Samples obtained from farms experiencing PED or TGE were quantified between $10^2$ and $10^5$ RNA copies. In conclusion, this assay provides an effective etiological diagnostic tool for detecting and quantifying viral loads. The assay may also prove useful for detecting infections, ultimately leading to better disease control on farms.

Keywords: Transmissible gastroenteritis virus (TGEV); Porcine epidemic diarrhea virus (PEDV); Multiplex real-time RT-PCR; Quantification

1. Introduction

Porcine transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV), members of the family Coronaviridae, are causes of viral enteritis, which destroys villus enterocytes in pigs of all ages (Debouck and Pensaert, 1980; Pensaert, 1999; Saif and Wesley, 1999). Both diseases are spread by the oral uptake of feces from infected pigs, resulting in vomiting, watery diarrhea, dehydration, and high mortality in nursery piglets (Pensaert, 1999; Saif and Wesley, 1999).

Conventional diagnostic assays use either antibodies or viral antigens to detect viral gastroenteritis. Several vaccines used for sows in Korea elicit immunoglobins (Igs) in maternal milk that help to protect offspring from infection. Therefore, serological examination cannot be used with these animals, since the vaccine does not distinguish between vaccinated animals and those infected with the wild-type virus. Several diagnostic assays are available for detecting viral antigens, including virus isolation, fluorescence assay, immune electron microscopy, in situ hybridization, and enzyme-linked immunosorbent assay (ELISA; Callebaut et al., 1982; Van Nieuwstadt et al., 1988; Sueyoshi et al., 1995; Pensaert, 1999; Shibata et al., 2000; Kim and Chae, 2001; Rodák et al., 2005). However, none of these techniques can be used for early detection of the viral antigen because they require clinical samples from the intestines of dead pigs and are cumbersome to use, resulting in rather long detection times. Reverse transcriptase polymerase chain reaction (RT-PCR), which includes single and multiplex RT-PCR, has proven to be a convenient and sensitive assay for detection of PEDV and TGEV (Paton et al., 1997;...
Kim et al., 2000, 2001). This technique also has its limitations; it has lower sensitivity (~10–100 times) than real-time PCR, viral loads cannot be measured, and identifying the virus by agarose gel electrophoresis is time consuming (Keyaerts et al., 2006). RT-PCR also poses the potential risk of cross contamination during mass screening within the reverse transcription step. These drawbacks reduce the overall effectiveness of using RT-PCR to detect virus in new outbreaks and previous outbreak sites (Van Rijn et al., 2004; Ophuis et al., 2006).

Real-time PCR has the advantages that it is very accurate and sensitive, allowing for high-throughput screening and quantification of viral loads using small volumes. Therefore, it shows considerable promise as a potential alternative to molecular assays for the detection of viral RNA in clinical samples (Giulietti et al., 2001; Bustin, 2000). Recently, real-time RT-PCR using light-upon-extension (LUX) fluorogenic primers have been reported for the detection of TGEV (Chen et al., 2004). In this study, multiplex real-time RT-PCR was developed for differential detection and quantification of the viral loads of TGEV and PEDV in diarrhea from both experimentally and naturally infected piglets using two sets of primers and different colored probes in a single reaction tube.

2. Materials and methods

2.1. Viruses

The TGEV 175L and PEDV SM98 strains were isolated from the intestines and feces of infected piglets, respectively. The TGEV 175L strain was cultured in swine testicle (ST) cells using alpha-minimum essential medium (α-MEM) supplemented with 5% fetal calf serum and antibiotic/mycotic solution. The PEDV SM98 strain was cultured in African green monkey kidney cells (Vero cells) using the same medium. The TGEV 175L and PEDV SM98P strains were titrated to 1 × 10^5.0 and 1 × 10^4.0 TCID50/ml, respectively. The specificity of the assay was evaluated with the PEDV CV777 strain (Kocherhans et al., 2001), TGEV Purdue and Miller strain (Kapke and Dermody et al., 1991), enterovirus Sukyung stain (isolated from the intestines and feces of infected piglets, respectively), rotavirus Gotffried, and OSU strain (Li and Gorziglia, 1993). Viral RNA was extracted from each sample using the RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions.

2.2. Designing primers and probes

The primers and probes used for multiplex real-time RT-PCR were designed and synthesized in cooperation with TIB MOLBIOL (TIB MOLBIOL Syntheselabor GmbH, Germany). The TGEV and PEDV sequences were aligned using Clustal X (version 1.81; Thompson et al., 1997), and the highly conserved regions within each virus genome were identified. Primers and probes corresponding to the conserved regions were designed using sequence alignments of the nucleocapsid (N) genes from 10 strains of TGEV (Purdue 46-MAD, NC002306; TO14, AF302264; TS, DQ201447; SC-Y, DQ443743; Miller M6, DQ811785; TH-98, AY676604; HN2002, AY587884; 96-1933, AF104420; FS772/70, Y00542) and from five strains of PEDV (CV777, AF353511; Chinju99, AF237764; CH/S, DQ35524; S, DQ35223; JS2001, AY539715). The four primers were designed with a similar Tm that was ~10°C lower than that of the probes to prevent any dimeric interactions from forming. For color multiplexing, the probe for TGEV was labeled with the 5’-reporter dye 6-carboxyfluorescein (FAM) and the 3’-quencher BHQ1, and the probe for PEDV was labeled with the 5’-reporter dye Cy-5 and the 3’-quencher BHQ2. The sequences and amplicon sizes of the primers and probes are listed in Table 1. The specificity of the primers and probes was confirmed against random nucleotide sequences using a BLAST search in GenBank databases located in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Standard curves and variability of the assay

Run-off transcripts were generated for use as standards in the assay with the MEGashortscript T7 kit (Ambion, USA) following the manufacturer’s suggestions. Briefly, a reaction mixture with a final volume of 20 μl containing 150 ng of the PCR product, 2 μl of each nucleotide triphosphate, 2 μl of enzyme mix, and 2 μl of reaction buffer was incubated at 37°C overnight for in vitro transcription. The cDNA was removed by digestion with 2 U of RNase-free DNase I for 15 min at 37°C. After precipitating with sequential ethanol solutions, the transcripts were dissolved in 200 μl of RNase-free H2O and stored at −70°C until used. The concentration of transcripts was determined using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, USA) at 260 nm, from which the molecular number was calculated (Fronhoffs et al., 2002). Tenfold serial dilutions of the transcripts were prepared at concentrations of 9 × 10^9 to 9 × 10^1 copies of TGEV.

| Virus  | Primer and probe name | Sequence (5’−3’) | Nucleotide position | Amplicon size (bp) |
|--------|-----------------------|------------------|---------------------|-------------------|
| TGEV   | TGENF/TGENR/TGE-FAM   | GCAAGTAAAGGTGATGTCAGAAA ACATTCCAGCAGTGTGGCTTAA 6FAM-TGGCACTGGATGGACTGCAGA-BHQ1 | 27,637−27,756a | 120 |
| PEDV   | PEDNF/PEDNR/PED-Cy5  | CGCAAAAGACTGACACCACTATATT TTGCCTCTTGTCTTCTTGAAGAT Cy5-TGTTGCACTGGACGACCTCTGC-BHQ3 | 26,679−26,876b | 198 |

a The position of the primer is based on the Purdue strain of TGEV.
b The position of the primer is based on the CV777 strain of PEDV.
and $7 \times 10^7$ to $7 \times 10^4$ copies of PEDV per 2 µl volume and stored at $-80^\circ$C. Multiplex real-time RT-PCR was performed using these standard solutions to obtain standard curves. Inter-assay variability was evaluated with three independent samples of each virus tested in triplicate, demonstrating copy numbers of $9 \times 10^5$, $9 \times 10^4$, and $9 \times 10^3$ for TGEV and $7 \times 10^7$, $7 \times 10^6$, and $7 \times 10^5$ for PEDV. Inter-assay variability was also determined by testing the same three samples in triplicate over 3 days.

2.4. Multiplex real-time RT-PCR

The quantitative one-step RT-PCR kit (Invitrogen Life Technologies\textsuperscript{TM}, USA) was used for multiplex real-time RT-PCR. Multiplex real-time RT-PCR was carried out in a 20 µl reaction containing 2 µl of RNA or transcripts, 0.5 µl of both TGEV forward and reverse primer, 0.5 µl of both PEDV forward and reverse primer, 0.5 µl of PEDV-Cy5 probe, 0.5 µl of TGEV-FAM probe, 0.8 µl of ThermoScript\textsuperscript{TM} plus/ Platinum\textsuperscript{®} Taq Enzyme Mix, 10 µl of 2 × ThermoScript Reaction Mix (a final concentration of 3 mM MgCl\textsubscript{2}), and 4.2 µl of water. The reaction took place using an iCycler IQ\textsuperscript{TM} multicolor real-time detection system (Bio-Rad, USA) under the following conditions: initial reverse transcription at 58 $\pm$ 3°C for 30 min, followed by initial denaturation at 95 $\pm$ 3°C for 5 min, 40 cycles of denaturation at 95 $\pm$ 3°C for 30 s, and annealing and extension at 60 $\pm$ 3°C for 1 min. The optical data were analyzed using iCycler iQ\textsuperscript{TM} Optical System Software (version 3.1). For each determination, the threshold lines used were the automatic settings at 185.2 and 72.9 relative fluorescence units (RFU) for specific product analysis of TGEV and PEDV, respectively.

To confirm the differential detection of TGEV and porcine respiratory coronavirus (PRCV), multiplex one-step RT-PCR was performed using a one-step RT-PCR kit (Qiagen). Primers were designed to amplify the spike gene, including the deletion region of TGEV, leading to differentiation from PRCV (forward, 5'-GCCATGGATTATGAGACA-3'; reverse, 5'-GTATAAACAATTCTCTGCGTG-3'). Briefly, multiplex one-step RT-PCR was carried out in a final reaction volume of 25 µl containing 5 µl of RNA, 1 µl each of the forward and reverse primers (0.8 pmol), 1 µl of dNTP mix (0.2 mM each dNTP), 1 µl of enzyme mix (2.5 U), 5 µl of 5× buffer, and 11 µl of RNase-free water. PCR was performed under the following conditions: initial reverse transcription at 45 $\pm$ 5°C for 30 min, followed by initial denaturation at 94 $\pm$ 5°C for 15 min, and then 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min. The amplicons were a 782-bp fragment of TGEV and 161-bp fragment of PRCV.

2.5. Clinical samples from experimentally infected piglets

Samples of diarrhea were taken from 3-day-old piglets inoculated with each virus. Two piglets were inoculated orally with 1 ml of virulent TGEV (Jeon3 strain, Korean isolated strain, $10^{6.0}$ PID\textsubscript{50}/ml) and another two with PEDV using 1 ml of virulent PEDV (SM98 strain, $10^{2.0}$ LD\textsubscript{50}/ml). The two groups of piglets were maintained in separate isolation units with their respective sows. Neither sow used in this study had antibodies for TGEV or PEDV, as confirmed by a serum neutralization test. Stool samples were collected with cotton swabs from piglets with diarrhea daily until death. The samples were diluted 1:10 in phosphate buffered saline (PBS, 0.1 M, pH 7.2), vortexed, and clarified by centrifugation at 10 min at 4000 × g to eliminate fecal debris. Viral RNA was extracted from 300 µl of the diluted sample and eluted in 30 µl RNase–DNase free water. RNA was extracted to quantify the viral load by multiplex real-time RT-PCR.

2.6. Clinical samples from naturally infected piglets

Stool and intestinal specimens were obtained from the Provincial Institute for Animal Health; these had been collected from infected swine on six Korean farms (farms KS, KB, KK, KD, IY for PED, and KL for TGE) between December 2005 and May 2006 (Table 2). The institute initially screened the samples as PEDV or TGEV positive with an indirect fluorescent assay using cryocut-sections and RT-PCR. RNA was extracted by vortexing 1 g of stool sample with 10 ml of PBS or by grinding 1 g of intestine with 5 ml of MEM. The specimens and extracted RNA were stored at $-70^\circ$C until used. The viral load of each sample was measured in duplicate using multiplex real-time RT-PCR.

Table 2

| Farm | No. of sows | Percentage mortality | Result of diagnosis | Viral loads (log10 copies.) | Sample |
|------|-------------|----------------------|--------------------|---------------------------|--------|
| KS   | 88          | 48.3 (290/600)\textsuperscript{a} | PED                | 2.66                      | Feces  |
| KB   | 516         | 41.2 (140/340)       | PED                | 2.94                      | Feces  |
| KK   | 100         | 50.0 (10/20)         | PED                | 4.63                      | Feces  |
| KD   | 1000        | 66.6 (1000/1500)     | PED                | 2.97                      | Feces  |
| IY   | 56          | 100.0 (100/100)      | PED                | 4.82                      | Feces  |
| KL   | 100         | 20.0 (200/1000)      | TGE                | 2.87                      | Intestine |

\textsuperscript{a} The number of dead piglets/number of infected piglets.
the probe/primer concentrations delayed the Ct-value at entire high concentrations of transcript and lowered sensitivity toward both viruses. Standard conditions were used for the annealing temperature and cycle number (as stated in Section 2) for the multiplex real-time RT-PCR when evaluating the optimal working conditions for the primers and probes. The specificity of the assay was evaluated using other diarrhea-causing viruses such as enterovirus, rotavirus, and reovirus. No cross-reactivity was detected between the viruses in the multiplex real-time RT-PCR (data not shown).

3.2. Evaluation of multiplex real-time RT-PCR

Transcripts of TGEV (9 × 10^{12}) and PEDV (7 × 10^{12}) were used for quantifying the viral RNA load. Standard curves were plotted using 10-fold serial dilution stock solutions for the multiplex real-time RT-PCR (Fig. 1). As shown in Fig. 1, the standard curves of TGEV and PEDV were plotted with slopes of −3.214 and −3.219, respectively. The curves were used to determine the viral load of each sample, with detection limits at 9 × 10^{1} TGEV and 7 × 10^{1} PEDV copies.

The reproducibility and precision of the assay was confirmed by evaluating the variations in the intra-assay using transcript standards with 10^{7}, 10^{4}, and 10^{2} range copies for each virus in three independent runs with three samples.

The intra-assay variability of TGEV was low with a coefficient of variation (CV) ranging between 0.78 and 1.85% at 9 × 10^{2} copies, between 0.25 and 2.14% at 9 × 10^{4} copies, and between 0.53 and 2.22% at 9 × 10^{6} copies. The variability for PEDV was also low, in the range of 0.50–3.43% for each copy. Inter-assay variability was also evaluated using the same transcript standards in three assay runs performed over 3 days. The variability of CV was low, with values between 1.56 and 1.83% for TGEV and 2.21 and 3.33% for PEDV. The 10-fold serial dilutions of TCID_{50}/ml (between 10^{2} and 1.0) were assayed for both TGEV and PEDV. The 10^{5.0} TCID_{50}/ml TGEV had an RNA viral load of 10^{7.72} copies, while the 10^{4.0} TCID_{50}/ml PEDV had 10^{6.89} copies.

3.3. Application of multiplex real-time RT-PCR to clinical samples

Piglets inoculated with virulent TGEV shed the virus on either the 2nd or 3rd day, followed by profuse diarrhea that persisted until death 3–5 days after inoculation. One of the piglets died earlier than the other piglets, 3 days after inoculation. The load of TGEV shed in diarrhea was 10^{9.80} copies on the 2nd day, peaking at 10^{5.34} copies on the 4th day, and then decreasing until death (Fig. 2).

At peak levels, more PEDV was shed than TGEV from the 1st day after inoculation until death. Approximately 10^{6.78} copies of PEDV were shed in diarrhea on the 1st day, with the copy number decreasing over the course of the experiment (Fig. 2).

Once the assay conditions were established, the assay was applied to clinical samples from naturally infected piglets. The amount of PEDV RNA from clinical samples varied between 10^{2} and 10^{5} copies (Table 2). TGEV had 10^{2.87} copies by real-time RT-PCR (Table 2). Non-specific reactions sometimes occurred with samples with late Ct values (Ct > 35), which were considered artifacts. The viral load for only one clinical sample of TGEV was evaluated, since there have been only a few reported TGE outbreaks in Korea over the past several years.

4. Discussion

PED is a major cause of viral diarrhea in piglets that results in heavy economic losses in Korea each year. Data on PED outbreaks have been compiled by the Animal Infectious Disease Data Management System (AIMS) of the National Veterinary Research & Quarantine Service.
starting 1 or 2 days after inoculation with each virus, continuing until death. The viral loads of TGEV were first calculated on day 2, peaked on day 4, and then decreased until death. For PEDV, the viral load reached its highest level on day 1 and then decreased until death. In this study, the virus shedding time, viral loads, and period of viral persistence differed from those previously reported due to differences in titers, the size of the viral inoculum, and the ages of the experimental pigs. De Arriba et al. (2002) reported that PEDV peaked after 5 days as determined by fecal swabs and decreased for 8 days after inoculation.

The viral loads of PEDV and TGEV obtained from samples in naturally infected piglets varied by farm. According to Song et al. (2006), viral levels and piglet mortality show a low degree of correlation; some of our data were consistent with that report. Nevertheless, a correlation was noted: the larger the viral load detected by multiplex real-time RT-PCR, the higher the rate of piglet mortality. The results show that this assay can be used to determine viral loads in pig herds, which makes it an additional factor for evaluating the status of the affected farms. Furthermore, farms experiencing a disease outbreak can use information on viral loads to determine the most appropriate treatment, such as selection of vaccine type to protect their herds against future epidemics as described for quantitative RT-PCR using a PEDV internal control by Song et al. (2006). To optimize calculation of viral load by this assay, virus loads must be evaluated on farms where outbreaks are just beginning or ending to elucidate the potential transmission of both viral infections on pig farms.

It is very important to differentiate the two viral diarrhea diseases rapidly, as they can be difficult to distinguish clinically in infected piglets, so that veterinarians can quickly control outbreaks and understand the entire epidemiology. This study showed that multiplex real-time RT-PCR with standard curves is a useful method for differential and quantitative diagnosis of piglets infected with diarrhea-causing TGEV and PEDV. Therefore, this assay may be useful as an alternative to the current diagnostic tools used for the detection of viruses, which tend to underestimate the infection status of outbreaks on farms.

Acknowledgment

This study was financed by a grant from the National Veterinary Research and Quarantine Service, Korean Ministry of Agriculture and Forestry.

References

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Callebaut, P., Deboeck, P., Pensaert, M., 1982. Enzyme-linked immunosorbent assay for the detection of the coronavirus-like agent and its antibodies in pigs with porcine epidemic diarrhea. Vet. Microbiol. 7, 295–306.

Chae, C., Kim, O., Choi, C., Min, K., Cho, W.-S., Kim, J., Tai, J.H., 2000. Prevalence of porcine epidemic diarrhea virus and transmissible gastroenteritis virus infection in Korean pigs. Vet. Rec. 147 (21), 606–608.

Chen, R., Huang, W., Lin, Z., Zhou, Z., Yu, H., Zhu, D., 2004. Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus. J. Virol. Method 122, 57–61.
De Arriba, M.L., Carvajal, A., Pozo, J., Rubio, P., 2002. Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody response in serum of conventional pigs inoculated with PEDV. Vet. Immunol. Immunopathol. 84, 1–16.

Debouck, P., Penzaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV 777. Am. J. Vet. Res. 41, 219–223.

Dermody, T.S., Schiff, L.A., Nibert, M.L., Coombs, K.M., Fields, B.N., 1991. The S2 gene nucleotide sequences of prototype strains of the three reovirus serotypes: characterization of reovirus core protein sigma 2. J. Virol. 65, 5721–5731.

Frouhoffs, S., Totzke, G., Stier, S., Wernert, N., Rothe, M., Bruning, T., Koch, B., Sachinidis, A., Vetter, H., Ko, Y., 2002. A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction. Mol. Cell. Probe 16, 99–110.

Giulietti, A., Overbergh, L., Decallonne, B., Bouillon, R., Mathieu, C., 2001. An overview of real-time quantitative PCR applications to quantify cytokine gene expression. Methods 25, 386–401.

Jung, K., Chae, C., 2005. RT-PCR-based dot blot hybridization for the detection and differentiation between porcine epidemic diarrhea virus and transmissible gastroenteritis virus in fecal samples using a non-radioactive digoxigenin cDNA probe. J. Virol. Method 123, 141–146.

Kapke, P.A., Brian, D.A., 1986. Sequence analysis of the porcine transmissible gastroenteritis coronavirus nucleocapsid protein gene. Virology 151, 41–49.

Keyaerts, E., Vijgen, L., Maes, P., Duson, G., Neyts, J., Van Ranst, M., 2006. Viral load quantitation of SARS-coronavirus RNA using a one-step real-time RT-PCR. Int. J. Infect. Dis. 10, 32–37.

Kim, B., Chae, C., 2001. In situ hybridization for the detection of transmissible gastroenteritis virus in pigs and comparison with other methods. Can. J. Vet. Res. 65, 33–37.

Kim, O., Choi, C., Kim, B., Chae, C., 2000. Detection and differentiation of porcine epidemic diarrhea virus and transmissible gastroenteritis virus in clinical samples by multiplex RT-PCR. Vet. Rec. 146, 637–640.

Kim, S.Y., Song, D.S., Park, B.K., 2001. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. J. Vet. Diagn. Invest. 13, 516–520.

Kocherhans, R., Bridgen, A., Ackermann, M., Tobler, K., 2001. Completion of the porcine epidemic diarrhea coronavirus (PEDV) genome sequence. Virus Genes 23, 137–144.

Kweon, C.H., Kwon, B.J., Jung, T.S., Kee, Y., Hur, D.H., Hwang, E.K., Rhee, J.C., An, S.H., 1993. Isolation of porcine epidemic diarrhea virus (PEDV) in Korea. Korean J. Vet. Res. 33, 249–254.

Li, B., Gorziglia, M., 1993. VP4 serotype of the Gottfried strain of porcine rotavirus. J. Clin. Microbiol. 31, 3075–3077.

Ophuis, R.J., Morrissey, C.J., Boyle, D.B., 2006. Detection and quantitative pathogenesis study of classical swine fever virus using a real-time RT-PCR assay. J. Virol. Method 131, 78–85.

Paton, D., Ibita, G., Sands, J., McGoldrick, A., 1997. Detection of transmissible gastroenteritis virus by RT-PCR and differentiation from porcine respiratory coronavirus. J. Virol. Method 66, 303–309.

Pensaert, M.B., 1999. Porcine epidemic diarrhea. In: Straw, B.E., D’Allaire, S., Mengeling, W.L., Taylor, D.I. (Eds.), Diseases of Swine, 8th ed. Iowa State University Press, Ames, pp. 179–185.

Pritchard, G.C., Paton, D.J., Wibberley, G., Ibita, G., 1999. Transmissible gastroenteritis and porcine epidemic diarrhoea in Britain. Vet. Rec. 144, 616–618.

Pugnale, P., Latorre, P., Rossi, C., Crovatto, K., Pazienza, V., De Gottardi, A., Negro, F., 2006. Real-time multiplex PCR assay to quantify hepatitis C virus RNA in peripheral blood mononuclear cells. J. Virol. Method 133, 195–204.

Rodák, L., Válček, L., Šníd, B., Nevránková, Z., 2005. An ELISA optimized for porcine epidemic diarrhea virus detection in faeces. Vet. Microbiol. 105, 9–17.

Saif, L.J., Wesely, R.D., 1999. Transmissible gastroenteritis and porcine respiratory coronavirus. In: Straw, B.E., D’Allaire, S., Mengeling, W.L., Taylor, D.I. (Eds.), Diseases of Swine, 8th ed. Iowa State University Press, Ames, pp. 295–325.

Sánchez, C.M., Jiménez, G., Laviada, M.D., Correa, L., Suné, C., Bullido, M., Gebauer, F., Smerdou, C., Callebaut, P., Escrivan, J., Enjuanes, L., 1990. Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. Virology 174, 410–417.

Shibata, I., Tsuda, T., Mori, M., Oshino, M., Sueyoshi, M., Uruno, K., 2000. Isolation of porcine epidemic diarrhea virus in porcine cell cultures and experimental infection of pigs of different ages. Vet. Microbiol. 72, 173–182.

Song, D.S., Kang, B.K., Lee, S.S., Yang, J.S., Moon, H.J., Oh, J.S., Ha, G.W., Jang, Y.S., Park, B.K., 2006. Use of an internal control in a quantitative RT-PCR assay for quantitation of porcine epidemic diarrhea virus shedding in pigs. J. Virol. Method 133, 27–33.

Stram, Y., Kuznetzova, L., Guini, M., Rogel, A., Meirion, R., Chai, D., Yadin, H., Brenner, J., 2004. Detection and quantitation of akabane and aino viruses by multiplex real-time reverse-transcriptase PCR. J. Virol. Method 116, 147–154.

Sueyoshi, M., Tsuda, T., Yamazaki, K., Yoshida, K., Nakazawa, M., Sato, K., Minami, T., Iwashita, K., Watanabe, M., Suzuki, Y., Mori, M., 1995. An immunohistochemical investigation of porcine epidemic diarrhoea. J. Comp. Pathol. 113, 59–67.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Van Nieuwstadt, A.P., Cornelissen, J.B., Vreeswijk, J., 1988. Solid phase immune electron microscopy for diagnosis of transmissible gastroenteritis in pigs. Res. Vet. Sci. 44 (3), 286–294.

Van Rijn, P.A., Wellenberg, G.J., Hakez-van der Honing, R., Jacobs, L., Moonen, P.L., Feitsma, H., 2004. Detection of economically important viruses in boar semen by quantitative real-time PCR technology. J. Virol. Method 120, 151–160.

Wesley, R.D., Woods, R.D., Cheung, A.K., 1990. Genetic basis for the pathogenesis of transmissible gastroenteritis virus. J. Virol. 64, 4761–4766.