The development of a reverse transcriptase-polymerase chain reaction (RT-PCR) for diagnosis of porcine epidemic diarrhea virus

by

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INTRODUCTION

The United States is responsible for 10% of the world's pork, is the second largest pork exporting and producing country, and is the third largest importer of pork and pork-related products (USDA Foreign Agricultural Service, 1997). Pork production contributes approximately 64 billion dollars to the economic activity in the United States through annual farm sales, retail pork sales to consumers, corn and soybean purchases for feed, and over 600,000 pork production related jobs (National Pork Producers, 1998). Entrance of new disease agents into U.S. pig herds would cause severe economic losses. Severe outbreaks of explosive diarrhea resulting in high morbidity and mortality emerged in Europe in the 1970s, Japan in the 1980s, and Korea in the 1990s (Pensaert, 1992). Porcine epidemic diarrhea virus (PEDV) was isolated and found to be the causative agent of the diarrheal episodes. No documented cases of PEDV have occurred in the United States.

PEDV is a member of *Coronaviridae* of the order *Nidovirales* (Cavanagh, 1997). Members of the *Nidovirales* have a linear nonsegmented positive sense single stranded RNA genome of 13-31 kbp. The genome encodes five or six major proteins: 5'-polymerase, hemagglutinin-esterase (HE) that may not be present in some viruses, spike/peplomer (S), small membrane (sM), integral membrane (M), and nucleocapsid (N). Some genomes contain nonstructural proteins at various locations downstream of the polymerase gene. Members produce a 3' co-terminal nested set of subgenomic mRNAs from which only the 5' unique region is translated. Genomic RNA serves as the mRNA for translation of the polymerase gene. Virus particles are pleomorphic with prominent spikes.

PEDV and transmissible gastroenteritis virus (TGEV), both members of the *Coronaviridae*, are responsible for viral scours in pigs (Wood, 1979). Symptoms of both viruses includes a profuse watery diarrhea, vomition, depression, anorexia or subclinical infection. The severity of the disease is influenced by the age of the animal infected and the immune status of the herd. Morbidity and mortality rates range from as low as 1% to as high as 100%. Laboratory diagnosis is required to differentiate PEDV from TGEV.

Polymerase chain reaction (PCR) was first applied to the diagnosis of the DNA viruses of veterinary interest in the 1980s (Reubel and Studdert, 1998). PCR is a method that uses

specific oligonucleotides and a DNA polymerase to enzymatically amplify a DNA target in a sample by repetitive cycling (Mullis, 1990). A modification of PCR to include a second stage of amplification with oligonucleotides internal to the first pair increases the sensitivity and specificity of the test (Reubel and Studdert, 1998). The amplification of the first stage product ensures that the product is the DNA of interest. It is also useful in diluting any PCR inhibitors that may have been present in the test sample. Theoretically, as little as one copy of a DNA molecule has the potential to be amplified. The development of RT-PCR methods allowed the detection of RNA virus, greatly expanding the diagnostic capabilities of PCR (Kawasaki, 1990).

The gradual emergence of PEDV as the causative agent in outbreaks of porcine diarrhea in the major pork producing countries is a threat to the U.S. pork producers. The development of an accurate diagnostic test that can clearly differentiate between PEDV and TGEV is necessary to ensure the safety of the U.S. swine herds.

Thesis organization

This thesis consists of a general introduction, review of the literature, materials and methods, results and discussion, conclusions, summary and literature cited.

LITERATURE REVIEW

Emergence of PEDV

Porcine epidemic diarrhea virus (PEDV) causes acute outbreaks of diarrhea in pigs of all ages (Pensaert, 1992). The disease was first recognized in the early 1970s in feeder pigs in the United Kingdom (Oldham, 1972). Beginning in the mid-1970s, outbreaks of diarrhea in pigs of all ages were reported in Belgium and the United Kingdom (Pensaert and Debouck, 1978; Wood, 1977). Early in the 1980s, problems with diarrhea in swine herds were reported in the Federal Republic of Germany (Hess et al., 1980). Similar diarrhea episodes in pigs were reported from Canada (Turgeon, 1980; Dea et al., 1985) and Japan (Takahashi et al., 1983). Korea first recognized the presence of PEDV in the early 1990s (Kweon et al., 1993).

Morphology and growth characteristics

Electron microscopy (EM) of negatively stained feces or intestinal preparations from pigs experiencing diarrhea revealed pleomorphic coronavirus-like particles (CLP) that ranged from 80 to 190 nm in diameter with 10 to 20 nm projections (Chasey and Cartwright, 1978; Pensaert and Debouck, 1978). The CLP had electron dense central cores, double membranes, and distinct surface projections. The early attempts to characterize PEDV were hindered by the inability to propagate the virus in cell culture. The German isolate V215/78 and the Belgian isolate CV777 were adapted to the Vero cell line ATCC 1587 in the presence of trypsin (Hoffman and Wyler, 1988). Release of virus into the media peaked at 17 hours after adsorption. The virus repeatedly grew to high titers between $10^{5.3}$ and $10^{6.5}$ PFU per ml.

Other isolates have been adapted to cell culture. The Japanese isolate 83P-5 was adapted to serial passage on Vero cell culture by the addition of trypsin to the media (Kusanagi et al., 1992). Attempts to propagate the cell culture adapted isolate on primary pig cell cultures were not successful due to trypsin degeneration of the cell monolayer. Established cell lines resistant to the trypsin action (MA104, minature pig kidney, cloned porcine kidney cells, and embryonic swine kidney cells) would support virus replication only after the isolate had first adapted to the Vero cell line, evident by specific immunofluorescence

when reacted with rabbit anti 83P-5 serum. Diploid human lung cells adapted to grow in trypsin were able to support the CV777 Vero-cell-adapted isolate (Utiger et al., 1995). Unlike the other isolates, Korean isolate KPEDV-9 did not replicate to high titers until more than 90 passages in cell culture (Kweon, 1993).

Physical and chemical characteristics

The buoyant density of CV777, purified from an intestinal perfusate, was 1.17 gram per ml (Egbrink et al., 1988). Cell cultured adapted CV777 was confirmed to be an enveloped RNA virus by its ability to replicate in the presence of 50 and 200 μg per ml of 5-iodo-2'-deoxyuridine and loss of infectivity when treated with ether or chloroform (Hofmann and Wyler, 1989). Cell cultured adapted CV777 retains infectivity at temperatures of 50°C for 30 minutes, at pH ranges of 5 to 9 at 4°C and pH ranges of 6 to 8 at 37°C, and is not sensitive to freezing and thawing or ultrasonication. Cell cultured adapted CV777 is inactivated by heating at 60°C for 30 minutes and at pH values less than 4 or greater than 9. Cell cultured adapted CV777 has an average buoyant density of 1.18 gram per ml in sucrose (Hofmann and Wyler, 1988).

Analysis of cDNA sequence from the British isolate 1/87 and the Belgian isolate CV777 showed some common coronavirus motifs (Bridgen et al., 1993; Tobler et al., 1994; Duarte et al., 1994; Tobler and Ackermann, 1995; Tobler and Ackermann, 1996). Analysis revealed a 3' co-terminal nested structure that contained a hexameric sequence 5'-XUA(A/G)AC upstream of each potential coding region and the highly conserved coronavirus signal sequence, 5'-UGGAAGAGCG, located upstream of the 3' end. Five open reading frames (ORF) were identified, four of which encode the structural proteins: spike (S), integral membrane (M), small membrane (sM), and the nucleocapsid (N) proteins (Duarte et al., 1994). The proteins encoded by PEDV share homology with other coronaviruses (17-61%). The N protein contained an additional 45 amino acids rich in asparagine in the middle of the protein (Bridgen et al., 1993; Duarte et al., 1994). A potential internal ORF of 336 nucleotides was observed in the nucleocapsid gene. The S gene encodes a 1383 amino acid polypeptide with 29 potential glycosylation sites (Duarte and Laude, 1994). It lacks the

proteolytic sites found in some coronaviruses. An ORF between the S and M genes had the potential to encode a nonstructural protein (Duarte et al., 1994). The initiation codon of ORF3 was deleted in the cell culture adapted PEDV as compared to the wild type PEDV (Tobler and Ackermann, 1995). The role of ORF 3 in pathogenesis is not known. The sequence data and protein homologies suggested that PEDV belongs in the *Coronaviridae* family and is more closely related to human coronavirus 229 E (HCV 229E) than TGEV (Bridgen et al., 1993; Tobler et al., 1994; Duarte et al., 1994).

Molecular weight estimates have been reported for PEDV proteins. Three major proteins of 58 kd, 85 kd, 135 kd and a minor protein cluster of 20 kd, 26 kd, and 32 kd have been detected by radioimmunoprecipitation of PEDV purified from intestinal perfusate (Egbrink et al., 1988). Immunoblot analysis with porcine anti-PEDV serum only displayed reactivity with the 58 kd protein and the low molecular weight cluster. SDS-PAGE and immunoblot analysis of the Korean isolate KPEDV-9 and cell culture adapted PEDV (Hoffman and Wyler, 1988) detected differences between the proteins from the two isolates (Kweon et al., 1993). The estimated molecular weights were 120 kd, 88 kd, 74 kd, and 70 kd in both strains. Slight differences were detected in the lower molecular weight proteins. The PEDV cell culture adapted strain had proteins of 58 kd, 54 kd, 44 kd, and 33 kd. The isolate KPEDV-9 had molecular estimates of 54 kd, 46 kd, 40 kd, and 32 kd. The convalescent serum from the source of the KPEDV-9 isolate was tested by immunoblot analysis. The serum reacted with the polypeptides of the same molecular weight with the exception of the 120 kd protein (Kweon et al., 1993).

Antisera directed against synthetic peptides from PEDV encoded genes were used to study the proteins of CV777 (Utiger et al., 1995). Antiserum specific for the NH_2-terminus of the N protein reacted with a 57 kd ^{32}P-orthophosphate labeled CV777 protein. The same antiserum also reacted with an antigen preparation from a baculovirus expressing the N gene. Only one phosphoprotein was detected in the antigen preparation. The 57 kd protein is the N protein (Utiger et al., 1995). Analysis of ^{14}C-glucosamine labeled virion preparations revealed three species of glycoproteins (Utiger et al., 1995). A doublet at 180/200 kd may represent the spike protein. The other glycoproteins had estimated molecular weights of 27 kd and 21

kd. A monoclonal antibody (mcAb204) raised to PEDV has been shown to recognize a family of four polypeptides of estimated molecular weights of 27 kd, a 24/23 kd double band, and a 19 kd band (Utiger et al., 1994). These proteins appeared over time in cell cultures infected with PEDV. The 27 kd protein was first detected at 6 to 8 hours postinfection. The 24/23 kd doublet appeared between 8 and 10 hours postinfection. The 19 kd polypeptide appeared at 10 to 12 hours postinfection. Both the 27 kd and the 24/23 kd bands could be detected in crude virus preparations, while the 19 kd protein was not detected (Utiger et al., 1994). Rabbit anti-peptide serum and expression studies have identified the 27 kd protein as the M protein of PEDV (Utiger et al., 1995). The M protein is located with its NH_2 terminus facing towards the exterior of the virus particle and the COOH terminus facing towards the interior, which is typical for coronavirus membrane proteins. The M protein is N-linked glycosylated (Utiger et al., 1995).

Antigenic relatedness of PEDV with other coronaviruses

Antigenic relatedness of animal coronaviruses was assayed by immunofluorescent and immunoelectron microscopy (Pensaert et al., 1981). The antigen preparations for immunoelectron microscopy were cell culture passage viruses (TGEV, hemagglutinating encephalomyelitis (HEV), neonatal calf diarrhea coronavirus (NCDCV), canine cornavirus (CCV), allantoic fluid (infectious bronchitis virus [IBV]), and intestinal perfusion (PEDV). Immunoelectron microscopy did not detect any antigenic relatedness between PEDV and any of the other coronaviruses tested. A two-way antigenic relationship was shown for HEV and NCDCV (Pensaert et al., 1981). A one-way antigenic relationship was shown for TGEV and CCV; TGEV specific antiserum precipitated both TGEV and CCV. The antigen preparations for immunofluorescence were infected cell cultures (HEV, NCDCV, CCV), frozen sections (PEDV, TEGV), and brain smears (feline infectious peritonitis virus [FIPV]). Immunofluorescent assays did not detect any antigenic relationships between PEDV and the other coronaviruses tested (Pensaert et al., 1981). A two-way antigenic relationship was shown between TGEV and CCV and between HEV and NCDCV. A one-way antigenic

relationship was detected with FIPV and TGEV; positive immunofluorescence was detected when FIPV antiserum was reacted with FIPV and TGEV antigens (Pensaert et al., 1981).

Immunoblot analysis of coronavirus antigens was assayed to determine antigenic relationships between CCV, FIPV, PEDV, TGEV, and a putative mink coronavirus (MCV) (Have et al., 1992). Immunoreactivity with TGEV antigens N and M was detected with MCV-, FIPV-, CCV-, and TGEV-specific antisera. Immunoreactivity with CCV antigens N and M was detected with CCV-, FIPV-, and TGEV-specific antisera. A weak reactivity was detected with the anti-MCV serum (Have et al., 1992). Immunoreactivity with PEDV antigens N and M proteins was detected with MCV- and PEDV-specific antiserum. The PEDV antiserum also reacted with the spike protein of PEDV (Have et al., 1992). A weak reactivity with PEDV N protein was detected with CCV- and TGEV-specific antisera. A weak cross-reactivity was detected between FIPV and PEDV when PEDV infected gut sections were reacted with ascites fluids from a cat that had succumbed to FIPV (Yaling et al., 1988).

Human and feline sera were tested for cross-reactivity with PEDV antigens by ELISA (Utiger et al., 1995). Antigen recognition occurred with 4% of the human sera and 20% of the feline sera. The HCV-229 E and FIPV immune status of the sera was not known.

Epidemiology

PEDV is transmitted by the oral-fecal route (Pensaert, 1992). The incubation period lasts 5 to 8 days in naturally acquired infections (Wood, 1977). Diarrhea lasts for 3 to 5 days, but the animals usually remain listless for up to a week (Wood, 1977; Pensaert and Debouck, 1978). Morbidity within a unit ranged from 50 to 95%. Mortality was usually restricted to young animals and in severe cases approached 40% (Wood, 1977; Pensaert and Debouck, 1978). In units of high mortality, the sow and the litter scoured simultaneously.

Serum samples collected from 158 herds with histories of diarrhea outbreaks (1979-1981) were assayed for PEDV antibodies by indirect immunofluorescent staining of PEDV positive tissue sections and for TGEV antibodies by virus neutralization (Prager and Witte, 1983). PEDV and TGEV were detected in 22% and 23% of the herds. Antibodies to both

viruses were detected in 4% of the herds. Random serum samples from sows and fattening pigs (2,853) were examined by indirect immunofluorescent staining (IFATS) of PEDV positive tissue sections for the presence of PEDV antibodies (Hofmann and Wyler, 1987). PEDV antibodies were detected in 1.6% of the herds. PEDV antibodies were detected in 50% (7 out of 14) of the fattening herds with diarrhea problems. In the positive herds, the number of positive animals ranged from 17% to 100%. An endemic form of PEDV developed in a breeding and finishing herd following an outbreak of PEDV (Pijpers et al., 1993). PEDV seronegative replacement gilts entered the herd monthly. Virus was mainly detected in replacement gilts and 6- to 10-week-old pigs (Pijpers et al., 1993). Every group of replacement gilts, a total of 87% of the animals, seroconverted to PEDV.

The ELISA was used to estimate the prevalence of PEDV infection in pigs shortly after entering into fattening units (Van Reeth and Pansaert, 1994). At the time of entrance, 93% of the replacement pigs had no measurable PEDV antibodies. PEDV antibodies were detected in pigs in 41% of the units. Episodes of diarrhea occurred during the first week after entrance of replacement pigs into the antibody positive units. In the units that seroconverted, 89 to 100% of the animals had detectable levels of antibodies (Van Reeth and Pansaert, 1994). Morbidity approached 100% in two of the groups while others remained unaffected. PEDV antigen was detected in 4 out of the 7 units tested and the range of antigen-positive animals was 10 to 67%.

Pathogenesis

Experimental oro-nasal inoculation of pigs with the CV777 isolate resulted in diarrhea in 2 to 3 days (Chasey and Cartwright, 1978; Pensaert and Debouck, 1978; Debouck and Pensaert, 1980; Debouck et al., 1981). The virus passed through the tonsils and stomach and infected the villous epithelium of the small intestine, large intestine, and mesenteric lymph node. PEDV specific immunofluorescent staining of the cytoplasm of epithelial cells covering the small intestine was first detected at 18 hours after inoculation. Similar amounts of staining were seen in all parts of the small intestine. No histological changes were observed in inoculated pigs during the incubation period of 24 to 38 hours (Ducatelle et al., 1981). The

onset of diarrhea and positive immunofluorescence coincide with histological changes. Histological lesions were first observed in pigs 24 hours postinoculation and 2 to 6 hours after the onset of diarrhea (Pospischil et al., 1981; Coussement et al., 1982). The villi of the jejunum appeared shortened and occasionally fused. Pores and clefts had developed between the epithelial cells (Ducatelle et al., 1981). The enterocytes were extremely vacuolated. Desquamation of the epithelial cells caused a release of cell debris into the intestinal lumen (Debouck and Pensaert, 1980; Debouck et al., 1981; Pospischil et al., 1981; Coussement et al., 1982). The enterocytes of the crypt epithelium were not infected and maintained the ability to replace infected epithelial cells. CV777 antigens were detected in 90-100% of the intestinal epithelial cells in pigs 4 days after the onset of diarrhea. The increase of immunofluorescencing cells was attributed to the infection of regenerated epithelial cells (Debouck and Pensaert, 1980; Debouck et al., 1981).

Viral particles were seen at 18 hours after inoculation in the absorptive cells covering the villi of the terminal part of the jejunum by electron microscopy (Ducatelle et al., 1981; Ducatelle et al., 1982). Viral particles were seen in all of the infected pigs examined from the onset of diarrhea and continued for 4 days (Chasey and Cartwright, 1978; Ducatelle et al., 1981; Ducatelle et al., 1982). Viral infection of the epithelial cells resulted in swelling of the mitochondria, distention of the endoplasmic reticulum, and loss of electron density of the cytoplasm. Shortening and loss of microvilli was observed (Chasey and Cartwright, 1978; Ducatelle et al., 1981; Ducatelle et al., 1982). Three types of virus containing cells were detected (Ducatelle et al., 1981; Ducatelle et al., 1982). These included the epithelial cells of the villi, cubodial cells, and columnar cells with highly vacuolated cytoplasm and irregular villi. The viral particles ranged from 60-90 nm in diameter with a 40-70 nm dense inner core. Virus assembly occurred by budding through the cytoplasmic membranes into the cisternae of the smooth endoplasmic reticulum (Ducatelle et al., 1981; Ducatelle et al., 1982). In vivo morphogenesis occurred in two phases. The first phase of attachment, entry, replication, and budding occurs rapidly. The second stage or late phase is a result of infection of regenerated epithelial cells and is a slower phase.

A decrease in the cell function of the intestines was documented. Few fat droplets were observed in the absorptive cells of the colonic surface epithelium and a decrease in staining of intestinal enzymes was noted. The decrease in the alkaline phosphatase correlates with the shortening of microvilli, and the decrease in acid phosphatase may reflect a decrease in lysosomes or lysosomal actions (Pospischil et al., 1981; Coussement et al., 1982).

Control

The attenuated strain, KPEDV-9 (Kweon et al., 1994), was used as a vaccine during outbreaks of PEDV (Kweon et al., 1996). Pregnant sows received two intramuscularly inoculations with 10^6 $TCID_{50}$ per ml at 2 to 3 week intervals. The animals were housed on eight separate farms with and without a history of PEDV. Antibody responses were monitored by ELISA. No clinical disorders were observed after vaccination. The study was not controlled and the effects of vaccination on the immune status could not be evaluated.

Laboratory diagnosis

Clinical signs are not sufficient for a preliminary diagnosis of PEDV. PEDV and TGEV cause similar clinical signs in infected pigs. Laboratory diagnosis is necessary to differentiate between the two viruses. The gold standard for PEDV diagnosis is the isolation of PEDV in cell culture or the identification of PEDV antigens in tissue sections or fecal suspensions (Pensaert, 1992). A variety of antibody detection assays have been utilized to detect PEDV-specific antibody titers (Prager and Witte, 1981; Witte and Prager, 1987; Callebaut et al., 1982; Hofmann and Wyler, 1990; Knuchel et al., 1992; Van Nieuwstadt and Zetstra, 1991). A rise in PEDV antibody titers in paired serum samples is also used for diagnosis of PEDV.

The virus isolation technique is labor intense (Hofmann and Wyler, 1988). Fecal or intestinal suspensions are adsorbed onto trypsin adapted cells. Three or more blind passages and daily media changes are necessary. Cells are observed for syncytia formation and virus is identified by immunfluorescence. The procedure can take up to 3 weeks and only a limited

number of isolates have been obtained (Hofmann and Wyler, 1988; Kusanagi et al., 1992; Kweon et al., 1993).

Fluorescent antibody staining of cryostat sections (FATS)

Immunofluorescent staining techniques have been utilized for the detection of PEDV in cryostat sections of intestines from experimentally infected pigs (Debouck et al., 1981; Kweon et al., 1994). PEDV antigens are detected by direct staining with fluorescein isothiocyanate (FITC)-labeled anti-PEDV serum. A comparison was made between FATS and EM detection (Debouck et al., 1981). The epithelial cells covering the small intestinal villi exhibited positive fluorescence in all 13 experimental pigs sampled at different stages during the first 4 days of diarrhea. When examined by EM, consecutive fecal samples collected from the same pig were sometimes positive and sometimes negative. If multiple samples from the same pig were examined by EM, coronavirus particles could be detected in 90% of the experimental pigs (Debouck et al., 1981). EM diagnosis of cornavirus is of limited value as it does not differentiate between PEDV and TGEV, while FATS is only useful for post mortem testing.

Streptavidin-biotin immunohistochemical technique

The streptavidin-biotin immunohistochemical technique (SAB) was used to examine formalin-fixed tissues from an outbreak of PEDV (Sueyoshi et al., 1995). Positive staining of the cytoplasm was detected in the enterocytes of the small intestine, cecum, and colon. The positive cells were scattered along the epithelial layer with distinct borders between uninfected and infected cells. Infected cells were seen in the crypts. The distribution of the positive staining cells varied with the degree of atrophy of the intestinal tract. SAB is only useful for post mortem testing.

A double sandwich ELISA for the detection of PEDV antigens in feces

An ELISA method has been developed for the detection of PEDV antigens in feces (Callebaut et al., 1982). Viral particles in fecal suspension were trapped by a swine anti-PEDV serum bound to the plate and detected with an enzyme labeled swine anti-PEDV serum. Bound particles were detected in a blocking assay by 50% reduction of control. ELISA and EM results were compared on samples from seven experimental animals. Virus particles were detected by EM on postinoculation days 1 through 4; samples from day 2 were not tested. Virus particles were observed in 6 out of 16 samples from days 1 through 4. PEDV antigens were detected by ELISA on postinoculation days 3 through 7. PEDV antigens were detected in 14 out of 20 samples tested.

The PEDV antigen detection ELISA has been modified (Carvajal et al., 1995). Monoclonal antibodies with different specificities were used as the capture and detecting antibodies (Van Nieuwstadt and Zetstra, 1991). PEDV virus was detected in experimentally infected pigs from day 3 to day 11. No virus was detected on days 12 through 14 (Carvajal et al., 1995).

IFATS for the detection of PEDV-specific antibodies

An indirect immunofluorescent staining of tissue sections for the detection of PEDV-specific antibodies was applied to cryostat sections from pigs experimentally inoculated with PEDV (Prager and Witte, 1981). Indirect staining showed poor reproducibility because of the heat lability of the antigen and antibody. PEDV antibodies seemed to be affected by storage conditions. Antibody half-life values, when assayed by indirect staining, were 1 day at 37°C, 2 days at 20°C, and 12 days at 4°C. Heat fixation destroyed the antigens. Acetone-fixed tissue sections were stable for 2 days at -70°C. Tissues were stored in egg whites for long-term storage. With this assay, antibodies were first detected in experimentally inoculated pigs on day 10 postinoculation in one animal and day 12 postinoculation in three animals (Prager and Witte, 1981). Antibodies were not detected in one animal until day 17 postinoculation, but this animal did not exhibit clinical signs until 10 days postinoculation. IFATS testing detected a secondary immune response in pigs that were

reinfected. A blocking FATS assay has also been evaluated on samples from experimentally infected pigs (Witte and Prager, 1987). Antibodies were detected in the experimental animals 10 to 21 days after exposure, and titers peaked 17 days later. Antibodies were detected up to 54 weeks postexposure.

Double sandwich ELISA for the detection of PEDV-specific antibodies

An ELISA method utilizing the double antibody sandwich technique has been developed for the detection of PEDV antibodies in convalescent serum (Callebaut et al., 1982). Test sera are first reacted with a fecal suspension containing CV777 virus particles; CV777 antigen not blocked by antibody was captured on the ELISA plates and detected by an enzyme labeled swine anti-PEDV serum. Samples that exhibited 50% reduction of the control were considered antibody positive. The first measurable antibodies were detected in experimentally inoculated pigs on day 43 postinoculation (Callebaut et al., 1982). In a separate study, low antibody titers were detected by the ELISA at 3 weeks after infection and reached maximal titers at 4 to 6 weeks (Debouck and Pensaert, 1984). A second inoculation 5 months later produced diarrhea and a secondary immune response evident by an increase in antibody titers detected by ELISA (Debouck and Pensaert, 1984).

Direct ELISAs for the detection of PEDV-specific antibodies

Three different methods for purification of cell culture propagated PEDV have been evaluate for ELISA (Hofmann and Wyler, 1990). The antigens were prepared by sucrose gradient, sucrose cushion, or NP40 solubilization. The optimal antigen was produced by the ultracentrifugation through a sucrose cushion. An ELISA assay was established and used to evaluate 1,024 pig sera previously tested by IFATS (Hofmann and Wyler, 1990). There was 77% agreement between the two tests. Of the IFATS negative sera, the ELISA identified 9% of the samples as questionable for PEDV antibody and 15% of the samples as positive for antibodies. Of the IFATS positive sera, the ELISA identified 11% as negative for PEDV antibodies and 4% as questionable. The immune status of 8% of the total sera tested was questionable by ELISA.

The N and S proteins of PEDV were separated based on their specific solubility (Knuchel et al., 1992). The S protein was soluble at pH 4, and the N protein was soluble at pH 9. The proteins were used in an ELISA format to determine the development of antibodies to the specific proteins. No antibodies were detected in the preimmune sera. Antibodies to both proteins were detected at 3 weeks postinoculation. Antibodies against the S protein remained detectable for longer periods of time than antibodies to the N protein.

A modified double sandwich ELISA for the detection of PEDV-specific antibodies

An improved blocking ELISA has also been developed (Van Nieuwstadt and Zetstra, 1991). This ELISA was similar to the ELISA developed by Callebaut (Callebaut et al., 1982), but monoclonal antibodies with different specificities were used as the capture and detecting antibodies and cell culture propagated PEDV was used as the antigen. A comparison was made to a fixed cell ELISA (IPT). In the fixed cell ELISA, PEDV-infected cells were fixed, reacted with the test serum, reacted with the conjugated detection antibody, reacted with substrate, and read microscopically (Van Nieuwstadt and Zetstra, 1991). The ELISA test did not detect any antibodies in any of the negative samples tested (127 sera). The IPT test did not detect any antibodies in 126 of the negative samples. One negative sample stained cellular proteins. The ELISA test detected antibodies in 87 out of 89 serum samples from experimental pigs 7 or more days postinoculation (Van Nieuwstadt and Zetstra, 1991). The IPT test detected antibodies in 80 of the samples. An analysis of serum samples from postexposure days 7 through 14 revealed that the ELISA test found 8 out of 11 samples containing antibodies. The IPT test did not detect antibodies in any of the sera. The IPT test was specific for IgG while the blocking ELISA will detect both IgG and IgM antibodies (Van Nieuwstadt and Zetstra, 1991).

Detection of PEDV RNA in fecal samples

RT-PCR was examined as a method to detect PEDV in fecal samples (Kweon et al., 1997 and Ishikawa et al., 1997). A primer pair derived from the M gene was used to amplify a 334 base pair fragment from a cDNA generated with a M gene-specific primer (Kweon et

al., 1997). The RT-PCR detected up to 10^4 $TCID_{50}$ per ml of PEDV. PEDV was detected in 10% suspensions from pooled fecal samples collected on days 1 to 6 after inoculation with PEDV. Another study used a larger segment for amplification by RT-PCR. A primer pair was derived from the M gene sequence to amplify a 854 base pair fragment (Ishikawa et al., 1997). The RT-PCR detected 100 $TCID_{50}$ of PEDV in a sample spiked with PEDV. The RT-PCR was compared to virus isolation and SAB (Ishikawa et al., 1997). In eleven clinical samples, four samples were positive for PEDV by all three techniques. The ability of RT-PCR, FATS, ELISA, and SAB to detect PEDV in samples collected from the intestinal tract at 6 to 60 hours after onset of diarrhea was compared (Guscetti et al., 1998). Large amounts of virus were present in the gut during the 54-hour period. The SAB technique detected PEDV antigens in all but two samples tested; these two samples were the cecum and colon (Guscetti et al., 1998). The ELISA test detected PEDV at all 6 time points sampled. The ELISA was tested in triplicate for each time point. On four of the time points, only two of the samples tested positive. The RT-PCR gave negative results when other tests were positive, so the test was discontinued (Guscetti et al., 1998).

Study Objectives

Because PEDV is seldom isolated from clinical specimens and FATS can only be performed on post mortem samples, the objective of this study was to develop a fast and accurate RT-PCR that would detect PEDV RNA in clinical samples. Since only one serotype of PEDV is recognized (Pensaert, 1992), a second objective was to utilize RT-PCR to look at genetic diversity in the spike protein of PEDV.

MATERIALS AND METHODS

Cells and virus

The PEDV strains used in this study were CV777 and NK94P6. Cell culture adapted CV777 virus was propagated on the Vero 76 (ATCC 1587) cell line and NK94P6 was propagated on the KY-5 cell line (National Institute of Animal Health, Ibaraki, Japan) derived from Vero cells. PEDV infectious inoculum was obtained from the Central Veterinary Laboratory, Weybridge, the United Kingdom, and the Laboratory of Veterinary Virology, University of Gent, Belgium (Pensaert). The propagation of Vero cells and PEDV was performed as previously described (Hofmann and Wyler, 1988). The Miller TGEV 88-13 challenge strain (CVLB) was used for the swine inoculations. Swine testicle cells (ST) and the Purdue strain of TGEV were used for the serum neutralization tests. Other viruses used in assaying the specificity of the PCR primers were TGEV (Purdue strain), feline infectious peritonitis viruses (FIPV) type I (UCD-1 strain) and type II (1146 strain), CCV (NVSL field isolate), porcine rotavirus (Bohl strain), and bovine viral diarrhea virus (BVDV) C24V strain.

Preparation of PEDV stocks

Three 150 cm^2 flasks of Vero 76 or KY-5 cells were seeded at a cell density to produce a monolayer after 48 hours of incubation at 37°C. Forty-eight hours after seeding, monolayers were washed twice with MEM containing 10% tryptose phosphate broth and 10 μg/ml of trypsin (VM). One ml of a 1:10 dilution of virus was inoculated onto the cell monolayer and allowed to absorb at 37°C for 1 hour. VM was added directly to the flask without removal of the viral inoculum. Cells were incubated until 90% of the cells exhibited cytopathic effects (CPE). Flasks were frozen at -70°C and thawed at room temperature. Virus-containing supernatant was centrifuged for 15 minutes at 800 X *g* (Beckman J-6) at 4°C to remove cell debris. Viral stocks were aliqouted and stored at -70°C.

Titration of PEDV

Vero 76 or KY-5 cells were seeded into 24-well culture vessels and incubated at 37°C for 48 hours. Virus stocks were serially diluted tenfold in VM. Cell monolayers were washed twice with VM; all media were removed from the wells. Three wells were used for each dilution of virus. Wells were inoculated with 0.2 ml of the virus dilution and incubated at 37°C in an atmosphere of 5% carbon dioxide and high humidity for one hour. Cells were washed one time, and 0.5 ml VM replacement media were added. Cells were incubated for 3 to 4 hours after which 0.5 ml of MEM containing 5% FBS were added to each well. These media were replaced after an additional 48 hour incubation with VM. Cytopathic effect was recorded at 72 hours, and 50% tissue culture infectious dosage endpoints were calculated by the Reed-Muench method (Reed and Muench, 1938).

Preparation of PEDV antigen for western blotting

Vero 76 cells were seeded into 850 cm^2 roller bottles at a cell density to reach confluency at 48 hours. Cells were washed twice with VM, all media were removed, and cells were inoculated with 10 ml of a 1:10 dilution of stock CV777 virus. Virus was allowed to absorb for 1 hour at 37°C with gentle rolling. An additional 100 ml of VM were added to each bottle. Cells were incubated until 80-100% CPE was observed. Virus-containing supernatant was centrifuged for 15 minutes at 800 X *g* (Beckman J-6) at 4°C to remove cell debris. The supernatant was layered over a 40% glycerol cushion and ultracentrifuged in a SW28 (Beckman) rotor at 100,000 X *g* for 90 minutes (Hofmann and Wyler, 1990). The supernatant was decanted, and the tube was allowed to drain. A sterile gauze was used to remove any residual liquid at the lip of the tube. The viral pellets from six tubes were suspended in a total volume of 1.5 ml of 0.01 M PBS pH 7.2. The viral suspension was centrifuged in a TL-100.3 (Beckman) rotor for 30 minutes at 27,000 X *g*. The supernatant was removed and the pellet was suspended in 500 µl of HS/TNE (500 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% Triton X-100 and Complete® protease inhibitor cocktail-Boehringer Mannheim) buffer, incubated on ice for 15 minutes, and sonicated. The viral lysate was diluted twofold, treated with an equal volume of 2X Laemmli treatment buffer, and

boiled for 5 minutes. The antigen was stored at -20°C. Uninfected cells were processed in a similar manner.

Production of PEDV hyperimmune antiserum

Gnotobiotic pigs (22 days old) were inoculated intramuscularly with 5 ml of a lipid emulsion of betapropiolactone inactivated PEDV-containing fecal suspension and the adjuvant avridine (6mg/ml). On day 16, pigs were inoculated intramuscularly with 3.5 ml of PEDV-containing fecal suspension emulsified with lipid and avridine. On day 41, pigs were inoculated with 10 ml of PEDV-containing fecal material. Pigs received PEDV intravenously on days 50 and 63. The pigs were euthanized and exsanguinated on day 73, and the blood was processed for serum. The serum was stored at -20°C.

Indirect immunoperoxidase or immunofluorescence antibody detection

Vero-76 cells were seeded into 96-well microtiter plates or chamber slides and incubated for 48 hours. Cells were washed twice with VM, inoculated with PEDV at a multiplicity of infection (MOI) of 0.01, and incubated at 37°C for 48 hours. The media were removed, and the infected monolayers were washed with 0.01 M PBS, pH 7.2 and fixed with 70% acetone for 15 minutes. The acetone was removed, and the plates or slides allowed to air dry. Plates or slides were stored at -20°C until needed. Test sera were diluted in 0.01 M PBS containing 0.05% Tween 20. Plates/slides were brought to room temperature and rinsed once with 0.01 M PBS with 0.05% Tween 20. Diluted test serum, 100 µl, was added to the appropriate well. Plates/slides were incubated at 37°C in an atmosphere of high humidity for 1 hour. Plates/slides were washed 5 times with 0.01 M PBS with 0.05% Tween 20. Two different detection systems were utilized. Horse-radish peroxidase labeled recombinant Protein-G (HRP-labeled rec-Protein G - Zymed) at 1:1000 dilution or FITC-labeled goat anti-porcine immunoglobulin, at 1:75 dilution, was added to the appropriate wells, and the plates/slides were incubated for 1 hour at 37°C in an atmosphere of high humidity. Plates/slides were washed 5 times with 0.01 M PBS with 0.05% Tween 20. The last wash was left on wells reacted with FITC- labeled goat anti-porcine immunoglobulin. One hundred

microliters of substrate, 3-amino-9-ethylcarbazole, (AEC,Vector) were added to plates/slides treated with HRP-labeled rec-Protein G and incubated at 37°C for 10 minutes. Both sets of plates/slides were washed with distilled water, air dried, and examined microscopically.

Serum neutralization test for transmissible gastroenteritis virus

A microtitration format was used and paired wells were used for each dilution. MEM with Earle's salts and 3X antibiotics (penicillin 250 units/ml, streptomycin 150 µg/ml, gentamicin 75 µg/ml, amphotericin B 3 µg/ml) was used as the diluent. Serum was diluted 1:2 and heat inactivated at 56°C for 30 minutes. Fifty microliters of the inactivated serum (1:2) were added to two wells of a 96-well microtiter plate. Serial twofold dilutions were performed with a multichannel pipette. Stock virus was diluted to a concentration of 100 to 300 $TCID_{50}$ per 25 µl of inoculum. The serum dilutions were inoculated with 25 µl of virus per well, vibrated to mix the virus and serum dilutions, and incubated for 1 hour at 37°C. Swine testicle cell suspension, 150 µl, was seeded directly into the serum-virus mixture. The plates were incubated at 37°C in 5% CO_2 and high humidity for 3 to 6 days. Plates were examined for CPE. The highest serum dilution demonstrating 100% neutralization of viral infectivity in duplicate wells was designated as the serum neutralizing titer.

RNA extraction

Method 1: Trizol® LS reagent (Gibco BRL) was used for the extraction of RNA. A 300 µl sample of test specimen was added to a microcentrifuge tube containing 900 µl of Trizol® LS. The sample was vortexed and incubated for 5 minutes at room temperature; 240 µl of chloroform was added to each tube and vortexed. After a 15-minute incubation at room temperature, the samples were vortexed followed by centrifugation at 10,000 X *g* (IEC microcentrifuge) for 5 minutes to separate the phases. The aqueous phase was transferred to a new tube containing 600 µl of isopropyl alcohol and 1.5 µl of yeast t-RNA. The tube was inverted 10 times and incubated at room temperature for 10 minutes. The RNA was pelleted by centrifugation at 16,250 X *g* (IEC microcentrifuge) for 15 minutes. The RNA was washed once with 80% cold ethanol. RNA was stored at -70°C in 100% ethanol. Pelleted RNA was

air dried for 10 minutes and solubilized in 15 or 20 µl of RNase/DNase-free water by incubating at 56°C for 10 minutes and immediately placed on ice.

Method 2: Catrimox-14™ (Iowa Biotechnology Corp/Qiagen) was used for the extraction of RNA (Macfarlane and Dahle, 1997). A 300 µl sample of test specimen was added to a tube containing 1 ml of Catrimox-14™ and 5 µl of yeast t-RNA, vortexed, and incubated at room temperature for 10 minutes. The sample was centrifuged at 16,250 X *g* (IEC microcentrifuge) for 10 minutes. The supernatant was discarded; the pellet was suspended in 500 µl of 2 M LiCl in 35% ethanol and centrifuged at 16,250 X *g* (IEC microcentrifuge) for 10 minutes. The RNA pellet was washed with 80% ethanol. RNA was stored at -70°C in 100 % ethanol. Pelleted RNA was air dried for 10 minutes and dissolved in 15 µl of RNase/DNase-free water by incubating at 70°C for 10 minutes and immediately placed on ice.

RT-PCR

Three sets of nested primers were designed to amplify regions in the nucleocapsid and spike genes of PEDV. Sequence information was obtained from GenBank accession numbers Z14976 and Z25483. A primer pair was designed to amplify a region in the spike gene of TGEV Miller strain. Sequence information was obtained from GenBank accession number S51223. Primer sequences are listed in Table 1. The first stage of the nested PEDV reaction, a single tube RT-PCR, was optimized with 2 µl of RNA extracted from cell culture adapted virus. Master mixes were prepared that contained 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.2 mM of each dATP, dCTP, dGTP, dTTP; 10 units of RNasin® (Promega), varying concentrations of $MgCl_2$ (1.8 mM, 2.0 mM, 2.5 mM, 3.0 mM), varying concentrations of primer (0.25 µM, 0.5 µM, 0.75 µM, 1.0 µM), 25 or 50 units of reverse transcriptase (Superscript®- Gibco BRL), and 1.25 units or 2.5 units of Amplitaq Gold polymerase (Perkin Elmer). Annealing temperatures of 53°C and 58°C were evaluated. Reactions were carried out in two different thermocyclers simultaneously.

Table 1. Primer sequences used in RT-PCR for the detection of PEDV

Stage	Position[a]	Sequence
PEDV primers		
Set 1: Nucleocapsid	(Z14976)[b]	
First	253	5'-GGC ATT TCT ACT ACC TCG GA
First	992	5'-ATA GCC TGA CGC ATC AAC AC
Second	447	5'-AAC ACA CCT CCT GCT TCA CG
Second	787	5'-TCC TGC TTA GGC TTC TGC TG
Set 2: Spike Gene	(Z25483)[b]	
First	61	5'-CAG CCT ACC ACA AGA TGT CA
First	724	5'-TAC ATT GGC AGC GTA ACC AG
Second	218	5'-GCT AGT GGC GTT CAT GGT AT
Second	442	5'-TAG GCA GTT ACG ACC TGT TG
Set 3: Spike Gene	(Z25483)[b]	
First	1581	5'-CCA ATC TCG TTG CAT CTG AC
First	2127	5'-ACA TAT GCA GCC TGC TCT GA
Second	1784	5'-TTG GCT GGT GCT TGT ACC AT
Second	1895	5'-GCG TGC CAG TAA TCA ACT CA
TGEV primers		
Set 1: Spike Gene	(S51223)[b]	
First	1018	5'-ctc gct cga gGG TAA CCA TTG GAA TCT CAT
First	1623	5'-gcc agg cct aac AGG ATT AAA CCA CCA AAG

[a]The position is based on the sequence deposited in GenBank data base.
[b]The numbers correspond to GenBank data base accession numbers.

Reaction conditions were as follows: 1 cycle (reverse transcription) of 50°C for 60 minutes, 95°C for 10 minutes; 40 cycles (PCR) of 94°C for 25 seconds, 53°C or 58°C for 20 seconds, 73°C for 60 seconds; 1 cycle (final extension) of 73°C for 3 minutes. PCR products were evaluated by gel electrophoresis, ethidium bromide staining, and UV visualization. The second stage reaction was optimized with 1.5 µl of the optimized first stage PCR product. Master mixes contained 1X PCR buffer, 0.2 mM of each of the dNTPs, varying concentrations of $MgCl_2$ (1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM, 4.0 mM), varying concentrations of primer (0.2 µM, 0.4 µM, 0.6 µM), and 1.25 units of Amplitaq Gold polymerase. Reaction conditions were as follows: 1 cycle of 95°C for 9 minutes; 35 cycles of 94°C for 25 seconds, 60°C for 25 seconds, 73°C for 45 seconds; 1 cycle of 73°C for 3 minutes. The sensitivity of the primers was assayed by amplification of tenfold dilutions of CV777 virus. The specificity of the primers was evaluated by amplification of RNA from TGEV (Purdue strain), FIPV types I (UCD-1 strain) and II (strain 1146), CCV (NVSL field isolate), porcine rotavirus (Bohl strain), and BVDV (C24V strain).

Electrophoresis

LE agarose, 1.6 grams, was allowed to swell in 80 ml of 0.0445 M Tris-borate, 0.0445 M boric acid, 0.001 M EDTA (0.5 X TBE) buffer. The mixture was heated in a microwave oven for 1 minute intervals until the agarose was dissolved (Maniatis et al., 1989). The agarose was allowed to cool in a 65°C waterbath for 15 minutes, and ethidium bromide was added to a final concentration of 0.5 µg/ml. The solution was poured into a wide mini apparatus (BioRad) containing a 20- or 30- well comb. The gel was allowed to solidify for 30 minutes at room temperature. The set gel was placed in the electrophoresis tank containing 600 ml of 0.5 X TBE buffer with 0.5 µg/ml of ethidium bromide. Ten microliters of PCR product were mixed with 3 microliters of sample loading buffer. Samples were loaded into the gels along with a molecular marker and electrophoresed at 70 volts for 1-1/2 hours. The PCR products were visualized on a UV light box and photographed.

Direct sequencing of PCR products

PCR products that migrated at the correct molecular size were sequenced to confirm their identity. A PCR product was added to a Microcon™ 100 concentrator RNase/DNase-free H_2O added to bring the volume to 500 µl. The concentrator was centrifuged for 8 minutes at 500 x *g*. The flow through was removed; 500 µl of H_20 was added and centrifuged for an additional 8 minutes. The filter was washed with 20 µl of H_2O, inverted into a clean tube and centrifuged for 3 minutes. The PCR product was quantitated by spectrophotometer and diluted to the appropriate concentration for sequencing (5 ng/100 bases in 2 µl). The sequence of the PCR product was determined by the dideoxy sequencing method (Sanger et al., 1977) with automated cycle sequencing (Applied Biosystems) at the DNA Synthesis and Sequencing Facility at Iowa State University.

Animal inoculations (Table 2)

Group A: Two caesarean-derived, colostrum-deprived (CDCD) pigs (pig 1 & pig 2) were orally inoculated at 19 days of age with 5 ml of fecal material containing PEDV (Weybridge). Fecal samples were collected twice per day. Pigs were euthanized 72 hours after inoculation. The intestinal contents from the small and large intestine were collected.

Group B: CDCD pig 3 was inoculated with two different PEDV preparations: a 10% suspension of intestinal contents from pig 1 (filtered through a 0.22 µm filter) and fecal material containing PEDV (Weybridge). Pig 3 was orally inoculated at 5 days of age with 5 ml of the 10% intestinal suspension (pig 1) and at 7 days of age with 5 ml of fecal material containing PEDV (Weybridge). The pig was euthanized at 72 hours after the initial inoculation. The intestine was collected and a 50% homogenate was prepared in VM media containing 3X antibiotics (penicillin 250 units/ml, streptomycin 150 µg/ml, gentamicin 75 µg/ml, amphotericin B 3 µg/ml).

Group C: Thirteen weaned pigs (20 days old) from a commercial source (TGEV free/nonvaccinated herd) were randomly divided into 3 groups (4/5/4). The groups were housed in separate rooms and handlers showered upon exiting the rooms. Each of the pigs in group C1 (pigs 103, 107, 112, and 120) were orally inoculated with 5 ml of a 10%

Table 2. Experimental inoculation of pigs with PEDV

Pig ID	Group	Inoculum 1[a]	Inoculum 2
1	A	PEDV CV777 Weybridge	None
2	A	PEDV CV777 Weybridge	None
3	B	PEDV 10% suspension pig 1	PEDV CV777 Weybridge[b]
103	C1	10% PEDV/TGEV negative feces	PEDV NK94P6[c]
107	C1	10% PEDV/TGEV negative feces	PEDV NK94P6[c]
112	C1	10% PEDV/TGEV negative feces	PEDV NK94P6[c]
120	C1	10% PEDV/TGEV negative feces	PEDV NK94P6[c]
102	C2	PEDV, 10% suspension pig 3	None
105	C2	PEDV, 10% suspension pig 3	None
106	C2	PEDV, 10% suspension pig 3	None
111	C2	PEDV, 10% suspension pig 3	None
116	C2	PEDV, 10% suspension pig 3	None
101	C3	TGEV Miller	None
104	C3	TGEV Miller	None
109	C3	TGEV Miller	None
110	C3	TGEV Miller	
115	D	PEDV, 50% suspension pig 3	None
119	D	PEDV, 50% suspension pig 3	None
125	D	PEDV, 50% suspension pig 3	None
122	E	PEDV CV777 Gent	None
123	E	PEDV CV777 Gent	PEDV CV777 Gent[d]
124	E	PEDV CV777 Gent	PEDV CV777 Gent[d]

[a]Inoculum administered on day zero.

[b]Inoculum administered two days after the first inoculation.

[c]Inoculum administered 18 days after the first inoculation.

[d]Inoculum administered 51 days after the first inoculation.

PEDV/TGEV negative fecal suspension filtered through a 0.22 μm filter (pig 2 - day 0 collection). Each of the pigs in group C2 (pigs 102, 105, 106, 111, and 116) were orally inoculated with a 10% suspension of PEDV containing intestinal contents filtered through a 0.22 μm filter from pig 3 (72 hours postinoculation). Each of the pigs in group C3 (pigs 101, 104, 109, and 110) were orally inoculated with 2 ml of a 1:10 dilution of Miller TGE 88-13

challenge strain (CVLB). Fecal samples were collected prior to inoculation and twice per day for 10 days. Blood samples were collected on days 0, 7, 10, 14, and weekly for the duration of the experiment. Pigs 104 and 106 were euthanized on day 4 postinoculation, and intestines were collected. Group C1 pigs were orally inoculated on day 18, intramuscularly on day 63, and intravenously on days 69 and 76 with 5 ml of low passage cell culture isolate NK94P6.

Group D: Three CDCD pigs (115, 119, and 125) were inoculated with 5 ml of the 50% intestinal homogenate from pig 3. Fecal samples were collected prior to inoculation and twice per day for 10 days. Blood samples were collected on days 0, 7, 10, 14, and weekly for the duration of the experiment. Pig 125 was euthanized at 24 hours postinoculation and the intestines collected.

Group E: Three ampoules of lyophilized PEDV inoculum (University of Gent) were reconstituted in 16 ml of 0.01 M PBS, pH 7.2. Three CDCD pigs (122, 123, and 124) were orally inoculated with 5 ml of the PEDV inoculum. The remaining PEDV inoculum was aliquoted and stored at -70°C for RNA extraction. Fecal samples were collected prior to inoculation and twice per day on days 1 through 9 and once on day 15. Blood samples were collected on days 0, 7, 10, 14 and weekly for the duration of the experiment. Pig 122 was euthanized at the onset of diarrhea. Two ampoules of lyophilized PEDV inoculum (University of Gent) were reconstituted in 20 ml of 0.01 M PBS, pH 7.2. Pigs 123 and 124 were inoculated with 10 ml of reconstituted PEDV (oro-nasally 5 ml and intramuscularly 5 ml) on day 51.

Collection and processing of blood and fecal samples

Blood samples were collected from the orbital sinus. Blood was allowed to clot and centrifuged at 1200 X *g* (Beckman J-6) for 25 minutes. Serum was removed and stored at -20°C. Fecal samples were collected from individual pigs and from the floor. Pigs were restrained and a fresh fecal sample was caught in a sterile tube. If a fresh fecal sample could not be obtained a rectal swab was taken. Fresh feces on the floor of the pen or concrete floor below the pen were scooped into the tube with a sterile swab. Samples not immediately processed were stored at -70°C. A 10% (V/V) suspension was made in VM media containing

3X antibiotics (penicillin 250 units/ml, streptomycin 150 µg/ml, gentamicin 75 µg/ml, amphotericin B 3 µg/ml). All swabs were suspended in 7 ml of VM media containing 3X antibiotics. Suspensions were vortexed and centrifuged in the J-6 centrifuge (Beckman) for 25 minutes at 1200 X *g* at 4°C. Supernatant was removed and 4 - 300 µl aliquots were placed in 1.5 ml centrifuge tubes and stored at -70°C for RNA extraction. An additional 3 ml was removed for virus isolation. The homogenate was mixed, 2 to 3 ml removed for electron microcospy, and the remainder placed in dram vials for storage at -70°C.

Collection and processing of intestine

Immediately after being euthanized pigs 1, 2, 106, 109, 122, and 125 were necropsied. The abdominal cavity was opened and the intestines were removed. One- to two-inch sections of the small and large intestine were cut for fluorescent antibody staining. The specimens were filled with Tissue Tek® (Sakura) and frozen. The intestinal contents were stripped from the remaining intestine, and a 10% homogenate was made in VM with 3 X antibiotics and processed like the fecal samples. Individual sites of pig 122 were sampled. A section of the ileum, jejunum, duodenum, distal duodenum, distal colon, and cecum was secured prior to removal. The tissue and its contents were collected in a sterile tube for RNA extraction. The intestinal tissues and a piece of the mesenteric lymph node were added directly to 2 ml of Trizol- LS and processed for total RNA extraction.

Fluorescent antibody staining of frozen tissue sections

Eight micron sections were cut from each tissue sampled. The sections were placed on a microscope slide, fixed in acetone for 10 minutes, and air dried. The tissue was flooded with FITC-labeled anti-PEDV or anti-TGEV antiserum. The slides were incubated at 37°C for 30 minutes in high humidity. The slides were washed in 0.01M PBS, pH 7.2, a quick rinse, followed by three 5-minute soaks, and a final rinse in distilled water. The slides were air dried, mounted, and examined microscopically.

Electron microscopy.

Two to three ml of a 10% fecal suspension in VM media was submitted for negative staining electron microscopy. The fecal suspension was adjusted to a total volume of 5 ml with sterile water and vortexed. The sample was centrifuged at 1200 X *g* (Beckman J-6 centrifuge) for 20 minutes. Three ml of the supernatant were added to 3 ml of distilled water, mixed, and centrifuged at 1200 X *g* for 20 minutes. The supernatant was removed and ultracentrifuged at 100,000 x *g* for one-half hour. The supernatant was discarded, and the pellet was suspended in 15 drops of sterile distilled water, and centrifuged at 800 x *g* for 3 minutes. One drop of supernatant was diluted with 2 drops of 2 % phosphotungstic acid (PTA), 1 drop of bovine serum albumin (BSA), and 20 drops of distilled water. A Pasteur pipette was used to mix the sample. After 2 minutes, the mixture was placed in a glass nebulizer and sprayed on a 200 mesh copper grid that had been coated with collodion and carbon. The grid was air dried and examined.

Immunoblot analysis

The viral protein preparations were thawed at room temperature, incubated at 37°C for 5 minutes, and vortexed. The antigen was separated on 10 to 20% gradient SDS-PAGE mini gels at 30 mA per gel. The separated proteins were transferred to a 0.45 μm nitrocellulose membrane in 25 mM sodium phosphate buffer, pH 6.8 at 0.5 amp for 30 minutes, 1.0 amp for 2 hours, and 1.5 amp for 30 minutes (Whetstone et al., 1991). The membranes were blocked overnight in 10 mM Tris-HCl, 0.5 M NaCl (TS) with 0.05% Tween 20, pH 8.6. The membrane was cut into strips or tested in a multiscreen (BioRad) apparatus. The test sera were diluted in TS with 0.05% Tween 20 and reacted with the membrane for 2 hours on an orbital rocker at room temperature. The membrane was washed once with TS with 0.05% Tween 20 for 5 minutes and twice with 10 mM Tris-HCl, 0.5 M NaCl (TBS) with 0.05% Tween 20 for 5 minutes each wash. The membranes were incubated for 1 hour with HRP- anti-swine immunoglobulin or HRP-labeled rec-Protein G (Zymed) diluted 1:1000 and 1:500 in TBS with 0.05% Tween 20 at room temperature. The membrane was washed four times in TBS with 0.05% Tween 20. The membranes were reacted with 4-chloro-1-naphthol

(4CN) for 10 to 20 minutes. The membranes were rinsed in water and dried between filter papers.

RESULTS AND DISCUSSION

Reference stocks of two PEDV cell-culture-adapted isolates, CV777 and NK94P6, were produced and titrated. The CV777 isolate replicated in both the Vero 76 and the Vero KY-5 cell lines. The NK94P6 isolate would only replicate in the Vero KY-5 cell line. Viruses were titrated on the cell line to which they had been adapted. The titer of the high cell culture passage CV777 stock was $10^{6.4}$ $TCID_{50}$ per ml. The titer of the low cell culture passage NK94P6 isolate was $10^{2.5}$ $TCID_{50}$ per ml.

Optimization of RT-PCR

RNA extracted from the CV777 virus stock was used in the selection of the RT-PCR parameters with the primers specific for the nucleocapsid gene of PEDV. In order to limit the number of manipulations of the test sample, the production of cDNA and its amplification were carried out in a single tube (Ridpath et al., 1994). First stage 50 µl reactions containing 25 units of Superscript ™ RNase H⁻ reverse transcriptase (Gibco BRL), 1.25 units of AmpliTaq Gold™ polymerase (Perkin-Elmer), and varying concentrations of $MgCl_2$ and primers were tested in duplicate at two different annealing temperatures. RT-PCR reactions were evaluated for the presence of a 740 base pair band by gel electrophoresis, ethidium bromide staining, and UV visualization (see Figure 1). The results of the RT-PCR are presented in Table 3.

RT-PCR at a 53°C annealing temperature produced more positive responses than at 58°C annealing temperature. RT-PCR products were produced over the range of $MgCl_2$ concentrations tested. Primer concentration influenced the RT-PCR outcome. The first stage reaction parameters of 53°C annealing temperature, 3.0 mM $MgCl_2$, and 0.75 µM of primer were selected for further evaluation. First stage product was utilized as the amplicon for optimization of the second stage PCR reaction in a checkerboard titration of $MgCl_2$ and

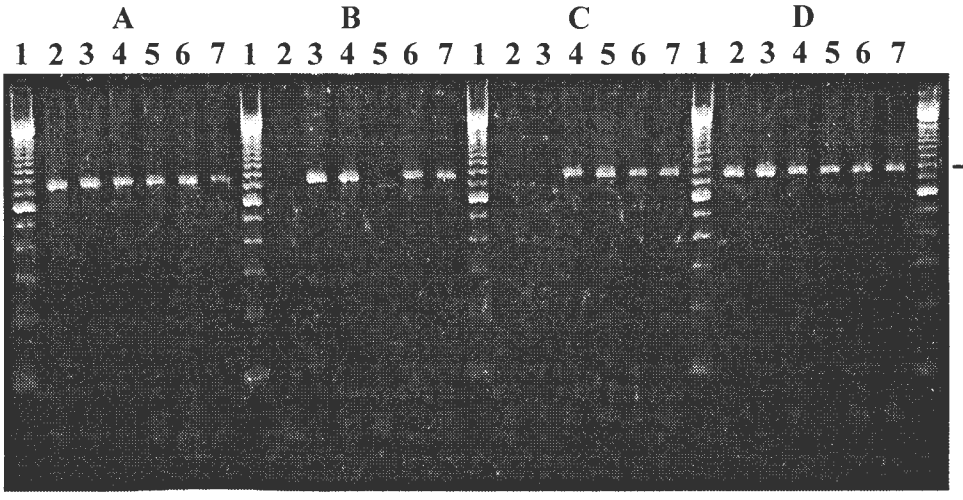

Figure 1: Effect of $MgCl_2$ concentration and primer concentration on the first stage RT-PCR at 53°C annealing temperature.

Panel A: 1.8 mM $MgCl_2$.

Panel B: 2.0 mM $MgCl_2$.

Panel C: 2.5 mM $MgCl_2$.

Panel D: 3.0 mM $MgCl_2$.

Lane 1: 100 base pair marker.

Lane 2, 3: 0.25 µM primer.

Lane 4, 5: 0.50 µM primer.

Lane 6, 7: 0.75 µM primer.

Note: Primer concentration influenced RT-PCR outcome.

Table 3. First stage RT-PCR annealing temperature, primer, and $MgCl_2$ concentration evaluation

		Number of RT-PCR positive reactions[a]			
	Annealing	Primer concentration			
$MgCl_2$ concentration	Temperature	0.25 μM	0.50 μM	0.75 μM	Total[b] (n=6)
1.8 mM	53°C	2	2	2	6
2.0 mM	53°C	1	1	2	4
2.5 mM	53°C	0	2	2	4
3.0 mM	53°C	2	2	2	6
Total[c] (n=8)		5	7	8	
1.8 mM	58°C	1	0	0	1
2.0 mM	58°C	0	0	1	1
2.5 mM	58°C	1	0	0	1
3.0 mM	58°C	0	1	0	1
Total[c] (n=8)		2	1	1	

[a]Reaction conditions were tested in duplicate.

[b]The number of RT-PCR positive reactions at a particular concentration of $MgCl_2$.

[c]The number of RT-PCR positive reactions at a particular primer concentration.

primer concentrations at an annealing temperature of 60°C. Second stage 50 μl PCR reactions containing 1.25 units of AmpliTaq Gold™ polymerase and varying concentrations of $MgCl_2$ and primers were tested in duplicate. PCR reactions were evaluated for the presence of a 341 base pair band by gel electrophoresis, ethidium bromide staining, and UV visualization (see Figure 2). Second stage PCR results are shown in Table 4. The optimal second stage reaction conditions selected were 3.0 mM $MgCl_2$ and 0.4 μM of primer.

Feces contain inhibitors to PCR that are extracted with the RNA. The extraction of RNA from fecal samples with Catrimox-14 and LiCl (method 2) has been reported to reduce the inhibitory effects during PCR (Uwatoko, 1996). To test the ability of the RT-PCR to detect PEDV RNA in fecal samples and if the nested RT-PCR reaction was influenced by the RNA extraction method, a 10% fecal suspension from a CDCD pig was used as the diluent and PEDV cell culture adapted virus was diluted tenfold. The same stock of virus

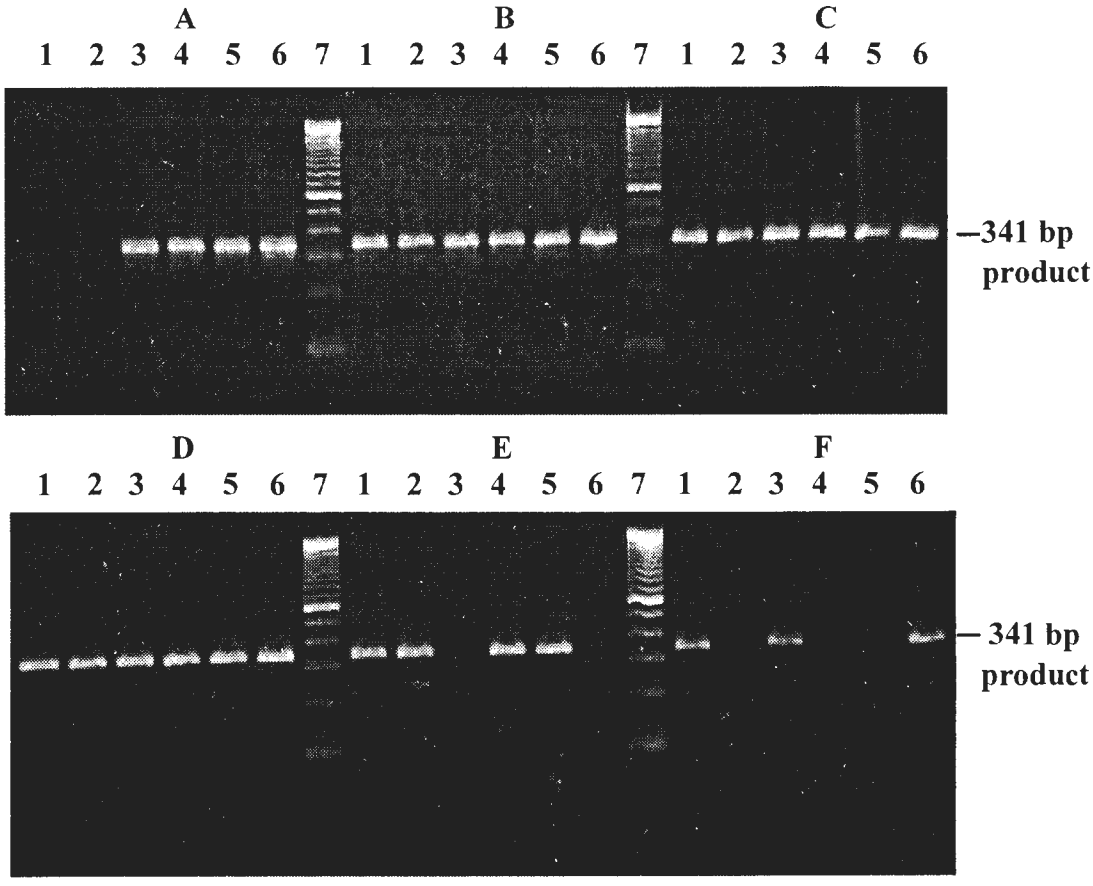

Figure 2: Effect of $MgCl_2$ and primer concentration on second stage RT-PCR at 60°C annealing temperature for the detection of PEDV nucleocapsid gene.

Panel A: 1.5 mM $MgCl_2$.

Panel B: 2.0 mM $MgCl_2$.

Panel C: 2.5 mM $MgCl_2$.

Panel D: 3.0 mM $MgCl_2$.

Panel E: 3.5 mM $MgCl_2$.

Panel F: 4.0 mM $MgCl_2$.

Lane 1, 2: 0.2 µM primer.

Lane 3, 4: 0.4 µM primer.

Lane 5, 6: 0.6 µM primer.

Note: $MgCl_2$ concentrations up to 3.0 mM were optimal. Higher concentrations gave inconsistent results.

Table 4. RT-PCR second stage primer and $MgCl_2$ concentration evaluation

$MgCl_2$ concentration	Number of RT-PCR positive reactions primer concentrations[a]		
	0.2 μM	0.4 μM	0.6 μM
1.5 mM	0	2	2
2.0 mM	2	2	2
2.5 mM	2	2	2
3.0 mM	2	2	2
3.5 mM	2	1	1
4.0 mM	1	1	1

[a]Reaction conditions were tested in duplicate.

was diluted tenfold in VM. RNA was extracted from both sets of dilutions with Trizol® LS (method one) or Catrimox-14™ (method two) and evaluated by RT-PCR. Low levels of PEDV diluted in feces or in VM were detected by RT-PCR when the samples were extracted by method 1. Contrary to the reported results (Uwatoko, 1996), when fecal samples containing PEDV were extracted by the Catrimox-14/LiCL method, low levels of PEDV were not detected. Trizol-LS was selected as the extraction method for the remainder of the study. Nested RT-PCR results from CV777 RNA extracted by methods one and two are shown in Table 5.

Table 5. Comparison of extraction method on RT-PCR outcome.

Dilution of CV777	Number of PEDV RT-PCR positive reactions[a]			
	Method one: Trizol LS		Method two: Catrimox-14	
	VM	10 % fecal	VM	10% fecal
10^{-1}	2	2	2	1
10^{-2}	2	2	2	1
10^{-3}	2	2	2	0
10^{-4}	2	2	2	0
10^{-5}	2	2	2	0
10^{-6}	2	1	1	0
10^{-7}	2	2	1	0
10^{-8}	0	2	0	0

[a]Reaction conditions were tested in duplicate.

Two additional primer sets, derived from PEDV spike gene sequence, were designed (Table 1). A 750 nucleotide sequence that is absent from HCV229 E and a 270 nucleotide sequence that has sequence divergence from other coronaviruses have been identified in the spike gene (Duarte and Laude, 1994). Primer sets were designed to amplify these regions in order to determine if two geographically separate isolates, CV777 and NK94P6, have sequence divergence.

The sensitivity and specificity of the three PEDV nested primer sets were evaluated. To compare the sensitivity of the primer sets, RNA was extracted from serial tenfold dilutions of CV777 by method one. Duplicate samples of the RNA were subjected to nested RT-PCR with all three primer sets. Second step products were evaluated for the appropriate size products (see Figure 3). All three primer sets detected PEDV-specific cDNA prepared from the 10^{-7} dilution of viral stock. The viral stock contained $10^{5.9}$ $TCID_{50}$ per 300 µl. Since the $TCID_{50}$ is statistically equivalent to a theoretical 0.69 of an infectious unit, the RT-PCR has the ability to detect less than one infectious unit. The results of the sensitivity trial are shown in Table 6.

Table 6. Sensitivity of PEDV primer sets

Dilution of PEDV CV777	Number of RT-PCR positive reactions[a]		
	primer set 1[b]	primer set 2[c]	primer set 3[d]
10^{-1}	2	2	2
10^{-2}	2	2	2
10^{-3}	2	2	2
10^{-4}	1	1	1
10^{-5}	2	2	2
10^{-6}	2	2	2
10^{-7}	1	1	1
10^{-8}	0	0	0

[a]Primer sets were tested in duplicate.

[b]PEDV primer set 1 amplifies a 341 base pair product from the nucleocapsid gene.

[c]PEDV primer set 2 amplifies a 225 base pair product from the spike gene.

[d]PEDV primer set 3 amplifies a 112 base pair product from the spike gene.

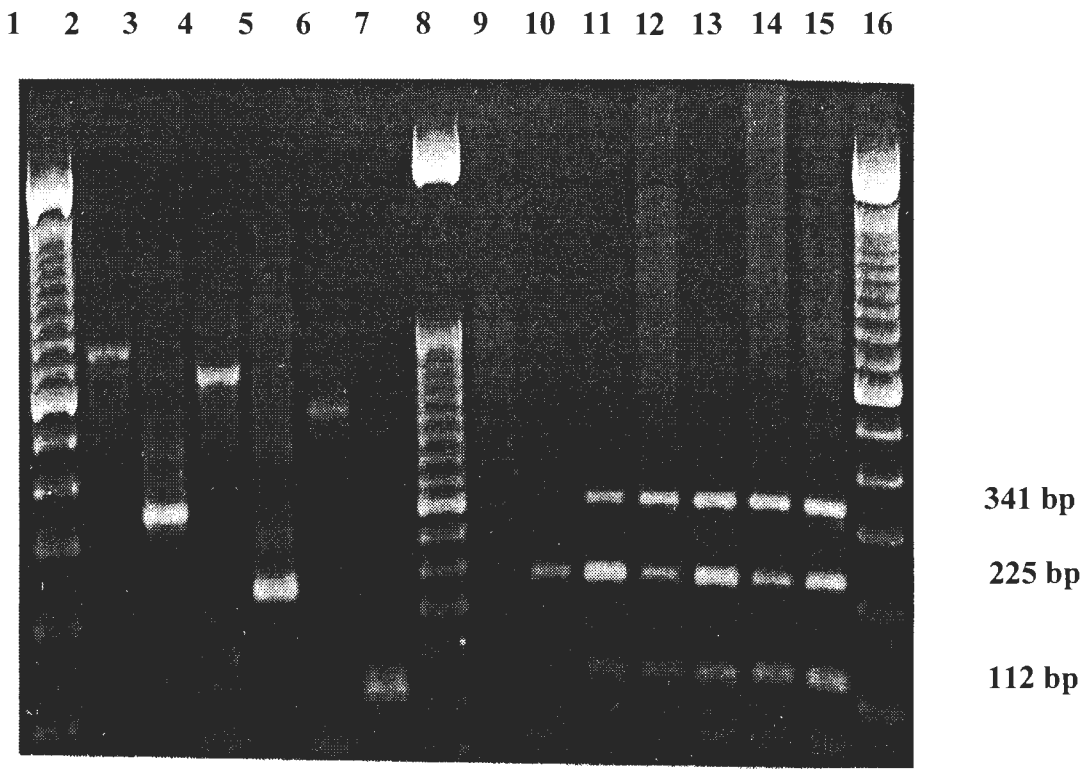

Figure 3: RT-PCR products of amplified dilutions of PEDV CV777.

Lanes 1 to 7 represent RT-PCR products with undiluted CV777.

Lane 1, 16: 100 base pair marker. Lane 8: 50 base pair marker.

Lane 2: First stage product of 740 bp with primer set 1.

Lane 3: Second stage product of 341 bp with primer set 1.

Lane 4: First stage product of 664 bp with primer set 2.

Lane 5: Second stage product of 225 bp with primer set 2.

Lane 6: First stage product of 547 bp with primer set 3.

Lane 7: Second stage product of 112 bp with primer set 1.

Lanes 9 to 15 represents second stage RT-PCR products from varying dilutions of CV777 with all three primers sets.

Lane 9: 10^{-8}. Lane 10: 10^{-7}. Lane 11: 10^{-6}. Lane 12: 10^{-5}.

Lane 13: 10^{-4}. Lane 14: 10^{-3}. Lane 15: 10^{-2}.

Note: Each nested primer set was assayed as a separate reaction and the products from the three reactions for a particular dilution were pooled for illustration in lanes 9 to 15.

RNA from serial tenfold dilutions of isolate NK94P6 was assayed with the nucleocapsid primer set (data not shown). PEDV RNA was detected in the 10^{-5} dilution of the virus stock ($10^{2.0}$ $TCID_{50}$ per 300 µl). To determine the specificity of the primer sets, RNA was extracted from viral stocks of BVDV, CCV, FIPV types I and II, HEV, TGEV, and porcine rotavirus and assayed by nested RT-PCR with all three primer sets. No PCR products were detected by UV visualization (data not shown).

Although the RT-PCR was extremely sensitive, reproducibility of detection at low levels was a concern. During the primer sensitivity analysis, it was noted that all three primer sets missed one of the duplicate samples in the 10^{-4} dilution, while the next dilution resulted in duplicate samples being positive. The reproducibility of the assay was evaluated by comparing RT-PCR results from multiple trials of the RT-PCR on CV777 cell culture virus dilutions. Two different reaction volumes and concentration parameters were examined. Set one conditions consisted of a first stage 100 µl reaction containing 1X PCR buffer, 200 µM of each dNTP, 50 units of RT, 2.5 units of AmpliTaq Gold polymerase, 1 µM each primer, and 2.0 mM $MgCl_2$. Set two conditions consisted of a first stage 50 µl reaction containing 1X PCR buffer, 200 µM of each dNTP, 25 units of RT, 1.25 units of AmpliTaq Gold polymerase, 0.75 µM each primer, and 3.0 mM $MgCl_2$. Standard conditions were utilized for the second stage reaction. Reproducibility of the assay results decreased as the number of infectious virus particles decreased. No attempt was made to determine if the RNA extraction step or the RT-PCR step contributed to the decrease in reproducibility. Testing in duplicate and multiple gene targets may offset the decrease in reproducibility. The results are presented in Table 7.

RNA was extracted from isolate NK94P6 and assayed by RT-PCR with all three primer sets. RT-PCR reactions were evaluated by gel electrophoresis, ethidium bromide staining, and UV visualization. Primer sets specific for the nucleocapsid gene (Z14976: nucleotides 253-992) and the spike gene (Z25483: nucleotides 61-724) produced bands of the appropriate sizes. The PCR product from the spike gene (Z25483: nucleotides 1581-2127) first and second stage reactions migrated at a higher molecular size than the CV777 PCR

Table 7. Reproducibility of RT-PCR to detect the nucleocapsid gene of PEDV

Dilution of PEDV CV777	RT-PCR products[a] Condition 1[b]	Percent	Condition 2[c]	Percent	Total (1 +2)
undiluted	4/4	100	2/2	100	100
10^{-1}	3/3	100	2/2	100	100
10^{-2}	4/4	100	4/4	100	100
10^{-3}	6/6	100	4/4	100	100
10^{-4}	6/6	100	3/4	75	90
10^{-5}	6/6	100	3/4	75	90
10^{-6}	6/6	100	3/4	75	90
10^{-7}	2/6	33	3/4	75	50
10^{-8}	0/1	0	1/4	25	20

[a]Number of RT-PCR positive/ number of replications tested.

[b]First stage 100 µl reaction contained 1X PCR buffer, 200 µM of each dNTP, 50 units of RT, 2.5 units of AmpliTaq Gold polymerase, 1 µM each primer, 2.0 mM MgCl2, followed by a 50 µl second stage reaction containing 1X PCR buffer, 200 µM of each dNTP, 1.25 units of AmpliTaq Gold polymerase, 0.4 µM of primer, and 3.0 mM $MgCl_2$

[c]First stage 50 µl reaction containing 1X PCR buffer, 200 µM of each dNTP, 25 units of RT, 1.25 units of AmpliTaq Gold polymerase, 0.75 µM each primer, 3.0 mM $MgCl_2$ followed by a 50 µl second stage reaction containing 1X PCR buffer, 200 µM of each dNTP, 1.25 units of AmpliTaq Gold polymerase, 0.4 µM of primer, and 3.0 mM $MgCl_2$

products in an agarose gel (see Figure 4). The RT-PCR products from CV777 and NK94P6 were sequenced and the sequence aligned with the Align software program (Scientific & Educational Software, NC). The nucleotide sequence of CV777 served as the reference for comparison purposes. An analysis of the nucleocapsid gene RT-PCR products sequence alignment revealed eight nucleotide substitutions (see Figure 5). Two of the nucleotide changes cause amino acid changes in the nucleocapsid protein. At nucleotide 564, a G is changed to a T, causing a glutamate to histamine amino acid change. At nucleotide 650, a T is changed to a G, causing a asparagine to lysine amino acid change. In both instances, a polar amino acid was substituted by a different polar amino acid.

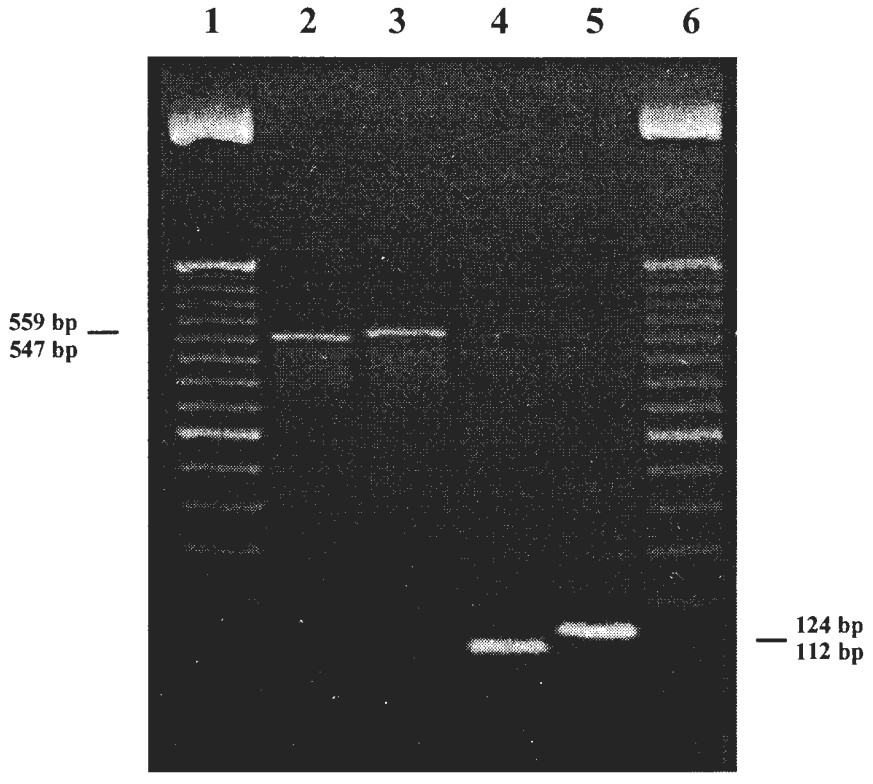

Figure 4: Comparison of the RT-PCR products of PEDV isolate CV777 and NK94P6 with spike gene primer set 3. The difference in the migration patterns was accounted for by sequence variation in the two isolates.

Lane 1, 6: 50 base pair marker.

Lane 2: CV777 first stage product.

Lane 3: NK94P6 first stage product.

Lane 4: CV777 second stage product.

Lane 5: NK94P6 second stage product.

Panel A

```
CV777       (   446) taacacacctcctgcttcacgtgcaaattcgcgtagcaggagtcgtggca
NK94P6      (   446) ..............................................t.

CV777       (   496) atggcaacaataggtctagatctccaagtaacaacagaggcaataaccag
NK94P6      (   496) ..........c.....c.........................c......

CV777       (   546) tcccgtggtaattcacagaatcgtggaaataaccagggtcgtggagcttc
NK94P6      (   546) ................t...........c...................

CV777       (   596) tcagaacagaggaggcaataataataacaataacaagtctcgtaaccagt
NK94P6      (   596) ..................................................

CV777       (   646) ccaataacaggaaccagtcaaatgaccgtggtggtgtaacatcacgcgat
NK94P6      (   646) ....g.............................................

CV777       (   696) gatctggtggctgctgtcaaggatgcacttaaatctttgggtattggaga
NK94P6      (   696) ........................c.........................

CV777       (   746) aaatcctgacaggca
NK94P6      (   746) ...............
```

Figure 5. Alignment of sequence of RT-PCR products of genes of PEDV. A dot indicates a matching base, while a dash indicates a missing base.

Panel A: Alignment of the sequence of the nucleocapsid gene RT-PCR products of PEDV isolates CV777 and NK94P6 amplified with PEDV primer set 1. The nucleotide sequence of CV777 (Z14976) served as the reference for comparison purposes.

Panel B

```
CV777       (  114) tcttttcaaaatttaatgttcaggcacctgccgtcgtcgttttgggtggt
NK94P6      (    1) ........................g.....a.....t..ac....c...

CV777       (  164) tacctacctagta-tgaac-----------tcttcta---gctggtactg
NK94P6      (   51) ..t.........gg.....aacagggtggg...g.accac.t........

CV777       (  199) tggcacaggcattgaaactgctagtggcgttcatggtatttttctcagct
NK94P6      (  101) ...tggccaaca................................t...c

CV777       (  249) acatcgattctggtcagggctttgagattggcatttcgcaagagccgttt
NK94P6      (  151) .t..ta.agg......t...............................

CV777       (  299) gatcctagtggttaccagctttatttacataaggccactaatggtaacac
NK94P6      (  201) ..c.....................................c........

CV777       (  349) taatgctactgcacgactgcgcatttgccagtttcccgataataaaacat
NK94P6      (  251) ...........g.......................agc..........

CV777       (  399) tgggccctactgtta---atgatgttacaacaggtcgtaactgcctattc
NK94P6      (  301) .......c....c..gtg..............................t

CV777       (  446) aacaaagccattccagcttatatgcgtgatggaaaagatattgttgtcgg
NK94P6      (  351) .................c.....a....------.c...g.........

CV777       (  496) cataacatgggataatgatcgtgtcactgtttttgctgacaagatctatc
NK94P6      (  395) .............................................

CV777       (  546) atttttatcttaaaaatgattggtcccgcgttgcgacaagatgttacaat
NK94P6      (  445) ............................t.........a.........c

CV777       (  596) cgcagaagttgtgctatgcaatatgtttatacacctacctactacatgct
NK94P6      (  495) a.tg..g.....................cga.....tt...........

CV777       (  646) taatgttactagtgcaggtgaggatggcatttattat
NK94P6      (  545) ..............t...........t....c....
```

Figure 5. Continued.

Panel B: Alignment of the sequence of the spike gene RT-PCR products of PEDV isolates CV777 and NK94P6 amplified with PEDV primer set 2. A dot indicates a matching base, while a dash indicates a missing base. The nucleotide sequence of CV777 (Z25483) served as the reference for comparison purposes.

Panel C

```
CV777       (  1601) actactatcaatgggtttagttctttctgtgttgacactagacaatttac
NK94P6      (     1) ................c.............................

CV777       (  1651) cattacactgttttataatgttacaaacagttatggttatgtgtctaaat
NK94P6      (    51) ..............c..c...............n.............

CV777       (  1701) cacaggatagtaattgtcctttcaccttgcaatctgttaatgattacctg
NK94P6      (   101) .....c....c.....c..............................

CV777       (  1751) tcttttagcaaattttgtgtttcaaccagcctttggctggtgcttgtac
NK94P6      (   151) ..................................................

CV777       (  1801) catagatctttttggttaccctgcgttcggtagt-----------ggtg
NK94P6      (   201) ........................t.........tcctttggtggt....

CV777       (  1839) ttaagttgacgtccctttattttcaattcacaaaaggtgagttgattact
NK94P6      (   251) .......t........c................g...............

CV777       (  1889) ggcacgcctaaaccacttgaaggtatcacagacgtttcttttatgactct
NK94P6      (   301) ........................g....g...................

CV777       (  1939) ggatgtgtgtaccaagtatactatctatggctttaaaggtgagggtatta
NK94P6      (   351) ......t...........................................

CV777       (  1989) ttacccttacaaattctagcattttggcaggtgtttattatacatctgat
NK94P6      (   401) ....................t...........t.................

CV777       (  2039) tctggacagttgttagcctttaagaatgtcactagtggtgctgtttattc
NK94P6      (   451) ..................................................

CV777       (  2089) tgtcacgccatgttctttttcagagcaggctgcatatgt
NK94P6      (   501) ...t..............................
```

Figure 5. continued

Panel C: Alignment of the sequence of the spike gene RT-PCR products of isolates CV777 and NK94P6 amplified with PEDV primer set three. A dot indicates a matching base, while a dash indicates a missing base. The nucleotide sequence of CV777 (Z25483) served as the reference for comparison purposes.

An analysis of the NK94P6 spike gene sequence amplified with PEDV primer set 2, nucleotides 61 through 772, revealed substitutions, deletions, and insertions when compared with CV777 sequence. No band shift in the agarose gel was observed. An analysis of the NK94P6 sequence revealed one insertion of 11 nucleotides, two insertions of 3 nucleotides each, a single nucleotide insertion, and a deletion of 6 nucleotides. The total nucleotide difference between the two sequences was 12 nucleotides. A shift should have been detected in the first stage product, as most of the differences were located between nucleotide 176 and nucleotide 191 of the Z25483 published sequence. The second stage product for NK94P6 contained only 3 more bases than the CV777 product, resulting in no band shift under the analysis conditions utilized.

An analysis of the NK94P6 spike gene sequence amplified with PEDV primer set 3, nucleotides 1581 through 2127, revealed 16 substitutions and a 12-nucleotide insertion. The insertion was located in the second stage product. Electrophoresis revealed a band shift by the first and second stage products (Figure 4). A synopsis of the changes in the spike protein is shown in Table 8. Even with all of the sequence variation in the regions of NK94P6 sequenced, the sequence remains in frame.

Table 8. Changes in the spike protein of PEDV isolate NK94P6

Amino Acid Position[a]	PEDV isolate CV777 (Z25483)[a]	PEDV isolate NK94P6
55-68	mnsss-----wycgtgie	geqqggsaprwycggqhe
80-90	syidsgqgfei	shikgghgfei
125-140	pdnktlgptvnd-vttg	psnktlgptasddvttg
150-160	paymrdgkdiv	pahm--sehsv
190-200	atrcynrrsca	atkcynsggca
205-210	ytptyy	yeptiyy
223	y	s
605-614	afgs----gvklts	afgssfgggvkft
665-675	ssilagvyyts	ssflagyytsd

[a]PEDV protein translated from GenBank sequence data base, accession number Z25483.

Titration of antibody in PEDV and TGEV hyperimmune control sera

Cell culture systems were used to determine the antibody titer of hyperimmune sera to PEDV and TGEV. Diluted serum (1:20 through 1:2560) was reacted with acetone-fixed, infected monolayers. Antibodies were detected by HRP-rec-Protein G conjugate (IPT) and FITC-goat anti-porcine immunoglobulin conjugate (IFA). The last dilution to give a positive reaction was considered the endpoint titer of the serum. Anti-CV777 serum (GP57) had an endpoint of 320 by both IPT and IFA when reacted with PEDV infected Vero 76 cells. No antibodies to TGEV were detected in the serum. Anti-TGEV serum (lot 8701) did not reach an endpoint by IPT or IFA when reacted with TGEV infected ST cells (>2560). Anti-TGEV serum (lot 8701) had an endpoint of 1:20 when reacted with PEDV infected Vero 76 cells.

Animal Studies

In order to evaluate the ability to detect PEDV RNA in clinical samples by RT-PCR and to evaluate the ability to differentiate between PEDV and TGEV infection, it was necessary to generate clinical samples. A limited amount of PEDV infectious inoculum was available. CDCD pigs 1, 2, and 3 (groups A and B) were used for the production of infectious inoculum. Group A pigs (pigs 1 and 2) began to exhibit clinical signs 22-24 hours after oral inoculation. Vomit was present on the floor, but no diarrhea was observed. A watery diarrhea was observed at 28 hours postinoculation. The piglets were observed for an additional 48 hours. A watery diarrhea continually dripped from the tip of the tail and vomit was occasionally observed in the water dish. No PEDV was isolated in cell culture from fecal or intestinal material processed. Using electron microscopy, coronavirus particles were observed in the intestinal contents recovered from pig 1. PEDV viral RNA was detected in intestinal contents from pig 1 and 2 by RT-PCR with primers specific for the nucleocapsid gene.

Intestinal material from pig 1 orally inoculated into pig 3 failed to induce clinical signs by 48 hours. In published experimental infections (Pensaert and Debouck, 1978), clinical signs were always observed 22 to 30 hours postinoculation. Pig 3 was inoculated with the remainder of the PEDV CV777 Weybridge inoculum. Twenty-four hours after the second

inoculation, pig 3 presented with a watery diarrhea. Upon onset of diarrhea, pig 3 was euthanized. No PEDV was isolated from a 50% intestinal homogenate from pig 3, but numerous coronavirus particles were observed by electron microscopy. No PEDV viral RNA was detected by RT-PCR. Due to import restrictions limiting the source of inoculum and because Weybridge could no longer provide infectious inoculum, the intestinal contents of pig 3 were utilized for further studies.

Group C pigs were inoculated to determine the ability of the RT-PCR and serological tests to distinguish between PEDV and TGEV infected pigs. Group C1 served as the control pigs and subsequently as low cell culture passaged PEDV inoculated pigs. Control pigs did not develop any clinical signs as a result of the sham inoculation with a PEDV/TGEV negative fecal suspension filtrate. After inoculation with the isolate NK94P6, no diarrhea or vomiting was observed. Pigs 112 and 120 developed body tremors that lasted for 4 days. Pig 112 developed a persistent head tilt. Neither PEDV nor PEDV RNA were detected in the fecal samples collected. Serum samples were tested for antibodies to PEDV and TGEV. No PEDV antibodies were detected prior to booster inoculations. TGEV virus neutralization and PEDV IPT results are presented in Table 9.

Group C2 pigs, inoculated with 10% PEDV intestinal homogenate filtrate from pig 3, did not exhibit any clinical signs of disease. Neither PEDV nor PEDV RNA were detected in fecal samples from day 1 nor day 2, and none of the pigs seroconverted to PEDV by IPT or IFA. A single fecal sample, collected on day 2 from the floor of the pen, gave weak fluorescence for TGEV isolation on ST cells. Repeat staining did not confirm this result. Serum samples were tested for TGEV neutralizing antibodies and the results are reported in Table 9.

All the pigs in group C3, inoculated with TGEV, had labored breathing 24 hours after inoculation. Restraining the pigs for fecal collection aggravated the respiratory condition of the pigs and caused them to wheeze. TGEV was isolated on day 1 from pigs 101 and 104; on day 2 from pigs 101, 104, and 109; and on day three from pig 109. Serum samples were tested for antibodies to PEDV and TGEV. No PEDV antibodies were detected. TGEV antibody neutralizing titers are reported in Table 9. TGEV neutralizing antibodies were

Table 9. TGEV neutralization and PEDV IPT antibody titers in PEDV or TGEV inoculated animals

	Group C1[a]				Group C2[b]					Group C3[c]			
Day PI	103	107	112	120	102	105	106	111	116	101	104	109	110
TGEV neutralization titers[d]													
0	32	128	128	256	128	64	128	128	64	128	256	128	128
4	32	128	64		64	64	16	64	64	128	256	16	16
7	32		64			32		64		16	64		32
10	32	32	128	16	32	16		64	32	64	64		32
14	8	32	16		32	32		32	8	256	32		16
21	8		32	8	32	32		16	16	512	32		8
24	8	16	16	8	32	64		64	16				
27	neg	16	8	8						512	16		8
31	neg	8	8										
38	neg	neg	neg										
49					8	32				256	8		neg
62					neg	16		8	8	256	8		neg
98	neg	neg	neg										
PEDV IPT Titers[e]													
98	64	64	128	32	neg	neg	neg	neg	neg	neg	neg	neg	neg

[a]Inoculated with a 10% PEDV/TGEV negative fecal suspension on day zero, followed by 5 ml of cell culture isolate NK94P6 on postinoculation (PI) day 18.

[b]Inoculated with a 10% intestinal suspension from PEDV pig 3 on day zero.

[c]Inoculated with TGEV challenge virus.

[d]The lowest dilution tested was 1:8.

[e]The lowest dilution tested was a 1:16.

detected in all preinoculation serum samples. The TGEV titers in the negative control group decreased over the duration of the experiment, until no antibodies were detected at a 1:8 dilution on day 38. A decline in titer was observed in the PEDV inoculated pigs, but two of the three animals maintained detectable antibody titers as late as day 62. One PEDV inoculated animal had no detectable antibodies on day 62. One animal in the TGEV group seroconverted and high neutralizing titers were detected. Two of the animals in the group showed a decrease in titer; one had no detectable antibodies on day 62. It was evident from the control group that the pigs had colostral antibody at the start of the experiment. The colostral antibody may have neutralized the TGEV challenge and promoted viral clearance. The TGEV neutralization titers in the PEDV inoculated group were a concern. TGEV antibodies do not cross protect (Debouck and Pensaert, 1984).

An additional group of CDCD pigs, group D, were inoculated with a 50% suspension from PEDV pig 3. Group D pigs presented with a profuse diarrhea at 24 hours postinoculation. The diarrhea lasted for 5 days. Pig 119 had to be treated for a rectal prolapse. Neither PEDV nor viral RNA were detected. Coronavirus-like particles were observed on days 1, 2, and 3 by electron microscopy. Antibodies to PEDV were not detected by the IPT assay, but TGEV antibodies were detected by virus neutralization. TGEV virus neutralization test revealed a classical response to TGEV infection (Table 10).

After the seroconversion to TGEV by CDCD pigs inoculated with the 50% suspension from PEDV pig 3 was noted, fecal samples from Group D pigs were tested by a single stage RT-PCR with primers specific for the spike gene of Miller TGEV. TGEV-specific primers did not amplify HEV, PEDV, or porcine rotavirus. TGEV viral RNA was detected in fecal samples from pigs 115 and 119 at 24 hours postinoculation. RT-PCR results are presented in Table 11. The inocula used in the previous pig studies were assayed by RT-PCR with PEDV- and TGEV- specific primers. PEDV and TGEV RNA were detected in the original Weybridge inoculum and in the intestinal homogenate from pig 3. PEDV RNA was detected in pigs 1 and 2. No TGEV RNA was detected in pig 1 or 2. TGEV was isolated from the 50% intestinal homogenate from pig 3. The sensitivity of the TGEV primers was not evaluated.

Table 10. TGEV virus neutralization titers in group D[a]

Days post-inoculation	Pig 115	Pig 119	Pig 125
0	neg[b]	neg	neg
5	Toxic	neg	
7	Toxic	128	
10	64	>512	
14	8	>512	
21	>512[c]	>512	
35	>512	>512	
42	256	>512	
49	>512	>512	
56	>512	>512	
63	>512	128	
70	>512	128	

[a]Group D pigs were inoculated with a 50% intestinal homogenate from PEDV pig 3.

[b]No antibodies were detected at the lowest dilution tested (1:8).

[c]The highest dilution tested was 1:512.

Table 11. Detection of PEDV or TGEV RNA by RT-PCR

Sample	PEDV primers[a]	TGEV primers[b]
PEDV CV777 (Weybridge)	+[f]	+
Pig 1 intestinal homogenate[c]	+	-
Pig 3 intestinal homogenate[d]	-	+
fecal pool day (Group D)[e]	-	-
pig 115 day 1 (Group D)	-	+
pig 115 day 3 (Group D)	-	-
pig 115 day 7 (Group D)	-	-
pig 119 day 1 (Group D)	-	+
pig 119 day 3 (Group D)	-	-
pig 119 day 7 (Group D)	-	-
pig 125 day 1 (Group D)	-	-

[a]PEDV nested RT-PCR amplifies a 341 base pair product from the nucleocapsid gene.

[b]TGEV single stage RT-PCR amplifies a 628 base pair product from the spike gene.

[c]Pig 1 was inoculated with PEDV CV777 (Weybridge).

[d]Pig 3 was inoculated with material from pig 1 and PEDV CV777 (Weybridge).

[e]Pigs in group D were inoculated with a 50% intestinal homogenate from pig 3.

[f]+ = RT-PCR product of the appropriate size, while a - = no RT-PCR product detected.

An additional PEDV inoculum was obtained from the University of Gent. Group E pigs, inoculated with CV777 (University of Gent), developed subtle clinical signs. At 20 hours postinoculation, inappetence was evident. A watery stool dripped slowly from the tail of the pigs, but no puddles of liquid feces were noticed on the floor. PEDV antibodies were detected by IPT starting on day 14. No TGEV neutralizing antibodies were detected. PEDV antibody titers are presented in Table 12. No virus was isolated in cell culture. CLP were

Table 12. PEDV IPT Titers in pigs inoculated with PEDV isolate CV777 (Gent)

Postinoculation day	Pig 123	Pig 124	Pig 122
0	neg[a]	neg	neg
7	neg	neg	
14	40	20	
21	80	40	
28	80	40	
35	20	40	
49	20	40	

[a]No antibodies detected in a 1:20 dilution (lowest dilution tested).

detected by electron microscopy in fecal suspensions from pig 123 on days 2, 3, 4, 5; from pig 124 on days 2 and 3; and from samples collected off the floor on days 2 and 5. PEDV RNA was detected in fecal samples from day 1 through day 9. PEDV RNA was detected in the intestines and mesenteric lymph node of pig 122. A summary of RT-PCR results is presented in Table 13 (see Figure 6). Samples from pigs 122 and 124 tested positive with the spike primer set 3 on day 0 (Figure 6. Lane 2). The original fecal suspensions were thawed and the testing repeated. The second RT-PCR assay did not detect viral RNA in the samples. The original positive reactions were most likely due to contamination. Contamination is a serious risk in any diagnostic assay, but nested PCR is prone to contamination if any breaks occur in the quality control of the testing (Neumaier, 1998). During the RT-PCR testing, three separate reactions for each primer set were set up at the same time, resulting in a large number of tubes being handled at the same time. This may have contributed to the false positive results.

Table 13. PEDV RT-PCR on RNA extracted from fecal samples of pigs inoculated with PEDV CV777 (Gent)

	Pig 122[a]			Pig 123			Pig 124			Samples from the floor		
	PEDV primer			PEDV primer			PEDV primer			PEDV primer		
	Set 1[b]	Set 2[c]	Set 3[d]	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Day 0	0/3[e]	0/3	2/3*	0/2	0/2	0/2	0/4	0/4	2/4*	0/2	0/2	0/2
Day 1	3/4	4/4	4/4	1/5	3/5	3/5	0/2	2/2	2/2	4/4	4/4	4/4
Day 2	1/2	2/2	2/2	4/4	4/4	4/4	2/2	2/2	2/2	2/2	2/2	2/2
Day 3				4/4	4/4	4/4	0/2	2/2	2/2			
Day 4				4/4	4/4	4/4	1/3	3/3	3/3			
Day 5				4/4	4/4	4/4	2/2	2/2	2/2			
Day 6				0/4	4/4	2/4	2/4	4/4	4/4			
Day 7				0/4	2/4	0/4	2/4	4/4	4/4			
Day 8				0/4	0/4	0/4	0/4	2/4	2/4			
Day 9				1/4	2/4	0/4	2/4	2/4	3/4			
Day 15				0/2	0/2	0/2	0/2	0/2	0/2			

[a]Pig 122 was euthanized on day 2 postinoculation.

[b]Primer set 1 amplifies a 341 base pair fragment from the nucleocapsid gene of PEDV.

[c]Primer set 2 amplifies a 225 base pair fragment from the spike gene of PEDV.

[d]Primer set 3 amplifies a 112 base pair fragment from the spike gene of PEDV.

[e]Number of positive samples/number of replicates tested.

*These samples were tested and positive results observed. The original fecal suspension was thawed, and RNA extracted, and assayed by RT-PCR. No RT-PCR products were detected on the repeat testing.

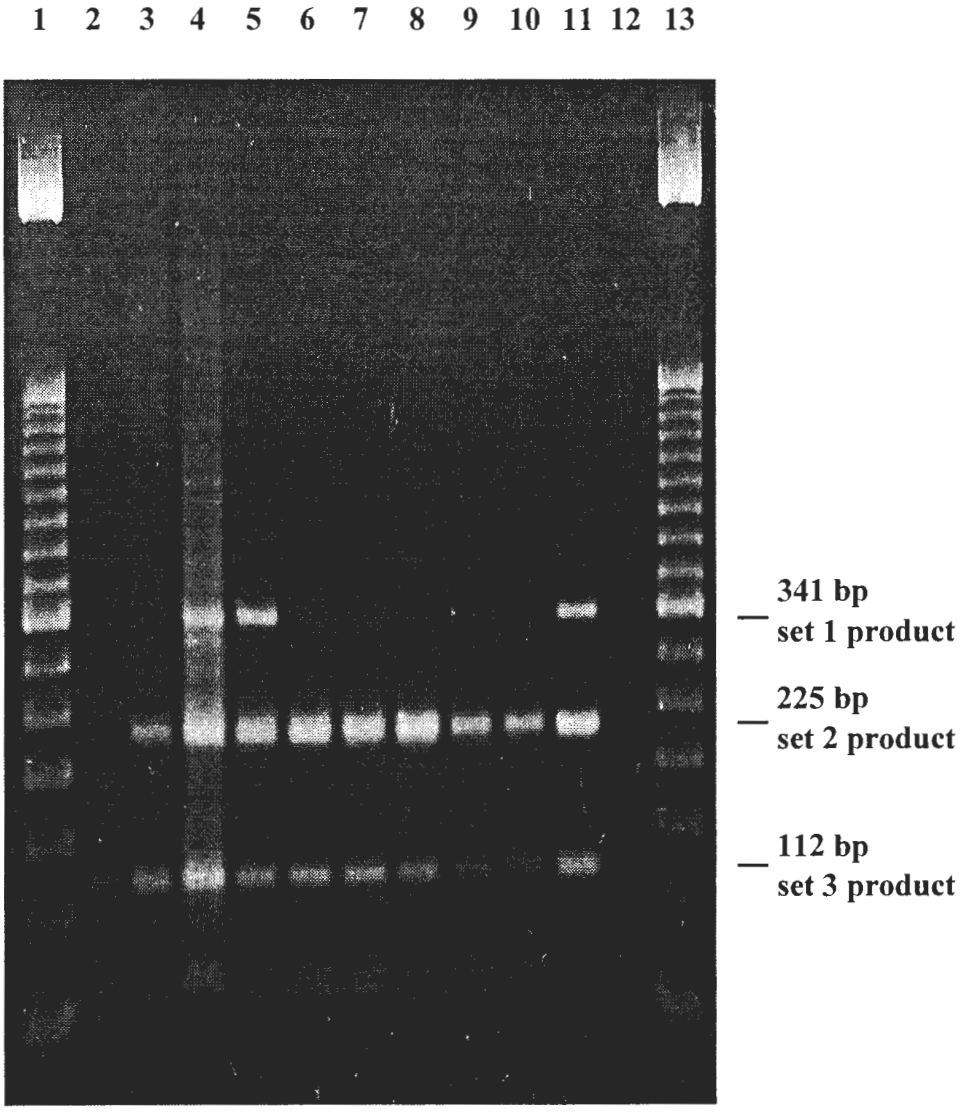

Figure 6: Second stage RT-PCR products amplified with all three PEDV primer sets from fecal samples collected from PEDV inoculated pig 124.

Lane 1, 13: 50 base pair marker. Lane 2: Day 0.

Lane 3: Day 1. Lane 4: Day 2.

Lane 5: Day 3. Lane 6: Day 4.

Lane 7: Day 5. Lane 8: Day 6.

Lane 9: Day 7. Lane 10: Day 8.

Lane 11: Day 9. Lane 12: Day 15.

Note: RT-PCR assays were carried out separately and the products from the three reactions on daily fecal collections were pooled for illustration .

FATS and histopathologic examination of intestines from experimental pigs

Histopathological examination revealed shortening of the villi and sloughing of cells into the lumen (Gidlewski, personal communication). Cryostat sections from experimental animals were examined by FATS for the detection of PEDV or TGEV antigens. Positive fluorescence was detected in individual cells in cryostat sections from pigs 1, 2, 3, and 122. A blocking method was utilized to determine if the staining was specific. The staining patterns did not vary between slides blocked with negative serum and slides blocked with positive serum. Pig 3 was positive for TGEV by FATS.

Antigenic Relationships

PEDV hyperimmune serum and TGEV hyperimmune sera were assayed by immunoblot analysis (see Figure 7). PEDV and TGEV hyperimmune serum did not react with any proteins in the cell control preparation. PEDV hyperimmune serum reacted with two major bands and two minor band clusters. The molecular weights were estimated by linear regression analysis of the prestained molecular markers migration. The estimated molecular weights of the two major bands are 116 kd and 62 kd. The estimated molecular weights of the minor band clusters are for cluster one: 59 kd, 55 kd, 52 kd, and 50 kd; and cluster 2: 40 kd, 38 kd, 35 kd, and 32 kd. TGEV hyperimmune serum reacted with the 62 kd PEDV protein.

Convalescent antiserum from TGEV challenged pigs (kindly provided by Jon Katz, NVSL, USDA) was assayed for reactivity with PEDV antigens (see Figure 8). No reactivity was detected in the preinoculation sera. All six of the pigs seroconverted to the 62 kd protein by day 28 postinoculation. One animal was evaluated on day 14 postinoculation and seroconversion to the 62 kd was detected. Convalescent antiserum from PEDV inoculated group E pigs was assayed for reactivity with PEDV antigens (see Figures 9 and 10). No reactivity was detected with antigens in the cell control preparation (see Figure 9, Lane 2).

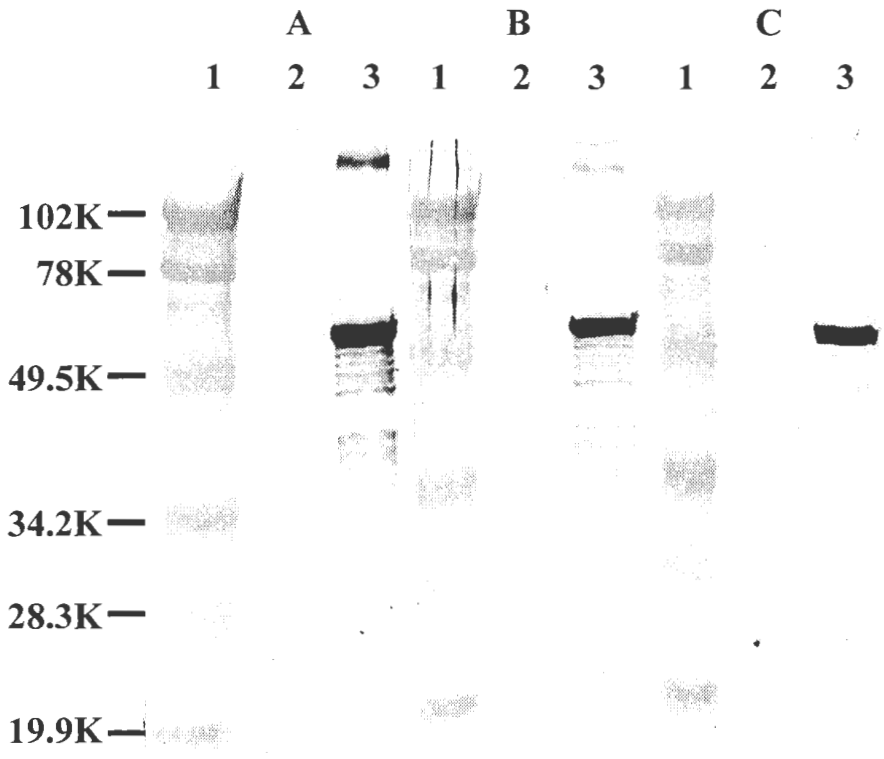

Figure 7: Immunoblot of PEDV antigens with PEDV and TGEV hyperimmune antisera.

Lane 1: Molecular marker.

Lane 2: Cell culture control.

Lane 3: PEDV antigens.

Panel A: Antigens reacted with a 1:10 dilution of swine anti-CV777 serum.

Panel B: Antigens reacted with a 1:50 dilution of swine anti-CV777 serum.

Panel C: Antigens reacted with a 1:50 dilution of swine anti-TGEV serum.

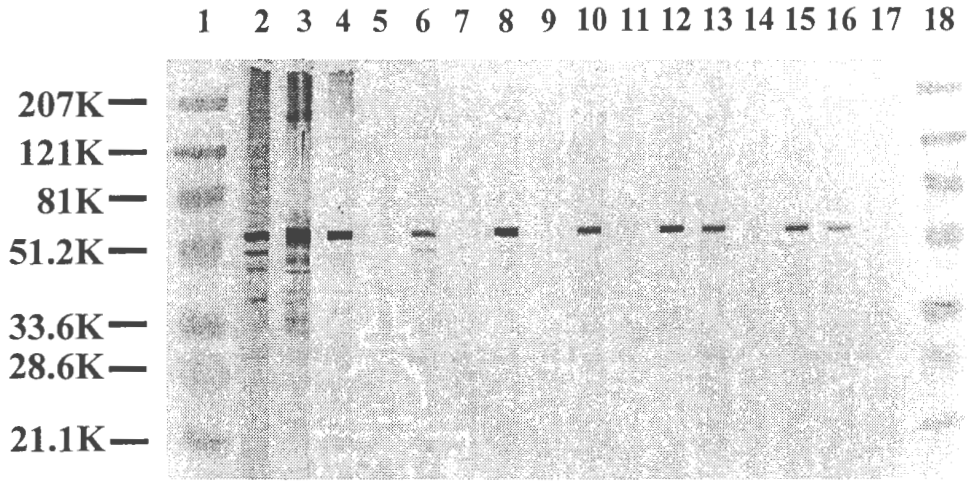

Figure 8: Preparative immunoblot of PEDV antigens with sera from six TGEV challenged pigs.

Lane 1,18: Prestained molecular marker.

Lane 2: TGEV hyperimmune serum 11-79.

Lane 3: CV777 hyperimmune serum.

Lane 4: TGEV hyperimmune serum 8701.

Lanes 5, 7, 9, 11, 14, 17: Day 0.

Lanes 6, 8, 10, 12, 16: Day 28 after inoculation with TGEV.

Lane13: Day 14 after inoculation with TGEV.

Lane 15: Day 35 after inoculation with TGEV.

Note: Hyperimmune sera were tested at a 1:100 dilution.

TGEV sera were tested at a 1:20 dilution.

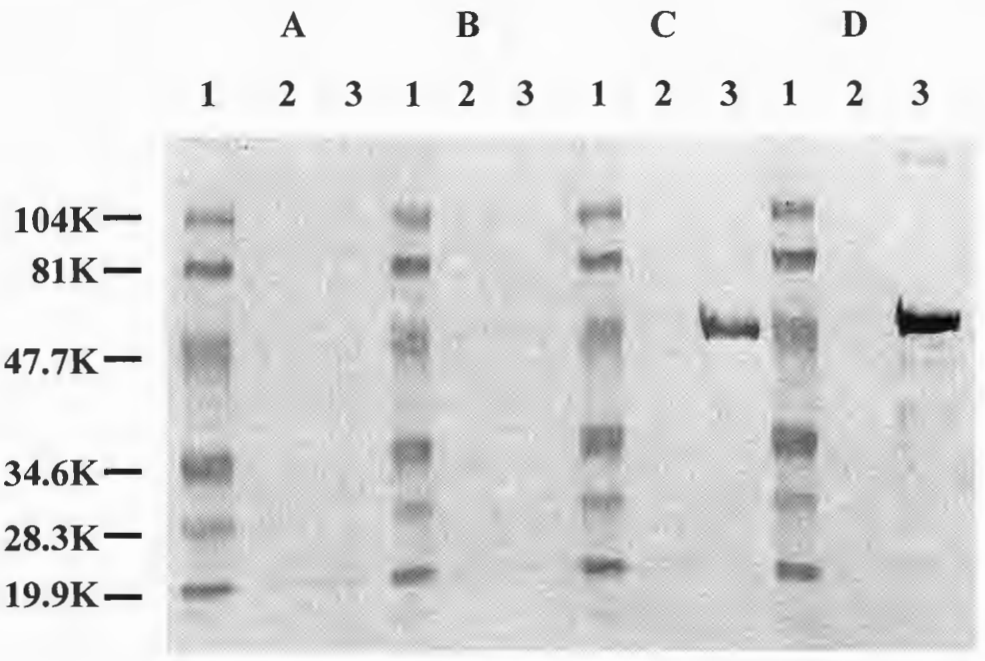

Figure 9: Immunoblot of PEDV antigens with sera from PEDV inoculated pig 123.

Lane 1: Molecular marker.

Lane 2: Cell culture control.

Lane 3: PEDV antigens.

Panel A: Day 0 serum.

Panel B: Day 7 serum after PEDV inoculation.

Panel C: Day 58 serum after PEDV inoculation.

Panel D: CV777 hyperimmune serum.

Note: CV777 hyperimmune serum was tested at a 1:100 dilution and sera from pig 123 were tested at a 1:20 dilution.

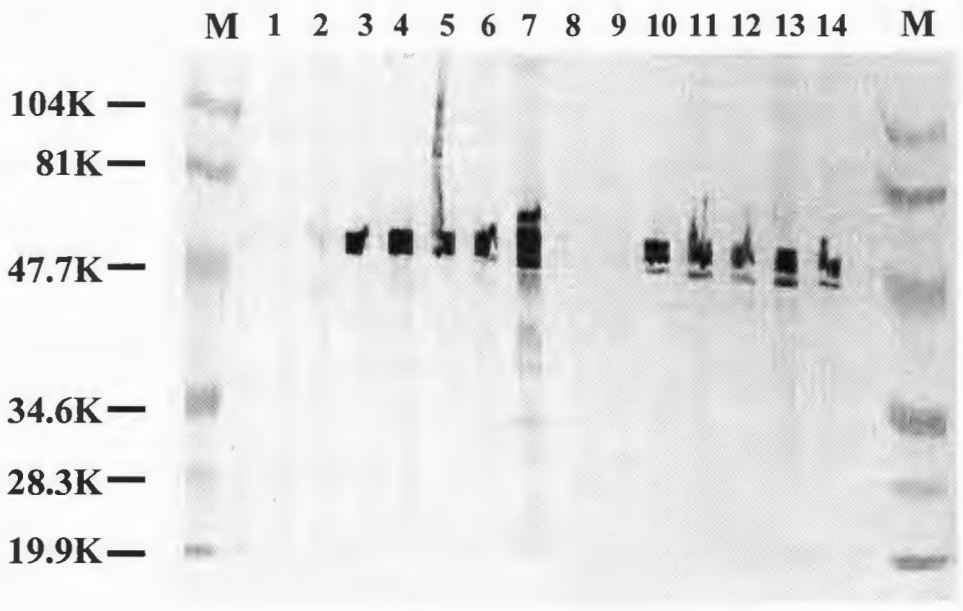

Figure 10: Immunoblot of PEDV antigens with sera from CV777 inoculated pigs. M: Prestained molecular marker.

Lanes 1 to 7 represent reactivity of sera from pig 123 and lanes 9 to 13 represent reactivity of sera from pig 124 at different times after infection with CV777.

Lane 1, 8: Day 0. Lane 2, 9: Day 7. Lane 3: Day 14. Lane 4, 11: Day 28. Lane 5, 10: Day 35. Lane 6, 12: Day 50. Lane 7, 13: Day 58. Lane 14: CV777 hyperimmne serum.

Note: CV777 hyperimmne serum was tested at a 1:80 dilution and sera from inoculated pigs were tested at a 1:20 dilution.

Antibodies specific for the 62 kd protein were detected on day 14 postinoculation. No reactivity was noted to the 116 kd in either animal after a single exposure to PEDV. A weak reactivity with the 116 kd protein was detected 7 days after a second inoculation (see figure 10, Lane 7) in one animal.

CONCLUSIONS

Meeting the gold standard for PEDV diagnosis is complicated by the difficulties in isolating virus in cell culture (Hofmann and Wyler, 1988) and the post mortem testing of tissue sections (Pensaert, 1992). RT-PCRs assays have been developed (Kweon et al., 1997; Ishikawa et al., 1997) for the diagnosis of PEDV with limited success when testing individual animal samples (Guscetti et al., 1998). The goal of this study was to develop a reliable nested RT-PCR for the diagnosis of PEDV.

A nested primer set was derived from the PEDV nucleocapsid gene sequence deposited in GenBank data base (Z14976) and the RT-PCR reaction conditions were optimized. The assay detected 0.1 to 100 $TCID_{50}$ of PEDV cell culture isolate CV777 in 75% of the aliquots tested and 1000 $TCID_{50}$ in 100% of the aliquots tested. These results are similar to the results of Kweon (Kweon et al., 1997) and Ishikawa (Ishikawa et al., 1997). Low levels of PEDV detection can be achieved by testing clinical samples in triplicate. The nested RT-PCR was specific for PEDV as no products were observed when other RNA viruses were assayed.

Two additional nested primer sets were derived from the PEDV spike sequence deposited in GenBank data base (Z25483). These primer sets were designed to amplify the NH_2 terminus of the spike protein, where sequence variation has been documented in other coronaviruses (Duarte and Laude, 1994). The spike primer sets detected 0.1 $TCID_{50}$ of PEDV cell culture isolate CV777. The reproducibility of the detection limits of the spike primer sets was not evaluated. The spike primer sets were specific for PEDV, as no product was detected when BVDV, CCV, FIPV I and II, HEV, TGEV, and porcine rotavirus were assayed. The spike primer set 3 (nucleotides 1581-2127) was useful in differentiating the high passage isolate CV777 and the low passage isolate NK94P6. Further studies are needed to

determine if the sequence differences, illustrated by the increase size of the PCR fragment, are a result of attenuation of the virus by cell culture passage or genetic diversity between two geographically separated isolates.

Attempts to generate PEDV infectious inoculum were not successful, but did demonstrate the ability of RT-PCR to differentiate between PEDV and TGEV. Although the TGEV infection was eventually detected by a rise in TGEV-specific neutralizing antibody titers, the diagnosis was not available until the pigs had recovered. Thus, RT-PCR can be used for early detection or screening of diarrheal outbreaks. The PEDV nested RT-PCR detected PEDV-specific RNA in fecal suspensions from pigs experimentally inoculated with PEDV on postinoculation days 1 to 9. PEDV RNA was also detected in samples of the small intestine, large intestine, and the mesenteric lymph node that were collected from CV777 inoculated pig 122 (only one animal was sampled).

Antigenic relatedness of the coronaviruses nucleocapsid protein has previously been documented with hyperimmune sera (Yaling et al., 1988; Have et al., 1992). Immunoblot analysis of the sera from the pigs inoculated with intestinal contents from pig 3, positive for TGEV by RT-PCR, revealed a faint reactivity with the 62 kd protein of PEDV. The immune response of TGEV inoculated pigs was similar to the immune response of PEDV inoculated pigs when reacted with PEDV antigens. The cross-reactivity of antisera from TGEV inoculated pigs with PEDV antigens excludes immunoblot analysis for diagnostic testing.

The serological response of the PEDV inoculated pigs was evaluated by IPT and immunoblot. PEDV-specific antibodies were first detected on day 14 by IPT. Antibody titers peaked on day 21. A decrease in titer was detected by day 35 in one pig. No decrease in antibody titer between days 21 and 35 was observed in the second animal. Immunoblot analysis revealed a similar pattern. Antibodies to a 62 kd protein developed on day 14 and were present in the serum for the duration of the experiment (Figure 10). Immunoblot analysis of serum collected 7 days after a second inoculation detected reactivity with additional PEDV proteins.

SUMMARY

In summary, sensitive nested RT-PCR assays were developed that accurately detected PEDV virus in the fecal and tissue samples from experimentally infected pigs. The RT-PCR assay with PEDV spike primer set 3 (corresponding to nucleotides 1581 to 2127 of GenBank accession number Z25483) was useful in detecting variation in the spike proteins of two PEDV isolates by gel electrophoresis. Sequence information of the spike PCR products revealed variation between the two geographically separated isolates. The significance of the nucleotide changes needs further study. Future development of a multiplex RT-PCR with the nucleocapsid and the spike primer sets would reduce the number of tests needed to be run on an individual sample. Inclusion of two gene targets in a diagnostic test would assure an accurate diagnosis. RT-PCR is a reliable, rapid diagnostic test that can be used to prevent the entry of PEDV into the U.S. and monitor porcine diarrhea outbreaks for PEDV.

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