Transmissible gastroenteritis in Cuba: experimental reproduction of the disease and molecular characterization of the virus

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Abstract

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease of pigs caused by an enteropathogenic coronavirus, TGEV. The sequence of the 5' end of the spike glycoprotein (S) gene best distinguishes this virus from related coronavirus. Since February 2003, a large number of unexplained outbreaks of gastroenteritis have occurred on pig farms in Havana. The problem was identified as TGE for the first time in May 2003. This paper describes the virological and molecular studies that led to this diagnosis. The disease was experimentally reproduced in susceptible piglets, a sow, and in weaned pigs. TGEV recovered from the diarrheic faeces of the sick piglets was identified by electron microscope negative staining, viral isolation in secondary swine kidney cultures, and by RT-PCR using previously reported primers. The PCR product amplified from the 5' end of the S gene was directly sequenced. The nucleotide sequence and the deduced amino acid residues of the amplified region are reported. A phylogenetic analysis was performed by comparing a 393-414 nucleotide stretch near the 5' end of the S gene in 36 viruses from different countries and with different isolation dates. The virus causing the outbreak in Cuba seems to be closely related to a TGEV previously isolated in the US Midwest. The source of infection remains unknown.

Additional key words: coronavirus, phylogenetic analysis, RT-PCR, spike protein, TGEV.

Resumen

Gastroenteritis transmisible en Cuba: reproducción experimental de la enfermedad y caracterización molecular del virus

La gastroenteritis transmisible (TGE) es una enfermedad entérica y altamente contagiosa de los cerdos causada por un coronavirus enteropatogénico, el TGEV. El extremo 5' del gen de la glicoproteina S de las espículas de la envoltura, difiere en gran medida de otros coronavirus relacionados. A partir de febrero del 2003, se produjeron numerosos brotes de gastroenteritis en granjas de cría de cerdos de la provincia de La Habana; la enfermedad fue caracterizada como TGE y reportada por primera vez en mayo del 2003. Se presentan los resultados de los estudios virológicos y moleculares que condujeron a este diagnóstico. La enfermedad fue reproducida experimentalmente en cerditos susceptibles, una cerda y su camada. El TGEV fue aislado de heces diarreicas provenientes de cerditos enfermos recién nacidos; se confirmó su identidad por tinción negativa al microscopio electrónico, aislamiento viral en cultivo primario de riñón de cerdo y RT-PCR usando cebadores específicos previamente descritos. El producto de PCR amplificado a partir del extremo 5' del gen de la proteína S fue secuenciado directamente. Se reporta la secuencia nucleotídica y la de aminoácidos deducida de la región amplificada. El análisis filogenético comparando las secuencias de un fragmento de 393-414 nucleótidos del extremo 5' del gen S de 36 virus de diferentes países y fechas de aislamiento, muestra que el virus que causó los brotes en Cuba parece estar muy relacionado con un aislado del medio oeste de EEUU, pero la fuente de la infección permanece sin esclarecer.

Palabras clave: análisis filogenético, coronavirus, proteína de la espícula, RT-PCR, TGEV.

Introduction

Transmissible gastroenteritis (TGE) is a highly contagious enteric disease affecting pigs. Mortality can be as high as 100% among newborn piglets when the virus spreads within a susceptible herd (Enjuanes and Van der Zeijst, 1995). TGE virus (TGEV) belongs to the *Coronaviridae* family (Enjuanes *et al.,* 2000). The viral membrane contains three proteins: S (220 kD), M (29-36 kD) and minor E (10 kDa). The spike glycoprotein (S) initiates infection by binding to the cell receptor via the aminopeptidase N binding site. It also shows sialic acid binding activity, perhaps providing a second binding site. This second receptor mechanism may partly account for the enteropathogenicity of the different strains (Schwegmann-Webels *et al.,* 2002).

The first report of clinical TGE dates from 1946 (Doyle and Hutchings, 1946). TGEV is very prevalent in the USA and Europe according to serological surveys (USDA, 1997; Krempl *et al.,* 2000), but in Cuba a lack of clinical and pathological evidence of the disease has not warranted serological screening. Even in electron microscope screenings conducted by Frías Lepoureau (1985) in diarrhoea samples from pigs, no coronavirus-like particles were found (only Rotavirus was detected).

Nevertheless, since February 2003, outbreaks of gastrointestinal disease have been recorded in the province of Havana. On affected farms, 100% of recently farrowed sows and their litters became sick with diarrhoea. The clinical signs of the newborns included very liquid and fetid, yellowish faeces plus vomiting, leading to serious dehydration and mortality rates close to 100%. At the onset of the disease, the sows showed a lack of appetite followed by vomiting and agalactia, but all recovered. The weaned and fattening pigs of these farms presented severe clinical signs, although only 8% lethality was reached. At *post mortem,* the intestines were seen to be distended by gas and the intestinal wall was transparent. Undigested coagulated milk was also seen in the piglets. Histologically, a loss of jejunal villi was consistently observed. These conditions were the main features for recognising the disease in the field. At first, mortality was associated with changes in concentrate/meal and food poisoning due to the presence of vomitoxin in some feed samples. However, the worsening epizootiological situation eventually involved almost all the farms in the province; this led to an infectious aetiology being proposed. The disease was readily transmitted to other farms on the island. In Havana province alone there were 15 outbreaks with 23,201 sick animals and 10,547 deaths. A further 5,256 animals had to be sacrificed (IMV, 2003).

This paper reports the results of experimental transmission studies leading to the diagnosis of TGEV as the etiological agent of these epizootics. The partial nucleotide sequence of the S glycoprotein gene is reported and the phylogenetic position of the Cuban isolate discussed.

Material and Methods

Experimental infection

A healthy, very recently farrowed sow, her litter of 7 piglets, and 10 weaned pigs (weighing between 12 and 15 kg), were obtained from a non-exposed farm. These animals were orally administered the homogenised intestinal tissue of sick animals (2 ml to the newborn piglets and 10 ml to the weaned pigs). The donors were 19 piglets with typical signs of clinical TGE (confirmed *a posteriori* by the indirect immunoperoxidase test) from two farms in Havana at the peak of the outbreak. These piglets were euthenised and their small intestines ligated. A pooled 10% suspension of these tissue and the intestinal content was then produced. This was clarified by centrifugation at 3500g for 15 min at 4°C, treated with antibiotics (1000 UI ml^{-1} penicillin, 1000 μg ml^{-1} streptomycin) and filtered (0.22 μm). Two newborn piglets and four weaned pigs were inoculated with phosphate-buffered saline (PBS) to act as in-contact controls.

For virus isolation, faecal samples were taken from the newborn piglets at the onset of diarrhoea. Two clinically affected animals were euthenised and samples of their intestinal tract collected for immunochemical diagnosis. All surviving animals were killed at 15 days post-infection (dpi). Blood samples were obtained at 0, 7 and 15 dpi for the detection of specific antibodies.

Electron microscope diagnosis

The intestinal homogenates from the experimentally infected piglets that developed disease and were killed for necropsy were clarified by centrifugation at 3,500g for 15 min at 4°C. The virus was concentrated by ultracentrifugation at 73,000g (SW-30, Beckman

HS75 rotor) for 90 min and purified in a discontinuous sucrose gradient in PBS (60, 40 and 20% w v^{-1}) at 100,000 g for 4 h. The virus collected was re-pelleted and dissolved in PBS.

The purified samples were negatively stained with phosphotungstic acid (2%, pH 7.0) or uranyl acetate (2%).

RT-PCR

RNA from purified virus and from the TGEV vaccine strain (Intervet) was extracted using Tripure isolation reagent (Boehringher Mannheim) and dissolved in 10 μl of nuclease-free water (Promega). The vaccine RNA was used as a positive control.

The primer sequences used for direct detection of the virus in faecal samples and sequencing assays (053 and 054; nt positions 20314-20332 and 21152-21171 respectively from TGE Purdue strain) were those of Ali and Reynolds (2000). The specific primers F1174 and R950 (Paton and Lowings, 1997) were used for the identification of isolated viruses in cell culture.

Reverse transcription was performed in a 20 μl volume reaction, using 5 μl of RNA, 1 μl of random hexamers (30 ng μl^{-1}, Promega), 1 μl of dNTP (10 mM), 4 μl of 5x RT buffer (Promega), 0.5 μl of RNAsin (20 U, Promega) and 0.5 μl of AMV reverse transcriptase (5 U μl^{-1}, Promega). Samples were incubated at 42°C for 40 min and then at 99°C for 5 min, before cooling to 4°C. PCR was performed in a total reaction volume of 50 μl containing 5 μl of 10x buffer (Promega), 5μl MgCl$_2$ (25 mM, Promega), 1μl dNTP (10 mM), 1 μl of each primer (stock of 0.2 μg μl^{-1}) and 0.2 μl of Taq polymerase (2.5 U, Promega). The temperature profile involved a first step at 94°C for 2.5 min, 32 cycles of 45 s at 94°C, 1 min at 53°C, and 1 min at 74°C. A final extension time of 5 min at 74°C was allowed. A 10 μl aliquot of each PCR product was visualized by agarose gel electrophoresis in Tris-borate buffer (TBE). RT-PCR was performed in triplicate and amplified products were pooled and cleaned using the Wizard PCR Preps Kit (Promega).

Sequencing and phylogenetic analysis

The nucleotide and deduced amino acid sequences from the 5' end of the S glycoprotein gene (750 bp including antigenic sites C and B) were determined from the PCR product (expected size: 858 bp).

Sequencing was performed using an ALF Express automated sequencer (Amersham Pharmacia Biotech).

A nucleotide stretch near the 5' end of the fragment corresponding to nucleotides 208-608 was used in sequence comparisons. A total of 399 nt were aligned with 36 published sequences for TGEV isolates, ranging in length from 393 to 414 nt depending on the presence of insertions and/or deletions. Nucleotide data editing and analysis and the prediction of amino acid residues were undertaken using the DNAMAN program (DNAMAN 4.2 *Lynnon Biosoft,* 1998). The distance between each pair of nucleotides was estimated according to the Jukes and Cantor method (1969). The corresponding dendrogram was constructed by the neighbour-joining method (Saitou and Nei, 1987) using MEGA 2.1 software (Kumar *et al.,* 2001). The reliability of the tree, i.e., the confidence levels for its branching order, was determined by the bootstrap method (Felsenstein, 1993) with 100 replicates of the original set of sequences.

Virus isolation

The material for virus isolation included diarrheic faeces and ligated intestine loops. The diarrheic faeces were collected from piglets with clinical signs 48 h post inoculation (hpi); the intestinal loops were taken from two sacrificed piglets. Homogenates of each sample were prepared with Dulbecco MEM (DMEM) tissue culture medium plus antibiotics (300 UI ml^{-1} penicillin, 300 μg ml^{-1} streptomycin sulphate, 7.5 μg ml^{-1} fungizone) to produce a 10% suspension. These were clarified, filtered and treated with an equal volume of heat-inactivated bovine serum following standard methods (OIE, 2000). The samples were inoculated into secondary pig kidney cell monolayers, previously washed with PBS and treated with DMEM plus 10 μg ml^{-1} trypsin and 20 mM Hepes (Paton *et al.*, 1997) (DMEM HT). After incubation at 37°C for 2 h, the cell sheets were washed twice with DMEM without trypsin and overlaid with DMEM HT.

Indirect immunoperoxidase test (IPI)

Cryostat sections of small intestine from 10 sick animals (naturally infected) were used to prepare

inocula. Two clinically affected piglets (experimentally infected) were analysed for the presence of TGEV antigens using the IPI test. The IPI test was also used to identify the virus isolated in tissue cultures. Isolated viruses were allowed to multiply in secondary pig kidney cell monolayers in plastic 4-well plates (Nunc) (inoculum dilution 1:10 or 1:100). At 18 hpi the cells were washed twice with PBS and fixed with 30% acetone for 10 min. The cryostat sections were also fixed with acetone. TGEV-specific monoclonal antibody (Ingenasa, S.A.) was added at a dilution of 1:50 in 10 mM Tris Buffer, pH 8 (OIE, 2000). After 1 h at 37°C, samples were washed three times with the same buffer. Anti-mouse peroxidase conjugate (Zymed) (1:100 diluted) was then added and incubation allowed to proceed at 37°C for 1 h. Aminoethyl carbazole was used as the chromogen in the H_2O_2 substrate solution.

Detection of TGEV specific antibodies by ELISA

A commercial kit (Ingezim Corona Differential, Ingenasa, S.A) was used to detect specific antibodies in serum samples from the experimentally infected animals. Four samples from the Republic of Cuba Serum Bank (CENSA, Havana) were also tested as part of a retrospective study. Two of these samples had been obtained from a farm in Havana in 1991; the other two came from sows brought from the USA for commercial exhibition prior to the outbreaks.

Results

Experimental infection

Clinical signs were successfully reproduced and the virus recovered from faecal and intestinal samples of infected newborn piglets. These showed the characteristic explosive, fetid, yellowish diarrhoea and depression at 18 hpi. Severe dehydration and death were seen at 5 dpi. At *post mortem,* the main pathological findings were the gas-filled stomach and intestine, milk coagulates, a translucid appearance to the intestine, and intestinal swelling and congestion. Histopathological analysis showed the loss of villi in

the jejunum and ileum. The infected sow suffered vomiting and agalactia but recovered after 5 days. The weaned pigs were less susceptible than the newborns; these suffered diarrhoea but all recovered before slaughter at 15 dpi. The in-contact animals showed the same clinical signs as the inoculated animals, but signs of disease onset occurred 24 h later. The sow, the surviving inoculated weaned pigs and the in-contact controls were negative for specific anti-TGE antibodies before inoculation, but all had seroconverted at 15 days. The samples from the Serum Bank were negative for specific antibodies according to ELISA.

Demonstration of the implication of TGEV

— Immunohistochemistry: In the IPI test, the cryostat sections showed extensive areas of immunoreactive enterocytes in the villi, providing evidence for the presence of TGEV antigens in both the animals used to provide the inoculum and in the experimentally infected pigs.

— Electron microscopy: Opalescent bands were observed after sucrose gradient centrifugation of all intestinal homogenates. Coronavirus-like particles of 170 nm were observed on grids after negative staining of samples from sick piglets (Fig 1).
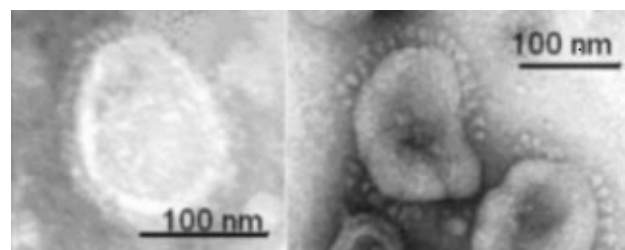

Figure 1. Coronavirus-like particles observed under the electron microscope (purified intestinal material from experimentally infected piglets negatively stained with 1% uranyl acetate).

— RT-PCR: The RT-PCR results for the intestinal contents corroborated the electron microscopy observations, with the amplified product showing the expected size (858 bp) (Fig. 2). This result allowed the conclusion that the causal agent of the outbreaks was TGEV.
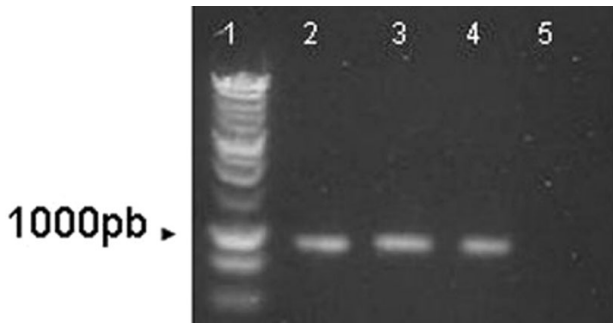
Figure 2. RT- PCR amplification with primers 053/054 of the RNA extracted from purified virus from intestinal homogenates of experimentally inoculated piglets. The figure shows the results of 2% agarose gel electrophoresis after staining with ethidium bromide. Lines: 1, 1 kb molecular marker (Promega); 2, positive control, TGE vaccine strain; 3 and 4, samples from piglets; 5, negative control, water.

— Virus isolation: At 48 hpi, a cytopathic effect (CPE) was observed in the cell cultures inoculated with the faecal samples taken 24 and 48 hpi from experimentally infected piglets and from the intestinal homogenates from the two piglets killed in the terminal stage of infection. The cells became rounded and syncitia were observed. The monolayers were destroyed after 72-96 h, the supernatants were
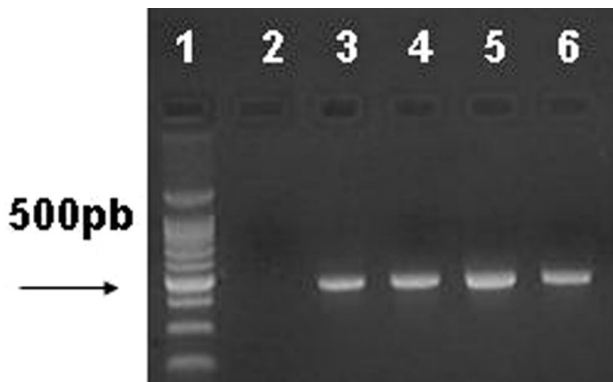

Figure 3. Identification of cell culture-isolated viruses from the diarrhoea of three sick piglet experimentally infected after three passages in swine kidney cells. Amplifications were performed by RT-PCR using primers F1174 and R950. Lanes: 1, size marker 100pb (Promega); 2, negative control; 3, Vaccine strain Intervet; 4, cell culture isolate VB 266; 5, cell culture isolate VB 267; 6, cell culture isolate VB 268.

harvested and second and third passages were performed to adapt the virus to tissue culture growth.

The supernatant of the third passage, which showed a stronger CPE, was selected for analysis by the IPI test. A TGEV-like isolate was identified from the positive cytoplasmic immunoreaction observed in the monolayers inoculated with the isolated virus or with the 1:10 dilution after 18 hpi, and from the absence of any reaction in the control wells with non-inoculated cells.

RT-PCR confirmed the identification of the isolated virus, the product showing the expected size of 476 bp (Fig 3). The CPE was neutralized by the sera of 10 field-recovered animals found reactive in ELISA tests (data not shown).

Molecular and phylogenetic analysis of the viral isolate

After PCR product sequencing, the 750-nucleotide sequence obtained (Accession: AJ884686) was compared with those held in the GenBank database. The sequence identity with TGEV ranged from 96-98%. The isolates and strains of TGEV used in the phylogenetic analysis have insertions and deletions in this region. The present sequence has deleted nucleotides, none of which altered the reading frame of the S gene.

The phylogenetic analysis of 36 TGEVs isolated from different countries and on different dates showed the present virus to locate in a defined cluster with TGEV BW021898B. The latter was isolated in 1999 from a Midwestern farm in the USA, suggesting that these strains have a common ancestor (Fig. 4). A number of other clusters were obtained for the remaining isolates. For instance, one group of viruses was closely related to the prototype Purdue isolate, another group included V344, V345 and V355 from Bulgaria and Russia, and another included Erica, Slagharen and V63, three closely related isolates that are geographically and temporally linked. The Korean and UK viruses clustered independently. Based on the partial sequence analysis of the S glycoprotein gene, the TGEV strains isolated in Korea seem to be different from all other TGEV isolates.
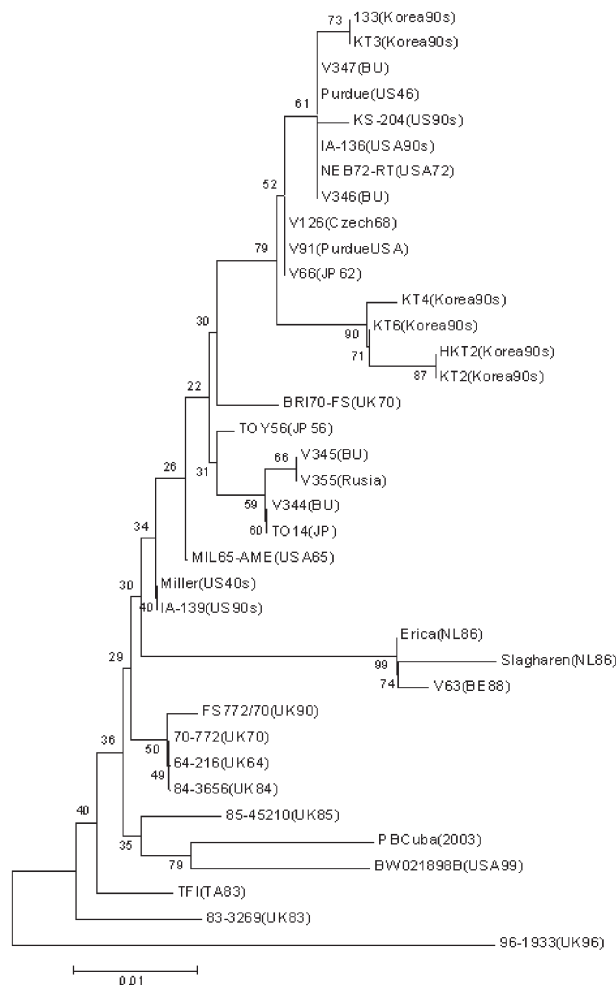
Figure 4. Dendrogram for the TGEV that caused the outbreak of TGE in Cuba. The sequences used for comparison were: FS772/70 (X53128); TFI (Z35758); W021898B (AF179882); TOY56-165 (M94103); PUR46-MAD (M94101); NEB72-RT (M94099); 96-1933 (AF104420); IA-139, IA-136, V346, V347, V66, V91, V126, 70-772, 84- 3656, 64-216, V355, V345, V344, KS-204, Erica, Slagharen, V63, 85-45210, 83-3269 (Paton and Lowings, 1997), Miller (S511223); BRI70-FS (X53128); MIL65-AME (S51223), 133 (AF481365), HKT2 (AF481366), KT2 (AF481360), KT3 (AF481361), KT4 (AF481362), KT6 (AF481364) and TO14 (AF302263).

Discussion

The experimental reproduction of clinical TGE in pigs inoculated with intestinal material from animals with naturally-acquired gastrointestinal illness shows the disease to be infectious and naturally transmissible. Another important observation was the transmissibility of infection to the in-contact pigs. It is remarkable how the different clinical signs correlated closely with the age of the animals, as previously described (Enjuanes and Van der Zeijst, 1995). The experimental piglets showed profuse diarrhoea, dehydration, and eventually died, while older animals (the sow and weaned pigs) became sick but recovered and seroconverted (as indicated by ELISA results). The experimentally- induced disease was remarkably similar to that observed in the field. Newborn piglets were the most affected and showed 100% mortality. The sows and weaned pigs became ill with vomiting and diarrhoea, but all recovered in about 5 days. In both the experimental and natural disease, the main pathological finding was the translucid appearance of the intestinal wall, the swelling of the intestine with gas and the appearance of milk coagulates. Histopathological analysis showed the loss of villi in the jejunum and ileum. The virus was recovered as early as 24 hpi in experimentally infected newborn pigs, and at 24 h after the onset of diarrhoea in naturally sick piglets. Sestak and Saif (2002), however, reported the shedding of TGEV at 72 hpi in experimentally inoculated pigs.

The presence of coronavirus-like particles in the intestinal samples indicated the need for a differential diagnosis between TGEV and pig epidemic diarrhoea (PED), whose etiological agent belongs to the same genus and causes similar clinical problems (Pensaert, 1999; Sestak and Saif, 2002). The agent causing the outbreaks was confirmed as TGEV by: 1) the amplification of specific genomic sequences by RT-PCR; 2) the high homology between the nucleotide sequence of the Cuban isolate and other TGEVs and; 3) the isolation of TGEV from the faecal samples of sick piglets with experimentally-induced disease. These results confirm the presence of transmissible gastroenteritis in Cuba, as reported in May 2003 by the country's veterinary authorities (OIE, 2003).

The ELISA results provided further evidence of this first appearance of TGEV in Cuba. No specific antibodies to TGEV or coronaviruses were found in samples from non-exposed farms in a serological survey conducted *a posteriori* to determine the geographical limits of the problem (Acevedo Beiras *et al.,* pers. commun.). At time 0, no specific antibodies were detected in the sample. Neither were any detected in the samples obtained from the serum bank.

The size of the PCR products amplified by the primers 053 and 054 from the 5' end of the S protein gene allowed discrimination between TGEV and the closely related porcine respiratory coronavirus (PRCV) since PRCV shows a nucleotide deletion in this region (Laude *et al.,* 1993; Paton and Lowings, 1997). RT-PCR is useful for detecting TGEV and has the advantage that no isolation of the virus is required. The viral nucleotide sequence was obtained directly from the virus in clinical samples. This made the phylogenetic analysis more reliable since variation from the original source caused by genomic RNA mutations during culture is obviated by direct sequencing of the sample (Krempl *et al.,* 2000). The studied sequence differs from those of most canine and feline coronaviruses and PRCV. Yet other coronaviruses have been found to contain large deletions in the studied part of the S gene (Kwon *et al.,* 1998). The results show the Cuban isolate to be more closely related to TGEV than to related coronavirus causing epidemic diarrhoea or from those of dogs and cats.

The present results are similar to those reported by Paton and Lowings (1997) who compared the same nucleotide stretch of the S gene belonging to 20 TGEV isolates. The Purdue cluster seemed to be exclusively formed by the Purdue strains, at least according to the distances between TGEV isolates based on these S gene sequences (Penzes *et al.,* 2001). A cluster was also seen for the Korean viruses (Kwon *et al.,* 1998; Kim *et al.,* 2003) which were isolated from similar geographical locations, and the Cuban isolate was found to be very similar to a relatively recent American isolate, BW021898B, currently circulating in pig populations in the USA and other American countries. How it reached Cuba, however, remains unclear. Certainly there is no current pig trade between American nations and Cuba. Serum samples from two sows brought to Cuba from the USA (prior to the outbreaks) for a commercial exhibition showed no antibodies to TGE or coronavirus.

The virulence of TGEV strains is linked to their enteropathogenicity, which depends on the sialic acid binding site on protein S (Krempl *et al.,* 1997, 2000) linking with the cell surface sialoglycoprotein (Schwegmann-Webels *et al.,* 2002). In comparisons of this S gene region of the Cuban isolate with that of Purdue 48 MAD, only 6 amino acid residues substitutions were found at positions 122, 180, 183, 207, 216 and 217. These are not, however, the locations for mutations thought associated with the abolishment of enteropathogenicity in the TGEV strain (Sánchez *et al.,* 1999).

The recent outbreaks of TGE in Cuba could be an example of a «virgin soil epidemic» (Murphy *et al.,* 1999). The pigs were completely susceptible and in the field the virus was highly virulent. The aim of the present work was not, however, to study the virulence of the virus but to seek diagnostic confirmation and to identify the source of the virus. It would be interesting to compare the virulence of the Cuban TGEV strain with that of others and to complete the characterization of the isolated virus for its use as a reference in laboratory diagnostic tests.

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