

# Diagnosis to Detect Porcine Transmissible Gastroenteritis Virus (TGEV) by Optical and Transmission Electron Microscopy Techniques

Diagnóstico para Detectar el Virus de la Gastroenteritis Porcina Transmisible por Técnicas de Microscopía Óptica y Electrónica de Transmisión

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MARTINS, A. M. C. R. P. F.; BERSANO, J. G.; OGATA, R.; AMANTE, G.; NASTARI, B. D. B.; CATROXO, M. H. B. Diagnosis to detect porcine transmissible gastroenteritis virus (TGEV) by optical and transmission electron microscopy techniques. *Int. J. Morphol.*, 31(2):706-715, 2013.

**SUMMARY:** The porcine transmissible gastroenteritis is a highly severe contagious disease, caused by virus of the Coronaviridae family, genus Coronavirus. Its epizootic shape can reach a rate of up to 100% mortality in piglets under two weeks of age as a result of severe dehydration. In this study fragments of small intestine and stool samples were collected from 75 autopsied pigs from properties. The samples of the fragments were frozen and sent to the Laboratory of Electron Microscopy, Instituto Biológico, SP, Brazil, for histological and transmission electron microscopic analyses. According to histological H&E technique, atrophy, villous necrosis and destruction of the enterocytes were observed in 35 (46.6%) out of the 75 fragments of the small intestine samples. On the immunohistochemistry technique 19 (25.3%) fragments were positively stained with DAB in the Ag-Ac reaction (MabTGEV). In 19 (25.3%) positive samples analyzed by *in situ* hybridization, a brown stain of enterocytes was observed, mainly in the epithelial cells of the villi. By the negative staining technique, we visualized enveloped, pleomorphic coronavirus particles, with typical radial projections resembling solar corona, with 140 nm diameter in 21 samples (28%) of the small intestine fragments and in 16 (21.3%) stool samples. In the ultrathin sections of 21 (28%) samples of small intestine, complete coronavirus particles with 80 nm diameter were seen among the microvilli and in the cytoplasm of epithelial cells. Immature particles with 60 nm diameter, budding from cell membrane and from a rough endoplasmic reticulum and also inside the vacuoles were visualized. In 19 (25.3%) out of 75 analyzed samples of small intestine, the antigen-antibody interaction was characterized by aggregation of viral particles in the immunoelectron microscopy technique. In the immunocytochemistry technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the coronavirus in 19 (25.3%) out of the 75 samples of fragments of small intestine examined.

**KEYWORDS:** Porcine Transmissible Gastroenteritis; Coronavirus; Histopathology; Transmission Electron Microscopy.

## INTRODUCTION

The porcine transmissible gastroenteritis is described as a highly contagious disease caused by coronavirus. It belongs to the Nidovirales order, Coronaviridae family, *Alphacoronavirus* genus, group Ia (Woo *et al.*, 2009). They are single-stranded, positive sense RNA viruses. Morphologically they are pleomorphic, with radial projections with a form like-club giving an aspect of solar corona. They have a diameter of 75-160 nm (Cappellaro *et al.*, 1998; Cleri *et al.*, 2010). These viruses are prone to genetic evolution by accumulation of point mutations in genes coding for structural and non-structural proteins and by analogous recombination between members of the same antigenic group (Decaro *et al.*, 2010).

The viral envelope is composed of five proteins (M, sM, HE, S and I) (Schwegmann-Webels *et al.*, 2002). The binding cell receptor to the viral spike glycoprotein (S) via aminopeptidase initiates infection. A second mechanism mediates binding cell receptor to the sialic acid seems to play a role in different strains of enteropathogenicity (Schwegmann-Webels *et al.*, 2002). Additionally, the 5'-terminal sequence differentiates porcine transmissible gastroenteritis virus (TGEV) from other existing coronavirus (Barrera-Vale, 2005).

This disorder was first described in 1946 by Doyle & Hutchings. It affects pigs of all ages and presents as characteristic symptoms, severe watery diarrhea

accompanied by vomiting (Wyllie *et al.*, 1984; Sur *et al.*, 1998). When compared with the respiratory variant form, porcine transmissible gastroenteritis occurs in most sporadic intervals. The respiratory variant form has spread over the world since 1986.

In the TGEV epizooty the high mortality rates, up to 100%, affecting piglets of less than 2 weeks of age is a result of severe dehydration (Saif & Wesley, 1999).

The replication of these viruses can occur both in the digestive and respiratory tracts as in mammary tissue of the pigs (Kemeny *et al.*, 1975). The most important goal of these agents is the epithelial cells of the small intestinal villi (Pensaert *et al.* 1970). The resulting atrophy of the infected epithelium results in severe intestinal disorders, which can be fatal in the neonatal period (Haelterman, 1972). Also the reduction of intestinal surface can make the digestion and absorption processes difficult and trigger diarrhea (Shoup *et al.*, 1996).

The mode of TGEV transmission still remains unclear, suggesting contamination by breastfeeding, oral-fecal, fomites by and through hosts as dogs, cats and foxes. It is likely that under tropical climates the role of fomites is less important. Usually, the disease occurs due to the introduction in properties of pigs subclinically infected, infected or already recovered. The fact that these animals eliminate the virus in feces for 2-3 weeks, may lead to propagation of these in the place (Saif & Sestak, 2006).

The low temperature of the northern countries is an ideal condition for the maintenance of viral activity. Exportation of fresh and frozen pork contaminated by TGEV, allows that these type of food could act as a potential source of viral transmission. The marketing of semen and embryos of pigs, according to OIE guidelines, no longer presents any evidence of contamination risk (Geering *et al.*, 1995; Saif & Wesley, 1999; OIE, 2008).

As a notifiable disease, the porcine transmissible gastroenteritis causes significant economic losses in the pig industry, and has been reported in several countries as Europe, America and Asia (Saif & Wesley, 1999; World Animal Health, 2001; Sestak & Saif, 2002; OIE, 2008).

In the TGEV epizooty, to take immediate actions demand a fast diagnosis of the presence of the viral agent.

This study aimed to standardize the histopathologic and electron transmission microscopic techniques for the use in diagnosis routine and in outbreaks and epizooty of

porcine transmissible gastroenteritis, thus collaborating with the National Porcine Agribusiness.

## MATERIAL AND METHOD

Fragments of small intestine from 75 necropsied pigs were collected from April 2009 to June 2012 in São Paulo properties of the following cities Jacarei, Pedra Bela, Franco da Rocha, Boituva, Descalvado, Embu Guaçu, São José dos Campos and in Minas Gerais, in the city Onça do Pitangui, Brazil.

All fragments were packed in ice and sent to the Laboratory of Electron Microscopy of the Instituto Biológico, SP, Brazil, to be hystopathologically processed (routine histological technique (H&E), immunohistochemistry and in situ hybridization) and by transmission electron microscopy technique (negative staining, resin embedding, immunoelectron microscopy and immunocytochemistry).

### Optical microscopy

**Routine histological technique.** All 75 fragments of the small intestine were fixed in 10% buffered formalin, dehydrated, diaphonized and embedded in paraffin. 5 µm thick sections were performed and stained with hematoxylin and eosin technique.

**Immunohistochemistry Technique.** Sections of the 75 fragments of small intestine with 5mm thick were deparaffinized and rehydrated. Antigen retrieval was done at room temperature in which they were applied 100 µl Proteinase K (Dako - S3020) for each cut for 5 minutes and washed with distilled water. The blocking of endogenous peroxidase aiming to minimize unspecific reactions was processed with 200 µl of 10 volume hydrogen peroxide at 3% in distilled water for 20 minutes. Next, slides with cuts were rinsed with distilled water, followed by a wash with phosphate buffer (0.1 M PBS). Thereafter, 100 µl of primary antibody monoclonal for TGEV (6AA6: Porcine Transmissible Gastroenteritis INGEZIM TGEV 2.0 11.TGE) diluted at 1:10, with Dako background reducing components (Code S3022) applied at room temperature for each cut and incubated in a humid chamber for 18 hours in refrigerator (2-8°C). Subsequent to this period, there were two washes with phosphate buffer (0.1 M PBS) for 1 minute. The visualization system used was the LSAB ® + System-HRP (Dako - code. K0690) adapting the protocol suggested by the manufacturer. The incubation time was 20 minutes for

each of the reagents interspersed with two washes with phosphate buffer (0.1 M PBS) for 1 minute. The substrate-chromogen system was the Liquid DAB+ Substrate Chromogen System (Dako - code. K3468) with 5 minutes incubation and rinsed in distilled current water. Counter-staining was performed with hematoxylin. Negative controls were due to lack of addition of primary antibody fragments of small intestine of healthy pigs (Shoup *et al.*)

### **In situ hybridization**

The RNA probes utilized for Porcine Transmissible Gastroenteritis were:

#### **Primers**

TGEV-T2 A sequence 5' -CACUAACCAACGUCCAACUA -3 '  
TGEV-T2 G sequence 5'-G CACUAACCAACGUCCAGCUA 3 '

**5' Modification biotin (Invitrogen).** The fragments of the small intestine of 75 pigs were collected and fixed in 10% formalin for 36-48 hours, dehydrated in increasing gradients of alcohols (70°, 80°, 95° and absolute), diaphanized with xylene and mounted in paraffin bath in a hot bath at 58°C for 18 hours. 4 mm thick cuts were placed on slides marked and kept at room temperature. Next, they were deparaffinized with xylene and rehydrated in decreased gradients of alcohols (absolute, 95°, 80°, 70°) and distilled water. For antigen recovery, a pretreatment was adopted a hot bath at 96° C and diluted buffer (Dako S1699) for 40 min. When slides with cuts were cooled, endogenous peroxidase was blocked at room temperature for 20 min. and, next, the enzymatic digestion of the tissues with proteinase k (Dako) at room temperature for 5-15 min was realized.

The biotinylated probes were mixed including specific target RNA on the fragments of the small intestine and a cover glass was laid over it. Samples from these fragments and probes were denatured and hybridized overnight (18 hours) in Dako hybridization system (denaturation at 96°C and hybridization at 37°C). After stringency, a wash with TBST (Tris-buffered saline / Tween) was performed. The visualization system used was the primary streptavidin dilution buffer (Dako - Kit cod. K0690) for 30 minutes in a humid chamber with Biotinyl Tyramide reagent for 15 min at room temperature and then the secondary streptavidin for 15 min. The procedures were intercalated with two washes of TBST buffer for 5 minutes. The substrate-chromogen system was the Liquid DAB+ Substrate Chromogen System (Dako - code. K3468), and incubation performed for 5 minutes at room temperature, followed by wash in distilled current water. Counter-staining was performed

with hematoxylin (Braissant & Wahli, 1998; Kim & Chae, 2001).

### **Transmission Electron Microscopy.**

**Negative staining technique (rapid preparation).** In this technique, samples of small intestine fragments and samples of feces of 75 pigs were suspended in 0.1 M phosphate buffer, pH 7.0. Drops of the obtained suspensions were placed in contact with metallic copper grids, stabilized with carbon supporting film of 0.5% in collodium amyl acetate. Next, the grids were drained with filter paper and negatively stained at 2% ammonium molybdate, pH 5.0 (Brenner & Horne, 1959; Hayat & Miller, 1990; Madeley, 1997).

**Resin embedding technique.** All 75 fragments of small intestine samples were fixed in 2.5% glutaraldehyde in 0.1 M, pH7.0 phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer. After dehydration in cetonic series, the fragments were embedded in Spurr resin (González-Santander 1969; Luft, 1961). Ultrathin sections were cut on the LKB ultratome and mounted on copper grids. The sections were contrasted with uranyl acetate-lead citrate (Watson, 1958; Reynolds, 1963).

**Immunoelectron microscopy technique.** In this technique, copper grids, previously prepared with collodion film and stabilized with carbon were first incubated with protein A (1µl/ml) placed in contact with the virus-specific antibody. After, grids were washed in PBS drops, incubated with the viral suspension of the 75 samples of small intestine, washed with drops of water and negatively stained with 2% ammonium molybdate, pH 5.0 (Berthiaume *et al.*, 1981; Katz & Kohn, 1984; Doane & Anderson, 1987; Hayat & Miller, 1990; Pa-drón, 1998).

**Immunocytochemistry technique.** At the immunolabeling technique with colloidal gold particles for negative staining, the copper grids were placed in contact with viral suspension of the 75 samples of small intestine fragments and, after removing excess with filter paper, the same were put on specific primary antibody drops. After successive washings in PBS drops, the grids were incubated in protein A drops, in association with 10 nm colloidal gold particles (secondary antibody). Grids were then contrasted at 2% ammonium molybdate, pH 5.0 (Knutton, 1995).

All grids submitted to the above reactions were observed in a Philips EM 208 electron micro-scope, at 80 kV.

## RESULTS

### Histopathology

Small intestine fragment of 75 pigs were processed and examined by the histopathology techniques (H & E, immunohistochemistry and *in situ* hybridization).

**Routine histological technique (H&E).** At the histological examination, 35 (46.6%) out of the 75 fragments of the small intestine presented atrophy and necrosis of villi in small intestine with destruction of enterocytes and presence of large hemorrhagic areas, inflammatory and monolymphocytic cells and a great number of eosinophils present in the affected areas (Figs. 1 and 2).

**Immunohistochemistry technique.** By immunohistochemistry it was visualized that 19 (25.3%) total of fragments showed positive DAB in the Ag-Ac reaction (MabTGEV) (Figs. 3, 4).

***In situ* hybridization technique.** This technique used RNA probes. It was verified that 19 (25.3%) fragments were positive by *in situ* hybridization with brown stain in enterocytes and mainly in villi of epithelial cells (Figs. 5, 6, 7, 8).

### Transmission Electron Microscopy.

**Negative staining technique (rapid preparation).** On transmission electron microscopy using the negative staining technique (rapid preparation), it was found enveloped, pleomorphic coronavirus-like particles, measuring about 140 nm and containing radial typical

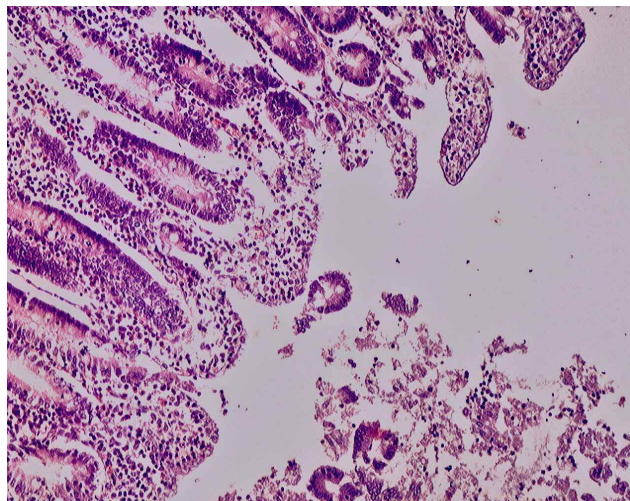


Fig. 1. Photomicrograph of the small intestine of swine. Observe desquamative and necrotic enteritis severe. X60.

projections resembling solar corona, in 21 (28%) out of the 75 samples of small intestine fragments and in 16 (21.3%) out of 75 fecal samples analyzed (Figs. 9, 10, arrow).

**Resin embedding technique.** In ultrathin sections, 21 (28%) out of the 75 samples of small intestine fragments it was observed rounded coronavirus particles, measuring approximately 80 nm in diameter, located between the microvilli and in the cytoplasm of epithelial cells. Immature particles measuring approximately 60 nm in diameter, budding from cell membrane (Fig. 11, big arrow) and from rough endoplasmic reticulum (Fig. 12, big arrow) and located inside the intracytoplasmic vacuoles contoured by a membrane (Fig. 11, small arrow) and complete particles in the cytoplasm (Fig. 11, blue arrow, Fig. 12, minor arrow) were observed. It was found in the intestinal cells, the occurrence of shortening and reduction or destruction of the microvilli (Figs. 13, 14, big arrow). The organelles were altered or empty (Figs. 13, 14, minor arrow). Some nuclei were pyknotic, deformed with densely packed chromatin (Fig. 14, blue arrow). The cisternae of the rough endoplasmic reticulum (site of virus replication) were dilated (Fig. 15, arrow).

**Immunoelectron microscopy technique.** In 19 (25.3%) out of 75 small intestine samples analyzed the antigen-antibody interaction was characterized by aggregation of viral particles (Fig. 16, arrow).

**Immunocytochemistry technique.** In this technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the coronavirus in 19 (25.3%) samples of fragments (Fig. 17, arrow), confirming the results of immunoelectron microscopy technique.

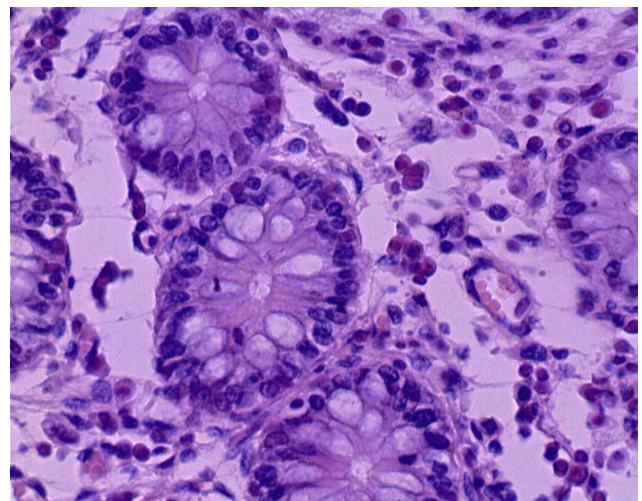


Fig. 2. Photomicrograph of the small intestine of swine with monolymphocytic and eosinophilic enteritis severe. X160.

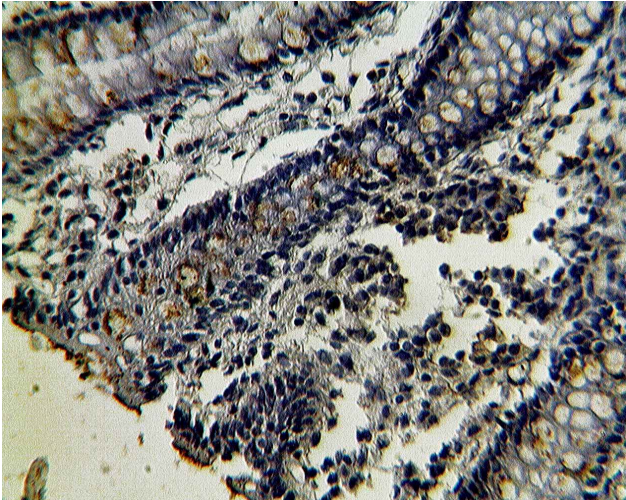


Fig. 3. Photomicrograph of the small intestine of swine. Observe numerous cells stained by the reaction antigen-antibody by immunohistochemistry technique. X160.

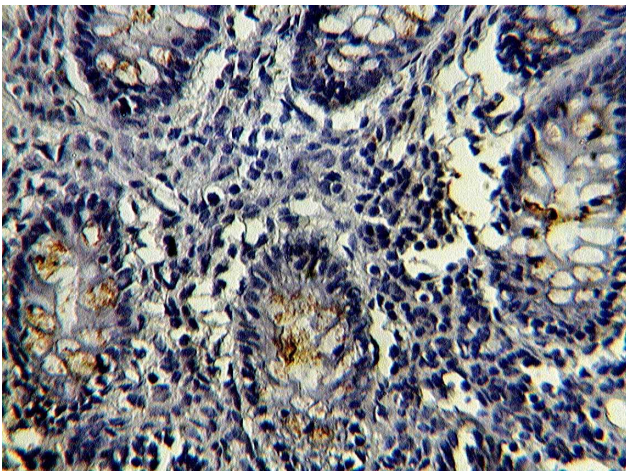


Fig. 4. Photomicrograph of the small intestine of swine. Observe numerous cells stained by the reaction antigen-antibody by immunohistochemistry technique. X160.

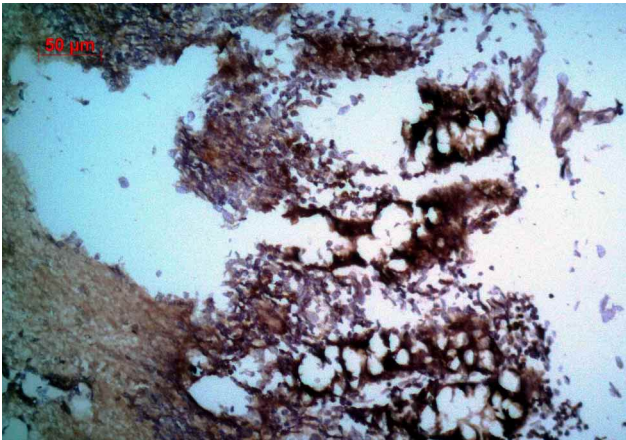


Fig. 5. Photomicrograph of the small intestine of swine with desquamative and necrotic enteritis. Observe stained brown mRNA of TGEV in the epithelial cells of the villi by in situ hybridization technique. X160.

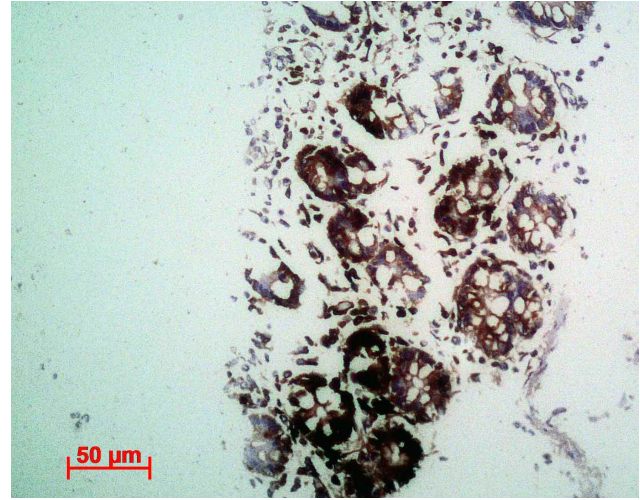


Fig. 6. Photomicrograph of the small intestine of swine. Observe stained brown mRNA of TGEV in the epithelial cells of the villi by in situ hybridization technique. X160.

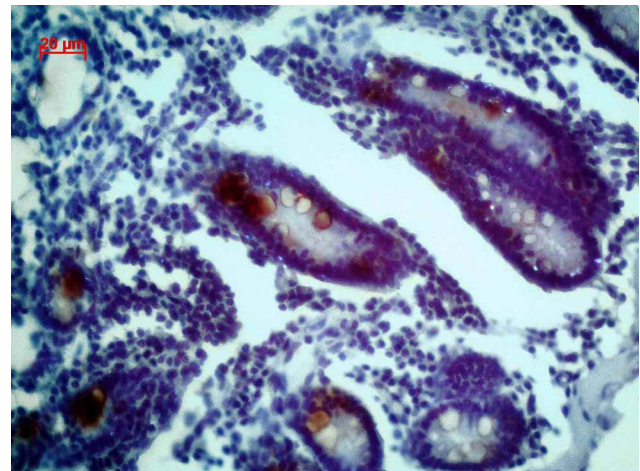


Fig. 7. Photomicrograph of the small intestine of swine. Observe stained brown mRNA of TGEV in the epithelial cells of the villi by in situ hybridization technique. X400.

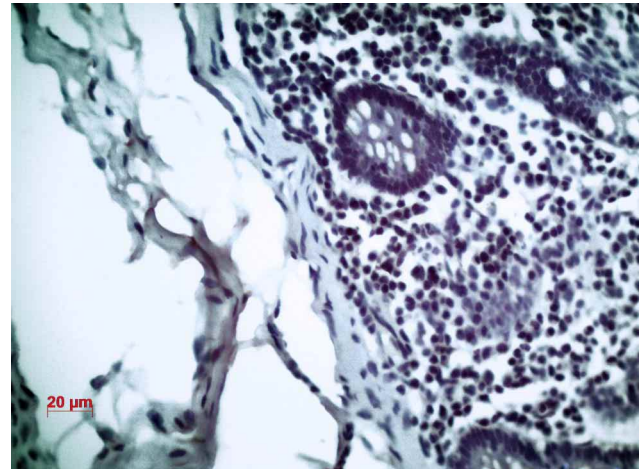


Fig. 8. Photomicrograph of the small intestine of swine. TGEV negative control. X400.

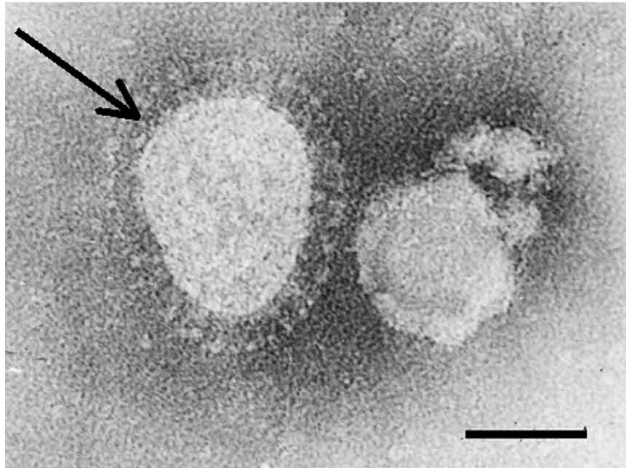


Fig. 9. Electron micrograph of coronavirus particles in small intestine by negative staining, showing characteristic envelope-shaped corona (arrow). Bar: 80nm.

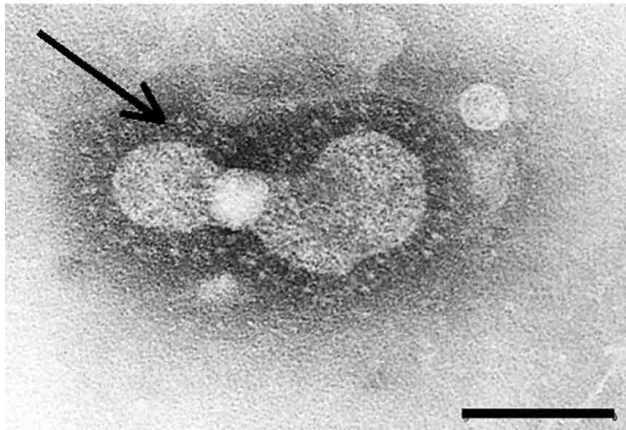


Fig. 10. Electron micrograph of coronavirus particle in small intestine by negative staining with a solar corona-shaped envelope (arrow). Bar: 80nm.

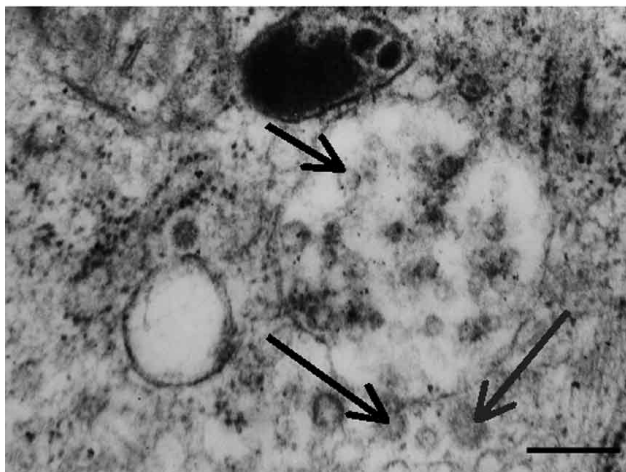


Fig. 11. Electron micrograph showing small intestine epithelial cell. Observe coronavirus particles, incomplete, within intracytoplasmic vacuoles (minor arrow); viral particles budding from the cell membranes (big arrow) and complete particles (arrow blue). Bar: 200 nm.

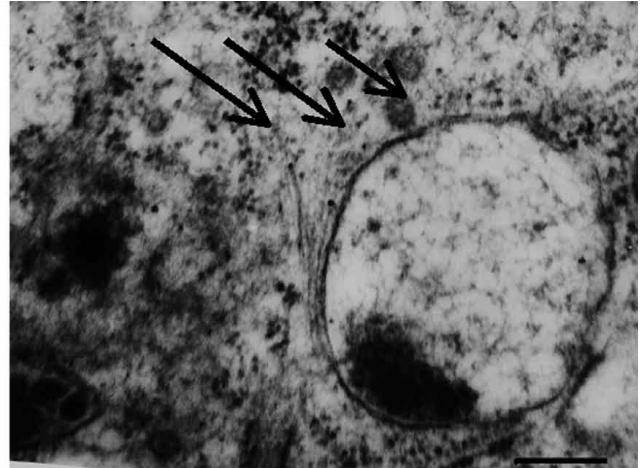


Fig. 12. Electron micrograph of the small intestine cell. Observe incomplete viral particles budding from rough endoplasmic reticulum (big arrow) and complete particles (minor arrow). Bar: 100 nm.

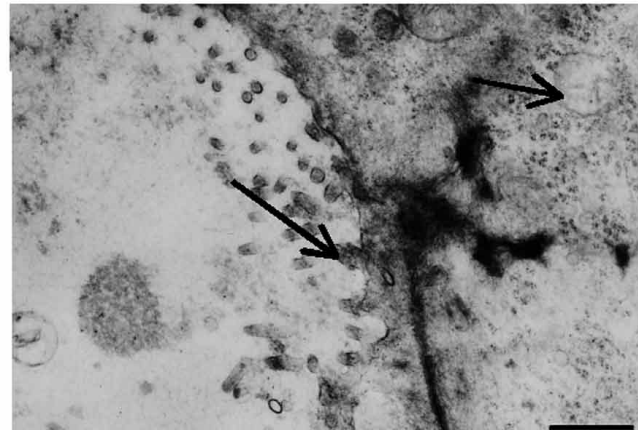


Fig. 13. Electron micrograph of the small intestine cell. Observe of shortening and reduction of the microvilli (big arrow) and empty mitochondria (minor arrow). Bar: 720 nm.

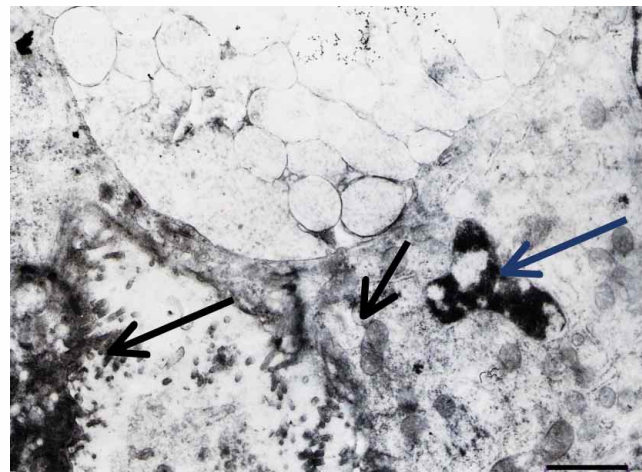


Fig. 14. Electron micrograph of small intestine cell showing altered microvilli (big arrow), empty organelle (minor arrow), picnotic and deformed nucleus (arrow blue). Bar: 1540 nm.



Fig. 15. Electron micrograph of small intestine cell showing dilated cisternae of the rough endoplasmic reticulum (seta). Bar: 200nm.

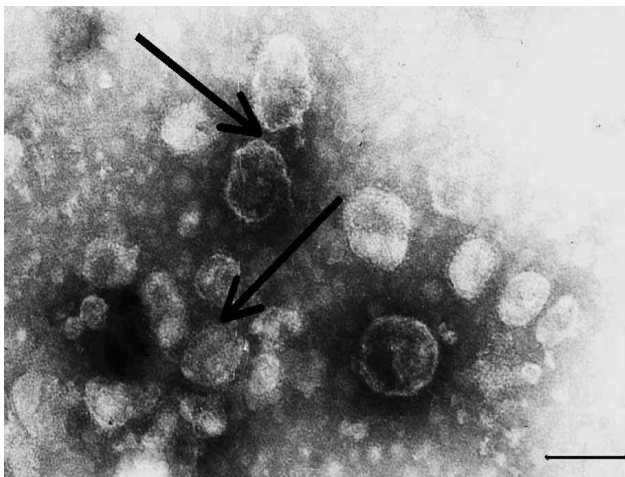


Fig 16. Electron micrograph showing coronavirus particles aggregated by antigen-antibody interaction in the immunoelectron microscopy technique. Bar: 140 nm.

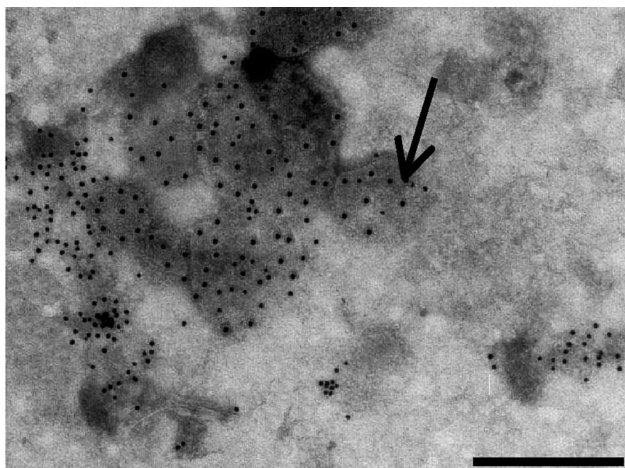


Fig.17. Electron micrograph of TGEV particles enhanced by colloidal gold particles in the immunocytochemistry technique. (arrow). Bar. 120 nm.

## DISCUSSION

The histological analysis of 75 cuts of fragments of small intestine stained with H&E showed in 35 (46.6%) of these, the presence of desquamative and necrotic enteritis caused by various non-specific pathogens. The observed lesions since are not pathognomonic to the porcine transmissible gastroenteritis, is necessary to complement with immunostaining techniques to diagnose the virus.

The immunohistochemical techniques using TGEV specific-antibodies and *in situ* hybridization with probes, both with high specificity and sensitivity, demonstrated the presence of these viruses in 19 (25.3%) samples, in accordance with the results of Shoup *et al.* (1996); Sirinarumitr *et al.* (1996); Chae *et al.* (1999); Kim & Chae (2001); Jung *et al.* (2003); Kim & Chae (2007).

The analysis under transmission electron microscope using the technique of negative stain showed in 21 (28%) of samples of fragments of the small intestine and in 16 (21.3%) stool samples, the presence of an increased number of pleomorphic coronavirus particles, measuring 140 nm in diameter, with radial club-like projections, giving the viral particle the appearance of solar corona. These features were described in other studies (Phillip *et al.*, 1971; Chasey & Cartwright, 1978; Dea *et al.*, 1985; Callebaut *et al.*, 1988; Benfield *et al.*, 1991; Risco *et al.*, 1995; Cappellaro *et al.*, Bersano *et al.*, 2003; Barrera-Valle *et al.*; Nogales *et al.*, 2012).

Atrophy and shortening of the microvilli observed in the intestinal cells in ultrathin cuts were consistent with the findings of other researches (Bauk & Stone, 1985; Pospischil *et al.*, 1981; Cappellaro *et al.*). In these cuts, coronavirus particles were located freely in the cytoplasm of epithelial cells of the microvilli, within intracytoplasmic vacuoles and budding from membranes of the rough endoplasmic reticulum, as described in the analyzed literature (Horvath & Mocsari, 1981; Cappellaro *et al.*; Risco *et al.*; Nogales *et al.*). It was possible to observe the dilated aspect of cisternae of the rough endoplasmic reticulum, site of viral replication, as mentioned by Salanueva *et al.* (1999).

In the immunoelectron microscopy technique, a positive antigen-antibody reaction, performed with a monoclonal antibody specific for TGEV, was detected in 19 (25.3%) samples of fragments of small intestine as a result of agglutination of a high number of viral particles. This technique was used previously by other researchers (Jensen *et al.*, 1980; Saif & Wesley, 1999).

The immunocytochemical technique showed in 19 (25.3%) samples of fragments of the small intestine extensive marking particles with colloidal gold, obtaining confirmation of the viral strain (TGEV), being these results similar to other publications (Garzon & Dea, 1991; Larochelle & Nagar, 1993; Risco et al. 1995).

According to the OIE (2008), techniques of transmission electron microscopy and *in situ* hybridization were chosen to identify TGEV by allowing differentiating them from those that cause epidemic porcine diarrhea and of the porcine respiratory coronavirus due to the use specific monoclonal antibodies.

In the present study the positive results for TGEV by

immunohistochemistry and *in situ* hybridization techniques were consistent with the results obtained by immunoelectron microscopy and immunocytochemistry by transmission electron microscopy.

In conclusion it is believed that the application of these techniques in routine diagnosis or during outbreaks or TGEV epizooty may help to develop appropriate measures for prevention and control of porcine transmissible gastroenteritis, collaborating with National Porcine Agribusiness.

This is the first report of identification of porcine transmissible gastroenteritis virus in São Paulo, Brazil through immunolabelling techniques.

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**RESUMEN:** La gastroenteritis transmisible porcina se caracteriza por ser una enfermedad altamente contagiosa y aguda, causada por virus de la familia Coronaviridae, género Coronavirus. Su forma epizootica puede alcanzar una tasa de hasta 100% de mortalidad en lechones con menos de dos semanas de edad, como resultado de deshidratación severa. En este trabajo se recogieron 75 fragmentos de intestino delgado y muestras de heces de 75 cerdos autopsiados. Las muestras se congelaron y se enviaron al Laboratorio de Microscopía Electrónica, del Instituto Biológico, SP, Brasil, para el análisis histológico y por microscopía electrónica de transmisión. Por la técnica histológica de H&E fue observado atrofia y necrosis de la vellosidades además de la destrucción de los enterocitos en 35 (46,6%) de las 75 muestras de fragmentos del intestino delgado. Por inmunohistoquímica 19 (25,3%) de los fragmentos se tiñeron positivamente por el DAB en la reacción Ag-Ac (MabTGEV). En 19 (25,3%) muestras positivas mediante hibridación *in situ*, se observó tinción marrón de los enterocitos, principalmente en las células epiteliales de las vellosidades. Por la técnica de coloración negativa, se observaron partículas de coronavirus, encapsuladas, pleomórficas con proyecciones radiales típicas, en forma de corona solar, midiendo alrededor de 140 nm de diámetro en 21 (28%) muestras de fragmentos del intestino delgado y en 16 (21,3%) muestras de heces. En cortes ultra finos de 21 (28%) fragmentos de intestino delgado fueron visualizadas partículas de coronavirus completas con 80 nm de diámetro entre las microvellosidades y en el citoplasma de las células epiteliales y partículas inmaduras de 60 nm de diámetro brotando de las membranas celulares y del retículo endoplasmático rugoso y en el interior de las vacuolas. En 19 (25,3%) de las 75 muestras de intestino delgado, la interacción antígeno-anticuerpo se caracterizó por agregación de las partículas virales en la técnica de microscopía inmunoelectrónica. En la técnica de inmunocitoquímica, la interacción antígeno-anticuerpo fue fuertemente reforzada por las densas partículas de oro coloidal sobre el coronavirus en 19 (25,3%) de los 75 fragmentos del intestino delgado examinados.

**PALABRAS CLAVE:** Gastroenteritis Transmisible Porcina; Coronavirus; Histopatología; Microscopía Electrónica de Transmisión.

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Received: 10-03-2013

Accepted: 04-04-2013