Diagnostic Techniques for porcine Circovirus Type 2 (PCV-2) by Optical and Transmission Electron Microscopy

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***Abstract*—** *The porcine circovirus type 2 is the etiologic agent of the post-weaning multisystemic wasting syndrome (PMWS) or post-weaning multisystemic cachetizing syndrome. Swine circovirosis is considered an emerging disease that can become a limiting factor for the development of the porcine industry worldwide. This study is aimed at detecting the PCV-2 presence in organ fragments of 65 terminated pigs from slaughterhouses in São Paulo, SP, Brazil, using histopathology and transmission electron microscopy techniques. Microscopic lesions were found in 84 (18.46%) organ fragments from 12 necropsied pigs. Various degrees of lymphoid depletion and diffuse infiltration of histiocytes, presence of giant cells and basophilic intracytoplasmic inclusions were the main lesions observed by histological technique of H & E. Using the immunohistochemistry technique, 84 (18.46%) organ fragments were positively stained in brown by DAB, varying in intensity and location according to the selected tissue. Following the in situ hybridization technique, in all 84 (18.46%) organ fragments positive cells were found in the inflammatory infiltrates, including macrophages. By the negative staining technique, non-enveloped, isometric, circovirus-like particles were found, characterized as "complete" and "empty", measuring 17 nm in diameter in 84 (18.46%) organ fragments. The antigen-antibody interaction was characterized by aggregation of circovirus particles in the immuno-electron microscopy, in all positive samples. Using the immunocytochemistry technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the circovirus. The results of the histopathological techniques were similar to those of the transmission electron microscopy techniques, both being satisfactory for detection of PCV-2.*

Keywords*— *Histopathology, Swine, Transmission electron microscopy, Type 2 Circovirus.

I. INTRODUCTION

The porcine circovirus type 2 was first described in Canada in 1991, associated with post-weaning multisystem syndrome of pigs, in animals without clinical signs of disease, and in animals with dermatitis and porcine kidney disease syndrome, reproductive disorders, porcine respiratory complex syndrome, proliferative and necrotizing pneumonia and congenital tremor (Allan and Ellis, 2000; Larochelle *et al*., 1994).

But it was in 1974, that Tischer *et al*. first described the porcine circovirus (PCV- Porcine circovirus), using electron microscopy, a virus morphologically similar to a picornavirus, responsible for persistent infections in pig kidney cultures (PK-15) and without causing cytopathic changes. Subsequently it was demonstrated to be a single-stranded DNA virus, approximately 1.76 Kb, ambisense, circular, covalently closed (Tischer *et al*., 1982). It is the smaller animal virus described in both sizes in the genome, non-enveloped, with icosahedral symmetry and measuring 15 to 17 nm in diameter. Its replication capability is autonomous in mammalian cells, necessarily occurring during cell replication, since the virus is dependent on proteins produced in S phase of mitosis (Studdert, 1993). Faced with different genotypic and phenotypic characteristics, two types of porcine circovirus are being described, PCV-1, consisting of the contaminant sample cell cultures and considered apathogenic, and PCV-2, represented by the samples detected in sick animals (Allan *et al*., 1999). The seroconversion occurs in pigs between 3 and 4 weeks, under normal conditions, the antibodies being detected at different stages of breeding (Allan and Ellis, 2000). The viremia takes place between 7 and 16 weeks of age, with low prevalence in matrices. According to Segales and Domingos in 2002, the individual variation in the PCV-2 DNA detection takes place between 5 and 21 weeks, suggesting that some pigs have developed a persistent viremia. The pigs may also maintain PCV-2 in the nasal cavity, regardless of viremia or clinical signs of disease. This observation leads us to ask whether the PCV-2 of the nasal cavity is the result of viral replication in the nasal mucosa or the result of high levels of the pathogen in the environment. The viral presence in nasal swabs, tonsillar, fecal and urinary, bronchial, suggests that transmission can be oral-nasal, fecal and urinary (Segales and Domingos, 2002). PCV-2 was also detected in aborted fetuses in late gestation and

stillbirths (Lyo *et al*., 2001). The occurrence of type 2 circovirus was described in several European, Asian and North American countries (Allan and Ellis, 2000). In Brazil, PCV-2 was detected at the beginning of 2000, through viral isolation studies, detection by PCR and serological surveys in swine herds (Ciacci-Zanella *et al*., 2003). During a study conducted in the state of São Paulo, Bersano *et al*. (2003) used those same techniques and reported the occurrence of co-infection with PCV-2 and coronavirus. In 2004 Ruiz *et al*., while searching in 8 Brazilian states the occurrence of porcine circovirus and possible co-infections with PVS (porcine parvovirus), detected in the state of São Paulo, by PCR, a pig reagent for both 4 viruses (PCV-1 / PCV-2), 33 for PCV-1 and 2 for PCV-2, in 146 studied samples. The association between PVC-2 and other pathogens was described in the literature as a circovirosis, an immunosuppressive disease that made swine more vulnerable to other agents capable of causing respiratory and enteric diseases, leading to an increase of losses (Ristow *et al*., 2000; Moreno *et al*., 2003). These findings were also reported by Bersano et al., 2008, as related to the presence of PCV-2 in multiple bacterial infections. The circovirosis occurs endemically in technified farms, affecting mainly piglets at the end of the nursery phase and the first month of age. Mortality is usually between 3% and 10%, but it can reach values up to 35%. In Brazil, there are no wide studies to verify the frequency of the disease in many regions. Estimates based on unpublished data indicate a frequency of the disease in 62.05% of nursery and 66.75% of terminations in technified farms, with mortality rates ranging from 2% to 10%. Besides the impact on commercial pig farming, the circovirus is important in xenotransplantation. In several countries this technique has been the solution for the lack of organs (Boneva *et al*., 2001). The swine is one of the most studied animal species, due to the size of its organs, compatible with that of an adult human. Agents which do not normally infect humans can infect them after xenotransplantation. Despite the xenozoonosis by circovirus being low-risk, in cultures of human cell lines, this pathogen can be infectious (Tucker *et al*., 2003).

This study was aimed at investigating the presence of porcine circovirus type 2 (PCV-2) in apparently healthy terminated pigs from slaughterhouses of São Paulo, SP, Brazil, using histopathology and transmission electron microscopy techniques, collaborating with the vision of the National Porcine Agribusiness.

II. MATERIAL AND METHOD

2.1 Animals

Samples of lung, lymph nodes, thymus, spleen, intestine, kidney and liver from necropsy of 65 pigs were collected from April 2009 to June 2012. The animals came from various slaughterhouses of the cities of São Paulo, SP, Brazil (Ibiúna, Jacarei, Pedra Bela, Franco da Rocha, Boituva, Descalvado, Embu Guaçu, e São José dos Campos). Organ fragments, kept on ice, were sent to the Electron Microscopy Laboratory of Biological Institute, to be processed for transmission electron microscopy by negative staining, immunoelectron microscopy and immunolabeling with colloidal gold techniques and tissues fixed in 10% formalin for histopathology (technique routine histological (H & E), immunohistochemistry and *in situ* hybridization).

2.2 Histopathology

2.2.1 Routine Histological Technique

All 455 organ fragments of 65 pigs were fixed in 10% buffered formalin, dehydrated, diaphanized and embedded in paraffin. 5 µm thick sections were stained with hematoxylin and eosin technique.

2.2.2 Immnunohistochemistry Technique

Sections of the 455 organ fragments, 5 µm thick, were deparaffinized and rehydrated. Antigen retrieval was performed at room temperature, by applying 100 µl of Proteinase K (Dako - S3020) for each cut for 5 minutes, followed by a wash with distilled water. The blocking of endogenous peroxidase, aimed at the minimization of unspecific reactions, was obtained with 200 µl of hydrogen peroxide, 10 volumes at 3% in distilled water for 20 minutes. Sections were then rinsed with distilled water, followed by a wash with phosphate buffer (0.1 M PBS). Thereafter, 100 µl of primary antibody monoclonal for PCV-2 (PCV 36A9: lot 170309, Ingenasa) diluted at 1:500. The Dako background reducing components (Code S3022) were applied at room temperature for each cut and incubated in a humid chamber for 18 hours in a refrigerator (2-8ºC). After this period, two washes with phosphate buffer (0.1 M PBS) for 1 minute were performed. The visualization system used was the LSAB® + System-HRP (Dako-code K0690), following the protocol recommended by the manufacturer. The incubation time was 20 minutes, at room temperature, for each of the reagents, alternated with two washes with phosphate buffer (0.1 M PBS) for 1 minute. The substrate chromogen system used was the Liquid DAB+ Substrate Chromogen System (Dako - code K3468), with 5 minutes incubation at room temperature, followed by a rinsing in distilled running water. The counterstaining

was performed with hematoxylin. Negative checks were due to the lack of addition of primary antibody fragments of lung, spleen and small intestine of healthy pigs (Shoup *et al*., 1996).

2.2.3 In situ hybridization

The RNA probes utilized for Porcine Circovirus type-2 were: Primers CVS2-**Rc2 CGCACTTCTTTCGTTTTCAG -** 50N scale synthesis. Modification of 5´ biotin (Invitrogen). The organ fragments (lung, lymph nodes, thymus, spleen, intestine, kidney and liver) of 65 pigs were collected and fixed in 10% formalin for 36-48 hours, dehydrated with an increasing concentration of alcohols (70º, 80º, 95º and absolute), diaphanized with xylene and put in a paraffin bath in a stove at 58ºC overnight. The 4 µm thick cuts were placed on marked slides and kept at room temperature. Prior to use, they were deparaffinized with xylene and rehydrated in decreasing concentration of alcohols (absolute, 95º, 80°, 70°) and distilled water. For antigen recovery, a pretreatment was performed, using a hot bath at 96º C and diluted buffer (Dako S1699) for 40 min. When the slides with cuts were cooled, the endogenous peroxidase was stopped 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 performed. The specific biotinylated probes were mixed, including specific target DNA on the organ fragments, and a cover glass was laid on them. Samples from these fragments and probes were denatured and hybridized overnight (18 hours) in a Dako hybridization system (denaturation at 96ºC and hybridization at 37ºC). After stringency, a wash with TBST (Tris-buffered saline / Tween) was performed. The visualization system used was the primary streptavidin in a diluting buffer (Dako – Kit cod. K0690) for 30 minutes in a humid chamber, Biotinyl Tyramide reagent for 15 min. at room temperature and then the secondary streptavidin for 15 min. All the procedures were alternated with two washes of TBST buffer for 5 minutes. The substrate-chromogen system used was the Liquid DAB+ Substrate Chromogen System (Dako - code. K3468), incubation was performed for 5 minutes at room temperature, followed by a wash in distilled running water. Counter-staining was performed with hematoxylin (Braissant and Wahli, 1998; Kim and Chae, 2001).

2.3 Transmission Electron Microscopy

2.3.1 Negative staining technique (rapid preparation)

In this technique, 455 samples of organ fragments of 65 pigs were suspended in 0.1 M phosphate buffer at pH 7.0. Drops of the obtained suspensions were placed in contact with metallic copper grids, previously covered with a film of 5% collodium amyl acetate and stabilized with carbon. Next, the grids were drained with filter paper and negatively stained with 2% ammonium molybdate at pH 5.0 (Brenner and Horne, 1959; Hayat and Miller, 1990; Madeley, 1997).

2.3.2 Immunoelectron microscopy technique

In this technique, the copper grids, previously prepared with collodium film and stabilized with carbon, were first incubated with protein A (1ml/ml) placed in contact with the virus-specific antibody. After that, grids were washed with drops of phosphate buffer 0.1M at pH 7.0, incubated with the viral suspension of the 65 samples of small intestine, washed with drops of distilled water and negatively stained with 2% ammonium molybdate at pH 5.0 (Berthiaume *et al.*, 1981; Doane and Anderson, 1987; Hayat and Miller, 1990; Katz and Kohn, 1984; Padrón, 1998).

2.3.3 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 455 samples of organ fragments and, after removing the excess with filter paper, the same were put on specific primary antibody drops. After further washing 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 with 2% ammonium molybdate at pH 5.0 (Knutton, 1995). All grids submitted to the above reactions were observed in a Philips EM 208 transmission electron microscope, at 80 kV.

III. RESULTS AND DISCUSSION

3.1 Necropsy

At necropsy, a wide range of lesions in the examined organs was observed; lung with adhesion of the pleura, heart stuck to the pericardial sac, severe enteritis, some with bleeding mucus diarrhea and gastritis, urinary bladder full. Anemia, jaundice, cough and dyspnea were also observed.

3.2 Histopathology

3.2.1 Routine Histological Technique (H&E)

At the histological examination, 84 samples of organ fragments (18.46%) out of the 12 pigs showed histological lesions. The main histological lesions were located in the lymphoid organs and lungs, but were also observed in the kidney and liver. In lymphoid organs lymphocyte depletion was observed with a decrease in lymphoid follicles, and the presence of many eosinophils (**Figure 1, minor arrow**). Another important finding was the presence of multinucleate cells (**Figure 1, big arrow**) and basophilic intracytoplasmic inclusions (**Figure 1, arrowhead**), mainly in histiocytes, which are pathognomonic of the disease. The lungs presented interstitial multifocal pneumonia, with monolymphocytic infiltration and several foam cells (foamy macrophages). The kidney showed interstitial multifocal monolymphocytic nephritis. The liver, however, showed mono lymphocyte hepatitis mainly in the portal zone, with varying degrees of intensity and multifocal necrosis of the hepatocytes groups.

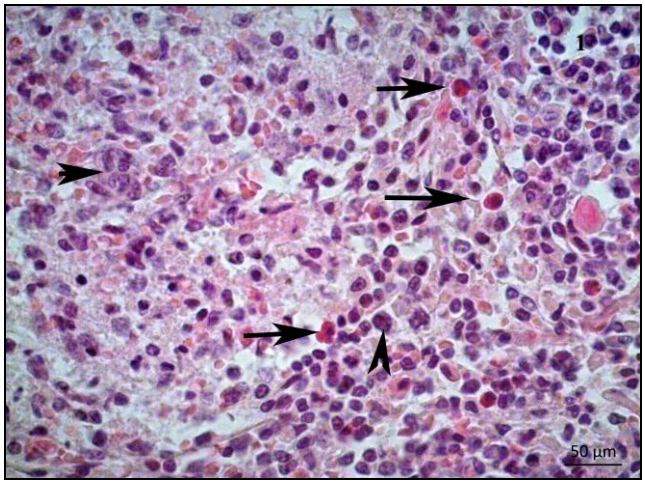

FIGURE 1: PHOTOMICROGRAPH OF THE SPLEEN OF SWINE. OBSERVE GIANT CELL (BIG ARROW), EOSINOPHILS (MINOR ARROW) AND BASOPHILIC INTRACYTOPLASMIC INCLUSION (ARROWHEAD). X630.

3.2.2 Immunohistochemistry Technique

By immunohistochemistry it was visualized that 84 (18.46%) out of all the organ fragments showed positive DAB in the Ag-Ac reaction (anti PCV-2 - Ingenasa) varying in intensity and location according to the selected tissue. In all organs, the positive cells are in the inflammatory infiltrates, including macrophages (**Figures 2, 3**).

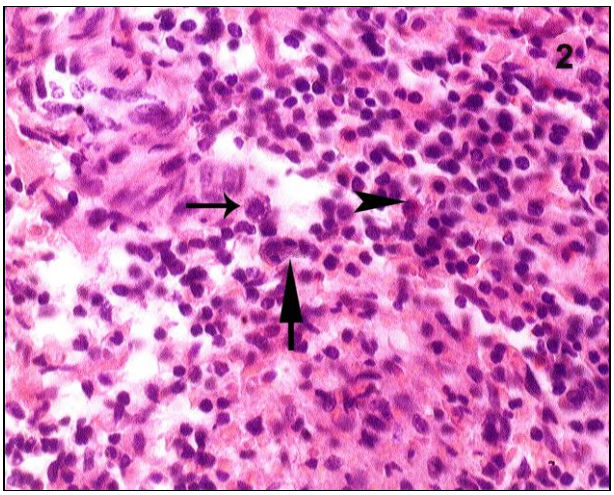

FIGURE 2: PHOTOMICROGRAPH OF THE LUNG. OBSERVE CELLS STAINED BROWN BY THE REACTION ANTIGEN-ANTIBODY BY IMMUNOHISTOCHEMISTRY TECHNIQUE. X200

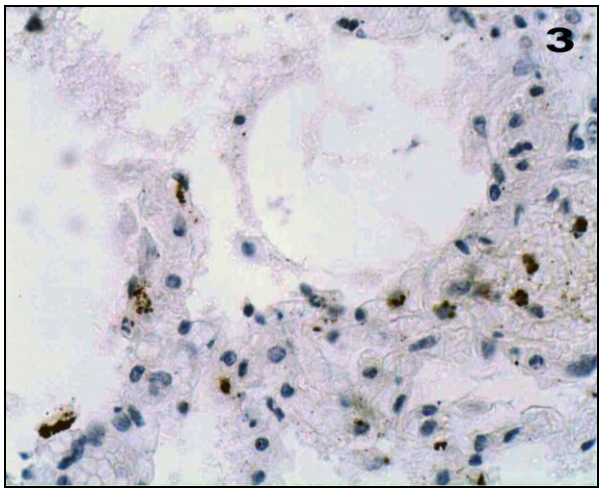

FIGURE 3: PHOTOMICROGRAPH OF THE SPLEEN. OBSERVE CELLS STAINED BROWN BY THE REACTION ANTIGEN-ANTIBODY BY IMMUNOHISTOCHEMISTRY TECHNIQUE. X400

3.2.3 In *situ* hybridization Technique

This technique used DNA probes. It was verified that 84 (18.46%) organ fragments of the 12 pigs were positive by in situ hybridization, with brown stain within macrophages (**Figure 4**).

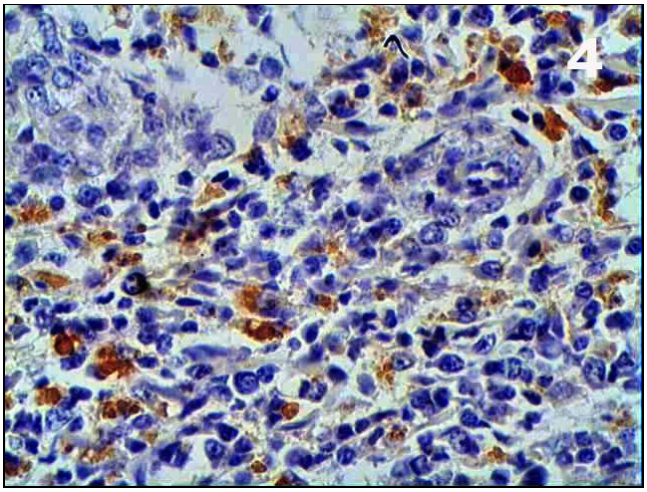

FIGURE 4: PHOTOMICROGRAPH OF THE LUNG. OBSERVE STAINED BROWN DNA OF CIRCOVIRUS TYPE 2 IN THE WITHIN MACROPHAGES BY IN SITU HYBRIDIZATION TECHNIQUE. X400.

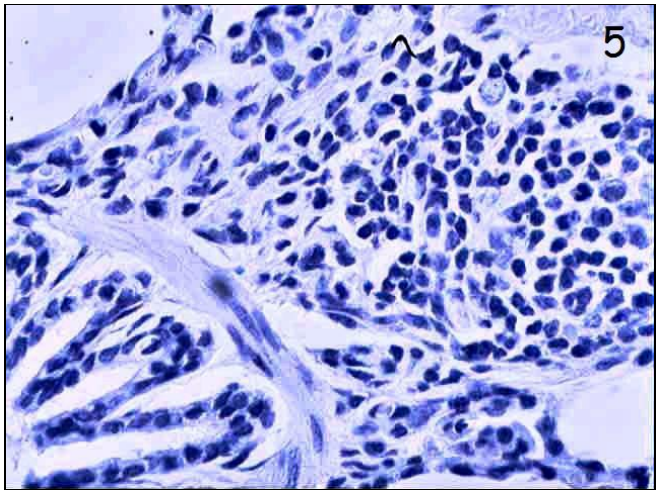

FIGURE 5: PHOTOMICROGRAPH OF THE LUNG. CIRCOVIRUS TYPE 2 NEGATIVE CONTROL. X400.

3.3 Transmission Electron Microscopy

3.3.1 Negative Staining Technique (Rapid Preparation)

On transmission electron microscopy, using the negative staining technique (rapid preparation), non-enveloped, isometric, circovirus-like particles were found, characterized as "complete" (**Fig. 6, big arrow**) and "empty" (**Fig. 6, minor arrow**), measuring about 17 nm in diameter, in 84 (18.46%) out of the 455 samples of organ fragments analyzed.

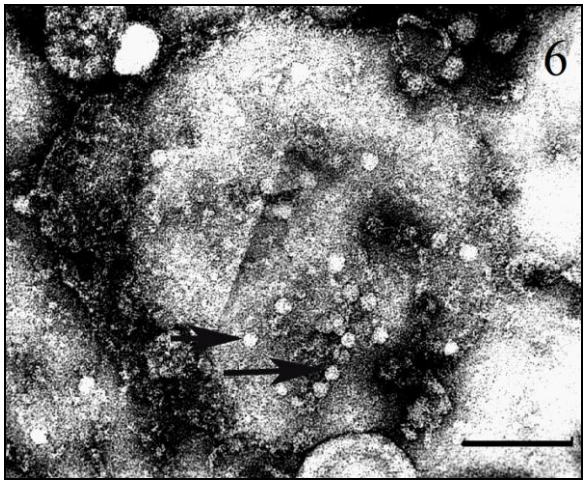

FIGURE 6: ELECTRON MICROGRAPH OF CIRCOVIRUS IN LUNG SUSPENSION BY NEGATIVE STAINING, SHOWING "COMPLETES" (BIG ARROW) AND "EMPTY" (MINOR ARROW) PARTICLES. BAR: 160 nm.

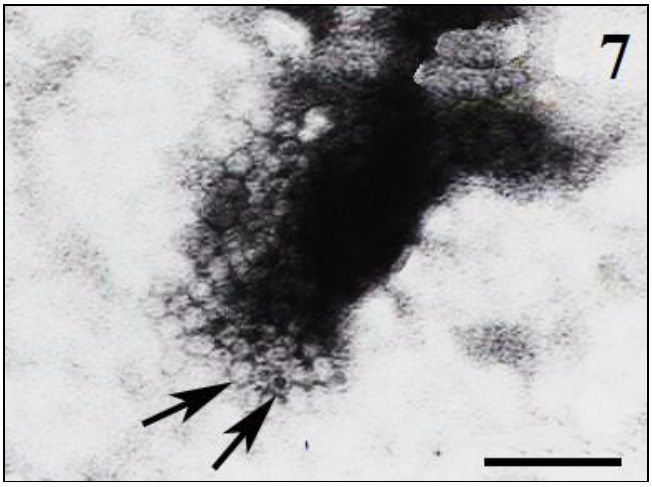

FIGURE 7: ELECTRON MICROGRAPH SHOWING CIRCOVIRUS PARTICLES AGGREGATED BY ANTIGEN-ANTIBODY INTERACTION IN THE IMMUNOELECTRON MICROSCOPY TECHNIQUE. OBSERVE "COMPLETES" (BIG ARROW) AND "EMPTY" (MINOR ARROW) PARTICLES. BAR: 108 nm.

3.3.2 Immunoelectron Microscopy Technique

In 84 (18.46%) out of 455 organ fragments samples analyzed, the antigen antibody interaction was characterized by the aggregation of viral particles (**Fig. 7**).

3.3.3 Immunocytochemistry Technique

In this technique, the antigen-antibody interaction was strongly enhanced by the dense colloidal gold particles over the circovirus in 84 (18.46%) samples of fragments (**Fig. 8, arrow**), confirming the results of immunoelectron microscopy technique.

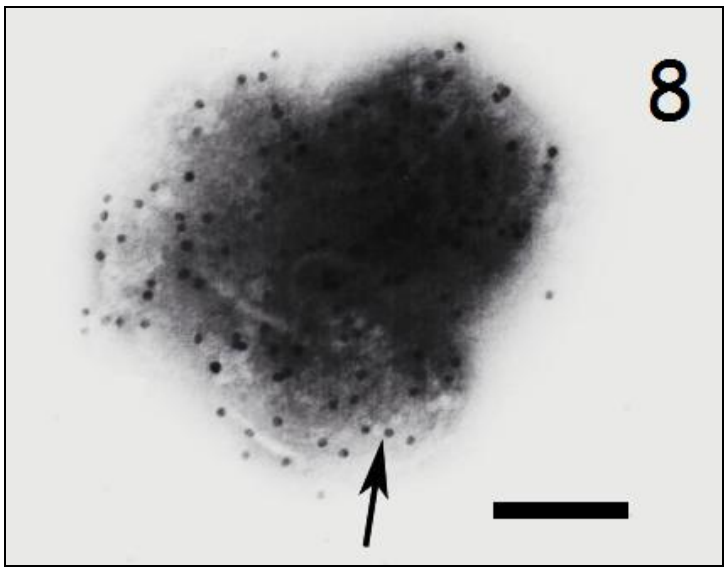

FIGURE 8: ELECTRON MICROGRAPH OF CIRCOVIRUS TYPE 2 ENHANCED BY COLLOIDAL GOLD PARTICLES IN THE IMMUNOCYTOCHEMISTRY TECHNIQUE (ARROWS). BAR: 160 nm.

The present study was aimed at finding the PVC-2 presence in slaughtered pigs from slaughterhouses of São Paulo, SP, Brazil, apparently healthy, using histopathology techniques and transmission electron microscopy.
Similar studies, using immunohistochemistry technique, reported that PCV-2 infection occurs in the early post-weaning period and are also recognized in slaughtered pigs 12-19 weeks old (Carman *et al*., 2008; D'Allaire *et al*., 2007; Desrosiers, 2007; Fachersen *et al*., 2008; Hansen *et al*., 2010).

The positive staining obtained in the immunohistochemistry technique was followed in 12 animals (18.4%), since it was used by several authors for the PCV-2 detection (Choi *et al*., 2000; Ciacci-Zanella, 2006; Correa *et al*., 2006; Ellis *et al*., 1998; Hansen *et al*., 2010; Seo *et al*., 2014; Szczotka *et al*., 2011; Szeredi and Deim, 2006; Wen *et al*., 2010).

Hansen *et al*. (2010) reported that 61% of the slaughtered pigs of their study were positive for PCV-2 antigen, because rates of 85% and 75% were reported by Hines *et al*. (1995) and D'Allaire *et al*. (2007), respectively.

It was noted that the intensity of the brown staining was variable, depending on the selected tissue.

Chianini *et al*. (2003), Krakowka *et al*. (2005) and Quintana *et al*. (2001) reported that the severity and distribution of lesions were related to the amount of the virus present and the stage of the disease and, therefore, there may be no specific histological changes if the amount of virus is low or zero, which also occurs in subclinical infection.

The necropsy on positive animals showed hemorrhagic enteritis with diarrhea mucus, gastritis, anemia, jaundice, cough and dyspnea, also observed in other studies of PCV-2 (Ellis *et al*., 1998, Harding *et al*., 1998; Harms, 1999; Jansen *et al*., 2006; King and Chae, 2005; Opriessning, *et al*., 2007; Stevenson, *et al*., 2001).

In the lymphoid organs, lymphoid depletion with reduction of lymphoid follicles and the presence of eosinophils was observed.

These findings were also reported in 10-14 week old pigs, subclinically infected with PCV-2 infection (Hansen *et al*., 2010; Nielsen *et al*., 2008; Quintana *et al*., 2001) and in newborn pigs (Karuppannan *et al*., 2014).

The presence of antibodies against the PCV-2, in clinically healthy animals, indicates that the pigs were infected with PCV-2, but managed to fight this viral infection. Several authors as Alam *et al*. (1999), Choi and Chae (2000) and Harms *et al*. (2001) also demonstrated that the PCV-2 is essential to cause disease, but for the development of PMWS, concomitant factors are required, such as co-infection with parvovirus or respiratory and reproductive syndrome porcine virus, or immunostimulatory cofactors (Krakouka *et al*., 2001; Kyriatis *et al*., 2002).

The PCV-2 induces lymphohistiocytic inflammation with multinucleated cells, especially in lymphoid organs, but also in liver, kidney and lungs. These pathognomonic changes are associated with the presence of corpuscles of intracytoplasmic inclusion in macrophages and giant cells, as it was also reported by Chae (2004), France *et al*. (2005) Harding and Halbur (2002), and Opriessnig *et al*. (2007).

As the infected animal cannot have an effective immune response, the PCV-2 may develop in other non lymphoid organs, causing various injuries and worsening the medical status. Furthermore, there may be the loss of homeostasis of immune mediators, death of lymphocytes and replacement of lymphoid cells, leading to immunosuppression (Ciacci -Zanella, 2007).
The presence of kidneys with interstitial monolymphocyte multifocal nephritis in the animals of this work was also confirmed in other studies of PCV-2 in slaughtered pigs (Hansen *et al*, 2010; Rutten *et al*, 2006) and in newborn pigs (Karuppannan *et al*., 2014).

The findings of PCV-2 antigen in the renal tubular epithelium provides evidence for dissemination of PCV-2 from slaughtered pigs to the environment, which could result in horizontal transmission to susceptible animals (Hansen *et al*., 2010; Rutten *et al*., 2006; Sarli *et al*., 2008).

The presence of interstitial pneumonia and hepatitis was also mentioned by Allan and Ellis (2000) and, by Harding and Clark (1997).

In the *in situ* hybridization, the DNA from PCV-2 inside macrophages was stained in brown, corroborating the findings of other researchers (Cadar, 2007; Ellis et al., 1998; Ellis, 2014; Kim *et al*., 2010; O'Dea, 2010; Seo *et al*., 2014; Szczotka *et al*., 2011).
When the negative staining technique for transmission electron microscopy is used, non-enveloped, isometric, circovirus-like particles are obtained, characterized as "complete" or "empty", measuring about 17 nm in diameter, in 84 (18.46%) out of the 455 samples of organ fragments analyzed.

The morphological characteristics of circovirus that are described were also reported in other PCV-2 studies (Allan *et al*., 1998; Allan and Ellis, 2000; Chi *et al*., 2014; Crowther *et al*., 2003; Ellis, 2014; Ellis *et al*., 1998; Ellis *et al*., 2000; Hines and Lukert, 1995; Karuppannan *et al*., 2014; Khayat *et al*., 2011; Opriessnig *et al*., 2007; Wen *et al*., 2012).

By the immunoelectron microscopy technique, using a monoclonal antibody, specific for PCV-2, the antigen-antibody interaction was obtained by agglutination of circovirus particles in 84 (18.46%) samples of organ fragments.
Guo *et al*. (2010; 2011) and Wen *et al*. (2012) demonstrate the specificity of the PCV-2 setup by immunoelectron microscopy.

The immunocytochemical technique showed, in 84 (18.46%) samples of organ fragments, an extensive marking of the particles with colloidal gold, confirming the presence of PCV-2, as reported by Chi *et al*. (2014).

In this study, the results of histopathological techniques were similar to those of transmission electron microscopy techniques, both being effective in the detection of PCV-2.

The serology should not be used either as a diagnostic tool or as a prognosis of infection. The detection of viral DNA by nested PCR in the samples, can lead to an overestimate of the risk of developing the disease, once the sub clinically infected

animals may be viremic, and also because DNA can be present in excretions and secretions of normal pigs (Kim and Chae, 2004).

Immunohistochemistry (IHC) and *in situ* hybridization (ISH) are usually used for confirmation of diagnosis. The comparison of the two techniques, ISH and IHC, for the detection of PCV-2 in tissues, has shown that ISH is more sensitive than IHC (Kim and Chae, 2004; Szczotka *et al*., 2011). In the laboratory routine, however, IHC is more commonly used, because it is less complex (Kim and Chae, 2004).

For these reasons, to make a definitive diagnosis of the disease, the presence of macroscopic and microscopic lesions should be considered, associated with the demonstration of viral protein or viral DNA by IHC or HIS, or by electron microscopy, where the presence of viral particles is observed in various techniques (Kim and Chae, 2004; Ellis, 2014).
These results report the first identification of PCV-2, using transmission electron microscopy techniques in São Paulo, SP, Brazil.

IV. CONCLUSION

Considering that pos-weaning multisystemic wasting syndrome is a multifactorial disease, causing severe growth retardation, weight loss and reproductive failure, the development of rapid and reliable techniques is essential to devise measures for prevention and control during outbreaks of the disease, collaborating with National Porcine Agribusiness.

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