COMPARATIVE EVALUATION OF REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND IMMUNOHISTOCHEMISTRY FOR THE DIAGNOSIS OF PORCINE EPIDEMIC DIARRHEA IN LUZON, PHILIPPINES

Karina Marie G. Nicolas1*, Helen A. Molina2 and Amadeo A. Alcantara3

ABSTRACT

Reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were used to demonstrate the presence of porcine epidemic diarrhea virus (PEDV) antigen in the intestinal cells of suckling pigs in Luzon. The PEDV antigen was detected in 11 (13.75%) and 32 (40%) out of 80 intestinal samples using RT-PCR and IHC, respectively. RT-PCR generated a 412-bp cDNA probe which amplified the viral RNA encoding the membrane protein of PEDV from the intestinal segments of the jejunum. Immunohistochemistry revealed positive cells in the jejunum as indicated by the brown staining in the cytoplasm of infected cells. Comparative evaluation of the two tests revealed a fair agreement. Histopathological changes observed include vacuolation of enterocytes, villous atrophy as exemplified by 2:1 villous:crypt height ratio and exfoliation of enterocytes which are associated with the clinical signs of PED such as watery diarrhea, dehydration and acidosis. RT-PCR may be used as a screening test for PEDV antigen detection using jejunal tissue with feces because of the shorter duration of processing and testing. IHC, on the other hand, can be performed as confirmatory test using formalin-fixed jejunal samples.

INTRODUCTION

Porcine epidemic diarrhea (PED) is a viral enteric disease of swine caused by a single stranded, positive sense, enveloped RNA virus with approximately 28 kb viral genome called Porcine Epidemic Diarrhea Virus (PEDV). PEDV is categorized under the genus Alpha coronavirus, family Coronaviridae, order Nidovirales (Pan et al., 2012). Transmission occurs via feco-oral route after the introduction of infected animals or contaminated materials (Carvajal et al., 1995). The disease is characterized by vomiting, watery diarrhea and dehydration in all ages of swine and manifests as sporadic outbreaks (Song et al., 2012) causing significant economic losses in the swine industry. PED has been reported to cause a 100% morbidity and mortality rate in less than 5 days old neonatal piglets and 10% mortality in infected piglets older than 10 days old (Kim and Chae, 2002). Methods for detection of PED infection are necessary prerequisite for assessing the current epidemic situation in herds and for subsequent immunoprophylactic measures (Roda’k et al., 2005). Many diagnostic methods have been used for the detection of...
PEDV such as immunofluorescence tests, direct electron microscopy, enzyme linked immunosorbent assays (ELISA), in situ hybridization, immunochromatographic assay kits, reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC).

In the Philippines, the immunochromatographic assay kit, immunofluorescence antibody test and RT-PCR using different primers are used to detect PED. However, immunochromatographic assay kits have been reported to be less accurate in detecting the disease with only 92% sensitivity (Song et al., 2012). Employing highly sensitive, specific, more reliable and rapid methods of detection of PEDV-specific nucleic acid in fecal and intestinal samples such as RT-PCR and in formalin-fixed tissues for IHC, would be a useful and accurate method for the detection of PEDV antigen. This will also strengthen the disease reporting in the country which is important for disease control.

The study aimed to demonstrate the presence of PEDV antigen in naturally infected pigs using RT-PCR of intestinal samples and immunohistochemistry (IHC) in formalin-fixed paraffin-embedded intestinal tissues, determine the level of agreement of RT-PCR and IHC in the diagnosis of PED and to describe the histopathological lesions in the jejunum of PED infected piglets.

MATERIALS AND METHODS

Sample collection
Intestinal samples were collected from 80 diarrheic piglets, 4-6 days old, from a cooperating commercial farm in Southern Luzon. A one inch long segment of the jejunum was collected from each piglet and placed in 10% neutral buffered formalin for fixation and histopathological examination. Tissue samples were sectioned at 4 µm thickness. Samples were processed for the routine Hematoxylin-Eosin technique for the histopathological description. The specimens for immunohistochemistry were placed on poly-L-lysine coated glass microscope slides. Tissues were dehydrated in an oven at 60°C for an hour and deparaffinized with xylene, rehydrated with graded alcohols and washed with distilled water.

Enzymatic antigen retrieval method was carried out for 10 min at 30°C. Succeeding immunohistochemistry procedures followed the manual staining protocol of the Dako Envision® + Dual Link System- HRP DAB+ (Dako Denmark A/S). Tissue samples were incubated with the monoclonal antibody in antibody diluent at 1:100 dilution for 1 hour and 60°C for an hour and deparaffinized with xylene, rehydrated with graded alcohols and washed with distilled water.

Routine histopathological examination
Jejunal samples were processed using the routine paraffin technique and stained with Hematoxylin-Eosin stain. The lesions were described in terms of presence or absence of vacuolation of enterocytes, villous atrophy and exfoliation of enterocytes: sloughing off of the epithelial cells covering the villi. Average villous lengths and crypt depths were determined in selected areas in which the villi and crypts are continuous and sectioned through their entire length. At that point, the mean length of the three longest villi and mean depths of three adjacent crypts were determined using an ocular micrometer.

Diagnostic comparison
The diagnostic performance of the two tests used for the detection of PEDV antigen field samples were compared and the level of agreement between RT-PCR and IHC in detecting PED infected animals was computed using the kappa-test to determine the % agreement. Presently, there is no "gold standard" diagnostic method for detecting PED-infected animals. Gold standards are established by the OIE. As a result, pigs were considered infected if they tested positive by RT-PCR or by IHC in the intestinal sections. Data were encoded in Microsoft Excel 2010 and analyzed using the Win Episcope 2.0.
RESULTS AND DISCUSSION

Eighty 4–6 day old diarrheic piglets were necropsied and examined in parallel to demonstrate the presence of PEDV antigen in the fresh and formalin-fixed jejunal segments of each piglet for RT-PCR and IHC respectively. Out of the 80 samples examined, 11 samples (13.75%) were positive for the PEDV antigen using RT-PCR and 32 samples (40%) using IHC. Physical examination showed piglets with emaciation, dehydration, anemia evidenced by pale mucous membrane, rough hair coat and soiling of perianal area with yellowish fecal material. Some of the piglets for necropsy were also observed vomiting. Examination of the gut revealed thin-walled, fluid and gas-filled yellowish to greenish small intestines with multifocal congestions. These findings coincide with the gross morphologic descriptions made by Sueyoshi et al. (1995); Lee et al. (1999) and Pensaert and Yeo (2006).

Figure 1 shows the 412-bp cDNA probe (Lanes 3, 7 and 10) generated by RT-PCR for the viral RNA encoding the membrane protein of PEDV from the intestinal segments of the jejunum. The presence of the band (amplicon) in the 400bp row indicates the presence of PEDV antigens in the intestinal sample. The test could also have been affected by the presence of inhibitors in the intestinal and fecal samples although the mechanism of the inhibition is unknown but may be related with the variable amounts of bilirubin and bile salts which inhibit the thermostable activity of the DNA polymerase (Kim and Chae 2002).

The results of this study agree with the results of Kim and Chae (2002) who found positive enterocytes distributed in the jejunal samples. In this study, positive staining cells were mostly found in the sloughed-off cells of the lining epithelium, lamina propria and in some macrophages of the villi of the jejunum. These results agree with that of Lee et al., (2000) who reported that most of the infected cells were not continuously arranged in the epithelial layer and the borders between infected cells were distinct. The number of viral antigens could also vary depending on the stage of the disease process because infected enterocytes tend to decrease with increasing severity of villous atrophy which may be either caused by PEDV leaving the enterocyte or caused by erosion of the infected cells (Sueyoshi et al., 1995).

Test results are summarized and plotted in a 2 x 2 table as shown in the Table. It
Table. Comparison between results obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) tests for diagnosis of porcine epidemic diarrhea virus.

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