Original Article

Discovery of Chicken Proventricular Necrosis Virus from Transmissible Viral Proventriculitis Outbreak in Broilers, Thailand

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Abstract

Transmissible viral proventriculitis (TVP) is an infectious disease-causing retarded growth in chickens. In Thailand, many stunted chickens have been found on commercial broiler farms since 2017. Significant post-mortem lesions are found only in the proventriculus. This study aims to investigate the causative agents of TVP outbreaks focusing on chicken proventricular necrosis virus (CPNV), the possible significant cause of TVP. CPNV was reproduced in specific pathogen-free (SPF) chickens. Affected chickens that have undergone proventriculitis are CPNV-positive by RT-PCR and nucleotide sequencing followed by phylogenetic analysis. The result showed that proventriculitis could be experimentally reproduced by inoculation of proventricular homogenate obtained from the affected chickens in the amniotic sacs of embryonic eggs. However, no cytopathic effect in inoculated primary chicken embryo fibroblasts was found. The viral genomes could be detected at the first passage of both egg inoculation and the cell culture by RT-PCR. Thai CPNV was in the same group as the reference strain from the previous report. Histopathologically, proventriculitis with infiltration of inflammatory cells was found in the glandular part of the inoculated chickens. By in situ hybridization with a CPNV-specific probe, positive signals were found in mucosal epithelium and necrotic cells. This study is the first report on CPNV discovery in broilers in Thailand.

Keywords: transmissible viral proventriculitis, chicken proventricular necrosis virus, broilers, Thailand

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Introduction

Transmissible viral proventriculitis (TVP) is an infectious disease that affects the growth performance of broilers, broiler breeders and commercial layer hens, resulting in economic loss in the poultry industry (Marusak *et al*., 2012). The infected flocks do not show significantly high mortality. The disease commonly affects three- to six-week-old broiler chickens but it has been detected in nine-to-twenty-week-old broiler breeders and layer hens (Dormitorio *et al*., 2007; Marusak *et al*., 2012).

The TVP-affected chickens typically showed pathological lesions of the proventriculus. For gross lesions, the affected proventriculus was enlarged with thickening of the mucosa and a mottled appearance. Undigested or poorly digested feed was also found in the lumen of the proventriculus and feces (Goodwin *et al*., 1996). Other organs were not affected (Pantin-Jackwood *et al*., 2004). TVP causes increased fragility of the proventriculus and is easily ruptured during carcass processing, resulting in a loss of profit because of the higher number of reprocessed carcasses and condemnations (Goodwin *et al*., 1996). Microscopically, TVP-associated cases were identified following four microscopic lesions: ductal epithelial degeneration and hyperplasia, diffuse infiltration of the glandular interstitium by lymphocytes and replacement of glandular epithelium by hyperplastic ductal epithelium (Pantin-Jackwood *et al*., 2004; Guy *et al*., 2011[b]; Śmiałek *et al*., 2017).

To date, chicken proventricular necrosis virus (CPNV) has been identified as the etiology of TVP (Guy *et al*., 2011[a]). CPNV was initially believed to be an adenovirus-like virus (AdLV) because of size, morphology and electron microscopic detection within intranuclear spaces in the glandular epithelium of proventriculi of TVP-affected chickens (Goodwin *et al*., 1996; Guy *et al*., 2005; Guy *et al*., 2007). Although CPNV is described as a novel birnavirus based on physical and genetic characteristics, the virus still has been an unclassified virus in the family of *Birnaviridae* (Delmas *et al*., 2019). The genome of CPNV was bi-segmented dsRNA, segment A for 4.0 kbp and segment B for 3.6 kbp in size (Guy *et al*., 2011[a]). The structure of virions is non-enveloped with a single-shelled icosahedral symmetry capsid of approximately 65-70 nm in diameter. The capsid is formed by two major structural proteins, VP2 and VP3. The VP2 builds up the external surface that forms spikes projecting radially from the capsid and carries the neutralizing epitopes responsible for antigenic variation and host-protective immunogen (Fahey *et al*., 1989; Brown *et al*., 1994). The VP3 or ribonucleoprotein is found in the inner surface of the capsids and forms a ribonucleoprotein complex with genomic RNA and RNA-dependent RNA polymerase (RdRp) or VP1 (Böttcher *et al*., 1997; Lombardo *et al*., 1999). The RdRp is a multifunctional protein that responds to the replication of genome and mRNA synthesis (Spies *et al*., 1987). CPNV associated with the TVP was reported in the USA (Guy *et al*., 2011[a,b]; Hauck *et al*., 2020), the UK (Grau-Roma *et al*., 2017; Grau-Roma *et al*., 2020), Poland (Śmiałek *et al*., 2021), Brazil (Leão *et al*., 2021), and Spain (Wali, 2021). CPNV-causing proventriculitis was able to reproduce in both naturally occurring situations and experimentally infected chickens (Guy *et al*., 2011[b]). To date, little is known about the prevalence and pathobiology of this virus.

Many stunted chickens associated with TVP have been found in broiler farms in Thailand since 2017. The culling rate of chicken in farms with poor feed conversion rate (FCR) and lack of flock uniformity ranged between three and eight percent. A post-mortem examination found TVP-compatible gross lesions with enlarged proventriculi and thickening of proventricular walls. In some cases, fibrinous necrotic material covered the proventricular mucosa and hemorrhages were also present. Histopathological lesion found marked inflammation in submucosal glands with lymphocytic infiltration and ductal metaplasia of the glandular epithelium. This study aims to investigate the causative agents of TVP outbreaks by focusing on CPNV. The discovery of CPNV in this report describes the identification, propagation and confirmation of the presence of the virus in TVP-affected proventriculi from broiler farms in Thailand.

Materials and Methods

Ethics statements: The animal experiments and handling procedures of this study were conducted with the Ethics Committee for Animal Experiment of National Institute of Animal Health (NIAH), Department of Livestock Development, Thailand following the regulations on the protection of animals used for scientific purposes.

Case history and clinical specimens: A chicken which had undergone the problems of decreasing performance characterized by poor feed conversion, less body weight, poor feathering and flock non-uniformity with prominent lesion in proventriculus was defined as a suspected TVP case. Fourteen proventriculi samples were collected from TVP-affected chickens at two to four weeks of age from commercial broiler farms located in Nakhon Pathom and Ratchaburi provinces in 2017. The specimens were collected in 10% buffered formalin for histopathology and stored fresh specimens at -80°C freezer upon use.

Proventricular homogenates: The proventriculi of affected broilers from -80°C freezer were homogenized mixed with 10% suspension in phosphate-buffered saline (PBS) solution, pH 7.2. The homogenate was centrifuged at 10,000 x g for 15 mins at 4°C twice. The supernatant was carefully collected from the pellet and filtrated by 0.22 µm syringe filters. The infiltrates were stored at – 80°C freezer upon use.

SPF Chickens: Fourteen one-day-old specific–pathogen-free (SPF) chicks were purchased from Thai SPF Co. Ltd, Nakhon Nayok province, Thailand. The parent flock and all progenies were free from common poultry diseases. Feed and water were provided *ad libitum*. The experimental design was modified by Guy *et al*. (2007).

Experimental infection of SPF Chickens with proventricular homogenates: Fourteen one-day-old

SPF chicks were randomly allocated into two groups with approximately the same mean body weight for the proventricular homogenate inoculum group and the negative control group. Eleven chicks were orally inoculated with the 0.5 ml inoculum and the remaining chicks were orally inoculated with PBS, serving as the negative control group. Each group was placed in a separate container in isolation rooms with controlled access at NIAH. Chickens were examined daily for clinical signs of illness and mortality till the end of the study. At 17 days of age, the chicks were euthanized by cervical dislocation and necropsied. Pieces of proventriculi and visceral organs were collected from each bird and fixed in 10% neutral buffered formalin. The tissues were processed and embedded in paraffin by routine histologic techniques. The remaining proventriculi were pooled within groups and collected in sterile containers. The homogenates were prepared for nucleic acid amplification as described above followed by CPNV detection by RT-PCR and DNA sequencing when found positive.

Virus isolation in cell culture and embryonated eggs: The isolation of CPNV was evaluated for the presence of viruses by inoculation onto primary chicken embryo fibroblast (CEF) cells and in the SPF embryonated chicken eggs. In detail, the proventricular homogenates were prepared from inoculated chickens that showed CPNV-positive results. CEF cells were prepared from 11-day-old embryonated eggs. The cells were grown at 37°C in a 5% CO_2 incubator in Dulbecco's Modified Eagle medium (DMEM) and 5% fetal calf serum. The proventricular homogenates were inoculated onto the CEF cells at 1:10 dilution and made 10-fold serial dilution to 1:10,000 in 24-well plates. The cells were incubated at 37°C with 5% CO_2 and passaged 3 times with 4 days per passage. The inoculated cell cultures were observed daily for cytopathic effect (CPE) and supernatants were examined for the presence of the virus by RT-PCR each passage. The freeze-thawed supernatant was separated from sediments by centrifugation at 2500 x g for 30 mins at 4°C. The supernatant was used for cell inoculation and RT-PCR. The infected embryonated chicken eggs (8-10 days of incubation) were inoculated a total of 4 passages by the amniotic route, allantoic route and chorioallantoic membrane (CAM) with 0.1 ml of proventricular homogenates. The eggs were examined daily for embryonic death and lesion in embryos at the end of each passage. After 7 days post-inoculation of each passage, the eggs were chilled at 4°C for 2-4 hours to kill the embryos and the amniotic fluid, allantoic fluid, CAM and embryonic proventriculus were collected. Each sample was pooled in the same collecting tube according to the route of inoculation. All samples were ground and centrifuged at 5000 x g for 5 mins followed by filtration with 0.2 µm syringe filters and stored at -80°C before reinoculation. CPNV was then performed by RT-PCR each passage.

RNA extraction: The viral RNA was extracted from the proventriculi, pooled samples of egg inoculums, and virus-infected cell culture supernatant fluid using Foodproof Magnetic Preparation kit VI (Biotecon Diagnostics GmBH, Germany) with the foodproof RoboPrep 32 automated extraction machine (Biotecon Diagnostics GmBH, Germany) following the manufacturer's instructions. The extracted RNA was stored at – 80°C until used.

CPNV detection by RT-PCR: The RT-PCR protocol to amplify a 169 bp fragment of the CPNV *vp1* gene was modified from the study of Guy *et al*. (2011[a]). The primers were modified after the determination of matching between primer and CPNV sequences from this study based on *vp1* gene region using SnapGene Viewer software version 5.3.2 (Insightful Science, USA). The primers were CPNV-F: 5′-GCGGTCGCCATACAGATACA-3′ and B2F: 5′-CGTAGACCTCGTCCT TCTGC-3′. The RT-PCR was performed with the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, USA). A total of 25-µl reaction was set up containing 3 µl of RNA, 12.5 µl of 2X reaction buffer containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 1 µl of a 5 mM magnesium sulfate solution and 10 pmol of each primer. All oligonucleotides were synthesized by Metabion International, Germany. Thermal cycling was performed at 50°C for 30 mins for reverse transcription, followed by 95°C for 2 mins, and then 35 cycles of 95°C for 30 secs, 58°C for 45 secs, and 68°C for 45 secs. The final extension was done at 68°C for 7 mins and then cooled to 15°C. PCR products were analyzed on a 1.5% agarose gel with Safe-Green™ loading dye (Applied Biological Material, Canada). The positive CPNV genome synthesized from the NGS sequence data in this study was mapped to the CPNV genome sequence (GeneBank accession number HM038436) by in vitro transcription of pUC57-BSaI-Free plasmid (2710 bp) (Gene Universal, USA). Positive and negative control samples of amplification were added to each batch of the sample tested.

DNA sequencing and phylogenetic analysis: The *vp1* gene of CPNV from positive samples was analyzed by DNA sequencing. The amplified products were purified using FavorPrep® Gel/PCR purification kit (Favorgen, Taiwan). The sequencing reactions were performed with Bigdye® terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) following the manufacturer's instructions. The sequencing reactions were analyzed using ABI 3500 DNA genetic analyzer (Applied Biosystems, USA).

The nucleotide sequences of CPNV were compared with the other reference sequences from the GenBank database to determine nucleotide identity using Basic Local Alignment Search Tool for nucleotide (BLASTN) tool on the National Center for Biotechnology Information (NCBI) web interface. The nucleotide sequences were assembled and analyzed with BioEdit Alignment Editor version 7.2.5 (Hall, 1999). Multiple alignments of nucleotide and deduced amino acid sequences were analyzed using the ClustalW algorithm. Phylogenetic trees were constructed by the best model determination for phylogenetic tree construction using the MEGA11 (Tamura *et al*., 2021). The robustness of different nodes

was assessed using 1,000 replicates for bootstrap analysis. The distance between branches based on the number of nucleotide differences was calculated using the same software.

Histopathology: The proventriculi collected from the TPV-affected chickens and the experimental infection of SPF chickens were observed as a gross lesion and processed under routine histopathology. The tissues were fixed in 10% neutral-buffered formalin, processed and embedded in paraffin. The formalin-fixed paraffin-embedded (FFPE) tissues were then sectioned three µm in thickness and taken up on glass slides for staining with hematoxylin and eosin (H&E).

***In situ* hybridization (ISH)**

Generation of the biotin-labeled probe: The ISH procedure was performed to assess the presence of viral genome fragments in FFPE proventriculus sections from clinical chickens. We first synthesized CPNV biotin-labeled probe using Biotin PCR Labeling Core Kit (Jena Bioscience, Germany) according to the manufacturer's instructions. The CPNV template DNA for probe labeling was synthesized from the sequence data of the CPNV by in vitro transcription of pUC57-BSaI-Free plasmid (2710 bp) (Gene Universal, USA). The primers were designed based on the CPNV genome sequence (GeneBank accession number HM038436) by SnapGene Viewer software version 5.3.2 (Insightful Science, USA). The mixture was prepared with 1 µM of primers CPNV-F: 5′-GCGGTCGCCAT ACAGATACATC-3′ and CPNV-R3: 5′-GCACCCCATCACTAGGTCATTA AT-3′. The product indicated for these primers was 708 bps.

Hybridization of tissue samples: Three µm-sections of proventriculi were placed on positively charged microscope slides (Jiangsu Huida Medical Instruments, China). The unstained slides were incubated at 60°C for 30 mins. The slides were deparaffinized with xylene and rehydrated in a series of aqueous ethanol solutions followed by incubation with DAKO proteinase K antigen retrieval solution (Agilent Technologies, USA) in humid boxes for 10 mins. After washing, the slides were treated with 0.3% H_2O_2 in methanol for 20 mins. The slides were rinsed in sterile water and air-dried. The Frame-Seal vapor-tight sealing chambers (Bio-Rad, USA) were placed on the slide sections. The CPNV biotin-labeled probe was prepared at 1:10 dilution in a hybridization solution and applied to the slides before covered with the siliconized coverslips. The covered slides were incubated in hybridizer (DAKO, USA) at 95°C for 10 mins and 42°C overnight. On day two, the coverslips were removed by soaking in phosphate-buffered saline with 0.05% tween (PBST) and washed sequentially with standard sodium citrate (SCC) buffer (Sigma - Aldrich, USA) at 42°C. The slides were incubated with 3% normal horse serum (Vector Laboratories, USA) in PBST at room temperature for 10 mins. The Dako REAL Streptavidin–horseradish peroxidase (HRP) (Agilent Technologies, USA) was applied to the slides and incubated for 15 mins at room temperature. After washing with PBST, the slides were incubated in the chromogen HRP Color Development Reagent diaminobenzidine (DAB) solution (Bio-Rad Laboratories, USA) at 1:50 dilution with H_2O_2 in PBS for 5 mins in dark conditions. The sterile water was applied to the slides to stop the reaction. The slides were counterstained with hematoxylin for 20 secs and rinsed in running tap water followed by mounting with Dako Glycergel Mounting Medium (Agilent Technologies, USA). The stained slides were examined with the Nikon Eclipse Ni microscope with a digital camera DS-Fi2 (Nikon, Japan).

Results

Evaluation of case materials: The proventriculi from the TVP-affected chickens from the infected farms showed significant lesions. The most frequently observed lesions were proventricular enlargement, swelling and dilation. The proventricular walls were thickened and had occasional hemorrhages in the glandular part (Fig. 1). In control chickens, the proventriculi showed no lesions or only minimal lesions (not shown).

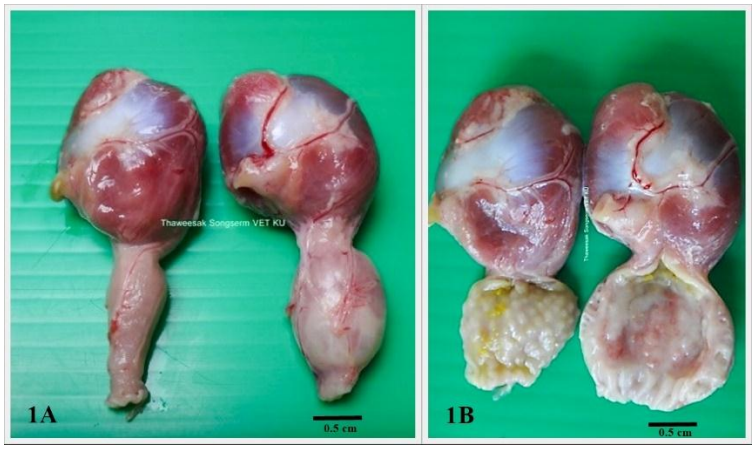

Figure 1 The thickening of proventricular wall of 20-day old broiler chickens from a TVP-affected farm (A) showed a thickening wall compared to a normal proventriculus (B).

Experimental infection of SPF chickens: No clinical signs or mortality occurred in either the homogenate proventriculus inoculum groups or the negative control group. The weight of chickens between these two groups was not significantly different ($P > 0.05$). At necropsy, the proventriculus of the inoculated group showed enlargement and thick wall (Fig. 2). No lesion was found in the proventriculus of the control group.

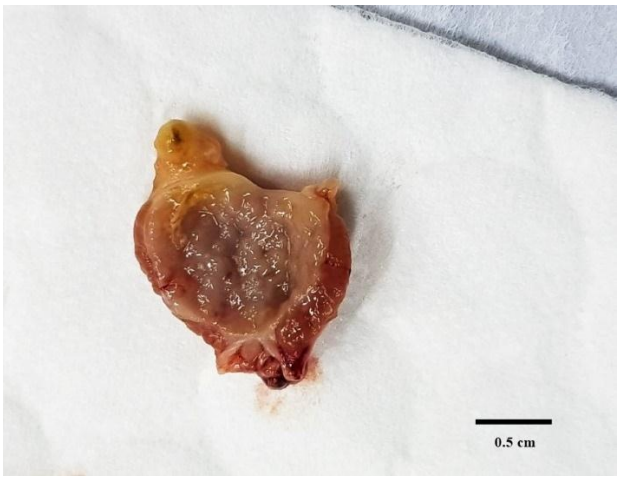

Figure 2 Proventricular lesion from chickens of the infected proventricular inoculated group showed a thickening-wall and swelling on day 16 post-infection.

Virus isolation in cell culture and embryonated eggs: No cytopathic effects were observed in CEF cell cultures inoculated with the proventricular homogenates during 3 passages (data not shown). For egg inoculation, prominent pathognomonic changes were found in the embryos of amniotic route eggs and appeared as early as the second passage. The embryos were stunted and curled with the presence of urate deposition. The proventriculus of the chicken embryos showed congestion and thickening walls (Fig. 3).

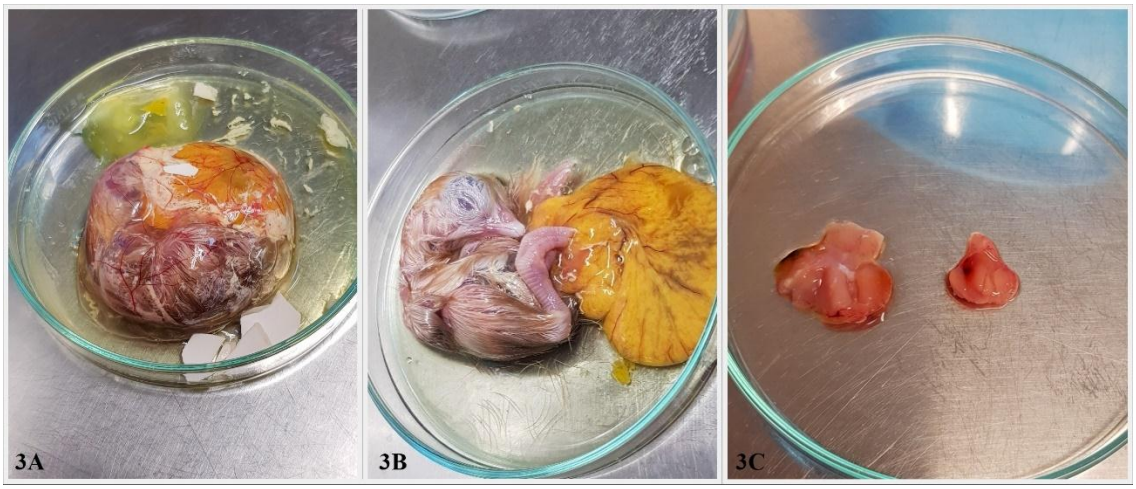

Figure 3 CPNV infected chicken embryo (A) and uninfected control (B) at 7 dpi. The infected embryo was stunted and curled covered with urate deposition. The proventriculus of an infected embryo showed congestion and thickening of the wall (C).

RT-PCR and nucleotide sequencing: The 169-bp partial *vp1* gene of CPNV was detected in some of the freshly collected proventriculi from the TVP-affected chickens in the infected farms. The result showed that only two of 14 chickens showed CPNV positive by RT-PCR, whereas all proventriculi showed TVP-associated lesions. For experimentally infected chickens, CPNV was detected by RT-PCR in four of 11 chickens which proventriculi exhibited TVP-associated lesions, whereas other samples were negative. The RT-PCR was performed to determine the CPNV after cell culture and egg inoculation. The CPNV was detected in 1:10 dilution of the first passage of inoculated CEF cell culture and presented an uncleared band in 1:100 dilution, whereas the following passages all showed negative. The CPNV was found in the embryonic samples inoculated by the amniotic route only at the first passage but the other routes were negative (Fig. 4). The nucleotide sequencing was performed with the use of samples from the inoculated chickens, positive supernatant of cell culture, and egg inoculation. The nucleotide sequence data from the RT-PCR products corresponded to the CPNV *vp1* gene.

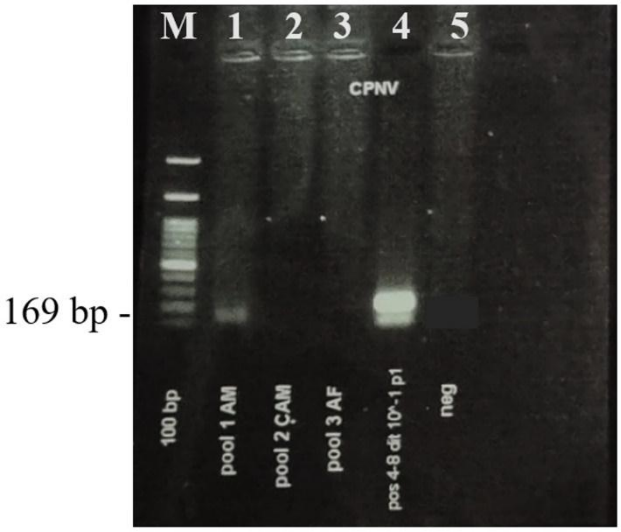

Figure 4 CPNV detection by RT-PCR from the samples collected from embryonated eggs inoculated with CPNV-positive proventricular homogenate. Lane M: 50 bp molecular weight marker (Promega, USA), lane 1: 169 bp CPNV positive result from the embryonated eggs inoculated by amniotic route, lane 2-3: negative results from the embryonated eggs inoculated by CAM and allantoic route, lane 4: positive control of CPNV affected chickens, and lane 5: negative control.

The sequence of the partial *vp1* gene of CPNV in this study (CPNV/chicken/TH/KU-NIAH/2016) showed 83.71% and 82.83% identity to CPNV isolated R11/3 from the USA (GenBank no. HM038436) and CPNV isolate UK_B15_124 from the UK (GenBank no. MT036105), respectively. For phylogenetic analysis, two clusters of CPNV were observed based on the study of Devaney *et al.* (2016), and were categorized by clusters I and II. The CPNV in this study was grouped into cluster I with CPNV reference strain R11/3 from the USA in 2004, the CPNV from Spain in 2004 and 2011 and the CPNV from UK in 2013-2015. Another group, cluster II consisted of the CPNV sequences obtained from UK in 2009-2012 (GeneBank no. MK531122 to MK531127) (Fig. 5)..

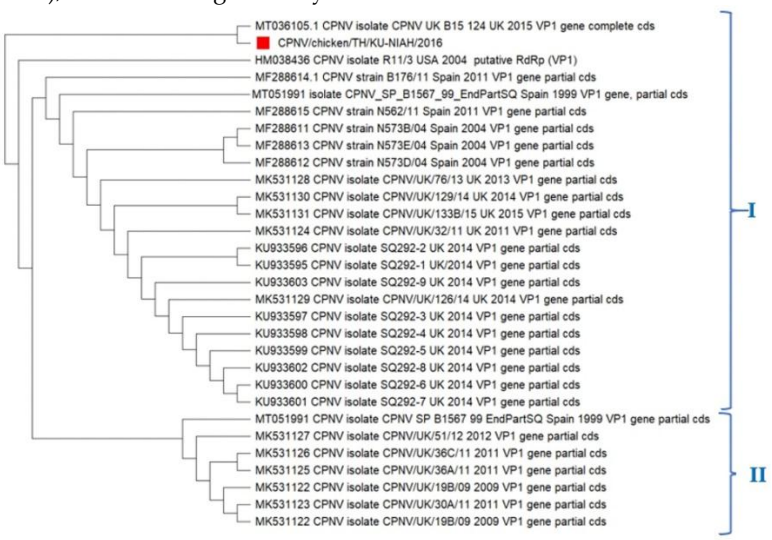

Figure 5 Phylogenetic analysis based on partial vp1 gene sequences of CPNV in this study (red square). The trees were constructed by the maximum likelihood method and Kimura 2–parameter model plus gamma (+G) correction. The Evolutionary analyses were conducted in MEGA11 with 1,000 bootstrap replications.

Histopathological examination: For TVP-affected chickens, we found moderate to severe necrosis of oxynticopeptic cells in all samples. The necrotic areas showed nuclear fragmentation, karyorrhexis, karyolysis and pyknosis of the cells presenting as small clusters within the lumen of proventricular alveoli and collecting ducts. Necrotic debris and sloughed cells appeared in the dilated lumen of the ducts. All samples had dramatic mononuclear cell infiltration with diffused and multifocal patterns within the interstitium of the proventricular glands. The prominent inflammatory cells were lymphocytic, with a few plasma cells and macrophages. The ductal epithelial cells were hyperplasia and metaplasia in all necrotic areas (Fig. 6A-6C). For the experimental chickens, multifocal lymphoid cell infiltration was found in the interstitium of the proventriculus (Fig. 6D). The control group showed no lesion in the proventriculus (Fig. 6E).

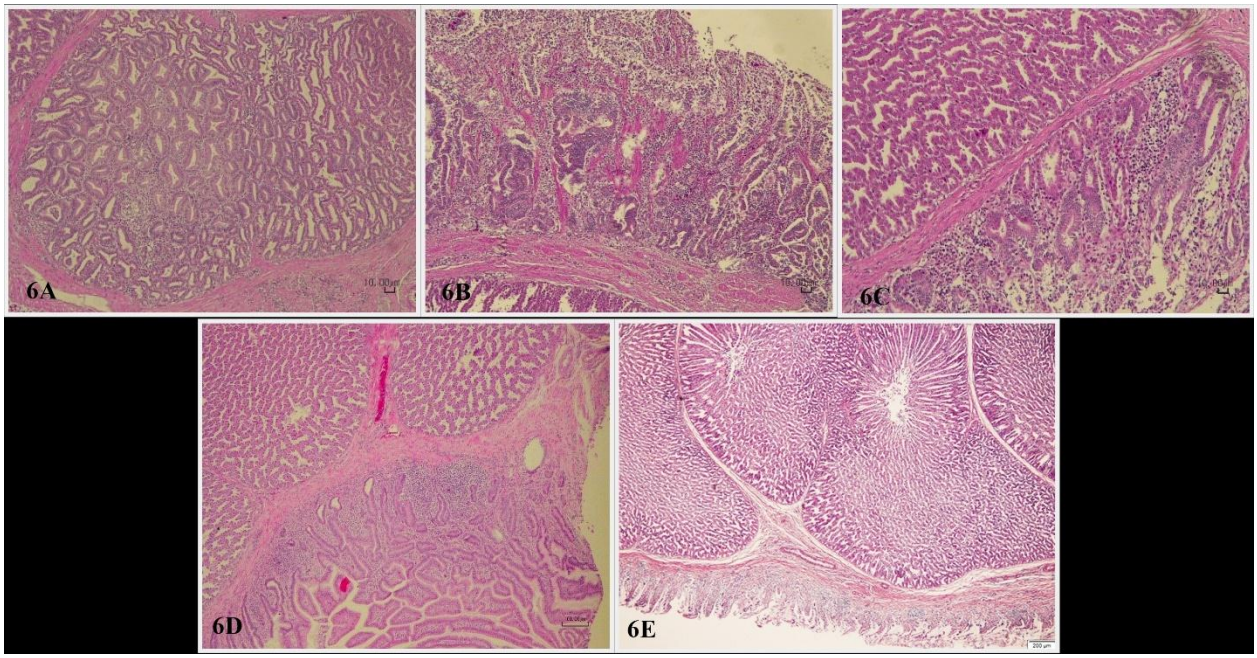

Figure 6 Histopathological examination of proventriculi of TVP-affected chickens. The typical lesions showed the necrosis of the glandular epithelium with mononuclear cell infiltration areas of glandular necrosis (A-C). The histopathology of proventriculus of experimental chickens showed lymphoid cell infiltration in the interstitium of the proventriculus (D). The normal proventriculus illustrated multilobular glands lined the oxynticopeptic cells (E). (A-C) Bar = 10 μm, (D) Bar = 100 μm, (E) Bar = 200 μm.

CPNV detection by ISH: The results of ISH using our designed CPNV-specific probe were observed in the proventriculus of TVP-infected chickens obtained from the farms. Two of the 14 samples with CPNV-positive proventriculi were performed in the ISH. Only one slide showed a positive signal in the proventricular section. The specific reaction was seen as a brown coloration in the necrotic area. Positive ISH staining was observed in ductal and mucosal epithelium including necrotic tissue debris inside the lumens (Fig. 7).

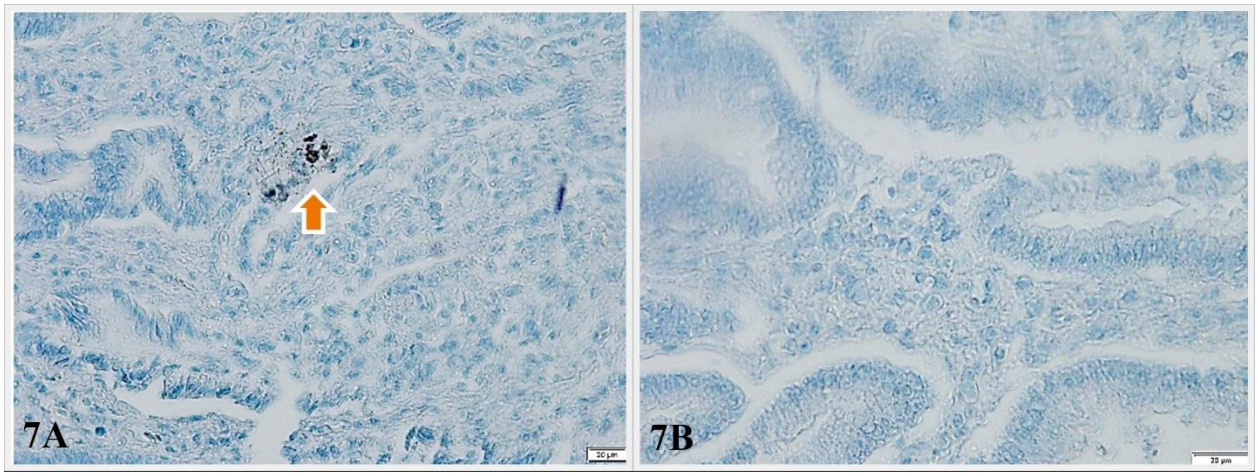

Figure 7 *In situ* hybridization detection of CPNV in proventriculi of a TVP-affected chicken. Positive staining was scattered throughout necrotic area and localized to mucosal epithelium (A) (orange arrow). The negative tissue showed no staining (B). Bar = 20 μm.

Discussion

CPNV was assumed as a significant causative agent of TPV in chickens. Some studies reported the presence of viruses in proventriculi and the virus was reproduced in experimental chickens (Guy *et al*., 2007; Guy *et al*., 2011[b]). Our study indicated that we could reproduce CPNV experimentally in SPF chickens using an inoculum derived from proventriculi of TVP-affected chicken as well as the study of Guy *et al*. (2005). The experimentally infected chickens showed no clinical signs and no difference in weight gain between the inoculated group and control group as in the previous study (Guy *et al*., 2007).

The isolation of CPNV was performed previously by Guy *et al*. (2005, 2007, and 2011[b]) and the virus was called adenovirus-like viruses (R11/3) at that time. Guy *et al*. (2005), found that CPNV could not be propagated in various avian and mammalian cell cultures as well as an inoculation to embryonated

chicken eggs by yolk, allantoic or CAM routes. The viruses were propagated by amniotic inoculation of embryonated eggs and were detected by histopathological techniques in proventriculi and intestinal contents of the hatched 2-day-old chicks at 8 dpi. In the present study, the presence of CPNV was detected by RT-PCR and was found only in the chicken embryos at the first passage and disappeared afterwards. This indicated that the infectivity was lower when time passed or there was a maternal antibody presented in some SPF chicken embryonated eggs. We could not detect antibodies against CPNV in the affected chickens' sera so far. Antibody detection of CPNV has tried to be developed in our laboratories in the future.

For CPNV detection by RT-PCR, the most widely used primers at present were from Guy *et al*. (2011[b]). The sensitivity and specificity of those primers in freshly collected proventriculi was 88% and 83%, respectively (Guy *et al*., 2011[b]). We found those primers were not matched to the CPNV sequences properly, therefore we modified the primers and aligned them with SnapGene Viewer software version 5.3.2 (Insightful Science, USA). Our study agreed with Grau-Roma *et al*., (2020) in categorizing the partial sequences of *the vp1* gene into two groups. The CPNV sequence was closely related to the CPNV reference strain from the USA, UK strain, and the Spanish strain. The result of the phylogenetic analysis was consistent with nucleotide identity.

For RT-PCR detection, there is a small number of CPNV-positive samples from TVP-affected farms and the experimental chickens at 17 days post-exposure (PE). CPNV could be detected by RT-PCR in the proventriculi presenting typical lesions of TVP with fulfilling the TVP-diagnostic criteria (Grau-Roma *et al*., 2020; Guy *et al*., 2011[b]). In the previous study, Guy *et al*. (2011[b]), found that TVP-characteristic proventriculi were positive against CPNV by RT-PCR at 72.73% (8/11) and RT-PCT could not detect CPNV before three days and after 14 days PE in experimentally infected chickens. It is possible that the presence of CPNV is for a brief period and might disappear when time passes. Moreover, TVP at chronic stages with lymphocytic infiltration might show CPVN-negative results (Guy *et al*., 2011[b]; Grau-Roma *et al*., 2020). These findings indicated that the timing of sample collection for CPNV detection should be considered for epidemiologic studies (Guy *et al*., 2011[b]).

Most of the previous studies diagnosed TVP-affected chickens by pathology, immunohistochemistry and molecular techniques. In this study, we could not show the antigen of CPNV by immunostaining because we do not have virus-specific antibody against CPNV. *In situ* hybridization can be used to localize the CPNV nucleotides by a designed CPNV-specific probe and can be used to confirm the presence of CPNV in chicken proventricular tissues. The signals were illustrated in necrotic tissues and mucosal epithelium same as CPNV detection by immunohistochemistry by Guy *et al*. (2007). Our study is the first report on CPNV detection and identification by *in situ* hybridization. This study will lead to improved awareness and knowledge of the diagnosis of CPNV and which will allow forecasting of the source and the monitoring of the TVP outbreaks in broiler farms.

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