Universitat Autònoma de Barcelona

STUDIES ON TRANSMISSIBLE VIRAL PROVENTRICULITIS IN CHICKENS

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Studies on Transmissible Viral Proventriculitis in chickens

Tesi doctoral presentada per Nabil Ali Wali per optar al grau de Doctor en Veterinària dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Natàlia Majó i Masferrer.

Bellaterra, 2021

La Dra. NATÀLIA MAJÓ i MASFERRER, professora titulars del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigadora adscrita a l'Institut de Recerca i Tecnologia Agroalimentàries-Programa de Sanitat Animal (IRTA-CReSA),

Certifica:

Que la memòria titulada "**Studies on transmissible viral proventriculitis in chickens"** presentada per Nabil Ali Wali per a l'obtenció del grau de Doctor, s'ha realitzat sota la seva direcció i supervisió i, considerant-la acabada, n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra,

Dra. Natàlia Majó i Masferrer	Nabil Ali Wali
Directora	Doctorand

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Dedication

This thesis is dedicated in memory of my parents and martyrs' brothers Samir, Munir, Amir and all humanity everywhere.

SUMMARY

Transmissible Viral Proventriculitis (TVP) is an emergent viral infectious disease that affects mainly broiler chickens. It is characterized by impaired feed digestion, poor growth, and poor feed conversion rate, causing economic losses to the poultry industry. Enlargement, thickening, fragility, and paleness of the proventriculus, together with weakness and dilation of gastric isthmus is observed in TVP cases. Although proventricular gross lesions could be indicative of TVP, they are not specific. The disease is characterized by its histologic lesions: necrosis of oxynticopeptic cells, inflammation with a predominance of lymphocytes, and replacement of glandular epithelium by hyperplasic ductal epithelium. The etiology of TVP identified to date is a new species of birnavirus, called Chicken proventricular necrosis virus (CPNV). This virus was identified as birnavirus based on physical characterisation (non-enveloped, 70 nm, icosahedral, bi-segmented, double-stranded RNA) and a partial sequence genome analysis. Recent studies have confirmed the association between CPNV and TPV in several countries, such as USA, France and most recently UK, but the number of studied cases is still scarce and there is little information available about the epidemiology and pathogenesis of this disease. Therefore, the main objective of this thesis was to determine the presence of the disease and the new viral agent in Spanish poultry farms and further characterize this viral agent by ultrastructural studies and next generation sequencing techniques.

In the first study, the presence of CPNV in TVP clinical cases from Spanish poultry farms was retrospectively evaluated from 1999 to 2019 in FFPE proventricular tissue. Histopathological examination, CPNV RT-PCR, and partial sequence genome of positive cases obtained using Sanger sequence was carried out in 42 clinical cases. In adition, a new ISH technique was set up as a new method to detect the virus. The study identified the presence of CPNV in Spanish chicken farms since at least 1999. Moreover, ten proventriculi from seven different clinical cases were positive to CPNV RT-PCR and ISH, and all of them showed the characteristic histopathological features of TVP (necrosis of oxynticopeptic cells and gland interstitial inflammation). Phylogenetic studies showed

that the Spanish CPNV partial sequences were very closely related to the available UK and USA CPNV sequences.

The second study of this thesis aimed to identify, visualize, and localize the causative agent of TVP by using TEM. Proventricular samples from twelve different clinical cases were used. Eight of the samples were positive by RT-PCR and ISH to CPNV, while the other three, although showing gross and microscopic lesiosn consistent with TVP, were negative to CPNV ISH and RT-PCR. Icosahedral, 70 nm, non-enveloped, intranuclear and/or intracytoplasm viruses were observed in four samples. Two of these samples were negative to CPNV by molecular methods, while the other two gave positive results to RT-PCR and ISH CPNV techniques. These results, together with the finding of virions in the nuclei of infecetd cells, a finding which is not usually seen in RNA viruses, raised the question whether TVP could also be caused by another viral agent simultaneoulsy or without the contribution fo CPNV.

To further undesrtand this last hypothesis, a third study was done to further characterize the genome of the virus/es involved in the TVP cases. Eight TVP proventriculi samples were studied by NGS and partial or complete sequences of CPNV Segment B were found in all of them, furter confirming the involvement of this virus in TVP clinical cases. However, in five of the cases, partial sequences of Avian adenovirus A were found, particularly the two cases where intranuclear virions had been observed by TEM. These results confirm the hypothesis that Avian adenovirus A can be present in TVP clinical cases, although its role in the development of the disease needs further studie.

In conclusion, the obtained results of this thesis provide relevant data on etiology, epidemiology and pathogenesis of TVP. In addition, new techniques were set up in order to further understand the disease and, finally, complete and partial sequencing data of CPNV was released in the public domain to promote research on this new viral agent.

RESUMEN

La proventriculitis viral transmisible (TVP) es una enfermedad infecciosa viral emergente que afecta principalmente a los pollos de engorde. Se caracteriza por una digestión deficiente de los alimentos, retraso en el crecimiento y un .índice de conversión elevado, lo que provoca pérdidas económicas para la industria avícola. En los casos de TVP se observa incremento de tamaño, engrosamiento, fragilidad y palidez del proventrículo, junto con debilidad y dilatación del istmo gástrico. Aunque las lesiones macroscópicas proventriculares podrían ser indicativas de TVP, no son específicas. La enfermedad se caracteriza por sus lesiones histológicas: necrosis de células oxinticopépticas, inflamación con predominio de linfocitos y sustitución del epitelio glandular por epitelio ductal hiperplásico. La etiología de TVP identificada hasta la fecha es una nueva especie de birnavirus, llamado virus de la necrosis proventricular del pollo (CPNV). Este virus se identificó como birnavirus basándose en la caracterización física (ARN sin envoltura, 70 nm, icosaédrico, bisegmentado, bicatenario) y un análisis parcial de la secuencia genómica. Estudios recientes han confirmado la asociación entre CPNV y TPV en varios países, como EE. UU., Francia y más recientemente Reino Unido, pero el número de casos estudiados aún es escaso y hay poca información disponible sobre la epidemiología y patogénesis de esta enfermedad. Por tanto, el objetivo principal de esta tesis fue determinar la presencia de la enfermedad y el nuevo agente viral en las granjas avícolas españolas y caracterizar este agente viral mediante estudios ultraestructurales y técnicas de secuenciación de última generación.

En el primer estudio, se evaluó retrospectivamente la presencia de CPNV en casos clínicos de TVP de granjas avícolas españolas de 1999 a 2019 en tejido proventricular fijado en formol e incluido en parafina. En 42 casos clínicos se realizó examen histopatológico, CPNV RT-PCR y secuenciación parcial del genoma de los casos positivos. Además, se estableció una nueva técnica de ISH como un nuevo método para detectar el virus. El estudio identificó la presencia de CPNV en las granjas avícolas españolas desde al menos 1999. Además, diez proventrículos de siete casos clínicos diferentes fueron positivos a CPNV RT-PCR e ISH, y todos mostraron los rasgos histopatológicos característicos de TVP (necrosis de oxinticopeptic células e inflamación intersticial de la glándula). Los estudios filogenéticos mostraron que las secuencias

parciales españolas de CPNV estaban estrechamente relacionadas con las secuencias de CPNV disponibles en Reino Unido y EE. UU.

El segundo estudio de esta tesis tuvo como objetivo identificar, visualizar y localizar el agente causante de TVP mediante el uso de TEM. Se utilizaron muestras proventriculares de doce casos clínicos diferentes. Ocho de las muestras eran positivas mediante RT-PCR e ISH a CPNV, mientras que las otras tres, aunque mostraron lesiones macroscópicas y microscópicas compatibles con TVP, eran negativas a CPNV ISH y RT-PCR. Se observaron virus icosaédricos, de 70 nm, no envueltos, intranucleares y/o intracitoplasmáticos en cuatro muestras. Dos de estas muestras resultaron negativas a CPNV por métodos moleculares, mientras que las otras dos dieron resultados positivos a las técnicas RT-PCR e ISH CPNV. Estos resultados, junto con el hallazgo de viriones en los núcleos de las células infecciosas, un hallazgo que no suele observarse en los virus de ARN, plantearon la cuestión de si la TVP también podría ser causada por otro agente viral simultáneamente o sin la contribución del CPNV.

Para comprender aún más esta última hipótesis, se realizó un tercer estudio para caracterizar el genoma de los virus involucrados en los casos de TVP. Se estudiaron por NGS ocho muestras de proventrículos con TVP y en todas se encontraron secuencias parciales o completas del Segmento B de CPNV, confirmando la participación de este virus en casos clínicos de TVP. Sin embargo, en cinco de los casos, se encontraron secuencias parciales de adenovirus aviar A, en particular los dos casos en los que se habían observado viriones intranucleares mediante TEM. Estos resultados confirman la hipótesis de que el adenovirus aviar puede estar presente en casos clínicos de TVP, aunque son necesarios más estudios para clarificar su papel en el desarrollo de la enfermedad.

En conclusión, los resultados obtenidos de esta tesis aportan datos relevantes sobre la epidemiología, etiología y patogénesis de la TVP. Además, se establecieron nuevas técnicas para conocer mejor la enfermedad y, finalmente, se dieron a conocer al dominio público datos de secuenciación completa y parcial de CPNV para promover la investigación sobre este nuevo agente viral.

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Abbreviation

aa	amino acids
AdLV	Aden-Like virus
AMV RT	Avian Meyloblastosis Virus Reverse Transcriptase
APHA	Animal and Plant Health Agency-Weybridge
AI	Avian influenza
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSNV	Blotched snake head virus
CPNV	Chicken proventricular necrosis virus
CReSA	Centre de Recerca en Sanitat Animal
cRNA	Complementary RNA
CUG	corrosive ulcerative gizzard
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate (nucleotides)
DPX	Dibutyl-phthalate Polystyrene Xylene
ds RNA	double strand RNA
EID	Emerging Infectious Diseases
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FAdV	Fowl Adenovirus
FCR	Feed Conversion Rate
FFPE	Formalin Fixed Paraffin Embedded
HE	Haematoxylin/eosin
HS	Hydropericardium syndrome
HT1	Hybridisation buffer for NGS
IBH	Inclusion Body Hepatitis
ICTV	International committee on Taxonomy of viruses
ICV	Intra cytoplasmic virus
INV	Intra nuclear virus
IPNV	Infectious pancreatic necrosis virus
IRTA	Institut de Recerca i Tecnologia Agroalimentàries
ISH	*In Situ* Hybridisation
MEGA X	Molecular Evolutionary Genetics Analysis version X
mRNA	Messenger RNA
N	Nucleotide
NCBI	National Centre for Biological Information
NGS	Next generation sequencing
NDV	Newcastle disease virus
NA	Non-available

LP	Lymphocytic proventriculitis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RdRP	RNA dependent RNA polymerase
RT	Room temperature
RT-PCR	Reverse Transcriptase-polymerase chain reaction
RSS	Runting stunting syndrome
RNA	Ribonucleic acid
SAMtool	Sequence Alignment Map tool
SGB	Servei de Genomica i Bioinformatica at UAB
SPAdes	St. Petersburg genome assembly
SPF	Specific pathogen free
SSC	standard sodium citrate buffer
ss cDNA	single strand cDNA
TEM	Transmission electron microscope
TAE	Tris acetate EDTA
TVP	Transmissible viral proventriculitis
UAB	Universidad Autónoma de Barcelona
UK	United Kingdom
USA	United States of America
VP1	Virus protein 1

Chapter 1

General Introduction

1.1. Emerging diseases and viruses

An emerging infectious disease (EID) is a disease caused by a pathogen that has not been observed previously within a population or geographic location. Viruses are a major cause of EIDs (Louten, 2016). An interesting study revealed that between 1980 and 2001 most of the emerging pathogens (67%) were viruses, suggesting a faster evolution than other pathogens. Moreover, an 84% of them were RNA viruses and mostly associated with an animal reservoir (Woolhouse and Gaunt, 2007).

Newly emerging or re-emerging pathogens have threatened the survival of humans and animals for centuries. Historically, some emerging diseases have been responsible for widespread deadly outbreaks, such as the 1918 influenza pandemic caused by a H1N1 strain that resulted in approximately 50 million human deaths, and the more recently HIV/AIDS-related illnesses have caused around 35 million deaths so far (https://www.avert.org/global-hiv-and-aids-statistics). Nowadays, a novel coronavirus, SARS-CoV-2, is threatening the globe.

Many reasons led to emerging new diseases developed along with intensification and changes in husbandry, genetic, hygiene and production system (Davies, 2012). The evolution of emerging diseases is associated with some factors embedded in the concept host-agent-environment triangle (One Health concept). In principle, the genetic factors of the virus and the host breed and the external factor of size and density of the human and animal population and intensification of farming and international trade play a role in this pathogen evolution (Davies, 2012).

Emerging and re-emerging diseases have a significant effect on socio-economy stability of societies and public health issues. Emerging diseases drastically affect animal populations, especially in food-producing animals. Livestock production in large communities (for example, poultry flocks) represents an excellent environment to facilitate the transmission and maintenance of viral infections within a population, leading to pathogen genome modifications. The intensive rearing of livestock since 1950 has probably been one of the main factors that contributed both to the emergence of new pathogens leading to changes in the epidemiology and presentation of diseases (Davies, 2012).

Most of the knowledge acquired on viral agents come from *in vitro* studies based on the gold-standard method for viral isolation, i.e. the propagation of the virus in cell culture. However, some viruses cannot be propagated in the cellular platforms, which is a limiting factor to increase the knowledge on multiple aspects (disease reproduction, pathogenesis, immunity, etc.). In the last decades, several viral discoveries occurred due to the advance and development of technologies, especially sequencing. Initially, to sequence a pathogen genome or part of it was dependent on previous knowledge of at least part of the genetic material of the pathogen (Sanger *et al.*, 1977). However, the advent on new technologies, such as the next generation sequencing (NGS), sidesteps both the un-culturability and the requirement on previous knowledge on the viral genome. NGS allows analysing the genomic sequences of a completely unknown organism and accurately characterizing the genetic composition of the individual (Wood and Salzberg, 2014).

1.2. Avian digestive system and proventriculus

The digestive system of a bird is composed of oral cavity, pharynx, oesophagus, crop, proventriculus, ventriculus or gizzard, small intestine, large intestine and cloaca. The avian gastrointestinal tract is much shorter compared to the mammalian gastrointestinal tract and the average transit time is less than 3.5 hours (Denbow, 2000).

The avian stomach consists of proventriculus, intermediate zone (isthmus) and ventriculus or gizzard (Figures 1 and 2). The proventriculus is the glandular part of stomach, which is functionally equivalent to mammalian stomach. It is a fusiform-shaped organ, located between the distal esophagus and the gizzard and lying on the liver. It is approximately 4-6 cm long and 2 cm diameter in an adult chicken. The intermediate zone (isthmus) between the proventriculus and the gizzard is very short, about 0.75 cm, and the histological structure is intermediate to that of proventriculus and ventriculus. The isthmus functions mainly when contracted as a barrier separating the proventriculus from the gizzard.

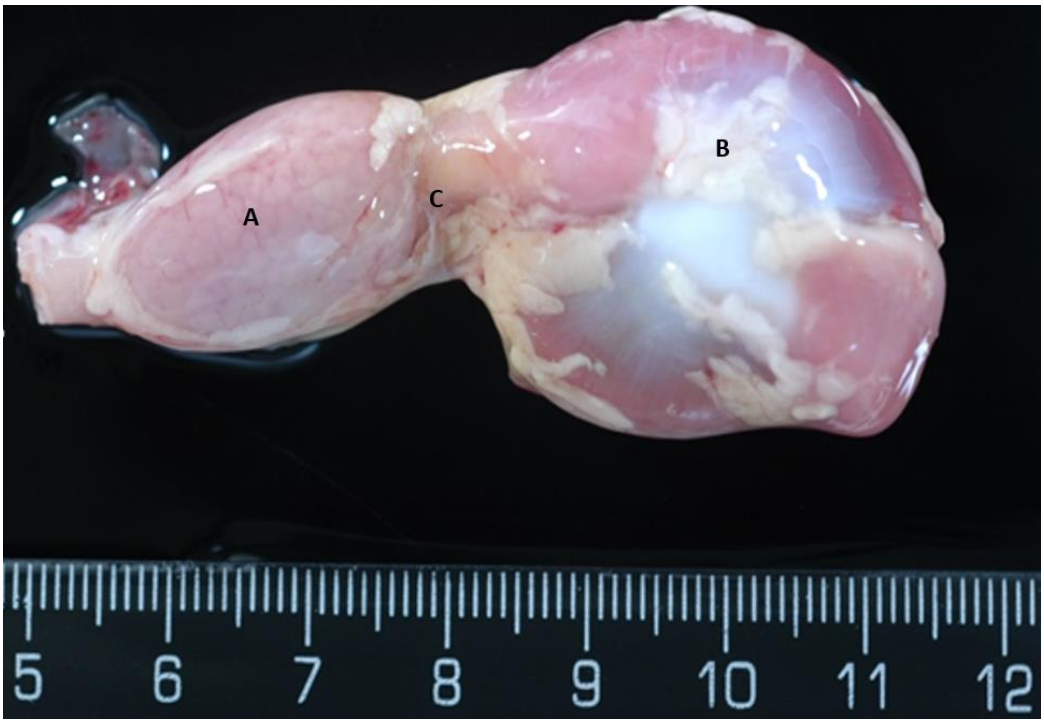

Figure 1. Chicken stomachs consisting of a glandular stomach or proventriculus (A) and a muscular stomach, named also ventriculus or gizzard (B). Between both, there is the isthmus (C).

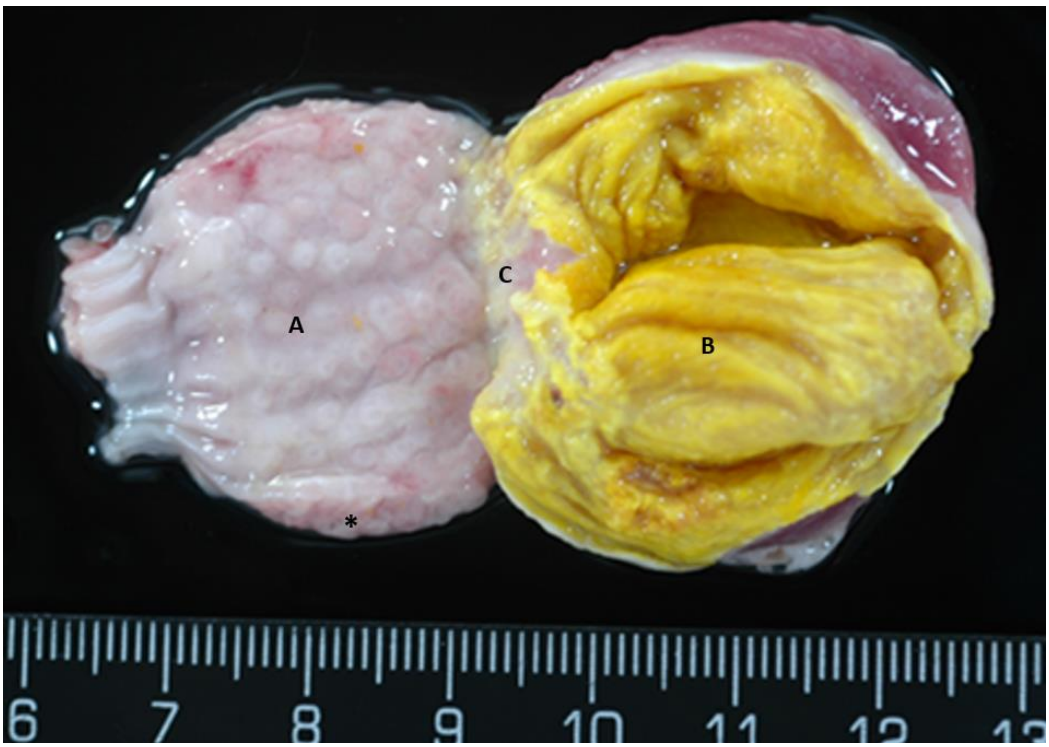

Figure 2. The inners surface or mucosa of the proventriculus (A), isthmus (C) and gizzard (B). In the proventricular mucosa, the papillae of the proventricular glands can be observed.

Microscopically, the proventriculus consists of four layers: mucosa, submucosa, muscular and serosa (Figure 3). The proventricular mucosal surface is not smooth but covered by several projections or papillae of gastric glands where the gastric secretion is released (Hodges, 1974).

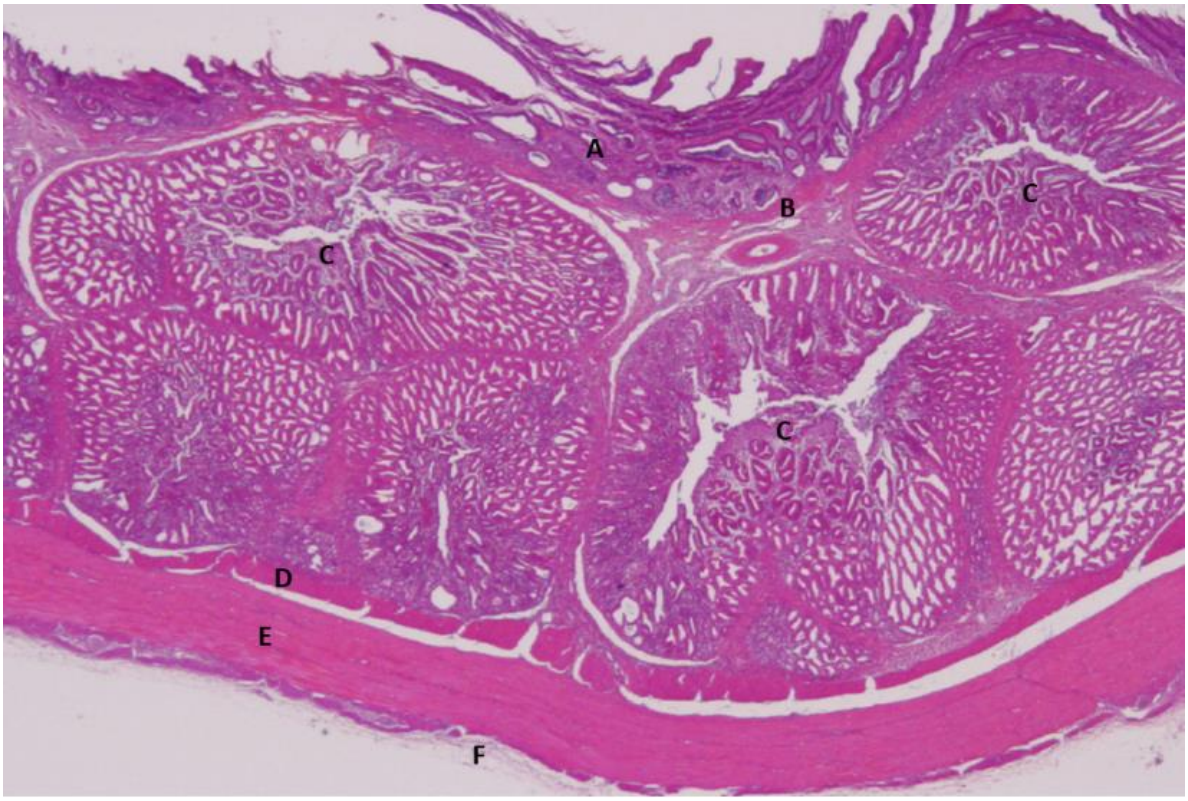

Figure 3. Cross section of chicken proventriculus tissue strained with H&E showing the layers of the proventriculus. (A) Columnar mucous secreting epithelium; (B) lamina propria; (C) multilobular glands and lined mainly by one type of cell, the oxynticopeptic cell, which secretes both pepsin and hydrochloric acid; (D) inner circular muscles layer; (E) outer longitudinal muscle layer; (F) serosa.

The mucosal layer is consisting of simple villi lined by columnar ductal mucous secreting cells where the nucleus is at the basal cytoplasmic area of the cells (Fig.3). The submucosa is filled with proventriculus glands. The mass of glands makes up the greater part of the thickness of the proventriculus wall (Reece and Frazier, 1990). The glands are composed of numerous rounded or polymorphic lobules which are arranged in small groups and separated by connective tissue septa, consisting of collagenous and elastic fibres with a few muscle fibres, blood vessels and nerves. The lobules consist mainly of alveoli

radiating out from central cavity. The central cavity is drained to a secondary duct; all secondary ducts are uniting to make the primary ducts that open to the orifice in the lumen of the proventriculus (Hodges, 1974).

The alveoli are lined by primary epithelial cells mainly oxynticopeptic cells which are responsible for secreting pepsin and hydrochloric acid (HCl), while the secondary and primary ducts are lined by simple columnar ductal mucous secreting cells to lubricate the feed and protect the inner surface of the proventriculus from high acidity (Hodges, 1974). The muscular layer is composed of an inner circular muscle layer and an outer longitudinal muscle layer. Finally, the serosa is the more external part of the proventriculus.

The primary function of the proventriculus is the production and release of the gastric secretions, pepsin, hydrochloric acid, and mucus. Although, the main mechanical and enzymatically food digestion occurs in the ventriculus where the pH 2.2-3.2 and accomplished through the strong contractions of the asymmetrical ventricular muscle masses and abrasive action of the cuticle. The proventriculus and ventriculus act as a unit, pushing the ingesta back and forth between the two components to optimize mechanical and enzymatic digestion (Denbow, 2000). Gastric acid facilitates the digestion of protein as well as the absorption of iron, calcium, vitamin B12 and the high acidity kills ingested microorganisms and enteric infection (Schubert, 2015). The proventriculus and ventriculus do not represent a major site for absorption of nutrients. The primary site of nutrient (carbohydrates, amino acids and peptides, fatty acids, electrolytes, vitamins) absorption is the small intestine, and to a lesser degree the large intestine (Denbow, 2000).

1.3 Proventriculitis

1.3.1. Definition and history

Proventriculitis is the inflammation of the proventriculus and is a frequent pathological process of commercial birds. It usually affects food digestion and consequently productivity, performance, and feed conversion in the chicken. It is characterized by the enlargement of the proventriculus and fragility of the gastric isthmus which may rupture during mechanical evisceration in the processing plant, resulting in carcass contamination and condemnation (Dormitorio *et al.*, 2007). Proventriculitis may be caused by dietary

causes, such as oral exposure to biogenic amines (Barnes *et al.*, 2001), mycotoxins (Dorner *et al.*, 1983), lack of dietary fibre (Goodwin *et al.*, 1996) or excessive copper sulfate (Bayyari *et al.*, 1995b), and it has also been associated to large number of infectious agents, such as bacteria, fungi, cryptosporidia, and viruses (Goodwin, 1995; Lenz *et al.*, 1998; Huff *et al.*, 2001; Pantin-Jackwood *et al.*, 2003; Tomaszewski *et al.*, 2003; Yu *et al.*, 2017).

In 1978, Kouwenhoven and co-workers suggested that an infectious agent might have been the cause of the runting and poor feed conversion associated to proventriculitis observed in a large broiler farm in the Netherlands. The authors described diffuse swelling of the proventriculus. In some cases, mucosa was covered with fibrinous necrotic material and in other cases, mucosal haemorrhages were observed. Microscopically, glandular necrosis, haemorrhages, fibrosis and leukocyte infiltration in the mucosa and glandular tissue were observed. They could reproduce the clinical picture by inoculating birds with a filtered homogenate of the proventriculus of affected chickens. The filtered agent was cell-free and since it passed 450 and 100 nm filters, it was probably a virus. Adenovirus was isolated from some of the tissues, but a clear etiological role could not be established. This was the first description of an infectious proventriculitis causing runting in chickens. (Kouwenhoven *et al.*, 1978).

Later, Bayyari *et al.* (1995a) were also able to reproduce proventriculitis by using a filterable agent found in proventricular homogenates from field cases. Proventriculitis was produced without an effect on growth, suggesting that proventriculitis and stunting syndrome could have a different etiology. One year later, Goodwin *et al.* (1996) were the first to describe intralesional virus and suggested a causal relationship between the virus and the lesion in its host. They found hexagonal virus particles in intact nuclei, associated with unbound condensed chromatin and within vacuolated spaces in the cytoplasm. The average size of the viral particles was 65-70 nm. The virus was not isolated nor identified beyond its transmission electron microscopic appearance and detection of adenovirus or polyomavirus nucleic acid by *in situ* hybridization was not successful, but this work lead to the designation of this condition as **Transmissible Viral Proventriculitis (TVP)**.

1.3.2. Etiology

Since these first studies mentioned above, many different viral agents have been proposed as possible etiological agents of TVP, being the Infectious bursal disease virus (IBDV)

(Pantin-jackwood *et al.*, 2003), Infectious bronchitis virus (IBV) (Zheng *et al.*, 2000; Kutkat *et al.*, 2010; Yu *et al.*, 2017), adeno virus (Kouwenhoven *et al.*, 1978; Goodwin *et al.*, 1996; Lenz *et al.*, 1998), and adeno -like virus (Guy *et al.*, 2005, 2007) the most frequently involved. In 2010, an IBDV-like virus, sharing some characteristics with IBDV, was suggested to be associated to TVP (Grau-Roma *et al.*, 2010) and, later on, Guy and co-workers described a new birnavirus, named Chicken proventricular necrosis virus (CPNV), as the cause of TVP (Guy *et al.*, 2011a, 2011b). They were able to reproduce to detect CPNV in naturally-occurring cases as well as in experimentally infected chickens (Guy *et al.*, 2011b).

Nowadays, even if there are other research groups that have identified by molecular techniques other viruses that may be involved in this pathological process, such as a picornavirus (Kim *et al.*, 2015) and the Gyrovirus 3 (Li *et al.*, 2018), the CPNV has been widely accepted as the causative agent of TVP (Guy *et al.*, 2011a, 2011b; Marguerie *et al.*, 2011; Marusak *et al.*, 2012; Hafner *et al.*, 2013; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2016, 2020).

1.3.2.1. Virus taxonomy and classification

CPNV was identified as a birnavirus based on virion size and morphology, buoyant density in cesium chloride, a genome comprised by bisegmented double-stranded RNA and the nucleotide sequence analysis (Guy *et al.*, 2011a).

Birnaviridae is a family of viruses that infect a wide variety of vertebrate and invertebrate species, but not mammals. The Birnaviridae family has four different genera: Avibirnavirus, Aquabirnavirus and Blosnavirus that may replicate in vertebrates and the Entomobirnavirus genus that infect insects. This family also comprises seven unclassified species, including CPNV, on the basis of the viral structure and genome sequence (Figure 4). Infectious bursal disease virus (IBDV) is one of the most important virus and the representative of this family (Delmas *et al.*, 2019). The CPNV VP1 protein, the viral RNA-dependent RNA polymerase (RdRp), has a unique permuted RdRp sequence motif arrangement characteristic of Birnaviruses; however, phylogenetic analyses based on VP1 demonstrated that CPNV is deeply divergent from other Birnaviruses (Guy *et al.*, 2011a).

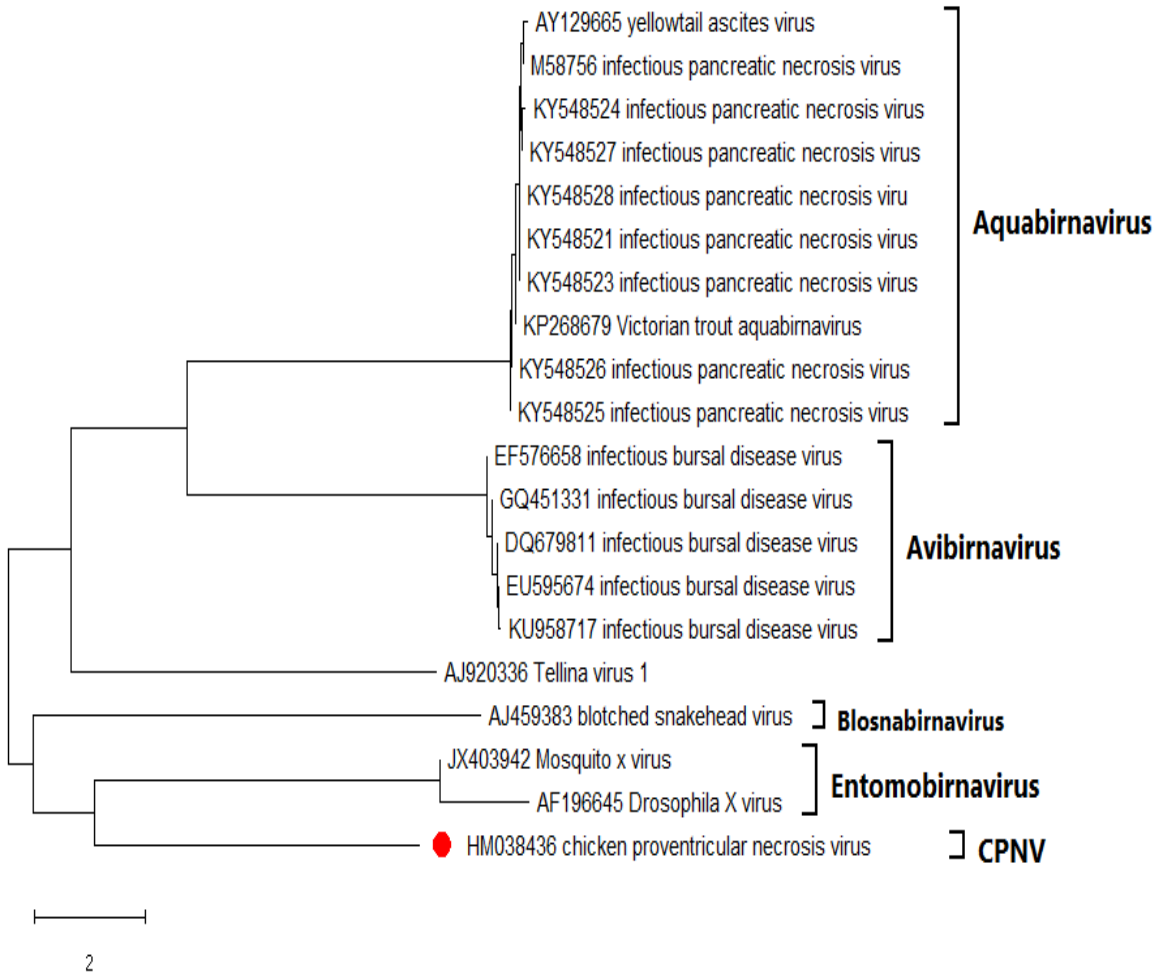

Figure 4. Distance tree representing the phylogenetic relationships of VP1 for members of the various genera and species in the family Birnaviridae, including new Birnaviruses and the only sequence available of CPNV. The amino acid alignment was done with muscle and adjusted visually. Phylogenetic analysis was performed with MEGA X (Kumar *et al*, 2016) using the JTT substitution matrix and a gamma distribution of rate variation between sites.

1.3.2.2. Viral structure

Viruses belonging to the Birnaviridae family are all non-enveloped, single shelled particles with a diameter of about 65 nm (Figure 5A). The capsid possesses icosahedral geometry and comprises a single capsid protein, VP2, clustered in trimers and forming 260 projections at the virus surface (Figure 5B). Inside the virus particle, there are the ribonucleoprotein complexes made by the two genome segments associated with multiples copies of a ribonucleoprotein (VP3) and the RNA-dependent RNA polymerase (Figure 5C).

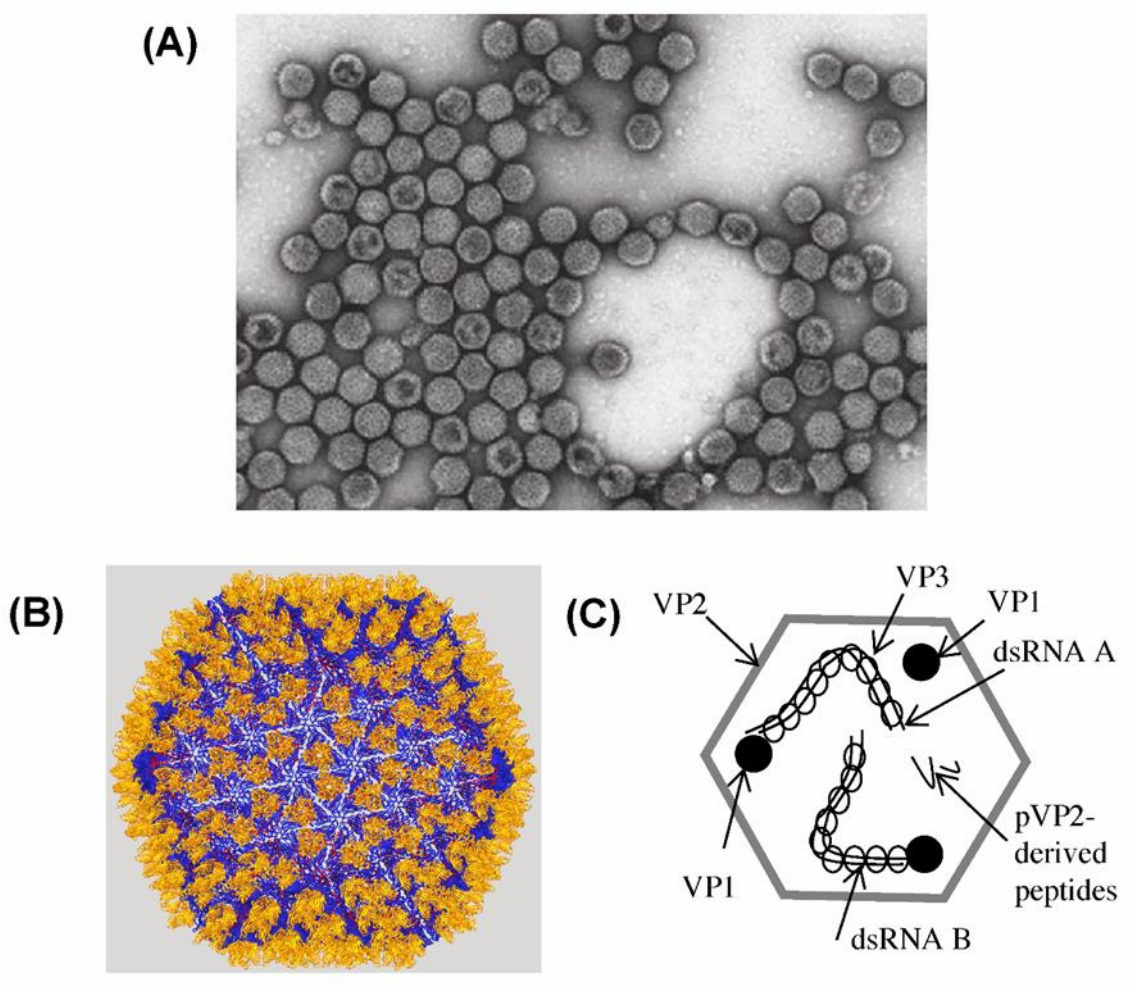

Figure 5. Birnavirus (infectious bursal disease virus) particle structure (A) Negative-contrast electron micrograph of particles. (B) A three-dimensional model of the virion. (C) Diagrammatic representation showing the distribution of the polypeptides and the genome in the virus particle. Adapted from (Delmas *et al.*, 2019)).

Regarding CPNV, few data exists about its structure. Guy *et al.* (2011a), demonstrated its icosahedral morphology and a diameter of approximately 75 nm. They also showed that the virus was resistant to chloroform treatment indicating absence of a lipid envelope.

1.3.2.3. Viral genome and proteins

The genome of the birnaviruses comprises two linear double-stranded RNA genomic segments A and B. The larger segment A is 3.1-3.6 Kbp and encodes the polyprotein

preVP2-VP3-VP4. Segment B is 2-8 to 3.3. kbp and encodes VP, the RNA-dependent RNA-polymerase (RdRp). (Fig. 6) (Delmas *et al.*, 2019).

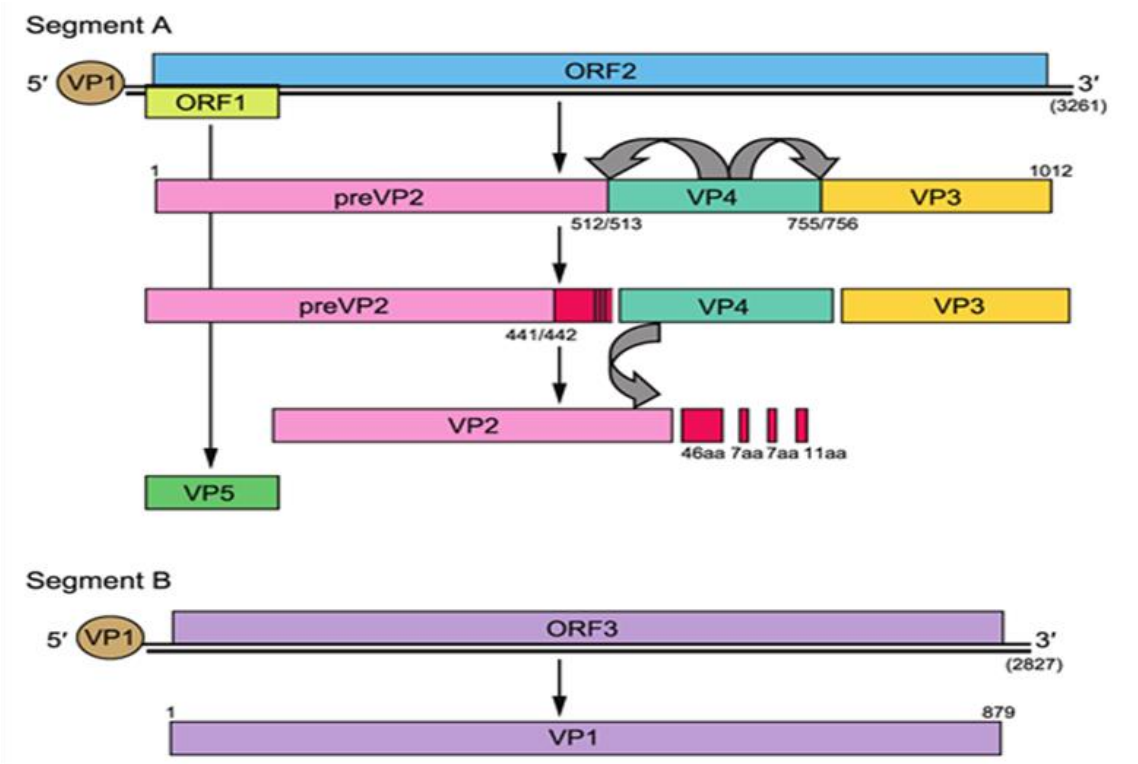

Figure 6. Schematic representation of the genome of infectious bursal disease virus illustrating processing of the encoded proteins. Numbers in parentheses indicate the nucleotide lengths of the two genomic segments. Adapted from Delmas *et al.* (2019).

Birnaviruses share no nucleic acid sequence similarity with other taxa. The birnavirus capsid protein VP2 has no sequence similarities with the corresponding capsid proteins of any other virus family.

Regarding CPNV, Guy and co-workers (2011a) reported the presence of a virus with a linear double-stranded RNA genomic segments (A and B) associated to TVP cases. The type of nucleic acid comprising the genome of CPNV was determined based on susceptibility to nuclease treatment (RNase, DNase and S1 nuclease) prior to agarose-gel electrophoresis. They proved that the virus was RNA because CPNV was digested with RNase but not with DNase or S1 nuclease. CPNV RNA appeared as two distinct

equimolar bands A and B of 3.6 and 2.9 kbp respectively by non-denaturing polyacrylamide gel electrophoresis (Guy *et al.*, 2011a).

In addition, in a pairwise alignment of CPNV VP1 with other birnavirus, VP1s revealed only 16% identity. In contrast, pairwise alignment of VP1s of IBDV, Infectious Pancreatic Necrosis Virus (IPNV), Drosophila X Virus (DXV), Blotched Snakehead Virus (BSNV), and Tellina Virus 1 (TV-1) ranged from 24% (DXV x TV-1) to 51% (IBDV x BSNV). The branching structure of the phylogenetic tree of CPNV was deeply divergent from other known birnaviruses and for that reason CPNV was suggested to be a new, the fifth genera, of Birnaviridae family (Guy *et al.*, 2011a). The ICTV finally recognised CPNV as a species in the Birnaviridae family (Delmas *et al.*, 2019).

Regarding viral proteins, the birnavirus have five proteins. VP1, the RNA-dependent RNA polymerase of the virus, is present in the virion as a free polypeptide and as a genome-linked protein. It plays a key role in the encapsidation of the viral particles (Pantin-Jackwood, 2003). The structural protein VP2 is the major protein component of the viral capsid. VP2 is the host protective antigen containing the antigenic region responsible for the induction of neutralizing antibodies and for serotype specificity in IBDV. VP3 is also a structural protein and is only found on the inner surfaces of the virus. This protein plays a role in the assembly of viral particles, and packaging of the viral genome. VP4 is a proteolytic enzyme-like protein and it is essential for the proteolytic processing of the polyprotein and either itself plays a role in the activation of VP1. Finally, VP5 protein plays an important role in viral pathogenesis (Pantin-Jackwood *et al.*, 2003).

1.3.2.4. Viral replication

Regarding birnavirus replication, much of the information that is currently available refers to IBDV replication. An IBDV single cycle of replication takes about 4–8 h. The mode of entry is binding at the cell surface, proteins such as heat shock protein 90 and α4β2 integrin have proposed to serve as functional receptors in various types of chicken cells. One of the small structural IBDV peptides, pep46, are able to induce pores in target membranes, suggesting a role in virus entry (direct penetration) (Delmas *et al.*, 2019). After delivery into the cytoplasm, the two steps of replication include synthesis of nucleic acids and proteins are. The viral RdRP becomes activated and produces two mRNA

molecules from each of the dsRNA genome segments. Virus RNA is transcribed by a semi-conservative strand displacement mechanism in vitro and produce positive-single strand RNA. There is no information on negative-strand RNA synthesis. Synthesis of protein in segment A mRNA is translated to yield VP2, VP3 and VP4 and segment B mRNA is translated to VP1 (the viral RdRP). VP1 is found in virions in both a "free" and a genome-linked form Figure (6). As a general the RNA virus particles assemble and accumulate in the cytoplasm but the mechanism of virus release is unknown (Delmas *et al.*, 2019).There is no information available about CPNV replication.

1.3.3. Epidemiological aspects and transmission

As said, the first description of a case of infectious proventriculitis was in 1978 in The Netherlands (Kouwenhoven *et al.*, 1978) and it was subsequently reported in the United States of America (Page *et al.*, 1980; Guy *et al.*, 2005, 2011b, 2015; Marusak *et al.*, 2012; Rute-Noiva *et al.*, 2015), Spain (Grau-Roma *et al.*, 2010), France (Marguerie *et al.*, 2011), Egypt (Kutkat *et al.*, 2010), Venezuela (Garcia *et al.*, 2012), South Korea (Kim *et al.*, 2015), Poland (Smilek *et al.*, 2017), China (Li *et al.*, 2018) and the UK (Grau-Roma *et al.*, 2017, 2020).

Most of the TVP outbreaks have been described in broilers (3-8-week-old), and more commonly in 4 to 5 week old chickens (Hafner and Guy, 2013). There is only one reported case of TVP in broiler breeder (9-20 weeks of age) and layer hens (14- 16 weeks of age) in the USA (Marusak *et al.*, 2012a). Although the definitive distribution and prevalence of TVP is not available and it is still too early to indicate the prevalence of infection with TVP, the frequency of outbreaks and viral detection by PCR in chicken according to the collected samples around the world is displayed in Figure 7.

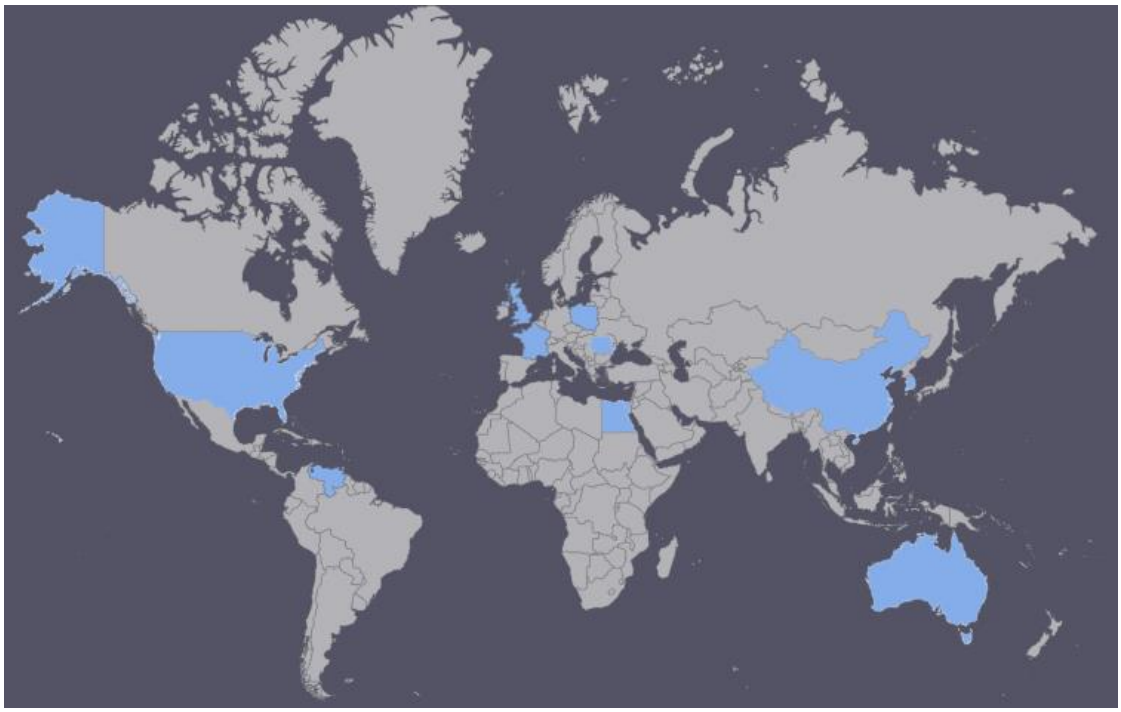

Figure 7. Map of the countries where TVP has been described.

The prevalence mean of infection is described to be around 2% - 5% and this percentage require reprocessing due to carcass contamination at slaughterhouses (Thayer and Walsh, 1993; Bayyari *et al.,* 1995), but the mortality of infected chicken is not increased (Reece, 2002).

There is no seasonality and the pattern of disease spread is not clear but most digestive viral diseases spread via fecal oral, and TVP was reproduced by oral inoculation of filtrate (0.2 um) homogenized infected proventriculi (Kouwenhoven *et al.,* 1978; Huff *et al.,* 2001; Guy *et al.,* 2007, 2011b; Grau-Roma *et al.,* 2010; Kutkat *et al.,* 2010). TVP has also been experimentally reproduced in chickens with CPNV administered by eye drop (Guy *et al.,* 2007, 2011b). There is no evidence of rodent or birds as a reservoir for the virus, but sanitation and clean feed and farm biosecurity and personal biosafety are important factors of any viral disease occurrence.

1.3.4. Clinical signs

In chickens the most frequently reported clinical signs are poor growth (Figure 8), impaired food digestion, poor feed conversion and pallor (Dormitorio *et al.,* 2007). The incubation period of TVP, based on experimental CPNV inoculation and detection of

gross and microscopic lesions, was determined to be 5-7 days, although no clinical were observed (Guy *et al.*, 2011a, 2011b).

In broiler breeders and layers, the few cases of TVP described until now, did not present any clinical sign (Marusak *et al.*, 2012b). Morbidity of TVP is unknown and there is no increase in mortality (Hafner and Guy, 2013). Some authors have described TVP and CPNV associated to Runting and Stunting Syndrome (RSS) or malabsorption syndrome, a poorly understood disease characterized by growth retardation, ruffled feathers and diarrhoea resulting in considerable economic losses. The etiology of this process is considered as multifactorial (Marguerie *et al.*, 2011; Noiva *et al.*, 2015).

Figure 8. Five -week-old chickens. TVP-affected chicken (on the right) shows marked growth retardation. Source: CESAC.

The incubation period for TVP was determined by detection of characteristic microscopic lesions 3-4 days post-inoculation with CPNV and gross lesions of paleness and enlargement of the proventriculus was seen at 7 days post-inoculation (Guy *et al.*, 2007, 2011b).

1.3.5. Gross and microscopic lesions

The most characteristic gross lesion is enlargement and/or dilation of the proventriculus (Figure 9), that may show pale, whitish areas on the serosal surface. The lobular pattern may be pronounced with pale polygonal areas representing affected glands seen from the serosal surface. The isthmus might be dilated and soft. Mucosal haemorrhages may be seen infrequently. No lesions are observed in other organs (Goodwin *et al.*, 1996; Hafner & Guy, 2013).

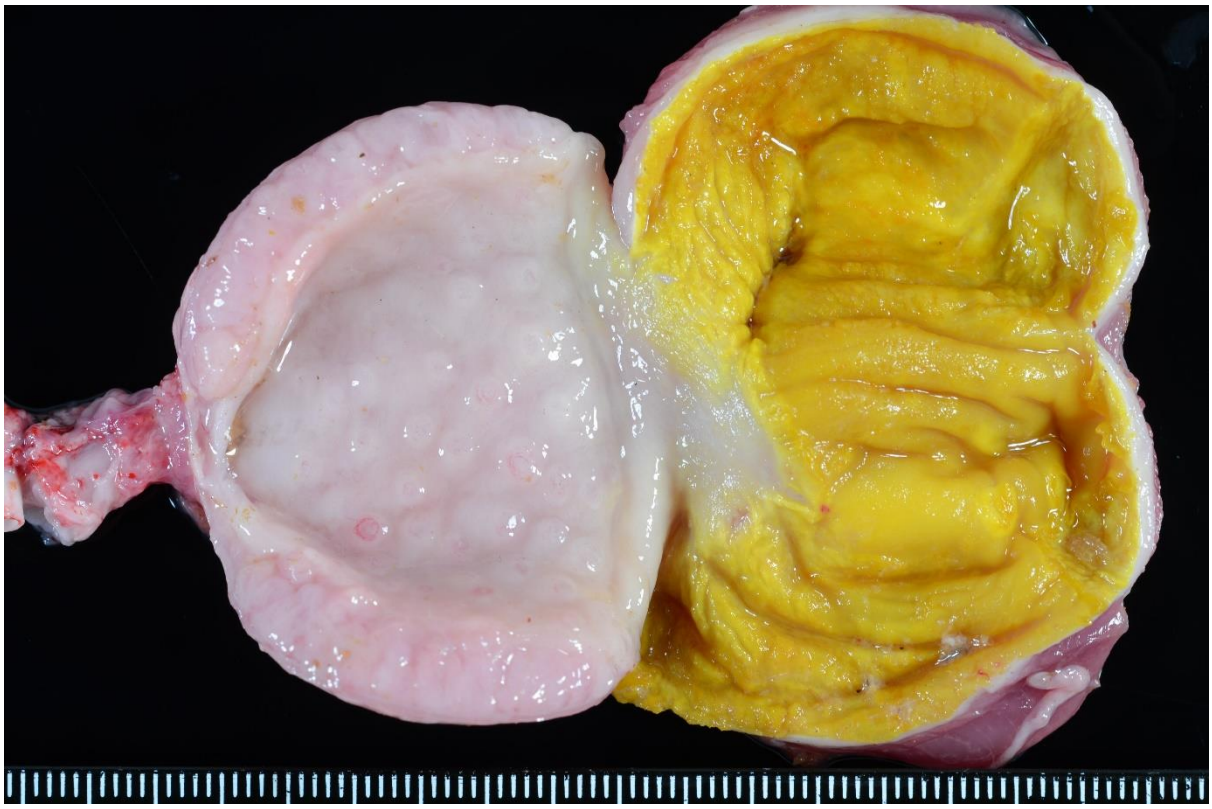

Figure 9. Increase of thickness of the proventricular wall in a broiler chicken with TVP.

Regarding microscopic lesions, Guy and co-workers (2011b) defined that the most characteristic lesions of TVP were: 1) necrosis of oxynticopeptic (proventricular gland) cells, 2) lymphocytic infiltrates in the glandular interstitium, 3) ductal epithelial degeneration and hyperplasia and 4) replacement of glandular epithelium by hyperplasic ductal epithelium. These lesions were also observed in broiler breeders and layer hens with TVP (Marusak *et al.*, 2012). It is worth to mention, that in several studies authors describe also intranuclear inclusion bodies in oxynticopeptic cells (Guy *et al.*, 2005, Grau-Roma *et al.*, 2010; 2020).

Experimental infection with CPNV demonstrated that acute oxynticopeptic cells necrosis is apparent at 5-7 days post infection, followed by lymphocytic, macrophagic and plasma cell infiltration in the gland stroma. At 14 days post infection, replacement of glandular epithelium by columnar duct-like epithelium is observed together with lymphocytic infiltration that may be severe. Up to 80% of the glandular epithelial cells might be destroyed in natural as well as experimental TVP cases (Hafner & Guy, 2013).

On the other hand, Grau-Roma *et al.* (2017) in a prospective study of TVP and CPNV presence in UK, described that TVP-compatible gross lesions could correspond microscopically to three histological lesions: (i) TVP-affected chickens: lymphocytic infiltration and glandular necrosis (ii) lymphocytic proventriculitis (LP)-affected chickens: lymphocytic infiltration without necrosis and (iii) without proventriculitis (WP): no lymphocytic infiltration or necrosis. Interestingly, CPNV genome was detected by PCR not only in the chickens showing necrosis and lymphocytic infiltration, but also in chickens showing only inflammation (LP-affected cases). Similar results were obtained when performing a retrospective study on UK samples (Grau-Roma *et al.*, 2010).

Regarding the inflammatory component of the lesion, Pantin-Jackwood *et al.* (2004) did a characterization of the lymphocytic infiltrates present in the proventriculus in PVT experimentally reproduced cases and found that most of the lymphocytes were CD3+ T cells, being most of these CD8+. B cells and Cd4+ cells were found in aggregates in chronic proventriculitis.

1.3.6. Diagnosis

As said previously, there are several infectious and non-infectious causes that have been associated with proventricular enlargement and proventriculitis. TVP diagnosis is therefore based on the concurrence of macroscopic, microscopic features and CPNV detection.

A reverse transcriptase polymerase chain reaction (RT-PCR) technique was described to specifically detect CPNV genome and targets a partial sequence of the segment B of the CPNV (VP1 gene) (Guy *et al.*, 2011b). This technique has been widely used to detect

CPNV in clinical cases of TVP in many countries (Marguerie *et al.*, 2011; Marusak *et al.*, 2012b; Hafner *et al.*, 2013; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017, 2020).

1.3.7. Differential diagnosis

Differential diagnoses for TVP include non-infectious proventriculitis caused by the ingestion of toxins such as biogenic amines (Huff *et al.*, 2001), and mycotoxins (Dorner *et al.*, 1983), which often contaminate poultry feed, A diet with high copper sulfate (Bayyari *et al.*, 1995b). Otherwise, Infectious proventriculitis caused by avian infectious agents can produce proventricular lesions including; Bacteria (Goodwin, 1995; Huff *et al.*, 2001; Guy *et al.*, 2005), Fungi (Schulze and Heidrich, 2002; Tomaszewski *et al.*, 2003), Yeast (Goodwin, 1995) and Viruses; Newcastle disease virus (NDV), Infectious bursal disease virus (IBDV) (Bayyari *et al.*, 1995a; Huff *et al.*, 2001; Pantin-jackwood *et al.*, 2003; Elkady *et al.*, 2009; Kutkat *et al.*, 2010), Infectious bronchitis or coronavirus (Zheng *et al.*, 2000; Kutkat *et al.*, 2010; Yu *et al.*, 2017), Stunting syndrome (Reovirus) (Page and Fletcher, 1980; Kouwenhoven *et al.*, 1988; Apple *et al.*, 1991; Awandkar *et al.*, 2017), Adeno like virus AdLV (Guy *et al.*, 2005, 2007) and Adenovirus (Kouwenhoven *et al.*, 1978; Lenz *et al.*, 1998) were suspected as a causative agent of TVP.

1.3.8. Viral isolation

Isolation of CPNV by cell culture procedures has only been successful by inoculation of embryonating chicken eggs with suspensions of proventricular tissues from suspect TVP-affected chickens (Guy *et al.*, 2005). Proventriculi are homogenized in minimal essential medium (MEM), clarified by centrifugation, and filtered through a 0.45μm filter. Embryonating chicken eggs (day 15 of embryonation) are inoculated by the amniotic cavity route and returned to the incubator, and eggs are allowed to hatch. CPNV is recovered in proventriculi of hatchlings and has determined by RT-PCR.

1.3.9. Control and prevention

There are no specific control measures or treatments are currently recommended for TVP (Hafner & Guy, 2013). The general prevention is by application measures of sanitation and biosecurity of cleaning and prevention of diseases. There are no specific prevention and control measures for TVP. The CPNV (Birnaviridae) is a non-enveloped virus, it is known to be resistant to many physical and chemical agents. In fact, Birnaviruses resistant to heat (less than 60ºC), chloroform, ether, extreme pH (3-12) and some phenol components (Delmas *et al.*, 2019). Thus, the virus exists for a long time in poultry barns (Alexander *et al.*, 1998). However, the Birnavirus is sensitive to sodium hydroxide, the iodinated and chlorinated derivatives disinfectants, as well as the aldehydes (formaldehyde, glutaraldehyde) are also active (Berg *et al.*, 2000). The infectivity of the virus is markedly reduced by formaldehyde which is acting on proteins by denaturation and on nucleic acids by alkylation (Maris *et al.*, 1995). Although vaccination is the best option for viral diseases control, implementation of proper biosecurity and sanitation measures are very important to avoid the introduction pathogen into poultry premises (Cardona, 2012). The biosecurity measures included but not limited to, clean and disinfect appropriately poultry houses and equipment, strict control access of vehicles, equipment's, and people, onto the farm and most importantly a comprehensive biosecurity program. Biosecurity is the single most important investment in disease prevention that a farmer, a company, or a country can make (Cardona, 2012).

Chapter 2

Hypothesis and objectives

TVP is a disease described since many decades in different countries. In 2011, a novel birnavirus named CPNV, was detected in chickens with TVP and, since then, it has been described associated to TVP clinical cases around the world. In spite of this, CPNV isolation in cell culture has not been successful so far and the final cause of TVP is still under debate. Therefore, investigations on the new development of diagnostic methods, epidemiology and pathogenesis are fields of major interest for this emerging virus.

The hypothesis of the present thesis is that CPNV might found associated to TVP in Spanish poultry farms and is the main cause of this pathological process in studied TVP clinical cases.

The main specific objectives of the present thesis were:

1- To study the presence of CPNV associated to TVP in Spanish poultry farms by a retrospective study on TVP clinical cases (*Chapter 3*).
2- To assess the presence of CPNV in TVP paraffin-embedded proventriculi by an *in situ* hybridization technique (*Chapter 3*).
3- To analyze the ultrastructural changes associated to CPNV infection in proventriculi from TVP clinical cases (*Chapter 4*).
4- To detect and characterize CPNV using next generation sequencing (NGS) in proventriculi from TVP clinical cases (*Chapter 5*).

Chapter 3

Study 1: Retrospective study on Transmissible Viral Proventriculitis in Spanish poultry farms

(manuscript submitted)

3.1. Introduction

Transmissible Viral Proventriculitis (TVP) is an emergent viral infectious disease that affects mainly broiler chickens. It is characterized by impaired feed digestion, poor growth and poor feed conversion rate (FCR). Economic losses associated to TVP happen owing to poor FCR (Kouwenhoven *et al.*, 1978; Bayyari *et al.*, 1995a; Goodwin, 1995; Goodwin *et al.*, 1996; Huff *et al.*, 2001; Reece, 2002; Pantin-jackwood *et al.*, 2003; Pantin-Jackwood *et al.*, 2005; Guy *et al.*, 2005; Marguerie *et al.*, 2011; Grau-Roma *et al.*, 2017, 2020), but affected proventriculus may also rupture during mechanical evisceration in the processing plant, resulting in carcass contamination and condemnation (Thayer and Walsh, 1993; Goodwin *et al.*, 1996; Dormitorio *et al.*, 2007). At necropsy, enlargement, thickening, fragility and paleness of the proventriculus, together with weakness and dilation of gastric isthmus is observed in TVP cases. Multifocal haemorrhages in the proventricular mucosa might also be noted (Guy *et al.*, 2007). Although proventricular gross lesions could be indicative of TVP, they are not specific. The disease is characterized by its histologic lesions: necrosis of oxynticopeptic cells, inflammation with a predominance of lymphocytes, and replacement of glandular epithelium by hyperplasic ductal epithelium (Hafner *et al.*, 1995; Goodwin *et al.*, 1996; Huff *et al.*, 2001; Pantin *et al.*, 2005; Guy *et al.*, 2005, 2007, 2011b; Dormitorio *et al.*, 2007; Margueri *et al.*, 2011; Coman *et al.*, 2011; Marusak *et al.*, 2012; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2010, 2017, 2020).

The etiology of TVP identified to date is a new species of birnavirus, called Chicken proventricular necrosis virus (CPNV) (Guy *et al.*, 2011b, 2011a; Marguerie *et al.*, 2011; Marusak *et al.*, 2012; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017, 2020). CPNV was first associated to TVP based on isolation from proventriculi of TVP-affected chickens. CPNV was identified as birnavirus based on physical characterisation (non-enveloped, 70 nm, icosahedral, bi-segmented, double-stranded RNA) and the partial genome sequence analysis (Guy *et al.*, 2011a).

Recent studies, have confirmed the association between CPNV and TPV in several countries, such as USA, France and most recently UK (Guy *et al.*, 2011b, 2011a; Marguerie *et al.*, 2011; Grau-Roma *et al.*, 2017, 2020), but the number of studied cases

is still scarce and there is little information available about the epidemiology and pathogenesis of this disease.

Although the occurrence of TVP caused by CPNV was suspected in Spanish poultry farms several years ago (Grau-Roma *et al.,* 2010), studies to confirm the presence and importance of this new viral agent have not been carried out. In order to further characterize this disease, a retrospective study of proventriculitis cases submitted to the Veterinary Pathology Service of the *Universitat Autònoma de Barcelona* (UAB, Barcelona, Spain) was performed. In addition, a new method of detection of the viral genome in tissues, by means of an *in situ* hybridization (ISH) technique, was developed with the aim to further characterize this infection.

3.2. Materials and methods

3.2.1. Samples

The retrospective study was performed on the database of cases received from January 1998 to April 2019 at the Veterinary Pathology Service of the UAB. All cases diagnosed as "proventriculitis" on the clinical history or on the morphologic diagnosis were selected and classified as suspected TVP cases.

3.2.2. Histological scoring

The following histopathological parameters characteristic of TVP (12, 13) were re-evaluated in the selected samples: 1) necrosis of oxynticopeptic cells, 2) glandular interstitial lymphocytic inflammation and 3) replacement of glandular epithelium by hyperplasic ductal epithelium (ductal epithelial metaplasia). A semi-quantitative scoring similar to that reported by Grau *et al.* (2017) was used as follows for each of the above mentioned parameters: negative - (none of the glands affected), mild + (>0 to 10% of the glands affected), moderate ++ (>10 to 50% of the glands affected), severe +++ (>50% of the glands affected).

According to the histological results, chickens were divided into two case status: 1) Transmissible Viral Proventriculitis (TVP)-affected chicken: lymphocytic infiltration and

necrosis present in proventriculus; 2) Lymphocytic Proventriculitis (LP)-affected chicken: lymphocytic infiltration without necrosis present in the proventriculus.

3.2.3. RNA extraction and RT-PCR for CPNV

Viral RNA was extracted using RNA extraction kit (Qiagen RNeasy® FFPE kit) from all formalin-fixed paraffin embedded (FFPE) proventriculi samples and all samples were tested by RT-PCR for CPNV. Five sections of 25 micrometre (μm) were used to extract viral RNA from each sample following the manufacturer's instructions (RNeasy FFPE Kit, Qiagen, CA, USA). Briefly, the samples were incubated 5 minutes with Xylol (twice) in an Eppendorf tube followed by centrifugation. Pellet re-suspension was performed with ethanol 100% and centrifuged (3 times). The sample was left to dry at room temperature (RT) for 40 minutes. Then, Proteinase K (Roche, Mannheim, Germany) and PKD buffer were used as a digestion buffer. The samples were incubated in a thermal block at 56° C and 80° C for 15 minutes each consecutively and then cooled in freezer for 3 minutes before being centrifuged for 15 minutes. The supernatant was transferred to a new tube and mixed with DNase booster buffer and DNase I stock solution and centrifuged briefly and incubated at RT for 15 min. The binding condition was adjusted by adding RBC buffer and mixed with absolute ethanol. The solution was then transferred to the RNeasy mini elute spin column and centrifuged. The RPE buffer was added to the column and centrifuged. The column was inserted into a new tube and 12 ul of RNase-free water was added to elute the RNA by centrifugation 1 min. The extracted RNA was stored at -80° C until use.

The RT-PCR protocol to amplify a 171 nucleotide fragment of the VP1 gene of CPNV was performed using designed primers B2F 5'-GGGCGTAACCATTCAGATA-3' and B2R 5'-CGTAGACCTCGTCCTTCTGC-3' and RT-PCR protocols described previously (Guy *et al.*, 2011b) and by using a commercially available reverse transcriptase reaction kit (Promega Corp., Madison, USA). A UK positive CPNV (case B15-124-2, Grau-Roma *et al.*, 2017) was used as positive control. Positive and negative controls of extraction and amplification were added to each batch of samples tested.

3.2.4. Sequencing of RT-PCR products and phylogenetic studies

The amplified products from the positive CPNV RT-PCR cases were purified using Mini Elute Gel Extraction Kit (Qiagen, CA, USA). Sequencing reactions were performed with

BigDye Terminator Cycle Sequencing v.3.1 Ready Reaction (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The sequencing reactions were analyzed using an ABI PRISM 3730 Genetic Analyzer (Applied Biosystem® Foster City, CA, USA) following the manufacturer's protocol. This Sanger sequence verification was done at the Genomic and Bioinformatics Service of the UAB.

Partial VP1 CPNV sequences obtained from positive samples were compared with the sequences available in the GenBank. Sequences and chromatograms were manually explored to trim bad-quality bases with BioEdit 7.2 software (Hall, 1999). All sequences were assembled, analyzed, and the phylogenetical tree was generated using MEGA X (Molecular Evolutionary Genetics Analysis version X) software (Kumar *et al.*, 2018) with the Maximum-Likelihood (ML) method using the JTT substitution matrix and a gamma distribution of rate variation between sites. Robustness of the ML tree was evaluated by analysis with 100 bootstrap replicates. The partial VP1 CPNV sequences reported in this work have been deposited at the GenBank under accession numbers MF288606 to MF288615.

3.2.5. ISH technique

To perform the ISH test, we first synthesized a CPNV Digoxigenin-labelled probe using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) following manufacturer's instruction with minor modifications.

For the ISH test only the animal showing the most severe lesion per case was assessed and positive ISH Porcine circovirus type 2 (PCV2) sample number 79/09 D and its specific designed probe was used as positive sample (Baró *et al.*, 2015). Case #8 of the present study positive by RT-PCR to CPNV and case #41 negative by RT-PCR to CPNV were also used as positive and negative cases, respectively. Four μm-sections of each sample were placed on positively charged slides (SuperFrost plus, Braunschweig, Germany) and left overnight to dry. Samples were then deparaffinised with xylene and rehydrated by transfer to a series of aqueous ethanol solutions of 70%, 90%, 100% and then H_2O and PBS for 5 min each. The samples were kept in humid boxes during the process and were treated with a solution containing 3% pepsin in buffer (pH2) for 10 minutes at 37 °C and then 8 minutes at 90 °C. Afterwards, they were washed with PBS thoroughly and 100% Formamide was added for 5 minutes at 90° C. The previously

prepared CPNV- labelled probe was diluted 1:10 in hybridisation solution and preheated for 10 minutes at 90° C. Samples were then incubated at 90 °C for 5 minutes with the probe mixture before an overnight incubation at 45 ºC. After incubation, samples were washed with 0.5 standard sodium citrate (SSC) buffer for 5 minutes at RT, then PBS buffer for 5 minutes and another 5 minutes in a Standard Sodium Citrate (SSC) 0.25 at 37° C. Finally, they were washed in buffer 1 at RT. The polyclonal Anti- Digoxigenin-Alkaline Phosphatase antibody was diluted with buffer 1 (1:500) and dropped on samples and incubated for 1 hr at 37 °C. The samples were dried and washed 3 times with Buffer 1 (pH7.5) and PBS followed by Buffer 2. The chromogen solution NBT/BCIP stock solution was diluted (1:50) with buffer 2 (pH 9.5) and covered the sample and incubated at 37° C for 50 minutes in dark conditions. Samples were then immersed in Buffer 3 (pH 8) and finally a counterstaining with Fast Green for 10 minutes was performed. The slides were rehydrated and mounted by DPX and cover slipped followed by examination with a Nikon Eclipse 80i microscope with Nikon digital camera DS-Qi1Mc.

3.3. Results

3.3.1. Epidemiological and gross examination data

A total of 160 proventriculi from 42 clinical cases fulfilled the histopathological criteria and were diagnosed as TVP cases between January 1998 to April 2019 (Table 1). All samples corresponded to broiler chickens between 7-52 days, except one case that corresponded to slow-growing chickens of 70 days of age (case #20). The first case was diagnosed in 1999 and the latest in 2016. The year with the highest frequency of cases (26%) was 2011. Samples were collected from different geographical areas in Spain. The percentage of of positive TVP cases in our studied samples was 17%.

Regarding clinical signs, 60% of the cases reported with poor growth or lack of uniformity, anorexia and increased mortality. In 30% of the cases there was concurrent bursal atrophy and in 26% of the cases hepatitis was diagnosed together with the proventriculitis.

Table 1. Epidemiological and clinico-pathological data of clinical cases diagnosed as TVP from 1998 to 2019. All cases were submitted to the Veterinary Pathology Service of the UAB from January 1998 to April 2019. NA: non-available.

Clinical Case	Internal Code	Date	Origin	Age (days)	Clinical case history	Concurrent lesions and/or diseases
#1	N-548/99	17/06/1999	Lleida	39	High mortality at the end of the fattening period	Pneumonia, airsacculitis, and tracheitis, thymus and bursal atrophy, mild intestinal coccidiosis
#2	B-1567/99	11/06/1999	Lleida	37	Proventriculitis	Bursal atrophy
#3	B-683/00	17/02/2000	Lleida	NA	Proventriculitis	Bursal atrophy, splenic hyperplasia
#4	B-684/00	17/02/2000	Lleida	35	Proventriculitis	Bursal atrophy, splenic hyperplasia, non-specific hepatitis
#5	B-685/00	17/02/2000	Lleida	NA	Proventriculitis	Bursal atrophy, non-specific hepatitis
#6	B-198/01	15/01/2001	Barcelona	18	Suspected toxicity	None
#7	B-600/03	04/03/2003	Madrid	7	Nervous signs	Encephalomalacia
#8	N-573/04	22/10/2004	Barcelona	25	Apathy, hemorrhagic bursitis	Infectious bursal disease
#9	B-0263/05	01/02/2005	Madrid	45	Lesions in crop, proventriculus, and gizzard. Fungal infection suspected.	Gizzard erosions
#10	B-284/05	02/02/2005	Madrid	29	Depression, high mortality and hemorrhages in thighs and bursas	Infectious bursal disease
#11	B-1009/05	19/04/2005	Girona	20	Suspected Marek's or Leukosis	Bursal atrophy
#12	B-1095/05	27/04/2005	Girona	10	Suspected Marek's or Leukosis	Bursal atrophy

#13	B-1125/05	28/04/2005	Girona	28	Stunting chicken	Bursal atrophy, tubulointerstitial nephritis
#14	N-295/06	23/05/2006	Catalunya	27	Crop dilatation	Crop dilatation, fungal granuloma
#15	B-1259/06	03/11/2006	Galicia	42	Severe digestion problem	None
#16	N-489/09	12/11/2009	Valencia	27	Enteritis and suspected mycotoxins	Suppurative bursitis, ulcer in gizzard and infectious bronchitis
#17	N-505/09	20/11/2009	NA	26	Lameness	Necrotizing hepatitis, pericarditis, bursal atrophy
#18	N-85/10	09/02/2010	Lleida	23	Respiratory signs, enteritis, and stunting	Infectious bronchitis, bursal atrophy
#19	B-266/10	08/03/2010	Galicia	NA	Suspected Infectious bursal disease	Mild bursal atrophy, tubulointerstitial nephritis
#20	B-285/10	12/03/2010	Barcelona	70	Haemorrhage in skin and proventriculus. Suspected Marek's	Encephalitis, skin hemorrhages
#21	N-566/10	24/11/2010	Barcelona	30	Stunting	Necrotizing hepatitis, catarrhal enteritis, mild bursal atrophy
#22	B-176/11	10/02/2011	Barcelona	40	Stunted low growth and lack of uniformity	None
#23	B-232/11	25/02/2011	Barcelona	28	Low growth, stunting, 5% mortality,	Inclusion body hepatitis, bursal atrophy
#24	N-87/11	01/03/2011	Barcelona	52	Increased mortality	Septicemic colibacillosis, splenic follicular hyalinosis, necrotizing hepatitis, pneumonia, catarrhal tracheitis, bursal atrophy
#25	B-776/11	08/08/2011	Barcelona	40	White dots on liver, bursal atrophy	Chronic hepatitis, bursal atrophy

#26	B-961/11	13/10/2011	Barcelona	32	Stunted low growth and lack of uniformity, coccidiosis	Multifocal necrotizing hepatitis
#27	B-1069/11	11/11/2011	Murcia	45	Suspected Infectious bronchitis	Necrotic ventriculitis
#28	N-562/11	18/11/2011	Murcia	18	Mortality and poor flock uniformity	None
#29	B-1106/11	21/11/2011	Barcelona	NA	Proventriculitis	None
#30	B-1170/11	14/12/2011	Barcelona	44	High mortality	None
#31	B-1212/11	23/12/2011	Barcelona	NA	Poor flock uniformity and mortality	None
#32	B-1214/11	27/12/2011	Murcia	15	Anorexia, enteritis	None
#33	B-428/12	02/05/2012	Girona	28	Mortality 3% and poor flock uniformity	None
#34	B-681/12	09/07/2012	Salamanca	NA	NA	Splenic follicular hyalinosis, catarrhal tracheitis.
#35	N-69/13	21/02/2013	Palma de Mallorca	30	Mortality, increase FCR, accumulation together in groups, bad feather shape	Catarrhal enteritis
#36	B-5/14	07/01/2014	Barcelona	24	Mortality 1.5% and anorexia	Necrotizing hepatitis
#37	N-8/14	08/01/2014	Barcelona	20	Poor growth and anorexia	None
#38	B-101/14	06/02/2014	Barcelona	34	Chick slow growth, colibacillosis	None
#39	B-135/14	18/02/2014	Barcelona	18	NA	Hepatitis
#40	B-173/14	05/03/2014	Barcelona	19	NA	Hepatitis
#41	B-174/14	05/03/2014	Barcelona	22	NA	Hepatitis
#42	B-1245/16	15/12/2016	Zaragoza	35-49	Proventriculitis	None

3.3.2. Pathological findings and ISH

Proventricular gross and histopathological lesions are shown in Table 2. Regarding gross lesions, they were observed or reported in 25 cases (60%), while 17 cases (40%) showed no macroscopic proventricular changes or they were not described. The most commonly reported lesion was thickening and/or enlargement of the proventriculus (Figure 10) (22 cases; 88%) followed or concomitantly with mucosal haemorrhages (Figure 11) (6 cases; 24%), ulceration and presence of subserosal white spots and irregular mucosal surface were also noted (each being observed in 1 case; 4%).

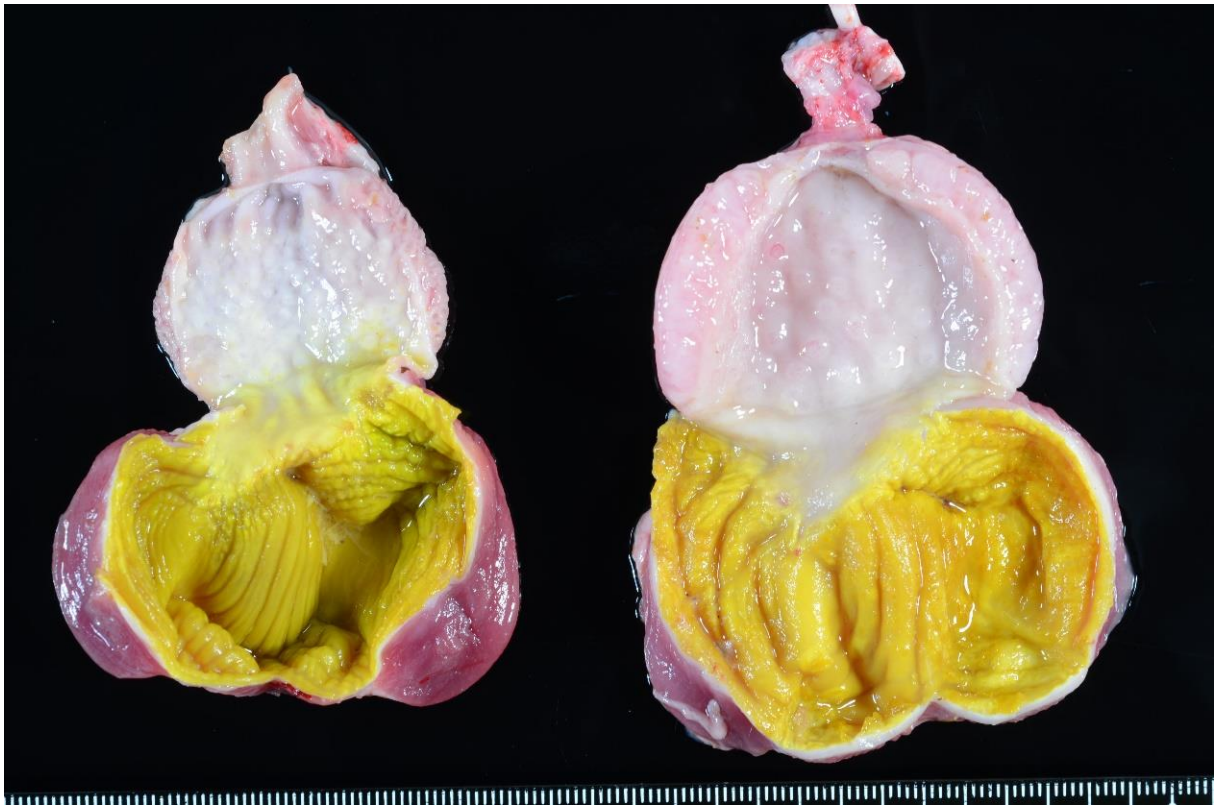

Figure 10. Proventricular thickening and enlargement in a TVP clinical case (case #35), compared to a normal proventriculus (left).

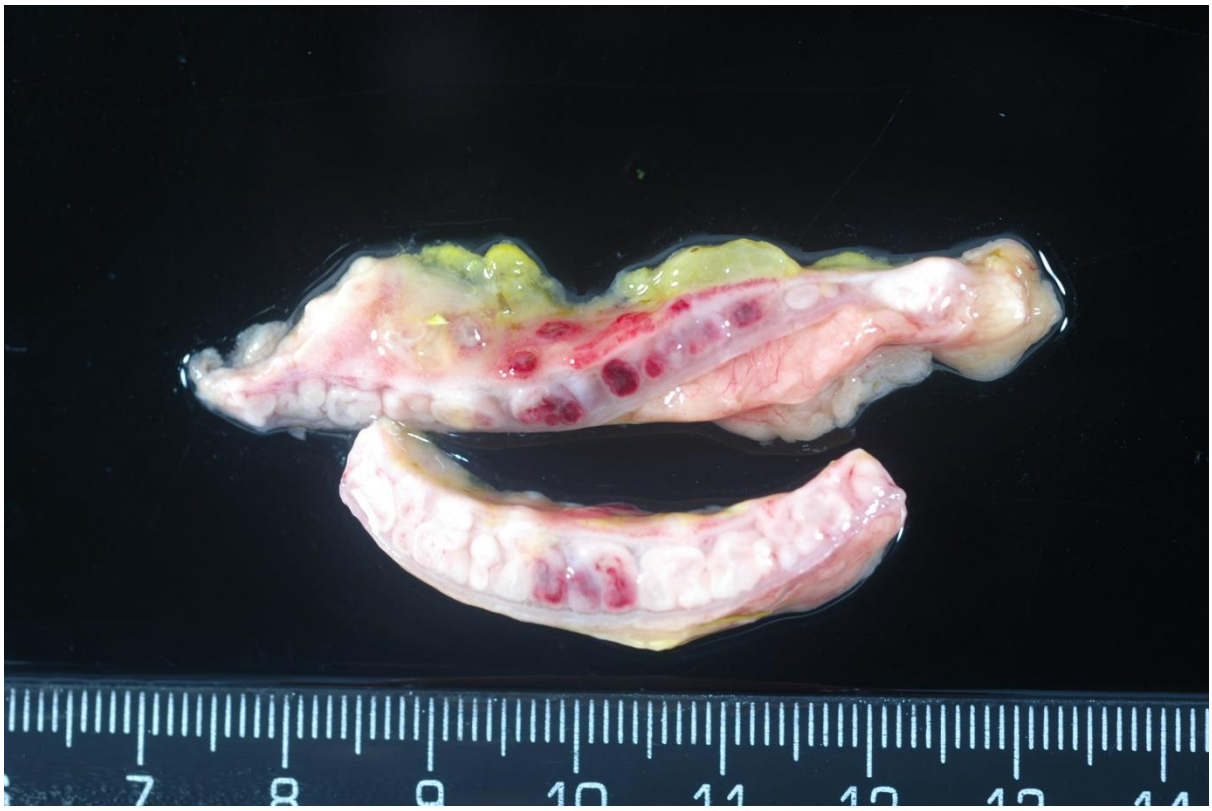

Figure 11. Haemorrhage in the proventricular mucosa in a TVP clinical case (case #24).

Histologically, out of the 42 cases studied, a total of 28 cases (66%) were classified as TVP-affected chickens and 14 cases (33%) as LP-affected chickens. When there was more than one proventriculi assessed per case, the one showing the most severe microscopic lesions was choosen to be shown as representative (Table 2) and to perform the ISH technique.

All the TVP-affected chicken showed mild to moderate necrosis of proventricular epithelium (oxynticopeptic) cells of one or more proventricular glands (Figure 12). The necrotic sloughed cells were usually seen as small clusters within the lumen of the proventricular gland (Figure 13). Lymphocytic infiltration of TVP cases was severe in 7 cases (25%), moderate and mild scores were observed in 19 cases (76%) and no inflammation was observed in 1 case (8%). Mild to moderate hyperplasia or replacement of oxynticopeptic cells by columnar ductal epithelial cells was observed in 21 TVP cases (84%).

In the LP cases (Figure 14), one sample showed severe inflammation (7%) and the rest were moderate and mild scores (93%). Lymphocytic infiltration was usually multifocal

and, in some cases, lymphocytic cells formed nodular aggregates located within the interstitium of the proventricular glands. Moderate and mild ductal hyperplasia was observed in 11 LP cases (78%) and three cases without changes in of ductal hyperplasia epithelial cells (22%).

Table 2. Results of clinical cases comprising gross and microscopic data, ISH and RT-PCR techniques. Note; TVP (Transmissible Viral proventriculitis), LP (lymphocytic proventriculitis), Histopathological scoring system: negative - (none of the glands affected), mild + (>0 to 10% of the glands affected), moderate ++ (>10 to 50% of the glands affected), severe +++ (>50% of the glands affected).

Case	**Proventricular macro lesions**	**Proventricular micro lesions**			**PCR**	**ISH**	**Case status**	**GenBank Accession number***
		Inflammation	**Necrosis**	**Metaplasia**				
#1	Enlargement	+++	-	++	-	-	LP	
#2	None	+++	+	++	+	+	TVP	**MF288606**
#3	Enlargement	+++	+	+	+	+	TVP	**MF288607**
#4	Enlargement	+++	+	++	+	+	TVP	**MF288608**
#5	Enlargement	+++	+	++	+	+	TVP	**MF288609 MF288610**
#6	Haemorrhage	+	+	-	-	-	TVP	
#7	None	-	+	+	-	-	TVP	
#8	Enlargement/ haemorrhage	+	+	+	+	+	TVP	**MF288611 MF288612 MF288613**
#9	Enlargement	++	-	+	-	-	LP	
#10	None	+	+	++	-	-	TVP	
#11	None	+++	+	++	-	-	TVP	
#12	None	+	++	+	-	-	TVP	
#13	Haemorrhage	+	+	+	-	-	TVP	

#14	None	+++	+	++	-	-	TVP	
#15	Enlargement	+++	-	++	-	-	LP	
#16	Enlargement	+	+	-	-	-	TVP	
#17	Ulcer	++	+	++	-	-	TVP	
#18	None	++	+	++	-	-	TVP	
#19	None	++	+	++	-	-	TVP	
#20	Enlargement	++	-	++	-	-	LP	
#21	Enlargement	++	+	++	-	-	TVP	
#22	None	++	+	-	+	+	TVP	**MF288614**
#23	Enlargement/ haemorrhage	++	-	+	-	-	LP	
#24	Enlargement/ haemorrhage	+	++	++	-	-	TVP	
#25	Enlargement	+	-	-	-	-	LP	
#26	None	++	-	++	-	-	LP	
#27	Enlargement/ haemorrhage	++	+	+	-	-	TVP	
#28	Enlargement	+++	+	+	+	+	TVP	**MF288615**
#29	Enlargement	+	+	-	-	-	TVP	
#30	None	++	-	+	-	-	LP	
#31	Enlargement	++	-	-	-	-	LP	
#32	Enlargement	+	+	+	-	-	TVP	
#33	Enlargement/ haemorrhage	++	+	-	-	-	TVP	
#34	None	+	-	-	-	-	LP	
#35	Enlargement	++	-	+	-	-	LP	
#36	Enlargement	++	+	++	-	-	TVP	
#37	Enlargement	++	+	++	-	-	TVP	
#38	None	+	++	-	-	-	TVP	
#39	None	-	++	-	-	-	TVP	

#40	None	++	-	+	-	-	LP
#41	None	++	+	++	-	-	TVP
#42	None	++	-	+	-	-	LP

(*: some clinical cases had more than one proventriculi positive to CPNV RT-PCR, and more than one accession number is shown).

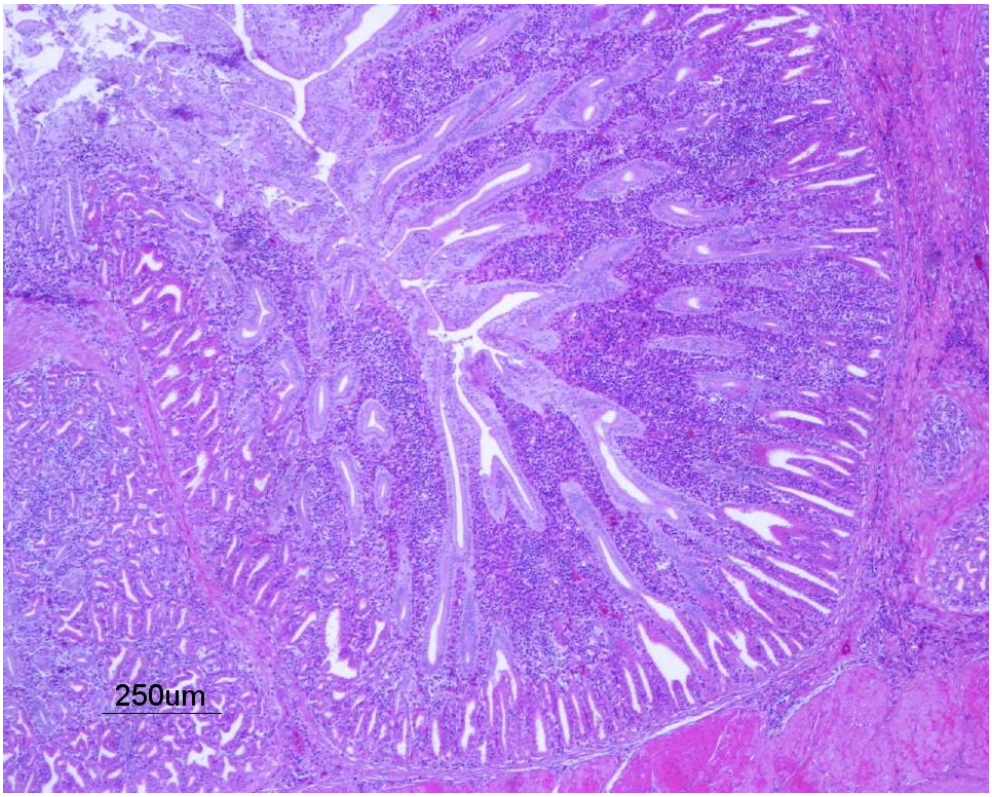

Figure 12. Histopathological changes in proventriculus with TVP (Transmissible Viral proventriculitis) case #5 (N-685/00) showing the three characteristic lesions of lymphocytic inflammation, metaplasia of ductal epithelial cells and necrosis of glandular epithelial cell (oxynticopeptic) cells of one or more proventricular glands, these cells mainly secretes pepsin and hydrochloric acid.

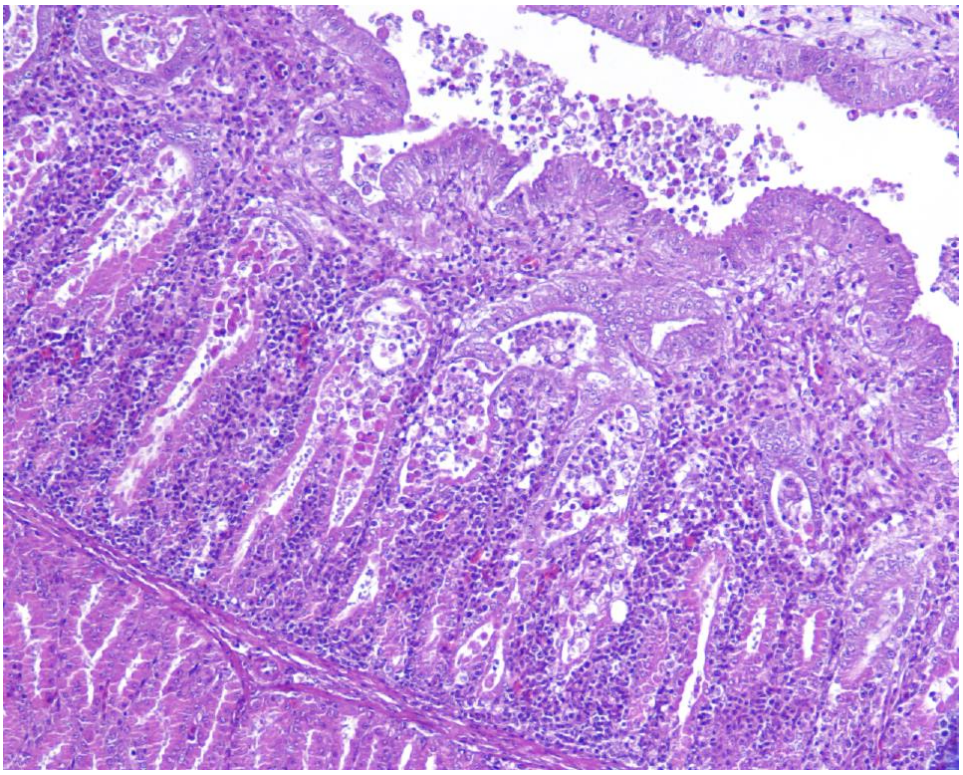

Figure 13. Histopathological changes in proventriculus with TVP (Transmissible Viral proventriculitis) case #5 (N-685/00) showing necrosis of oxynticopeptic cells. Necrotic cells are sloughed off in the lumen of the gland.

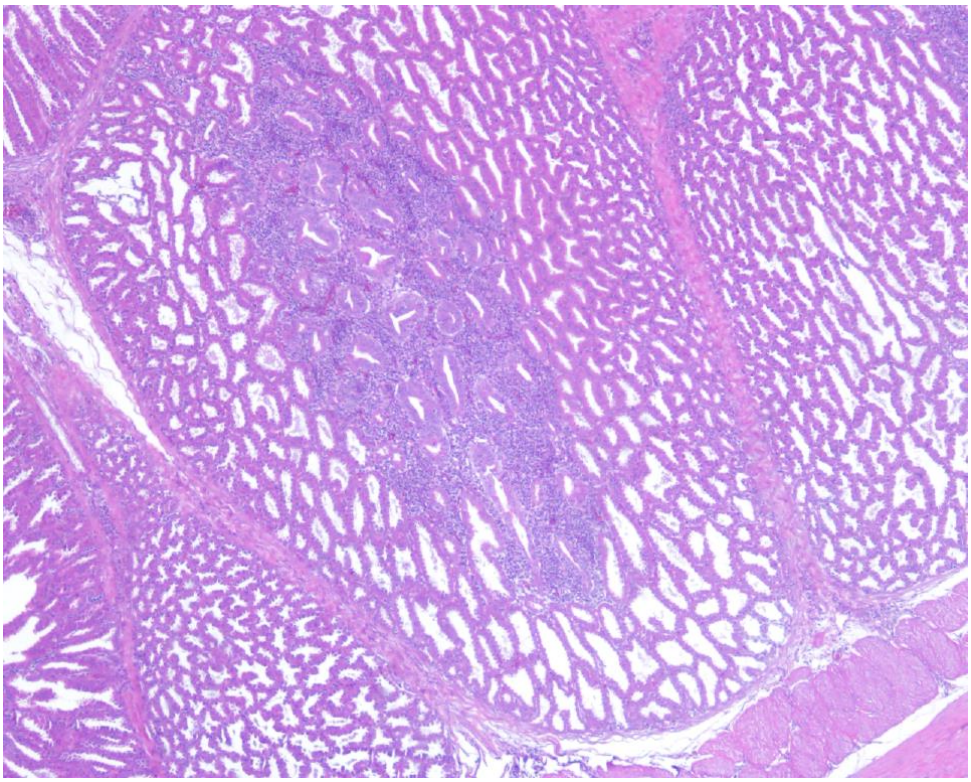

Figure 14. Histopathological changes in proventriculus with LP (lymphocytic proventriculitis) cases status, LP characterised by lymphocytic infiltration and hyperplasia of epithelial columnar ductal cells.

Regarding the ISH technique, we were able to produce specific PCR- Digoxigenin probe for CPNV from the CPNV PCR products. The probe length was 171 nucleotides conjugated with digoxigenin (Figure 15).

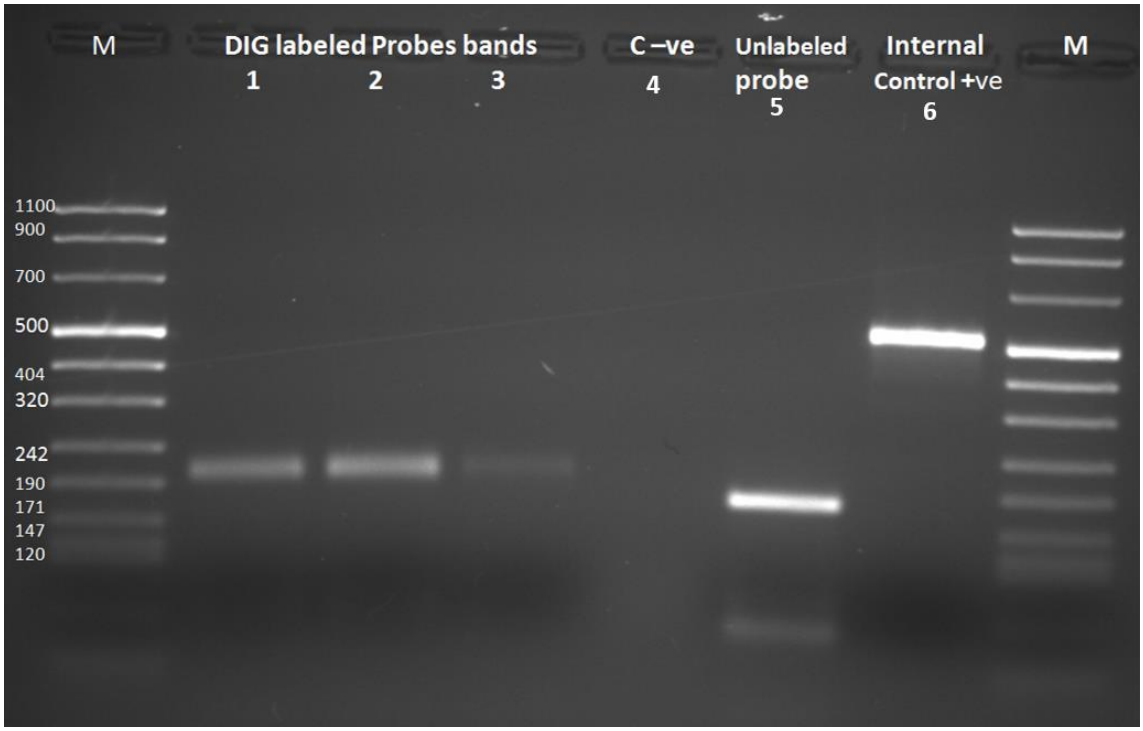

Figure 15. Electrophoresis gel showing the PCR probe digoxigenin-labeled (lane 1-3) and the unlabeled probe (lane 5).

Following the ISH technique, positive staining was observed in the nucleus of oxynticopepetic cells in 10 proventriculi belonging to seven clinical cases (Table 2). Positivity was only observed in areas where oxynticopeptic cell necrosis was evident (Figure 16).

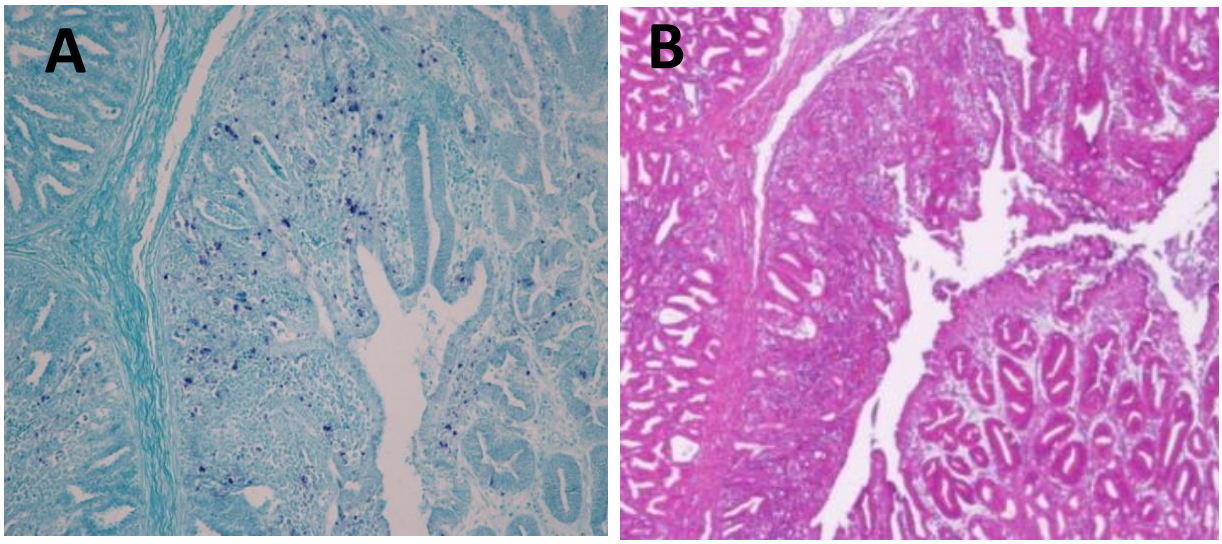

Figure 16. Proventricular glands of TVP by H/E technique (left) comparing to ISH technique (right and down) case #28 which showed the presence of viral genome in the necrotic are of infected TVP positive CPNV RT-PCR chicken (blue spots).

3.3.3. CPNV RT-PCR and phylogenetic studies

Ten broiler chickens belonging to seven clinical cases resulted positive for CPNV RT-PCR in the proventriculus (Table 2). All the cases corresponded to TVP-affected chickens and were positive by ISH to CPNV genome detection. The first CPNV RT- PCR positive sample dated back to 1999 (case #2) with accession number MF288606. It is important to mention that in five out of the seven positive cases macroscopic lesions, mainly enlargement, were seen and all positive RT-PCR cases showed severe inflammation, mild necrosis and mild to moderate tubular metaplasia at the microscopic examination.

Partial sequences were obtained from the ten CPNV RT-PCR positive samples that comprised part of the VP1 gene including a fragment of 171 nucleotides (n) (1080 to 1251 nucleotide positions including the forward and reverse primers). The phylogenetic analysis comparing the available CPNV sequences in the Genbank (UK sequences and the USA reference sequence) showed a 97-99% of identity among the Spanish strains and also between the Spanish strains and other already published CPNV sequences. The Spanish sequences formed a cluster and were closer to the USA reference sequence than

the UK sequences (Figure 17). Within the group of the Spanish sequences, the three sequences that came fom the same clinical case (MF288611-MF288613) were identical. A temporal distribution was observed within the Spanish sequences; the ones from earlier cases clustered together, while the two last cases formed a separate branch with the USA reference sequence.

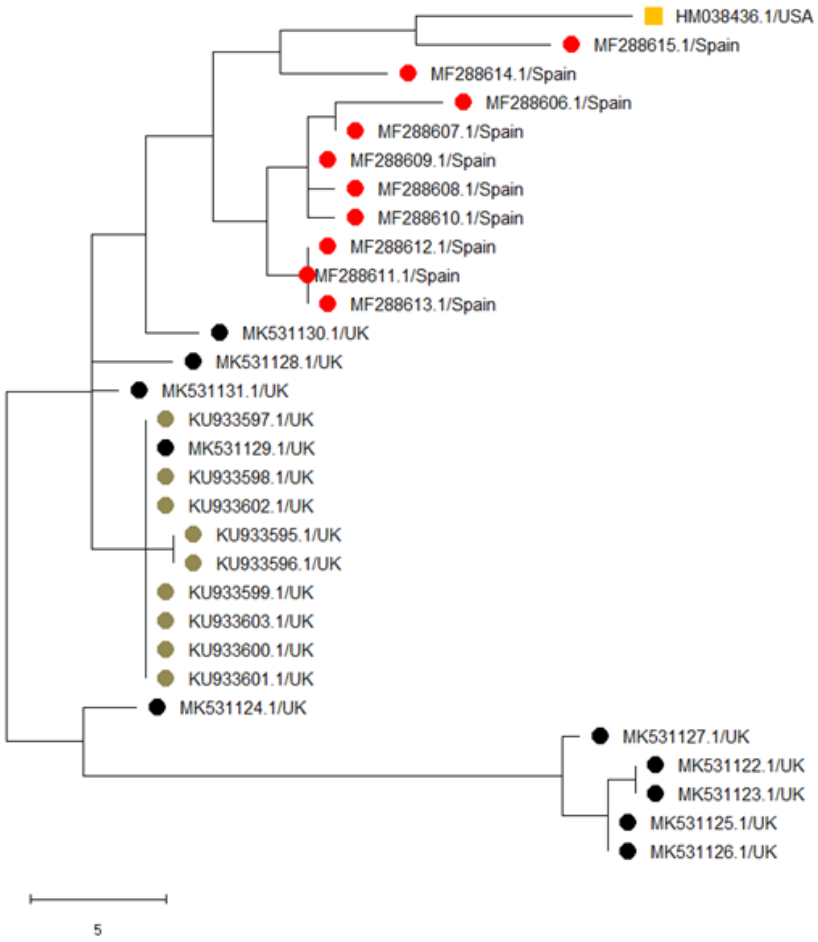

Figure 17. Phylogenetic tree of the partial genomes of the VP1 gene of CPNV Spanish strains and CPNV freely available sequences at GenBank. The brown and black circles indicate a previous CPNV from UK sequence reported by Grau-Roma *et al.* 2017 and 2020, and the red circles indicate the CPNV Spanish samples obtained in this study compared to the reference sequence HM038436.1/USA (yellow square).

When comparing CPNV sequences with other sequences from the four different genera of the Birnaviridae family, all CPNV sequences clustered together forming a unique branch, while the other genus (Avibirnavirus, Aquabirnavirus, Blosnabirnavirus and

Entomobirnavirus) form another branch (Figure 18). The overall average similarity of Spanish partial VP1 genome fragment of 171 n was 97, while the overall average similarity with USA reference was 95%. Fortunately, these nucleotide differences occurred in the middle of the VP1 171 n fragment and not in the primer's location, while comparing to the family the evolutionary distances was 40% divergent using the p-distance method (Kumar and Nei, 2001).

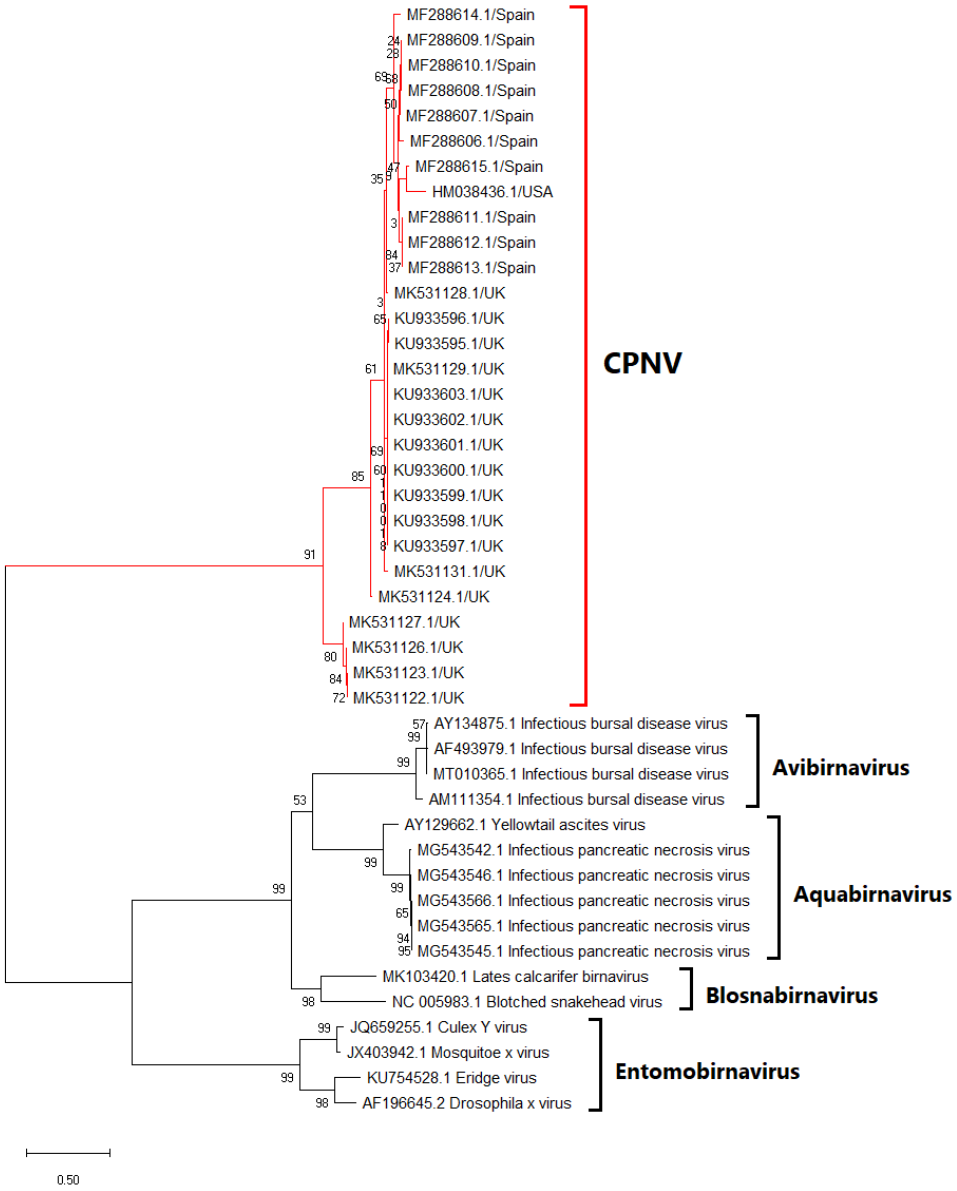

Figure 18. Phylogenetic tree of CPNV sequences based on the partial sequence of the VP1 gene obtained in this study compare to all partial CPNV sequences available in GenBank and to the complete B segment genome of reference strain HM038436.1/USA and all genera of Birnaviridae.

3.4. Discussion

Since its first description, TVP has been reported in many countries. The only work reporting the presence of TVP in Spain, indicated that the disease was in the country, at least since 2009 (Grau-Roma *et al.*, 2010). The present retrospective study indicates that TVP is present in broiler farms in Spain since at least 1999, ten years earlier.

TVP is a disease characterized by proventriculitis associated with poor weight gain, impaired feed digestion and increased FCR resulting in considerable economic losses (Kouwenhoven *et al.*, 1978; Thayer *et al.*, 1993; Bayyari *et al.*, 1995a; Goodwin, 1995, 1996; Huff *et al.*, 2001; Reece P., 2002; Pantin-Jackwood *et al.*, 2005; Guy *et al.*, 2005, 2011b; Marguerie *et al.* 2011; Marusak *et al.*, 2012; Hafner and Guy, 2013; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017, 2020). These clinical signs were present in 60% of the cases detected in the present study further confirming the clinical picture already described. Moreover, the percentage of positive CPNV RT-PCR and ISH cases in this study (17%) was almost equavilant to recent study in UK (19%) by Grau-Roma co-workers in (2020).

All cases reported in this study were from broiler farms. Most of the poultry submission to the diagnostic service correspond to broiler chickens, so it cannot be rule out that TVP cases in broiler breeders or layer hens could have been also occurring in Spain, as it has been already described in other countries (Marusak *et al.*, 2012). Further studies including more samples from these types of birds should be carried out in order to confirm or not the presence of the disease.

In addition, most of the cases included in this study came from farms located in Catalonia. This was likely influenced by the fact that it is one of the regions with the highest density of poultry farms in Spain, but also is the area where the veterinary pathology service receiving the clinical cases is located, so it cannot be concluded that this region is more affected, because it may be that it is overrepresented in the present study.

Enlargement and thickening of the proventriculus was the most common finding observed in TVP cases as it has also been described by other authors (Hafner *et al.*, 2013). However, 40% of the cases in the present study did not show or it was not described as

showing apparent gross lesions. This point out that TVP cannot be ruled out when gross proventricular lesions are absent.

The specific microscopic lesions that are described associated with this disease were observed in the present study. For comparison purposes, we used the more recent system established by Grau-Roma *et al.* (2017, 2020) where the authors grouped the cases into three groups based on the histological lesions: (i) TVP-affected chickens: lymphocytic infiltration and glandular necrosis (ii) lymphocytic proventriculitis (LP)-affected chickens: lymphocytic infiltration without necrosis and (iii) without proventriculitis (WP): no lymphocytic infiltration or necrosis. In the retrospective study with the UK samples, Grau-Roma *et al.* (2020) found a similar distribution of TVP cases (73%) and LP cases (26%) compared with the present study. However, the distribution of RT-PCR CPNV positive cases was different. Grau-Roma *et al.* (2020) found 22% of the TVP cases and 11% of the LP cases as being RT-PCR positive, while in the present study all positive cases were from TVP-affected chickens. In both studies, the high number of negative proventriculus may correspond to chronically CPNV infected chickens (Guy *et al.*, 2011b), although it has also to be taken into account that FFPE samples are less sensitive compared to fresh sample for the PCR reaction because of the formalin fixation procedures (Klopfleisch *et al.*, 2011). Finally, it cannot be ruled out that other infectious agents might cause a similar clinocopathological picture.

An ISH technique was set up in the present work to further confirm and study this disease. The ISH is a sensitive method that help to determine the precise localization of the infected cells. It has been used for many years to detect viral genomes in tissue sections (Furuta and Shinohara, 1990). In this study, positivity was observed in the nucleus of oxynticopeptic cells in necrotic areas, further confirming the association between the virus and the microscopic lesions observed, which is relevant to understand the pathogenesis of CPNV infection. In addition, ISH results correlated with RT-PCR results, which also supports the role of CPNV in TVP.

Sequences obtained from positive RT-PCR cases confirmed that the circulating CPNV Spanish strains are very similar to strains from UK and USA. However, the sequence obtained is a short nucleotide sequence (171 n) within the VP1 gene. It would be necessary to perform larger studies, increasing the number of sequences and their length

using for example next generation sequencing (NGS), to confirm these initial observations of CPNV genetic lineages.

In conclusion, the present study has considerable implications for a better understanding on the occurrence and an improvement in the diagnostic of TVP. Definitively, more studies trying to confirm or rule out a potential relationship of CPNV loads with disease in poultry, additional investigations and more fresh samples are needed in order to determine the incidence and prevalence of TVP in Spain and globally. Lastly, this study identified the presence of TVP in Spain chicken populations since at least 1999 as a silent disease and identified ISH as a new method to detect the virus.

Chapter 4

Study 2: Detection and identification of Chicken Proventricular Necrosis Virus (CPNV) using transmission electron microscopy in field cases

4.1. Introduction

The etiology of proventriculitis has been a question of debate for many years, since the first description of this pathological process in 1978. From the beginning, several viruses were identified as being the putative etiological agent of this process, and it was not until 2011, that Guy and coworkers described a new birnavirus, named Chicken proventricular necrosis virus (CPNV), as the cause of TVP and were able to reproduce the disease (Guy *et al.*, 2011a, 2011b).

In these works, the authors demonstrate that the viral agent associated to the proventricular lesions was not an adenovirus, as the same group described years before (Guy *et al.*, 2005, 2007), but a new birnavirus. This was based on the finding that the new viral agent had a genome comprised of a bisegmented, double-stranded RNA, characteristic of birnaviruses, and on the analyses of the nucleotide sequence, specially the sequence of the putative CPNV VP1, in which a high conserved region among birnavirus VP1 proteins was found. In addition, a negative staining of a partially-purified CPNV preparation examined under transmission electron microscopy, revealed non-enveloped, icosahedral viral particles of approximately 75 nm in diameter (Guy *et al.*, 2011a).

Since these findings, many authors have reported the detection of CPNV in proventricular samples from clinical TVP cases, mainly by molecular methods (RT-PCR) (Guy *et al.*, 2011a, 2011b; Marguerie *et al.*, 2011; Marusak *et al.*, 2012; Hafner *et al.*, 2013; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017, 2020), however no further information on the characteristics of this new viral agent have been reported.

The aim of the present study was to provide additional information on this viral agent, about its morphology, replication site and ultrastructural changes that the virus may cause to the infected cell in proventricular tissue from natural TVP clinical cases by using conventional Transmission Electron Microscopy (TEM) examination.

4.2. Materials and Methods

4.2.1. Samples

Formalin fixed paraffin embedded (FFPE) proventricular samples from 8 clinical cases from those assessed in study 1 were used. Five of them (case #1, #3, #4, #8 and #28) showed characteristic TVP histopathological lesions and were positive by ISH technique and RT-PCR to CPNV, while the remaining three cases (case #16, #21 and #33) showed also characteristic TVP histopathological lesions but were negative to CPNV RT-PCR and ISH test. In addition, three more samples from other laboratories were also examined. Two samples from France (Fr 1635/03 & Fr1493/99) kindly offered by Dr. Jean-Luc Guerin and one sample from UK (B15.124.2) kindly given by Dr. Llorenç Grau-Roma.

Prior to prepare for TEM, a histopathology procedure was done for all the samples. All tissues were fixed in 10% neutral-buffered formalin and paraffin embedded for histopathology processing and staining with hematoxylin and eosin (H&E). All samples were sectioned again and examined by the ISH technique detailed in the previous study. In the clinical cases where more than one proventriculi was available, the one showing the more severe lesions and/or the highest positivity by ISH was chosen. In addition, a RT-PCR to detect CPNV genome following the procedures described in study 1 was also performed in selected proventriculi.

4.2.2. TEM procedure

After histological evaluation, the proventricular areas showing clear histopathological lesions (i.e. necrosis) and/or positivity by ISH were chosen and sections of 1-2 mm^3 of paraffin-embedded proventriculi were deparaffinized with xylene for 3x15 minutes and then rehydrated by decreasing ethanol baths 100%, 90%, 80%, 50% for 15 minutes, three times each. Then, samples were fixed with glutaraldehyde 2.5% for 2 hr and post-fixed overnight with osmium tetroxide 1% in Potassium hexoferrocianyed 0.08% in water. Third stage was dehydration, by increasing ethanol baths: 50, 70, 90 and 100% to enable

infiltration with a liquid resin. Finally, all samples were embedded in epoxy resin Epon12 (Ted Pella, CA, USA) that is not miscible with water and polymerized at 60°C for 48 hrs.

Blocks were then sectioned with a Leica ultracut UCT microtome (Leica Microsystem GmbH, Wetzlar, Germany) and semithin sections were stained with 1% (w/v) aqueous Toluidine blue solution. The semithin sections were observed with a light microscope to identify suitable areas for further ultrathin sectioning (Figure 19).

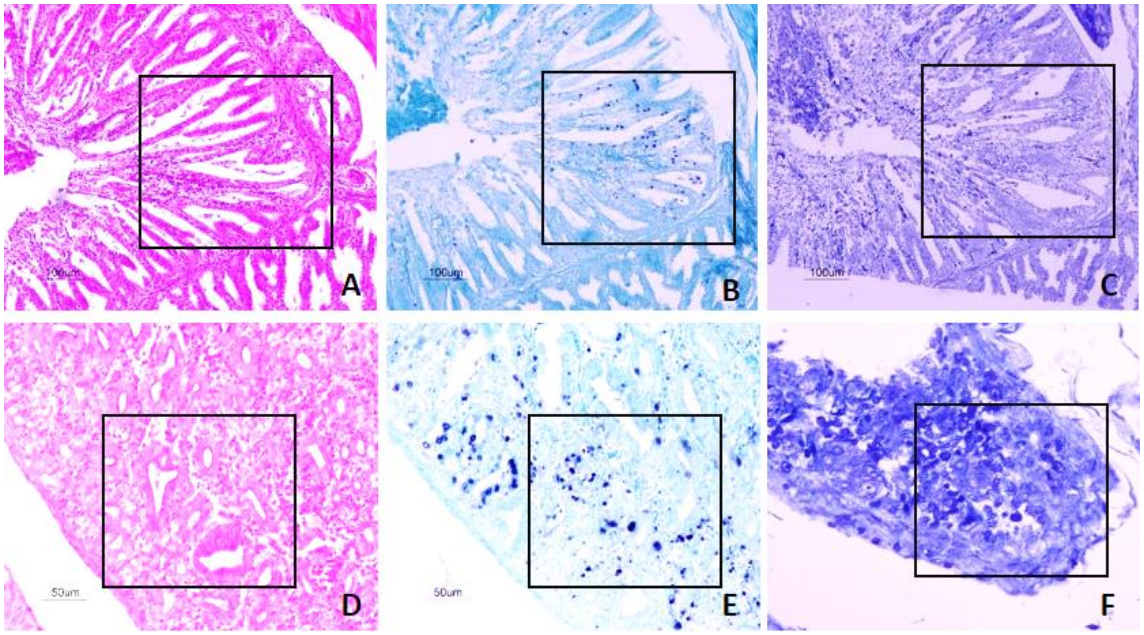

Figure 19. Microscopic preparation steps method to select exact area for ultrathin sectioning of sample FR-1635-03 (A, B, C) and sample #28 (D, E, F). H&E (A, D) and ISH (B, E) slides were visualized and the area with higher positivity was chosen for semithin sectioning, stained with Toluidine blue solution, (C, F) and further ultrathin sectioning.

The last stage was ultrathin sectioning (70-100 nm in thickness). These sections were also done with a Leica ultracut UCT microtome (Leica Microsystem GmbH, Wetzlar, Germany) with a diamond knife, and placed on non-coated 200 mesh copper grids. Then sections were stained with ready to use 2% uranyl acetate solution (Thermo fisher, USA) for 30 min and Reynold's lead citrate solution (Thermo fisher, USA) for 5 min. Sections were observed with a Jeol 1400 transmission electron microscope (Jeol LTD, Tokyo, Japan) and photographed with a Gatan Ultrascan ES1000 CCD Camera at the Electron Microscopy Unit facility of the UAB.

4.3. Results

4.3.1. Histology, ISH and RT-PCR

Microscopic lesions consistent with TVP were identified in all selected cases and are summarized in Table 3. All cases showed necrosis of oxynticopeptic cells and most of them had moderate to severe inflammation, with only case #8 and the two French cases showing mild inflammation. Tubular metaplasia was variable.

Regarding ISH and CPNV RT-PCR results, all cases except case #16, #21 and #33 were positive to both techniques.

4.3.2. TEM

Non-enveloped icosahedral viral particles 70 nm of diameter were observed in scattered degenerated and non-degenerated oxynticopeptic cells in the proventricular glands of cases #16, #21, #28 and Fr 1635/03 (Table 3).

Table 3. Samples used in this study and results obtained by histopathology, RT-PCR, ISH, and TEM. ICV=intracytoplasmic virions, INV=intranuclear virions.

Clinical case	**Internal code**	***Proventricular histopathology***			**RT-PCR**	**ISH**	**TEM**
		Inflammation	**Necrosis**	**Metaplasia**			
#2	B-1567/99 A	+++	+	++	+	+	Not detected
#3	B-683/00 B	+++	+	+	+	+	Not detected
#4	B-684/00	+++	+	++	+	+	Not detected
#8	N-573/04 D	+++	+	+	+	+	Not detected
#16	N-489/09 C	+	+	+	-	-	ICV
#21	N-566/10 C	++	+	++	-	-	ICV & INV
#28	N-562/11 T	+++	+	+	+	+	ICV
#33	B-428/12	++	+	-	-	-	Not detected
	UKB15.124.2	++	+	+	+	+	Not detected
	Fr1493/99	+	+	+	+	+	Not detected
	Fr1635/03A	+	+	+	+	+	ICV & INV

Virions had variable electron density appearance, and, in all cases, they were observed in the cytoplasm, organized in aggregations of variable size and shape and para-crystalline arrays close to cytoplasmic organelles such as mitochondria or lysosomes (Figures 20, 21, 24). In some cells, virions were free in the cytoplasm, while in others they were surrounded by a membrane. In cases #21 and Fr 1635/03 virions were also observed in the nucleus of oxynticopeptic cells. The virions found in the nucleus were irregularly distributed within the nucleus (Figure 22) or were arranged in paracrystalline arrays in some cells (Figure 23). In any case, nuclei were enlarged and showed the heterochromatin marginated at the inner nuclear. In some cases, cell degeneration, as well as apoptotic features such as cell shrinkage, membrane blebbing and chromatin condensation and nuclear fragmentation were observed in infected cells (Figures 23 and 24).

In all cases, the number of cells with virions was scarce and did not show a clear relationship with the number of cells positive by ISH in the same tissue area.

In the remaining cases (#2, #3, #4, #8, #33, Fr1493/99 and UKB15.124.2) no virions or viral structures were observed in oxynticopeptic cells

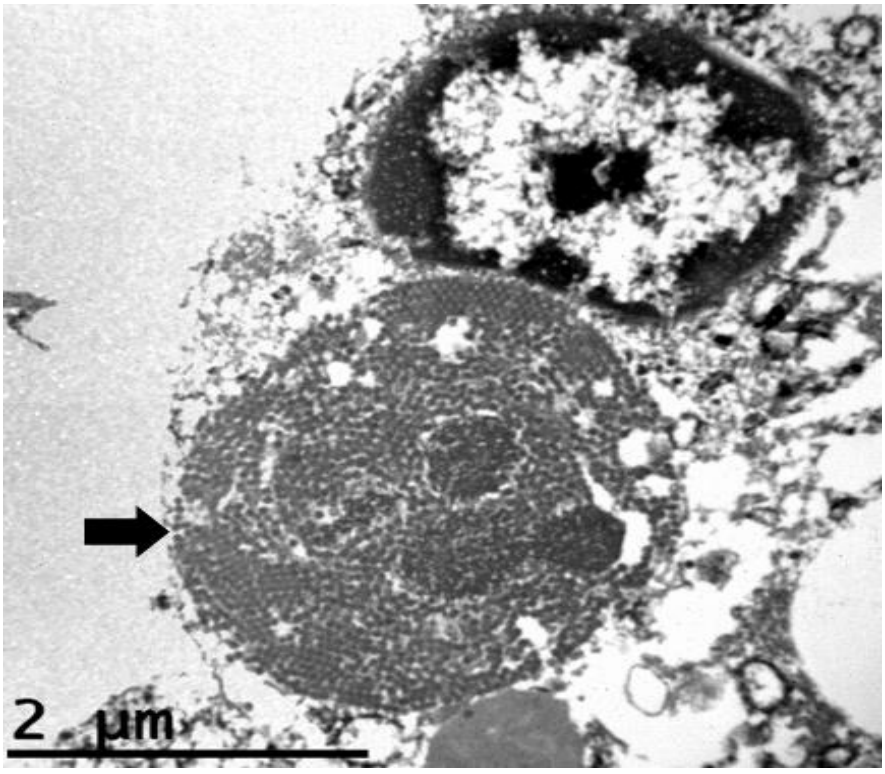

Figure 20. Electron microscope image of sample Fr-1635-0 showing an oxynticopeptic cell with an intracytoplasmic paracrystalline array (black arrow) of icosahedral virions.

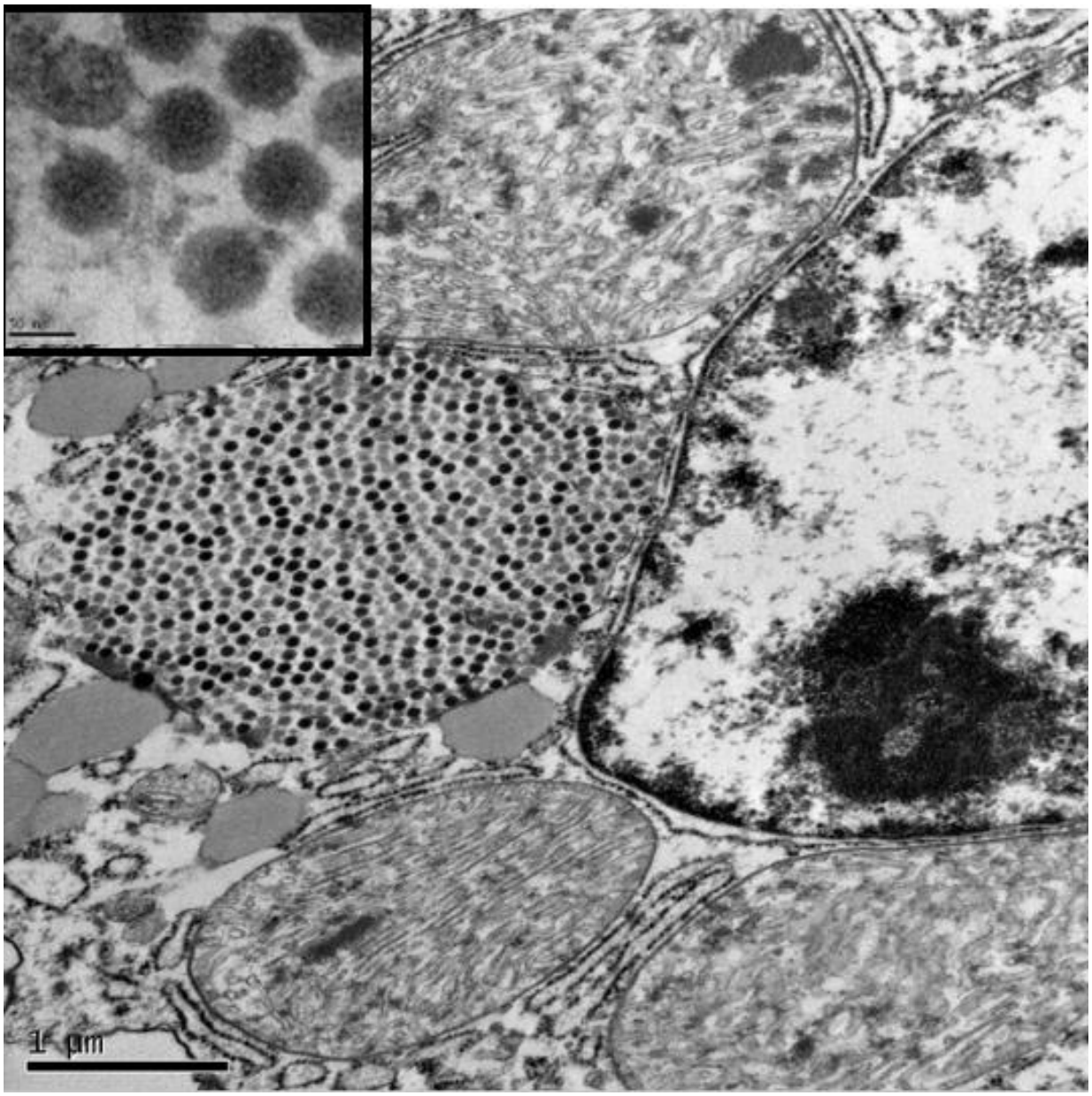

Figure 21. Electron microscope image of case #16 showing an oxynticopeptic cell with an intracytoplasmic aggregation (white arrow) of icosahedral virions. Viruses are icosahedral and approximately 70 nm of diameter (inset).

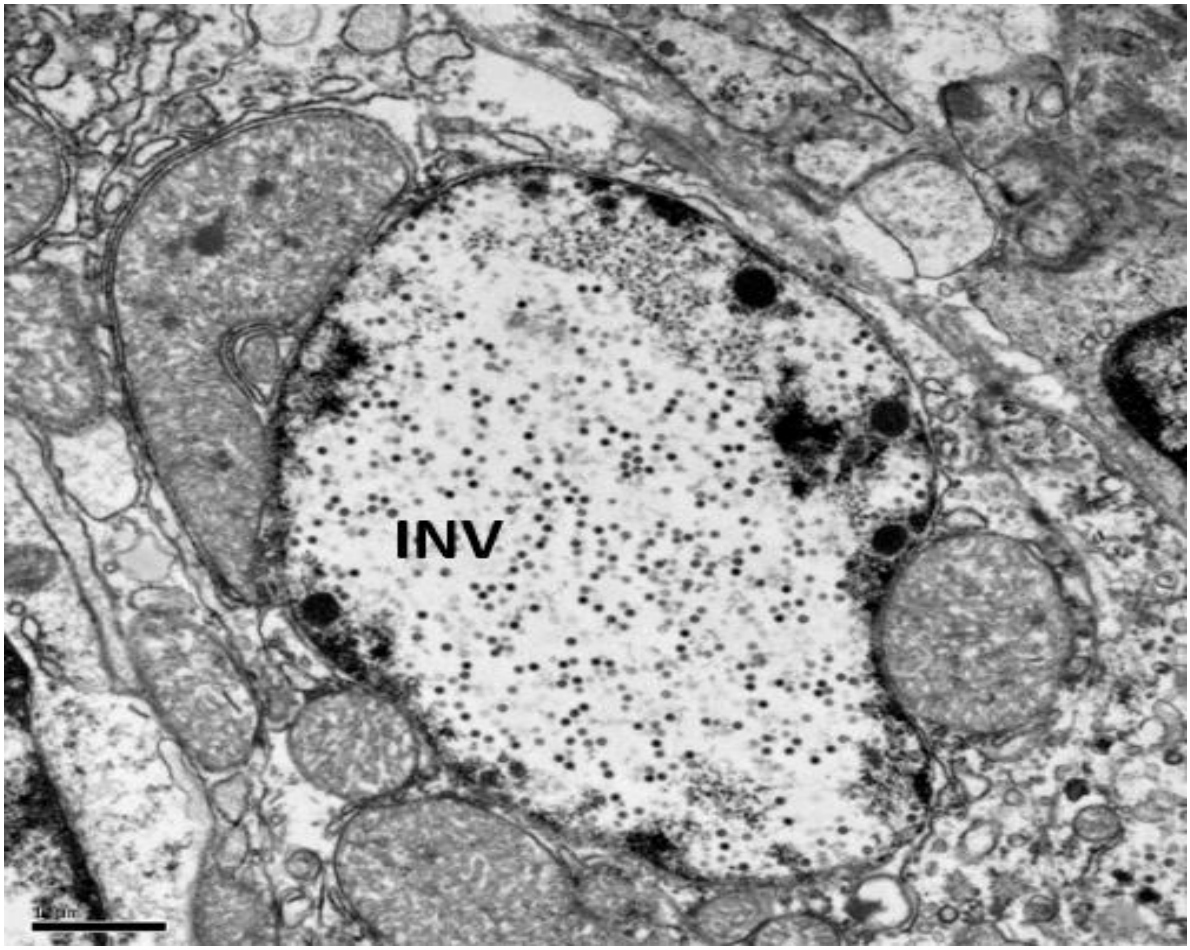

Figure 22. Electron microscope image of case #21 showing an oxynticopeptic cell with intranuclear scattered icosahedral virions (INV) and marginated heterochromatin.

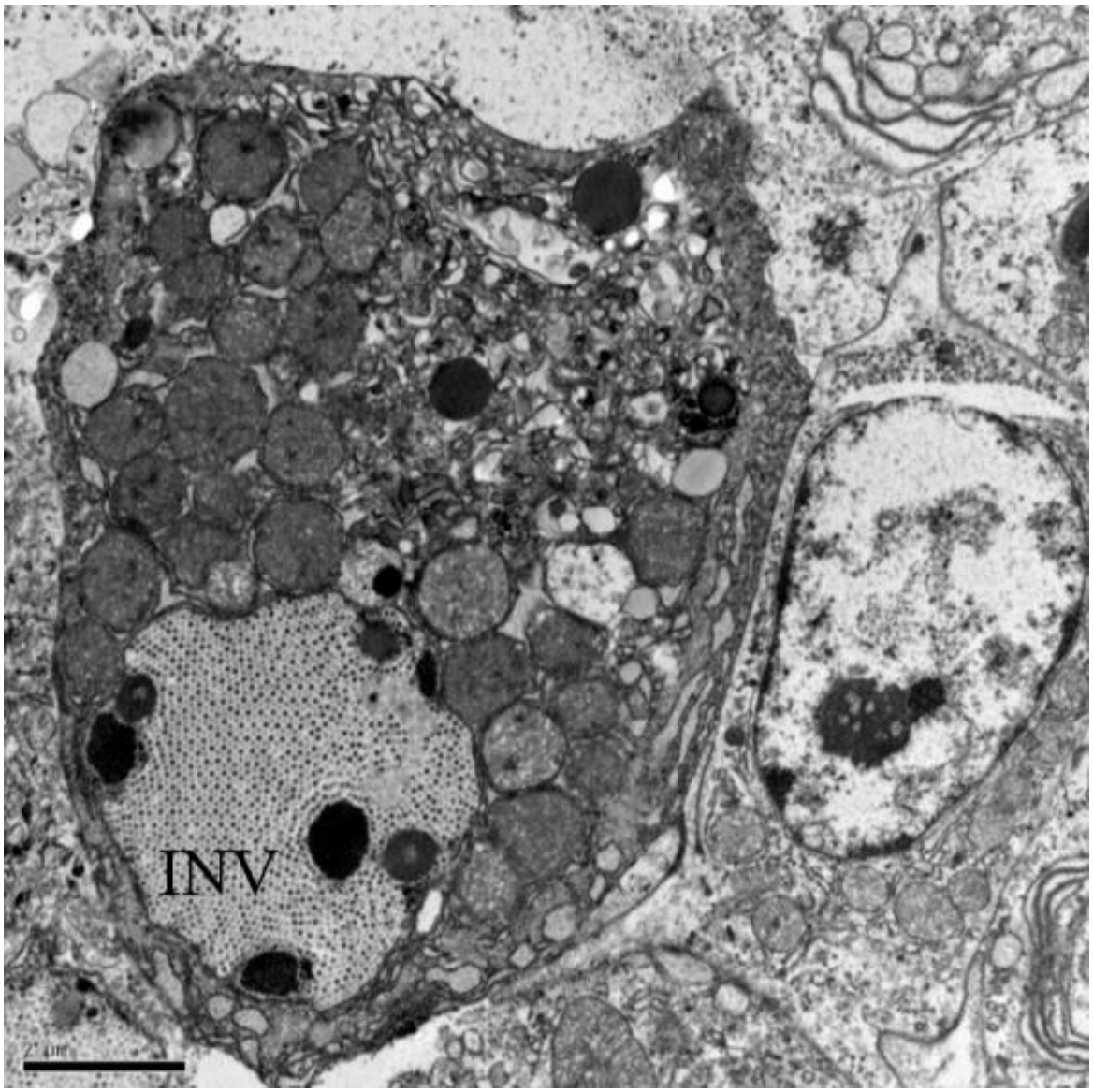

Figure 23. Electron microscope image of case #21 showing an oxynticopeptic cell with intranuclear paracrystalline array of icosahedral virions (INV) and marginated heterochromatin.

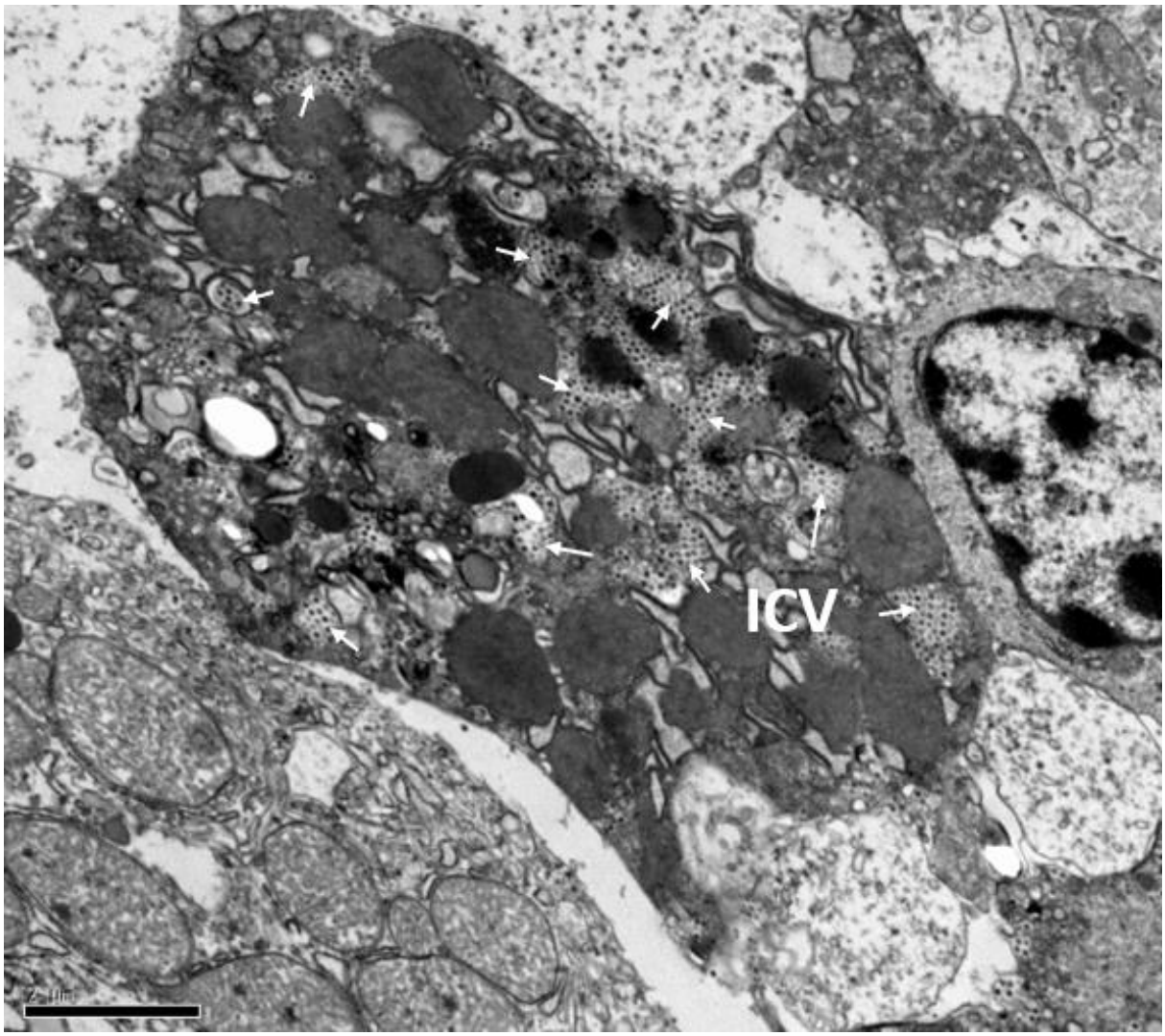

Figure 24. Electron microscope image of case #21 showing a degenerated oxynticopeptic cell with intracytoplasmic icosahedral virions (ICV, white arrows).

4.4. Discussion

The etiology of TVPs has been under debate since the first description of the disease. Different authors have shown by electron microscopy studies the presence of viral structures of 70 nm. of diameter in the nucleus and cytoplasm of oxynticopeptic cells and for that reason for many years the most likely cause was thought to be a virus, but it was not until 2011, that Guy and coworkers (2011a, 2011b) were able to characterize a new birnavirus, named CPNV, as the main cause of this disease.

From that moment, many retrospective and prospective studies focused on detecting CPNV in suspected TVP clinical cases have been performed. These studies mainly relied on histopathological characterization as well as CPNV detection by RT-PCR in proventricular samples (Marguerie *et al.*, 2011; Marusak *et al.*, 2012; Hafner *et al.*, 2013; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017, 2020), and although CPNV has been associated to many of the cases there are still clinical cases showing characteristic histopathological lesions in which no CPNV has been detected. In the previous study, we analyzed 42 clinical cases and only in 7 of them we were able to detect CPNV by RT-PCR. As already discussed, this can be due to the sample analyzed (FFPE in most cases) or the stage of the infection in which the sample was taken (Noiva *et al.*, 2015), but still the question remains on the role of CPNV on TVP. For that reason, an ultrastructural study on samples from different countries showing not only the characteristic proventricular histological lesions, but also positivity to CPNV genome by ISH and RT-PCR was carried out and compared with some samples that also showed histological features of TVP, but were negative to CPNV by molecular methods.

Viral structures with the same characteristics as those described for CPNV (non-enveloped, icosahedral, approximately 70 nm. in diameter) were observed in 4 clinical cases in the present study. Two of them (case #21 and Fr1635/03A) had been positive to CPNV detection by RT-PCR and ISH and thus the detection of viral particles inside oxynticopeptic cells would further confirm the causative relationship between CPNV and TVP. The other two cases (case #16 and #28), where viral particles showing morphological features of CPNV were observed, were negative to both ISH and RT-PCR techniques. It can be argued that in these cases, alteration of the viral genome due to the histological processing could have a negative effect on the sensitivity of the molecular techniques used or that changes in the targeted specific region of the viral genome (VP1

gene) could cause negative results to RT-PCR and ISH techniques. Further studies using more sensitive techniques such as NGS could clarify this. On the other hand, in six cases that were positive by molecular methods to CPNV no viral structures were observed. The failure to see virions in RT-PCR and ISH positive cases could be due to the limited number of cells that were observed under TEM.

Another controversial observation in this study is the visualization of viral structures in the cytoplasm and also the nuclei of infected oxynticopeptic cells. In the present study, intracytoplasmic viruses were observed in the four samples where viral structures were seen and in two of them intranuclear virions were also observed. These findings are in accordance with previous TEM studies on TVP cases where authors describe intracytoplasmic as well as intranuclear arrays of viral particles of the same morphological characteristics of the present study. In fact, these characteristics together with the intranuclear location of the virus pointed for many years to the assumption that an adenovirus was the most likely cause of TVP, but neiter the putative adenovirus could be isolated nor detected following standard molecular methods (Goodwin *et al.*, 1996; Guy *et al.*, 2005, 2007).

Unfortunately, no studies about the morphogenesis of CPNV have been carried out until date. The nearest virus species to CPNV is IBDV in the Avibirnavirus genus and, in this case, different studies related to viral morphogenesis and replication cycle do exist. IBDV mechanism of entry and release is not well understood, however the heat shock protein 90 and α4β2 integrin have been suggested as receptors in various types of chicken cells. IBDV peptides (pep46) could also make openings in target cell membranes, suggesting a role in virus entry (Delmas *et al.*, 2019). After introduction into the cytoplasm, the viral RNA dependent RNA polymerase (RdRP) enzyme plays an essential role in the replication in host cells and has no host cell homolog (Wei *et al.*, 2020). It becomes activated and produces two mRNA molecules from each A and B genome segments (Delmas *et al.*, 2019). Segment A mRNA is translated to VP2, VP4, and VP3 polypeptides, while segment B mRNA is translated to a viral VP1 RdRP. VP1 which is found in virions in both a “free” and a genome-linked form (Delmas *et al.*, 2019). Virus particles assemble and accumulate in the cytoplasm. Encapsidation of the RdRP VP1 is mediated by its interaction with VP3 (Delmas *et al.*, 2019). Infectious necrotic pancreatic virus is another member of the Birnaviridae family whose replication cycle has been studied in depth (Villanueva *et al.*, 2004). In this case also, viral particles assemble and

mature in the cytoplasm. It is therefore, highly probable that CPNV, as other birnaviruses and as other RNA viruses, does replicate, assemble and encapsidate in the cytoplasm. And then the question arises again on whether the virus that has been seen in the nucleus of some cells in two of the clinical cases can be a virus different from CPNV. The size, morphology and location of the viral particles point out again to an adenovirus as one of the most likely viruses. As said before, adenovirus was suspected as a causative agent of TVP in the past (Goodwin *et al.*, 1996; Guy *et al.*, 2005, 2007; Kouwenhoven *et al.*, 1978; Lenz *et al.*, 1998), but then Guy and coworkers (2011a, 2011b) based on virion size and morphology, buoyant density, bisegmented, double-stranded RNA genome and nucleotide sequence analyses of B segment described that a new birnavirus was the cause of TVP.

The present study showed the presence of non-enveloped, icosahedral, 70 nm viral particles in some TVP cases. However, correlation between histopathological lesions, RT-PCR and ISH techniques, and TEM results was not conclusive. Indeed, the presence of viral particles inside the nucleus is controversial when an RNA virus such as CPNV is suspected. Taken together the possibility of a virus, different from the CPNV, causing the same clinico-pathological picture of TVP should still be considered.

Chapter 5

Study 3: Virus detection and identification using Next Generation Sequencing (NGS) method on TVP field cases

5.1. Introduction

Next generation sequencing or NGS is an efficient way of sequencing the total nucleic acid content of samples and subsequent identification of pathogens by bioinformatics tools (Radford *et al.*, 2012; Wu *et al.*, 2015). NGS technique for detection, identification, and analyses of pathogens is better than conventional methods because the sequences produced can be used for more accurate detection and characterization of pathogens (Radford *et al.*, 2012; Datta, 2015; Berry *et al.*, 2016, 2019, 2020; Hadidi *et al.*, 2016; Temmam *et al.*, 2016; Dimitrov *et al.*, 2017; Goldstein *et al.*, 2018) and it is appropriate for novel viruses, while the PCR techniques are more appropriate for discovering the new genotypes (Chiu, 2013). NGS eliminates the need for prior knowledge of genomic sequences and provides advantages over traditional methods such as PCR amplification or *in situ* hybridization which are dependent on target specific primers and probes (Allander *et al.*, 2001). Until recently, genome sequencing of viruses has been performed using overlapping genome amplification with primer pairs. This approach is hard, depends on preexisting information, and produces two reads of less than 600bp only, while NGS can produce millions in each run. For all these reasons, currently, the study of viral genomes (entire nucleotide sequence) relies on deep sequencing, NGS data and public domain databases like GenBank and unique virus specific databases (Ramamurthy *et al.*, 2017).

The use of NGS on FFPE tissue samples allows retrospective studies and molecular investigations of infectious organisms to be carried out (Carrick *et al.*, 2015). NGS includes three basic steps: library preparation, sequencing, and data analysis. In the case of RNA viruses, RNA is converted to ds cDNA and then those three steps start to determine the RNA (RNA converted to double-stranded cDNA) sequence of short fragments of genomic DNA and then computationally reassembling them in the correct order. The principal is that genomic DNA or ds cDNA here is randomly broken into small fragments (typically less than 300 bp) before ligating sequencing primers to each end. Each of these ligated fragments is immobilized and clonally amplified before denaturing. The millions of short DNA sequences are known as raw data or sequence reads and are produced, then bioinformatic computationally re-assembled to provide the DNA

sequence of the original genome, with the aid of the reference genome using special *in-house* pipeline tools (Seekings *et al.*, 2019).

In the previous study, non-enveloped icosahedral viruses of 70 nm of diameter were observed in the cytoplasm and nuclei of proventricular oxynticopeptic cells in 4 out of 11 TVP clinical cases studied by TEM. Two of these cases were negative by RT-PCR and ISH to CPNV. In addition, intranuclear viral assembly has not been described before for other birnaviruses or RNA viruses. Altogether, these results raised the question whether TVP may be caused by other viruses in combination or not with CPNV.

Fowl Aviadenoviruses for example are ds DNA, unenveloped, icosahedral viral particles of 70-90 nm of diameter, and replicate in the nucleus (Niczyporuk, 2016). Avian adenoviruses are ubiquitous (very common) in chicken and fall into three genera, *Aviadenovirus*, *Siadenovirus*, and *Atadenovirus*. The aviadenoviruses affecting fowl are further subdivided into 5 species (A–E) and 11 serotypes. Many of the aviadenoviruses are subclinical and may only produce disease when birds have other concurrent infections, with the notable exceptions of strains of fowl adenovirus-1 producing gizzard erosions, strains of fowl adenovirus-4 producing hydropericardium syndrome, certain strains of fowl adenovirus species D and E producing inclusion body disease, and strains of fowl adenovirus-1 producing quail bronchitis virus (Fitzgerald, 2020).

The aim of this study was to characterize the causative agents' genome of TVP using NGS from FFPE proventricular samples, of TVP clinical cases, in some of which intranuclear and intracytoplasmic icosahedral 70 nm viruses were observed by TEM. Lastly, the study would also add new sequencing data in the public domain to promote research on TVP-CPNV disease.

5.2 Materials and Methods

5.2.1. Samples

Formalin-fixed paraffin embedded proventricular samples from 6 cases of study 1 were used (case #2, #3, #8, #16, #21 and #28) and two samples from other laboratories were also examined. One sample from France (Fr 1635/03) Kindly offered by Dr. Jean-Luc

Guerin (Université de Toulouse) and one sample from UK (B15.124.2) kindly given by Dr. Llorenç Grau-Roma (University of Nottingham). All showed positive ISH, positive RT-PCR to CPNV except two cases: case #16, #21, that were negative to CPNV RT-PCR and ISH, but were included because viral particles were observed by TEM (Study 2).

5.2.2. RNA extraction

Four micrometers thick tissue sections were collected in 1.5 ml centrifuge tubes and deparaffinized by Xylene. Total RNA was extracted using Qiagen RNeasy FFPE Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Briefly, deparaffinized tissue was digested by proteinase K, treated with DNase, precipitated by 100% ethanol, adsorbed to a RNeasy MinElute spin column, washed twice, and eluted in 12 µl RNase-free water directly from the spin column membrane. The RNA was cleaned using Invitrogen Turbo DNA-free kit to treat any contaminating DNA, reagent and to completely remove DNase without phenol treatment or heating according to manufacturer manual (Thermo Fisher scientific, Waltham, MA, USA). Nine µl of RNA per sample were used for NGS analysis.

5.2.3. NGS Library preparation and sequencing

NGS was performed using an Illumina system NextSeq 550 platform (Illumina, San Diego, CA, USA) at the Animal and Plant Health Agency (APHA) in Weybridge (UK).

One library for each sample was prepared using 1 ng of purified cDNA with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol.

Prior to NGS, RNA samples were converted to double-stranded complementary DNA (ds cDNA). Briefly, the protocol involved the synthesis of both the first strand and second strand of DNA from total extracted RNA by using a First strand cDNA synthesis kit, (Roche Mannheim, Germany) containing random hexamer primer, a master mix of oligo primer, AMV RT enzyme, $MgCl_2$, and buffer. Nine µl of RNA from each sample were mixed with two µl (400 uM) of random hexamer primer in a PCR tube and incubated at 70 ̊C for 10 minutes. A 10 µl of a master mix above containing RT AMV enzyme was prepared and added to the sample, and a thermal cycle programmed at 25 ̊C for 10 minutes followed by 42 ̊C for 1 hour was used. The second strand or ds cDNA synthesis using

cDNA synthesis system (Roche, Mannheim, Germany), 55 µl of the master mix 2 of [buffer, dNTPs, water, and 2ed strand synthesis enzyme (Mixture of DNA polymerase I, E. coli ligase, RNase H)], was added to the above result and incubated at 16 ̊C for two hours. Finally, added nine µl T4 DNA polymerase in 16 ̊C for 5 minutes then the reaction was stopped using nine µl of EDTA (0.2 M). The ds cDNA products were purified and cleaned with 150 µl AMPure® XP beads (Beckman Coulter, Brea, CA, USA) by incubating at room temperature for 5 min followed by three washes with 500 µl of 70% ethanol on a magnetic stand. The tubes with the magnetic beads were air-dried for 15 min at room temperature and the library products have recovered in 10 µl of Resuspension Buffer. The ds cDNA was ready for quantification and normalization. The Quantiflour ds DNA system and fluorescent kit (Promega, Madison, WI, USA) was used and the Qubit® fluorometer was used for measuring the concentration of the dsDNA libraries. The standard concentration required for each sample was 0.2 ng/µl.

5.2.4 Sequencing library preparation

A Nextera XT library and index kits (Illumina, San Diego, CA, USA) to identify the sample and allow DNA or RNA to adhere to the sequencing flowcell was used. Ten µl of buffer and 5 µl of Amplicon Tagment mix were added to 5 µl of sample DNA and incubated at 55 ̊C for 5 minutes for chopping and first primer ligation. The reaction was stopped using five µl of NT buffer. A 5 µl of each index primers and 15µl of PCR master mix was added to each sample. The PCR program was: 72 ºC for 3 min and 95 ºC for 30 seconds followed by 12 cycles of 95ºC /10 s, 5 ºC /30 s and 72ºC /30 s and extension at 72ºC for 10 minutes.

All libraries for NGS were normalized and quantified to 2 nM concentration using the above Quantiflour dsDNA system and fluorescent kit (Promega, Madison, WI, USA). One µl of 2N NaOH were mixed with six µl of the library (2 nM) with 993 µl HT1 (hybridization buffer) prior to sequencing. The library was diluted further to 10 pM by transferring 160 µl of the 20 pM denatured libraries to a new tube. Then 1150 ul HT1 and 2 ul of PhiX library were added. The library pool (1300 µl) was loaded in the flow cell of the NextSeq Reagent Kit v2 (Illumina, San Diego, CA, USA). Twenty-four hours after automated cluster generation in NextSeq, the sequencing reads were processed, and all statistical data generated by the instrument were collected and summarized.

5.2.5. Bioinformatic analysis

Both *de novo* and mapping methods were used for bioinformatic analysis of NGS output. Sample reads were mapped to a reference genome, and the reads were placed based on the best match and alignment to the reference (Milne *et al.*, 2013). The *de novo* genome assembly relied upon connecting the sample reads to each other using sequence match overlaps to generate longer sequences (contigs).

An *in-house* pipeline mapping tool was designed and used on Linux operation system (Seekings *et al.*, 2019). The chicken (*Gallus gallus*) genome sequence was downloaded from GenBank as a host reference estimated 1 billion bp (Hillier *et al.*, 2004). Briefly, raw reads quality was assessed using fastQ. Bowtie software was used to create an index of *Gallus gallus* genome sequence and all reads were mapped to *Gallus gallus* in order to identify and filter host read contamination (decrease background noise) and increase the frequency of pathogen reads (Grard *et al.*, 2012; Langmead and Salzberg, 2012). Samtool (Sequence Alignment Map) was used to resynchronize and recover all remain reads which were no longer synchronized due to trimming and filtering with *Gallus gallus*. Finally, new raw sequencing data reads fastQ file were assembled with Spades v1.13 (St. Petersburg genome assembly) bioinformatic software. *De novo* assembly was performed using spades too. An in-house pipeline tool containing reference mapping and *de novo* methods, both specific for the CPNV was used. Segment A & B of all genera of Birnaviridae family were used as a reference of suspected virus including, reference CPNV VP1 segment B (accession number HM038436.1) as high suspected specific causative virus reference. In addition, because of the results obtained on study 2, all samples were referenced to Fowl adenovirus A, B, C, and D complete genome using mapped alignment method only. The Tablet software (Figure 25) was used as graphical viewer for visualization of NGS assemblies and read mappings to analysis of these data (Milne *et al.*, 2010, 2012). The genome assembly we used for CPNV followed procedures already described.

Figure 25. NGS assembly visualization (Tablet software) showing Illumina reads of Sample UK B.15-124-2 against CPNV sequence reference HM038436.1 and viewing graphical sequences of all sequences and contigs reads with the reference. Reads are coloured according to nucleotide type.

5.2.6. Phylogenetic analyses

Viral sequences reads were assembled and visualized using Tablet software (Milne *et al.*, 2009) as showed in Figure 25. The contigs were translated and aligned with other amino acids and nucleotides sequences of Birnaviridae family, and also with partial or complete sequences of CPNV from GenBank database using Muscle algorithm in the Molecular Evolutionary Genetics Analysis program version X (MEGAX) (Kumar *et al.*, 2018). The final trees were constructed using the maximum likelihood (ML) method as implemented in MEGA X and 100 replicate bootstraps was used. On the other hand, reads were also mapped against Avian adenovirus reference type A, B, and C complete genome free available at NCBI and a phylogenetic analysis was done using the same procedure as described above.

5.3. Results

5.3.1. Next-generation sequencing of FFPE tissue samples

In the eight samples submitted for sequencing, CPNV sequences were identified in all except one case, case #3, that had poor RNA quality. A complete CPNV genome segment B (2887 bp) was obtained from sample UK- B.15-124-2 and was submitted to GenBank (accession number MT036105). In another sample, case #2, a large sequence of CPNV genome segment B was obtained (2180 bp) and was also submitted to GenBank (accession number MT024788). The only published complete CPNV segment B shared 93% identity at the nucleotide level with UK.B.15.124-2, and 89% identity with the partial sequence of sample case #2 (CPNV-Spain-1567-99). The remaining samples were with less reads. Sample #28 had 86 reads, #21 and #16 had 40 reads each, sample #8 and Fr.1635/03, only 10 reads. The reads length was at least 19 to 150 n. Regarding segment A of the viral genome, in case # 8, #21, and #28 only 14 reads each were found, while in case #16 there were 26 reads, and in case Fr.1635/03 only 11 reads. Case #2 had only one read. Most of the read lengths were around 19 n.

The complete genome sequencing of the UK.15/124 samples produced 10,871,780 in each two raw reads of 35-150 bases sequence length. The assembly analyses showed that 495 reads were mapped to CPNV segment B and only three small reads to segment A. The mean fragment lengths ranged from 35 to 150 nucleotides and the assembled genomes had a percent coverage ranging from 92 to 96% of the reference. The mean depth was 9–1498.

In five samples (#8, #16, #21, #28, and Fr.1635/03) Avian adenovirus A partial sequences were also found. Case 8# had 200 reads, # 16 and #21 had 400 reads and, finally, #28 and Fr.1635/03 showed 200 reads. Each sequence read was composed of 19-45 n. The assembly analyses results read were mapped to avian adenovirus A. All results are summarized in table 4.

Table 4. Summary of results from samples used in this study. ISH= In Situ hybridization, TEM = Transmission Electron Microscopy, NGS=Next Generation Sequencing. ICV=Intracytoplasmic Virions, INV= Intranuclear Virions. *GenBank Accession numbers.

case #	Internal Code	CPNV RT-PCR	CPNV ISH	TEM	NGS	
					CPNV seq	ADV seq
#2	B-1567/99 A	+	+	-	+ MT024788*	-
#3	B-683/00 A	+	+	-	-	-
#8	N-573/04 D	+	+	-	+	+
#16	N-489/09 C	-	-	+ ICV	+	+
#21	N-566/10 C	-	-	+ ICV-INV	+	+
#28	N-562/11 T	+	+	+ ICV	+	+
FR	Fr 1635/03 A	+	+	+ ICV	+	+
UK	UK B.15-124-2	+	+	-	+ MT036105*	-

5.3.2. Phylogenetic analyses

The phylogenetic analysis of the partial or complete CPNV segment B sequences obtained by NGS from seven of the samples showed the clustering of all these sequences in one group which was divergent from other birnaviruses (Figure 26). Both CPNV-UK B.15-124-2 and CPNV-Spain-1567-99 (case #2) samples sequences showed a high similarity with the reference sequence CPNV USA HM038436 (93% & 89%, respectively).

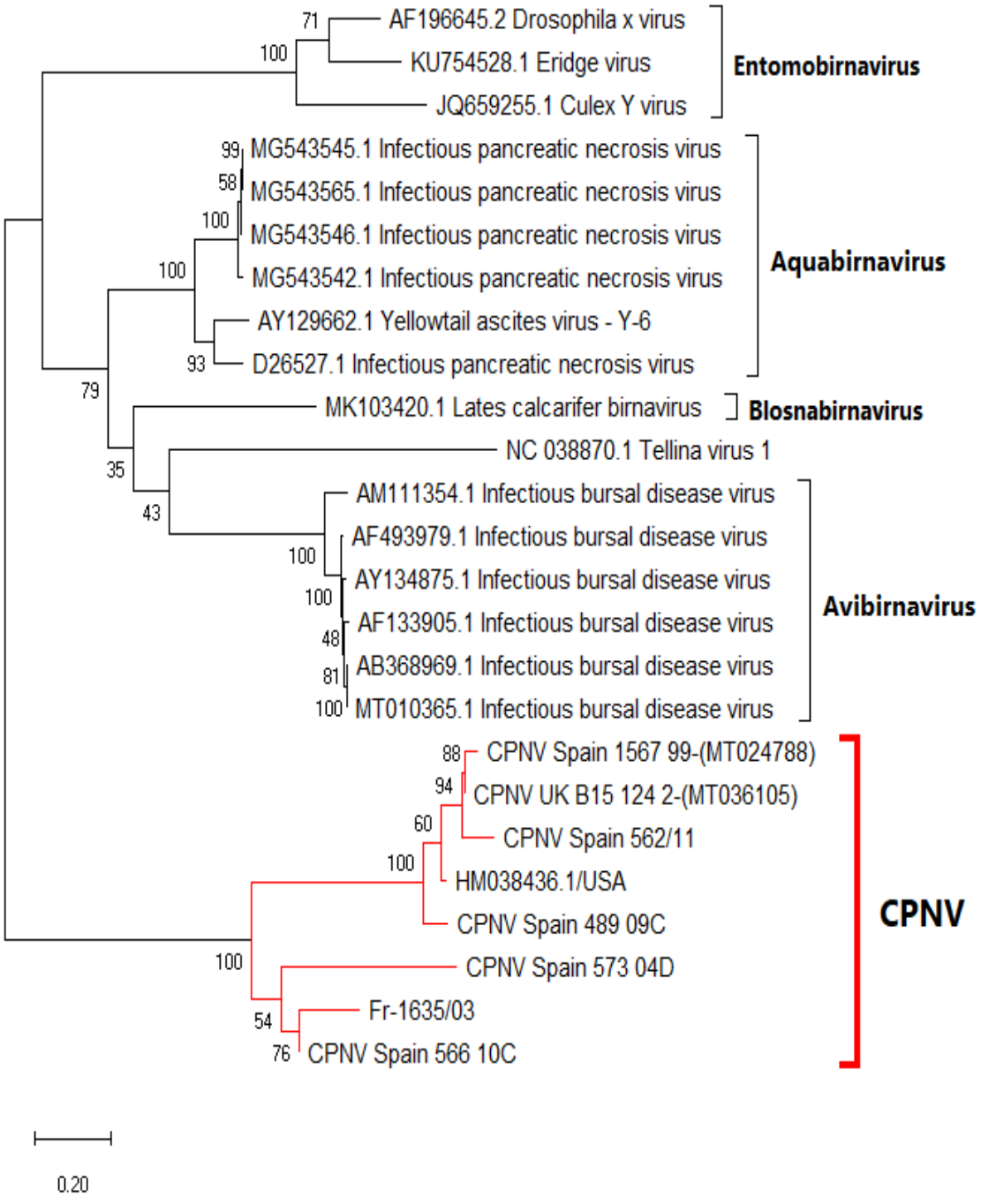

Figure 26. A distance tree representing the phylogenetic relationships members of the various genera and species in the family including new seven CPNV detected by this study (red bracket). The tree represents complete segment B VP1 for all showed samples and six not complete sequences of our samples.

On the other hand, the sequences obtained in five of the samples, clustered in the phylogenetic analysis with the reference sequences of the genera of Aviadenoviruses type A (Figure 27) and differed from other species of Fowl aviadenoviruses species B, C, and D.

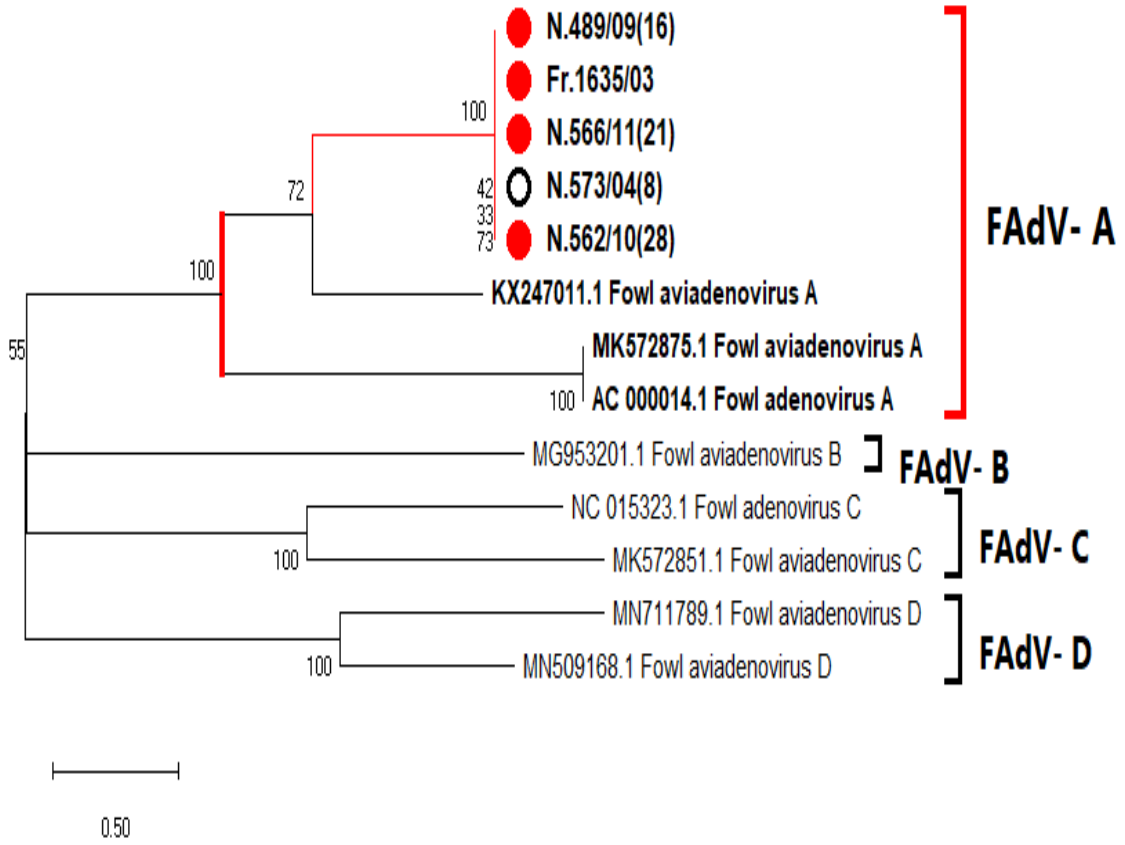

Figure 27. Fowl avian adenovirus phylogenetic relationships of partial sequences compare to full genome of members of the various genera and species in the family. (red circle) is positive TEM intracellular, (white circle) is negative TEM result, (number) is # case number in this study.

5.4. Discussion

For many years, the cause of TVP has been under debate, and even with the finding of a novel birnavirus as the main cause of TVP, the fact that nor the isolation of this viral agent by conventional methods such as cell culture and egg inoculation has been possible until date (Guy *et al.*, 2011b), neither the experimental reproduction of the disease, encouraged us to use NGS in this study. NGS has become a universal approach for detection and identification of many novel and known viruses (Grard *et al.*, 2012; Hang *et al.*, 2012; Chiu, 2013; Neill *et al.*, 2014;

Hadidi *et al.*, 2016; Berry *et al.*, 2020). NGS is faster, because it involves devastating the whole genome into pieces, sequencing the fragments (pieces) and assembling them by computer, and in overnight can generate many millions of bases of sequence (Barzon *et al.*, 2011; Radford *et al.*, 2012; Wu *et al.*, 2015). However, NGS is not widely used because cost estimates and inefficient utilization of freely available bioinformatics tools (Dimitrov *et al.*, 2017).

Two bioinformatic approaches can be used in analysis of sequence read data. If a reference genome is available, then the sequences can be mapped directly to this reference using a mapper such as BWA (Radford et al., 2012). The second approach, when a suitable reference genome is not available, is that individual sequencing reads can be assembled *de novo* using another software such as Velvet (Radford *et al.*, 2012), which uses algorithms to find overlapping information between reads, leading to the generation of continues sequence referred as a contigs. This approach allows discovery of novel genes and sequences. Both the *de novo* and mapping strategies may not cover the entire genome, due either to insufficient depth of coverage or the read length is smaller than the genomic repeat because of gaps in the genome assembly (Radford *et al.*, 2012). Therefore, and because of the limited computational capacity and with low sequencing throughput, we used a reference-based assembly which is a very useful and accurate tool for assembly of known genomes (Berry *et al.*, 2020). Combining reference mapping and *de novo* assembly can be a good strategy for increasing the accuracy of the overall genome (Hang *et al.*, 2012). In the present study, the host contamination of viral RNA was very high, about 98%, and this percentage is normal because the RNA extraction was from FFPE of chicken proventriculus of field cases and not from tissue culture or isolated viruses of chicken embryonated eggs which is cleaner in NGS sequences method. Also, as obligate intracellular organisms, viral preparations are usually heavily contaminated by host nucleic acid, and many of the resulting sequence reads are of host origin rather than viral. Under these circumstances, only a small percentage (1 % or less) of the reads would typically be of viral origin (Radford *et al.*, 2012).

The results of the present study, using NGS molecular analysis, confirmed that CPNV was present in all studied samples, even in those that were negative to CPNV RT-PCR and ISH, possibly because the sensitivity of these techniques is much lower than NGS. This result is relevant since it further confirms the involvement of CPNV in TVP clinical cases and demonstrates the effectiveness of NGS approach for using in pathogen discovery and obtaining viral genomes from total nucleic acid extracts without purification or enrichment of viruses, and without knowing nucleotide sequences for amplification. Unfortunately, just the complete

sequence of the segment B of one of the samples and a large, but partial, sequence also from the segment B of another sample was obtained. Genome sequences of segment A of our samples was not identified here because of low sequence reads (10 reads), small read length (19 n) and not depth, as NGS often fails to represent all regions accurately (Schmutz and Grimwood, 2004; Barzon *et al.*, 2011). In any case, the lack of a successful detection of the complete sequence of segment A in all the cases is an intriguing result that needs further investigation.

The phylogenetic tree of CPNV sequences showed a clear clustering of all CPNV sequences that were divergent from other birnavirus genomes available at the GenBank from around the world. The Spanish sequences were in the same cluster that others from UK, France, and USA showing CPNV is a new species of Birnaviridae and confirmed the previous studies (Guy *et al.*, 2011a; Grau-Roma *et al.*, 2017, 2020). In the present study and these authors used a fragment of 171 nucleotides of VP1 gene of segment B which is suitable for screening the diseases but not very suitable for geographical differentiation between the viruses because of the small size of the fragment (171 n). Even with this limited fragment size, these data also reinforcing the idea that CPNV does not show independent molecular evolution in the particular areas of the world where it has been detected to date but confirm the divergent from other birnaviruses as a specific species or new genera of Birnaviridae family. The whole genome or long sequence without gaps is favorable in phylogenetic analysis to give exact differentiation between viruses' species and genera in a family and geographical classification. The phylogenetic analysis of genomes is a complement to laboratory analysis with a distinct advantage (Janies and Pol, 2002).

The results obtained in study 2 showed intranuclear and intracytoplasmic 70 nm icosahedral non- enveloped virions in oxynticopeptic cells of proventriculi from TVP clinical cases. The morphology and dimensions above could refer to Adenoviridae and Birnaviridae families respectively, although the location of viral particles in the nuclei observed in two of the samples prompted us to consider the involvement of a DNA virus, at least in these cases. NGS results showed that in five cases, avian adenovirus sequences that clustered together with FAdV- A were also present together with CPNV. These five cases included the two cases were intranuclear virions were observed (case #21 and F1635/03 A), so it is very likely that the icosahedral viral particles that were seen in those proventriculi were actually adenoviruses. FAdV-A is mainly associated to corrosive ulcerative gizzard (CUG) in broiler (Fitzgerald, 2020). Interestingly, these results are in accordance with old studies on TVP where an

adenovirus (Kouwenhoven *et al.*, 1978; Goodwin *et al.*, 1996; Lenz *et al.*, 1998) or adeno-like virus (Guy *et al.*, 2005, 2007) was hypothesized to be the principal agent involved. Unfortunately, although FAdV-A sequence reads by NGS were identified, no complete genome was obtained. The reason for this might be that the FAdV cDNA might have been generated due to the usage of random hexamer primers which could amplify some of the remaining degraded FAdV DNA to reproduce and synthesize cDNA that would finally generate partial sequences of FAdV detected by NGS.

The use of FFPE tissues provided several advantages in the current study even that the RNA is more degraded by FFPE blocks samples than fresh samples (Jt *et al.*, 2013). The archived FFPE tissue, which was once considered unusable, are becoming increasingly accessible to genomic analyses, molecular investigations of infectious organisms, and allows for retrospective studies (Tang *et al.*, 2009; Carrick *et al.*, 2015). In this study, the NGS and bioinformatic approach allowed complete genome sequence of CPNV segment B characterization or partial sequences of CPNV and avian adenoviruses from FFPE specimens sampled 5 to 20 years ago, which is of significant value. On other hand, NGS can be successfully used as a complementary test for precision diagnostics of infectious diseases in FFPE samples because the limitation of other available techniques and pre requirements information. Previously, the genome of the 1918 pandemic influenza A virus was determined after a 9-year effort by overlapping RT-PCR from postmortem samples; while using NGS, the complete genome was obtained in a single run in one day (He *et al.*, 2018).

This the first time to find a full segment B genome from CPNV in proventricular samples using NGS. Further, we found that TVP might be also associated to avian adenovirus combined with CPNV, although further studies are needed to clarify the role of FAdV in TVP. Also, one of our goal of genomic sequencing study are to put sequencing data in the public domain to promote research. Particularly, there is only a complete sequence of CPNV available in GenBank (HM038436) by Guy *et al.* (2011). It is worth mentioning that NGS can provide only genetic information, but no viable virus that could be isolated for further characterization. Indeed, more isolates from different geographic sites and time periods should be sequenced for phylogenetic analyses and to find the full genome of CPNV segment A which has not found by any researchers yet. Further phylogenetic and evolutionary studies with a higher number of CPNV and Avian adenoviruses sequences, and isolation of the viruses by cell culture are needed to ascertain those hypotheses.

Chapter 6

General Discussion

Chickens represent the predominant species in poultry production; around 137 million ton was produced in 2020 worldwide and it is anticipated to expand year by year (FAO, 2020). The poultry sector continues to grow in the world and Spain is one of the biggest producer (1.4 million tons/2020) and consumer country in EU (USDA, 2020).

TVP is a viral disease that affects mainly on the proventricular glandular epithelial cells (oxynticopeptic) of chickens which are responsible for secreting pepsin and hydrochloric acid for food digestion. Any effect on these cells leads to defect in digestion and consequently reduces financial benefit. TVP is an economic disease due to losses associated to poor FCR, poor flock uniformity and possibility of proventriculus rupture during mechanical evisceration in the processing plant (Kouwenhoven *et al.*, 1978; Apple *et al.*, 1991; Bayyari *et al.*, 1995; Goodwin *et al.*, 1996; Huff *et al.*, 2001; Guy *et al.*, 2005; Dormitorio *et al.*, 2007; Marusak *et al.*, 2012; Hafner *et al.*, 2013; Noiva *et al.*, 2015). Many investigations have demonstrated the presence of TVP in different countries around the world and, more recently, with the description of a new viral agent, CPNV, associated to this pathological process, this virus has also been detected in several countries (Kouwenhoven *et al.*, 1978, 1988; Page and Fletcher, 1980; Bayyari *et al.*, 1995a; Goodwin *et al.*, 1996; Lenz *et al.*, 1998; Zheng *et al.*, 2000; Huff *et al.*, 2001; Pantin-Jackwood, 2003; Dormitorio *et al.*, 2007; Grau-Roma *et al.*, 2010; Kutkat *et al.*, 2010; Kim *et al.*, 2015). However, the lack of success in isolating and better characterizing this new pathogen still raises some questions on the final etiology of TVP. So, any advances in diagnosis methods would increase the opportunity to identify the causative agent, control, and prevent the disease. Therefore, with the purpose of filling knowledge gaps in TVP and its causative agent, the main objectives of this thesis were to investigate the possibility of occurrence of TVP caused by CPNV in Spanish poultry farms and further characterize the cause of this disease. In this thesis, we conducted three studies to detect and identify the causative agent of TVP. Five different laboratorial methods were used: histopathological examination, RT-PCR and Sanger sequencing, ISH, TEM, and NGS.

To achieve the proposed goals, we first carried out a retrospective study on clinical cases where proventriculitis had been diagnosed. Histopathological examination following a published score, and molecular tools based on RT-PCR and Sanger sequencing, and a new modified ISH technique were used in study 1 (*Chapter 3*). Following CPNV RT-PCR, it was shown that the virus was present in Spanish poultry farms at least since 1999, suggesting that this is not a new

virus, although went unnoticed for years. The virus was only detected in seven out forty-two TVP clinical cases, similar to the other only retrospective study that has been published so far (Grau-Roma *et al.*, 2020). Many different reasons, that have been exposed in the discussion section of Study 1 could explain these results, however the possibility that proventriculitis may be caused by other agents apart from CPNV had still to be considered.

As CPNV is a newly discovered virus, there are still very limited diagnostic methods available. The CPNV isolation has not been successful in the past and these limitations lead us to develop an ISH technique to detect and localize the virus in the tissue samples and confirm the RT-PCR results. The viral cDNA PCR products were used to establish specific CPNV probes and ISH was proven to be very useful for detecting CPNV and confirming the results of RT-PCR. Moreover, the localization of viral genome was essential to be able to conduct the second study of this thesis, since it allowed to choose specific tissue areas positive to viral detection for TEM examination.

Therefore, in the second study, we used TEM, which is a method not usually used for routine viral diagnosis, but it is very important to study new emergent viruses or diseases with unknown etiology. As said, the ISH test was very helpful to select the exact location where the viral agent could be found, and these were the areas chosen for ultrathin sectioning. Twelve samples were examined by TEM (8 of them positive to CPNV by ISH and RT-PCR, and 3 negative), but only in four samples (two of them negative to CPNV RT-PCR and ISH), viral particles were seen. The size and morphology of the observed virions were consistent with birnavirus, but the intranuclear location in some cells was controversial. RNA viruses transcribe viral mRNA by RdRp and this is further translated to viral proteins by host ribosomes and new virions can be formed in the cell cytoplasm. dsRNA viruses contain an RdRp in VP1 that is carried into the cell within the virion (Louten, 2016). For different RNA viruses, RNA replication occurred on membrane of intracellular organelles, but reovirus happened within the cytoplasm on non-membrane cytoskeletal element, while for CPNV, IBDV, and IPNV is unknown (Villanueva *et al.*, 2004). Usually RNA viruses do not enter the nucleus of an infected cell. For this reason, the finding of intranuclear virions raised the question whether TVP was the viral agent observed in the nucleus of oxynticopeptic cells and, therefore, if another virus could be involved in this pathological process. Particularly, adenovirus have the same morphology of Birnaviridae, but they are DNA viruses and normally replicate in the nucleus because they need host DNA for transcribed to mRNA by a DNA-dependent RNA

polymerase(Choi, 2012). Interestingly, until 2011, many researchers reported avian adenovirus (FAdV) as the most likely causative agent of TVP (Kouwenhoven *et al.*, 1978; Goodwin *et al.*, 1996; Guy *et al.*, 2005, 2007; Lenz *et al.*, 1998).

To further clarify the role of the different viruses in TVP, an NGS analysis was carried out in the third study. NGS results indicated the CPNV was involved in those TVP cases (CPNV sequences were identified in all cases), but in five of them FAdV-A sequences were also found which, on one hand, correlates with the finding of intranuclear virions, and on the other hand raises the question whether this virus can also play a role on TVP etiology or its observation was an incidental finding. It is worth mentioning that two of these cases where FAdV-A sequences were found had concomitant lesions usually associated to FAdV-A infection (CPNV #16: ulcerative ventriculitis and case #21, necrotizing hepatitis), so it is difficult to ascertain if FAdV-A plays an essential role in TVP or not. Also, the limited number of tested samples (8) does not allow establishing a definitive causative agent of the disease.

Genetic characterization was done in two studies of this thesis through Sanger sequencing and NGS. CPNV genomes sequenced were found in a cluster when compared with sequences of other member of the Birnaviridae family available in the GenBank. In addition, the nucleotide identity among these sequences and those already existing in the GenBank was really high, indicated by the phylogenetic analysis. In consequence, and considering the analyses done with sequences of this thesis were from a 6-year period, it seems that CPNV has remained fairly stable over the years without an independent molecular evolution according to specific areas of the world (comparing to USA, UK and France sequences only). In fact, the fragment size of VP1 was limited (171 n) from all partial sequences obtained in study 1 and were analyzed by pairwise distance showing that similarity among the partial genome was higher than 95% and in full genome was 89% both comparing to reference USA sequence. This indicates the importance of NGS to get the full genome in future studies to check these differences between strains of diverse geographical origin (overall differences of Spanish partial VP1 fragment average was 0.04 or 4% different nucleotides or 7 nucleotides different in total of 171n fragment). The findings regarding the high similarity among CPNV sequences detected in these three countries and in the study 1 suggest that this virus does not really follow such high mutation rate at least in selected fragment of 171 n. If such mutation rate would be high, it would have generated a higher variation of the genome, which should have been detected at least in the retrospective study (*Chapter 3*). Further studies on the evolution on CPNV are vital by using NGS.

Although over four decades have passed since the first report of TVP, little is known about the disease epidemiology and causative agent. Results obtained from the present thesis demonstrated that TVP and CPNV were present in Spanish poultry farms more than twenty years ago, yet the disease went unnoticed. In addition, the results obtained by TEM studies and NGS analysis suggest that although CPNV may play a principal role in this pathological process, other viral agents, particularly a FAdV-A might also be involved. Further studies characterizing the CPNV genome and setting up an isolation method for this viral agent would be of great help to ascertain the putative role of CPNV and/or FAdV in TVP cases.

Chapter 7

Conclusions

1- TVP associated to CPNV is present in Spanish brioler chicken farms since at least 1999. Clinical signs, gross and microscopic lesions observed in TVP clinical cases are similar to those reported in other countries.
2- Only 16% of the TVP clinical cases studied were positive by RT-PCR and ISH to CPNV. This is similar to other retrospective studies and might be due to i) sample used (FFPE) ii) chronicity of the lesions iii) involvement of other viral agents in TVP.
3- ISH tecnhique to detect CPNV genome in FFPE samples was proven to be very useful to confirm the RT-PCR results and localize the presence of the genome of CPNV.
4- In 4 out of 11 TVP cases, ultrastructural examination revealed intranuclear and /or intracytoplasmic icosahedral, 70 nm, non-enveloped viruses in oxynticopeptic cells, even in 2 cases that were negative by RT-PCR and ISH.
5- CPNV genome was found by NGS in all TVP samples analysed (8), confirming the involvement of this virus in TVP. Phylogenetic analysis of segment B confirmed that CPNV are divergent from other genera of Birnaviridae.
6- FAdV-A genome was detected in some TVP cases analysed (5 out of 8). Two of these cases showed intranuclear virions at TEM examination, suggesting a double infection with CPNV and FAdV-A in some TVP cases. Whether FAdV-a plays a role in TVP together with CPNV or was an incidental finding should be further studied.

Chapter 8

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Annex

Figure 1. The full genome sequence segment B VP1_gene_complete_CDS (2887 n) of CPNV_UK_B15_124 (NCBI Accession number MT036105)

GGGACCCAGCGGCGGCAGAGGACGAGTCCAAATCGAAACTGACAGCTCCAGCCATGGATGAGGCCAGAGAGGAGCTGTCTC
GCAGCCGTGCGACCCGCAATCCCACACTACGGCCAATCACTCTAAACAAGCGCAGGCGTGCGGAGCCCGAACCCGCGCCAA
CTACCCCACTATCCGAGGGACTAACCCTGCGTAGCGATTCGGAGCTGGAGCTGGCGCCCAGCCCCACCCTATCATTAGCGGC
GAGTGACAACCTGTCGCTCAACCCTGAAACCCCGGTCTCCACAATACCCACTCCCAAAACCGTCGAGACCGTTGACGCTACA
TCCGGGCCCAGTCTAGACTATAATGAGATTTACGTGTATATTGACAATGAAGGCAAGGGCATCACACCCCACGACATAGTGA
GAATGAAACAACAAGGGCTGAATATAGAGCAGATGAAAGCAGGCTGGACGAGCGTGTACACGCGGGCAGGAGTCCCACCG
CCACCCGGTAGCGCACCACAGTGGGCGCTGGCGGAGTGTACCAAGAATGAACCCAAAGCCTCATACCTCAGGCTGGAACTG
GGGAAAGCCGGACTGAGACTGGTGGCAGCAAGTGACTACATAAGGGCACCCACCCTGGCCCAGCTCAATGCACCCCCAGAC
TTCATCATGAAGTTCGTGGAGGAGGCAGGCACAAATACACTTGAATTAGCGCCCATGGGCATGCCAGGGCAGCACCTGAGTG
ACTTGAACTACCACCAGAGGGCGAGGGTGGGCAAACCGCTGAAGCTAATATTTTCATCATGTGCGATGGACATAATCAGGTC
GAAGGGAGGAGAGAAGGCCGGCCTCCAAGAGCTATACCTTGACCTACTGAAGACTGCATCCTCCAAGGTCAGGGGAGTGGC
AGGAACCAACGCCATAGTGCCCAGGATGCAAGCCCTAGAGGAAGTGAGGCAGTGGTCAGAAACAAACAATGTGCTGATGAA
GGATGTTGCCCAGATGGCCAGGCGGATGATTAAGGTTTATGACAAAGTTGCCGAGCCCAAGAACAGCGCCTCATTTGAACCA
AAGAGGCTGCTCCTAGACCCTGAGTGTCTAGAGACAACCAGTGGGGAAAGGGCGGTAACCATTCAGATACACCCCAACTCA
GCAGCAGGGCCAGGGTTTGGCACTGGCAAGCAGAAGGAGGCATGGCTGTCCGCAGTCGCGCTGGCCCAGGAGATGTGGGAG
GGCAAATGGCATCAGCTGATGAAAGTGTGCTATGCCAAACAGAAGGATGAGGTCTACGAGGCAGACAAATTCGGCCGAACC
AGGATTATTTCAGTGAGGTGCATGGCGGCCGAAATTAGCGGCGGCGCCCTGGCCCGGCTCTTCTACAAGTGCACGAGGGCTG
AGGGCTGGTCAAGCACAAGCAACCGCTCACTGAGAGGCTTTAACCCCCTTGGTCCGGATCTACAGTCCATACTACACAGGAC
CGAAAACGGGGAGAAGCTGGTGCTACACTATGCTGACAACCTGTACTTCATCAGCCGGGACCGGGTTGTGAGCTACGACGCA
AGCAAGTTCGAAGCGTCGCACAGCGGGAACAAGATAGAAGCCGCAGTGAAGGCCATGCTGGCGGTGGTGGGCGGCGGGAG
CCTGGACTCCCTAGGCAGAGTTGTGGGCACGACCAAATTGACCGACAAGAGCGTTAGGTTCATTCTAGGCCCCTACCTGAAC
AGCCTGACCGACAGTGTCAGTCTACGCAACACCACTCAATGGAAGACACCAGGGATGCCGTCAGGCACCTCACTCACCTTCC
TGATCAACGACCTGGTGATGGGGTGTGCAGTCCAGCTGGCCCTTGAAAGTGGCGTAAACCTAGCAGACGCCAACTCCACCAT
TGAATTCATGGCCAAGTGCGGCATCAAGCTAAAGGTTGAGTGCGAAAGCGATCTCAACCCCAGTCTAACCGCCTTCAATGGC
CTGGGTTCCCCGGGCAGACTTGACTTGCTGGGCTTTGACCTCATGGGCATGCGAGTGTCGAACCAGGAGGCCAGGTTCCTAG
TCCTCAATGAGGACAGGTGCATGGATTCCCTAGTGTGGGGCAGAAGCGACGCCAACCAAGAGGAACTCGACGATCTGATCA
GTGCGAAGGGCAAGCAGCTGTCCATGCTGGTGAGCGCATTACTGAACGGCCAGGGCACATACAAACCAATAGAAGGGCCGA
TTCGCACGGCTGTGAGGCGACTCCAGGAGGAATTAACCGTCGAAATTGACGAAGCTGCTGTGGCGGGTGGCGTGCAGTCGA
GCTGGATCACCAGCAACGGTGGGCCGAGCTGTGCAGTAATAGTCGGCATGAGGCTGGGGGTGGAGCCCGGCTCGGAGGAGG
CCTGGTCGCTGATGAGGGAGGCGAGCAGGGCTCCGGCCGCGGGCATCCGCCCCCCTGACCTCGCGCGCTCCCAAGCGGGGCT
CCGGCGCCGTGGCCGAGGTCCCGCCGGCCAAGCTCACCAAAACAGTGGCGCAGGCCACCTTCAAGGAAATATTTCAATCCAG
GAGAGGGGAGGGTCCACGACGCCCAGCCACAGGAGAAGAAATTCAAAGAGTGAGAGACCTGCTGACAAGCAGGCCCAATC
AGTCCCTGCTGGGTCCCCCGGGCAGGGAGATACTGGGCCGGCCACTGCGCGTGCCAGCCGACACCGCAGAGGCCACACTAC
GCCTGTTGGCGCTGTGCACAAAGGCACCTCTATCAGAGGTGGTGAAGAAGCTGCTACCCGAGGCCACAGAGGCCGCTAACA
GGGGGCTCCTTCACTACCAACCAACAACAGATCAGACAGAATGGGGCAGGTGGACCATCCCCGACTCACACACGGCCGGTG
CGTCGGGGCCTATGTAAAGTCATTCTTTTGTCGACGCGG

Figure 2. Translated Amino Acids (aa) of CPNV-UK_B15-124-2 VP1 gene complete CDS (903 A.A)

translation="MDEAREELSRSRATRNPTLRPITLNKRRRAEPEPAPTTPLSEGLTLRSDSELELAPSPTLSLAASDNLSLNPETPVSTIPTP
KTVETVDATSGPSLDYNEIYVYIDNEGKGITPHDIVRMKQQGLNIEQMKAGWTSVYTRAGVPPPPGSAPQWALAECTKNEPKASY
LRLELGKAGLRLVAASDYIRAPTLAQLNAPPDFIMKFVEEAGTNTLELAPMGMPGQHLSDLNYHQRARVGKPLKLIFSSCAMDIIR
SKGGEKAGLQELYLDLLKTASSKVRGVAGTNAIVPRMQALEEVRQWSETNNVLMKDVAQMARRMIKVYDKVAEPKNSASFEPK
RLLLDPECLETTSGERAVTIQIHPNSAAGPGFGTGKQKEAWLSAVALAQEMWEGKWHQLMKVCYAKQKDEVYEADKFGRTRIIS
VRCMAAEISGGALARLFYKCTRAEGWSSTSNRSLRGFNPLGPDLQSILHRTENGEKLVLHYADNLYFISRDRVVSYDASKFEASHS
GNKIEAAVKAMLAVVGGGSLDSLGRVVGTTKLTDKSVRFILGPYLNSLTDSVSLRNTTQWKTPGMPSGTSLTFLINDLVMGCAVQ
LALESGVNLADANSTIEFMAKCGIKLKVECESDLNPSLTAFNGLGSPGRLDLLGFDLMGMRVSNQEARFLVLNEDRCMDSLVWG
RSDANQEELDDLISAKGKQLSMLVSALLNGQGTYKPIEGPIRTAVRRLQEELTVEIDEAAVAGGVQSSWITSNGGPSCAVIVGMRL
GVEPGSEEAWSLMREASRAPAAGIRPPDLARSQAGLRRRGRGPAGQAHQNSGAGHLQGNISIQERGGSTTPSHRRRNSKSERPAD
KQAQSVPAGSPGQGDTGPATARASRHRRGHTTPVGAVHKGTSIRGGEEAATRGHRGR*"

Figure 3. The partial genome sequence segment B_VP1_gene_partial_CDS (2067 n) of CPNV_Spain_N1567_99 (NCBI Accession number MT024788)

AGCCAGGGAAGAGCTGTCTCGCAGCCGTGCGACCCGCAATCCCACACTACGGCCAATCACTCTAAACAAGCG
CAGGCGGGTTAAACCCGAGCCGTCCCCGTCCACACCATTGTCCGAGGGACTAACCCTGCGTAGCGATTCGGAG
CTGGAGCTGGCGCCCAGCCCCACCCTATCATTAGCGGCGAGTGACAACCTGTCGCTCAACCCCGAGACCCCAG
TCTCCACAATCCCCACACCCAAAACTGTGGAGGCGTCCGAGACCGCTTCCGGGCCCAGTCTAGACTATAATGA
GATTTACGTGTATATTGACAATGAAGGCAAGGGCATCACACCCCACGACATAGTGAGAATGAAACAACAAGG
GCTGAATATAGAGCAGATGAAAGCAGGCTGGACGAGCGTGTACACACGAGCAGGGGTTACACCCCCACCCNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAACCGTCGAATGAACCCAAAGCCTCATACCTCAGGCTGGAAC
TGGGGAAAGCCGGACTGAGACTGGTGGCAGCAAGTGACTACATAAGGGCACCCACCCTGGCCCAGCTCAATG
CACCCCCAGACTTCATCATGAAGTTCGTGGAGGAGGCAGGCACAAATACACTTGAATTAGCGCCCATGGGCAT
GCCAGGGCAGCACCTGAGTGACTTGAACTACCACCAGAGGGCGAGGGTGGGCAAACCGCTGAAGCTAATATT
TTCATCATGTGCGATGGACATAATCAGGTCGAAGGGAGGAGAGAAGGCCGGCCTCCAAGAGCTATACCTTGA
CCTACTGAAGACTGCATCCTCCAAGGTCAGGGGAGTGGCAGGAACCAACGCCATAGTGCCCAGGATGCAAGC
CCTAGAGGAAGTGAGGCAGTGGTCAGAAACAAACAATGTGCTGATGAAGGATGTTGCCCAGATGGCCAGGCG
GATGATTAAGGTTTATGACAAAATTGCCGAGCCCAAGAACAGCGCCTCATTTGAACCAAAGAGGCTGCTCCTA
GACCCTGAGTGTCTAGAGACAACCAGTGGAGACAAGGCGGTGGCCATTCAGATACACCCCAACTCGGCGGCA
GGACCGGGGTTTGGCACGGGCAAGCAAAAGGAGGCATGGCTATCTGCAGTGGCACTGGCTCAGGAGATGTGG
GAAGGCAAATGGCACTCGCTAATGAAAGTGTGCTATGCCAAACAGAAGGATGAGGTCTACGAGGCAGACAAA
TTCGGCCGAACCAGGATTATTTCAGTGAGGTGCATGGCGGCCGAAATTAGCGGCGGCGCCCTGGCCCGGCTCT
TCTACAAGTGCACGAGGGCTGAGGGCTGGTCAAGCACAAGCAACCGCTCACTGAGAGGCTTTAACCCCCTTGG
TCCGGATCTACAGTCCATACTACACAGGACCGAAAACGGGGAGAAGCTGGTGCTACACTATGCTGACAACCT
GTACTTCATCAGCCGGGACCGGGTTGTGAGCTACGACGCAAGCAAGTTCGAAGCGTCGCACAGCGGGAACAA
GATAGAAGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNGGCACGACAAGACTGACCGACAAGAGCGTTAGGTTCATTCTAGGCCCCTACCTGAACAGCCTGACCGA
CAGTGTCAGTCTACGCAACACCACTCAATGGAAGACACCAGGGATGCCGTCAGGCACCTCACTCACCTTCCTG
ATCAACGACCTGGTGATGGGGTGTGCAGTCCAGCTGGCCCTTGAAAGTGGCGTAAACCTAGCAGACGCCAACT
CCACCATTGAATTCATGGCCAAGTGCGGCATCAAGCTAAAGGTTGAGTGCGAAAGCGATCTCAACCCCAGTCT
AACCGCCTTCAATGGCCTGGGTTCCCCGGGCAGACTTGACTTGCTGGGCTTTGACCTCATGGGCATGCGAGTG
TCGAACCAGGAGGCCAGGTTCCTAGTCCTCAATGAGGACAGGTGCATGGATTCCCTAGTGTGGGGCAGAAGC
GACGCCAACCAAGAGGAACTCGACGATCTGATCAGTGCGAAGGGCAAGCAGCTGTCCATGCTGGTGAGCGCA
TTACTGAACGGCCAGGGCACATACAAACCAATAGAAG

Figure 4. Translated Amino Acids (A.A) of HM038436.1VP1 gene complete CDS (903 A.A) which is only reference available data in NCBI, CPNV codon start from nucleotide 54 and finished in 2765 nucleotides, base count ,739 A, 811 C, 873 G, and 464 T.

translation="MDEAREELSRSRATRNPTLRPITLNKRRRAEPEPAPTTPLSEGLTLRSDSELELAPSPTLSLAASDNLSLNPETPVSTIPTP
KTVETVDATSGPSLDYNEIYVYIDNEGKGITPHDIVRMKQQGLNIEQMKAGWTSVYTRAGVPPPPGSAPQWALAECTKNEPKASY
LRLELGKAGLRLVAASDYIRAPTLAQLNAPPDFIMKFVEEAGTNTLELAPMGMPGQHLSDLNYHQRARVGKPLKLIFSSCAMDIIR
SKGGEKAGLQELYLDLLKTASSKVRGVAGTNAIVPRMQALEEVRQWSETNNVLMKDVAQMARRMIKVYDKVAEPKNSASFEPK
RLLLDPECLETTSGERAVTIQIHPNSAAGPGFGTGKQKEAWLSAVALAQEMWEGKWHQLMKVCYAKQKDEVYEADKFGRTRIIS
VRCMAAEISGGALARLFYKCTRAEGWSSTSNRSLRGFNPLGPDLQSILHRTENGEKLVLHYADNLYFISRDRVVSYDASKFEASHS
GNKIEAAVKAMLAVVGGGSLDSLGRVVGTTKLTDKSVRFILGPYLNSLTDSVSLRNTTQWKTPGMPSGTSLTFLINDLVMGCAVQ
LALESGVNLADANSTIEFMAKCGIKLKVECESDLNPSLTAFNGLGSPGRLDLLGFDLMGMRVSNQEARFLVLNEDRCMDSLVWG
RSDANQEELDDLISAKGKQLSMLVSALLNGQGTYKPIEGPIRTAVRRLQEELTVEIDEAAVAGGVQSSWITSNGGPSCAVIVGMRL
GVEPGSEEAWSLMREASRAPAAGIRPPDLARSQAGLRRRGRGPAGQAHQNSGAGHLQGNISIQERGGSTTPSHRRRNSKSERPAD
KQAQSVPAGSPGQGDTGPATARASRHRRGHTTPVGAVHKGTSIRGGEEAATRGHRGR*"