Retrospective study on transmissible viral proventriculitis and Chicken proventricular necrosis virus (CPNV) in the UK

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Abstract

Chicken proventricular necrosis virus (CPNV) is a recently described birnavirus, which has been proposed to be the cause of transmissible viral proventriculitis (TVP). The understanding of the epidemiology of both the virus and the disease is very limited. A retrospective investigation on TVP and CPNV in broiler chicken submissions from the UK from between 1994 and 2015 was performed with the aims of assessing the longitudinal temporal evolution of TVP and CPNV and to review the histological proventricular lesions in the studied chickens. Ninety-nine of the 135 included submissions (73.3%) fulfilled the TVP-diagnostic criteria, while the remaining 36 submissions (26.7%) displayed only lymphocytic proventriculitis (LP). The first detection of CPNV by PCR dated from 2009. Results showed a rise in the number of both TVP and positive CPNV RT-PCR submissions from 2009 with a peak in 2013, suggesting that they may be an emerging or re-emerging disease and pathogen, respectively. Twenty-two out of the 99 submissions displaying TVP lesions (22%) and 4 out of the 36 (11%) ones with LP gave positive CPNV RT-PCR results, further supporting the association between CPNV and TVP and confirming that CPNV is present in a low proportion of proventriculi that do not fulfil the TVP diagnostic criteria. In addition, intranuclear inclusion bodies were observed in 22 of the submissions with TVP. The vast majority of these cases (21 of 22, 96%) gave negative CPNV RT-PCR results, raising the question of whether another virus different from CPNV is responsible for some of these TVP-affected cases.

Research highlights

- TVP and CPNV are present in the British broilers since at least 1994 and 2009, respectively
- TVP and CPNV seem to be an emerging and re-emerging disease and pathogen, respectively
- CPNV was detected in proventriculi with both TVP and LP-lesions
- Other viruses different from CPNV may be responsible for some TVP-affected cases

Keywords: Birnavirus; Chicken proventricular necrosis virus (CPNV); transmissible viral proventriculitis (TVP); natural infection; poultry; Retrospective study.

Introduction

Transmissible viral proventriculitis (TVP) typically affects broiler chickens and is characterized by specific histological lesions, which include oxynticopeptic cell necrosis, lymphocytic inflammation and ductal epithelial cell hyperplasia of the submucosal glands (Hafner & Guy, 2013). Because of the lesions in the glandular stomach, affected birds suffer from maldigestion, poor feed conversion and stunted growth (Dormitorio *et al.*, 2007). The aetiology of the disease has been debated since its first description in 1978 (Kouwenhoven *et al.*, 1978). In 2011, Guy et al. described the detection of a new birnavirus in field and experimentally reproduced cases affected by the disease, which they tentatively named *Chicken proventricular necrosis virus* (CPNV) (Guy *et al.*, 2007; J. S. Guy *et al.*, 2011b). Later on, few other works supported the association between CPNV and TVP (Marguerie *et al.*, 2011; Noiva *et al.*, 2015; Grau-Roma *et al.*, 2017).

Although it has been suggested to have a significant economic impact, the available information about both TVP and CPNV is very limited (Dormitorio, et al., 2007; Hafner & Guy, 2013), and most of the TVP reports are based on low number of cases or on experimental infections (Guy *et al.*, 2011b; Kim *et al.*, 2015; Noiva, et al., 2015). In the past few years, TVP has been reported to occur in several countries from North and South America, Europe and Asia (Grau-Roma *et al.*, 2010; Guy, et al., 2011b; Marguerie, et al., 2011; Kim, et al., 2015), suggesting for it to be an emerging or re-emerging disease. A recent non-peer reviewed retrospective study performed in California broiler flocks shows that TVP is a frequent disease (Hauck et al., 2016). Even though lesions compatible to TVP had been seen in the UK since at least the 90s (Randall & Reece, 1996), the first peer-reviewed report describing the presence of the disease in the UK is very recent (Grau-Roma, et al., 2017). The latter work reported a strong association between the CPNV and the TVP-affected chickens, and detected the presence of CPNV in a low number of birds affected by lymphocytic proventriculitis (LP), which lacked the necrosis of oxynticopeptic cells and therefore did not fulfil all the TVP diagnostic criteria.

The understanding of the epidemiology of both the virus and the disease is scarce and, as far as the authors are aware, there is no retrospective study on TVP and CPNV. The present work is a retrospective investigation in broiler chicken submissions received by the Animal and Plant Health Agency (APHA) Lasswade with the following aims: (i) to assess the longitudinal temporal evolution of TVP, LP and CPNV in the studied British broiler chickens; (ii) To retrospectively review the proventricular histological lesions in a high number of TVP-affected proventricular sections; (iii) to assess the presence of CPNV in chickens affected by TVP or LP.

Material and Methods

Study design. A retrospective study in the archive of submissions received by the Animal and Plant Health Agency (APHA) Lasswade was performed between the years 2000 and 2015. The study was based on formalin-fixed, paraffin-embedded (FFPE) proventricular tissues of broiler chickens. All submissions containing the term ´proventriculitis´ on the microscopic description and/or on the morphologic diagnosis were selected. All histology slides were then re-examined by 1 Pathologist (LG) and these showing lesions compatible with TVP or LP were selected. Submissions compatible with TVP were those showing lesions of lymphocytic and necrotizing proventriculitis affecting the proventricular glands within the submucosa, while submissions of LP showed lesions of lymphocytic proventriculitis without oxynticopeptic cell necrosis (Grau-Roma, et al., 2017). In addition, a single case archived from 1994, which corresponded to the case illustrated by Randall end Reece (1996), was also included in the study. A total of 135 cases were identified. Each submission contained between 1 and 6 FFPE tissue blocks, with between 1 and 5 proventricular sections per block. Typically, each section corresponded to a different bird.

Histopathology. Proventricular sections were histologically re-examined by 2 European College of Veterinary Pathologists' board-certified veterinary pathologists (SdB, LG), and they were all assessed following a previously reported system with minor modifications (Grau-Roma et al., 2017). Briefly, proventriculi were assessed for the following 3 histopathological lesions: i) glandular lymphocytic

infiltration; ii) glandular hyperplasia and metaplasia; iii) necrosis of oxynticopeptic cells. These parameters were semi-quantified as follows: 0 (absence), 1 (>0% to 10% of the glands affected), 2 (>10–50% of the glands affected), and 3 (>50% of the glands affected). If only few (<10) small multifocal follicular aggregates of lymphocytes or few (<10) small multifocal clusters of necrotic cells were scattered through the proventricular glands, these parameters were graded as '1' even if they were present in more than 10% of glands. Based on the histopathological results, each section was classified as: i) TVP-affected chicken: lymphocytic infiltration and necrosis present in the proventriculus; ii) LP-affected chicken: lymphocytic infiltration without necrosis present in the proventriculus; iii) 'Without proventriculitis' (WP): neither lymphocytic infiltration nor necrosis present in the proventriculus. Subsequently, those submissions with at least one section fulfilling the TVP criteria were classified as such, while the remaining ones were classified as LP. No submissions contained only WP sections. All sections were histologically analysed blindly, without knowing the CPNV RT-PCR results.

RNA extraction, RT-PCR, sequencing and phylogenetic analysis. RNA was extracted from all the FFPE proventriculi and subsequently tested by reverse transcriptase polymerase chain reaction (RT-PCR) for CPNV. RNA extraction was done using four 25 µm-sections of each FFPE tissue following a previously described protocol (Grau-Roma et al., 2017). A RT-PCR procedure was performed to amplify a 171 nucleotide (nt) sequence within the VP1 gene of CPNV using primers and protocols described previously (Guy et al., 2011b). RNA extracted material from FFPE tissues known to be positive for CPNV from a previous study (Grau-Roma, et al., 2017) were used as positive controls.

For sequencing, the amplified products from the positive CPNV RT-PCR cases were purified using Mini Elute Gel Extraction Kit (Qiagen, Valencia, CA, USA). Sequencing reactions were performed with ABI Prism BigDye Terminator Cycle Sequencing v.31 Ready Reaction (Applied Biosystems, Foster City, CA, USA), and analysed using an ABI Prism model 3730 automated sequencer (Applied Biosystems). Positive and negative controls of extraction and amplification were added to each batch of samples tested. Partial VP1 CPNV obtained sequences were compared with the previously reported CPNV

sequences using MEGA X (Molecular Evolutionary Genetics Analysis version X Center for Evolutionary Medicine and Informatics, Biodesign Institute, Arizona, USA) software (Tamura et al., 2013). Sequences were compared with the partial VP1 sequence of the American CPNV isolate R11/3 (Guy et al., 2011a) available at Genbank under accession number HM038436.1 and 9 sequences from the United Kingdom (Grau-Roma et al., 2017) available under Genbank accession numbers KU933595 to KU933603. Sequences were aligned using muscle method. A nucleotide distance matrix between sequences was computed to infer phylogenies and a neighbour-joining phylogenetic tree was generated. The partial VP1 CPNV sequences reported in this work have been deposited at GenBank under accession numbers MK531122 to MK531131.

Statistical analyses. IBM SPSS Statistics software (version 24.0, Armonk, NY: IBM Corp.) was used for statistical analyses. The distribution of the age variable was assessed by the Shapiro-Wilk test. Mann-Whitney test was used to compare the age between the histopathological (TVP and LP) and CPNV RT-PCR categories of the studied submissions. Chi-square was used to compare the proportions of histopathological lesions and of CPNV RT-PCR results between groups.

Results

Table 1 summarizes the number of submissions within each histopathological category, its age and the CPNV RT-PCR results. One hundred and thirty-five submissions were identified. Ninety-nine of them (73.3%) had at least 1 chicken with lesions compatible with TVP. The remaining 36 submissions (26.7%) contained no TVP-affected proventricular sections but included at least 1 proventriculus with LP lesions. Thirty-one out of the 135 submissions (23%) contained a mixture of TVP as well as LP proventricular sections, while the remaining ones contained only either TVP or LP-affected proventriculi. The youngest submission was 13 days old, corresponding to a TVP-affected case, while the oldest one was 59 days old, corresponding to a LP-affected case. The mean±SD and range of age

149 within the TVP and LP groups was 28.2±8.0 (13 to 49) and 30.0±7.7 (15 to 56) days, respectively. No
150 statistically significant differences were found between the mean of age in each group (p=0.705).
151 Figure 1 depicts the yearly distribution of the total number of submissions as well as of CPNV RT-PCR
152 positive cases. Data shows less than 5 submissions per year until 2009, when there was a rise in the
153 total number as well as of CPNV RT-PCR positive submissions, reaching a peak in 2013. The earliest
154 CPNV RT-PCR positive submission dates from 2009, and the most recent one from 2015.

155 A total of 452 proventricular sections were assessed histologically. Two hundred and forty-eight of
156 them (54.9%) fulfilled the TVP diagnostic criteria, 166 (36.7%) were classified as LP and the remaining
157 38 (8.4%) showed no inflammation or necrosis and were classified as 'without proventriculitis' (WP)
158 (Grau-Roma, et al., 2017). Each submission contained between 1 and 17 proventricular sections. The
159 submissions consisted of formalin-fixed tissues with very variable, often limited, information about
160 the macroscopical findings. Therefore, no data about the macroscopic lesions of the proventriculi was
161 included in the study.

162 Table 2 summarizes the histopathological results in the 3 studied categories. Per definition, necrosis
163 of oxynticopeptic cells was only observed in the group of TVP-affected animals, and lymphocytic
164 infiltration was not present in the proventricular sections classified as WP. The proportion of lesional
165 grades of lymphocytic infiltration and tubular metaplasia/hyperplasia was significantly different
166 between the TVP and the LP-affected samples, with the TVP-affected proventricular sections showing
167 a higher proportion of moderate (2) and severe (3) lesions than in LP-affected ones in both categories
168 ($p<0.001$). Tubular metaplasia and hyperplasia was only present in 18% of the WP sections, and it was
169 graded as mild (1) in most (5 out of the 7 cases) of them.

170 The characteristic histological lesions of the TVP-affected proventricular sections were either
171 multifocal or diffuse, the former typically affecting several submucosal glands and sparing other
172 intermingled glands in between. In addition to the characteristic TVP-lesions, other histological lesions
173 were observed within the interstitium of the submucosal glands in both TVP and LP-affected

proventricular sections. These included the presence of oedema (in 10 out of the 99 submissions with TVP and in 3 out of the 36 submissions with LP), which in few cases was accompanied with fibrin deposition (in 4 and 1 of the submissions with TVP and LP, respectively), an increased number of spindle cells (likely fibroblasts) with or without fibrosis (in 1 and 2 of the TVP and LP-affected cases, respectively), and the presence of myxomatous stroma (2 TVP- and 2 LP-affected). No haemorrhages were observed. Moreover, in 25 out of the 99 submissions with TVP (22%), low to moderate numbers of oxynticopeptic cells within or adjacent to the areas of necrosis showed intranuclear structures compatible to inclusion bodies, which were characterized by a pale eosinophilic centre and peripheral margination of the chromatin (Figure 2). The inclusions were only observed in proventricular sections with TVP-compatible lesions. No inclusions were observed in the submissions containing only LP-affected proventricular sections.

Twenty-two out of the 99 submissions classified as TVP (22%) and 4 out of the 36 (11%) classified as LP gave positive CPNV RT-PCR results, showing no statistically significant differences between groups (p=0.148). The youngest CPNV positive case was 21 days old (a TVP-affected case), while the oldest one was 56 days old (a LP-affected case). The oldest CPNV-positive TVP-affected submission was 49 days old. The mean of age of the CPNV RT-PCR positive submissions (32.8±9.6) was statistically higher compared to the CPNV RT-PCR negative ones (27.7±7.2) (p=0.032). Within the positive CPNV RT-PCR submissions, the mean±SD and range of age in the TVP and LP groups was 32.1±8.7 (21 to 49) and 36.0±14.5 (27 to 56) days, respectively. Within the negative CPNV RT-PCR submissions, the mean±SD and range of age in the TVP and LP groups was 27.1±7.5 (13 to 49) and 29.2±6.3 (19 to 42), respectively. No statistically significant differences were found between the mean of age of these groups. Only 1 out of the 25 TVP-submissions with intranuclear inclusions (4%) showed positive CPNV RT-PCR results, making the proportion of positive CPNV RT-PCR submissions within the 'TVP-submissions without inclusions' significantly higher [(21 out of 74, (28%)] than the one in the group of 'TVP-submissions with intranuclear inclusions' (p=0.01). All the submissions with the additional above-described lesions (oedema, fibrin, fibrosis and myxoid stroma) gave negative CPNV RT-PCR results.

A total of 10 sequences were obtained from the positive CPNV RT-PCRs. Phylogenetical analysis is depicted in Figure 3, where two clear clusters are observed. One of them is composed by the UK sequences from samples obtained in an study conducted in 2014-15 (Grau-Roma et al., 2017), the American reference sequence and the four more recent sequences of the present study, obtained from samples taken from 2013-2015. The other cluster includes sequences obtained from the oldest samples of the present study, collected from 2009-2011. The British sequences obtained in the present study showed a 78-100% nt similarity among them, whereas the percentage of similarity compared with the reference CPNV American sequence ranges from 75-92%.

Discussion

As far as the authors are aware, this is the first retrospective study on both TVP and CPNV. The study includes a TVP-affected case from 1994 and dates the first detection of CPNV back to 2009. This is five years before its first detection in the UK, where CPNV-positive TVP-cases were reported in a single prospective study from 2014 (Grau-Roma, et al., 2017), and 2 years before its first detection outside the USA (Marguerie, et al., 2011). The work performed by Marusak et al. (2012) in the USA included cases from between 2005 and 2009, without specifying the year of collection of the CPNV-positive TVP cases. The other few works reporting CPNV detection in TVP field cases did not provide the year of collection of the studied animals (Guy, et al., 2011b; Noiva, et al., 2015).

Present results show a rise in the number of TVP and positive CPNV RT-PCR submissions from 2009, with a peak in 2013, which suggests that they may be an emerging or re-emerging disease and pathogen, respectively. However, data about the total number of submissions to the APHA Lasswade laboratory during the period of the study was not available and therefore an eventual increase in the number of submissions, for example related to an increased awareness of the disease, cannot totally be ruled out.

This study confirms the recent detection of CPNV not only in TVP-affected cases but also in LP-affected cases (Grau-Roma, et al., 2017). As reported in the previous work, it seems likely for the group of LP-

affected cases to correspond to a mixture of cases with different aetiologies. Amongst them, a number of LP-affected cases with negative CPNV RT-PCR results may correspond to chronically affected TVP cases, where the virus is not detectable (Guy, et al., 2011b). In addition, cases with only multifocal lymphoid aggregates in the proventricular glands are considered to be 'normal findings' (Fletcher & Riddell, 2008), which may be therefore the case of some LP-cases showing only mild follicular aggregates. The latter may also explain the lower proportion of histopathological scores 2 and 3 in the group of LP-affected cases compared to the TVP-affected ones.

VP1 Partial sequencing of the CPNV RT-PCR positive samples showed differences in terms of nt percentage similarity and clustering. A temporal distribution was observed, so that "old" UK strains (from 2009-2012) clustered together and "new" (from 2013 onwards) made another branch together with the reference American strain. The previously suggested geographical different lineages between the European CPNV and the American CPNV sequences was not observed in the present study (Grau-Roma et al., 2017). A larger study including more and larger sequences would be needed in order to make further conclusions about the spatiotemporal evolution of this virus.

The here presented 22% of CPNV RT-PCR detection within the submissions showing TVP is lower than the 47% reported in the previous prospective study (Grau-Roma, et al., 2017). In both studies, the CPNV detection was performed in FFPE blocks following similar protocols of RNA extraction and RT-PCR. As the cases are derived from field material, the post-mortem examination procedure, tissue handling, type of fixative used as well as the length of fixation and storage were not standardised and are likely to have reduced the sensitivity of the RT-PCR (Lewis et al., 2001). This may account for some of the differences in these results (Lewis et al., 2001). In any case, the number of TVP cases or submissions with negative CPNV RT-PCR results is relevant in both studies (53% in Grau-Roma et al., (2017) and 78% in the present one). Certainly, TVP cases with negative CPNV RT-PCR results may be due to chronic stages of the disease or to the reduced RT-PCR sensitivity on formalin-fixed paraffin-embedded tissues. However, given the relatively high number of them, it seems reasonable to also

consider the possibility that other pathogens different from CPNV may cause similar proventricular lesions to the ones attributed to CPNV. Indeed, the number of pathogen factors that have over the years been suggested as cause of TVP is broad, and includes several viruses, bacteria, fungi, or parasites (Dormitorio, et al., 2007). In this regards, in addition to the characteristic TVP histological lesions, we observed the presence of other proventricular lesions. These included oedema, fibrin deposition, spindle cell proliferation and a myxomatous stroma, all of which gave negative CPNV RT-PCR results. No haemorrhages, which were previously described in some TVP-affected cases with positive CPNV RT-PCR results (Grau-Roma, et al., 2017), were observed in this study. Interestingly, intranuclear inclusion bodies were observed in 22% of the TVP cases. The inclusions were similar to the viral inclusions occasionally described in other works (Goodwin *et al.*, 1996; Grau-Roma, et al., 2010). Certainly, the number of structures within each case was often not abundant, and some may be non-viral, alternatively corresponding to degenerative features associated to the areas of necrosis. Nevertheless, the fact that the vast majority of submissions with intranuclear inclusions gave negative CPNV RT-PCR results (all but 1) contrasted with a CPNV RT-PCR positivity of more than one fourth (28%) within the group of submissions with TVP which did not show intranuclear inclusions. All together raises the question of whether another virus, different from the CPNV, is related to some of these TVP-affected cases. On this line, several other virus including adenovirus (Goodwin, et al., 1996), Gyrovirus (Li *et al.*, 2018) and picornavirus (Kim, et al., 2015), have been reported to be associated with TVP, although none of them could be definitively proved as cause of TVP. The investigation of such potential different aetiologies was however beyond the aims of the current work, and further studies are needed to get further insights in this area.

In summary, the present retrospective study indicates that TVP and CPNV are present in the British broilers since at least 1994 and 2009, respectively. Both the virus and the disease seem to have emerged or re-emerged in the recent years. CPNV was detected in a significant number of submissons with TVP, providing further evidence for this virus to be cause of TVP.

Disclosure statement

No potential conflict of interest was reported by the authors.

Table 1. Number of submissions classified as transmissible viral proventriculitis (TVP) and lymphocytic proventriculitis (LP) as well as showing positive *Chicken proventricular necrosis virus* (CPNV) RT-PCR results.

	Number and percentage of submissions	Age (days, mean±SD)	Positive CPNV RT-PCR (%)
TVP	99 (73.3%)	28.2±8.0	22 (22%)
LP	36 (26.7%)	30.0±7.7	4 (11%)
Total	135	28.7±7.9	26 (20%)

Table 2. Histopathological scoring results in the 3 histologically established categories: transmissible viral proventriculitis (TVP), lymphocytic proventriculitis (LP) and without proventriculitis (WP). The values correspond to the studied proventricular sections and the percentage is given within each category.

	Score	TVP	LP	WP
Oxynticopeptic cell necrosis	0	0	166 (100%)	38 (100%)
	1	132 (53%)	0	0
	2	77 (31%)	0	0
	3	39 (16%)	0	0
Lymphocytic infiltration	0	0	0	38 (100%)
	1	15 (6%)	63 (38%)	0
	2	73 (29%)	67 (40%)	0
	3	160 (65%)	36 (22%)	0
Tubular metaplasia and hyperplasia	0	4 (2%)	22 (13%)	31 (82%)
	1	26 (10%)	26 (16%)	5 (13%)
	2	62 (25%)	46 (28%)	0 (0%)
	3	156 (63%)	72 (43%)	2 (5%)

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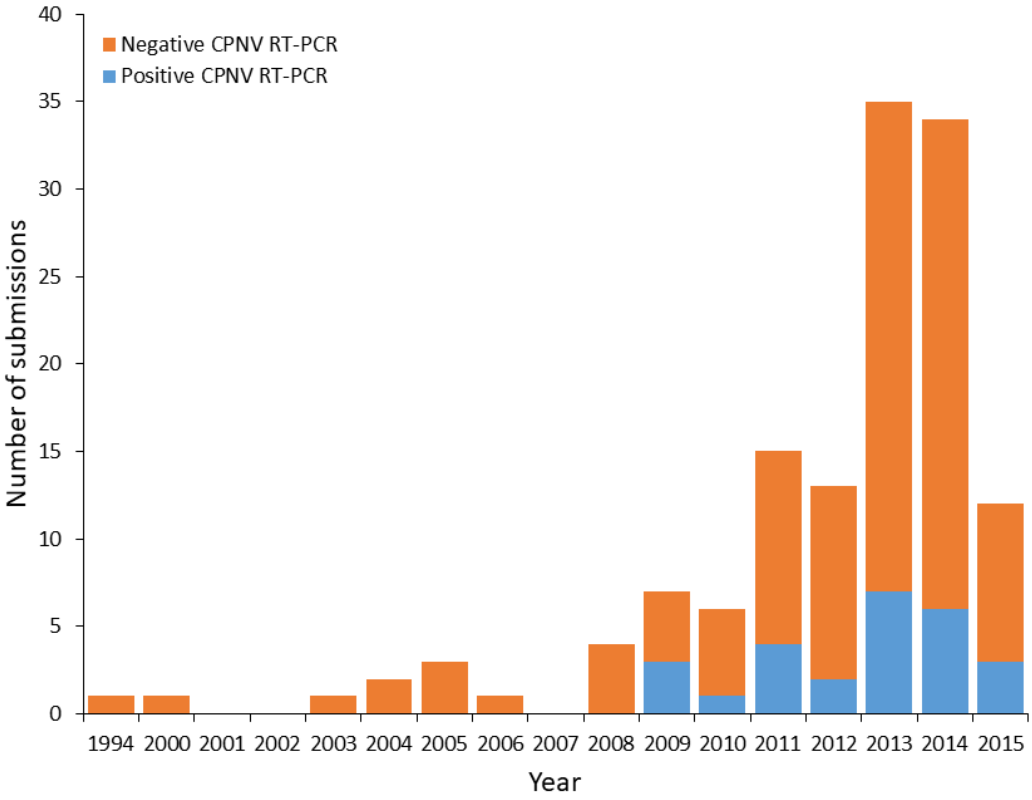


Figure 1. Number of submissions included in the study as well as number of positive Chicken proventricular necrosis virus (CPNV) RT-PCR per year.

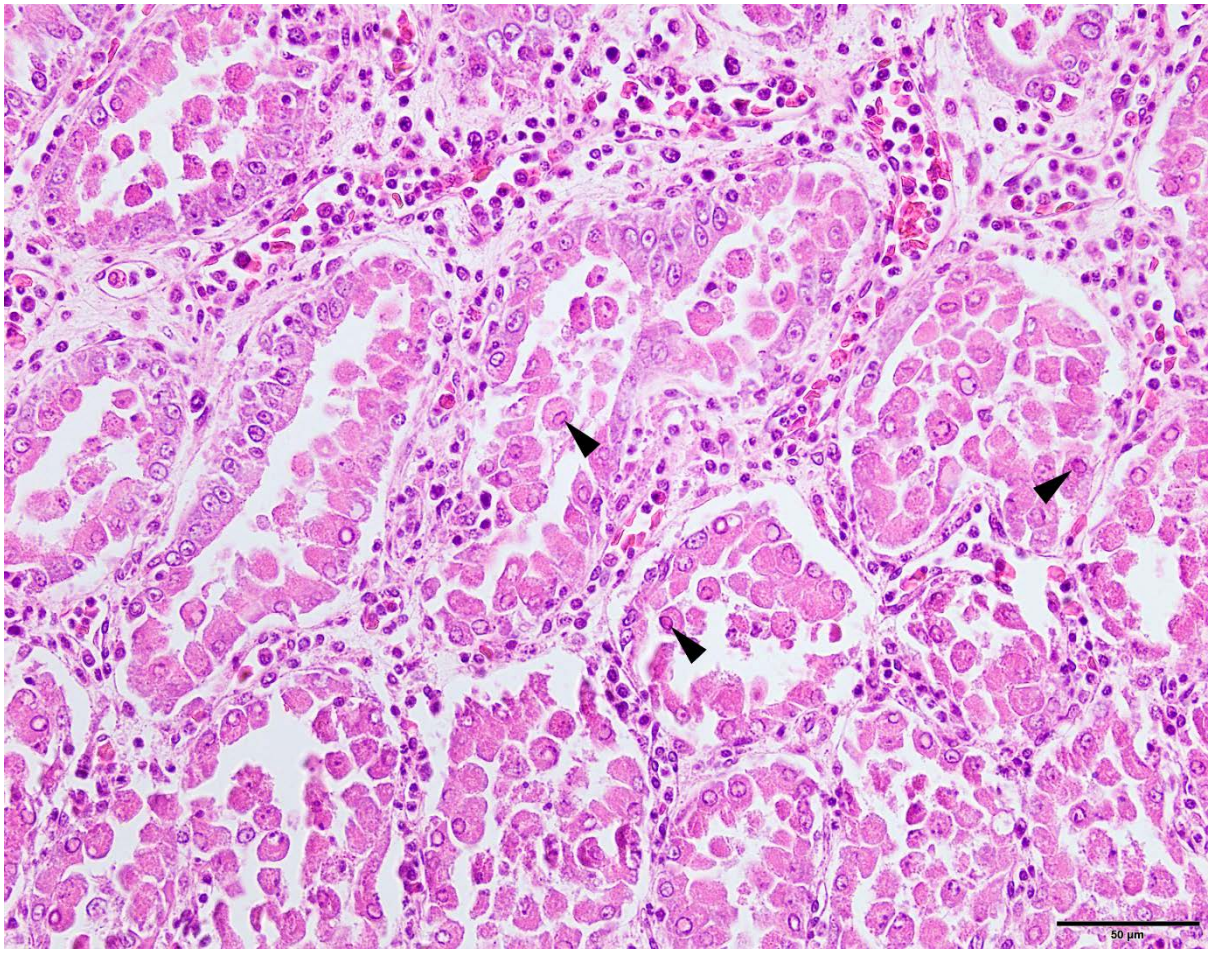
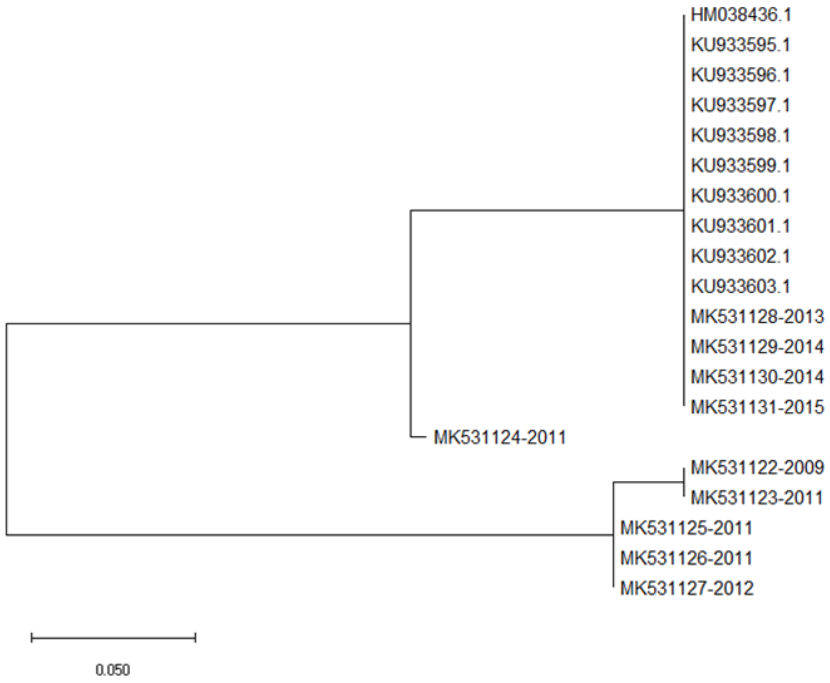


Figure 2. Proventriculus, transmissible viral proventriculitis-affected broiler chicken. Photomicrograph showing the presence of moderate numbers of pale eosinophilic intranuclear inclusion bodies with marginated chromatin in oxynticopeptic cells within areas of glandular necrosis (arrowheads). Haematoxylin and eosin.



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431 Figure 3. Phylogenetic tree-based on the neighbour-joining method for 20 partial (171 nt) VP1 CPNV

432 sequences. Sequences originate from: USA (HM038436.1), a previous study on UK samples (Grau-
433 Roma et al., 2017) (KU933595 to KU933603) and samples from the present study (MK531122 to
434 MK531131).

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