# Chicken proventricular necrosis virus related transmissible viral proventriculitis in broiler chickens in Poland

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## Abstract

# Background.

Transmissible viral proventriculitis (TVP) is an infectious disease reported in all production types of chickens. TVP is manifested in decreased body weight gains, wide weight diversity of birds in the flock and poor feed conversion. Histopathological examination seems to be the most reliable method for confirming the disease. Although TVP etiology has not been explicitly defined, a novel virus identified as a member of the *Birnaviridae* family, named chicken proventricular necrosis virus (CPNV) has been isolated from clinical cases of TVP and it is now considered as a potential factor of a disease. The study was undertaken in order to reproduce the disease under laboratory conditions and to evaluate the etiology of first described Polish case of TVP.

# Results.

Anatomopathological and histopathological evaluation reveled that we've succeeded to reproduce TVP in broiler chickens. Within 14 days after infection birds gained approximately 30,38% less body weight in comparison to Control group. In TVP infected group a seroconversion against FAdV and IBDV was recorded 14 days post infection (dpi). 14 dpi CPNV was detected in proventriculi, while FAdV in spleens and livers of infected birds.

# Conclusions.

We have demonstrated that CPNV was involved in the development of the disease. We did not record the presence of IBDV in the TVP or control birds, despite our recording a strong seroconversion against IBDV in the birds from the TVP group. CPNV belongs to the same family as IBDV, which allows us to assume serological cross-reactivity between them. This possibility of CPNV infections affecting IBDV antibody levels detected by commonly available ELISAs should be taken into account under poultry field conditions and diagnosis. The role of FAdV in the development of TVP needs further evaluation.

## Background

Transmissible viral proventriculitis (TVP) is an infectious disease reported in the production of all types of chickens and having significant impact on the poultry industry. The typical pathological lesions observed in the course of TVP affect the proventriculus and are described as proventricular enlargement, thickening of its walls and spotty discoloration in the cross-section. In isolated cases, small hemorrhagic changes are observed in the proventricular mucosa [1].

The first reports on TVP date back to 1978 and come from the Netherlands [2], when Kouwenhoven et al. reported a case of proventriculitis in commercial chicken broilers and proved that TVP was induced by an

infectious factor. Since then, TVP cases have been identified and reported in the USA, Australia, China, South Korea, Spain, France, the UK, and Poland among other countries [3–10].

The etiology of TVP has not been explicitly defined so far. Studies on TVP etiopathology imply the involvement of infectious bursal disease viruses (IBDV) of the *Birnaviridae* family, IBDV-like viruses, infectious bronchitis viruses (IB) of the *Coronaviridae* family, reoviruses (REO), picornaviruses, fowl adenoviruses (FAdV), adeno-like viruses, or mixed infections in the development of typical pathological lesions [3–6, 8, 10–15]. Recently a novel virus identified as a member of the *Birnaviridae* family has been isolated from clinical cases of TVP. Preliminary studies confirmed that this virus differed significantly from the *Avibirnavirus* genus IBDV. This virus was named chicken proventricular necrosis virus (CPNV), and an RT-PCR method has been developed which enables its detection [13]. Unfortunately, cases of TVP which were negative for CPNV as well as cases positive for CPNV presence without typical TVP changes has also been reported [12], which makes the etiology of TVP unresolved and further research are still necessary.

Clinically, TVP is manifested mainly in broiler chickens by decreased body weight gains, wide weight diversity of birds in the flock and an increased feed conversion ratio [1]. The disease, which usually affects up to 50% of the birds in the flock, can significantly reduce the cost-effectiveness of production.

Considering its problematic etiology, TVP diagnosis is difficult. Histopathological examination seems to be the most reliable method for confirming the disease. The histopathological lesions observed in the case of TVP exclusively affect the proventriculus and are manifested in a triad of lesions related to the necrosis of glandular epithelial cells (even up to 80% of cells in the proventricular mucosa), a strong lymphatic infiltration in the lamina propria of the mucosa and among the proventriculus glands, hypertrophy of the epithelial cells of the excretory ducts of proventricular glands with successive replacement of the epithelial glandular cells with hypertrophied cells of the excretory ducts. The severity of these lesions can vary depending on the duration of the disease [1, 16].

Given the recent cases of TVP recorded in Poland [9], a laboratory-conditions study was undertaken that attempted to reproduce the clinical course of TVP in broiler chickens by inoculating them with a homogenate of proventriculi from a confirmed TVP field case. The research was also aimed at identifying the etiological factor in domestic cases of TVP.

## Results

# Body weight

The mean body weight of the control and TVP-infected birds is summarized in Table 1. Over a 14-day span after the infection, the birds from the control group gained 1.58 kg on average, while the birds from the TVP group gained 1.1 kg. In other words, after the infection, the body weight gain in the TVP group was significantly less and lower by 30.38% than in the control group.

Table 1
Mean body weight of birds in TVP and Control groups.

| Group | Mean body weight ± SD at different time points after infection | |
|---|---|---|
| | 0 dpi | 14 dpi |
| Control | 1,54 ± 0,14 | 3,12 ± 0,23 |
| TVP | 1,56 ± 0,16 | 2,66 ± 0,42* |
| Birds of TVP group was infected with TVP-positive proventriculi homogenate on the 28th day of life (0 dpi). Within 14 days after the infection birds of TVP group gained approximately 30,38%% less body weight in comparison to Control birds. | | |
| * significant difference in the mean body weight between birds of the TVP and the control group at the same dpi. | | |

# Gross Lesions

No lesions typical of TVP were recorded in any of the groups at the time of TVP infection on the 24th day of life. In the TVP group at 14 days after the infection, enlargement of the proventriculus was noted in 6 out of the 8 birds examined, thickening of the proventricular wall was also observed in 6 out of 8, and discoloration was registered in 5 (Fig. 1). No TVP lesions were recorded in the control group at this time. The relevant data are summarized in Table 2. No other anatomopathological lesions were observed in any of the birds from either the control or TVP groups.

Table 2
Prevalence of gross lesion in birds of TVP and Control groups.

| Gross lesion recorded in proventriculus | Number of birds in the group with lesions at different dpi | | | |
|---|---|---|---|---|
| | Control | | TVP | |
| | 0 dpi | 14 dpi | 0 dpi | 14 dpi |
| Enlargement | 0/4 | 0/8 | 0/4 | 6/8*/** |
| Thickening | 0/4 | 0/8 | 0/4 | 6/8*/** |
| Discoloration | 0/4 | 0/8 | 0/4 | 5/8*/** |

Birds of TVP group were infected with TVP-positive proventriculi homogenate at 28 days of life (0dpi). In this group gross lesions associated with TVP, which were characterized as enlargement of the proventriculus (enlargement), thickening of the proventriculi wall (thickening) and discoloration recorded in the proventriculi wall cross-section (discoloration) were recorded at 14 dpi. No changes were observed in the proventriculi of Control birds.

* significant difference in the number of proventriculi with gross lesion in TVP group in comparison to the Control group at the same dpi.

** significant difference in the number of proventriculi with gross lesion within the group (TVP or Control) at 14 dpi in comparison to 0 dpi.

# Histopathological Lesions

Histopathology revealed no TVP lesions in the proventriculi of birds from either group on the day of infection. In the TVP group at 14 dpi, necrosis, hyperplasia, and infiltration (Fig. 2) were recorded in 7 out of the 8 proventriculi examined. No lesions were recorded in the control group at this time. The results of the histopathological examination are provided in Table 3.

Table 3
Prevalence of histopathological lesion in birds of TVP and Control groups.

| Histopathological lesion recorded in proventriculus | Number of birds in the group with lesions | | | |
|---|---|---|---|---|
| | Control | | TVP | |
| | 0 dpi | 14 dpi | 0 dpi | 14 dpi |
| Necrosis | 0/4 | 0/8 | 0/4 | 7/8*/** |
| Hyperplasia | 0/4 | 0/8 | 0/4 | 7/8*/** |
| Infiltration | 0/4 | 0/8 | 0/4 | 7/8*/** |

Birds of TVP group were infected with TVP-positive proventriculi homogenate at 28 days of life (0dpi). In this group histopathological lesions associated with TVP, which were characterized as necrosis of glandular epithelium (necrosis), hypertrophy and hyperplasia of ductal epithelium, replacement of glandular epithelium by hyperplastic ductal epithelium (hyperplasia) and multifocal to severe infiltration of lymphoid cells (infiltration) were recorded at 14 dpi. No histopathological changes were observed in Control birds.

* significant difference in the number of proventriculi with histopathological lesion in TVP group in comparison to the Control group at the same dpi.

** significant difference in the number of proventriculi with histopathologica lesion within the group (TVP or Control) at 14 dpi in comparison to 0 dpi.

# Serological Results

The results of the serological examination are summarized in Table 4. In the TVP group, a significant increase in the anti-FAdV and anti-IBDV antibodies level was recorded 14 dpi in comparison to their level on the day of infection. At the same time, levels of these antibodies were significantly higher in the TVP group than in the Control group 14 dpi.

Table 4
Mean IB, REO, FAdV and IBDV antibody levels ± SD in Control and TVP group at different dpi.

| Group | Mean S/P ± SD (positive/tested) at different time points after infection | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IB | | REO | | FAdV | | IBDV | |
| | 0 dpi | 14 dpi | 0 dpi | 14 dpi | 0 dpi | 14 dpi | 0 dpi | 14 dpi |
| Control | 0,08 ± 0,049 (0/8) | 0,188 ± 0,098 (0/8) | 0,624 ± 0,013 (0/8) | 0,404 ± 0,026 (0/8) | 0,126 ± 0,053 (0/8) | 0,118 ± 0,056 (0/8) | 0,093 ± 0,045 (0/8) | 0,036 ± 0,060 (0/8) |
| TVP | 0,060± 0,121 (0/8) | 0,212 ± 0,177 (0/8) | 0,760 ± 0,051 (0/8) | 0,399 ± 0,055 (0/8) | 0,092 ± 0,043 (0/8) | **0,536 ± 0,327*/** (4/8)** | 0,044 ± 0,043 (0/8) | **0,856 ± 0,881*/** (8/8)** |
| In birds of TVP infected group a strong seroconversion against IBDV and FaDV was recorded 14 dpi. | | | | | | | | |
| * significant difference in the specific antibody level in TVP group in comparison to the Control group at the same dpi | | | | | | | | |
| ** significant difference in the specific antibody level within the group (TVP or Control) at 14 dpi in comparison to 0 dpi. | | | | | | | | |

# Molecular Biology

The results of molecular studies are shown in Table 5. All samples collected at 0 and 14 dpi in the control group were negative in all assays. In the TVP group at 0 dpi, all samples were also negative. In this group at 14 dpi, the samples of proventriculi (100% of samples tested) were positive for CPNV, while those of spleens and livers were positive for FAdV.

Table 5
Results of molecular studies.

| Group | Internal organ tested | Number of positive samples / tested at different dpi | | | | | |
|---|---|---|---|---|---|---|---|
| | | IBDV | | FAdV | | CPNV | |
| | | 0 dpi | 14 dpi | 0 dpi | 14 dpi | 0 dpi | 14 dpi |
| Control | Proventriculus | 0/4 | 0/4 | nd.* | 0/4 | 0/4 | 0/4 |
| | Liver | 0/4 | 0/4 | nd. | 0/4 | nd. | nd. |
| | Spleen | 0/4 | 0/4 | nd. | 0/4 | nd. | nd. |
| | Intestines | 0/4 | 0/4 | nd. | 0/4 | nd. | nd. |
| | BF | 0/4 | 0/4 | nd. | nd | nd. | nd. |
| TVP | Proventriculus | 0/4 | 0/4 | nd. | 0/4 | 0/4 | **4/4** |
| | Liver | 0/4 | 0/4 | nd. | **3/4** | nd. | nd. |
| | Spleen | 0/4 | 0/4 | nd. | **3/4** | nd. | nd. |
| | Intestines | 0/4 | 0/4 | nd. | 0/4 | nd. | nd. |
| | BF | 0/4 | 0/4 | nd. | nd. | nd. | nd. |

Table presents the results of IBDV, FAdV and CPNV prevalence in the internal organs of birds of Control and TVP groups at different dpi.

* Not done

# Discussion

Clinically, TVP is manifested by lower body weight gains, wide weight diversity of birds in the flock and an increased feed conversion ratio [1]. Goodwin et al. [4] reported that suboptimal body weight gains could result from pepsinogen- and hydrochloric acid-producing cell destruction and that, in severe cases of TVP, 80% of these cells are destroyed due to necrosis caused by infection. In the course of TVP no increased mortality is observed in the flock, but the number of culled birds increases significantly. In our study no increased mortality was manifest but poorer body weight gain was recorded in the TVP group. Additionally, in this group both the anatomical and histopathological changes typical of TVP were observed. Given the fact that such lesions were not recorded in the control group and that no other lesions apart from those related to TVP were observed in any of the groups examined, we may conclude that we have succeeded in the reproduction of TVP under experimental conditions. Our study showed that over the 14-day period post infection the body weight gains of birds were lower by over 30% than those of the control birds.

To date, the etiological agent causing TVP has not been clearly established. However, the infectious nature of the disease has been emphasized many times, which is reflected in our work. Studies on TVP etiopathology imply the coaction of infectious bursal disease viruses, infectious bronchitis viruses, reoviruses, picornaviruses, adenoviruses, adeno-like viruses, IBDV-like viruses, or mixed infections in the development of characteristic lesions [3–6, 8, 10–15]. Recently, CPNV was suggested as a possible TVP causative agent [6]. With what is currently known about CPNV, it seems that the first description of this virus comes from 1996 when Goodwin et al. [4] observed hexagonal virus particles in the nuclei and cytoplasm of proventricular cells in a case of TVP [4]. However, a more detailed description along with the taxonomy of CPNV was provided later by Guy et al. [13]. Chicken proventricular necrosis virus is a twenty-walled, non-enveloped virus with a diameter of approximately 75 nm. As genetic material, it has double-stranded RNA, organized into two segments (c. 3.8 and 3.4 kbp). Within the putative viral protein-1–encoding gene (VP), CPNV has a motif characteristic of *Birnaviridae* encoding the RNA-dependent RNA polymerase. However, based on the phylogenetic studies of the VP1 gene, it has been established that this virus is different from other known members of this family of viruses. It is interesting that clinical TVP was reproduced with the use of isolated CPNV [7].

Recent research from the UK also suggests the involvement of CPNV in the development of TVP in chickens. In two studies, the authors reported that CPNV was detected in 22 and 47% of confirmed clinical cases of TVP [11, 12]. It remains unknown, however, if all cases of this disease are of the same infectious origin. There are cases of TVP in which CPNV cannot be identified, as well as disease cases in which CPNV can be identified but which do not meet the diagnostic criteria of TVP [7, 11, 12]. In addition, it is suggested that the pathogenesis of TVP could be more complex and may result from other viral, bacterial or fungal co-infections [1].

Due to seroconversion against FAdV and IBDV being observed in the studied TVP group, we performed a further molecular analysis to confirm the presence of these viruses as well as the CPNV. At 14 dpi, we confirmed the presence of CPNV in 100% of the proventriculus samples collected from birds from the TVP group, which indicates the involvement of this virus in TVP development. We also confirmed the presence of FAdV in the TVP-infected birds. It is difficult to clearly state the nature of FAdV's involvement in TVP development in our study. In our opinion, two hypotheses are likely. The first is that CPNV infection contributed to the activation of a latent FAdV infection in the experimental birds and the second is that these viruses were present in the infectious material. However, the fact that FAdV was not detected in the proventriculi of TVP-infected birds but only in spleen and liver samples supports the hypothesis of latent infection. The explanation of these relationships requires further research. Once again, these results highlight the complex nature of TVP etiology.

# Conclusions

In this experiment we were able to reproduce the clinical TVP in broiler chickens under laboratory conditions with the use of homogenates of proventriculus from confirmed, first described Polish case of TVP. We have demonstrated that CPNV was involved in the development of the disease. To our surprise,

we did not record the presence of IBDV in the TVP or control birds, despite our recording a strong seroconversion against IBDV in the birds from the TVP group. The putative etiological agent CPNV belongs to the same *Birnaviridae* family as IBDV, which allows us to assume serological cross-reactivity between them. This should be taken into account during serological evaluation under field conditions, as the possibility also should of CPNV infections affecting IBDV antibody levels detected by commonly available ELISAs. On the other hand, the possibility of using IBDV ELISA kits for TVP diagnosis might be considered. Undoubtedly, these issues, as well as the contribution of FAdV in the development of TVP require further research.

## Methods

# Birds

Commercial Ross 308 broiler chicks of both sexes, purchased from one hatchery and one hatch, were used in the experiment. The trial was conducted in isolated pens of the Pavilion of Experimental Poultry Infections, at the Department of Avian Diseases, University of Warmia and Mazury in Olsztyn, which are maintained at a biosafety PCL 3 conditions. Water and feed were given to birds *ad libitum*. Rearing conditions were consistent with Aviagen (Aviagen, USA) recommendations.

# Experiment Layout

The experiment was carried out with 24 commercial broiler chickens. The birds were reared until day 24 of life, after which they were divided into two groups (n = 12). One served as the control, and the other was infected with a proventriculus homogenate originating from a confirmed field TVP case. Before the infection (0 days post infection – dpi), 4 birds selected randomly from each group were euthanized and subjected to autopsy examination to check for macroscopic lesions typical of TVP. Afterwards, proventriculi were collected for histopathological examination, and samples of them and of other internal organs (intestines, spleen, liver, and bursa of Fabricius) were taken for molecular analysis. For histopathological examination, the proventriculus samples were fixed in a 10% formaldehyde solution. Samples for molecular analysis were frozen at − 20 °C until they were analyzed. The remaining birds from both groups were weighed and blood samples were collected for serological analysis. Blood was centrifuged at 1 500 × g for 15 minutes and the serum obtained was frozen at − 20 °C until it was analyzed. After infection, the birds were reared for the next 14 days. At 14 dpi, blood was sampled from all birds from both groups for serological analysis. Next, the birds were weighed and euthanized. Pathological lesions in the proventriculus were investigated and recorded during the anatomopathological examination, after which samples of this organ were collected for histopathological and molecular examinations. Production results were presented as the mean body weight (kg) +/− standard deviation (SD).

For euthanasia, chickens were placed in a chamber with Carbogen (95% O2 + 5% CO2). After 1 min, Carbogen was slowly replaced by 100% $CO_2$. This method of euthanasia is responsible for stress reduction in the birds.

# Proventriculi Homogenate And Infection

Proventriculi from the first Polish case of TVP [9] were used for the experimental infection. After homogenization, proventriculus samples were processed through three freeze–thaw cycles. After centrifugation (2 000 × g, 15 minutes), the supernatant was stored and used for the infection. Broiler chickens from the TVP group were infected with 5 mL of the supernatant per bird. The supernatant was given to birds *per os*, directly with a probe to the crop. At the same time, the birds from the control group were given PBS in the same way.

# Serological Analysis

A commercial kit of ELISA Ab Tests (IDEXX Laboratories, USA) was used to determine the titer of anti-IBV, anti-REO, anti-FAdV, and anti-IBDV specific IgY in broiler serum. Particular stages of the tests were performed with an Eppendorf epMotion 5075 LH automatic pipetting station (Eppendorf, Germany), a BioTek ELx405 automatic multi-well plate washer (BioTek, USA), and a BioTek ELx800 plate reader. Sample to positive (S/P) ratio, plus/minus standard deviation (SD) were computed for each group in each sampling period.

# Gross Lesion Evaluation

The birds were investigated for the presence of lesions typical of TVP in the proventriculus during an anatomopathological examination. Enlargement of the proventriculus coupled with thickening of its walls and their spotty discoloration in the cross-section were recorded. Results were collated as the number of birds with recorded pathological lesions relative to the total number of birds examined.

# Histopathology

During necropsy, samples of the central part of the proventricular wall were embedded in 10% formalin (pH 7.4) and processed for histopathological examination. After passing the samples through intermediate liquids (increasing concentrations of alcohol and xylene) they were embedded in paraffin blocks. Sections of the examined samples 4 µm thick were stained with hematoxylin–eosin and microscope samples were scanned with a Pannoramic MIDI scanner (3DHISTECH, Budapest, Hungary). Samples were considered TVP positive if the following histopathological lesions were registered during examination: necrosis of glandular epithelium (necrosis), hypertrophy and hyperplasia of ductal epithelium, replacement of glandular epithelium by hyperplastic ductal epithelium (hyperplasia) and

multifocal-to-severe infiltration of lymphoid cells (infiltration). For PCR analysis, five 4 um sections from each paraffin block (each bird) were put into xylene and prepared for PCR analysis.

# CPNV Identification

Paraffin fixed sections of the proventriculi were used for CPNV identification. One mL of xylene was added to each sample comprising five paraffin-fixed proventriculus sections (each 5 µm thick) and these were incubated for 5 minutes at 50 °C in order to remove paraffin residues. Further RNA isolation steps were performed with an Isolate II FFPE RNA/DNA Kit (Bioline, London, UK) according to the manufacturer's recommendations. Concentration and the purity of isolated RNA were established with the use of a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to transcribe the RNA. The reaction was performed with 2 µL of 10X RT Buffer, 0.8 µL of 25X dNTP Mix (100 mM), 0.5 µL of 100 µM 5'- GGGCGGTAACCATTCAGATA- 3' reverse primer, 1 µL of MultiScribe Reverse Transcriptase, 1 µL of RNase Inhibitor, 4.7 µL of nuclease-free water and 10 µL of RNA (previously incubated for 5 min at 99 °C). The PCR was performed as described previously [13]. The amplification of the target 171 bp CPNV gene was performed with the use of a HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) and carried out with 10 µL of HotStarTaq Plus DNA Polymerase, 0.1 µL of each 100 µM primer, 2 µL of CoralLoad PCR Buffer, 5,8 µL of RNase-free water and 2 µl of cDNA. After pre-denaturation at 95 °C for 5 min, the denaturation step was performed at 94 °C for 1 min, followed by primer annealing at 55 °C for 1 min, product elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. Thirty-five replication cycles were performed.

# IBDV Identification

Total RNA was extracted directly from the homogenised internal organs of the examined birds. Briefly, 700 µL of sterile PBS was added to each sample (0.2 g) and then samples were homogenized with the use of a TissueLyser II (Qiagen). Samples were centrifuged at 1 500 × g for 30 s. After centrifugation, 200 µL of the supernatant was used for further steps of RNA isolation, which were performed with an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Reverse transcription was performed with the use of High-Capacity cDNA Reverse Transcription Kit (Life Technologies). IBDV was detected in the samples adopting the method described previously by Lin et al. [17] again with the use of a HotStarTaq Plus Master Mix Kit (Qiagen) and the following set of primers: IBDV F (sense primer): 5' CCCAGAGTCTACACCATA 3' and IBDV R (antisense primer): 5' TCCTGTTGCCACTCTTTC 3'. The reaction was performed with 10 µl of HotStarTaq Plus DNA Polymerase, 0,1 µl of each 100 µM primer, 2 µL of CoralLoad PCR Buffer, 5,8 µl of RNase free water and 2 µl of cDNA. After pre-denaturation at 95 °C for 5 min, the denaturation step was performed at 94 °C for 1 min, followed by primer annealing at 55 °C for 1 min, product elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. Thirty-five replication cycles were performed.

# FAdV Identification

Total DNA was extracted directly from the internal organs of the examined birds. Extraction was performed using a DNA Mini Kit (Qiagen) according to the manufacturer's procedure. DNA templates were stored at − 20 °C until further analysis. DNA obtained from the reference strain type/species 2/D served as a positive control.

The specific oligonucleotide primers were used for the amplification of the loop L1 region of the hexon gene of all FAdV types. The primers were synthesised at the Genomed company (Warsaw, Poland). The sequences of nucleotide primers were as follows: FAdV F (forward primer) – 5'ATGGGAGCSACCTAYTTCGACAT 3' and FAdV R (reverse primer) – 5'AAATTGTCCCKR AANCCGATGTA 3'. The expected product size was 830 bp.

The reaction was conducted in a final volume of 25 µL, which contained 2.5 µL of 10X PCR buffer, 1 µL of dNTP (10 mM), 1.5 µL of each primer (10 µM), 2 µL of DNA template, and 11.5 µL of sterile water. After the pre-denaturation at 95 °C for 5 min, the denaturation step was performed at 94 °C for 45 s, followed by primer annealing at 55 °C for 1 min, product elongation at 72 °C for 2 min, and final elongation at 72 °C for 10 min. Thirty-five replication cycles were performed. Amplification was conducted in a basic gradient thermocycler (Biometra, Göttingen, Germany).

After amplification, electrophoresis was carried out in 2% agarose gel with 1 µg/mL of ethidium bromide. Electrophoresis was conducted in Tris borate EDTA buffer, pH 8.2, (150 V and 80 mA) for 50 min in a Mini-Sub Cell (Biorad, Hercules, CA, USA). After gel electrophoresis, the size of the amplicons was compared with the MassRuler Low Range DNA ladder to 1 031 bp (Fermentas/Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The results were visualised using a UV transilluminator, then photographed and analysed.

The results were considered positive when the DNA product obtained had the predicted size of 830 bp.

**Statistics** Statistical analysis was performed with GraphPad Prism 6.05 with the use of Mann Whitney *U* test. Differences were considered statistically significant at $p < 0.05$.

# Abbreviations

Ab - antibodies

cDNA - complementary DNA

CPNV - chicken proventricular necrosis virus

DNA - deoxyribonucleic acid

dpi - day post infection

ELISA - enzyme-linked immunosorbent assay

FAdV - fowl adenovirus

FFPE - formalin fixed paraffin embedded

IB - infectious bronchitis

IBDV - infectious bursal disease virus

PBS - Phosphate Buffered Saline

PCL - Process Control Laboratory

PCR - polymerase chain reaction

REO - reovirus

RNA - ribonucleic acid

RT-PCR - reverse transcriptase polymerase chain reaction

TVP - transmissible viral proventriculitis

## Declarations

# Ethics approval and consent to participate

The experimental procedures and animal handling procedures were conducted with the approval of the Local Ethic Committee for Animal Experiments in Olsztyn, Poland (resolution No. 50/2017).

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare no competing interests.

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# Authors' contributions

MŚ designed and performed the study, performed ELISA analysis, analyzed the data, wrote the manuscript, MG performed histopathological evaluation, DD performed IBDV and CPNV detection, JSN performed FAdV detection, AK supervised the study and helped write the manuscript. All authors have read and reviewed the manuscript. The author (s) read and approved the final manuscript.

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# Figures

**Figure 1**

Gross lesions. Proventriculi of TVP (left) and Control (right) broiler chicken examined at 14 dpi. Typical pathological lesions observed Proventriculi enlargement, thickening of its walls and spotty discoloration in the cross-section were recorded in 6 out of 8 TVP infected birds, which are the three characteristic gross lesions related to transmissible viral proventriculitis.

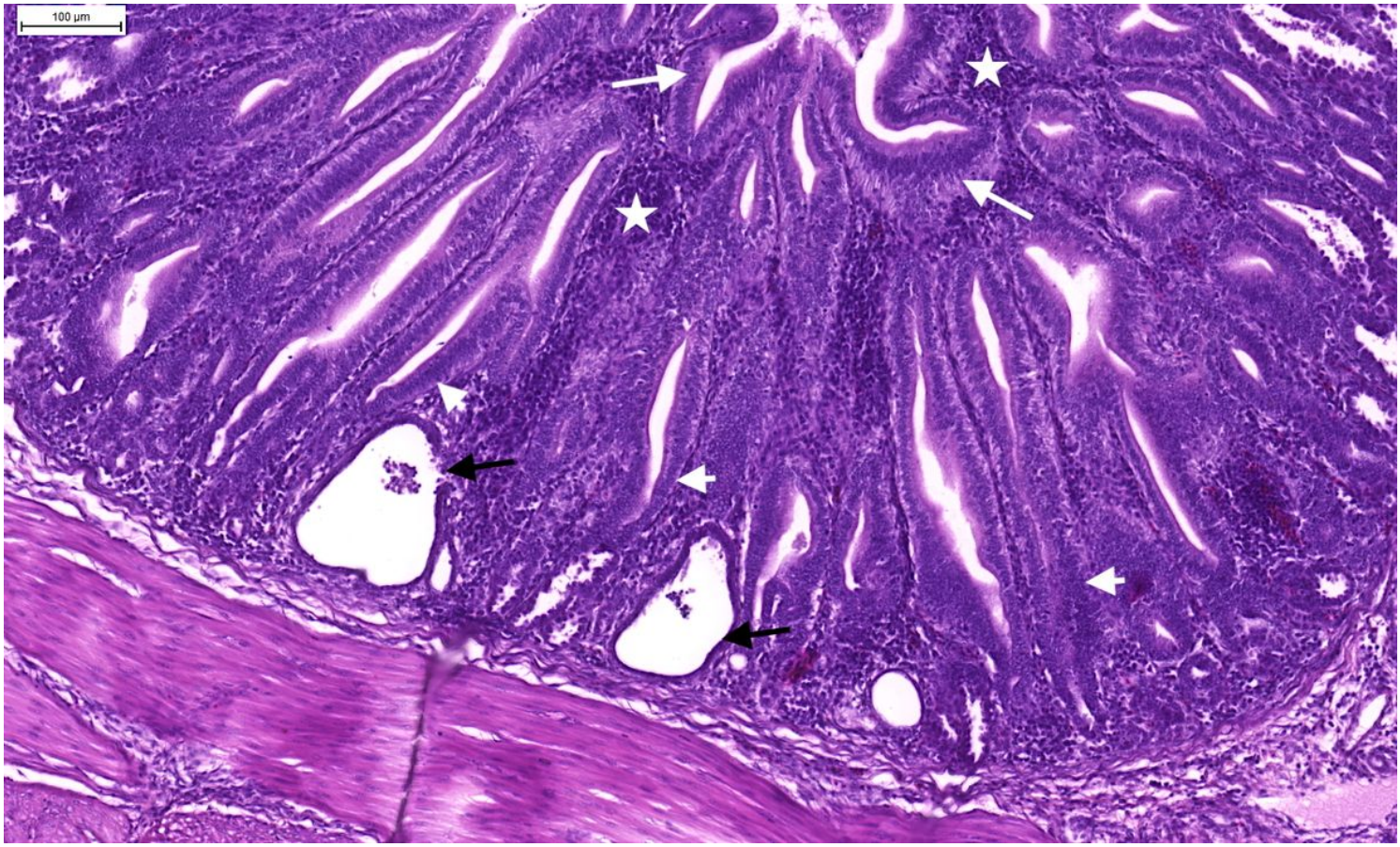


**Figure 2**

Histopathological lesions. Histopathological lesions typical for transmissible viral proventriculitis were recorded in 7 out of 8 examined proventriculi of birds from TVP group. The lesions concerned necrosis of glandular epithelium (black arrows), hypertrophy and hyperplasia of ductal epithelium (white arrow), replacement of glandular epithelium by hyperplastic ductal epithelium (white arrowhead) and infiltration of lymphoid cells (asterisks).

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