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Original article

Relationship between chicken proventricular necrosis virus prevalence and transmissible viral proventriculitis in broiler chickens in Poland

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Abstract

Transmissible Viral Proventriculitis (TVP) is a disease of chickens which contributes to significant production losses. Recent reports indicate the role of chicken proventricular necrosis virus (CPNV) in the development of TVP. However, the relationship between CPNV and TVP is inconclusive and it has been addressed in just a few reports.

Given the above, a study was conducted to identify the relationship between TVP and CPNV prevalence in broiler chickens in Poland.

The study was carried out on 35 proventriculi samples sent for histopathological (HP) examination to the Faculty of Veterinary Medicine in Olsztyn between 2017 and 2019. After HP examination, TVP positive samples were processed for CPNV identification by RT-PCR. TVP was the most common pathological condition of proventriculi (23 cases). CPNV was identified in 10 out of those 23 cases. The average HP score, and the average necrosis and infiltration score for CPNV-positive samples was significantly higher than in CPNV-negative ones. The average age of the CPNV-positive chickens was significantly lower than in CPNV-negative birds.

Our study confirms the role of CPNV in TVP pathogenesis and it seems that preservation of the proventriculi in the early stages of the disease, when the lesions are more pronounced, should result in a greater probability of CPNV detection.

Key words: transmissible viral proventriculitis, chicken proventricular necrosis virus, prevalence, broiler chickens

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Introduction

Transmissible viral proventriculitis (TVP), a viral infection of the proventriculus, has been described in chickens of all production types, and the disease causes significant production losses. It has recently been estimated that body weight gains can decrease in the course of TVP by more than 30% over 14 days post infection (Śmiałek et al. 2020). Since the first description of TVP in 1978, when it was diagnosed in the Netherlands, cases have been identified and described in the USA, Australia, China, South Korea, Spain, France, the UK, and Poland (Kouwenhoven et al. 1978, Goodwin et al. 1996, Yu et al. 2001, Dormitro et al. 2007, Grau-Roma et al. 2010, Marquerie et al. 2011, Hafner and Guy, 2013, Kim et al. 2015, Śmiałek et al. 2017).

To date, histopathological examination has been considered the most reliable means of TVP diagnosis. The histopathological lesions in the course of TVP recorded in the proventriculus are characterized by a triad of lesions related to the necrosis of glandular epithelial cells, lymphatic infiltration in the lamina propria and the hypertrophy of the epithelial cells of the excretory ducts with replacement of the epithelial glandular cells by those cells. What is more, histopathological examination enables differentiation of this disease from cases of other poultry diseases without proventriculus enlargement, which may be caused by intoxication with biogenic amines, zinc sulfate, or mycotoxins. It can also differentiate TVP from disorders with proventriculus enlargement, but without inflammatory manifestations, which may be caused by ingestion of low-fiber feed, for example (Hafner and Guy, 2013).

The etiology of TVP has not been explicitly defined to this day. TVP was recreated under experimental conditions by inoculating chickens with a homogenate of diseased proventriculi, hence it is known for certain that TVP is induced by an infectious agent transmitted by the horizontal route. Ample works addressing the etiopathogenesis of TVP suggest the involvement of the Gumboro disease and infectious bronchitis viruses, reoviruses, picornaviruses, adenoviruses, adeno-like viruses, IBDV-like viruses, or mixed infections in the development of lesions typical of TVP (Goodwin et al. 1996, Yu et al. 2001, Dormitro et al. 2007, Grau-Roma et al. 2010, Marquerie et al. 2011, Guy et al. 2011a, Kim et al. 2015). Recently, a virus belonging to the Birnaviridae family has been isolated from clinical TVP cases and identified, but its preliminary phylogenetic analysis showed that it differed significantly from the Gumboro disease viruses. It was defined as chicken proventricular necrosis virus (CPNV), and at the same time an RT-PCR method was developed to enable its detection (Guy et al. 2011b). The relationship between CPNV and TVP has been tentatively confirmed also in chicken broilers in the UK, Brazil and Poland (Grau-Roma et al. 2016, Grau-Roma et al. 2020, Śmiałek et al. 2020, Leão et al. 2021). So far, however, this relationship has been addressed in few scientific experiments, mostly due to the difficulty of CPNV propagation under laboratory conditions and the territorially limited nature of the disease. However, it has been emphasized many times that the extent of TVP spread is probably much greater than it appears, the disease very often not being diagnosed or being misdiagnosed. So far, investigations aimed at determining the frequency of CPNV identification from TVP field cases have been conducted only in the UK and Brazil. In two studies, Grau-Roma et al. (2016, 2020) showed that CPNV was identified in 22% (in the years 2000–2015) or 47% (in 2014–2015) of TVP cases. Additionally, in the study by Leão et al. (2021) the authors reported that CPNV was detected in 36% of TVP positive proventriculi samples collected between 2013 and 2017. These data suggest that TVP may also be caused by other infectious agents and that its successful clinical induction in chickens infected with a purified CPNV isolate is not conclusive for that virus being the pathogen uniquely responsible for TVP. On the other hand, these results indicate an increasing share of CPNV in TVP cases in recent years. Furthermore, it has been suggested that differences in results noted over the years may also be due to the longer fixation of proventriculus samples in paraffin blocks, or to the differences in field techniques used by veterinarians to collect material for diagnostic analysis (Grau-Roma et al. 2020).

It is noteworthy, however, that the above data come from a territorially limited region and these relationships remain unclear in other latitudes. Undoubtedly, the results of such studies would help to better define the magnitude of CPNV spread in the poultry population and its role in the pathogenesis of TVP. They could also contribute to the improvement of the diagnostic process of the disease, and, consequently, to an expansion of the scope of preventive measures.

Given this poverty of data, a study was carried out to identify the relationship between the occurrence of histopathological TVP lesions in the proventriculi in commercial flocks of broiler chickens in Poland and the prevalence of CPNV. In TVP-positive cases the age differences of birds and the intensity of histopathological lesions between CPNV - positive and negative samples were also analyzed.

Materials and Methods

Ethic Statement

According to information obtained from the Local Ethics Committee in Olsztyn, no special approval was necessary for the experiments performed under field conditions. All animal procedures and sample collection were performed during standard veterinary field inspection and observations.

Study design and sample collection

The study was carried out with proventriculus samples from chicken broilers in commercial flocks suspected of being affected by TVP. The samples were sent for histopathological examination to the Faculty of Veterinary Medicine in Olsztyn by field veterinarians from June 2017 until May 2019. Before sample collection, the veterinarians were informed of the preferred method of material preservation (fixation in formaldehyde). The proventriculi were dissected as whole organs and then cut lengthwise for better formalin penetration into the tissue. The volume ratio of formalin to tissue was 10:1. In the sampling period, 35 samples of broiler chicken proventriculi were tested. One sample consisted of 1 to 4 proventriculi, each from a different bird from a given flock. Whenever possible, the age of the birds was recorded on the day of sampling for histopathological examination. Only samples that met the histopathological criteria for TVP were used for molecular analysis for the presence of CPNV genetic material. Additionally, the TVP-positive samples were analyzed for the relationship between the identification of CPNV in the sample, the age of the birds on the day of sample preservation, and the severity of histopathological lesions.

Histopathology

Histopathological analysis was performed as described previously (Śmiałek et al 2017, Śmiałek et al. 2020). Briefly, during necropsy, samples of the central part of the proventricular wall were embedded in 10% formalin (pH 7.4). After the samples were passed through liquids with increasing concentrations of alcohol and xylene, they were embedded in paraffin blocks. Sections 4 μm thick of the examined samples were stained with hematoxylin–eosin, and microscope samples were scanned with a Pannoramic MIDI scanner (3DHISTECH, Budapest, Hungary). TVP-related histopathological lesions were characterized as necrosis of glandular epithelium (categorized as necrosis), hypertrophy and hyperplasia of ductal epithelium and replacement of glandular epithelium by hyperplastic ductal epithelium (categorized as hyperplasia), and infiltration of lymphoid cells (infiltration). If such lesions were registered during the examination, the sample was considered TVP-positive.

The result of the histopathological examination was also analyzed in terms of the severity of lesions, using the following scoring system: minor necrosis (less than 10% of glands) – 0, focal (10-25% of glands) – 1, multi-focal (25-50% of glands) – 2, diffuse (over 50% of glands) – 3, minor hyperplasia – 0, observed hypertrophy and hyperplasia – 1, observed hypertrophy and hyperplasia of ductal epithelium, replacement of glandular epithelium by hyperplastic ductal epithelium – 2, minor infiltration (less than 10% of glands) – 0, moderate (10-25% of glands) – 1, intensive (25-50% of glands) – 2, and diffuse (over 50% of glands) – 3. The results obtained for the individual components of the scoring procedure were totalled, and the scoring results obtained for bulk samples (containing samples of more than one proventriculus) were averaged per bird.

Five 4 μm-thick paraffin-embedded sections from each TVP-positive sample were put into xylene and thus prepared for PCR analysis. In the case of the bulk samples, at least one section was preserved from each tested proventriculus for molecular analysis.

Molecular identification of CPNV

Chicken proventricular necrosis virus identification was performed as described previously (Guy et al. 2011b). Briefly, 1 mL of xylene was added to each sample, and these were incubated for 5 minutes at 50°C to remove paraffin residues. RNA isolation was performed with an Isolate II FFPE RNA/DNA Kit (Bioline, London, UK) according to the manufacturer's recommendations. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to establish the concentration and purity of the isolated RNA, and for its transcription, a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used. The reaction was performed with 2 μL of 10X RT Buffer, 0.8 μL of 25X dNTP Mix (100 mM), with 0.5 μL each of 100 μM 5′- GGGCGGTAACCATTCAGATA-3′ reverse primer and 5'-CGTAGACCTCGTCCTTCTGC-3' forward primer, 1 μL of MultiScribe Reverse Transcriptase, 1 μL of RNase Inhibitor, 4.7 μL of nuclease-free water and 10 μL of RNA. The amplification of the target 171 bp CPNV gene was performed using a HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) 0.1 μL of each100 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 elonga-

Table 1. Results of histopathological examination.

Number of proventriculi samples examined	Number of TVP positive samples	% of TVP positive samples	Average birds age (days) in TVP positive cases	Average histopathological score of TVP positive samples
35	23	65.71	28.7	5.84

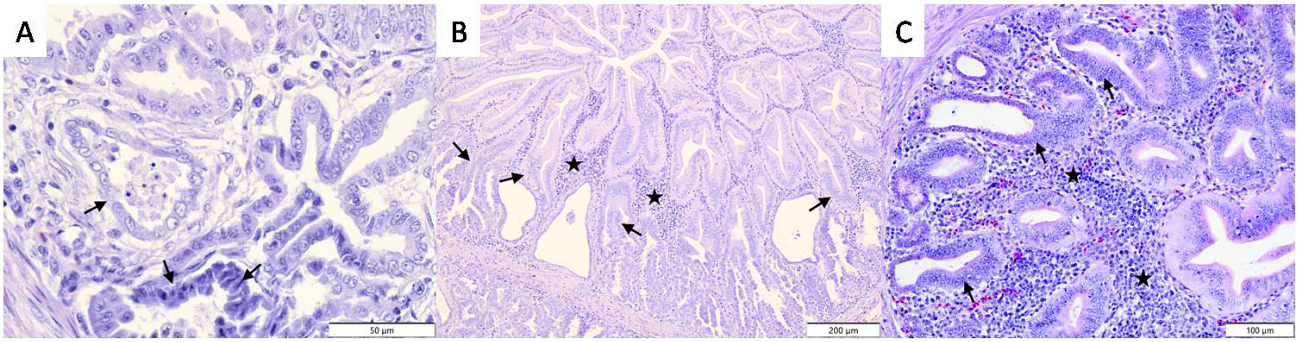

Fig. 1. Typical result of histopathological evaluation of a transmissible viral proventriculitis - positive proventriculi sample. Typical TVP lesions concerned necrosis of glandular epithelium (A; black arrow), replacement of glandular epithelium by hyperplastic ductal epithelium (B and C; black arrow) and infiltration of lymphoid cells (B and C; star).

tion at 72°C for 1 min, and final elongation at 72°C for 10 min. Thirty-five replication cycles were performed.

Statistical analysis

The significance of differences in age and the score of histopathological lesions between CPNV-positive and CPNV-negative samples was analyzed with Student's *t*-test for independent samples. All calculations were made using GraphPad Prism 6.05 software (GraphPad, San Diego, USA). Differences were considered statistically significant at $p<0.1$.

Results

Histopathology

The results of the histopathological examination are summarized in Table 1. Twenty three (65.71%) of the 35 proventriculi samples examined were TVP-positive (Fig. 1). In 20 of these 23 TVP-positive samples we were able to determine the age of the birds on the day of sample collection, and the average was 28.7 days, with the youngest being 21 days old and the oldest 42 days old. The average histopathological score for the TVP-positive samples was 5.84, with 3 being the lowest and 8 the highest. Histopathological results for individual samples are summarized in Table 2. The average score for the CPNV-positive TVP samples was significantly higher than for the CPNV-negative ones ($p=0.026$). Additionally, the average necrosis and infiltration scores for the CPNV-positive birds were also significantly higher ($p=0.027$ and 0.034, respectively). No statistical differences were recorded with regard to hyperplasia.

CPNV molecular identification in the TVP-positive samples and age differences

The results of molecular CPNV identification in the TVP-positive proventriculus samples are summarized in Table 3. CPNV was identified in 10 of the 23 (43.47%) TVP-positive samples. In 8 of the 10 CPNV-positive samples we were able to determine the age of the birds. The average age of the CPNV-positive chickens was 26 days, while that of the CPNV-negative birds was 30.5 days, and this difference was statistically significant ($p=0.0778$). The molecular results for individual samples are summarized in Table 2, together with the histopathological results.

Discussion

For nearly a decade, chicken proventricular necrosis virus has been considered the main etiological factor of TVP. Despite the significant impact of CPNV infection on the profitability of chicken production, mainly of broiler chickens, studies regarding the disease itself and the biology of the virus are very limited. Among the few studies into the relationship between TVP and CPNV, two deserve special attention. Their authors tried to establish the percentage of TVP and CPNV co-presence in broiler chickens. In a study conducted

Table 2. Results of histopathological scoring for individual samples (1-23).* significant difference between CPNV positive and CPNV negative groups.

Sample ID (chronologically)	CPNV result	Necrosis	Infiltration	Hyperplasia	Sum
1	Neg	1.50	2.00	2.00	5.50
2	Neg	2.00	2.00	2.00	6.00
3	Neg	1.15	1.80	1.40	4.35
4	Neg	2.00	2.50	2.00	6.50
5	**Pos**	2.50	2.75	2.00	7.25
6	**Pos**	3.00	3.00	2.00	8.00
7	**Pos**	1.75	2.00	1.75	5.50
8	Neg	1.00	2.00	2.00	5.00
9	**Pos**	3.00	2.00	2.00	7.00
10	Neg	3.00	2.00	2.00	7.00
11	**Pos**	3.00	2.5	2.00	7.50
12	Neg	2.00	2.00	2.00	6.00
13	**Pos**	1.00	2.00	1.50	4.50
14	**Pos**	2.00	2.00	2.00	6.00
15	Neg	1.00	1.00	2.00	4.00
16	**Pos**	2.00	2.00	1.50	5.50
17	**Pos**	2.67	2.67	2.00	7.34
18	**Pos**	2.00	2.67	1.67	6.34
19	Neg	1.50	0.50	1.00	3.00
20	Neg	1.00	2.00	1.34	4.34
21	Neg	2.00	2.67	2.00	6.67
22	Neg	2.00	2.00	2.00	6.00
23	Neg	1.67	2.00	1.34	5.01
Average	-	1.95	2.09	1.80	5.84
Average of CPNV positive	-	2.29*	2.36*	1.84	6.49*
Average of CPNV negative	-	1.68	1.88	1.78	5.33

Table 3. Results of RT-PCR analysis with in-depth analysis of CPNV positive and CPNV negative samples with regard to bird age and average histopatological score. * significant difference between CPNV positive and CPNV negative groups.

Number of TVP positive samples examined	Number (%) of CPNV positive samples	Number (%) of CPNV negative samples	Average birds age (days)		Average histopathological score	
			CPNV Positive	CPNV Neagtive	CPNV Positive	CPNV Negative
23	10 (43.47%)	13 (56.53%)	26*	30.5	6.49*	5.33

at the turn of 2014 and 2015, Grau-Roma et al. (2016) showed that CPNV was present in nearly 47% of the proventriculus samples with confirmed TVP. In another, retrospective, study, in which the authors tested proventriculus samples collected from 2000 to 2015 (and one sample from 1994), 22 out of 99 TVP-positive samples (22%) were found to contain the genetic material of CPNV (Grau-Roma et al. 2020). The CPNV detection in the cited research was carried out in a similar way to that described in this work and involved the

isolation of RNA from the proventriculi embedded in paraffin blocks. The common authors of both studies claim that the differences in their results might be due to differences in the methods of sample preservation for testing, the types of fixative, and the lengths of fixation, which are likely to have reduced the sensitivity of the RT-PCR (Grau-Roma et al. 2020). The results of the present research, performed using samples collected within a relatively short period (2017-2019), correspond to the results from the 2017 study by Grau-Roma et al. (2016), carried out with samples from a similarly short time interval. The present study showed that CPNV was found in 43.47% of the proventriculi with confirmed histopathological lesions typical of TVP.

Grau-Roma et al. (Grau-Roma et al. 2016, Grau-Roma et al. 2020) demonstrated a higher percentage of CPNV-positive samples in TVP cases (respective data from the earlier and later studies are 47% and 22%), compared with cases of lymphocytic proventriculitis (LP, 11%) recorded in broiler chickens. At the same time, in this study, the authors demonstrated higher histopathological scores for lymphocytic infiltration and tubular metaplasia and hyperplasia in TVP cases than in LP. In addition, it has already been suggested that LP may be classified as a chronic form of TVP in which the virus is most often not detected (Guy et al 2011a, Grau-Roma et al. 2020). Grau-Roma et al. (2020), in the study involving histopathological scoring of TVP cases, did not distinguish between CPNV-positive and CPNV-negative TVP cases, and to date, only one paper has analyzed TVP cases in the context of CPNV prevalence depending on the severity of histopathological lesions in the course of the disease (Leão et al. (2021). Considering the results of the present research, it should be concluded that the probability of CPNV detection in TVP cases is highest at the peak of the development of histopathological lesions. As pathological lesions regenerate (lowering the histopathological score) or the disease becomes chronic (LP), the probability of CPNV detection decreases. Although we are not able to precisely determine from the results of our research whether the relationship between the lower score and the lack of CPNV detection is indeed a corollary of the regeneration and healing period in the course of TVP, such a conclusion seems to be justified, taking into account the average 4.5-day-greater age of the birds providing CPNV-negative samples (of which the mean age was 30.5 days) than that of the CPNV-positive birds (26 days old). The results of our study are in some part comparable to thosereported by Leão et al. (2021). In this study the authors also recorded that the probability of CPNV identification in TVP positive proventriculi samples was higher in the case of younger birds (28 days old on average). In contrast, the authors reported that the peak of TVP histopathological lesions was recorded in older (33 days on average) birds. The differences between our study and the study by Leão et al. (2021) could be explained by a number of factors, including factors at a farm level and factors in the laboratory. Factors at the farm level could include: breed of chicken and their susceptibility to CPNV infection and TVP course intensity; biosecurity measures and CPNV infection rate; CPNV pathogenicity and genotype difference; and the sample collection scheme. Factors in the lababoratory could include: time span between the time when the samples were fixed and embadged and the time of RNA isolation (Leão et al. (2021) examined samples from a 5 year span); the difference in the reagents used in the study. Additionally, the relatively low number of analyzed samples in both our and Leão et al. (2021) studies could be the explanation for the differences in both studies. For example, in our study we found CPNV-negative samples among 21-day-old TVP positive birds and CPNV-positive samples among 35-day-old birds. More complication is added by the probability of TVP cases being induced by factors other than CPNV, which has been repeatedly suggested in the past.

What should be emphasized, however, is the fact that in the time frame described in this paper, the suspicion of TVP was the most common indication for proventriculus collection for the histopathological examination. Additionally, the results of this study confirmed that TVP was the most common pathological condition of the proventriculus in broiler chickens (65.71% of total submissions). The remaining submissions were diagnosed as bacterial proventriculitis (7 submissions, 20%) or yielded no histopathological findings (5 submissions, 14.29%) (data not shown).

Conclusions

The prevalence of TVP-typical pathology together with the high percentage of CPNV-positive samples among the TVP-positive samples (43.47%) makes the conclusion reasonable that TVP and CPNV represent an emerging disease and a pathogen in broiler chickens. In addition, it seems that early sampling and preservation of the material for testing, i.e. in the initial period of the infection when the lesions are more pronounced, should result in a greater probability of CPNV detection, provided, of course, that a given case of TVP is actually caused by this virus.

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References

Dormitorio TV, Giambrone JJ, Hoerr FJ **(2007)** Transmissible proventriculitis in broilers. Avian Pathol 36: 87-91.

Goodwin MA, Hafner S, Bounous DI, Latimer KS, Player EC, Niagro FD, Campagnoli RP, Brown J **(1996)** Viral proventriculitis in chickens. Avian Pathol 25: 369-379.

Grau-Roma L, Marco A, Martinez J, Chaves A, Dolz R, Majo N **(2010)** Infectious bursal disease - like virus in case of transmissible viral proventriculitis. Vet Rec 167: 836.

Grau-Roma L, Reid K, de Brot S, Jennison R, Barrow P, Sánchez R, Nofrarías M, Clark M, Majó N **(2016)** Detection of transmissible viral proventriculitis and Chicken proventricular necrosis virus in the UK. Avian Pathol 46: 68-75.

Grau-Roma L, Schock A, Nofrarías M, Ali Wali N, de Fraga AP, Garcia-Rueda C, de Brot S, Majó N **(2020)** Retrospective study on transmissible viral proventriculitis and chicken proventricular necrosis virus (CPNV) in the UK. Avian Pathol 49: 99-105.

Guy JS, West AM, Fuller FJ **(2011a)** Physical and genomic characteristics identify chicken proventricular necrosis virus (R11/3 virus) as a novel birnavirus. Avian Dis 55: 2-7.

Guy JS, West MA, Fuller FJ, Marusak RA, Shivaprasad HL, Davis JL, Fletcher OJ **(2011b)** Detection of chicken proventricular necrosis virus (R11/3 virus) in experimental and naturally occurring cases of transmissible viral proventriculitis with the use of a reverse transcriptase – PCR procedure. Avian Dis 55: 70-75.

Hafner S, Guy JS **(2013)** Proventriculitis and proventricular dilatation of broiler chickens. In: Swayne DE, Glisson JR, McDouglald LR, Nolan LK, Suarez DL, Nair VL (eds) Diseases of poultry. 13th ed., Wiley-Blackwell Publishing, Ames, USA, pp 1328-1332.

Kim HR, Yoon SJ, Lee HS, Kwon YK **(2015)** Identification of a picornavirus from chickens with transmissible viral proventriculitis using metagenomic analysis. Arch Virol 160: 701-709.

Kouwenhoven B, Davelaar FG, Van Walsum J **(1978)** Infectious proventriculitis causing runting in broilers. Avian Pathol 7: 183-187.

Leão PA, Amaral CI, Santos WH, Moreira MV, de Oliveira LB, Costa EA, Resende M, Wenceslau R, Ecco R **(2021)** Retrospective and prospective studies of transmissible viral proventriculitis in broiler chickens in Brazil. J Vet Diagn Invest 33: 605-610.

Marquerie J, Leon O, Albaric O, Guy JS, Guerin JL **(2011)** Birnavirus-associated proventriculitis in French broiler chickens. Vet Rec 169: 394-396.

Śmiałek M, Gesek M, Dziewulska D, Niczyporuk JS, Koncicki A **(2020)** Transmissible Viral Proventriculitis Caused by Chicken Proventricular Necrosis Virus Displaying Serological Cross-Reactivity with IBDV. Animals 11: 8, doi: https://doi.org/10.3390/ani11010008

Śmiałek M, Gesek M, Śmiałek A, Koncicki A **(2017)** Identification of Transmissible Viral Proventriculitis (TVP) in broiler chickens in Poland. Pol J Vet Sci 20: 417-420.

Yu L, Jiang Y, Low S, Wang Z, Nam SJ, Liu W, Kwangac J **(2001)** Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis 45: 416-424.