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**Origin of the chicken splenic reticular cells influences the effect of the infectious bursa disease virus (IBDV) on the extracellular matrix**

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Development of a SYBR Green Quantitative Polymerase Chain Reaction  
Assay for Rapid Detection and Quantification of Infectious Laryngotracheitis  
Virus

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## Abstract

Infectious laryngotracheitis is an acute viral respiratory disease of chickens with a worldwide distribution. Sensitive detection of the causative herpesvirus is particularly important because it can persist in the host at a very low copy number and be transmitted to other birds. Quantification of viral genome copy number is also useful for clinical investigations and experimental studies. In the study presented here, a quantitative PCR assay was developed using SYBR Green chemistry and the viral gene UL15a to detect and quantify infectious laryngotracheitis virus (ILTV) in ILTV inoculated chicken embryos or naturally infected birds. The specificity of the assay was confirmed using a panel of viral and bacterial pathogens of poultry. The sensitivity of the assay was compared to two conventional PCR assays, virus titration and an antigen detecting ELISA. The quantitative PCR developed in this study was highly sensitive and specific, and has potential for quantification of ILTV in tissues from naturally and experimentally infected birds and embryos.

## Introduction

Traditionally virus isolation has been used for the detection of herpesvirus infections, including infectious laryngotracheitis (ILT) (Kaleta, 1998), but it may take up to 10 days before a diagnosis can be made. Serological methods for the detection of antibodies against ILTV have also been described, but are generally of low sensitivity (York *et al.*, 1990; Keam *et al.*, 1991; Nagy, 1992; Key *et al.*, 1994). More rapid, methods to detect ILTV

based on the polymerase chain reaction (PCR) (Vogtlin *et al.*, 1999; Han & Kim, 2001; Humberd *et al.*, 2002) and the use of fluorogenic DNA probes (Keam *et al.*, 1991; Yilmaz *et al.*, 2004) have been reported in more recent years. Kits for antigen detection by enzyme linked immunosorbent assay (ELISA) are also available.

Recently, a real-time PCR followed by RFLP analysis of the PCR product, using the viral immediate-early gene of ICP4 as the target, has been used for detection and characterisation of viruses responsible for field cases of ILT (Creelan *et al.*, 2006). However, in this study no attempt was made to determine the viral genome copy number, nor was the method compared with conventional PCR for sensitivity.

In the work described here, a standard curve constructed using a dilution series of cloned ILTV DNA was used to assess the viral genome copy number in clinical samples and the sensitivity and specificity of the qPCR assay compared to viral isolation and other methods for detection of ILTV.

## Materials and methods

**Viruses.** The ILTV vaccine strains SA2 and A20 (Fort Dodge Animal Health, Baulkham Hills, NSW, Australia) were obtained from the manufacturer. The field strain CSW-1 is a historical strain of ILTV isolated in New South Wales (Bagust *et al.*, 1986), which was propagated by cell culture in LMH cells as previously described. The other field isolates were from outbreaks of ILT in different states of Australia including South Australia (prefix S), New South Wales (prefix N) and Victoria (prefix V) as shown in Table 2. The

ILTV vaccine strain SA2 was propagated on the chorioallantoic membranes (CAMs) of 10-day-old chicken embryos as described previously (Longenecker et al., 1977; Hitchner & Hirai, 1979; Kirkpatrick et al., 2006a).

**Pock assay.** The CAMs of five 9- to 12- day-old SPF chicken embryos were inoculated with a 0.1 mL suspension of viral inoculum and the eggs incubated at 37°C. At least 5 different dilutions of the inocula and 6 eggs per dilution were used for this assay. After three days, any pocks were counted by visual examination to determine the viral concentration as pock forming units per milliliter (PFU/mL).

**Extraction of DNA from ILTV vaccine and field isolates.** ILTV DNA was extracted from commercial vaccines, directly from swabs taken from infected tracheas or supernatants from homogenates of infected CAMs using a method described previously (Sykes et al., 2001). Tracheal swabs from clinical specimens were placed into 400 µl of RLT lysis buffer (Qiagen) containing 4 µl of β-mercaptoethanol (1.12 g/ml) and incubated at 4°C overnight. A total of 15 µl of Qiaex<sup>®</sup> II matrix (Qiagen) and 300 µl of 100% ethanol were added to the mixture and the matrix was resuspended by vortexing. This suspension was loaded into a Multispin MSK-100 column (Axygen Inc., Hayward, CA, USA). Columns were centrifuged for 30 sec at 10,000 × g in a microcentrifuge and the flow-through discarded. The matrix was washed once with 600 µl RW1 buffer (Qiagen) and twice with 500 µl RPE buffer (Qiagen). For each wash, columns were centrifuged for 30 sec at 10,000 × g. The columns were centrifuged for 90 sec at 18,000 × g to dry the matrix. The matrix was overlaid with 30 µl distilled water. After incubation at room temperature

for 1 min, columns were centrifuged for 1 min at  $10,000 \times g$ . The supernatant containing extracted DNA was used immediately or stored at  $-20^{\circ}\text{C}$  prior to use in the PCR.

Commercial vaccines (4000 doses) were reconstituted in 10 mL of PBS and centrifuged ( $120 \times g$  for 3 min at room temperature). A 100  $\mu\text{L}$  volume of the reconstituted commercial vaccines or 30  $\mu\text{L}$  of supernatant from homogenates of CAMs was placed into 400  $\mu\text{L}$  of RLT lysis buffer (Qiagen) containing 4  $\mu\text{L}$  of  $\beta$ -mercaptoethanol (1.12 g/ml) and DNA extracted as described above for tracheal swabs. In each run of DNA extraction, reconstituted ILTV SA2 vaccine and RLT lysis buffer only were used as positive and negative controls for extraction, respectively.

#### **Extraction of DNA and RNA from viruses and bacteria used as negative controls.**

DNA was extracted from fowl adenovirus 8b (Steer et al., 2009), avian leukosis virus J (Fenton et al., 2005), chicken anaemia virus strain CAU269/7 (Kaffashi et al., 2008), *Mycoplasma gallisepticum* strain S6 (Ghorashi et al., 2010), *Escherichia coli* DH5 $\alpha$ , and lysates of LMH and chick embryo kidney cells using the QIAamp DNA mini kit as described above and used as negative controls in the PCR.

Total RNA was extracted from a culture of infectious bronchitis virus strain Armidale (Hewson et al., 2009) using RNeasy Mini kits (Qiagen) according to the manufacturer's instructions. To generate cDNA, 1  $\mu\text{g}$  of total RNA was primed with 500 ng random hexamer primers (Geneworks, Adelaide, Australia) and reverse transcribed at  $37^{\circ}\text{C}$  for 1 h using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as described previously (Mardani et al., 2006).

**Quantitative polymerase chain reaction assay.** Two oligonucleotide primers, UL15a Forward (5'-TTGCTGTGCTATTTTCGCGTG-3') and UL15a Reverse (5'-GTAAATCGTTTGTAGTGCGGCAT-3'), were designed to amplify a 113 bp product from the ILTV UL15a gene and the reaction optimised to determine the concentrations of the forward and reverse primers that gave the highest efficiency and yielded the lowest cycle threshold (Ct) value. The reaction was conducted using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer's instructions. The final PCR reaction (20  $\mu$ L total volume) contained 0.3  $\mu$ L of each of the forward and reverse primers to give a final of 0.75  $\mu$ M each, 10  $\mu$ L of SYBR PCR Supermix containing uracil-DNA glycosylase (UDG) and Rox reference dye (for normalisation of background), 4.4  $\mu$ L of distilled water and 5  $\mu$ L of DNA template. Incubation and data acquisition were performed using a Stratagene Mx3000 qPCR Thermal Cycler (Agilent Technologies, Cedar Creek, TX, USA). Reactions were incubated for 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. To create a dissociation curve after the cycling was completed, reactions were incubated at 95°C for 1 min, 55°C for 30 s and then the temperature increased to 95°C over 30 min with readings taken every 30 s. The PCR products were visualised by electrophoresis through 2% agarose gels. Amplification efficiencies and Ct values were determined using the Stratagene Mx3000 1.0 software.

**Preparation of plasmid construct for use as a standard.** A 113-bp fragment of the ILTV UL15 gene was amplified from ILTV strain SA2 genomic DNA by PCR using oligonucleotide primers UL15a Forward and UL15a Reverse and the PCR protocol described above. Following PCR, the UL15a PCR product was purified using a Promega

Clean Up Kit (Promega, Madison, WI, USA). The purified PCR product was then cloned in the pGEM-T cloning vector (Promega) according to the manufacturer's instructions and this used to transform *Escherichia coli* DH5- $\alpha$ . White colonies were selected and examined for the presence of the insert by PCR, as well as by electrophoresis in an 0.8% agarose gel of the purified plasmid linearised with the restriction endonuclease *SacI* (New England Biolabs, Ipswich, MA, USA).

A standard curve was generated using a ten-fold dilution series of linearised pUL15a, (Figure 1). Plasmid DNA copy was measured by spectrophotometry at  $A_{260}$ . The number of copies of plasmid was calculated using the equation:

$$\text{Plasmid copies}/\mu\text{l} = [\text{plasmid amount (g}/\mu\text{L)} \times (6.022 \times 10^{23})] / [(\text{plasmid length in bp} \times 660)].$$

**Conventional PCR.** The TK Forward (5'-CTGGGCTAAATCATCCAAGACATCA-3') and Reverse (5'-GCTCTCTCGAGTAAGAATGAGTACA-3') primers were used to amplify 1480 bp of the thymidine kinase gene (TK, GenBank database accession number D00565) and gG Forward (5'-CCTTCTCGTGCCGATTCAATATG-3') and Reverse (5'-AACCACACCTGATGCTTTTGTAC-3') primers were used to amplify 2240 bp of the glycoprotein G gene (gG, GenBank database accession number U2883) of ILTV.

The 50  $\mu\text{L}$  PCR reactions contained 200  $\mu\text{M}$  of each of dATP, dCTP, dGTP and dTTP, 1 mM  $\text{MgCl}_2$ , 250  $\mu\text{M}$  of each of the primers, 1 U of *Taq* DNA polymerase (Promega), 5  $\mu\text{L}$  of 10 x *Taq* DNA polymerase buffer and 5  $\mu\text{L}$  of extracted DNA as template. The reaction mixture was incubated at 94°C for 3 min, then subjected to 35 cycles of 94°C for 15 sec, 60°C for 45 sec and 72°C for 150 sec, and finally incubated at 72°C for 3 min.

In each series of PCRs, a control tube containing sterile distilled H<sub>2</sub>O, instead of extracted DNA, was included as a negative control (no template control). All amplification reactions were performed in a thermal cycler (iCycler™, BioRad, Gladesville, NSW, Australia). The PCR products were separated by electrophoresis in 0.8% agarose gels, stained with SYBR Safe nucleic acid stain (Invitrogen) and exposed to ultraviolet light for visualization.

**Antigen ELISA.** Homogenates of infected CAMs were diluted in 1 mL of ELISA diluent, then ILTV detected and quantified using the Trop-Ag ILT ELISA Kit (TropBio Pty Ltd, Queensland, Australia) according to the manufacturer's instructions. ELISA diluent was used as negative control.

## Results

**UL15a primer design and optimisation.** The forward and reverse primers were designed to amplify 113 bp of the 3' region of the UL15a gene. Searches of the GenBank database did not reveal any significant matches between the UL15a primer pair and any other avian viruses or other accessions. The optimal ratio for the forward and reverse primers was found to be 1:1 (both 0.75 µM). The standard curve generated using serial ten-fold dilutions of pUL15a plasmid DNA indicated that the assay had an average efficiency of 96.36% with a coefficient of determination ( $R^2$ ) of 0.994 (Figure 1). The efficiency of amplification, estimated by performing the assay on a three-fold dilution series of viral DNA, was 97.5%, with an  $R^2$  of 0.999 (Figure 2 A and B). ILTV vaccine strains and field isolates generated a



single PCR product melting at approximately 80°C, as confirmed by melting (dissociation) curve analysis (Figure 2 C). The size of the PCR product was approximately 113 bp, as determined by electrophoresis through a 1.5% agarose gel.

**Specificity and reproducibility of the UL15a qPCR assay.** The specificity of the UL15a qPCR was assessed by attempting amplification from DNA or cDNA of different viruses and bacteria (including fowl adenovirus, avian leukosis virus J, chicken anaemia virus, infectious bronchitis virus, *Mycoplasma gallisepticum* and *E. coli*), as well as cellular DNA. No amplification was detected from any of these sources of DNA by visual examination of the amplification plots and agarose gels.

The reproducibility of the assay was assessed by examination of variation in Ct values. An average standard deviation of 1.59 was obtained in 9 independent experiments.

**Higher sensitivity of qPCR for detection of ILTV in vaccine dilutions compared to conventional PCR and ELISA.** Comparison of the conventional PCR assay, the ILTV ELISA and qPCR for detection of ILTV in serial two-fold dilutions of the SA2 vaccine revealed that qPCR was more sensitive than conventional PCR (Table 1). The qPCR assay detected viral DNA at dilutions down to 1: 327680 (102 copies), while conventional PCR failed to detect viral DNA at dilutions below 1:40960 (1660 copies). The Ag ELISA was not able to detect the presence of ILTV beyond a 1:80 dilution.

There was a high correlation between the dilution factor and the estimated DNA copy number by qPCR, although for the first four dilutions (1:10 to 1:80), the Ct values were beyond the range of the standard curve ( $10^2 - 10^8$  copy numbers). There was an

apparent lack of correlation between the copy numbers calculated using qPCR Ct values and the dilution factors beyond 1/5120, although the overall correlation for the last six dilutions was still moderately strong (Spearman's coefficient of correlation = 0.71).

**Higher sensitivity of qPCR for detection of ILTV in clinical samples compared to conventional PCR.** A total of 40 clinical samples from suspected cases of ILT (based on clinical signs, gross lesions and location of the farm in relation to areas where ILT was endemic), including samples from multiple chickens in the same flocks, were examined for ILTV using both qPCR and conventional TK and/or gG PCR assays (Table 2). The higher sensitivity of the qPCR assay was demonstrated by the successful detection of ILTV in all samples from the N4-04 and S4-04 outbreaks by qPCR, while the conventional PCR assay failed to amplify the target sequence in all but one sample from the S4-04 outbreak. In total, 28 samples were detected as positive by conventional PCR, compared to 33 positive samples by the qPCR assay, when all samples yielding Ct values outside the range of the standard curve (that is, less than 100 genome copies) were regarded as negative.

## Discussion

Sensitive detection of ILTV is pivotal in assessment of the progress of infection and thus in control of virus spread. Quantification of the viral copy number is also valuable for *in vitro* and *in vivo* experiments. The precise quantification of target DNA is particularly useful in growth curve analysis of different isolates of ILTV. A qPCR assay offers a higher

sensitivity than conventional PCR and the post-PCR processing step is avoided, allowing savings in both time and reagents.

In the work described here, a quantitative PCR assay was developed to amplify a 113 bp fragment of ILTV UL15a using the SYBR Green for detection of PCR product and the test was used to detect and quantify ILTV nucleic acid in vaccine strains and field isolates. SYBR Green technology was used because of its relatively lower cost compared to Taqman technology. The UL15a gene was chosen because it is known to be highly conserved within the herpesviruses (Baines & Roizman, 1992; Przech *et al.*, 2003). The genes gG and TK were selected for conventional PCR assays as they have been commonly used for conventional ILTV PCR assays (Abbas *et al.*, 1996; Chang *et al.*, 1997; Kirkpatrick *et al.*, 2006b). Virus titration was also compared to conventional PCR and qPCR.

The standard curve generated using serial ten-fold dilutions of the pUL15a plasmid or viral DNA suggested that the reaction had an average efficiency of at least 96 %. The high efficiency (close to 100%) of the UL15a primers indicated that they would offer a reliable and reproducible qPCR. The assay was also found to be highly reproducible throughout the study and was able to detect viral DNA in clinical specimens and cultivated virus in CAMs. The analytical sensitivity and specificity of the UL15a primers were also confirmed by amplification of the target gene from field isolates and vaccine strains of ILTV. In addition, the assay was specific when tested against DNA from several respiratory pathogens. The high sensitivity of the assay was demonstrated by its capacity to detect ILTV in samples of cultured virus at greater dilutions than those detected by ELISA or conventional PCR. It is notable that there appeared to be very little correlation between

copy numbers calculated from qPCR Ct values and the presence of a detectable DNA band generated in the conventional PCR assay. The reason for this discrepancy is unknown, although it should be noted that the conventional PCR assay and the qPCR assay described here target different genes for amplification and generate amplicons of different sizes. In addition, it is possible that the quality of extracted DNA may have varied from one specimen to another and that this variation may have favoured positive results in one PCR over another. These explanations are also applicable to the discrepancies observed in results obtained with case S4-04, where specimen 2 was positive in the conventional PCR assay and had low copy numbers detectable in the qPCR assay, whereas specimens 1 and 4 had high copy numbers detectable by qPCR but were negative in the conventional PCR assay. Inclusion of an internal control would have been ideal for the qPCR assay described in this study, but was considered too complicated to set up for a simple single tube SYBR Green-based qPCR protocol. There are a number of technologies, such as LUX™ (Invitrogen), that allow the detection of two sets of amplicons in the presence of an intercalating dye in a single tube. However, these technologies are currently very expensive and have proven to be difficult to set up and optimize and therefore would not be justifiable for a routine qPCR diagnostic protocol for poultry.

Previously, a SYBR Green assay targeting the ICP4 gene was developed for detection of ILTV (Creelan *et al.*, 2006), but the potential of the assay for quantification of ILTV was not examined and nor was a standard incorporated. In another study (Callison *et al.*, 2007), a Taqman qPCR assay using a probe for the gC gene and a standard was developed for the detection and quantification of naturally and experimentally infected chickens and the limit of quantification was determined to be 100 template copies, similar

to that of the qPCR assay designed in this study. The standard curve used in the assay described by Callison *et al.* (2007) maintained linearity for at least 5 orders of magnitude, with overall efficiency of 94.54% and coefficient of determination ( $R^2$ ) 0.994. When UL15a plasmid was used as template, the UL15a qPCR developed in our study maintained linearity for 7 orders of magnitude with an average efficiency of 96.36% and an average  $R^2$  value of 0.994. The linearity of the qPCR was also examined for the SA2 vaccine strain, and was shown to be maintained for at least 3 orders of magnitude.

The qPCR assay described here was primarily developed for use in investigations of the replication kinetics of different strains of ILTV (unpublished data) and monitoring experimental infections (Devlin *et al.*, 2006), but was also found to have value for detection and quantification of ILTV in clinical cases, using tracheal swabs or homogenates of CAMs. The sensitivity of this assay was significantly better than conventional PCR. The speed with which qPCR can detect ILTV makes this technique suitable for screening clinical samples in epidemiological studies. In addition, the test was found to be specific and reproducible.

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**Table 1.** Comparison of conventional PCR, ILTV ELISA and qPCR for detection of two-fold dilutions of the ILTV vaccine SA2

Dilution	log <sub>10</sub> PFU	ELISA result (OD)	PCR (TK)	Number of copies
1/10	6.10	+ (2.6)	NT <sup>A</sup>	AR <sup>B</sup>
1/20	5.80	+ (1.3)	NT	AR
1/40	5.50	+/- (0.4)	NT	AR
1/80	5.20	+/- (0.2)	NT	AR
1/160	4.90	- (0.16)	+	4677351
1/320	4.59	- (0.13)	+	2041738
1/640	4.29	- (0.14)	+	831764
1/1280	3.99	- (0.12)	+	549541
1/2560	3.69	- (0.14)	+	165959
1/5120	3.39	- (0.12)	+	34674
1/10240	3.09	- (0.14)	+	5754
1/20480	2.79	- (0.12)	+	7943
1/40960	2.49	- (0.14)	+	1660
1/81920	2.19	- (0.12)	-	3981
1/163840	1.89	- (0.14)	-	105
1/327680	1.58	- (0.12)	-	102

<sup>A</sup> “NT” not tested.

<sup>B</sup> “AR” indicates that the obtained Ct was above the range of the standard curve.

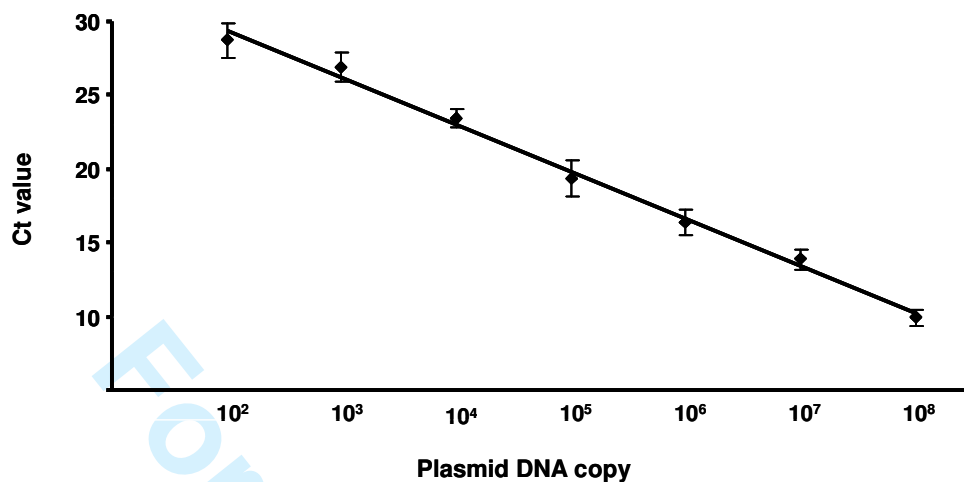
**Table 2.** Comparison of conventional PCR and qPCR for detection of ILTV in tracheal swabs from field cases.

Sample no.	Specimen ID	TK and/or gG PCR <sup>A</sup>	qPCR	
			Ct	Number of copies <sup>B</sup>
1	N1-03/1	+	16.0	1532318
2	N1-03/2	+	21.4	32145
3	N1-03/3	+	24.1	4656
4	N1-03/4	+	22.5	14630
5	N1-04/1	+	14.8	3616521
6	N1-04/2	+	16.5	1071412
7	N1-04/3	+	24.2	4334
8	N2-04/1	+	19.7	108504
9	N2-04/2	+	35.4	-
10	N2-04/3	+	15.0	3134252
11	N2-04/4	+	26.7	724
12	N3-04/5	+	39.7	-
13	N4-04/1	-	21.2	36757
14	N4-04/2	-	25.7	1482
15	N4-04/3	-	17.8	422606
16	N4-04/4	-	18.3	295490
17	S1-04/1	+	21.3	34529
18	S1-04/2	+	24.0	5001
19	S1-04/3	+	27.7	354
20	S1-04/4	+	15.9	1645991
21	S1-04/5	+	21.7	27748
22	S1-04/6	+	14.0	6410900
23	S2-04/1	+	19.9	94035
24	S2-04/2	-	17.0	749142
25	S2-04/3	+	23.4	7683
26	S3-04/1	+	15.5	2191500
27	S3-04/2	+	29.7	-
28	S3-04/3	+	19.1	166692
29	S3-04/4	+	18.5	256086
30	S3-04/5	-	20.3	70627
31	S4-04/1	-	17.2	649242
32	S4-04/2	+	27.4	439
33	S4-04/3	-	24.3	3958
34	S4-04/4	-	13.1	12207466
35	S4-04/5	-	29.3	113
36	V1-03/1	+	29.7	-
37	V1-03/2	-	31.7	-
38	V1-03/3	-	36.9	-
39	V1-03/4	+	36.0	-
40	V1-04/1	+	26.6	778

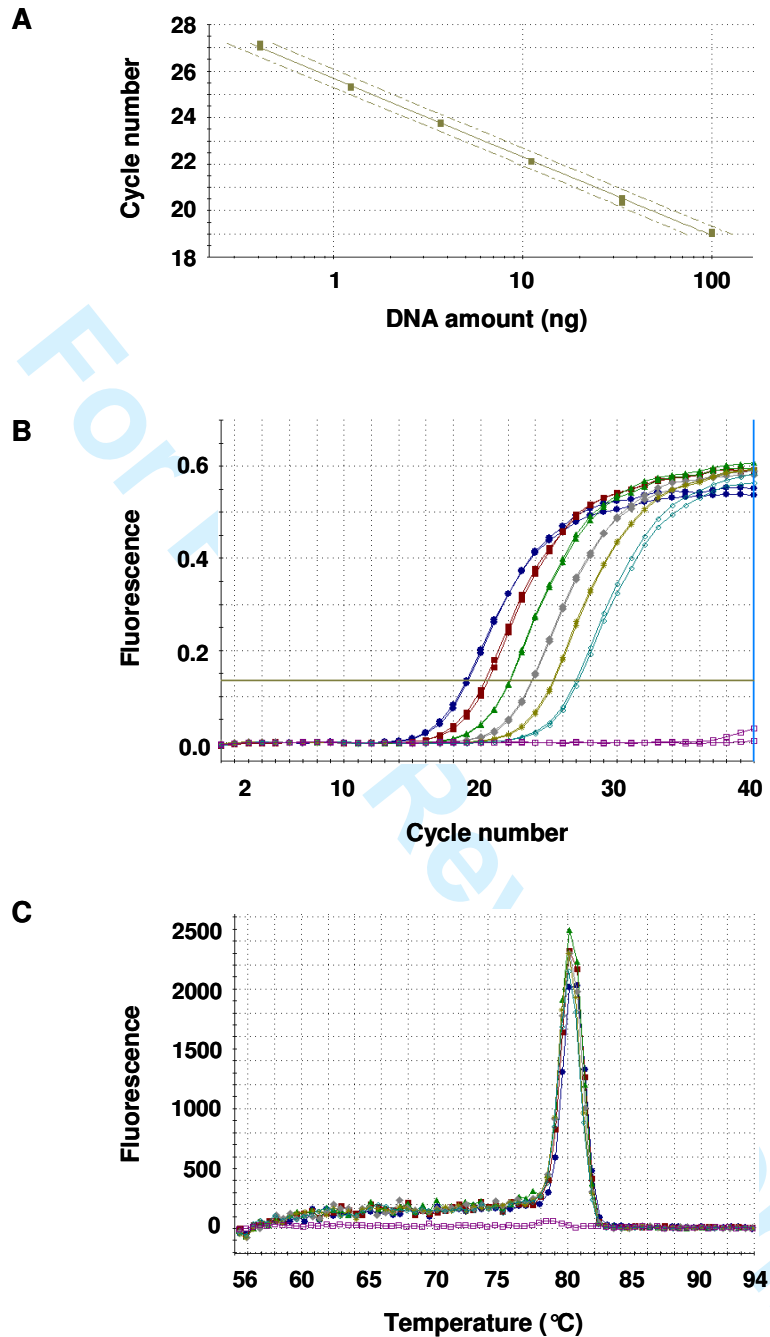
<sup>A</sup> Results of TK and gG PCR. “+” indicates detection of a DNA band in either the TK or the gG PCR. “-” indicates that no DNA bands were detected in either TK or gG PCRs.

<sup>B</sup> Samples were regarded as negative if Ct > 29.4 (< 100 genome copies), as this was the lower limit of the standard curve.

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**Figure 1.** Standard curve generated using pUL15a DNA ( $2 \times 10^7$  copies per microlitre) serially diluted from  $10^8$  to  $10^2$  copies. The average efficiency was 96.36% and the average coefficient of determination ( $R^2$ ) was 0.994.



**Figure 2.** Validation of the UL15a primer. (A) Standard curve generated from a three-fold dilution series of genomic DNA of ILTV  $\pm$  standard deviation with efficiency of 97.5% and  $R^2=0.999$ , (B) amplification plot (linear scale), showing duplicate amplification curves for each serial dilution, and (C) dissociation (melting) curve, showing the same single melting point for all serially diluted replicates.

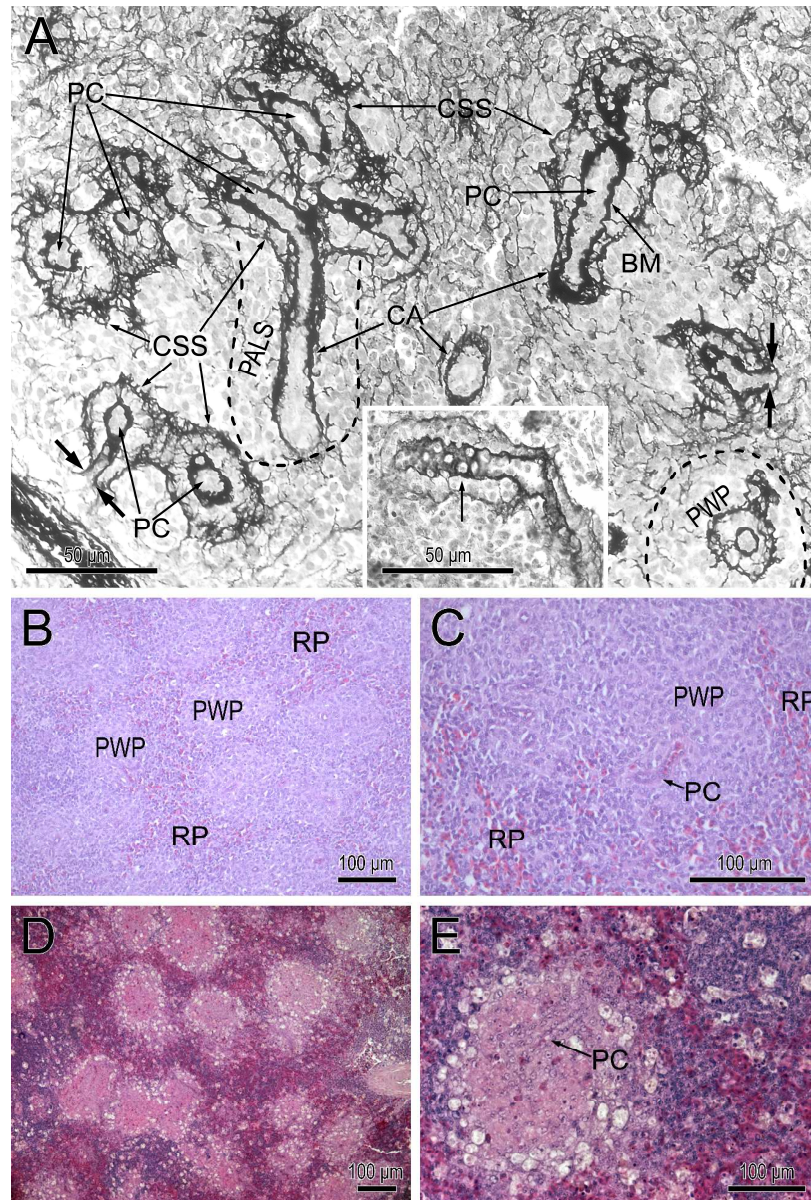


Figure 1. A) Control bird, silver impregnation; shows the reticular fiber pattern of the splenic antigen trapping region. The reticular fibers around the central artery (CA) form a thick layer, which splits for two layers at the beginning of the penicillar capillary (PC); the inner layer continues as a basal membrane (BM) of the PC, the outer one is the actual capsule of the Schweigger-Seidel sheath (CSS). The two layers fuse at the border of the red pulp, where the PC joins to the red pulp sinus (arrow). Periarteriolar lymphatic sheet (PALS) and periellipsoidal white pulp (PWP) are outlined. The cells (unstained) of the ellipsoid locate between the two rings. Inset: the round holes in the BM of the PC show the stomata through which the vascular channel communicates with the antigen trapping region. B) The antigen trapping regions (ellipsoid and PWP) appear as cell clusters embedded into the red pulp (RP). C) The penicillar capillary lined by cuboidal-shaped endothelial cells locates in the PWP. D) 3 dpi hematoxylin-eosin staining. The antigen trapping regions (ellipsoid+PWP) are outlined by pale-stained cells, some of them are scattered over the red pulp. E) Higher magnification of an antigen trapping region. The pale-stained cells demarcate the PWP



from the red pulp (RP). The PC is longitudinally sectioned.  
168x249mm (600 x 600 DPI)

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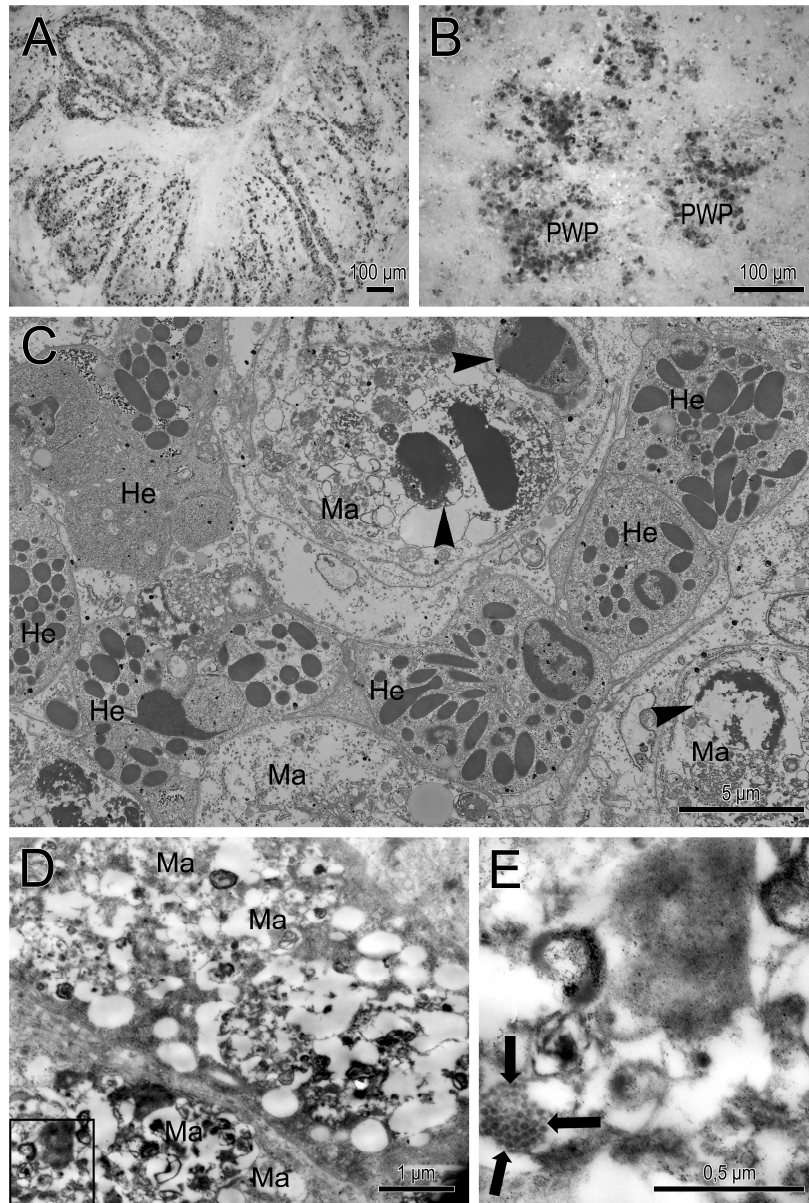


Figure 2. A) 3 dpi. Anti-IBDV monoclonal antibody staining. Bursa of Fabricius. Medulla of the follicles is highly depleted, scattered IBDV containing cells accumulate in the cortex. B) Spleen from the same animal as Figure 2A. IBDV positive cells outline the PWP. C) Electron microscopy indicates that in the PWP large number of heterophyl granulocytes (He) accumulates and intermingled with "swollen" macrophage - like cells (Ma). The latter cells contain detrites of apoptotic cells (arrowhead). D) The two macrophage-like cells contain mainly lipid droplets and less cell debris. In one of them a detail is outlined enlarged and shown on Fig. 2E. Crystallized virus particles are present in a vacuolized region.  
162x240mm (600 x 600 DPI)

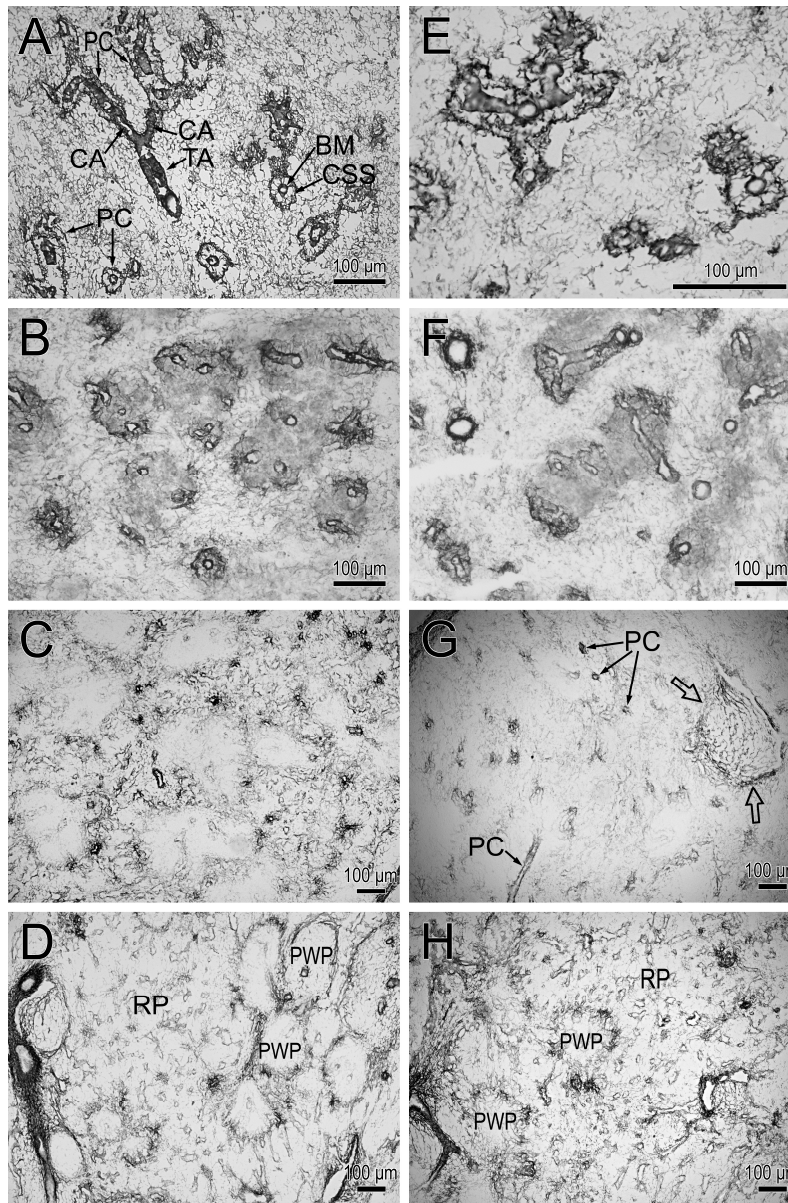


Figure 3. A, B, C and D: type I collagen immunostaining; E, F, G and H: type III collagen immunostaining. A) Control bird: a trabecular artery (TA) divides for two central arteries (CA), which continue as penicillar capillary (PC). Type I collagen is present in both the basal membrane (BM) of the PC and the capsule of the Schweigger-Seidel sheath (CSS). B) Three days post infection (d.p.i.), BM of the PC is intact, but the CSS partially or totally desintegrated. C) Seven d.p.i., the ECM of the ellipsoid disappeared. "Empty" unstained spots remained on the place of ellipsoid and periellipsoidal white pulp (PWP). D) Eleven d.p.i., accumulation take place around the "empty" PWP and the red pulp (RP) sinuses became significant. E) Control bird type III. collagen immunostaining shows similar staining pattern as type I collagen. F) Three d.p.i., the CSS almost completely disappeared and at the place of the ellipsoid and PWP a homogenous "mass" has formed. G) Seven d.p.i., the homogenous mass is cleared up around the BM. The reticular network of a perivenous lymphoid substance is intact (arrow). H) Eleven d.p.i. Type III collagen forms a clustered ringed - shaped demarcation around the PWP. Red pulp sinuses are well-stained.

168x254mm (600 x 600 DPI)

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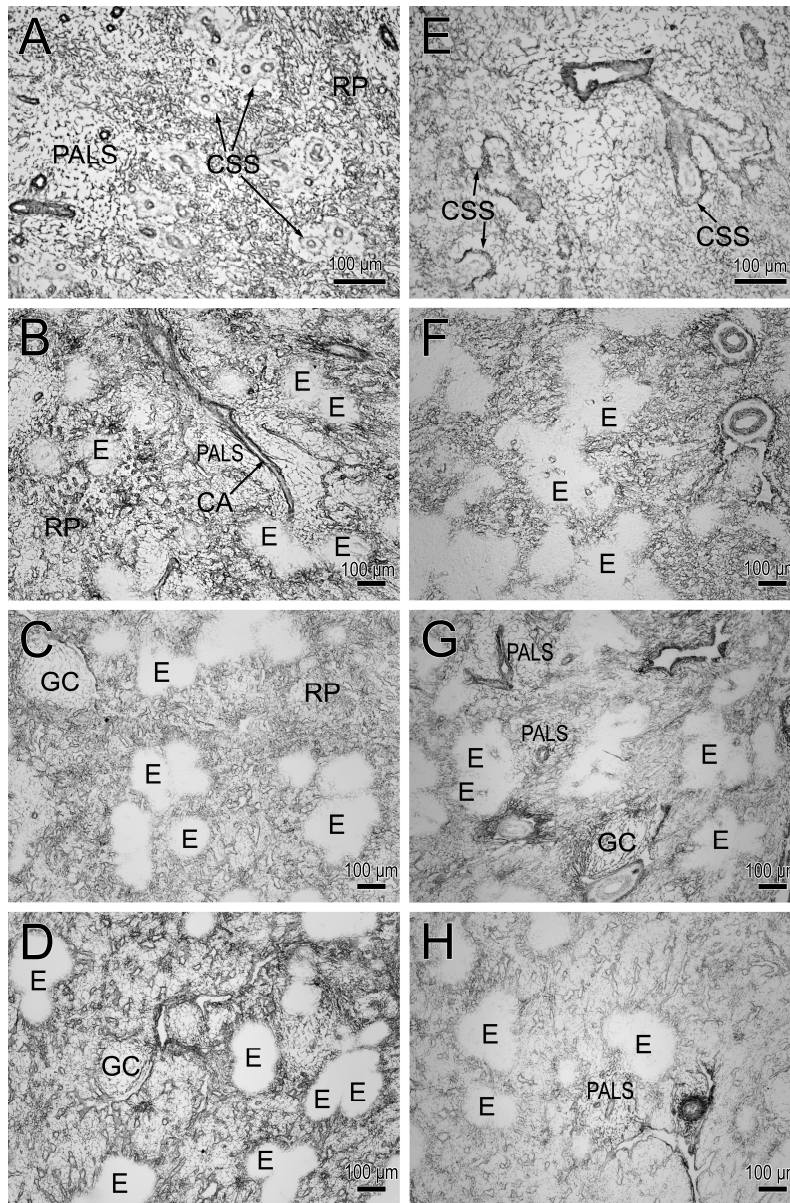


Figure 4. A, B, C and D: laminin immunostaining, E, F, G and H: tenascin immunostaining. A Control bird, anti-laminin immunostaining, BM of the PC appears in the center of the periellipsoidal white pulp (PWP), capsule of the Schweigger-Seidel sheath (CSS) is pale stained. PALS: periarterial lymphatic sheath. B) Three days post infection (d.p.i.), BM of the central artery (CA) is surrounded by a fine reticular network of PALS. The ECM of the ellipsoid (E) and PWP disappeared. C) Seven d.p.i., laminin is still present in the germinal center (GC) and red pulp (RP), but disappeared from the ellipsoid (E). D) Eleven d.p.i., laminin; BM of the sinuses of the red pulp are well-stained. E) Control bird, tenascin immunostaining is concentrated into the CSS. F) Three d.p.i., tenascin rapidly disappeared from the CSS but not from the red pulp. G) H) Seven and eleven d.p.i., tenascin is unchanged in the PALS, and GC and red pulp, but lacks in CSS of the ellipsoid (E).  
168x254mm (600 x 600 DPI)

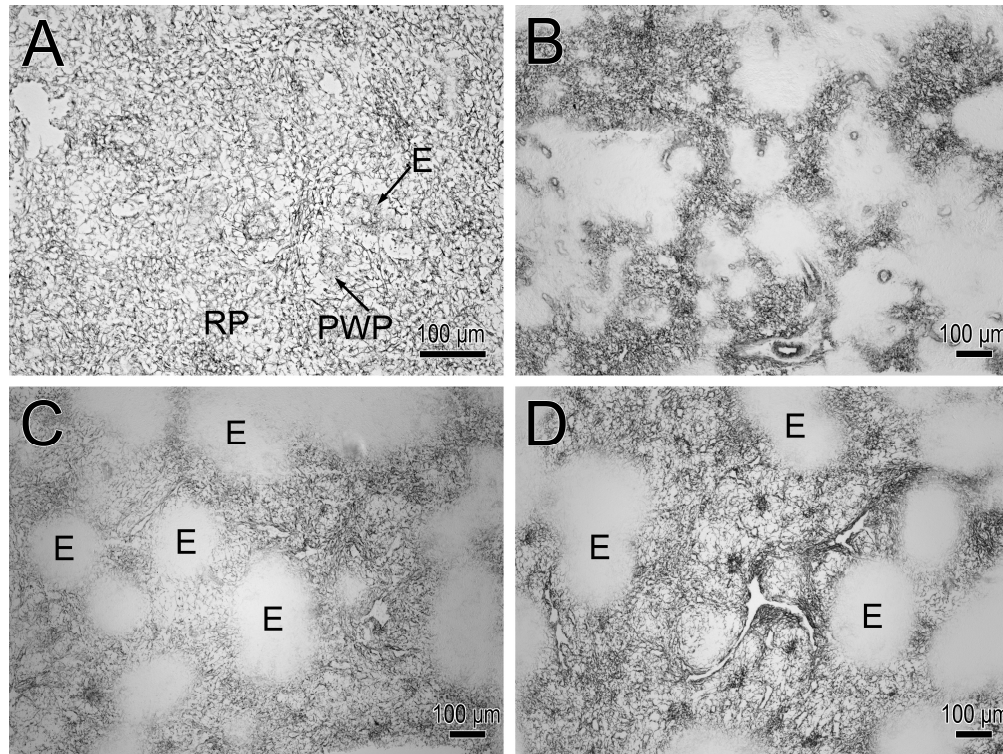


Figure 5. A, B, C and D: fibronectin immunostaining. A) Control bird, there is a dense 3D fibronectin network in the red pulp. The ellipsoid (E) appears as a fine hazy ring, surrounded by a less densely stained periellipsoidal white pulp (PWP). B) Three days post infection (d.p.i.), fibronectin rings of the ellipsoid disappeared and the 3D fiber pattern in the red pulp became blurry. C) Seven d.p.i. basically identical with that of the three d.p.i.. D) The blurry fiber patterns of 3 d.p.i. seems to be changed, fibers emerged again in the red pulp, but not in the PWP.

168x126mm (600 x 600 DPI)