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Approaches to develop an *in situ* hybridization and an immunohistochemistry for the specific detection and localization of avian reovirus in tissues

Diploma thesis

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submitted by

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1 Introduction and aim of the study

Avian reovirus (ARV) is a member of the genus *Orthoreovirus* in the family *Reoviridae* (Mertens 2002). The virus is ubiquitous and can be the cause of viral arthritis as well as other diseases like the malabsorbtion syndrome in broiler (Jones 2013). ARV is a pathogen with a high economic impact (Dobson and Glisson 1992). It is more prevalent in meat type chicken than in laying breeds (Jones and Kibenge 1984). Viral arthritis is the most prominent disease caused by ARV (Olson and Kerr 1966). The clinical changes of viral arthritis are poor growth, low uniformity, secondary infection and mortality. The pathological changes are marked edema of digital flexor tendon sheaths, rupture of the tendons, and a downgrading at the slaughterhouse (Jones 2013). During the last decades vaccination was applied using different serotypes of the virus (Jones 2013). Thereby, protection by vaccination is only effective against homologous serotypes (Rau et al. 1980). Frequently applied commercial vaccines can differ from circulating field strains which reduces protection against ARV (Sellers 2017). Recently several publications reported that strains of ARV newly emerge as a pathogen in poultry (Szilvia et al. 2016, Davis et al. 2013, Lu et al. 2015, Noh et al. 2018, Palomino-Tapia 2018, Ayalew et al. 2017, Souza et al. 2018, Troxler et al. 2013).

Usually an ARV associated disease is suspected by clinical signs, pathological and histopathological changes before specifically identified by reverse transcription polymerase chain reaction (RT-PCR) (Lee et al. 1998), by real-time reverse transcription polymerase chain reaction (qRT-PCR) (Tang and Lu 2016, Kumar et al. 2017) or by virus isolation (Guneratne et al. 1982). The pathological changes resemble lesions caused by other pathogens like *Staphylococcus* spp. or *Enterococcus* spp. (Jones 2013). Furthermore, a positive RT-PCR result on its own is not conclusive for the cause of disease because the virus is ubiquitous. Therefore, a method to visualize the pathogen in the tissue together with its pathogenic effect would provide a reliable diagnosis and could furthermore be of importance to investigate the pathogenesis of the disease.

Studies using *in situ* hybridization (ISH) and immunohistochemistry (IHC) of ARV were already published (Menendez et al. 1974, Liu and Giambrone 1997a, Liu and Giambrone 1997b, Liu et al. 1999, Songserm et al. 2000, Bhardwaj et al. 2003, Pantin-Jackwood et al. 2007, Shivaprasad et al. 2009, Lu et al. 2015, Chen et al. 2015, Zhong et al. 2016, Ayalew et al. 2017). However, the methods were only applied in tissues obtained from animal trials respectively only in turkey tissue or the signals were only visible with an electron microscope without giving details of the protocols that allow an accurate reproduction. Hence, the aim of

this study was to develop an ISH and an IHC to visualize the pathogen in infected tissues and to determine the causality between the virus and the clinical signs, respectively macroscopic and microscopic lesions.

2 Literature survey

2.1 Classification and morphology of avian reoviruses

ARV is a non-enveloped doubled stranded RNA (dsRNA) Virus and is a member of the family *Reoviridae*. The *Reoviridae* have a wide host range and contain many different important viruses like the blue tongue virus or rotaviruses. Inside the family *Reoviridae*, ARV is in the subfamily *Spinareovirinae* and in the genus *Orthoreovirus* (Benavente and Martinez-Costas 2007, Mertens 2004). The name "reovirus" is derived from respiratory enteric orphan virus. By definition, an orphan virus is not associated with disease (Jones 2013).

The dsRNA of the ARV is divided into 10 strands. They are refered to as L (large) 1–3, M (medium) 1–3 and S (small) 1–4 (Spandidos and Graham 1976). The genome of the ARV expressed 12 protein products of which 8 of these are structural proteins. The outer capsid protein sigma-C, which is encoded by the S1 gene, is the only protein that stimulates the production of ARV specific antibodies (Shapouri et al. 1996a).

The assembly and the replication of the virus occurs inside the cytoplasm before virus particles are released from host cell after cell death (Benavente and Martinez-Costas 2007). Recently, the ability for reassortment (antigenetic shift) of ARV was comprehensively reviewed (Attoui et al. 2011).

2.2 Diseases associated with avian reoviruses

ARV is involved in several diseases of poultry: It is the causative agent of viral arthritis (Walker et al. 1972) and involved in respiratory (Subramanyam and Pomeroy 1960) and enteric diseases (Page et al. 1982, Goodwin et al. 1993, Montgomery et al. 1997) but more than 80 % of ARV isolates are non-pathogenic (van der Heide 1977). Viral arthritis is the disease with the biggest economic impact caused by ARV and is recognised worldwide (Jones 2013).

2.2.1 Viral arthritis

In 1957, there was the first isolation of an agent that produced synovitis by Olson et al.. Later on it was described to be the "viral arthritis agent" (Olson and Kerr 1966).

Viral arthritis is economically an important disease which is mostly observed in broilers (Noh et al. 2018, Palomino-Tapia et al. 2018, Souza et al. 2018, Troxler et al. 2013), broiler-breeders (van der Heide and Kalbac 1975) but also layer type chickens and turkeys can be infected

(Macdonald et al. 1978, Gussem et al., 2017, Lu et al. 2015). In general, broilers have been reported to be more susceptible than laying breeds (Jones and Kibenge 1984).

The infection of ARV occurs mainly by horizontal transmission. Birds get infected via the oral route or via dermal injuries and shed the virus via faeces (Macdonald et al. 1978, Jones and Georgiou 1984). Furthermore, the virus can spread via vertical transmission (Menendez et al. 1975). An age resistance is assumed based on the fact that older birds show less severe clinical signs following infection (Jones and Georgiou 1984).

Acute infection by ARV can cause stunting of chicks whereas clinical signs for a chronic infection are lameness, usually recognised 4–5 weeks after hatching. The inflammation of the joints can be observed by swelling and increased temperature. If both legs get severely infected birds get immobilised. In most cases the mortality is low, however, morbidity can reach up to 100 % (Jones 2013).

Typically, gross lesion in chickens and turkeys are swellings of the gastrocnemius, digital flexor and metatarsal extensor tendons (Jones 2013). Some birds show green discolouration above the hock joint. This can be the consequence of a rupture of the gastrocnemius tendon (Jones and Kibenge 1984). The hock joint has usually a blood or straw-coloured content and can become purulent in some cases (Jones 2013).

Histological changes are edema, coagulative necrosis, accumulation of heterophil granulocytes, infiltration of lymphocytes, macrophages and proliferation of reticular cells in the acute phase of the infection. Chronical cases show a fibrosis of tendon sheaths, fibrineous content in the tendons and changes in chondrocytes (Jones 2013).

Causality between the presence of ARV and the disease must be proven by clinical signs, gross and histologic lesions. For diagnosis it must be considered that the described lesions are not pathognomonic for the disease. Pathogens that can cause similar lesions are *Mycoplasma synoviae*, *Staphylococcus* spp. or other bacteria that induce athritis (van der Heide 1977).

For prevention good management practice and biosecurity is important. Furthermore, vaccination can be applied. Chickens have the highest susceptibility to infection within the first two weeks of life. Later on, they develop an age-associated resistance against an infection. Maternal antibodies are capable of protecting the birds during the early stage of life, minimize the transovarian transmission and inhibit the replication of the virus in the gut (Jones 2013). To archieve a high amount of maternal antibodies in breeders the birds get vaccinated with an

inactivated vaccine after priming with a life vaccine (Wood et al. 1986). For that, different strains for vaccination are available but the most common vaccine strains are S1133, 1733 and 2408. The S1133 strain is commonly used as an attenuated live vaccine and the 1733 and the 2408 strain are used as inactivated vaccines with an adjuvant (Jones 2013). S1133 vaccine was developed by 235 serial passages of S1133 ARV strain in embryonated chicken eggs and 100 additional passages in chicken embryo fibroblast culture (van der Heide 1983). The vaccine is usually administered via the subcutaneous or intramuscularly route but also via coarse-spray application (Jones 2013).

2.2.2 Other diseases caused by avian reoviruses

2.2.2.1 Respiratory disease

The first ARV was isolated from birds with a chronical respiratory disease (Olson et al. 1957). This virus was called “Fahey Crawley Virus” (Fahey and Crawley 1954). It caused a mild mucoid nasal discharge in young birds. Anyhow, the virus has no abillity to cause severe respiratory disease (Subramanyam and Pomeroy 1960).

2.2.2.2 Malabsorptions syndrome

The Malabsorptions syndrome (MAS) is also described as “Runting-Stunting Syndrome”, “Brittle Bone Disease”, or “Helicopter Disease” (Jones 2013). Broilers in the second week of life are mostly effected (Pantin-Jackwood 2013). The clinical signs are an increased mortality, bone abnormalities, diarrhea with undigested food, retarded feathering and poor growth (Guy 1998). The primary etiological agents of MAS are Astroviruses, Parvoviruses and Coronaviruses. ARV, Adenoviruses and entero-like viruses are described to be secondary etiological agents of MAS (Jones 2013).

2.3 Detection methods of avian reovirus

The suspicion for an ARV infection is made by clinical, pathological and histopathological signs. The virus can be detected by electron microscopy (Shivaprasad et al. 2009, Zhong et al. 2016), by RT-PCR (Kant et al. 2003, Kumar et al. 2017, Pantin-Jackwood et al. 2008) or by qRT-PCR (Kumar et al. 2017, Tang and Lu 2016). Furthermore, the virus can be isolated and identified in cell culture (Grande and Benavente 2000) or in embryonated eggs (Guneratne et al. 1982).

Antibodies against ARV can be detected by the agar gel precipitin test (Jones 2013), indirect fluorescent antibody assay (Ide 1982), plaque test (Takase and Fujikawa 1996) and ELISA (Liu and Kuo 2002).

However, it has to be again mentioned that ARV is ubiquitous and the detection of ARV is not an evidence for causality between virus detection and disease (Jones 2013).

Since this thesis deals with the detection of ARV in tissues by ISH and IHC, it will be discussed in more detail below.

2.3.1 Detection of avian reoviruses in histological preparations

Histological changes can be observed in ARV infected birds (chapter 2.2.1), however, it is not possible to identify the virus by conventional histolopathology (Jones 2013).

2.3.1.1 *In situ* hybridization

Liu and Giambrone (1997a) reported the establishment of an ISH to detect ARV in tissues. The authors produced a digoxigenin (DIG)-labeled cDNA-Probe from the S1133 strain on the S1 gene using a cDNA library. For visualization, a sheep anti DIG-Fab (fragments of immunoglobulin G) antibody conjugated to alkaline phosphatase (AP) antibody was used with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates. The specifity of the probe was shown by a dot blot and the ISH. The probe was applied in a dot blot with the S1133, 1733, 2408 and CO8 strain and other viruses for hybridzation abilites. As a result the probe hybridized to the ARV strains but not to the other viruses. They applied the probe in paraffin embedded tissue samples obtained from an infection trial with chickens. In brief, one group of birds was infected at day 18 of life into the allantoic sac with the R2 strain. Another group of birds was infected after hatching subcutaneously also with same strain and one group of birds was infected intratracheally 7 days after hatching with the S1133 strain. Signals were observed in the liver, spleen, intestinal epithelium, tibiotarsal and hock joint, tendons and most frequently in the heart. To improve the sensitivity an *in situ* PCR was later on developed and tested on tissue obtained from animals which were expirimental infected with the S1133 and the 2408 strain (Liu et al. 1999).

Pantin-Jackwood et al. (2007) developed an ISH for reovirus of turkeys. They produced a 402 base pair probe by using RT-PCR. The probe was DIG-labeled. An anti-DIG antibody was used to detect the probe. Then streptavidin-alkaline-phosphatase conjugate was applied and NBT

and BCIP were used as substrates. SPF turkeys were experimentally infected with 1 of 4 different turkey field strains or with the 1733 chicken vaccine strain. The probe was applied in paraffin embedded tissue samples. Signals could be observed in the infected groups inside the cytoplasm of surface epithelial cells, stromal cells and macrophages of the bursa of Fabricius, in enterocytes at the tip and the middle of affected intestinal villi as well as in macrophages in the red pulpa of the spleen.

Details of the described ISH protocols are not available. Furthermore, the sequence of the probe established for the detection of the virus in chicken samples has not been published. For this reason the previous described procedures cannot be reproduced in samples of chickens.

2.3.1.2 Immunohistochemistry and immunofluorecence test

In 1974, Menendez et al. detected ARV antigen in an animal experiment using a fluorescent-labelled antibody on frozen sections. The used serum as well as the organs were obtained from chickens experimentally infected with the FDO-1 strain. Antigen was detected in the nasal turbinate, trachea, lung, air sac, gizzard, duodenum, jejunum, ileum cecum, cecal tonsil, rectum, cloaca, ovary, infundibulum, magnum, isthmus, uterus, flexor tendon and extensor tendon of infected birds. The immunological detection method was not as sensitive as the detection of the virus by cell culture. In 2003, Bhardwaj et al. used a similar protocol and were able to recognize intracytoplasmic fluorescens signals in liver, thymus, bursa, spleen and joints.

Songserm et al. (2000) developed an IHC to detect ARV in frozen intestinal tissue samples of experimentally infected broilers. The birds were infected with different ARV strains, WLS96, WLS88, MAS97-1, MAS97-2 and MAS80. The strains were isolated from cases showing MAS. Polyclonal rabbit anti-ARV antiserum were used as a primary antibody. ARV was detected in villi of the intestinal mucosa in birds that were infected with MAS97-1 and MAS97-2. In another animal trial, Songserm et al. (2003) used the same procedure and antibodies and were able to detect signals in the bursa and intestinal villi.

In 2015, Chen et al. performed an IHC with monoclonal antibodies applied on tissues from an animal trial. The monoclonal antibody H1E1 was directed against the ARV protein sigma-NS. The production of monoclonal antibodies was described by Shapouri et al. (1996b). The monoclonal antibody was used on paraffin embedded sections, on frozen sections and in a cell culture. In the paraffin sections a biotinylated goat anti mouse antibody was used as secondary antibody. Subsequently, the avidin-biotin peroxidase technique was used for signal

amplification. Diaminobenzidine was used as substrate for the color reaction. In the frozen sections, the H1E1 antibody was conjugated with Alexa 594. The fluorescence-cabled antibody was also used in an ARV-infected cell suspension. Specific signals were detected in the paraffin sections, in the frozen sections and in the cell culture.

Zhong et al. (2016) detected ARV field strains in a cell culture by monoclonal antibodies (1F4 against sigma-C). The antiodies were produced in their own laboratory detected by an fluorescens labelled secondary antibody (fluorescein isothiocyanate-conjugated anti-mouse IgG, Sigma-Aldrich)

Ayalew et al. (2017) investigated ARV signals in tendons (field samples) by using ultrathin sectioning and immunoglod labeling with anti-ARV primary antibody without detailed description, using negative staining TEM. Furthermore, ARV was detected in LMH cell culture by indirect immunoflurescence staining using an commercial available anti-ARV polyclonal antibody (Abcam, Cambridge, United Kingdom). Even though the manufacturer (Abcam) of the antibody indicate its suitablity exclusively for ELISA and neutralising assay.

Lu et al. (2015) detected ARV in infected LMH cells by flurescent antibody test. Virus was stained with an commercial available fluorescent tagged anti-ARV antibody (National Veterinary Services Laboratory, Ames, USA). According to the manufacturer, the antibody can be applied by IHC. The antibody is effective against ARV from the “Fahey Crawley Strain”. This strain has been described in connection with respiratory symptoms (chapter 2.2.2.1) (Fahey and Crawley 1954).

The two commercial anti-ARV antibodies were exclusively applied for immunofluorescence and are not described to be suitable for the use in tissue samples.

In the work of Pantin-Jackwood et al. (2007) convalescent or hyperimmune sera from ARV-inoculated turkeys or chickens were used to identify ARV-infected tissues. The IHC was applied in organs samples obtained from an animal trial. The turkeys were infected with different ARV strains. Four of the strains were isolated from turkeys and one from chickens. ARV specific signals were observed at the same location in the tissues of the birds as by ISH in the same work. Shivaprasad et al. (2009) identified ARV infection with IHC developed by Pantin-Jackwood et al. (2007) in the heart of turkeys with myocarditis as well as in other organs like bursa of Fabricius, spleen, intestine, lung and liver. However, the antibody is not

commercially available. This antibody was only used in turkeys and the application in chicken tissue was not reported.

3 Material and Methods

For the detection of ARV in tissue samples, protocols for ISH and IHC were set up and applied. ARV positive tissue samples and ARV grown in cell culture were taken from different field cases of ARV infected poultry. Additionaly one vaccine strain was used in cell culture for preparation of positive target cells. The field samples for the present trial have the protocoll numbers of the Clinic for Poultry and Fish Medicine, Unviversity of Veterinary Medicine, Vienna (Austria): PA 17/10253, PA 17/11463, PA 17/10812, PA 17/11025, PA 17/11024, PA 17/7376, PA 17/3942, PA 15/16103 and PA 14/5927 (**Tab. 1**). The vaccine strain (Nobilis® REO S 1133, MSD Animal Health, Makati City, Philippines) was included under the protocoll number PA 18/0014.

The viruses in the samples could be differentiated by their specific genetic cluster (**Tab. 1**). The allocation to the different clusters was done by sequencing of a part of the S1 gene. This gene encodes the protein sigma-C (Benavente and Martinez-Costas 2007). The S1133 vaccine strain belongs to the cluster 1. Other genetic analysed viruses used in this study belonged to cluster II–V and have been isolated from the field.

Tab. 1: Overview on the samples used for the detection of ARV: tissue samples of chickens suspected for ARV infection or cell pellets of ARV infected cell cultures.

Samples (Protocoll number)	Cluster sigma-C	Sample type	Origin	Diagnostic method
PA 14/5927	not applicable	tissue sample	tendons	histopathology
PA 15/16103			joint	
PA 17/3942	cluster 1		liver	cell culture (1. passage) and RT-PCR
PA 17/7376	cluster 4			
PA 17/10253	cluster 1	cell pellet	tendons	cell culture (2. passage) and RT-PCR
PA 17/10812	cluster 3			cell culture (1. passage) and RT-PCR
PA 17/11024	cluster 5			cell culture (2. passage) and RT-PCR
PA 17/11025	cluster 4			cell culture (1. passage) and RT-PCR
PA 17/11463	cluster 2		organs, pancreas, proventriculus	
PA 18/0014	cluster 1		Nobilis® REO S 1133 (MSD Animal Health)	verified by manufacturer

3.1 Sample preparation

3.1.1 Preparation of cell pellets

Cell pellets were prepared to establish positive control samples with a known virus concentration. In this process, chicken hepatocellular carcinoma cell line cells (LMH, CRL-2117TM, ATCC®, LGC Standards, Manassas, USA) were infected with different reovirus strains seperately ((PA 17/10253, PA 17/10812, PA 17/11024, PA 17/11025, PA 17/11463, PA 18/0014)) (**Tab. 1**). The LMH cells were seeded in 75 cm^2 bottles. The infected cells were incubated at 37 °C in 5 % CO_2 atmosphere. The virus titre was determined 3 days post infection (dpi) by plaque assay in the cell culture. The TCID 50 (tissue culture infection dose 50) was defined. After that cells were scraped off and centrifuged at 1,300 revolutions per minute (rpm) to obtain a cell pellet consisting of virus positive cells.

3.1.2 Preparation of paraffin sections

The pellets were resuspended for 10 minutes in formalin (formaldehyde 4 %, pH 7.2–7.4, SAV LP GmbH, Flintsbach am Inn, Germany) and then centrifuged at 4000 rpm for 10 minutes. This was followed by further fixation for 45 minutes in formalin in a biopsy cassette.

Formalin fixed tissue samples (PA 14/5927, PA 15/16103, PA 17/3942, PA 17/7376) and the above described pellets (PA 17/10253, PA 17/10812, PA 17/11024, PA 17/11025, PA 17/11463, PA 18/0014) were embedded in paraffin. According, the tissues were washed by rinsing in tap water for 15 minutes. Then, automated dehydration was performed (Histomaster 2065, Formafix GmbH, Düsseldorf, Germany) by increasing dilutions of alcohol before submerging it in Neoclear® (Merck, Darmstadt, Germany) and paraffin. Afterwards, the paraffin embedded samples were sectioned into 5 µm slices (Microm HM 360, rotating microtome, Microm International GmbH®, Germany) and mounted on Superfrost Ultra Plus® slides (Menzel-Gläser). Slices of the paraffin embeded cell pellets were examined by qRT-PCR (M1, developed by the Clinic for Poultry and Fish Medicine, unpublished work) on their ARV-RNA content to confirm the presence of viral RNA following preservation.

3.1.3 Preparation of frozen sections

The pellets (PA 17/10253, PA 17/10812, PA 17/11024, PA 17/11025, PA 17/11463, PA 18/0014) (**Tab. 1**) were embedded in OCT Tissue-Tek® (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) in a biopsy cassette. Then, the samples were snap frozen in liquid nitrogen (-80 °C). The pellets were sectioned into 5 µm slices using a cryomicrotom (CM 1800, Leica, Wetzlar, Germany) and mounted on Superfrost Ultra Plus® Slides (Menzel-Gläser, Thermo Scientific, Waltham, USA).

3.2 *In situ* hybridization

3.2.1 Probe preparation

3.2.1.1 Oligonucleotide Probes

Specific genetic regions of ARV were identified by using the GenBank® database. Mainly conserved regions in the genome (RNA encoding the reo sigma-NS, respectively the reo lambda-A) of ARV were targeted in order to detect different genetic variations of the virus.

The sigma-NS (non-structural) is, as the name suggests, a non-structural protein and is encoded by the S4 segment of the ARV genome (Schnitzer 1985, Varela and Benavente 1994, Jones 2000). The protein sigma-NS can bind single-stranded RNA in a nucleotide sequence unspecific manner (Lee and Yin 2015). The protein sigma-NS accumulate in viral factories of the infected cell. Therefore, there should be a high amount of complementary RNA inside the viral factory to bind the probe. In the cytoplasm of the infected cell it is associated to large ribonucleoprotein complexes (Touris-Otero et al. 2004).

The protein lambda-A is encoded by the L1 gene and is responsible to form the inner core shell. It is diffusely disseminated in the cytoplasm of infected cells (Benavente and Martinez-Costas 2007, Touris-Otero et al. 2004).

The specificity of the probes to ARV sequences was confirmed by Blast®. The sequences of the probes were sent to Eurofins Genomics (Ebersberg, Germany) for synthesising probes with a DIG label at the 3´end (**Tab. 2**).

Tab. 2: Sequences of the applied oligonucleotide probes.

Gene	Probe sequence	Accession number
Reo-sigma-NS	GCCATGGACAACACYGTGCGTGTTGGAGTTTCCCGCAACAC-DIG (3`–5`)	KP288866
Reo-lambda-A	GAGTTCGCGCAAAGTGGCTAGACGTCGTCATARGGATGCT-DIG (3`–5`)	KJ865904

3.2.1.2 RNA Probes

The protocol for establishing RNA-probes for ISH was applied according to Kidane et al. (2016) with slight modifications.

For that, total RNA was extracted from cell cultures using primary chicken liver cells infected with cluster 4 ARV (PA 17/11025) using the RNeasy® Mini kit (Qiagen, Hilden, Germany). Cluster I and cluster IV from two different ARV strains were used.

To obtain cDNA templates a conventional RT-PCR was performed using QIAGEN OneStep RT-PCR kit and two different primer pairs labelled P1P4 (Kant et al., 2003) and M1 (developed by the Clinic for Poultry and Fish medicine, unpublished work) (**Tab. 3**). The RT-PCR was

done using Engine Dyad® Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) and the specific programs. The reverse transciptase was activated at 50 °C for 30 minutes. Followed by denaturation of the reverse transcriptase at 94 °C (P1P4) or 95 °C (M1) for 15 minutes. The termal cycling conditions were 35 (P1P4) or 40 (M1) cycles of 94 °C (P1P4) or 95 °C (M1) for 30 seconds, 58 °C (P1P4) or 53 °C (M1) for 1 minute and 68 °C (P1P4) or 72 °C (M1) for 2 minutes (P1P4) or 1 minute (M1). The final step was 68 °C (P1P4) or 72 °C (M1) for 12 minutes (P1P4) or 10 minutes (M1). Afterwards the cDNA product was isolated by gel electrophoresis (1.5 % agarose gel) and then purified from gel by QIAcube (QIA-Gen, Hilden, Germany).

Tab. 3: Primers used for RT-PCR.

RNA target	Primer sequence 3`–5`	Product size (bp)
P1 (forward primer)[1]	AGT ATT TGT GAG TAC GAT TG	1088
P4 (reverse primer)[1]	GGC GCC ACA CCT TAG GT	
M1 (forward primer)[2]	TCA ACT GTA TCC CCR CGY CC	884
M1 (reverse primer)[2]	AC CTT AAC CGC ACG RAT RTC	

[1)] (Kant et al. 2003),
[2)] (developed by the Clinic for Poultry and Fish Medicine)

The cDNA product was ligated into a pCR®4-TOPO® vector following the instructor guidance (Invitrogen, Carlsbad, USA). The vector was then used to transform a chemicaly comptent *E. coli* which was grown on selective media (LB-Agar) containing Ampicilline (0.1 mg/ml) for 24 hours. The colonies were transferred to LB-broth also containing Ampicilline (0.1 mg/ml). The plasmid was determined for the presence of the insert by digestion using *Eco*RI and isolation of the fragments using gel electrophoresis. Plamids that contained the target fragment were sequenced by LGC-Genomics (Berlin, Germany) to verify the sequence and the orientation of the cloned product. The product was linearized with *Spe* I Restriction enzymes (New England BioLabs® Inc., Ipswich, USA). *In vitro* transcription of the probes was done by DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Rotkreuz, Switzerland) following the manufacture guidance. By this, a digoxigenin was inserted into the probe every 20-25 bases.

The concentration of the probe was quantified with NanoDrop spectrophotometry (Thermo Scientific). The size of the transcripted probe was verified by gel electrophoresis.

The success of the DIG-labeling was determined by dot blot following the modified instruction of the DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics). 1 µl of serial diluted probe was

spotted on a positive charged nylon membrane (Amersham HybondTM-N+, GE Healthcare, Chicago, USA) dried and UV linked. The strip was incubated in a "blocking solution" that contains 50 % of Buffer I 1x (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 0.3 % Triton® X-100 (Calbiochem, Merck) and 5% normal goat serum (Vector Laboratories, Burlingame, USA) in DEPC treated water, for 30 minutes. In the next step the stripe was incubated for another 30 minutes in "blocking solution" with 1:100 anti-DIG-AP-antibody (Roche Diagnostics). Then the stripe was washed two times in Buffer I diluted with an equal amount of DEPC treated water for 15 minutes and then for another 15 minutes in Buffer III (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl, final pH 9.5). For the colour reaction the stripe was incubated in Buffer III with 0.45 mg/ml of NBT and 0.175 mg/ml of BCIP (Roche Diagnostics) for 60 minutes. The reaction was stopped in TE-Buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 10 minutes. The development of dark spots confirmed the correct labelling of the probes.

3.2.2 *In situ* hybridization in paraffin sections

The ISH was performed based on a recentlys published protocol by Liebhart et al. (2006) with modifications to detect RNA in tissues. For the RNA probes and for the oligonucleotide probes the same protocol was applied.

The paraffin samples (**Tab. 4**) were deparaffined using Neoclear® (Merck) (two times) and rehydrated in a graded series of ethanol (2 x 100 %, 96 %, 70 %) each for 5 minutes. Previous to proteolysis the slides were rested in DEPC-treated water for another 5 minutes. The proteolysis was done in Tris-buffered saline (50 mM, pH 7.6) and 2.5 mg/ml proteinase K (Roche Diagnostics) for 30 minutes at 37 °C. The reaction was stopped by rinsing the slides two times in DEPC-treated water for a short time. Afterwards the tissues were dehydrated in 96 % and 100 % ethanol before dried by air. The hybridization solution contains 5 % herring-sperm DNA (Invitrogen), 50 % formamide, 20 % 20 x SSC, 2 % Denhardt 50 x (Sigma-Aldrich, St. Louis, USA), 10 % Dextransulfat (Sigma-Aldrich) and the probe. Two different oligonucleotide probes (lambda-A, sigma-NS) and two RNA probes (P1P4, M1) as described in chapter 3.2.1.1 and 3.2.1.2. The samples were heated with the hybridization mix at 95 °C for 6 minutes before cooled down on ice. Then the samples were incubated at 40 °C in a humidified chamber overnight.

On the second day stringency washing was performed in 2 x SSC for 30 minutes followed by a washing step at 40 °C for 30 minutes containing 16.6 % NaCl 3 M, 1 % Tris HCL 0.5 M,

0.2 % EDTA 0.5 M pH 8 (Merck), 0.01 % RNase A (10 mg/ml). The slides were put into 1 x SSC and in 0.1 x SSC for 10 minutes each.

Before the immunological detection of the hybrids an equilibration buffer containing Buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 5 % normal goat serum (5 %) (Vector Laboratories) and 3 % Triton® X-100 (10 %) (Calbiochem) was applied to the samples on the slides for 30 minutes for blocking any unspecific background.To detect the DIG-labeling of the probe anti-DIG-AP Fab fragments (Roche Diagnostics) was used. The anti-DIG-AP Fab fragments (Roche Diagnostics) were mixed (1:100) with the equilibration buffer. This mixture was applied to the sections for 30 minutes at room temperature. Then the slides were washed two times in Buffer I, each for 15 minutes. This was followed by a washing step in Buffer III (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl, final pH 9.5) for 15 minutes.

For visualization of the target the slides were overerlayed with Buffer III with 0.45 % NBT (Roche Diagnostics) and 0.35 % BCIP (x-Phosphat) (Roche Diagnostics) for up to 180 minutes. The reaction was stopped in TE-Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

For counterstaining the slides were put in distilled water for 5 minutes and afterwards dipped into Gill's hematoxylin (III) (Merck) for a short time. Then they were rinsed for 10 minutes with tap water and mounted with Aquatex® (Merck) before covering with glass coverslips.

As a control of the protocol the probe FAdV-D, (1:50, developed by the Clinic for Poultry and Fish Medicine, unpublished work) was hybridized to adenovirus positive paraffin embedded liver samples.

Tab. 4: Paraffin embedded samples used with different probes and different concentrations.

<table>
<tr><th>Samples (Protocoll number)</th><th>Oligonucleotide probes and concentration</th><th>RNA probes (P1P4, M1) and concentration</th></tr>
<tr><td>PA 14/5927 (tissue sample)</td><td rowspan="4">lambda-A: 1:10 (131.3 ng/ml)
sigma-NS: 1:10 (133.0 ng/ml)</td><td rowspan="6">not applied</td></tr>
<tr><td>PA 15/16103 (tissue sample)</td></tr>
<tr><td>PA 17/3942 cluster 1 (tissue sample)</td></tr>
<tr><td>PA 17/7376 cluster 4 (tissue sample)</td></tr>
<tr><td>PA 17/10253 cluster 1 (cell pellet)</td><td rowspan="2">lambda-A: 1:10 (131.3 ng/ml), 1:50 (26.3 ng/ml), 1:500 (2.6 ng/ml)
sigma-NS: 1:10 (133.0 ng/ml), 1:50 (26.6 ng/ml), 1:500 (2.7 ng/ml)</td></tr>
<tr><td>PA 17/10812 cluster 3 (cell pellet)</td></tr>
<tr><td>PA 17/11024 cluster 5 (cell pellet)</td><td rowspan="3">lambda-A: 1:10 (131.3 ng/ml)
sigma-NS: 1:10 (133.0 ng/ml)</td><td></td></tr>
<tr><td>PA 17/11025 cluster 4 (cell pellet)</td><td>1000, 500, 250 ng/ml</td></tr>
<tr><td>PA 17/11463 cluster 2 (cell pellet)</td><td>not applied</td></tr>
<tr><td>PA 18/0014 cluster 1 (cell pellet)</td><td>lambda-A: 1:10 (131.3 ng/ml), 1:50 (26.3 ng/ ml)
sigma-NS: 1:10 (133.0 ng/ml), 1:50 (26.6 ng/ml)</td><td>1000, 500, 250 ng/ml</td></tr>
</table>

3.2.3 *In situ* hybridization in frozen sections

For the ISH with frozen sections two different variations of the protocol used for paraffin sections (chapter 3.2.2) were applied.

In the first variation of the protocol the slides were stored at -20 °C before transferred to aceton for 20 minutes at -20 °C. After that they were dried by air for 30 minutes at room temperature and rehydrated in phoshate buffered saline (PBS) for 20 minutes. The proteolysis was done at room temperature for 10 minutes. The buffer used for proteolysis and the remaining protocol is given above (chapter 3.2.2). The applied probes are listed in **Tab. 5**.

In the second protocol, the slides were, as a pretreatment, warmed and dried for 20 minutes at 50 °C. The slides were placed into 4 % formalin in PBS (pH 7.4) for 20 minutes at room temperature followed by two times washing in PBS for 5 minutes each. The slides were then treated with 50 µg/ml proteinase K (Roche Diagnostics) in Tris-buffered saline (50 mM, pH 7.6) and 5 mM EDTA for 10 minutes at room temperature. After that the slides were washed again in PBS for 5 minutes and placed in 4 % formalin in PBS (pH 7.4) for 15 minutes. Before continuing with the next step the slides were shortly transferred into DEPC-treated water. The slides were treated with triethanolamine-HCl (0.1 M, pH 8.0) containing 0.25 % acetic anhydride. Afterwards the slides were washed again for 5 minutes in PBS. For prehybridization the slides were mounted with the prehybridization solution containing 50 % high grade formamide, 5 x SSC, 0.3 mg/ml yeast tRNA, Denhardt (Sigma-Aldrich), 0.1 % Tween-20 (Polysorbate 20), 5 mM EDTA for 2 hours at 55 °C in a humidified chamber. Then the slides were mounted with the prehybridization solution containing the probe (**Tab. 5**) and incubated over night at 55 °C in a humidified chamber.

On the second day the slides were rinsed two times in 2 x SSC at 55 °C for 15 minutes. For stringency washing the slides were placed into 2 x SSC with 10 µg/ml RNase A for 30 minutes at 37 °C. Then the slides were rinsed two times for 30 minutes with 2 x SSC at room temperature and washed two times in 0.2 x SSC at 55 °C. Before the antibody reaction was initiated the slides were washed in PBS for two times 15 minutes and then blocked in phoshate buffered saline + Triton® X (Calbiochem) (PBS + Triton®) with 10 % normal goat serum for 45 minutes. PBS + Triton® contains 1 x PBS, 2 mg/ml bovine serum albumin (BSA) and 0.1 % Triton® X-100 (Calbiochem). As antibody anti-DIG-AP Fab fragments (Roche Diagnostics) was used as a 1:2,000 dilution in PBS + Triton® with 1 % normal goat serum overnight in a humidified chamber.

On the third day the slides were washed in an alkaline-phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween-20) for 5 minutes and for another 5 minutes in alkaline-phosphatase buffer with levamisole (5 mM). For the colour development an alkaline-phosphatase buffer was prepared containing 0.1 % of NBT (75 mg/ml) and 0.35 % BCIP (50 mg/ml). The reaction was stopped after 4 hours in PBS with 10 mM EDTA 3 times for 5 minutes and fixed in formalin with 10 mM EDTA for 20 minutes.

Counterstaining and the mounting of the slides are given above (chapter 3.2.2).

As a control of the protocol, an already established probe (FAdV-D, 1:50) was hybridized to adenovirus positive frozen liver samples.

3.2.4 Verification of the probes using dot blot

The dot blot was used for validating the specify of the oligonucleotide probes and the RNA probes. The protocol was adapted to an puplished ARV dot blot protocol (Liu and Giambrone et al. 1997a). For this procedure, the RNA was extracted from cell cultures using ARV infected primary chicken liver cells. The RNA probes were localised at cluster 1 (PA 18/0014, vaccine, Nobilis® REO S 1133, MSD Animal Health) and cluster 4 (PA 17/11025, field strain) of the sigma-C Protein. As a reaction tube a 1.5 ml tube (Eppendorf, Hamburg, Germany) was used.

First, the ARV RNA was boiled using a thermomixer (Eppendorf Thermomixer comfort) before it was blotted on a membrane (Amersham HybondTM-N+, GE Healthcare) together with a negative control and, as a control for the "visualisation reaction", a DIG-labelled probe. The membrane was dried and UV linked. The prehybridization was done for 1 hour at a temperature of 42 °C (5 x SSC, 2 % normal goat serum (5 %) (Vector Laboratories), 50 % formamide, 0.1 % N-lauroylsarcosine, 0.02 % SDS). All probes used in this work were tested by dot blot (oligonucleotide probes: sigma-NS, lambda-A; RNA-probes: P1P4, M1). The probes were heated to 95 °C for 5 minutes in the thermomixer. 26 ng/ml of each probe were given to the hybridisation mix (like the prehybridization mix). The stripes were then incubated in the thermomixer overnight at 42 °C with a mild agitation.

On the second day two times 5 minutes steps of washing (2 x SSC, 0.1 % SDS) at room temperature followed by two times 15 minutes of washing (0.1 x SSC, 0.1 % SDS) at 68 °C were performed. The remaining steps were done at room temperature. Blocking was done with an equilibration buffer (Buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 5 % normal goat serum 5 %) (Vector Laboratories), 3 % Triton® X-100 10 % (Calbiochem)) for 30 minutes. After blocking the stripe was put in a 1:1,000 anti-DIG-AP Fab fragments (Roche Diagnostics) equilibration buffer solution for 30 minutes. Then the stripe was washed two times in Buffer I, each for 15 minutes. A washing step in Buffer III (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl, final pH 9.5) for 15 minutes was applied. The last steps were performed in darkness. For "visualization" of the probes the stripes were laid in Buffer III with 0.45 % NBT (Roche Diagnostics) and 0.35 % BCIP (x-Phosphat) (Roche Diagnostics) for 60 minutes. The reaction was stopped by putting the stripes in TE-Buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before drying overnight.

Tab. 5: Frozen samples used with different probes and different concentrations for ISH.

Samples (Protocoll number)	Oligonucleotide probes and concentration
PA 17/10253 cluster 1 (cell pellet)	lambda-A: 1:10 (131.3 ng/ml), 1:50 (26.3 ng/ml) sigma-NS: 1:10 (133.0 ng/ml), 1:50 (26.6 ng/ml)
PA 17/10812 cluster 3 (cell pellet)	
PA 17/11024 cluster 5 (cell pellet)	lambda-A: 1:10 (131.3 ng/ml) sigma-NS: 1:10 (133.0 ng/ml)
PA 17/11025 cluster 4 (cell pellet)	
PA 17/11463 cluster 2 (cell pellet)	

3.3 Immunostaining

3.3.1 Preparation of Antibodies

3.3.1.1 Rabbit raised antibodies

For the production of antibodies against ARV four rabbits (New Zealand white) were kept at the Institute for Parasitology, University of Veterinary Medicine, Vienna, Austria. The experiment was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ft. of Animal Experiments Act, Tierversuchsgesetz 2012 -TVG 2012 (license number GZ.38-205_0093-WF_V-3b_2016). They were initially immunized by 1 ml of the immunogen (cluster 1 (PA 18/0014, vaccine, Nobilis® REO S 1133, MSD Animal Health), cluster 4 (PA 17/11025, field strain)) solved in GERBU-Adjuvanz (GERBU Biotechnik GmbH, Heidelberg, Germany) by subcutaneous application. They were boostered 3 times in a 2–6 week intervall. Two of them were immunized with cluster 1 and two with cluster 4. Regularly, blood samples were taken to determine the antibody titre with a serum dilution neutralization test. They were kept for half a year before they were killed 18 weeks after the first immunization by blood deprivation under anaesthesia. The final titre of the serum was determined by the serum dilution neutralization test. The titres of the rabbits immunized with cluster 1 were 1:32 and 1:256 respectively. The rabbits immunized with cluster 4 had 1:32 and 1:128 respectively.

In order to obtain higher antibody concentrations, the rabbit sera were concentrated. The procedure was performed with a concentration kit (Amicon® Pro Affinity Concentration Kit – Protein G, Merck Millipore, Merck).

3.3.1.2 Chicken raised antibodies

Serum samples obtained from chickens infected or vaccinated with ARV were received from France. The first collection of sera came from a conventional breeder flock provided by the Institut National de la Recherche Agronomique (Nouzilly, France). Furthermore, sera from SPF chickens vaccinated twice with Nobilis® REO ERS inac (MSD Animal Health) were available. The applied vaccine contained the ARV strains 1733 and 2408 and was developed for a booster of a parent stock after priming with a live vaccine. In addition sera was available from SPF chickens vaccinated twice with TRI-REO® (Rhone Ma Holdings Berhad, Selangor, Malysia). This vaccine consists of the inactivated strains S1133, 2408 and 3005. Finally, sera from SPF chickens vaccinated twice with Nobilis® REO 1133 (MSD Animal Health) which contains the live strain S1133 were applicable. The sera were tested by ELISA (Biochek, Reeuwijk, Netherlands) and showed high antibody titers. The amount of antibodys in the samples belonged to the titer group 8–12 (maximum value = 18).

3.3.2 Immunohistochemistry

3.3.2.1 Immunohistochemistry in paraffin sections

For the detection of ARV the avidin biotin complex (ABC) technique (Vectastain ABC Kit, Vector Laboratories) was used:

IHC in paraffin samples containing ARV of cluster 1 (PA 18/0014), cluster 4 (PA 17/11025) and cluster 5 (PA 17/11024) was done after the modified protocol according to Singh et al. (2008). The modifications included different concentrations of the primary antibody (**Tab. 6**) and the incubation overnight at 4 °C. The initial concentrations of the sera were measured by ELISA and serum dilution neutralization test (chapter 3.3.1.1 and 3.3.1.2).

To determine the correct implementation of the protocol, already established antibodies were applied with the corresponding infected tissue. For that, an anti-*Histomonas meleagridis* (rabbit anti-Histomonas) (Singh et al. 2008) and an anti-FAdV-D antibody were used with the respective positive controls.

3.3.2.2 Immunohistochemistry in frozen sections

The IHC with frozen sections was performed with chicken antibodies. The same technique and protocol were applied as for the paraffin sections without dewaxing and the microwave treatment. IHC was done using cell pellets with ARV of cluster 1 (PA 18/0014) and cluster 4 (PA 17/11025) (**Tab. 6**).

As described above, the protocol was tested with established antibodies for its correct implementation. Again, an anti-*Histomonas meleagridis* (rabbit anti-Histomonas) (Singh et al. 2008) and an anti-FAdV-D antibody with corresponding positive control sections were used.

Tab. 6: Paraffin embedded and frozen samples used with different antibodies and different concentrations for IHC.

<table>
<tr><th>Samples (Protocoll number)</th><th>Unconcentrated rabbit antibodies and concentration</th><th>Concentrated rabbit antibodies and concentration</th><th>Chicken antibodies and concentration</th></tr>
<tr><td>PA 17/11024 cluster 5 (cell pellet)</td><td>not applied</td><td rowspan="2">1:500 (paraffin samles)</td><td>not applied</td></tr>
<tr><td>PA 17/11025 cluster 4 (cell pellet)</td><td rowspan="2">1:50, 1:500, 1:1000, 1:5000, 1:10000 (paraffin samples)</td><td rowspan="2">1:50, 1:500, 1:5000 (paraffin and frozen samples)</td></tr>
<tr><td>PA 18/0014 cluster 1 (cell pellet)</td><td>not applied</td></tr>
</table>

3.3.3 Immunofluorescence

LMH cells were grown on a 96 well plate. The cells were infected separately with cluster 1 (PA 18/0014) and cluster 4 (PA 17/11025) (**Tab. 7**). The infection was confirmed by observation of the cytopathic effect. 72 hours after infection and 70 % confluence the cells were rinsed with PBS. Then the cells were fixed with methanol at -20 °C for 5 minutes. After that, the cells were incubated in PBS for 10 minutes. After three washing steps with PBS (5 minutes each) the cells were blocked with 3 % BSA for 1 hour at room temperature. The rabbit antibodies were diluted 1:100, 1:500, 1:1,000, 1:10,000 and then incubated for 1 hour at room temperature. The process was stopped by three times washing with PBS for 5 minutes each. The second antibody (goat anti-rabbit IgG, Alexa fluor 568, Life technologies, Carlsbad, USA) was diluted 1:500 and incubated for 1 hour at room temperature. Before counterstaining with DAPI (1:1,000) the cells were rinsed with PBS (3 times each 5 minutes).

The procedure was done with infected cells (cluster 1 and cluster 4) and as controls with i) uninfected cells, ii) without a second antibody as well as iii) without a primary antibody.

Tab. 7: Cell cultures used with different antibodies and different concentrations for immunofluorescence.

Cell culture (Protocoll number)	Concentration of antibodies in rabbit serum
PA 17/11025 cluster 4	1:100, 1:500, 1:1000, 1:10000
PA 18/0014 cluster 1	

3.4 Haematoxylin and Eosin staining

Parrafin embedded sections on slides were dewaxed in Neoclear® (Merck) (two times for 5 minutes) before they were rehydrated in a descending series of ethanol (100 %, 96 %, 70 %) and distilled water each for 5 minutes.

Frozen samples were transferred to aceton for 20 minutes at -20 °C for 20 minutes before they were air dried for 30 minutes.

Frozen and paraffinized sections were stained by Haematoxylin solution, Gill III (Merck) for 3 minutes. After this step the slides were rinsed with tab water three to four times. To obtain the preferred colour intensity the slides were shortly dipped in a solution consisting of 1 % HCl in 70 % ethanol. The intensity of staining was controlled by microscopic observation. To develop a bright colour the slides were rinsed for 10 minutes with tab water. After dipping in destilled water they were stained by Eosin G-solution (0.5 % watery solution, Carl Roth GmbH + Co. KG®, Karlsruhe, Germany) for a short time. To remove the remaining eosin the slides were rinsed with tab water. Then the samples were dehydrated in an ascending series of ethanol (70 %, 96 %, 100 %) and Neoclear® (Merck). Finally, the tissue sections were covered using a glas cover slip with Neomount® (Merck) mounting medium.

3.5 Analysis of the samples

The tissue and the cell pellet samples were evaluated by microscope (BX 53, Olympus, Tokyo, Japan) and pictures were made with the Olympus DP 72 camera (Olympus). For the evaluation of the pictures the Olympus cellSens standard imaging software Version 1.4 (Olympus) was used.

For the immunofluorescence a inverted microscope Leica DM IRB was used and pictures were made with the Leica DFC 300 FX camera. For the evaluation of the pictures DFC Twain V7.7.1 software (Leica) was used.

4 Results

4.1 Virus concentration in cell pellets

The virus titre of the cell cultures used for the preparation of the cell pellets was determined 3 days post infection (dpi) by plaque assay in the cell culture. Plaques were found in all infected cell cultures. Virus concentrations are shown in **Tab. 8**.

Tab. 8: Virus concentration tested by plaque assay 3 days post infection.

Samples (Protocoll number)	Cluster sigma-C	Sample type	Virus concentration tested by plaque assay
PA 17/10253	cluster 1	cell pellet	$10^{3.3}$ pfu*/ml
PA 17/10812	cluster 3		$10^{3.7}$ pfu*/ml
PA 17/11024	cluster 5		$10^{3.7}$ pfu*/ml
PA 17/11025	cluster 4		$10^{3.3}$ pfu*/ml
PA 17/11463	cluster 2		$10^{2.7}$ pfu*/ml
PA 18/0014	cluster 1		$10^{8.4}$ pfu*/ml

* plaque forming units

4.2 *In situ* hybridization

4.2.1 *In situ* hybridization in paraffin sections

There were no specific signals observable by ISH after applying the oligonucleotide probes (**Tab. 9**) (**Fig. 1** and **Fig. 2**) or the RNA probes (**Fig. 3** and **Fig. 4**) on the tissues or the cell pellets. This was true for all probes in the different concentrations. Negative controls did not result in the development of an unspecific signal (**Fig. 4**, **Fig. 5** and **Fig. 6**). The already established probe (FAdV-D, 1:50), which was included as control for the entire protocol, clearly detected adenoviruses in the respective positive samples for FAdV (data not shown).

Tab. 9: Results of ISH on paraffin embedded samples with different probes and different concentrations.

Samples (Protocoll number)	Oligonucleotide probe	RNA probe	Result
PA 14/5927 (tissue sample)	lambda-A sigma-NS	not applied	no specific signals observed
PA 15/16103 (tissue sample)			
PA 17/3942 cluster 1 (tissue sample)			
PA 17/7376 cluster 4 (tissue sample)			
PA 17/10253 cluster 1 (cell pellet)			
PA 17/10812 cluster 3 (cell pellet)			
PA 17/11024 cluster 5 (cell pellet)			
PA 17/11025 cluster 4 (cell pellet)		P1P4 M1	
PA 17/11463 cluster 2 (cell pellet)		not applied	
PA 18/0014 cluster 1 (cell pellet)		P1P4 M1	

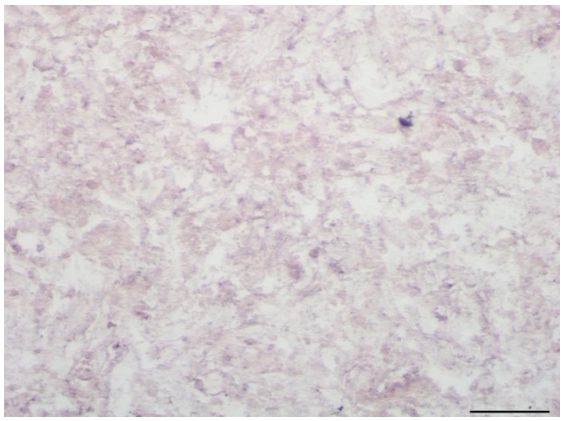

Fig. 1: No signals by ISH in a cell pellet with the lambda-A-probe, paraffin, infected cells (bar = 20 µm)

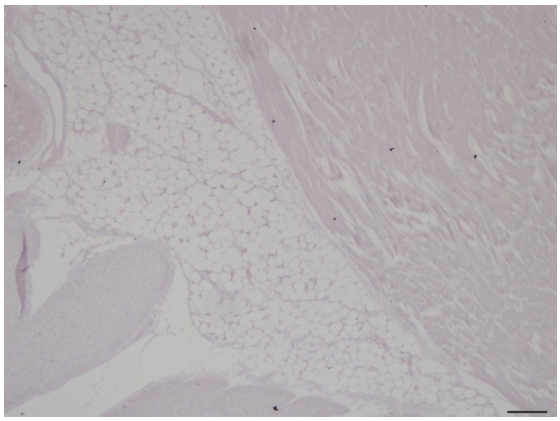

Fig. 2: No signals by ISH in a joint (PA 14/5927) with the lambda-A-probe, paraffin, ARV pos. (bar = 100 µm)

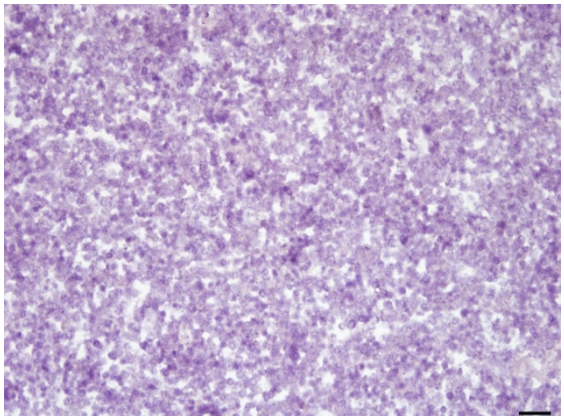

Fig. 3: No signals by ISH in a cell pellet with the P1P4 RNA-probe, paraffin, infected cells (bar = 50 µm)

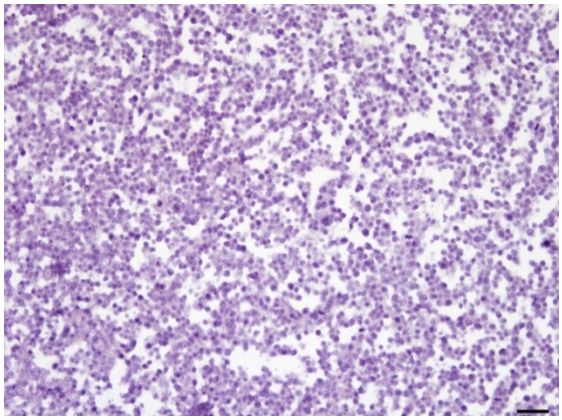

Fig. 4: No signals by ISH in a cell pellet with the P1P4 RNA-probe, paraffin, uninfected cells (bar = 50 µm)

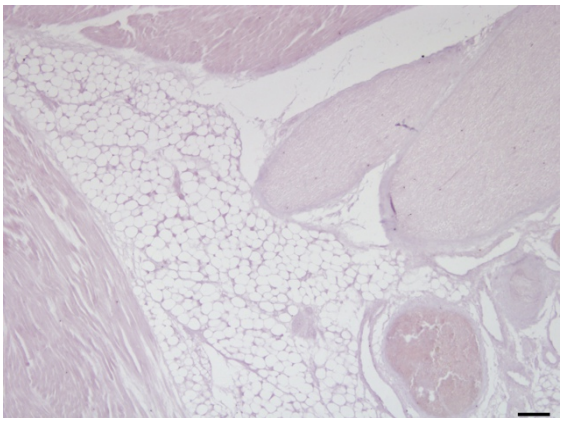

Fig. 5: No signals by ISH in a joint (PA 14/5927) without a probe, paraffin, ARV pos. (bar = 100 µm)

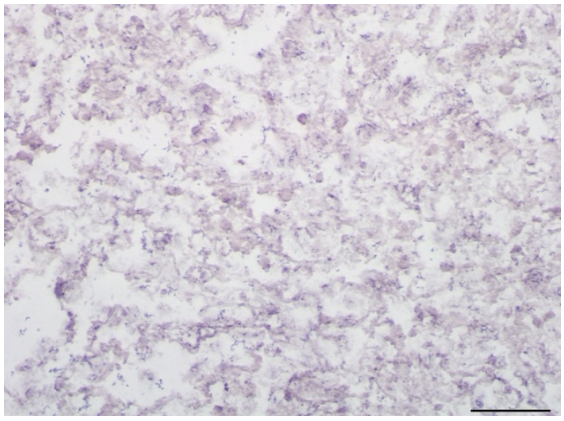

Fig. 6: No signals by ISH in a cell pellet without a probe, paraffin, infected cells (bar = 20 µm)

4.2.2 *In situ* hybridization in frozen sections

The morphology of the pellets was degraded by processing the samples. However, there was distinct cell material on the slide for applying the ISHs. Overall, no specific signals could be observed (**Tab. 10**).

As control of the protocol, the already established probe (FAdV-D, 1:50) was carried out. Thereby, adenovirus could be specifically detected in the respective FAdV infected cells.

Tab. 10: Results of frozen samples used with different probes and different concentrations for ISH.

Sample (Protocoll number)	Oligonucleotide probes	Result
PA 17/10253, cluster 1 (cell pellet)	lambda-A sigma-NS	no specific signals observed
PA 17/10812, cluster 3 (cell pellet)		
PA 17/11024, cluster 5 (cell pellet)		
PA 17/11025, cluster 4 (cell pellet)		
PA 17/11463, cluster 2 (cell pellet)		

4.2.3 Verification of the probes using dot blot

The correct labeling of the probes could be proven by dot blot. No specific signals, however, could be detected by dot blot for the oligonucleotide probes (sigma-NS and lambda-A) and RNA probes (P1P4 and M1) on the membrane. Negative controls did not indicate any unspecific reaction.

4.3 Immunostaining

4.3.1 Immunohistochemistry

The antibodies used for IHC produced in rabbit and chicken resulted in no signals using different concentrations of the antibodies on paraffin samples (**Tab. 11**). Unspecific signals were observed using rabbit and chicken antibodies against ARV in infected (**Fig. 7**) and non-infected cell pellets (**Fig. 8**). IHC of cell pellets without any primary antibody did not result in an unspecific staining (**Fig. 9**).

Tab. 11: Results of paraffin embedded and frozen samples used with different antibodies and different concentrations for IHC.

Sample (Protocoll number)	Antibodies	Result
PA 17/11024 cluster 5 (cell pellet)	concentrated rabbit antibody (paraffin samples)	no specific signals observed
PA 17/11025 cluster 4 (cell pellet)	unconcentrated rabbit antibody (paraffin samples) concentrated rabbit antibody (paraffin samples) chicken antibody (paraffin and frozen samples)	
PA 18/0014 cluster 1 (cell pellet)	unconcentrated rabbit antibody (paraffin samples) chicken antibody (paraffin and frozen samples)	

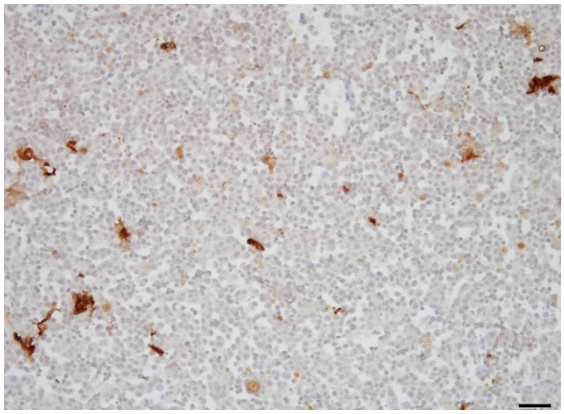

Fig. 7: Unspecific signals by IHC in a cell pellet with a rabbit antibody, paraffin, infected cells (bar = 50 µm)

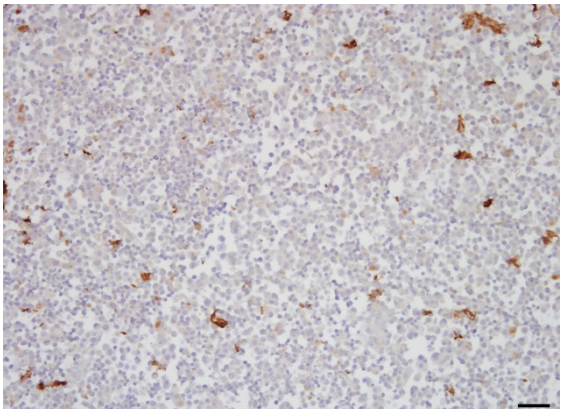

Fig. 8: Unspecific signals by IHC in a cell pellet with a rabbit antibody, paraffin, uninfected cells (bar = 50 µm)

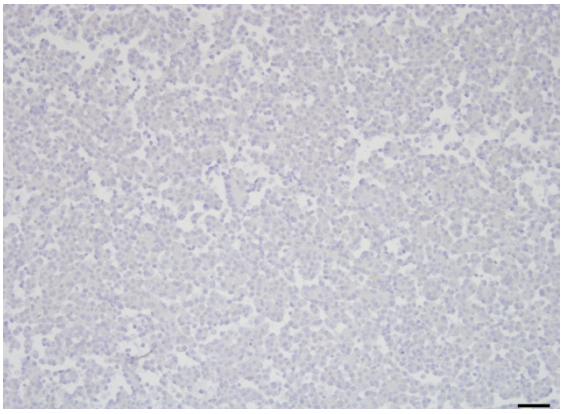

Fig. 9: No signals by IHC in a cell pellet without antibody, paraffin, infected cells (bar = 50 µm)

4.3.2 Immunofluorescence

No specific signal was observed in the investigated samples (**Tab. 12**). Using a high intensity of light, unspecific fluorescence appeared in infected as well as uninfected cells (**Fig. 10** and **Fig. 11**). No signal was observed in samples without application of antibody.

Tab. 12: Results of cell cultures used with different antibodies and different concentrations for immunofluorescence.

Cell culture (Protocoll number)	Antibodies	Results
PA 17/11025 cluster 4	unconcentrated rabbit antibodies	no specific signals were observed
PA 18/0014 cluster 1		

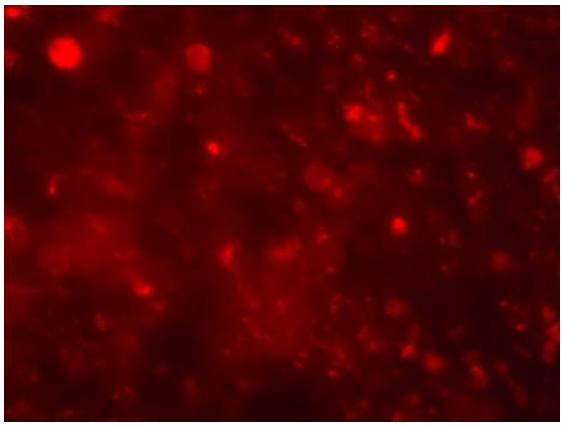

Fig. 10: Unspecific signals by immunofluorescence in infected cells with rabbit antibody

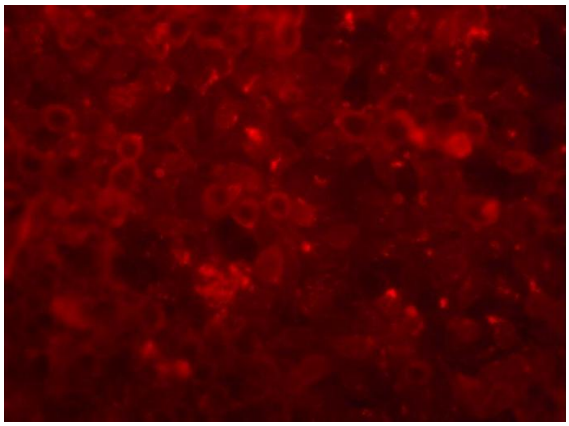

Fig. 11: Unspecific signals by immunofluorescence in uninfected cells with rabbit antibody

4.4 Haematoxylin and Eosin staining

In the HE staining clear changes in the infected cell pellets (**Fig. 12**) but also in the ARV positive tissue sections could be observed (**Fig. 13**). A cytopathic effect could be observed in the cell pellets whereas uninfected cell pellets showed no changes (**Fig. 14**). The infected tissue sections showed inflammation, mainly with lymphocytic infiltrations.

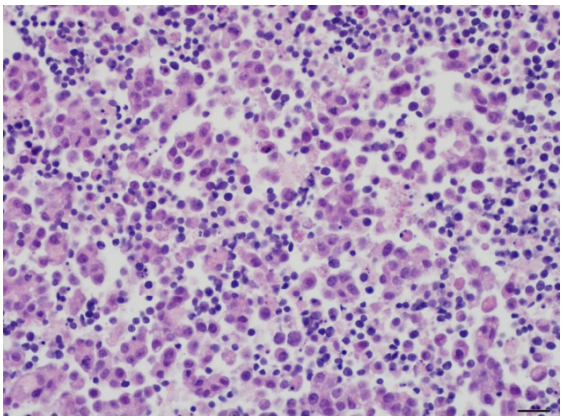

Fig. 12: Cytopathic effect in a cell pellet, HE, paraffin, infected cells (bar = 20 µm)

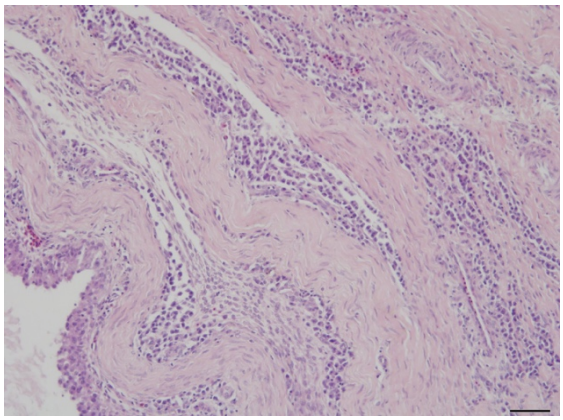

Fig. 13: Lymphocytic tenosynovitis in a joint, HE, 10-times enlarged, paraffin, ARV positive

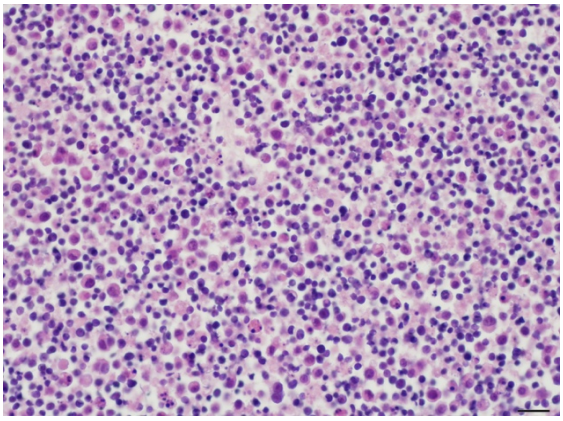

Fig. 14: No changes in a cell pellet, HE, paraffin, uninfected cells (bar = 20 µm)

5 Discussion

ARV is a pathogen in poultry that causes diseases like arthritis, MAS and respiratory diseases (Jones 2013). In the last years several cases were reported in which vaccination did not provide a proper protection (Szilvia et al. 2016, Davis et al. 2013, Lu et al. 2015, Noh et al. 2018, Palomino-Tapia et al. 2018, Ayalew et al. 2017, Souza et al. 2018, Troxler et al. 2013). Using the existing diagnostic tools like pathology, histopathology, RT-PCR, qRT-PCR and virus isolation, it is difficult to demonstrate a causality between ARV and the ARV-associated diseases (Jones 2013). In order to resolve this problem an ISH or an IHC would be very helpful to detect the infectious agent in tissue samples.

There are different publications available describing ISH and IHC of ARV in tissues. However, these protocols could not be directly reproduced due to various reasons. The previously described probes used for ISH are not commercially available and the sequences of the probes have not been published as well as the procedure of antibody production. The commercially available anti-ARV antibodies were only applied for immunofluorescence (Ayalew et al. 2017, Lu et al. 2015) and are not certified for IHC (for different strains) by the distributing companies. Furthermore, previous by established ISH and IHCs were only used in the context of specific animal trials and never applied in tissues of ARV infected chickens from the field. In the animal trials, the birds were only infected with a very limited number of different strains. Ayalew et al. (2017) detected ARV in tendons with the IHC. However, this was only done with electron microscopy. Therefore, it is not known whether the published methods can identify a broader spectrum of different strains. An IHC for the detection of ARV in tissues of birds infected in the field was reported by Shivaprasad et al. (2009), who used the antibody reported by Pantin-Jackwood et al. (2007), exclusively in turkey organs.

The intention of the present work was to develop an ISH and an IHC that can be applied in the diagnosis of field cases to facilitate a proper diagnosis. However, this aim could not be achieved even though different protocols, inclusively different probes, respectively antibodies have been tested for their suitability.

The application of ISH resulted in no specific signals in ARV infected tissues and cells from culture. The efficacy of the protocols was determined with an established probe and respective positive controls (FAdV-D, 1:50, probe was hybridized to adenovirus positive paraffin liver samples). Therefore, the failure of the ISH most probably arose due to mismatches of the targeted sequence and the probe.

A major problem was that the viral load in the tissue samples prepared for ISH was unknown. For this reason, cell pellets were prepared to determine the virus titer of the cell culture by plaque assay (chapter 3.1.1). Even though a distinct virus titer was measured (**Tab. 8**) and a clear propagation of the virus could be observed by cytopathogenic effects it might be possible that the amount of virus was degraded during preparation of the pellets used for ISH. Probably the virus concentration was below the detection limit which was described to be variable according to the applied probe (Nuovo 1997). Liu and Giambrone (1997a) showed that the signal observed by ISH was related to the virus concentration and that it was not detectable below a certain threshold value. Therefore, it can be speculated that the virus concentration and with it the signal might be below the detection limit. The improvement of the sensitivity to detect of ARV in tissues was indicated in the work of Liu et al. (1999) who developed an *in situ* PCR following the described ISH (Jen and Giambrone 1997). The application of *in situ* PCR is known to significantly increase the sensitivity compared to ISH (Nuovo 1997).

Furthermore, the choice of a fixation is crucial for the amount of target nucleic acid for ISH. A fixation with formalin improves the preservation of cellular morphology but also reduced the accessibility of the target (Leitch et al. 1994, Wilkinson 1995). It is possible that the fixative inhibited the binding of the probe or that the RNA of the ARV is already decomposed or destroyed by ribonuclease (Nuovo 1997). To determine the RNA content of the paraffin embedded cell pellets qRT-PCR was done. By that, ARV RNA was still detectable. Nevertheless, in order to exclude a degradation of nucleic acid in the samples by formalin fixation, frozen sections were additionally used, however, without success.

A probe should have a high sensitivity and specificity for the target nucleic acid. It was previously described that the type of the probe has an influence on its sensitivity (Wilkinson 1995). In the present work ARV oligonucleotide probes as well as RNA probes were applied as they have diverse characteristics. The oligonucleotide probes used in this work consist of 40 and 41 nucleotides respectively. Shorter probes produce stronger signals than longer probes. This is because short probes like these oligonucleotide probes can penetrate tissue better than longer probes (Wilkinson 1995). A disadvantage of short probes is their lower sensitivity compared to longer probes which have a higher number of signal molecules (Clark 1996). The oligonucleotide probe used in this work contains only one digoxigenin as signal molecule. The RNA probes used in this study were equipped with a digoxigenin every 20–25 nucleotides. As a result, more target molecules should be present than with oligonucleotide probe.

Beside that, the penetration is also depended on the type of the tissue, the fixative and the pre-treatment (Leitch et al. 1994, Nuovo 1997). In order to circumvent the specific failures of probes we applied short oligonucleotides and longer RNA probes in the present work.

In order to exclude possible interference factors, hybridization was carried out as a dot blot on a membrane according to a previous described protocol (Liu and Giambrone 1997). However, no signal could be observed by this method. This result indicated that the probes were inefficient in hybridizing with the spotted RNA.

Similar to the ISH, there are several influencing factors that impede the development of a specific signal by IHC. The problems are mainly associated with antigen-antibody binding. The reason for an undetectable antigen can be a low antigen concentration or a structural change of antigen during the handling procedure (Buchwalow and Böcker 2010). Based on the results of plaque assay the amount of virus and therefore also of the antigen was generally low.

In the literature only one study reported a successful IHC to detect ARV in chicken field samples which was evaluated by TEM (Ayalew et al. 2017). The authors observed signals after immunogold labeling using anti-ARV antibodies in tendon tissue that obviously could not be identified by light microscopy. This perhaps because of a too low virus concentration in the tissue. It might explain why it was not possible to detect specific signals in the present study. However, it was not intended to combine the IHC with electron microscopy in this work as the virus can be identified by its morphology even without IHC or ISH (Ayalew et al. 2017). IHC or ISH could be used to differentiate different strains using specific antibodies or probes. However, differentiation with RT-PCR and following sequencing is much more precise and therefore probably more useful.

Ayalew et al. (2017) and Lu et al. (2015) used the commercially available antibodies exclusively in cell culture by the immunofluorescence technique. It is not known if the mentioned antibodies can detect different strains of ARV. The distributor (Abcam) of the antibody used by Ayalew et al. (2017) indicates its suitablity exclusively for ELISA and neutralising assay. According to the manufacturer (National Veterinary Services Laboratory), the antibody used by Lu et al. (2015) can be applied by IHC. However, this was produced with the “Fahey Crawley Strain”, that is involved in respiratiory symptoms, and therefore probably has a narrow binding spectrum. The antibody is fluorescent labelled which severly reduced the application in paraffin embedded tissue due to autofluorescence.

The rabbit sera produced in the present work were tested by serum dilution neutralization test and the chicken sera were tested by ELISA. It was shown that in all of the samples ARV specific antibodies were present. However, the antibody concentration of the rabbit serum was found to be low. Directly used rabbit sera and following concentration did not yield in a positive antigen-antibody reaction by ISH.

The IHC protocol was applied not only with paraffin sections because it is possible that the fixation with formalin, the dehydration with alcohol or the paraffin embedding destroyed the antigen (Mulisch 2010). In order to exclude the described negative impacts during preparation of paraffin embedded samples frozen material was additionally used. The frozen sections were embedded by OTC. However, Mulisch (2010) reported that the embedding medium can also disturb the IHC process when the medium is not accurately removed. The paraffin also has to be extensively removed otherwise the antigens are covered and not free to bind with the antibody. The present protocol works reliably with other antibodies and positive samples. Therefore, it is unlikely that preparation or washing steps are the cause of the failure. Anyhow, the frozen section lost part of their morphological integrity during the cutting process which can be related to a loss of antigens (Buchwalow and Böcker 2010).

To bypass the negative effects of the treatment of the samples as the cause of an unsuccessful IHC an immunofluorescence directly was conducted. The immunofluorescence showed no specific signals as well. This could be due to non-binding antibodies or, again, due to a too low amount of virus.

5.1 Possible further strategies

In the present work it was not possible to establish an ISH or an IHC for the detection of ARV in tissue. Therefore, a different strategy is necessary to detect the virus in histological preparations.

It would be possible to search for signals of an ISH and an IHC with an electron microscope. This was already done by Ayalew et al (2017). The use of an electron microscope would be sufficient to visualize the pathogen in the infected tissue but the use of an electron microscope is very time-consuming, only available in a few laboratories, and expensive. For this reason, this method would probably be unsuitable for routine diagnostics but very interesting for special investigations and research. The use of a light microscope would of course be more practicable. Therefore, it would be useful to continue looking for a way to visualize ARV in tissues.

Another approach would be to implement an IHC using monoclonal antibodies. Monoclonal antibodies have already been applied by Chen et al. (2015) but are not applicable for the routine diagnosis. Monoclonal antibodies can be achieved by hybridoma technology in which the antibodies are produced in a cell culture (Buchwalow and Böcker 2010). Monoclonal antibodies are only directed against a single epitope and have therefore a very high specificity (Peters et al. 1988). However, as ARV is a very heterogeneous virus, it is likely that a monoclonal antibody does not detect multiple ARV strains. This might be avoided by combining different monoclonal antibodies. In this way a high specificity and a high sensitivity can be achieved (Reisner and Wick 1988).

Another possibility would be to amplify the used primary or secondary antibodies with amplifying reagents. An examples of this technology is the Tyramide Signal Amplification (Thermo Fisher Scientific, Waltham, USA). According to the manufacturer, these reagents can increase sensitivity up to 10 times.

Another possibility to amplify the signal would be the use of a probe with a radioactive label. Radioactive probes can be directly detected by measuring the radioactive radiation and do not require a color reaction. This increases the sensitivity (Wilkinson 1995). A major disadvantage, however, is the costly disposal and the endangerment of the user (Wilkinson 1995). For these reasons, radioactive probes can only be used in exceptional cases and are rarely used today due to the radioactive hazard.

The development of an *in situ* PCR seems to be a promising possibility according to the literature (Liu et al. 1997). The strength of *in situ* PCR is the combination of the high sensitivity by PCR with the possibility to localize the pathogen in tissue sections (Gu 1995). Therefore, it would be advantageous to develop this method by identifying relevant primers and get information on suitable protocols. A disadvantage, however, would be the relatively laborious protocol of an *in situ* PCR which might not be feasible for routine diagnosis (Nuovo 1997).

5.2 Conclusion

Despite extensive investigations to establish an ISH and an IHC for the detection of ARV, this aim could not be achieved. As a consequence, it was not possible to implement a protocol to detect the pathogen in the tissue samples for routine diagnosis. The etiological association between the pathogen and the disease still relies on the combination of clinical signs, pathological changes and virus detection by isolation as well as PCR.

6 Summary

The aim of the present work was the development of an *in situ* hybridization (ISH) and an immunohistochemistry (IHC).

An oligonucleotide probe was developed using the GenBank database to perform an ISH. It was tested with different protocols, in paraffin sections and frozen sections, in tissues infected with avian reoviruses (ARV) and cell pellets with different probe concentrations. However, no specific signal could be detected.

Furthermore, an RNA probe was developed. However, no specific signal could be generated here either.

For all probes, a dot blot was also carried out in which ARV was UV linked to a positively charged membrane. Also here the probes could not bind.

To develop an IHC, polyclonal antibodies were produced in rabbits. The rabbits were immunised with different ARV strains. Antibodies against ARV could be detected in the sera of the rabbits by a serum neutralization test, but the titre concentrations were low. In addition, antibodies against ARV produced in chickens were used for IHC.

By IHC no specific signals could be detected in ARV infected pellets at different concentrations of the sera, on paraffin or frozen sections.

The disfunction of ISH and IHC protocols are manifold and difficult to identify. However, an important factor for the failure of the methods described in the present work are most probably the low virus content in tissues and pellets.

7 Zusammenfassung

Das Ziel der vorliegenden Arbeit war die Entwicklung einer *In situ* Hybridisierung (ISH) und einer Immunhistochemie (IHC).

Zur Durchführung einer ISH wurden Oligonukleotidsonden mithilfe der GenBank® Datenbank entwickelt. Sie wurde mit verschiedenen Protokollen, auf Paraffinschnitten und Gefrierschnitten, in mit aviären Reoviren (ARV) infizierten Geweben und Zellpellets mit unterschiedlichen Sondenkonzentrationen getestet. Dabei konnte jedoch kein spezifisches Signal detektiert werden.

Weiters wurde eine RNA-Sonde entwickelt. Jedoch konnte auch hier kein spezifisches Signal in Zielzellen erzeugt werden.

Für alle Sonden wurde auch ein Dot Blot durchgeführt, bei dem ARV auf einer positiv geladenen Membran UV-verlinkt wurden, was ebenfalls zu keiner Bindungsreaktion führte.

Zur Entwicklung einer IHC wurden polyklonale Antikörper in Kaninchen produziert. Die Kaninchen wurden dazu mit verschiedenen ARV Stämmen infiziert. Es konnten in den Seren der Kaninchen Antikörper gegen ARV mittels Serumneutralisationstest nachgewiesen werden, jedoch waren die Titerkonzentrationen gering. Zusätzlich wurden noch Antikörper gegen ARV, die in Hühnern produziert wurden, mittels IHC getestet.

Bei der Durchführung der IHC konnten bei verschiedenen Konzentrationen der Seren, auf Paraffin- oder Gefrierschnitten keine spezifischen Signale in mit ARV infizierten Pellets nachgewiesen werden.

Die Ursachen für das Nichtfunktionieren der ISH und der IHC sind vielfältig zu identifizieren. Relevante Fehlerquellen bei den beschriebenen Verfahren sind jedoch niedrige Virustiter in Gewebe und Pellet.

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11 List of abbreviations

ABC	avidin biotin complex
AP	alkaline phosphatase
ARV	avian reovirus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
DIG	digoxigenin
dpi	days post infection
ds-RNA	double stranded RNA
Fab	fragments of immunoglobulin G
IHC	immunohistochemistry
ISH	*in situ* hybridization
MAS	Malabsorption syndrome
NBT	4-nitro blue tetrazolium chloride
PBS	phoshate buffered saline
PBS+Triton®	phoshate buffered saline + Triton® X-100
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
SPF	specific pathogen free