UNIVERSITÀ DEGLI STUDI DI MILANO

Graduate School of Animal Health and Production:

Science, Technology and Biotechnologies

Department of Health, Animal Science and Food Safety

PhD Course in Biotechnologies Applied to Veterinary and

Animal Husbandry Sciences

(Cycle XXV)

Doctoral Thesis

SET-UP AND APPLICATION OF BIOTECHNOLOGICAL METHODS IN AVIAN FIELD

(VET05)

Giovanni MANAROLLA

Nr. R08631

Tutor: Prof. Valerio BRONZO

Coordinator: Prof. Fulvio GANDOLFI

Academic Year 2011-2012

TABLES OF CONTENTS

The use of biotechnology in avian field has been increased in the last decade especially on the track of the complete sequencing of chicken genome and on the global threat of avian flu pandemic which alerted the scientific community worldwide. Nevertheless, it is still on the whole a minor and spotty approach in avian field. Matter-of-factly, birds can be roughly divided into two groups on a research point of view: poultry and non-poultry (companion and wild birds). The economic impact of the latter is usually scarce if compared to the former and to other veterinary and human contexts. Thus, routine use of laboratory diagnostic tools including biotechnological methods is usually limited to the more expensive psittacines species (i.e. DNA-based determination of sex, diagnosis of chlamydiosis etc.) or to endangered species. As for poultry, extensive use of biotechnology is performed in vaccine research and genetic selection with a secondary role in avian and comparative pathology. Besides, chicken has historically assumed the role of avian model. Consequently, birds are often considered an uniform class but they are not, and a deep knowledge of the huge diversity which can exist among the several avian species is fundamental to correctly approach and interpret avian research no matter using biotechnology or not. For all these reasons it seemed an unique occasion to match the documented experience and activity in avian field (acquired during a previous doctorate) to biotechnological methods as the subject of this doctoral thesis.

After a brief introduction revising fundamental principles of the molecular methods used, this thesis is divided into two parts. The first is focused on avian immunity and in particular on the TIR8-SIGIRR receptor which has been thoroughly investigate in chickens as a model for avian species and with an importance in itself as an ubiquitously diffused food production animal. The second part is made up of some diagnostic challenges arisen during the routine diagnostic activity of the Section of Avian Pathology of the Department of Veterinary Science and Public Health.

0 - INTRODUCTION

Biotechnology has potential applications across a wide range of scientific disciplines. It is already being widely used to advance developments in animal health and offers advantages for disease control, eradication programs (new vaccines, rapid diagnostic tests etc.). Other possible and future applications of animal biotechnology regards the commercial use of biotechnology-derived animals. In the last two decades molecular biological methods have become increasingly easy, safe, sensitive, reproducible and applicable on larger numbers of samples.

POLYMERASE CHAIN REACTION (PCR) AND REAL-TIME PCR

The PCR is based on DNA replication mechanisms. It can produce *in-vitro* large quantities of a desired sequence of DNA. PCR can amplify a selected region of a few to several thousand base pairs into billions of copies. The amplification of DNA by the PCR is based on cyclic incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers (short synthetic molecules of DNA complementary to both strands and flanking the target sequences) are then annealed to the single-stranded template at low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesized a new strand of DNA, the product is separated from the template by heating to a higher temperature. These cycles are repeated up to 40 times, resulting in amplification of the target DNA sequences. The key to successful PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as RT-PCR.

Any PCR product generated has, by definition, a characteristic size. Its identity is generally confirmed using DNA probes, or restriction digests, which can be used to provide RFLPs. More commonly, since the advent of automated cycle sequencing techniques, identification is via

direct sequencing of the PCR product. For example, sequencing has been used in the virulence typing of avian influenza A virus, in which virulence-associated structural motifs at the haemagglutinin gene cleavage site are reliable indicators of high pathogenicity in chickens. PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected. The sensitivity of a PCR may be enhanced by the use of a second set of primers to amplify a sub-fragment from the PCR product of the first reaction. This technique is commonly referred to as 'nested PCR' and has been used to detect low levels of the target sequence. However, the use of nested PCR can increase the rate of false-positive results.

PCR can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. It can also target and amplify unintegrated viral gene sequences. It is clear that PCR has a role in the testing of vaccines to detect contamination. PCR may prove to be very useful in the diagnosis of chronic-persistent infections, such as those caused by retroviruses (bovine leukaemia virus, caprine arthritis/encephalitis virus, etc.). These diseases present serious problems in terms of diagnosis and prevention since infected animals are a constant potential source for transmission.

However, PCR does not differentiate between viable and nonviable organisms or incomplete pieces of genomic DNA, and this may complicate interpretation of results and affect the applicability of PCR. Moreover, when PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the huge sensitivity of the technique can easily lead to false-positive results. Multicenter studies have shown that positive samples are detected consistently between laboratories, but that false positives are frequently obtained with known negative samples, indicating the continuing presence of contamination problems. Systems have been developed to deal with this problem, for example the dUTP-UNG system (d-uracil triphosphate and uracil-N-glycosylase). This system uses an enzymatic reaction to specifically degrade PCR products from previous PCR amplification (in which dUTP has been incorporated) without degrading native nucleic acid templates. This, of course, does not exclude

contamination of the sample with extraneous virus. It is also important to control for potential 'negative' results caused by the presence of interfering substances in the PCR reaction mixture or patient's sample by the inclusion of a template known to produce a PCR product (special attention must be paid, for instance, to PCR inhibitors in stool).

To expand its utility in veterinary diagnostics and pathogen identifications, PCR has been extensively modified in the past years. PCR using broadly conserved primers is designed for identification of classes of pathogens. The best example is the use of sequences of the 16s rRNA gene, an evolutionarily conserved gene in bacterial species. Using PCR primers that are complementary to these conserved sequence regions, one can determine the presence of any bacteria of a desired class from the sample. It must be noted that a positive PCR result needs to be further characterized by hybridization with species-specific probes, analysis by restriction enzyme digestion, or by sequencing. Similarly, consensus PCR is designed to use degenerate primers targeting conserved sequence regions or motifs of a group of related pathogens. Use of degenerate primers targeting the sequence regions of the herpesviral DNA polymerase gene has led to identification of many previously unrecognized herpesviruses in various animal species. On the other hand, multiplex PCR is designed to use two or more primer pairs directed at pathogen-specific unique sequences within a single reaction for simultaneous detection of multiple pathogens that are of interest. Multiplex PCR has the advantage of a high degree of sensitivity and specificity. However, there have been reports that multiplexing can reduce sensitivity compared with single reactions, because of competition. If it is important to have a very sensitive assay, this should be considered.

Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now being complemented and in some cases replaced with real-time PCR assays. Real-time PCR monitors the accumulation of PCR product during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. In other words, the assay can be used to reliably quantify the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more

rapid than conventional PCR techniques, has a closed-tube format therefore decreasing risk of cross-contamination, is highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. In many cases, the real-time PCR assays have proved to be more sensitive than existing reference methods. The recent development of portable real-time PCR machines and assays raises the exciting prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

In conclusion, Recombinant DNA technology has provided highly sensitive and specific tools for the diagnosis, prognosis and disease surveillance in humans and animals. (http://web.oie.int/boutique/index.php?page=ficprod&id_prec=92&id_produit=373&lang=en&fichrech=1&PHPSESSID=d0e340cde3bb3eb6de8e42c8a93d8872)

1 - PART ONE - INVESTIGATIONS ON TIR8-SIGIRR IN CHICKEN

This part includes our latest results on the innate immunity receptor TIR8/SIGIRR and focuses on the expression profile of this receptor during the embryonic stages in the chicken.

According to human and mouse studies, TIR8 receptor plays an important role in mucosal immune response as well as in the tuning of inflammation in different districts, but nothing is known about it during the embryonic development.

After the identification of the homologue molecules in different domestic animal species, we focused our interest on TIR8 receptor in the embryonic development.

The chicken species was chosen as a feasible model, due to the simple accessibility of avian embryos at all developmental stages. A deeper knowledge of TIR8 receptor in the development and in the function of the animal immune system could prove useful for developing innovative strategies and control infection and inflammation in veterinary farming.

1.1 - THE RECEPTOR TIR8/SIGIRR IS EXPRESSED DURING CHICKEN EMBRYO DEVELOPMENT

INTRODUCTION

The orphan receptor TIR8 (Toll-like/interleukin-1 receptor-8), also known as SIGIRR (Single Immunoglobulin IL-1R-Related molecule), belongs to the TIR super-family (Toll-like/ interleukin-1 receptors). Innate mechanisms of first-line defense against pathogens involve different members of the TIR superfamily, which includes Toll-like receptors (TLRs), IL-1Rs and adapter molecules, all sharing the intracellular TIR domain (Gangloff et al., 2003; Mantovani et al., 2004; Thomassen et al., 1999). TLR molecules are pattern recognition receptors, which sense specific microbial components (pathogen-associated molecular patterns, PAMPs) and activate signaling pathways leading to develop both innate and adaptive immune responses (Kaisho and Akira, 2006, O'Neill and Dinarello, 2000). The IL-1R subgroup includes receptors and accessory proteins (AcP) for IL-1 and IL-18, involved in the inflammatory process (Mantovani et al., 2004;

O'Neill and Dinarello, 2000; Thomassen et al., 1999). Some of the members of both groups are orphan receptors, such as TIGIRR-1, IL-1RAPL and TIR8 (Li and Qin, 2005; Liew et al., 2005; Medzhitov et al., 1997; Thomassen et al., 1999). Upon stimulation by their ligands, TLRs and IL-1Rs interact with specific adaptor proteins and initiate a signaling pathway leading to the synthesis of proinflammatory cytokines and chemokines (Takeda and Akira, 2004). The signaling complexes also trigger a cascade of cell kinases, which finally lead to the activation of transcription factors NFkB and IRF3 (Li and Qin, 2005). Signaling pathways of IL-1R/TLRs molecules are very complex and tightly regulated to ensure the appropriate modulation of the innate and inflammatory responses and limit dysregulated activation that could cause severe local or systemic inflammation or autoimmunity (Henson, 2005; O'Neill and Dinarello, 2000). For example, IL-1 regulation involves extracellular proteins such as IL-1R antagonists (IL-1Ra), other cell receptors such as the decoy receptor IL-1RII and intracellular factors, such as the intracellular signal transduction regulators IRAK-M and MyD88s (Mantovani et al., 2004; Mantovani et al., 2007). TIR8 acts as an intracellular inhibitor of IL-1R/TLRs signaling; it does not activate NFkB (nuclear factor kB) and IRF3 (interferon regulatory factor 3), therefore it behaves as an intracellular decoy for key components of the inflammatory pathways in the epithelial tissues and at mucosal sites, where it is highly expressed (Liew et al., 2005; O'Neill, 2003; Polentarutti et al, 2003; Quin et al., 2005; Wald et al., 2003). Its regulatory activity is crucial for inhibiting exaggerated immune response and for controlling chronic inflammatory diseases due to Th1 cytokine toxicity (Huang et al., 2006; Lech et al, 2008); indeed, some pathological conditions are caused by dysregulated proinflammatory responses (Garlanda et al., 2007b; Janeway and Medzhitov, 2000; Mantovani et al., 2004). TIR8 is expressed ubiquitously, with the highest levels observed in the kidney and in the gastrointestinal (GI) tract (Garlanda et al., 2004). Many findings support TIR8 role in dampening excessive inflammatory responses: it is moderately downregulated upon LPS stimulation in various mouse tissues; TIR8-deficient mice were more susceptible to intestinal inflammation and to colitis-associated cancer in models of chronic colitis and colon carcinogenesis; finally, TIR8-knock-out mice were more susceptible to Mycobacterium tuberculosis killing than WT mice (Garlanda et al., 2004; Garlanda et al.,

2007a; Mantovani et al., 2004; Xiao et al, 2007). Avian immune system is quite similar to the mammalian in terms of overall organization and mechanisms of immunity. Bird TLR repertoire consists of 10 genes, most of which have orthologues in mammalian genomes, whereas three elements are unique to birds (Temperley et al., 2008). To date, of the two Mammalian IL-1Rs (type I and II), only the type I form has been found in chicken; it shares high sequence homology, tissue distribution, function and role with the mammalian counterpart (Wang et al., 2003). Little is known about the other members of the TIR family. TIR8 investigation has been mainly done in mouse and human models (Garlanda et al, 2004; Garlanda et al, 2007a; Garlanda et al, 2007b; Lech et al, 2008; Mantovani et al, 2004; Polentarutti et al, 2003; Thomassen et al, 1999; Wald et al, 2003), and, more recently, also in other animals (Riva et al., 2009; Riva et al., 2010). However, there is no information regarding embryonic expression of TIR8 in any species. Chicken embryo has a long and distinguished history as a major model system for developmental studies in different fields, including genetic and immunology (Stern, 2005). It was the first economically important species for which a genetic linkage map was constructed, the first non-mammalian amniote genome to be fully sequenced and the first species where the T- (thymus) and B- (Bursa of Fabricius) lymphocytes were discovered. Chicken is a very powerful and advantageous experimental system available for investigations; its eggs are a common and accessible source of embryos, which can be observed at different stages of incubation for progressive studies. The attractiveness of this animal model for gene expression studies is due to the availability of embryonated eggs and the absence of any technical and ethical limitation in the use of chicken embryos. Chicken has been proved as a very valuable resource particularly for comparative genomic analysis, especially after the identification of highly conserved sequences regions and functional elements. Moreover chickens have been selected genetically either for improved feed conversion and rapid growth (broilers) or for production of eggs (egg-type). This selection for economically important production traits has led to two physiologically quite different types of chickens, which differ in body weight gain, productive lifespan and immunological responses (Praharaj et al, 1995). Meat-type chickens usually display a lower antibody and T-cell mediated immune response

when compared under the same conditions to layer-type strains (Koenen et al., 2002); alternatively the first ones require higher doses of stimuli to develop immune responses comparable to the second ones (Koenen et al., 2004). Due to the importance of TIR8 molecule in innate immunity and in inflammation, its high level of evolutionary conservation and the lack of data on TIR8 expression during embryo development, we focused our study on the investigation of this receptor during incubation till hatching. TIR8 expression was examined both at the transcriptional level (mRNA) by mean of quantitative RT-Real-Time PCR and at the protein level by Western Blot and immunohistochemistry. Organs from two different chicken breeds (meat-type and egg-type) were compared at different time-points. This is the first study on TIR8 receptor expression in embryos.

MATERIALS AND METHODS

Samples

Commercial embryonated eggs from broiler and layer breeders were incubated in laboratory incubators under standard conditions. The eggs were candled daily during incubation to check embryo development. At different time-points embryo organs were collected as detailed in Table I. The whole embryo was obtained at the beginning of the incubation (T0) (germinal disk) and at 3 days of incubation (T1) from 6 eggs; at 6 days (T2) the whole embryo except the cephalic region was taken from 5 eggs and at 8 days (T3) from 3 eggs. For the subsequent time-points an average of 0.3 cm^3 pieces were collected from an adequate number of embryonated eggs. At day 10 of incubation (T4) kidney, liver, intestine, proventriculus and gizzard of the embryos were obtained and for the subsequent timepoints T5, T6, T7 and T8 (12, 14, 16 and 20 days of incubation respectively) kidney, liver, intestine, proventriculus and gizzard and Bursa of Fabricius were separately sampled. The samples were immediately placed in sterile tubes containing 1.5 ml of RNA Later (Qiagen, Hilden, Germany) and stored at 4°C for 24 hours. The same organs from 4 extra eggs at day 14 of incubation were used for Western Blot analysis.

RNA Extraction

Total RNA was isolated from the samples by the guanidine isothiocyanate (4M) method with minor modifications. Briefly, the samples were homogenized in 1.5 ml of guanidine isothiocyanate using a rotor-stator system (Ultra Turrax T25 Ika-Werke, Staufen, Germany). The lysate was centrifuged overnight at 42,000 rpm at 18°C on a 5.7M cesium chloride layer (Optima TL ultracentrifuge, Beckman Instruments, Inc. Palo Alto, CA, USA). The pellet was dissolved in sterile water and the RNA was precipitated with absolute ethanol and sodium acetate 3M pH 5.4 in dry ice for 2 hours. After centrifugation in microcentrifuge (Eppendorf, Hamburg, Germany) at maximum speed for 30 minutes, the RNA pellet was dissolved in sterile water and stored at -20°C.

RNA Determination and Reverse Transcription

The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength. About 1 µg of total RNA from each sample was reverse transcribed to cDNA using the High Capacity cDNA Archive kit with random hexamers (Applied Biosystem, Foster City, CA, USA) and the resulting cDNA was stored at -20°C before Real-Time PCR assays.

Real-Time PCR

The cDNA obtained from each sample was used as a template for Real-Time PCR in optimized 25 µl reaction volume in MicroAmp optical 96-well plates. Each plate contained duplicates of each sample cDNA diluted 1:100 (9 µl), 2X Power Syber Green PCR Master Mix (12.5 µl) (Applied Biosystem, Foster City, CA, USA) and primers 300 nM each (0.3 µl of 10 µM solution). The species-specific primer pairs were designed using as target the most complete chicken sequence homologous to human and mouse TIR8 available in the NCBI nucleotide sequences database (gi:118091084). Primers were custom synthesized by Invitrogen (Carlsbad, CA, USA); their sequences are listed in Table II. To correct for variations of extracted mRNA amounts and cDNA synthesis efficacy in quantitative Real-Time PCR assays, primers for the detection of the

chicken housekeeping gene beta-actin were designed as well by Primer Express on the sequence accession number gi:45382926 (NCBI). Their sequences also are listed in Table II. In addition to the cDNA samples, duplicates of 10-fold serial dilutions of a known amount of chicken kidney cDNA were used as templates to generate standard curves for estimation of the expression in relationship to the calibrator (kidney 1:100) (relative quantification). A duplicate no-template control (NTC) was also included in each plate. Real-Time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) at the following thermal cycle conditions, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantification was determined after application of an algorithm to the data analyzed by the software of the 7000 Detection System (Applied Biosystem, Foster City, CA, USA). For each sample tested the expression level was calculated following the results of the calibrator of the same run. The chicken TIR8 expression was normalized using the calculated beta-actin cDNA expression (mean) of the same sample and run.

Software

We used the free software BLAST available on PubMed web site. Primer Express software (Applied Biosystem, Foster City, CA, USA) was used for the specific primer design.

Protein Extraction And Western Blot Analysis

Samples of kidney, liver, proventriculus and gizzard, intestine and Bursa of Fabricius were collected from 4 chicken embryos at day 14 of incubation and immediately processed for the extraction of proteins. Each organ was mechanically homogenized in 1 ml of cold lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% (v/v) NP40, 2% (v/v) TritonX100, 1% (w/v) Zwitterion, 1 mM EDTA) added with protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 minutes on ice. After centrifugation for 10 minutes at 13,000 x g at 4°C, the supernatant was collected and protein concentration was determined

spectrophotometrically using the Bradford micromethod (BioRad Protein Assay, BioRad Laboratories, GmbH, Munich, Germany).

Aliquots of lysed organs, corresponding to 40 µg of total protein content were loaded on 10% SDS-PAGE and Western blotted on nitrocellulose membrane. Immunodetection was carried out using a polyclonal goat anti-hSIGIRR Antibody (R&D Systems, Minneapolis, MN, USA) as the primary antibody at a concentration of 0.1 µg/µl (1:500 dilution from stock solution), overnight at 4°C. An HRP-conjugated anti-goat secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) was used (1:2000 dilution, 1 hour at room temperature). Positive bands were detected using chemiluminescent HRP substrate (Millipore, Molsheim, France).

Immunohistochemistry

Tissue samples (gizzard and proventriculus, kidneys, intestine, Bursa of Fabricius) from a selection of 18-day embryos of the two lines were fixed in 10% buffered formalin and routinely processed for histology. Four-µm-thick paraffin-wax sections were stained for demonstration of SIGIRR receptor using Avidin-Biotin-peroxidase Complex (ABC) immunohistochemistry (Hsu et al., 1981). Additional sections were included as negative control. Briefly, after the quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide in Phosphate Buffered Saline (PBS) pH 7.4 for 30 minutes, antigen retrieval was performed using microwave treatment at 750 W for 15 minutes (three cycles, 5 minutes each) in 10 mM citrate buffer (pH 6.0). After rinsing with PBS, 10% normal horse serum was applied for 30 minutes to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4°C with the primary antibody (polyclonal goat anti-hSIGIRR Antibody, R&D Systems, Minneapolis, MN, USA) diluted 1:100. Biotinylated horse anti-goat antibody (Vector Laboratories Inc., Burlingame, California) was used as secondary antibody, followed by ABC Vectastain Elite kit (Vector Laboratories Inc., Burlingame, California). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole (Vector Laboratories Inc., Burlingame, California).

RESULTS

TIR8 mRNA expression analysis by Real-Time PCR

TIR8 expression was analyzed by mean of quantitative Real-Time PCR assays in meat-type and egg-type embryo samples at different time-points of incubation. The panel of embryo organs tested for both chicken types is listed in Table I. Only at T4 (day 10 of incubation) and later it was possible to take biopsies from different organs in both the chicken types.

The complete mRNA sequence of chicken TIR8 (gi:118091084) was selected to design specific primers spanning two adjacent exons. The primer pair with the highest score localized in the middle of the sequence was used in Real-Time PCR assays to amplify the cDNA from each sample (Table II).

All the sampled organs resulted positive to the presence of TIR8 transcript, with different levels of expression. The results of Real-Time PCR quantification, expressed as ratio between each specimen and the commercially mature chicken kidney, which was chosen as experimental calibrator, are summarized in Figure 1. In all the assays, the kidney RNA from commercially mature chicken was used as positive control besides calibrator.

The expression pattern of TIR8 in meat-type chicken embryos was similar qualitatively (for the type of organ) and quantitatively (for the level of expression) to that found in egg-type layers chickens. Very low levels of expression were displayed until day 10 of incubation, corresponding to T4.

Liver and kidney were the organs showing the highest levels of TIR8 messenger RNA along all the embryonic development; intestine displayed a moderate level of the receptor transcript; stomachs and Bursa of Fabricius were also found positive, but expressed TIR8 at very low levels.

Interestingly, all the organs tested in the study (except Bursa of Fabricius) showed a progressive increase in TIR8 expression during the embryo growth. On the contrary, Bursa of

Fabricius, after an initial increase from day 12 to day 14, maintained constant low expression levels of messenger RNA up to hatching.

TIR8 protein expression analysis by Western Blot

The expression of TIR8 protein in different chicken embryo organs collected at day 14 of incubation as well as the presence of possible organ-specific isoforms, was assessed by Western Blot. A polyclonal goat anti-human TIR8 was used as primary antibody, after preliminary validation on chicken samples.

Results from Western Blot analysis presented in Figure 2, showed TIR8 protein in all the organs examined. A TIR8 form of about 45 kDa was observed in all the examined samples, very faint in Bursa of Fabricius. Some TIR8 bands, corresponding to different molecular weight, were also detected in some organs, such as kidney and liver. In these organs, the anti-human TIR8 antibody reacted with two proteins showing MW ranging from 20 to 30 kDa. Just the lowest molecular weight band of these two was detectable in intestine and stomachs. In liver the low molecular weight fraction appeared to be the major TIR8 component. Furthermore, Western Blot analysis also revealed specific heavier bands ranging from 75 to 100 kDa, which correspond to the expected molecular weight of the complete protein. TIR8 expression in Bursa of Fabricius went barely detectable by anti-TIR8 antibody. Taken together, results from this study showed that TIR8 receptor was widely expressed throughout all the chicken embryo organs tested.

Immunohistochemistry

The expression of TIR8 protein was assayed by immunohistochemistry with the same polyclonal anti-human TIR8 antibody used for Western Blot and validated for cross-reactivity with the chicken receptor.

Variably intense cytoplasm immunolabeling was observed only in scattered cells of the intestinal mucosa of the embryos of the two lines (Figure 3). The samples of kidney, liver, Bursa of

Fabricius, proventriculus and gizzard, and the negative controls showed no immunopositivity for TIR8 protein.

DISCUSSION

TIR8 mRNA was detected and quantified by Real-Time PCR in chicken embryos of two breeds (meat-type and egg-type) at 8 different developmental stages. In parallel, TIR8 protein was identified by both Western Blot analysis and Immunohistochemistry at a single developmental time-point for practical reasons. Interestingly, TIR8 mRNA was detected, at low levels, in very early embryos (T0), when the fertilized egg is at the blastodermal stage, characterized by thousands of undifferentiated cells forming the germinal disc (Eyal-Giladi and Fabian, 1980). The levels of TIR8 mRNA increase at remarkable levels in most selected organs after day 10 of incubation (T4) (Figure 1). Remarkably, the highest expression of TIR8 was observed in liver and in kidney of chicken embryo; mRNA was also detected in the intestine, the stomachs and the Bursa of Fabricius, at lower levels. Embryonic TIR8 expression resembles the pattern observed in growing and commercially mature chickens (Riva et al., 2009; data in publication). It is conceivable that this trait could be typical of birds, as the result of a specific evolutionary pressure, due to the environment and the pathogens. Indeed, the rudimental lymphatic system of birds (lack of lymph nodes) and the presence of a renal portal system could account for the high TIR8 expression in avian liver and kidney.

Since TIR8 acts as a negative regulator of the TIR family members, it could just undergo a similar regulation mechanism. TLRs signaling in embryos is tightly regulated as well as in post-hatching life (Belderbos et al., 2009; Merkerova et al., 2009; Michailidis et al., 2010; Yuan et al., 2009). The well-established increasing expression of TLRs during chicken embryo development could take part in a protection mechanism of embryos and newly hatched birds from pathogens, where TIR8 increases accordingly to the embryo development. It is well known that chicken ovary is a preferential site of microbial infection (for example due to *Salmonella enteritidis*) that can be subsequently transmitted to the eggs and embryos (Barua and Yoshimura, 2004). Different levels of TIR8 in different organs could suggest a complex regulatory mechanism for

the receptor expression in response to microbial infections, particularly depending on their duration. Since the embryo develops in a protected environment with few external stimulations of the immune system, the role of TIR8 in this context may be marginal or different than immune regulator. As a confirm, recent studies showed the expression of TLRs in ovary during maturation and in embryos during development (Michailidis et al., 2010), suggesting a new role for TLRs both in fertility and in protection of reproductive organs, possibly as a further way to protect newly forming eggs from microbial infection.

In contrast with the results of the other tested organs, TIR8 mRNA expression in Bursa of Fabricius remains low during the embryo development. Although TIR8 mRNA expression still needs to be ascertained in other avian lymphatic organs (thymus and spleen), present results agree with TIR8 mRNA expression patterns in mammalian lymphoid organs.

In conclusion, an ubiquitous expression of TIR8 was demonstrated in embryos of both meat-type and layer-type chickens at both transcriptional (mRNA) and translational (protein) levels. Although the meat-type breeds are known to have a lower degree of immunocompetence compared to the egg-type breeds, no substantial differences in TIR8 expression levels were observed in our study between them at embryo stages.

Our findings document for the first time that TIR8 expression also occurs during the embryonic stages of chicken development and supports the aim at performing similar studies in other species embryos.

References

- Barua, A., Yoshimura, Y., 2004. Ovarian cell-mediated immune responde to Salmonella enteritidis infection in laying hens (gallus domesticus). Poult. Sci. 83, 997-1002.
- Belderbos, M.E., van Bleek, G.M., Levy, O., Blanken, M.O., Houben, M.L., Schuijff, L., Kimpen, J.L.L., Bont, L., 2009. Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. Clin. Immunol. 133, 228-237.
- Eyal-Giladi, H., Fabian, B.C., 1980. Axis determination in uterine chick blastodiscs under changing spatial positions during the sensitive period for polarity. Dev. Biol. 77, 228-232.
- Gangloff, M., Weber, A.N.R., Gibbard, R.J., Gay, N.J., 2003. Evolutionary relationships, but functional differences, between the Drosophila and human Toll-like receptor families. Biochem. Soc. Trans. 31, 659-663.
- Garlanda, C., Riva, F., Polentarutti, N., Buracchi, C., Sironi, M., De Bortoli, M., Muzio, M., Bergottini, R., Scanziani, E., Vecchi, A., Hirsch, E., Mantovani A., 2004. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proc. Natl. Acad. Sci. USA. 101, 3522-3526.
- Garlanda, C., Di Liberto, D., Vecchi, A., La Manna, M.P., Buracchi, C., Caccamo, N., Salerno, A., Dieli, F., Mantovani, A., 2007a. Damping excessive inflammation and tissue damage in Mycobacterium tuberculosis infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. J. Immunol. 179, 3119-3125.
- Garlanda, C., Riva, F., Veliz, T., Polentarutti, N., Pasqualini, F., Radaelli, E., Sironi, M., Nebulosi, M., Omodeo Zorini, E., Scanziani, E., Mantovani, A., 2007b. Increased Susceptibility to Colitis-Associated Cancer of Mice Lacking TIR8, an Inhibitory Member of the Interleukin-1 Receptor Family. Canc. Res. 67, 6017-21.
- Henson, P.M., 2005. Dampening inflammation. Nat. Immunol. 6, 1179-1181.

- Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Hictochem. Cytochem. 29, 577-580.
- Huang, X., Hazlett, L.D., Du, W., Barrett, R.P., 2006. SIGIRR promotes resistance against Pseudomonas aeruginosa keratitis by down-regulating type-1 immunity and IL-1R1 and TLR4 signaling. J. Immunol. 177, 548-556.
- Janeway, C.A.J., Medzhitov, R. 2000. Innate immunity. New England J. Med. 343, 338-344.
- Kaisho, T., Akira, S., 2006. Toll-like receptor function and signaling. J. Allergy Clin. Immunol. 117, 979-987.
- Koenen, M.E., Boonstra-Blom, A., Jeurissen, S.H.M., 2002. Immunological differences between layer- and broiler-type chickens. Vet. Immunol. Immunopathol. 89, 47-56.
- Koenen, M.E., Kramer, J., van der Hulst, R., Heres, L., Jeurissen, S.H.M., Boersma, W.J.A., 2004. Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. British Poultry Sci. 45, 355-366.
- Lech, M., Kulkarni, O.P., Pfeiffer, S., Savarese, E., Krug, A., Garlanda, C., Mantovani, A., Anders, H.J., 2008. Tir8/Sigirr prevents murine lupus by suppressing the immunostimulatory effects of lupus autoantigens. J. Exp. Med. 205, 1879-1888.
- Li, X., Qin, J., 2005. Modulation of Toll-interleukin 1 receptor mediated signalling. J. Mol. Med. 83, 258-266.
- Liew, F.Y., Xu, D., Brint, E.K., O'Neill, L.A., 2005. Negative regulation of toll-like receptormediated immune responses. Nat. Rev. Immunol. 5, 446-458.
- Mantovani, A., Locati, M., Polentarutti, N., Vecchi, A., Garlanda, C., 2004. Extracellular and intracellular decoys in the tuning of inflammatory cytokines and Toll-like receptors: the new entry TIR8/SIGIRR. J. Leukoc. Biol. 75, 738-742.
- Mantovani, A., Garlanda, C., Locati, M., Rodriguez T.V., Feo S.G., Savino B., Vecchi A., 2007. Regulatory pathway in inflammation. Autoimmun. Rev. 7, 8-11.

- Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A.Jr., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 388, 394-397.
- Merkerova, M., Vasikova, A., Bruchova, H., Libalova, H., Topinka, J., Balascak, I., Sram, R.J., Brdicka, R., 2009. Differential gene expression in umbilical cord blood and maternal peripheral blood. Eur. J. Haematol. 83, 183-190.
- Michailidis, G., Theodoridis, A., Avdi, M., 2010. Transcriptional profiling of Toll-like receptors in chicken embryos and the ovary during sexual maturation and in response to Salmonella enteritidis infection. Anim. Reprod. Sci. 122, 294-302.
- O'Neill, L.A., 2003. SIGIRR puts the brakes on Toll-like receptors. Nat. Immunol. 4, 823-824.
- O'Neill, L.A., Dinarello, C.A., 2000. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol. Today. 21, 206-209.
- Polentarutti, N., Rol, G.P., Muzio, M., Bosisio, D., Camnasio, M., Riva, F., Zoja, C., Benigni, A., Tomasoni, S., Vecchi, A., Garlanda, C., Mantovani, A., 2003. Unique pattern of expression and inhibition of IL-1 signaling by the IL-1 receptor family member TIR8/SIGIRR. Eur. Cytokine Netw. 14, 211-218.
- Praharaj, N.K., Dunnington, E.A., Siegel, P.B.,1995. Growth, immunoresponsiveness, and disease resistance of diverse stocks of chickens reared under two nutritional regimens. Poultry Sci. 74, 1721-1729.
- Qin, J., Qian, Y., Yao, J., Grace, C., Li, X., 2005. SIGIRR inhibits interleukin-1 receptorand toll-like receptor 4-mediated signaling through different mechanisms. J. Biol. Chem. 280, 25233-25241.
- Riva, F., Polentarutti, N., Tribbioli, G., Mantovani, A., Garlanda, C., Turin, L., 2009. The expression pattern of TIR8 is conserved among vertebrates. Vet. Immunol. Immunopathol. 131, 44-49.
- Riva, F., Rahman, M.M., Turin, L., Ceciliani, F., Russo, S., Tribbioli, G., Lecchi, C., 2010. TIR8 receptor expression in bovine tissues. Vet. Immunol. Immunopathol. 136, 65-70.

- Stern, C.D., 2005. The chick: a great model system becomes even greater. Dev Cell 8, 9-17.
- Takeda, K., Akira, S., 2004. TLR signalling pathways. Semin. Immunol. 16, 3-9.
- Temperley, N.D., Berlin, S., Paton, I.R., Griffin, D.K., Burt, D.W, 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics 9, 62.
- Thomassen, E., Renshaw, B.R., Sims, J.E., 1999. Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. Cytokine. 11, 389-399.
- Wald, D., Qin, J., Zhao, Z., Qian, Y., Naramura, M., Tian, L., Towne, J., Sims, J.E., Stark, G.R., Li, X., 2003. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signalling. Nat. Immunol. 4, 920-927.
- Wang, R., Millam, J.R., Klasing, K.C., 2003. Distribution of interleukin-1 receptor in chicken quail brain. Comp. Biochem. Physiol Part A 136, 663-671.
- Xiao, H., Gulen, M.F., Qin, J., Yao, J., Bulek, K., Kish, D., Altuntas, C.Z., Wald, D., Ma, C., Zhou, H., Tuohy, V.K., Fairchild, R.L., de la Motte, C., Cua, D., Vallance, B.A., Li, X., 2007.
- The Toll-V-Interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. Immunity. 26, 461-475.
- Yuan, S., Huang, S., Zhang, W., Wu, T., Dong, M., Yu, Y., Liu, T., Wu, K., Liu, H., Yang, M., Zhang, H., Xu, A., 2009. An amphioxus TLR with dynamic embryonic expression pattern responses to pathogens and activates NF-kB pathway via MyD88. Mol. Immunol. 46, 2348-2356.

TABLES

Table I: Chicken embryo samples tested at different time-points for both layer- and broiler-type.

Time-point days of incubation	**Samples**
T0 (germinal disk)	whole embryo
T1 (3 days)	whole embryo
T2 (6 days)	whole embryo except cephalic region
T3 (8 days)	whole embryo except cephalic region
T4 (10 days)	kidney, liver, intestine, proventriculus and gizzard
T5 (12 days)	kidney, liver, intestine, proventriculus and gizzard, Bursa of Fabricius
T6 (14 days)	kidney, liver, intestine, proventriculus and gizzard, Bursa of Fabricius
T7 (16 days)	kidney, liver, intestine, proventriculus and gizzard, Bursa of Fabricius
T8 (20 days)	kidney, liver, intestine, proventriculus and gizzard, Bursa of Fabricius

Table II: The following primer pairs were designed and used to quantify TIR8 expression in Real-Time PCR assays.

Target gene	**Primers**
Chicken *TIR8*	5'-ACATTCCTGTCCGCAGTACCAG-3' (forward) 5'-TCCCAGTGTTTGTCCAGTAGGC-3' (reverse)
Chicken *β-actin*	5'-CCCACCTGAGCGCAAGTACT-3' (forward) 5'-GAGGCCAGGATAGAGCCTCC-3' (reverse)

Figure 1. Summary of the quantitative Real-Time PCR analysis results of TIR8 mRNA expression in chicken embryo organs. The TIR8 expression level of each sample is quantified as folds of the expression of the same gene in the chicken kidney (calibrator).

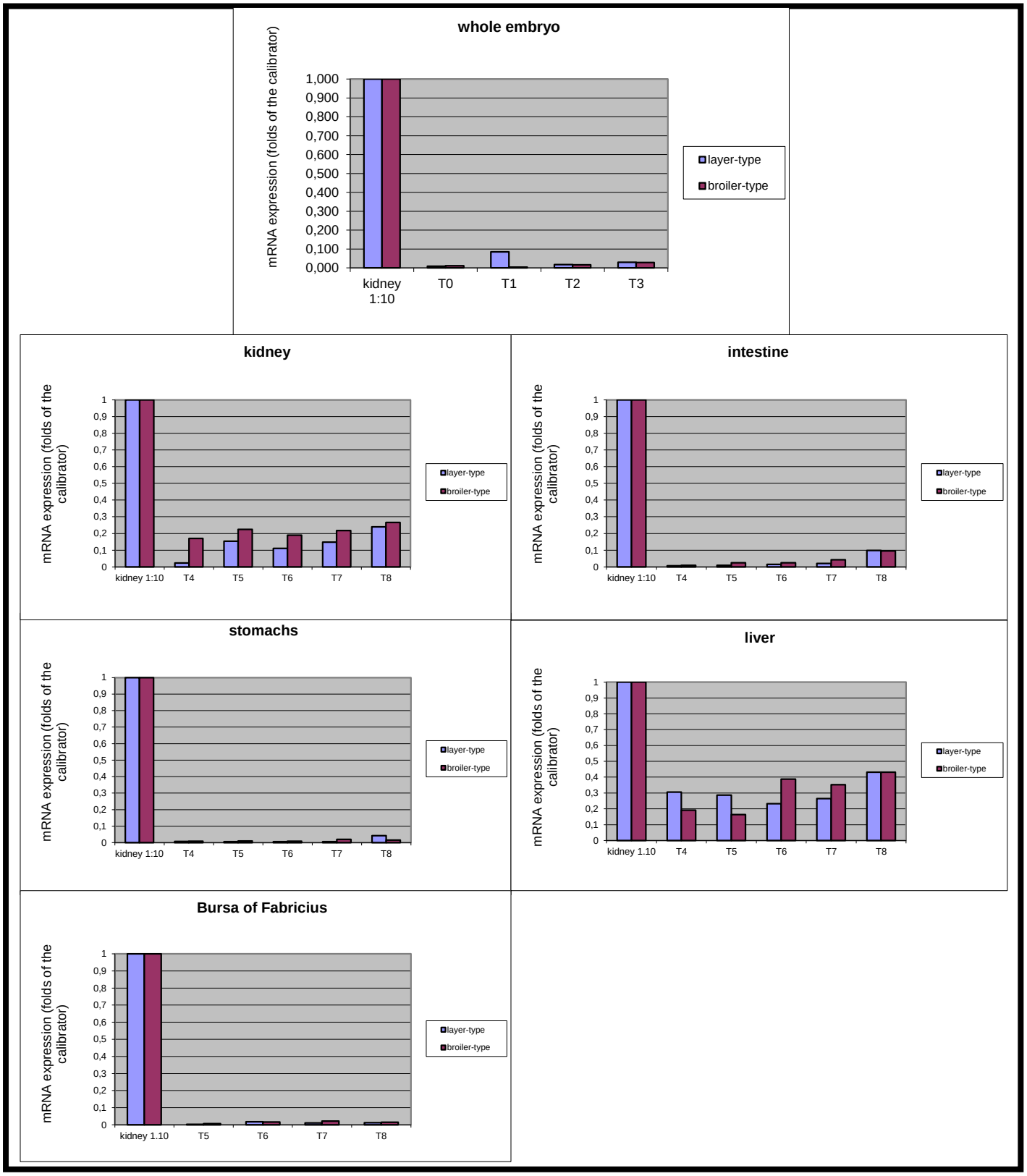


Figure 2. Western Blot analysis of TIR8 protein expression in different organs of chicken embryo. Homogenates of 40 µg of total protein extracted from each sample were loaded. Target protein was detected on the blots by staining with polyclonal goat anti-human SIGIRR antibody. A pool of 4-6 embryos was tested.

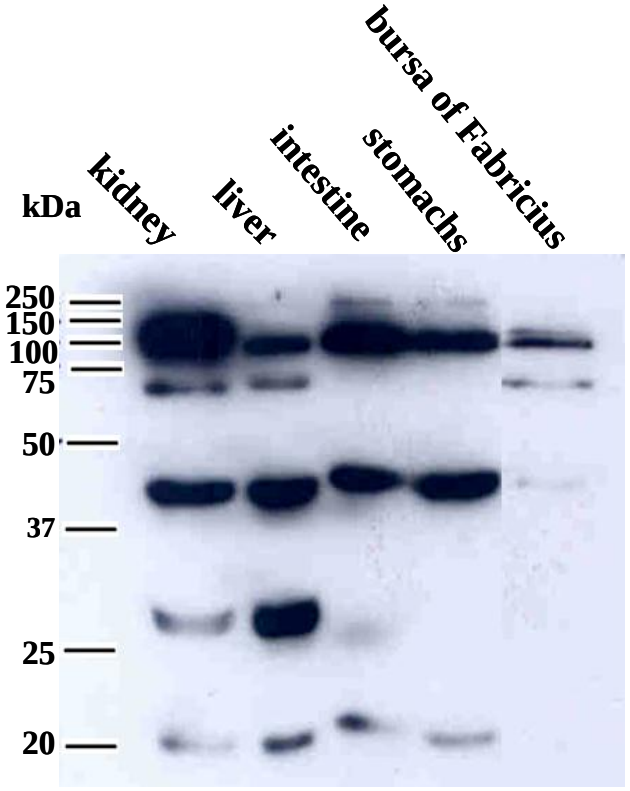
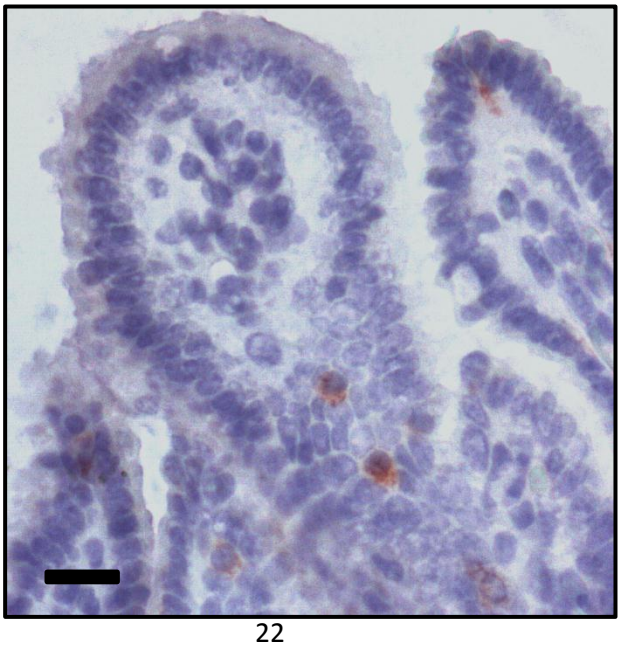


Figure 3. Section of intenstine of 18-day old egg type embryo. Cytoplasm positive immunolabeling of TIR8 protein in few cells of intestinal villi. Anti-SIGRR immunohistochemistry, Mayer's Haematoxylin counterstain. BAR: 40 µm.



1.2 - EXPRESSION OF TIR8 RECEPTOR IN CHICKEN TISSUES

INTRODUCTION

The Second part of the study on the orphan receptor TIR8 (Toll-like/interleukin-1 receptor-8), also known as SIGIRR (Single Immunoglobulin IL-1R-Related molecule), is focused on the expression of this receptor in tissues of commercially mature chickens. The main characteristics of TIR8 have been already described above in the section regarding the chicken embryos. Here we briefly describe the TIR8 features necessary as a basis for the study performed. TIR8 is expressed ubiquitously, with the highest levels observed in the kidney and in the gastrointestinal (GI) tract (7). Many findings support TIR8 role in dampening excessive inflammatory responses: it is moderately downregulated upon LPS stimulation in various mouse tissues; TIR8-deficient mice were more susceptible to intestinal inflammation and to colitis-associated cancer in models of chronic colitis and colon carcinogenesis; finally, TIR8-knock-out mice were more susceptible to *Mycobacterium tuberculosis* killing than WT mice (1, 7-9).

Birds immune system is similar to the mammalian in terms of overall organization and mechanisms of immunity. Bird TLR repertoire consists of 10 genes, most of which have orthologues in mammalian genomes, whereas three elements are unique to birds (10). Of the two Mammalian IL-1Rs (type I and II), only the type I form has been found to date in chicken; it shares high sequence homology, tissue distribution, function and role with the mammalian counterpart (11). Little is known about the other members of the avian TIR family.

TIR8 investigation has been mainly done in mouse and human models (1-2, 3, 4, 5-6, 7-8), and, more recently, also in other animals (12-13). However, it is poorly known in chickens.

Chicken has a long and distinguished history as a major model system for studies in different fields, including genetic and immunology. It was the first economically important species for which a genetic linkage map was constructed, the first non-mammalian amniote genome to be

fully sequenced and the first species where the T- (thymus) and B- (Bursa of Fabricius) lymphocytes were discovered.

Due to the importance of TIR8 molecule in innate immunity and in inflammation, its high level of evolutionary conservation and the lack of data on TIR8 in chicken, we focused our study on this receptor in such species. A detailed map of expression of TIR8, preliminary step to any investigation on its role in the pathogenesis, was the major aim of this study. We examined the distribution of TIR8 in a wide panel of chicken organs both at the transcriptional level (mRNA) by mean of quantitative RT-Real-Time PCR and at the protein level by Western blot and immunohistochemistry. The presence of isoforms was evaluated by Western blot analysis, after the validation of a poly-clonal antibody to human TIR8, which was cross-reactive with the chicken receptor.

MATERIALS AND METHODS

Animals and Samples

Samples (0.5 cm^3) of kidney, liver, pancreas, duodenum, proventriculus, caecum, colon, oviduct, cloaca, lung, heart, air sac, trachea, gizzard, adrenal gland, skin, Bursa of Fabricius, testicle, ovary, thymus, brain, spleen, cerebellum, Harder's gland, skeletal muscle, bone marrow, heterophils were collected at slaughterhouse from 56-day-old commercial broiler chickens (male and female), immediately placed in sterile tubes containing 3 ml of RNA Later (Qiagen, Hilden, Germany) and stored at 4°C for 24 h. Duplicate samples were fixed in 10% buffered formalin for immunohistochemistry.

Heterophils were isolated from blood collected from healthy chickens as reported (14). Briefly, blood from two chickens was collected as pool in tubes containing ethylenediamine-tetraacetic acid (EDTA) and mixed thoroughly. The blood diluted 1:2 with RPMI-1640 media containing 1% methylcellulose was centrifuged at 35 g for 15 min at 4°C. The supernatant was transferred to a new tube and diluted 1:2 with Ca++ and Mg++-free Hank's balanced salt solution, layered onto discontinuous Histopaque-1077 (Sigma Chemical Company, St.Louis, MO, USA) gradient and

centrifuged at 190 g for 1 h at 4°C. The heterophils layer was collected, washed with Hank's solution and resuspended in Phosphate Buffered Saline (PBS).

Organs were collected from one animal and frozen at -80°C to be used for Western blot experiments.

RNA extraction

Total RNA was isolated from the samples by the guanidine isothiocyanate (4M) method with minor modifications. Briefly, the samples were homogenized in 1.5 ml of guanidine isothiocyanate using a rotor-stator system (Ultra Turrax T25 Ika-Werke, Staufen, Germany). The lysate was centrifuged overnight at 42,000 rpm at 18°C on a 5.7M cesium chloride layer (Optima TL ultracentrifuge, Beckman Instruments, Inc. Palo Alto, CA, USA). The pellet was dissolved in sterile water and the RNA was precipitated with absolute ethanol and sodium acetate 3M pH 5.4 in dry ice for 2 hours. After centrifugation in a microcentrifuge (Eppendorf, Hamburg, Germany) at maximum speed for 30 minutes, the RNA pellet was dissolved in sterile water and stored at -20°C.

RNA determination and Reverse Transcription

The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength. About 1 μg of total RNA from each sample was reverse transcribed to cDNA using the High Capacity cDNA Archive kit with random hexamers (Applied Biosystem, Foster City, CA, USA) and the resulting cDNA was stored at -20°C before Real-Time PCR assays.

Real-Time PCR

The cDNA obtained from each sample was used as a template for Real-Time PCR in optimized 25 μl reaction volume in MicroAmp optical 96-well plates. Each plate contained duplicates of each sample cDNA diluted 1:100 (9 μl), 2X Power Syber Green PCR Master Mix (12.5 μl) (Applied Biosystem, Foster City, CA, USA) and primers 300 nM each (0.3 μl of 10 μM solution).

The species-specific primer pairs were designed using as target the most complete chicken sequence homologous to human and mouse *TIR8* available in the NCBI nucleotide sequences database (gi:118091084). Primers were custom synthesized by Invitrogen (Carlsbad, CA, USA); their sequences are listed in Table I.

To correct for variations of extracted mRNA amounts and cDNA synthesis efficacy in quantitative Real-Time PCR assays, primers for the detection of the chicken housekeeping gene beta-actin were designed as well by Primer Express on the sequence accession number gi:45382926 (NCBI). Their sequences also are listed in Table I.

In addition to the cDNA samples, duplicates of 10-fold serial dilutions of a known amount of chicken kidney cDNA were used as templates to generate standard curves for estimation of the expression in relationship to the calibrator (kidney 1:100) (relative quantification). A duplicate no-template control (NTC) was also included in each plate.

Real-Time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) at the following thermal cycle conditions, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantification was determined after application of an algorithm to the data analyzed by the software of the 7000 Detection System (Applied Biosystem, Foster City, CA, USA).

For each sample tested the expression level was calculated following the results of the calibrator of the same run. The chicken *TIR8* expression was normalized using the calculated beta-actin cDNA expression (mean) of the same sample and run.

Software

We used the free software BLAST available on PubMed web site. Primer Express software (Applied Biosystem, Foster City, CA, USA) was used for the specific primer design.

Protein extraction and Western blot analysis

Chicken tissues stored at -80°C were processed for the extraction of proteins. Each organ was mechanically homogenized in 1 ml of cold lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% (v/v) NP40, 2% (v/v) TritonX100, 1% (w/v) Zwitterion, 1 mM EDTA) added with protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 minutes on ice. After centrifugation for 10 minutes at 13,000 x g at 4°C, the supernatant was collected and protein concentration was determined spectrophotometrically using the Bradford micromethod (BioRad Protein Assay, BioRad Laboratories, GmbH, Munich, Germany).

Aliquots of lysed organs, corresponding to 40 μg of total protein content were loaded on 10% SDS-PAGE and Western blotted on nitrocellulose membrane. Immunodetection was carried out using a polyclonal goat anti-hSIGIRR Antibody (R&D Systems, Minneapolis, MN, USA) as the primary antibody at a concentration of 0.1 μg/μl (1:500 dilution from stock solution), overnight at 4°C. An HRP-conjugated anti-goat secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) was used (1:2000 dilution, 1 hour at room temperature). Positive bands were detected using chemiluminescent HRP substrate (Millipore, Molsheim, France).

Immunohistochemistry

Formalin fixed tissue samples (sciatic nerve, Bursa of Fabricius, thymus, spleen lung liver proventriculus, gizzard, intestine (duodenum, jejunum, cecum), harderian glands, kidneys) were routinely processed for histology. Four μm thick paraffin-wax sections were stained for demonstration of TIR8-SIGIRR receptor using Avidin-Biotin-peroxidase Complex (ABC) immunohistochemistry. Additional sections were included as negative control. Briefly, after the quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide in Phosphate Buffered Saline (PBS) pH 7.4 for 30 minutes, antigen retrieval was performed using microwave treatment at 750 W for 15 minutes (three cycles, 5 minutes each) in 10 mM citrate buffer (pH 6.0). After rinsing with PBS, 10% normal horse serum was applied for 30 minutes to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4°C with the

primary antibody (polyclonal goat anti-hSIGIRR Antibody, R&D Systems, Minneapolis, MN, USA) diluted 1:100. Biotinylated horse anti-goat antibody (Vector Laboratories Inc., Burlingame, California) was used as secondary antibody, followed by ABC Vectastain Elite kit (Vector Laboratories Inc., Burlingame, California). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole (Vector Laboratories Inc., Burlingame, California).

RESULTS

TIR8 mRNA expression analysis by Real-Time PCR

The expression pattern of *TIR8* was analyzed by mean of quantitative Real-Time PCR assays in a panel of 27 chicken samples.

The complete mRNA sequence of chicken *TIR8* (gi:118091084) was selected to design specific primers spanning two adjacent exons. The primer pair with the highest score localized in the middle of the sequence was used in Real-Time PCR assays to amplify the cDNA from each sample (Table I).

All the sampled organs resulted positive to the presence of *TIR8* transcript, with different levels of expression. In all the assays, the kidney RNA from chicken was the organ showing the highest level of *tir8* mRNA; therefore it was used as experimental calibrator besides as positive control. The results of Real-Time PCR quantification, expressed as ratio between each specimen and the kidney (calibrator), are summarized in Figure 1.

Besides kidney, TIR8 is highly expressed in heterophils, liver and in pancreas, followed by GI tract and oviduct; it is moderately expressed in respiratory tract, heart and muscular stomach. Low expression of TIR8 was detected in adrenal gland, skin, Bursa of Fabricius, testicle and ovary. All the other tissues analyzed displayed very low levels of *TIR8* messenger RNA.

Interestingly, a different expression of TIR8 was noted in the two chicken stomachs studied.

TIR8 protein expression analysis by Western blot

The expression of TIR8 protein in different chicken organs as well as the presence of possible tissue-specific isoforms, was assessed by Western blot.

A polyclonal goat anti-human TIR8 was used as primary antibody, after preliminary validation on chicken samples.

Results from Western blot analysis presented in Figure 2, showed TIR8 protein in all the organs examined. Moreover, Western blot analysis also revealed TIR8 forms with different molecular weight in most of the examined samples.

A TIR8 form of about 75 kDa was observed in most of the tissues and organs. TIR8 bands corresponding to a molecular weight of about 45 kDa were detected in some samples, such as caecum, colon, thymus, Bursa of Fabricius, spleen, liver, pancreas and glandular stomach. In other organs (cerebellum, brain, pancreas, muscular stomach, cloaca and adrenal gland) the anti-TIR8 antibody reacted with proteins showing a MW around 30 kDa.

Furthermore, Western blot analysis revealed TIR8 specific heavier bands of about 150 kDa in few samples (cerebellum, brain, Bburursa of Fabricius, pancreas. muscular stomach and glandular stomach).

The fraction with MW about 75 kDa, which correspond to the expected molecular weight of the complete protein, appeared to be the major TIR8 component in all the samples except kidney, brain and pancreas. In kidney, three TIR8 forms were identified, ranging from 70 to 90 kDa; brain displayed high MW forms, while in pancreas the band at about 45 kDa appeared to be predominant.

TIR8 expression in Harder's gland went barely detectable by anti-TIR8 antibody.

Taken together, results from this study showed that TIR8 receptor was widely expressed throughout all the digestive apparatus as well as in several other organs and tissues.

Immunohistochemistry

The expression of TIR8 protein was assayed by immunohistochemistry with the same polyclonal anti-human TIR8 antibody used for Western blot and validated for cross-reactivity with the chicken receptor. Nevertheless, positive immunolabeling was detected only in scattered cells of the intestinal mucosa (Figure 3) in some thymus epithelial cells and in oesophageal mural ganglia, whereas all the other samples were negative.

DISCUSSION

The negative regulator of the immune response TIR8 is gaining importance lately also in veterinary species animals, where inflammatory diseases have a negative impact on the productivity and consequently on the profit of the farm industry.

In this study, the expression of TIR8 was investigated in chicken at both transcriptional (mRNA) and translational (protein) levels. Messenger RNA was detected and quantified by Real-Time PCR, while TIR8 protein was revealed by both Western blot analysis and Immunohistochemistry. The two sets of data are substantially in agreement.

Present findings confirm some conservation in the expression pattern of this receptor between chicken and the other species studied so far, i.e. mouse, humans and cattle (2, 3, 12, 13).

The highest levels of *TIR8* expression were found in the kidneys and in the gastrointestinal tract; however, bird exhibited different expression levels in different organs, such as a high expression in liver and in oviduct. These peculiar traits may be linked to a different evolutionary pressure by the environment and pathogens. Indeed, the rudimental lymphatic system of birds (lack of lymph nodes) and the presence of a renal portal system could account for the high TIR8 expression in avian liver and kidney. It is well known that chicken ovary is a preferential site of microbial infection (for example due to *Salmonella enteritidis*) that can be subsequently transmitted to the eggs and embryos (14). Different levels of TIR8 in different organs could suggest a complex regulatory mechanism for the receptor expression in response to microbial infections, particularly depending on their duration.

Interestingly the different *TIR8* expression in the two stomachs of chicken studied was possibly correlated with the different relevance of the epithelial component in the two stomachs, compared to connective and muscular components. A similar observation was reported in other studies for the three stomachs of cattle (12, 13).

Remarkably, the expression study of chicken TIR8 mRNA by Real-Time PCR yielded results in agreement to those previously obtained by the same authors with Northern blot (12). High levels of hybridization with the species-specific probe were observed for the kidney, the liver, the glandular stomach and the cloacae; moderate levels were detected for the muscular stomach and for the Bursa of Fabricius (12).

In order to study the effective presence of TIR8 protein and the possibility of different post-translational modifications occurring in the same tissues, SDS-PAGE and Western blot were carried out on tissue homogenates. Protein distribution was in agreement with mRNA analysis. Similarly to results already shown in human and murine species, the high TIR8 expression observed in the whole digestive apparatus might support a role of local immune-modulator for chicken TIR8 (4). Indeed, the main activity attributed to TIR8 in the intestine, was that of dampening the immune response in cells physiologically exposed to microorganisms (6, 7, 9).

TIR8 expression pattern in chicken species was not found homogeneous, due to the detection of several isoforms after Western blot analysis. Since the antibody used throughout these experiments was highly specific for TIR8, the possibility of a cross-reactivity with other proteins seemed very remote. It should not to be ruled out that TIR8 mRNA could undergo alternative splicing and/or that the protein could host different post-translational modifications. The presence of TIR8 mRNA with different lengths was already reported in human and mouse species (2, 3) as well as by Northern blot analysis in chicken (12). The non-homogeneous pattern of TIR8 protein observed in electrophoresis could be also due to different post-translational modifications, such as tissue-specific glycosylation or proteolysis. This is not surprising, because TIR8 sequence displays seven glycosylation sites and the differences between predicted and actual molecular weight of TIR8 suggested extensive glycosylation (2).

Immunohistochemistry displayed low sensitivity showing immunolabeling only in a few organs. Nevertheless, positivity of scattered cells of the intestinal mucosa further confirm the central role of TIR8 expression in chicken intestine. Noteworthy, the immunolabeling of scattered cells in chicken thymus reveals an unexpected presence of SIGIRR-TIR8 in an organ showing only scarce TIR8 mRNA expression. In addition, oesophagus had not been included in the selection of organs to test. Nonetheless, some oesophageal tissue was found within trachea or thymus histological sections. The unexpected finding of TIR8 positive immunolabeling in some oesophageal ganglia needs further evaluation. Consequently, it would be of interest a full cytological characterization of the TIR8 expressing cells (including a double immunohistochemistry staining) in the aforementioned organs.

In conclusion, an ubiquitous expression of TIR8 was demonstrated in chickens at both transcriptional (mRNA) and translational (protein) levels. Given the relevance of intestinal diseases of infectious and non-infectious origin in chicken, these results support the interest in further investigating the role of TIR8 as a regulator of the immune response in this species.

References

1. Mantovani A, Locati M, Polentarutti N, Vecchi A, Garlanda C. Extracellular and intracellular decoys in the tuning of inflammatory cytokines and Toll-like receptors: the new entry TIR8/SIGIRR. J Leukoc Biol 2004; 75: 738-42.
2. Thomassen E, Renshaw BR, Sims JE. Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. Cytokine 1999; 11: 389-99.
3. Polentarutti N, Rol GP, Muzio M, Bosisio D, Camnasio M, Riva F, Zoja C, Benigni A, Tomasoni S, Vecchi A, Garlanda C, Mantovani A. Unique pattern of expression and inhibition of IL-1 signaling by the IL-1 receptor family member TIR8/SIGIRR. Eur Cytokine Net 2003; 14: 211-8.
4. Wald D, Qin J, Zhao Z, Qian Y, Naramura M, Tian L, Towne J, Sims JE, Stark GR, Li X. SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signalling. Nat Immunol 2003; 4: 920-7.
5. Lech M, Kulkarni OP, Pfeiffer S, Savarese E, Krug A, Garlanda C, Mantovani A, Anders HJ. Tir8/Sigirr prevents murine lupus by suppressing the immunostimulatory effects of lupus autoantigens. J Exp Med 2008; 205: 1879-88.
6. Garlanda C, Riva F, Veliz T, Polentarutti N, Pasqualini F, Radaelli E, Sironi M, Nebulosi M, Omodeo Zorini E, Scanziani E, Mantovani A. Increased Susceptibility to Colitis-Associated Cancer of Mice Lacking *TIR8*, an Inhibitory Member of the Interleukin-1 Receptor Family. Canc Res 2007b; 67: 6017-21.
7. Garlanda C, Riva F, Polentarutti N, Buracchi C, Sironi M, De Bortoli M, Muzio M, Bergottini R, Scanziani E, Vecchi A, Hirsch E, Mantovani A. Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proc Natl Acad Sci USA 2004; 101: 3522-6.
8. Garlanda C, Di Liberto D, Vecchi A, La Manna MP, Buracchi C, Caccamo N, Salerno A, Dieli F, Mantovani A. Damping excessive inflammation and tissue damage in

Mycobacterium tuberculosis infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. J Immunol 2007a; 179: 3119-25.

9. Xiao H, Gulen MF, Qin J, Yao J, Bulek K, Kish D, Altuntas CZ, Wald D, Ma C, Zhou H, Tuohy VK, Fairchild RL, de la Motte C, Cua D, Vallance BA, Li X. The Toll–Interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. Immunity 2007; 26: 461-75.
10. Temperley ND, Berlin S, Paton IR, Griffin DK, Burt DW. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics 2008; 9: 62.
11. Wang R, Millam JR, Klasing KC. Distribution of interleukin-1 receptor in chicken quail brain. Comp Biochem Physiol Part A 2003; 136: 663-71.
12. Riva F, Polentarutti N, Tribbioli G, Mantovani A, Garlanda C, Turin L. The expression pattern of TIR8 is conserved among vertebrates. Vet Immunol Immunopathol 2009; 131: 44-9.
13. Riva F, Rahman MM, Turin L, Ceciliani F, Russo S, Tribbioli G, Lecchi C. TIR8 receptor expression in bovine tissues. Vet Immunol Immunopathol 2010; 136: 65-70.
14. Barua A, Yoshimura Y. Ovarian cell-mediated immune response to Salmonella enteritidis infection in laying hens (gallus domesticus). Poult Sci 2004; 83: 997-1002.

FIGURE LEGENDS

Figure 1. Summary of the quantitative Real-Time PCR analysis results of *TIR8* mRNA expression in chicken tissues. The *TIR8* expression level of each sample is quantified as folds of the expression of the same gene in the chicken kidney (calibrator). Data were normalized to beta-actin expression in all the organs (multiple samples, repeatedly assayed samples and replicates were averaged).

Figure 2. Western blot analysis of TIR8 protein expression in different organs of chicken. Homogenates of 40 μg of total protein extracted from each sample were loaded. Target protein was detected on the blots by staining with polyclonal goat anti-human SIGIRR antibody.

Figure 3. Section of intestine of 18-day old egg type embryo. Cytoplasm positive immunolabeling of TIR8 protein in a few cells of intestinal villi (BAR: 40 μm). Inset 1: section of thymus showing TIR8 positivity in scattered epithelial cells (BAR: 120 μm). Inset 2: section of mural oesophageal ganglion with TIR8 immunolabeled cells (BAR: 80 μm). Anti-SIGRR immunohistochemistry, Mayer's Haematoxylin counterstain.

TABLES

Table I. The following primer pairs were designed and used to quantify TIR8 expression in Real-Time PCR assays.

Target gene	**Primers**
Chicken *TIR8*	5’-ACATTCCTGTCCGCAGTACCAG-3’ (forward)
	5’-TCCCAGTGTTTGTCCAGTAGGC-3’ (reverse)
Chicken *β-actin*	5’-CCCACCTGAGCGCAAGTACT-3’ (forward)
	5’-GAGGCCAGGATAGAGCCTCC-3’ (reverse)

Figure 1

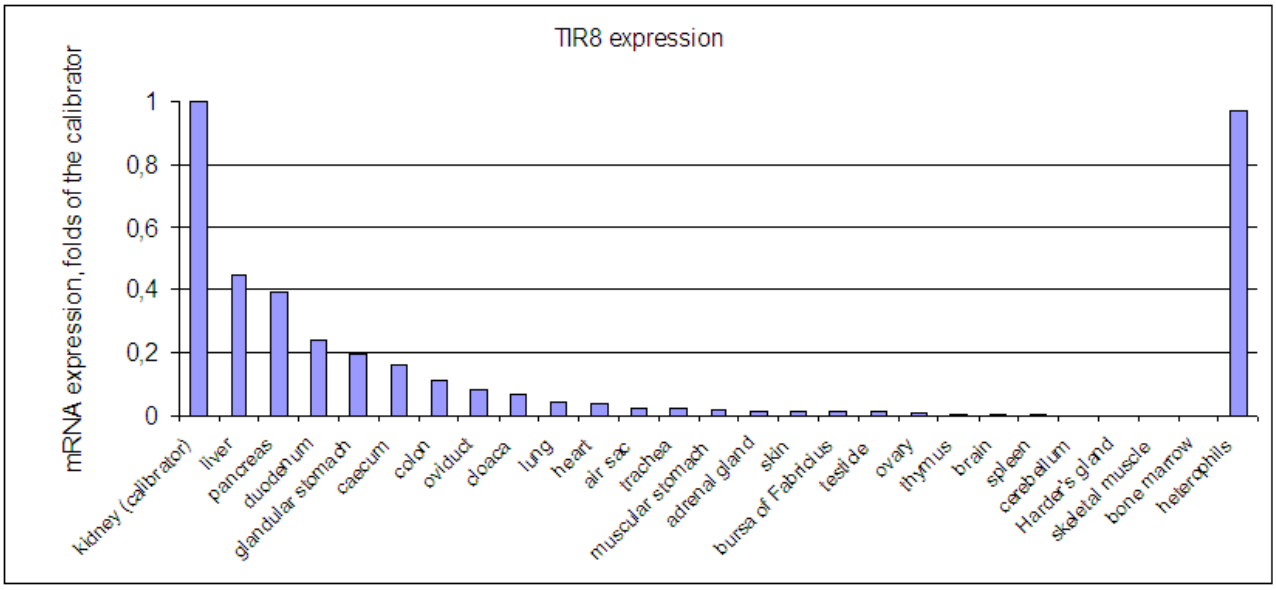


Figure 2

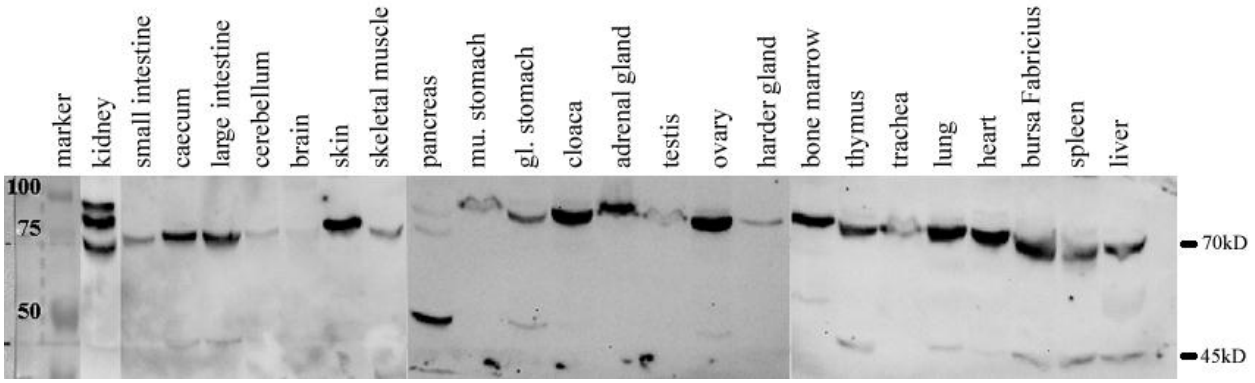
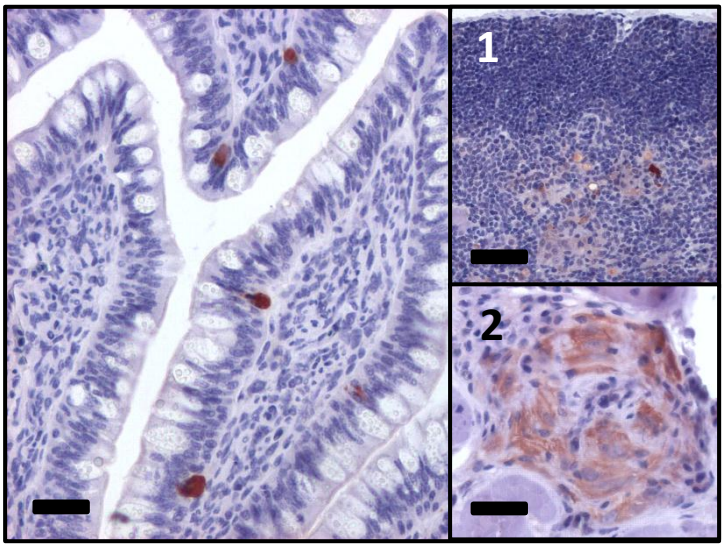


Figure 3



2. - PART TWO – MOLECULAR BIOLOGICAL METHODS IN THE PRACTICE OF AVIAN PATHOLOGY

2.1 - PARTIAL GENOME SEQUENCE OF PARVOVIRUS ASSOCIATED WITH HEPATITIS IN GREY PARTRIDGES (*Perdix perdix*)

INTRODUCTION

In avian species, parvoviruses are the responsible agents for Derzsy's diseases in young geese and are also found in Muscovy ducks (Gough, 2003). Recently, parvoviruses associated with enteric disease in turkeys and chicken have been partially (Zsak et al., 2008) and completely sequenced (Day and Zsak, 2010).

Increased mortality characterized by necrotizing hepatitis associated with parvovirus-like particles have been reported in flocks of grey partridges bred and raised as game birds in Northern Italy (Grilli et al., 2008). The affected birds were 1 to 2-month old and died acutely after a brief period of apathy.

Although histopathological and electron microscopic examinations reveal findings compatible with a parvovirus infection, the aetiology of these acute episodes of mortality has not been confirmed by more definitive methods.

In the present study molecular approaches were used to characterize the viral particles present in liver samples from partridges died with necrotizing hepatitis.

MATERIALS AND METHODS

Tissues

Liver samples were collected during necropsy of partridges with signs of necrotizing hepatitis (Grilli et al., 2008). Samples were stored at -20°C until use.

Attempts to isolate the responsible virus were performed on embryonated SPF eggs and on cell culture (CEF, QT-35, primary line from partridge embryo livers) but were all unsuccessful and are not reported in this thesis (details and data available on request).

DNA

25 mg pieces were cut from frozen samples and DNA extracted using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Purified DNA stocks were quantified and stored at –80°C.

PCR

Two PCR protocols were performed. For all reactions, PCR amplifications were carried out using 0.5 µg DNA added to 50 µl PCR buffer (Eppendorf, Hamburg, Germany) containing 1.5 mM MgCl2, 20 pmol each primer, 200 mM each dNTP and 1.25 U Taq polymerase (Eppendorf, Hamburg, Germany). For the first protocol, a primer pair that target consensus areas of the capsid protein for parvoviruses, including associated virus or dependovirus as previously described (Gao et al., 2002) was used. The primers were 5'-GGTAATGCCTCAGGAAATTGGCATT-3' and 5'- GACTCATCAACAACAATTGGGGATTC-3'and were expected to amply a 255 bp product. A gradient PCR protocol with annealing temperatures ranging from 55 to 65°C was used. Incubation conditions consisted of an initial incubation at 95°C for 3 min followed by 35 cycles at 95°C for 60 seconds, annealing at the desired temperature for 60 seconds, extension at 72°C for 45 sec and a final extension at 72°C for 15 minutes. Positive DNA from a case of Derzsy's disease was included as positive control. Primers specific for the NS protein of chicken and turkey parvoviruses (Zsak et al., 2008) were designed for the second protocol. The sense primer NS3F, 5'-CAACCAGTGAATCCATACTCATTTCTA-3' and antisense primer NS2R, 5'-CCAATAGGTCGATGGTTTCTGTAAGAT-3' were expected to amplify a 1370 bp product and were used at 95°C for 15 min, followed by 30 cycles of amplification of 94°C for 30 sec, 55°C for

1 min and 68°C for 3 min. After amplification, PCR products were separated by electrophoresis in a 2% agarose gel containing 1 µg/ml ethidium bromide.

Particle Associated Nucleic Acid Amplification

To detect viruses of unknown sequence in clinical samples, we used a random PCR approach allowing sequence-independent amplification (Allander et al., 2005; Stang et al., 2005). Frozen liver samples were homogenized and pooled. 300 and 500 mg and suspended in PBS (pH 7.2). Suspensions were centrifuged at 775 x *g* at room temperature and the supernatants were collected and filtered through 0.22 µm syringe filters (Merck-Millipore, Germany) to remove cellular debris and bacteria. Filtered supernatants were ultracentrifuged at 200000 x *g* for 90 min at 4°C. The resulting pellets were suspended in 200 µl molecular biology grade water and treated with 10U DNase I (Invitrogen, Milan) for 2 h at 37°C to remove unencapsitated DNA. DNA was extracted from 200 µl samples of the suspensions using a QIAamp DNA Mini Kit (Qiagen, Milan, Italy). DNA was eluted in 50 µl elution buffer.

Extracted DNA (20 µl) was mixed with 2.5 µl of 10× Ecopol buffer (100 mM Tris·HCl, 50 mM MgCl2; 75 mM DTT) (New England Biolabs), a 1-µl solution containing each dNTP at 10 mM, and 2 µl of primer FR26RV-N (GCCGGAGCTCTGCAGATATCNNNNNN) at 10 µM. The reaction was incubated at 94°C for 2 min and on ice for 2 min, after which 2.5 units (0.5 µl) of 3′-5′ exo- Klenow DNA polymerase (New England Biolabs) was added, and the reaction was incubated at 37°C for 1 h. This denaturation-annealing-elongation cycle was repeated once and then followed by an enzyme inactivation step at 75°C for 10 min. The DNA was purified with a PCR Purification kit (Qiagen, Milan, Italy) and eluted in 20 □l elution buffer (TE) provided by the manufacturer. Five µl of the eluted DNA was used as a template in a subsequent PCR. The 50-µl reaction mix consisted of 1× High Fidelity PCR buffer (60 mM Tris·SO4, pH 8.9; 18 mM Ammonium Sulfate) (Invitrogen, Milan, Italy), 2.5 mM MgCl2, 0.2 mM each dNTP, 40 pmol of the primer FR20RV (GCCGGAGCTCTGCAGATATC), and 2.5 units of Platinum Taq High Fidelity DNA polymerase (Invitrogen, Milan, Italy). After 10 min at 94°C, 40 cycles of amplification (94°C for 1 min, 65°C for 1 min, and 72°C for 2 min) were performed. PCR

amplicons were separated on 2% agarose gels and fragments between ≈600 and 1,500 bp in length were excised and extracted by QIAquick Gel Extraction Kit (Qiagen, Milan, Italy). The excised fragments were purified with Perfectprep Gel Cleanup kit (Eppendorf, Milan, Italy) and were subsequently cloned with the TOPO TA cloning kit (pCR 4 - TOPO Vector; Invitrogen, Italy) according to the manufacturer's protocol. Fifty to 100 colonies were picked and grown overnight at 37°C in LB medium (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl) with 50□g/ml ampicillin (SERVA GmbH, Heidelberg, Germany). Minipreps were prepared with the Wizard Plus Kit (Promega Italia, Milan, Italy) according to the manufacturer's instructions.

Sequencing

Sequencing reactions were performed by BMR Genomics (Padova, Italy) on an ABI377 sequencer by using the ABI PRISM dye-terminator cycle sequencing ready reaction kit with Amplitaq DNA polymerase (Perkin-Elmer, Applied Biosystems).The sequences obtained were compared with those in the GenBank database using the BLASTn and BLASTx algorithms (http:// www.ncbi.nlm.nih.gov/blast/Blast.cgi). For phylogenetic analysis, nucleotide and amino acid pairwise alignments were generated using the ClustalW programs (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 4.0 software package (Tamura et al., 2007) (http://www.megasoftware.net/mega4). The derived amino acids sequence was compared to sequence databases by BLASTP 2.2.24+.

PCR For Partridge Parvovirus

DNA from liver samples was also used as a template for a PCR protocol specific for the amplified sequence. PCR amplification was carried out as described above. The following primer pair was designed based on the sequence obtained with the particle associated nucleic acid amplification protocol: PP1f (TTTGCAATGCATCATGGAAC) and PP2r (CCCAGTATGCATCTATTAGA). The protocol consisted of 10 min initial denaturation at 95°C, followed by 30 cycles of amplification (94°C for 30 sec, 55°C for 1 min and 68°C for 3 min) were performed. PCR products were visualized after separation in an ethidium bromide stained

agarose gel. The amplicons were purified and sequenced as described above. The GenBank accession number for the sequences determined in this study is HM362436.

RESULTS

PCR targeting the capsid protein of parvoviruses and PCR specific for the NS protein of chicken and turkey parvoviruses gave negative results.

Particle associated nucleic acid amplification allowed the amplification of a 963 bp product from both the suspensions (Figure 1). Nucleotide sequences were compared by BLAST analysis which revealed the two sequences were identical but only distantly related to other published sequences. The derived amino acids sequence compared by BLAST revealed a correlation ranging from 65.5% and 68.6% with non-structural proteins of parvoviruses. 136 amino acids from the derived amino acids sequence were used for phylogenetic analysis which reveals the partridge sequence do not cluster with the other NS sequence from the other parvovirus considered (Figure 2).

PCR with primers designed on the specific nucleotide sequences derived from partridge parvoviral hepatitis successfully amplified a 963 bp product whose sequencing confirmed 100% identity with sequences from the random PCR.

DISCUSSION

The detection and identification of new viruses currently rely on a large range of techniques. Typically, the process starts by screening for a range of suspected ‘known’ viruses, using a panel of tests including electron microscopy and molecular methods such as PCR with degenerate primers. In the present study, histopathology and electron microscopy have previously demonstrated the presence of parvovirus-like particles in liver samples of these partridges (Grilli et al., 2008). We detected a 963 bp parvovirus sequence in liver samples from partridges died acutely with necrotizing hepatitis using a random PCR-based approach. This technique was successfully applied to detect novel bovine, human, chicken and turkey parvoviruses (Allander et al. 2001; Allander et al., 2005; Zsak et al., 2008; Day and Zsak, 2010).

The sequence analysis revealed no similarity with other known parvovirus. This could explained the negative results obtained with the PCR protocols using primers either for a consensus areas of the capsid protein for parvoviruses or for NS protein of chicken and turkey parvoviruses. On the contrary, the deduced amino acids sequence showed a certain similarity (65.5-68.6%) to the non-structural protein of parvoviruses.

Parvoviruses are small (20 nm), non-enveloped viruses with linear single-stranded DNA genome between 4.5 and 5.5 kb. Classification of parvoviruses is now based on their genomic structure rather than the previous classical criteria (antigenicity and host restriction). At present, parvoviruses infecting vertebrates have been classified into five genera: the parvoviruses, which include minute virus of mice as the prototype; the erythroviruses, of which the human parvovirus B19 is the prototype; the dependoviruses, which include the adeno-associated viruses and the goose and Muscovy duck parvoviruses; the amdoviruses, of which Aleutian mink disease virus is currently the only known member; and the bocaviruses, which include bovine parvovirus and canine minute virus (Tattersall et al., 2005).

The partial sequence of the partridge parvovirus represents approximately 18% of the complete genome and included a portion of the NS1 protein gene. Amino acid sequence alignments revealed a certain similarity with several members of the parvoviruses. Nevertheless, our results suggest that this parvovirus is representative of an independent member of the Parvovirinae subfamily.

Further comparative sequencing data will be required to determine the origin of this virus which has proven to cause mortality in flocks of partridges bred and raised as game birds. Nevertheless, the partial sequence we identified could be used to assess a PCR protocol on diagnostic purposes. Matter-of-factly, the epidemiology of this virus and its role as a pathogen needs to be thoroughly assessed not only for the economic loss it can cause to the breeders but primarily for its possible impact on the wild populations of partridges which are small and endangered.

Figure 1

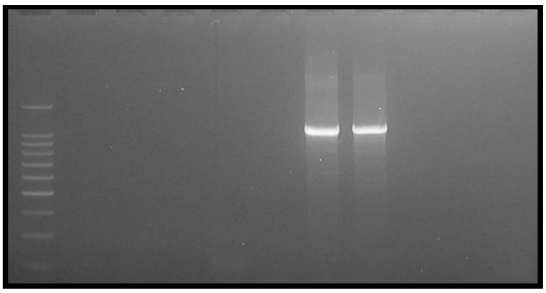


Figure 1: electrophoretic run showing two amplicons (960 bp) obtained from random PCR.

Figure 2

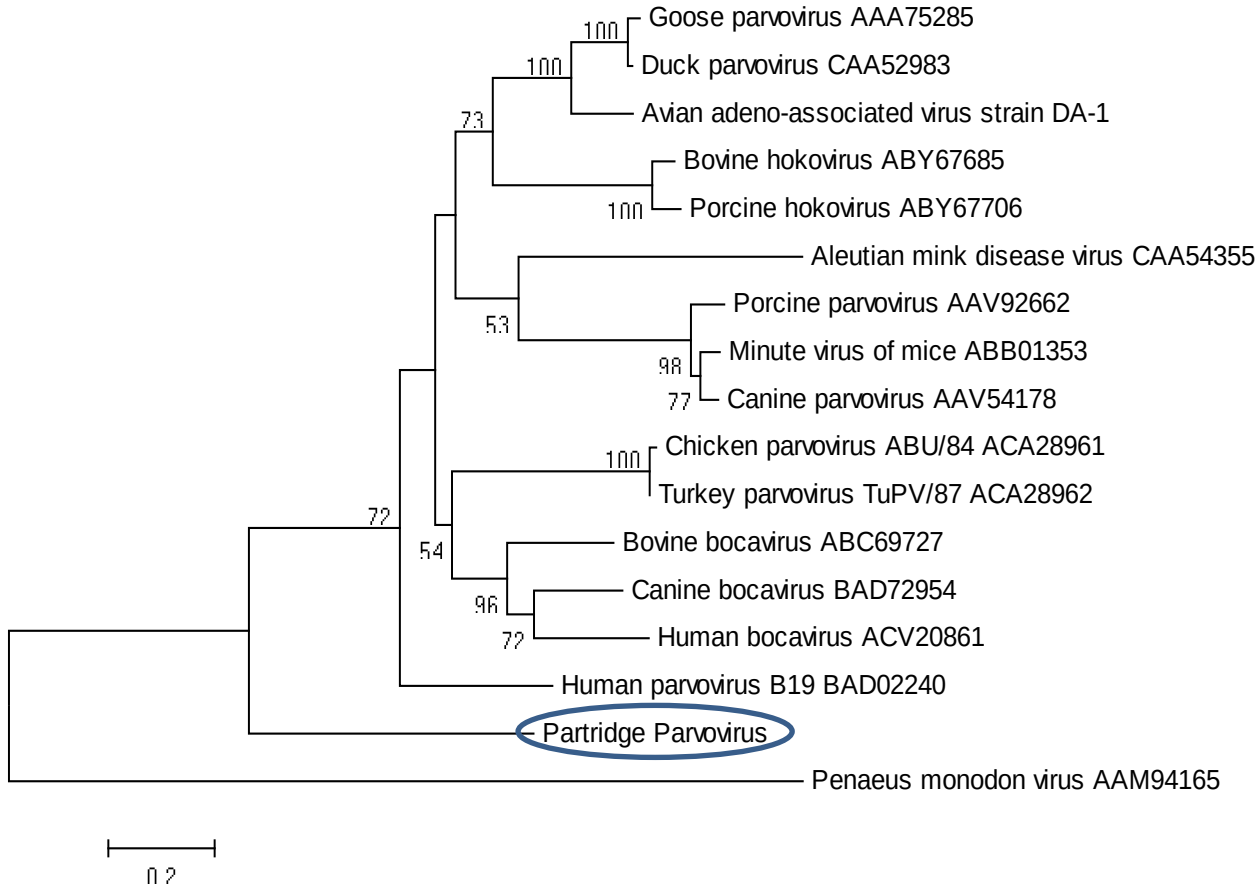


Figure 2: phylogenetic tree showing the relationship between the partridge parvovirus (encircled) and other members of the Parvovirinae subfamily. The tree was obtained by the Neighbor-Joining method calculated with the Jukes and Cantor model. Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 70 are indicated on the branches (as a percentage). Penaeus monodon virus AAM94165 was used as an outgroup.

References

- Allander T., Emerson S.U., Engle R.E., Purcell R.H. & Bukh J. (2001) A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. *Proceedings of the National Academy of Sciences of the United States of America*, 25, 11609-11614.
- Allander T., Tammi M.T., Eriksson M., Bjerkner A., Tiveljung-Lindell A. & Andersson B. (2005). Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 12891-12896.
- Day J.M. & Zsak L. (2010). Determination and analysis of the full-length chicken parvovirus genome. *Virology* 30, 59-64.
- Gao G.P., Alvira M.R., Wang L., Calcedo R., Johnston J., Wilson J.M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proceedings of the National Academy of Sciences of the United States of America,* 3, 11854-11859.
- Gough, R.E. (2003). Goose parvovirus infection. In Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, & D.E. Swayne (Eds.). *Diseases of Poultry* 11th edn (pp. 367-374). Ames: Iowa State Press.
- Grilli G., Ferrazzi V., Cislaghi G., Sironi G., Lavazza A. & Gallazzi D. (2008). Parvovirus-like hepatitis in Grey partridge (*Perdix perdix*): preliminary observation. In Proceedings of *Western Poultry Disease Conference and Poultry Health* (pp. 207-209). Puerto Vallarta, Mexico.
- Stang A., Korn K., Wildner O. & Uberla K. (2005). Characterization of virus isolates by particle-associated nucleic acid PCR. *Journal of Clinical Microbiology*, 43, 716-720.
- Tamura K., Dudley J., Nei M., Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.

- Tattersall P., Bergoin M., Bloom M.E., Brown K.E., Linden R.M., Muzyczka N., Parrish C.R. & Tijssen, P. (2005). Taxonomic structure of the family Parvoviridae". In C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, & L.A. Ball (Eds.). *Eighth Report of the International Committee on Taxonomy of Viruses* (pp.353-369). San Diego: Elsevier.
- Thompson J.D., Higgins D.G. & Gibson T.J. (1994).CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673-4680.
- Zsak L., Strother K.O. & Kisary J. (2008). Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. Avian Pathology. 37, 435-41.

2.2 – AVIAN BORNA VIRUS IN THE CASELOAD OF THE SECTION OF AVIAN PATHOLOGY

INTRODUCTION

Borna disease is a well-known disease in veterinary medicine since the end of the 18th century. It was usually characterized by nervous signs and reported in horses with abnormal behavior, explained at that time with sexual frustration and overfeeding. It took its name after a city in Saxony where in 1885 an outbreak of the disease was reported in horses and sheep. Thereafter, only sporadic cases were recorded mainly in Germany and Swiss.

In the first phase of the disease symptoms are vague: hyperthermia, anorexia, colics and constipation. In the acute phase the main signs are ataxia, circular movements, bruxism, nystagmus associated with abnormal reactions to stimuli such as hyperexcitability, aggressiveness or lethargy (Dauphin et al., 2002). The nervous signs are due to a immunomediated reaction involving T helper, CD4+, CD8+ lymphocytes (De la Torre, 2002). Histologically non purulent meningoencephalitis is observed. The disease progression takes one to three months with mortality rate up to 80% (Tomonaga e Carbone, 2002).

The viral etiology of Borna disease was demonstrated in 1927 at Giessen. From that time on, several other species have been proved to be naturally prone to this infection: cats, goats, rabbits, monkeys, llamas, alpacas, hippopotamus, lynx, fox and ostrich (Richt e Rott, 2001). Additionally, Borna disease virus has been proved to be able to infect all warm blooded animals when experimentally inoculated thus showing a huge range of possible hosts (Dauphin et al., 2002). The virus can be successfully isolated on several cell cultures. Serological surveys in horses revealed Borna virus diffusion is much larger than the area of its endemicity (Borna county and Swiss): seropositive horse have been found all over Europe, Iran, USA and Japan (Dauphin et al., 2002). Although Borna virus diffusion is particularly wide, genome sequencing revealed strict homology among the isolates from different parts of the world. Besides, the presence of seropositive horses in absence clinical signs has demonstrated that the infection can remain at a subclinical level.

Interestingly, in the ‘80s antibodies against Borna virus and Borna virus markers were detected at much higher levels in serum and cephalorachidian fluid of people suffering from different psychiatric diseases such as schizophrenia, autism, chronic depression (Rott et al., 1985). This renewed the scientific interest for this infection which could assume a zoonotic role though the actual involvement of Borna in the aforementioned psychiatric diseases is still debated.

BORNA VIRUS CHARACTERISTICS

Borna virus belongs to the family Bornaviridae, within Mononegavirales order, the taxonomic home of Filoviridae, Paramixoviridae e Rhabdoviridae. All these viral families have negative sense single stranded RNA with similar position, expression and codification of their genes (Pringle e Easton, 1997).

Bornaviridae family counted only one gender and species, Borna virus, until 2008 when several new species of avian origin were included with the name of Avian Bornavirus (http://www.ncbi.nlm.nih.gov/). Borna virus replicates in the cell nucleus and has proven to be particularly stable if compared to the other RNA viruses showing great homology among the isolates from different species in different times and Countries.

Borna virus genome encode six Open Reading Frames (ORF) divided into three transcriptional units: the first encode the nucleoprotein N (p40), the second the phosphoprotein P (p24) (polymerase cofactor) and X protein (p10), and the third the M protein (p16), GP protein (membrane GP protein) and the RNA polymerase (p180). The N nucleoprotein, the P protein and the polymerase are referred to as RPN and represent the viral replication complex. The GP protein is important for viral entry into the host cell and it ia activated through cleavage by host cell proteases. The P protein and the N protein show the most immunogenic activity and are the target of the serological test for Borna virus diagnosis. As common feature in RNA viruses, Borna virus genome increases its codification capability by the overlapping of the ORF and by post-transcriptional splicing which makes possible to get different proteins from the same

sequence. In mammals two main viral strains are known (V strain and He/80 strain) sharing 80% homology (Richt and Rott, 2001; Weissenböck et al, 2009).

AVIAN BORNAVIRUS AND PROVENTRICULAR DILATATION DISEASE

Avian Bornavirus infections have been recently related to the so-called Proventricular Dilatation Syndrome (PDD) (Kistler et al., 2008) which is considered one of the most critical health issue in psittacine breeding. This disease has been described in more than 50 species all over the world.

PDD affects the autonomic nervous system of the digestive tract and particularly the afferent fibers of oesophagus, crop, proventriculus, gizzard and duodenum. Clinically, affected birds show neurological signs such as ataxia, proprioceptive disorders associated with gastro-enteric signs, progressive weight loss, dysphagia, regurgitation and presence of stool with non-digested feed (Kistler et al., 2008).

Grossly, the main finding is variably dilated proventriculus. Microscopically, myoenteric ganglia are infiltrated by lymphocytes and plasma cells. Similar infiltration can be detected in brain, spinal cord, cardiac conduction tissues, cardiac and smooth muscle and adrenal glands. Brain lesions are usually associated by lymphoplasmacytic myositis and myocarditis.

Diagnosis is based on radiographic evidence of proventricular dilation (with or without contrast medium) and on histological evaluation of crop biopsies. As PDD is a progressive disease, identification of clinically healthy but infected birds has been recently made possible through non-invasive diagnostic methods such as Western blot (Villanueva et al., 2009), indirect immunofluorescence (Herzog et al., 2010) and RT-PCR on cloacal swabs (De Kloet e Dorrestein., 2009). Prognosis for infected birds is always fatal.

AVIAN BORNAVIRUS PECULIARITIES

A genetic variability greater than Mammals Borna virus seems to characterize Avian Borna virus which count several species. In addition, they are more successfully cultured on cell lines of

avian origin. Finally, Avian Bornaviruses show larger tropism than mammals Borna virus as they can infect several organs and tissues (i.e. intestine with a fecal-oral route of transmission) and not only nervous system.

Retrospective investigations on archived files and materials of the Avian Pathology Section with a presumptive diagnosis of PDD or with lesions compatible with PDD were re-evaluated through biotechnological approach as detailed hereafter.

MATERIALS AND METHODS

The paper and electronic files of the Section of Avian Pathology archive were searched from 1980 to 2009 to select cases from the diagnostic routine. Attention was focused on cases regarding psittacines species with gross or microscopic signs of gastritis compatible with PDD. Selected cases are listed in Table 1. For eight cases diagnosis was based only on histopathological evaluation of formalin fixed material. Paraffin embedded and/or frozen material for each case was also identified in the specific archives and used for analysis.

Necropsy

Psittacines submitted for diagnosis had been necropsied and samples of different organs had been collected, fixed in 10% buffered formalin and routinely processed for histopathology. For limited numbers of cases frozen material (-20°C) had been also collected.

Immunohistochemistry

From a selection of nine cases immunohistochemical investigations were also performed. Four µm thick paraffin-wax sections were stained for demonstration of P24 protein and P40 protein using Avidin-Biotin-peroxidase Complex (ABC) immunohistochemistry (Hsu et al., 1981). Briefly, after the quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide in Phosphate Buffered Saline (PBS) pH 7.4 for 30 minutes, antigen retrieval was performed using microwave treatment at 750 W for 15 minutes (three cycles, 5 minutes each) in 10 mM citrate buffer (pH 6.0). After rinsing with PBS, 10% normal horse serum was applied for 30 minutes to

block non-specific antibody binding. Subsequently, sections were incubated overnight at 4°C with the primary antibodies, 1:500 (mouse monoclonal, IZS Lombardia ed Emilia Romagna). Additional sections were included as negative controls. Biotinylated horse antimouse antibody (Vector Laboratories Inc., Burlingame, California) was used as secondary antibody, followed by ABC Vectastain Elite kit (Vector Laboratories Inc., Burlingame, California). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole (Vector Laboratories Inc., Burlingame, California)

RT-PCR INVESTIGATIONS

RNA extraction

RNA extraction was performed only on those cases with a histopatholoc diagnosis compatible with PDD and of which frozen samples were available. RNA was extracted using 750 µl Trizol (Invitrogen, Paisley, UK) on 50 µg of frozen materials always including proventriculus and brain. Frozen material were minced by sterile blades and placed into 1.5 ml sterile DNasi/RNasi free Eppendorf tubes (Eppendorf, Hamburg, Germany). After 5-minute incubation at room temperature, 200 µl chloroform were added. After 3-minute incubation the samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatants were transferred into new Eppendorf tubes where 500 µl isopropanolol were added with a 10 minute incubation. A second centrifugation at 12000 rpm for 10 minutes at 4°C was applied. The supernatant fluid was discarded and the pellets were washed with 75% ethanol at 4°C. After discarding the supernatant, the pellet containing the RNA was dried at room temperature and then re-suspended in 10 µl of water for molecular biology (Invitrogen, Paisley, UK).

Retrotranscription

SuperScript III RNase H-free reverse transcriptase (Invitrogen, Milan, Italy) kit was used for RNA retrotranscription following the manufacturer's instructions. Briefly, for each sample a total volume of 10 µl was prepared as follows: 1X RT Buffer, 0.5 mM of dNTPs, 10 mM of DTT, 0.5 M of oligo-dT primer 12-18, 40 U RNase OUT TM, 200 U of Superscript III. 2 µl of RNA were

added and the mix was incubated at 45°C for 1 hour. Then retranscriptase was inactivated placing the samples at 95°C for 5 minutes. cDNA was stored at -20°C until use.

Semi-nested PCR

A semi-nested PCR protocol was tested. First, Avian Borna virus sequences from literature were aligned by BioEdit (7.0.5). A sequence region crossing the genes of N protein, P protein and M protein was identified. For the amplification of this region the following primer were designed: Borna1F 5'-GCAAACTAATGAACATACG-3' (gene of N protein) and Borna3R 5'-CACCAATGTTCCGAAGACGA-3' (gene of M protein (matrix). For the second amplification the last primer was used with the primer Borna2F 5'-GAGCAAGTCAAGAAGAA-3' (gene of protein X (nucleocapsid). Amplifications were made in a total volume of 50 µl containing: 5 µl of PCR buffer (10x), 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 mM of dNTPs and 1.25 U of Taq polymerase (Eppendorf, Hamburg, Germany). Amplification protocol for each reaction was the following: 95°C for 4 minutes, followed by 35 cycles of 50 seconds each (40 seconds at 50°C) and 2 minutes at 72°C. Elongation was performed at 72°C for 5 minutes. 5 µl for each PCR sample was PCR products was visualized after separation in an ethidium bromide stained agarose gel. The amplicons of the expected size were sequenced by "ABI-PRISM 377 DNA sequencer" (Applied Biosytems, Foster City, California, USA). The sequences were submitted to GenBank and phylogenetically analyzed by BLAST in order to ascertain the percentage of similarity.

Phylogenetic Analysis

After manual editing to exclude primer regions, the sequences were aligned (clustalW; Thompson et al., 1994) with Borna virus sequences available in GenBank as reported by Lierz et al. (2009). In detail, four sequences of the most studied strains identified in horse and humans were considered: He80 (accession number: L27077.2), No/98 (accession number: AJ311524.1), "virus V" (accession number: U04608.1) and He/80/FR (accession number: AJ311522.1). In addition, other sequences of avian origin were included: FJ169440.1 (identified

in *Ara glaucogularis* in 2008), FJ169441.1 (identified in *Ara glaucogularis* in 2008), FJ932550 (identified in *Cacatua moluccensis* in Germany in 2009), FJ932551 (identified in *Amazona vinacea* in Germany in 2009), FJ770253.1 (identified in *Cacatua moluccensis* in Germany in 2009), ABVNL-001 (identified in *Cacatua alba* in Netherlands in 2008), EU781967.1 (identified in *Aratinga solstitialis* in 2008 in the USA) and WO2910006296 (laboratory avian strain developed in the USA in 2010). The sequences obtained with this study were included as follows: PA 139/05 from *Cacatua ophthalmica* (accession number: HM565487), and PA 67/09 from *Psittacus erithacus* (accession number: HM565488). Phylogenetic analysis was performed using Neighbor-Joining method (Jukes and Cantor model). The percentage of the similarity between sequences was also evaluated (MEGA 4.0.2).

RESULTS

Out of the protocols of the Avian Pathology Section 30 cases compatible with PDD were identified on the basis of the necropsy and/or microscopic findings covering a period ranging from 1980 and 2009 (Table 1). All the parrots included in the caseload had a diagnosis of PDD or necroscopic signs of proventriculus dilation. For each sample the histologic archive was checked to find the related slides and paraffin blocks when available. All the histologic samples were re-evaluated and 13 samples were identified as PDD positive. 6 out of the 13 cases had frozen samples available (Table 1: PA 139/05, PA 257/06, PA 88/07 B, PA 45/08, PA 67/09, PA 122/09).

Histologic Lesions

The thirteen cases were all characterized by mild to severe ganglioneuritis characterized by lymphocytes and plasma cells infiltrating mural ganglia of the digestive tract. Case PA 122/09 (*Nymphicus hollandicus* with a history of crop paralysis) was also characterized by crop involvement. For 5 cases sections of central nervous system were also available (Table 1, PA 185/97, PA 351/06 B, PA 45/08, PA 67/09, PA 122/09) showing mild to severe gliosis. Cases

PA 185/97, PA 45/08 e PA 67/09, were also characterized by perivascular cuffing of lymphocytes and plasma cells.

Immunohistochemistry

No clearly positivity immunolabeling was obtained. Cases PA 9907, PA 2/93, PA 257/06, PA 88/07B e PA 67/09 were negative. Weak immunolabeling was observed in a section of gizzard of case PA 351/06B (in the koilin layer), in a section of crop mucosa of case PA 122/09, and in some crop sections of case PA 139/05. Finally, in a section of central nervous system of case PA 45/08 strong positivity of a single neuron.

PCR

PCR investigations revealed only two positive cases for avian Borna virus (PA 139/05 e PA 67/09) (Figure 1). Case PA 122/09 gave a band of the expected size but very weak and consequently was not included.

The two sequences were submitted to the GenBank, accession numbers: HM565487 (PA 139/05) and HM565488 (PA 67/09).

Phylogenetic tree is reported in Figure 2. Both sequences of case PA 139/05 and PA 67/09 cluster with another Avian Borna virus strain identified in *Ara glaucogularis* (accession number: FJ169440.1). The percentage of similarity between the two sequences was 81.7% whereas between the two sequences and the strain identified in *Ara glaucogularis* the percentage of similarity was 84.2% (PA 67/09) and 85.7% (PA 139/05). The percentage of similarity between the cluster including the two sequences of the study and the other two clusters including other Avian Borna virus was 43.4% and del 42%, whereas it was 66.6% considering the cluster including mammal Borna viruses.

DISCUSSION

Thirty cases compatible with a diagnosis of PDD were extracted from the files of the archive of the Section of Avian Pathology (Table 1). For 13 cases histologic sections and paraffin-

embedded material were available to confirm the presumptive diagnosis of PDD and for re-evaluation. The cases for which frozen specimens had been stored were chosen to set up immunohistochemical and molecular biological evaluation through PCR. Samples from budgerigars with proventricular dilation in absence of microscopic findings of Macrorhabdos ornithogaster infection were also included though so far this is not a species prone to PDD infection. Matter-of-factly Avian Borna virus has been recently found in no psittacines species including canaries (*Serinus canaria*), (Weissenböck et al., 2009).

Immunohistochemistry

Two monoclonal antibodies (IZS Lombardia ed Emilia Romagna) directed against P24 (phosphoprotein P) protein and P40 protein (nucleoprotein N) of Borna virus isolated from horse were used. Immunolabeling was found only in few cases and appeared scarce. Nevertheless distribution and localization of the positive signal was compatible with history and lesions of the birds. For instance, case PA 45/08 had a history of nervous signs and immunohistochemically one single neuron appeared strongly positive. As for case PA 122/09, the parrot was submitted with a history of crop impaction and a week immunolabeling was found just in the crop sections. Similar results with monoclonal antibodies for Borna virus have been recently reported in literature thus confirming the importance to focus on polyclonal antibodies in similar investigations Weissenböck et al., (2009). It is conceivable that antigenic differences between Borna virus of mammalian and avian origin were strong as to prevent cross reaction. Moreover, it should be considered that none or scarce immunohistochemical positivity could be partially due to formalin over-fixation and/or long storage (especially for older samples) of paraffin embedded tissues. Other unmasking protocols (proteolysis with trypsin and pepsin) will be performed in parallel to testing of polyclonal antibodies.

PCR Investigations

Molecular biological analysis through a semi-nested PCR protocol was successful only for two out of the six cases with frozen material available. Sequencing and phylogenetic analysis

revealed both sequences belong to the same cluster where another sequence of avian origin is also included. Nevertheless, the percentage of similarity between the two sequences is 81.7%. This means that there is wide difference between the two PCR amplicons. Noteworthy, the percentage of nucleotidic between both sequences and the cluster of mammalian Borna virus is much higher (66.6%) than similarity evaluated between their cluster and the other two containing Borna virus sequences of avian origin obtained with this phylogenetic approach. This further confirm the great variability among Borna virus of avian origin in comparison to great homology observed among mammalian Borna virus (Kistler et al., 2008; Rinder et al., 2009).

As for the high numbers of negative cases in PCR analysis, it is necessary to underline the scarce resistance of Borna viruses especially considering the nature of their genome (RNA viruses). Moreover, the submitted material (frequently in poor condition with post mortal changes in most birds) and the storage at -20°C for long time (several years in some case) could have reduce the quantity of intact genome available for performing RCR analysis. Finally, it is necessary to stress that the viral load can vary significantly from bird to bird depending on the stage of infection, bird immunity, affected tissue, etc., and this can also affect the success of genome extraction and amplification.

Table 1

YEAR	PROTOCOL N.	SPECIES/SEX	AGE	PARAFIN-BLOCK	HISTO-SLIDES	FROZEN
1988	**PA 8560**	*Psittacus erithacus*	1 YEAR	NO	NO	NO
	PA 8636	*Agapornis fisheri*	..	NO	NO	NO
1991	**PA 9408**	*Amazona aestiva* ♂	..	NO	NO	NO
1992	**PA 9907***	*Ara macao*	..	YES	YES	NO
1993	**PA 2/93***	*Ara severa* ♀	Adult	YES	YES	NO
	PA 320/93	*Psittacus erithacus* ♀	Adult	YES	YES	NO
1995	**PA 157/95***	*Ara ararauna*	..	YES	YES	NO
	PA 345/95*	*Cacatua moluccensis*	..	YES	YES	NO
1997	**PA 185/97***	*Cacatua moluccensis*	18 MONTHS	YES	YES	NO
2004	**PA 264/04**	*Amazona aestiva* ♀	1 YEAR	YES	NO	NO
2005	**PA 102/05**	*Forpus coelestis* ♀	7 YEARS	YES	YES	YES
	PA 139/05	*Cacatua ophthalmica* ♀	1 YEAR	YES	YES	YES
2006	**PA 257/06***	*Ara militaris* ♀	Adult	YES	YES	NO
	PA 304/06 B	*Melopsittacus undulatus* ♀	14 DAYS	YES	YES	YES
	PA 351/06 B	*Melopsittacus undulatus* ♂	Adult	YES	YES	NO
2007	**PA 40/07**	*Melopsittacus undulatus* ♂	10 MONTHS	NO	NO	NO
	PA 46/07	*Bolborhynchus lineola*♀	4YEARS	YES	YES	YES
	PA 71/07	*Psittacus erithacus* ♂	12YEARS	YES	YES	YES
	PA 82/07 B	*Melopsittacus undulatus* ♂	14 DAYS	YES	YES	YES
	PA 88/07 B	*Melopsittacus undulatus* ♂	..	YES	YES	YES
	PA 100/07 D	*Melopsittacus undulatus*	13 DAYS	YES	YES	YES
	PA 101/07 C	*Melopsittacus undulatus* ♂	2 YEARS	YES	YES	YES
2008	**PA 30/08***	*Agapornis roseicollis* ♂	..	YES	YES	NO
	PA 32/08	*Amazona aestiva*	..	YES	YES	YES
	PA 45/08	*Psittacus erithacus* ♂	2 YEARS	YES	YES	YES
	PA 89/08	*Melopsittacus undulatus* ♀	9anni	YES	YES	YES
2008	**PA 100/08***	*Trichoglossus haematodus* ♀	10 MONTHS	YES	YES	YES
	PA 109/08	*Psittacus erithacus* ♀	2 YEARS	YES	YES	NO
2009	**PA 67/09**	*Psittacus erithacus* ♀	Adult	YES	YES	YES
	PA 122/09	*Nymphicus hollandicus* ♀	--	YES	YES	YES

Table 1: 30 cases selected considering the gross finding compatible with PDD. The cases without histologic analysis are highlighted in yellow. Cases submitted as formalin fixed material have been identified with *.

Figure 1

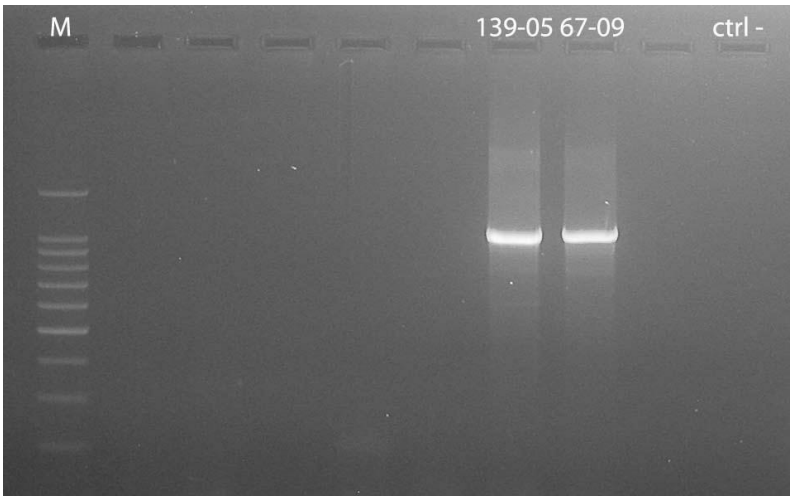


Figure 1: electrophoretic run showing the two positive samples identified in this study

Figure 2

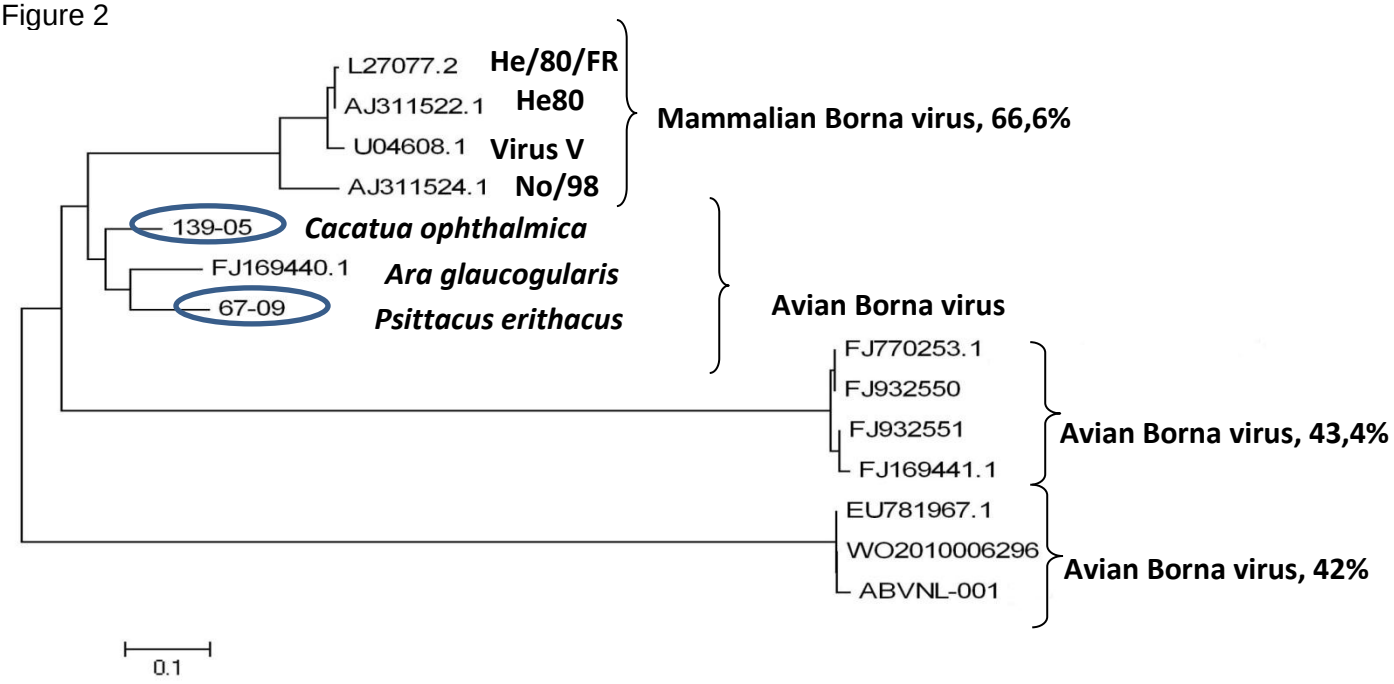


Figure 2: phylogenetic tree (Neighbor-Joining method, Jukes e Cantor model). Phylogenetic analysis was performed by bootstrap, 1000 resamples). Reference sequences derived from Lierz et al. (2009). PA 139/05 e PA 67/09 (encircled) are the two sequences obtained with this study.

References

- Dauphin G., Legay V., Pitel P.H., Zientara S. (2002). Borna disease: current knowledge and virus detection in France. Veterinary research, 33: 127-138.

- De la Torre J. C. (2002). Bornavirus and the Brain. The journal of Infectious Disease 186 (suppl.2) 241-247.

- De Kloet S.R. and Dorrestein G. M. (2009). Presence of Avian Bornavirus RNA and Anti-Avian Bornavirus Antibodies in Apparently Healthy Macaws. Avian Diseases, 53: 568-573.

- Herzog S., Enderlein D., Heffels-Redmann U., Piepenbring A., Neumann D., Kaleta E. F., Müller H.,Lierz M. e Herden C. (2010). Indirect immunofluorescence assay for intra vitam diagnostic of avian Bornavirus infection in psittacine birds. Journal of clinical microbiology, 48 (6); 2282-2284.

- Hsu S .M., Raine L., Fanger H. (1981). Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. Journal of Histochemistry and Cytochemistry, 29: 577-580.

- Lierz M., Hafez H. M., Honkavuori K. S., Gruber A. D., Olias P., Abdelwhab

E. M., Kohls, A., Lipkin, W. I., Briese T. and Hauck R. (2009). Anatomical distribution of avian Bornavirus in parrots, its occurrence in clinically healthy birds and ABV-antibody detection. Avian Pathology, 38 (6): 491-496.

- Kistler AL, Gancz A, Clubb S, Skewes-Cox. P.,Fischer K., Sorber K., Chiu C. Y., Lublin A.,Mechani S., Farnoushi Y., Greninger A., Wen C. C., Karlene S. B., Ganem D.and DeRisi J. L. (2008). Recovery of divergent avian Bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. Virolology journal, 5: 88.

- Pringle C. R. e Easton A. J. (1997). Monopartite Negative Strand RNA Genomes. Seminars in Virology, 8: 49-57.

• Richt J. A. e Rott R. (2001). Borna Disease Virus: a Mystery as an Emerging Zoonotic Pathogen. The veterinary journal, 161: 24-40.

- Rott R., Herzog S., Fleischer B., Winokur A., Amsterdam J., Dyson W., Koprowski H. (1985) Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science, 228(4700):755-6.

• Thompson J.D., Higgins D.G., Gibson T.J., (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-80.

• Tomonaga K. e Carbone K. M. (2002). Cap. 1, Borna disease virus: Spanning a century of science, in Borna Disease Virus and its role in Neurobehavioral disease. Editore Carbone K. M, pag. 1-21, ASM press, Washington, D. C., USA.

• Villanueva I., Gray P., Mirhosseini N., Payne S., Hoppes S., Honkavuori K. S., Briese T., Turner D. e Tizard I (2009).The diagnosis of proventricular dilatation disease: Use of a Western blot assay to detect antibodies against avian Borna virus. Veterinary Microbiology, 143 (2-4): 196-201

• Weissenböck H., Sekulin K., Bakoniy T., Högler S e Nowotny N. (2009). Novel Avian Bornavirus in a Nonpsittacine Species (Canary; Serinus canaria) with Enteric Ganglioneuritis and Encephalitis. Journal of virology, 83 (21):11367-11371.

2.3 - SEVERE ORAL CANDIDIASIS IN COMMERCIAL TURKEYS

INTRODUCTION

Candida species have a worldwide distribution and are part of the microflora of the healthy digestive system of humans, animals, and birds (**3**). Perturbance of the mucosal microflora, young age, concurrent infections and debilitation of the host can lead to candidiasis. Birds are particularly susceptible to oral and crop candidiasis, which resembles thrush in humans. In poultry production the occurrence of candidiasis is sporadic, but outbreaks can be costly. Diagnosis of candidiasis requires a multidisciplinary approach based primarily on conventional methods (direct microscopic examination of fresh smears, isolation, histopathology, etc.) often associated with molecular genetic techniques necessary to obtain more accurate and detailed insight (**2**).

MATERIALS AND METHODS

Animals – Seven 28-day-old commercial male turkeys out of a flock of 10000 were submitted for necropsy because of increased mortality and severe oral lesions compatible with a presumptive diagnosis of mycotoxicosis (trichothecene mycotoxins). Recent manual debeaking (second week of age) was reported as anamnestic data.

Histopathology - Samples of oral tissues (roof and floor of the buccal cavity and tongue), thymus, Bursa of Fabricius, spleen, liver, intestine and kidney were fixed in 10% buffered formalin and routinely included for histopathology. 4-micron sections were stained with haematoxylin and eosin (HE) and PAS.

PCR - DNA was retrospectively extracted from a selection of paraffin blocks from the archival tissue material shown to contain structures consistent with Candida on histopathology. Sterile razor blades were used to harvest 30-µg samples of tissue from the centres of the larger lesions. Samples were collected in sterile 1.5-ml tubes and dewaxed in 1 ml for 20 min, centrifuged at 1000 × *g* for 3 min, and washed twice with 100% ethanol. DNA extracted from the pellet by using a DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's

instructions. The identification of the yeasts was based on the amplification with universal primers (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2 5'-TCCTCCGCTTATTGATATGC-3') and sequence analysis of a region of the Internal Transcribed Spacer (ITS) of the rRNA gene as previously described (**4**). Negative and positive controls were included alongside the amplification reactions. Presence and size of the PCR products were assessed by agarose gel electrophoresis and ethidium bromide staining. PCR products of the expected length (218 bp) were purified from agarose gel slices using a QIAquick PCR purification kit (Qiagen) and sequenced using an ABI Prism 310 genetic analyser (Applied Biosystems, Foster City, California, USA). The sequence were submitted to the GenBank database (HQ845604). The percentage of similarity with reference sequences was evaluated by BLAST search in the NCBI website.

RESULTS

Necropsy – All the birds showed unsatisfactory growth associated with severe oral lesions characterized by erosions, ulcers and crusts extensively affecting the upper beak rim and the outer portions of the palate and tongue (Figures A-C). Signs of recent debeaking appeared evident in the seven birds. Mild entero-typhlitis with few oocysts in the cecal content was detected in two turkeys. Gizzards appeared normal. Mild fibrinous aerosacculitis was observed.

Histopathology - Diffuse, severe ulcerative glossitis and stomatitis with abundant necrotic debris associated with myriads of 3-6 µm diameter, roundish, pale staining, thin-walled yeasts (consistent with Candida yeast) often arranged in short chains of pseudohyphae (Figures E-F). Scattered aggregates of cocci were also found. Mild ballooning degeneration of the oral epithelium was focally observed in few sections. Mild to moderate lymphocytic depletion was found in all the spleen sections. As additional finding, mild, chronic typhlitis associated with coccidia at different developmental stages was observed in one section.

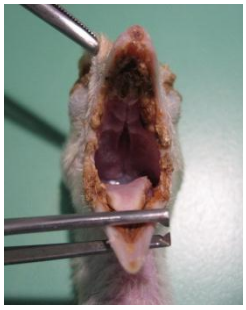
PCR and Sequencing – Molecular analysis identified *Candida albicans* (similarity of 95%) as the yeast massively present in oral lesions.

DISCUSSION

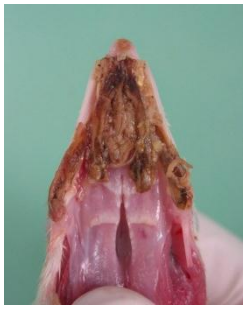
The presumptive diagnosis of trichothecene mycotoxicosis was discarded as the other gross lesions typically associated with the oral involvement (yellow-tan, friable livers, swollen kidneys, urate deposits in the ureters, focal ulceration and inflammation of crop mucosa, and a thickened, rough lining in the gizzard) were lacking (**1**). Histopathological evaluation of all the oral samples from each bird revealed erosions and ulcers were constantly and massively colonized by structures consistent with blastoconidia and pseudohyphae of Candida as retrospectively confirmed by molecular analysis. The differential diagnosis of fowlpox was ruled out for lack of typical microscopic lesions (epithelial hypertrophy and Bollinger bodies).

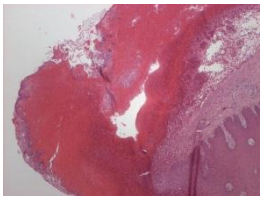
It was not possible to clearly assess which of the predisposing factors were primarily involved in this outbreak of oral candidiasis. Nevertheless, it is possible to hypothesize the recent manual debeaking played a major role. Matter-of-factly the oral lesions were significantly more severe in the upper beak which was completely affected from the debeaking scar to the beak commissure including the whole oral roof. On the contrary, in the oral floor the lesions were limited to the beak and tongue margins. The turkeys had mild aerosacculitis but it was not possible to ascertain whether they had received antibiotic treatment which could have enhanced Candida proliferation. Unfortunately, the isolation of this Candida strain was not performed. This could have been essential to characterize possible virulence factors related to the severity of this uncommon outbreak of candidiasis.

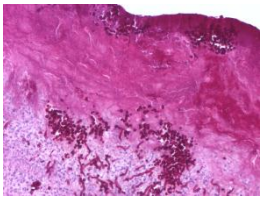
Figures


A


B


C


E


F

Legends

Figures A-B-C: erosions, ulcers and crusts extensively affecting the upper beak rim and the outer portions of the palate and tongue. Note in A and B the signs of recent debeaking. D: section of tongue with abundant necrotic debris associated with numerous yeast cells (HE stain, bar: 150 µm). E: section of tongue, aggregates of pseudohyphae and yeast cells within necrotic debris layer and infiltrating the tongue epithelium (PAS stain, bar: 50 µm).

References

1. Hoerr, F. J. Mycotoxicoses. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa, USA. pp. 1103-1109. 2003.

2. Kunkle, R. A. Fungal Infections. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa, USA. pp. 896-899. 2003

3. Schmidt, R. E., Schmidt R. E., Reavill D.R., Phalen D.N. Gastrointestinal system and pancreas. In: Pathology of pet and aviary birds, Iowa State Press, Ames, Iowa, USA. 2003

4. Williams, D. W., Wilson M. J., Lewis M.A., Potts A. J. Identification of Candida species in formalin fixed, paraffin wax embedded oral mucosa by sequencing of ribosomal DNA. Clin Mol Pathol. 49:M23-8. 1996.

2.4 - PERIOCULAR MYCOBACTERIOSIS IN COMMERCIAL LAYERS

INTRODUCTION

Avian mycobacteriosis is a well-known disease with a world-wide distribution. In poultry, the disease is usually termed tuberculosis and is principally caused by *Mycobacterium avium*. This disease is chronic and affected flocks are characterized by unthriftiness, decreased egg production, and finally death. In poultry, the onset of mycobacterial infections is usually confined to the gastro-intestinal tract (**1,2**). Tuberculosis in commercial poultry is now rare due to the modern husbandry practices of integrated poultry farming but it still occurs sporadically in backyard poultry and game birds. This report describes an episode of unusual avian mycobacteriosis in commercial brown layers.

MATERIALS AND METHODS

Animals – Eight 46-week-old commercial brown layers out of a flock with a history of egg drop and increased mortality were submitted for diagnosis. The animals were humanly euthanized and immediately necropsied.

Histopathology - Samples of periocular tissues, eyes, heart, lungs, spleen, liver and intestine were fixed in 10% buffered formalin and routinely included for histopathology. 4-micron sections were stained with Haematoxylin and Eosin and with Ziehl-Neelsen (ZN). Additional samples of conjunctiva were store at -20°C until use.

PCR - DNA was extracted from frozen tissues using a DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The identification of the mycobacterial species was based on the amplification and sequence analysis of a hypervariable region of the 16S rRNA gene as previously described (**1,3,4**). A 5 µL sample of the PCR products was electrophoresed in 2% agarose (Promega, Milan, Italy) and stained with ethidium bromide (Euroclone, Milan, Italy). PCR products of the expected length (555 bp) were purified from agarose gel slices using a QIAquick PCR purification kit (Qiagen) and sequenced using an ABI Prism 310 genetic

analyser (Applied Biosystems, Foster City, California, USA). The sequence were submitted to the GenBank database (FJ639163). The percentage of similarity with reference sequences was evaluated by BLAST search in the NCBI website.

RESULTS

Animal –The birds were in poor body condition. Three layers showed monolateral, moderate to extremely severe enlargement and thickening of the soft periocular tissues with chemotic conjunctivitis and subconjunctival nodular lesions (Figure 1). Disseminated nodular lesions of varying dimensions were also observed in intestine, spleen, liver, lungs and bone marrow of all the 8 birds. with ovary and oviduct. Ovaries and oviducts were atresic in all the birds.

Histopathology - The sections of both periocular and visceral nodular lesions were characterized by numerous macrophages and multinucleated giant cells surrounded by variable numbers of lymphocytes and lesser numbers of plasmacells and viable heterophils. Larger granulomas were centred on necrotic debris. The sections of periocular tissues showed the granulomas were immediately beneath the conjunctiva which was diffusely hyperplastic and infiltrated by large numbers of lymphocytes and plasma cells. Conjunctival erosions were detected in association with larger granulomas which merged inwardly with massive necrosis. Mild uveitis was observed in the eye sections.

ZN stain revealed scarce numbers of Acid Fast Bacilli (AFB) within the visceral granulomas whereas AFB were extremely rare in the periocular lesions (Figure 2).

PCR and Sequencing – Molecular analysis revealed *Mycobacterium avium* as the causative agent with a percentage of similarity of 99%.

DISCUSSION

Mycobacterium avium infections are only occasionally reported in commercial poultry as they are usually characterized by chronic course and clinical signs appear in the second year of age. Mycobacteriosis in poultry has a oral-faecal route of transmission with lesions affecting mainly the gastrointestinal tract. are typically characterized by disseminated granulomas associated

with numerous AFB. Here we describe an outbreak of tuberculosis caused by *Mycobacterium avium* in 46-week old commercial layers with peculiar involvement of the orbital tissues and scarce AFB within the lesions. It was not possible to ascertain the source of infection and no follow-up data were available. It is possible to hypothesize that the precocious onset of the disease with exuberant paucibacillary granulomas was due to a hyperergic response as well-known in mammals (**5**). As mycobacteria are usually shed via faeces from intestinal lesions, the uncommon orbital involvement can be explained with superinfection via aerosolized bacteria in the environmental dust. Extreme paucibacillarity of the orbital lesions and the low numbers of birds with these signs seems to sustain a secondary involvement of the periocular tissues.

Figure 1

Figure 2

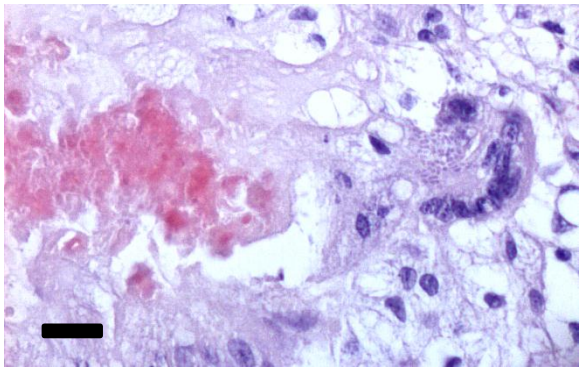


Figure 1: monolateral, extremely severe enlargement and thickening of the soft periocular tissues with chemotic conjunctivitis and subconjunctival nodular lesions in a layer.

Figure 2: ZN stain of a layer periocular lesion showing scarce numbers of Acid Fast Bacilli (AFB) within the ocular granulomas whereas (BAR: 50 µm).

References

1. Dumonceau, J. M., Fonteyne, P. A., Realini, L., van Gossum, A., van vVoren, J. P., Portaels F., 1995. Species-specific Mycobacterium genavense DNA in intestinal tissues of individuals not infected with Human Immunodeficiency Virus. J. Clin 33, 2514-2515.

2. Fulton R. M and C.O. Thoen. Other bacterial diseases. Tuberculosis. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa, USA. pp 836-844. 2003.

3. Hughes M. M. James G., Ball N., Scally M., Malik, R. Wigney D. I., Martin P., Chen S., Mitchell D., Love D. N., 2000. Identification by 16S rRNA gene analyses of a potential novel Mycobacterial species as an etiological agent of canine leproid granuloma syndrome. J. Clin. Microbiol. 38, 953-959.

4. Manarolla G., Liandris E., Pisoni G., Sassera D., Grilli G., Gallazzi D., Sironi G., Moroni P., Piccinini R., Rampin T., 2009. Avian mycobacteriosis in companion birds: 20-year survey. Vet. Microbiol., 133: 323-327.

5. Snyder P. W. Diseases of Immunity. In: Pathologic basis of veterinary disease, M. D. McGavin and J. F. Zachary, eds. Mosby Elsevier, St. Luis, Missouri, USA. pp 193-251. 2003.

6. Tell, L. A., Woods, L., Cromie, R. L., 2001. Mycobacteriosis in birds. Rev. Sci. Tech. Ser. Sci. Hum. (International Office of Epizootics). 20, 180-203.