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Immunohistochemical mapping of thymic microenvironment in sterlet (*Acipenser ruthenus*)

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Abstract: In this study, we describe the immunohistochemical characterisation of the thymus, the main lymphoid organ, in sturgeon. The wide range cytokeratin, vimentin, S-100 protein, LCA (CD45) and CD3 were selected as the immunohistochemical markers to map the thymus in juvenile sterlet (*Acipenser ruthenus*). The epithelial cells and Hassall's corpuscles were labelled with a wide range cytokeratin. The fibroblasts and connective tissue within the thin fibrous capsule on the thymic surface expressed vimentin positivity. The stromal reticular cells were S-100 protein positive. The Leukocyte Common Antigen LCA (CD45) was negative on the thymic lymphocytes. The CD3 was negative on the thymic lymphocytes with cross-reactivity on the non-targeted structures. In conclusion, the commercially available antibodies against the wide range cytokeratin, vimentin and S-100 protein can be used to differentiate components of the sturgeon thymus, while the LCA (CD45) and CD3 application failed. We suggest that further studies are needed to generate fish specific antibodies.

Keywords: CD3; Hassall's corpuscles; LCA (CD45); S-100 protein; vimentin; wide range cytokeratin

The thymus is considered as the primary lymphoid organ in sturgeons, *Acipenseridae* (Petrie-Hanson and Peterman 2005; Peterman and Petrie-Hanson 2006). The thymus is a paired lymphoepithelial organ situated on the upper inside edge of the operculum in close proximity to the branchial cavity and pharyngeal epithelium (Bowden et al. 2005; Salkova and Flajshans 2016). Thymi persist until the fish reach sexual maturity, later the thymus undergoes age-related atrophy. From an evolutionary perspective, the interaction of three embryonic germ layers (ectoderm, endoderm and mesoderm) takes place during the thymic development (Bowden et al. 2005). Histologically, the surface of the thymus is covered with a fine fibrous capsule. The thymus is lobulated and consists of an outer cortex and the inner medulla, with a diverse range of cell types within its microenvironment (Salkova and Flajshans 2016). The epithelial cells constitute a fine epithelial intra-thymic network (Mohammad et al. 2007; Gradil et al. 2014), while closely packed lymphocytes dominate the cortex. The medulla consists of sparsely organised lymphocytes, macrophages, reticular cells, fibroblasts and dispersedly distributed Hassall's corpuscles (Bowden et al. 2005; Petrie-Hanson and Peterman 2005; Peterman and Petrie-Hanson 2006; Salkova and Flajshans 2016).

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Currently, few studies have reported histological and ultrastructural examinations of the thymus in acipenserid fishes (Petrie-Hanson and Peterman 2005; Peterman and Petrie-Hanson 2006; Gradil et al. 2014; Salkova and Flajshans 2016). To the best of our knowledge, there is no literature concerning the immunohistochemical (IHC) mapping of the thymic microenvironment in sturgeons.

Immunohistochemistry is a method which detects expression of markers in tissues and organs by applying mono- or polyclonal antibodies against antigens (Ramos-Vara et al. 2008). Currently, Mohammad et al. (2007) have studied the application of IHC on the thymus of Australian lungfish, *Neoceratodus forsteri*. They described the immunohistochemical examination of the cytoskeletal proteins (cytokeratins, desmin, vimentin, actin and tubulin) in the thymic epithelial cells and found that the positivity of each antibody differed depending on the location within the thymus (capsule, cortex, medulla). The study helped to define seven categories of thymic epithelial cells in lungfish.

In the present study, a wide spectrum of markers was examined in order to characterise the specific cell types located within the thymus of sterlet (*Acipenser ruthenus*). This immunohistochemical study followed the study of Salkova and Flajshans (2016), describing a histological investigation of the thymus and Hassall's corpuscle in sturgeon. Moreover, this study examined application of commercially available antibodies in ichthyological practice. We applied antibodies against markers representing the main components of the thymic structure: a wide range cytokeratin to map the epithelial components, vimentin to highlight the mesenchymal components, the S-100 protein to map the neuroectodermal constituents, and the LCA (CD45) and CD3 to map the thymic lymphoid elements.

MATERIAL AND METHODS

Fish

A six-month-old functionally diploid (2n) specimen of sterlet (TL = 14 cm, BW = 28 g) was obtained from the hatchery of the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia (USB), Czech Republic for the histological and immunohistochemical examination. This study was conducted in accordance with Czech Law No. 246/1992 on animal welfare. The Institutional Animal Care and Use Committee (IACUC) of the University of South Bohemia, Faculty of Fisheries and Protection of Waters (USB FFPW) in Vodňany, supervised and approved the scientific protocol. The USB FFPW has approval from the Ministry of Agriculture of the Czech Republic for the handling and usage of experimental animals, Ref. No. 16OZ15759/2013-17214. Clove oil (30–40 mg/l) was used for the anaesthesia, while a double dose was used for the euthanasia.

Histological preparations

The whole specimen was fixed in 10% neutral-buffered formalin. After fixation, the sterlet was vertically cut. The tissues were embedded in paraffin, conventionally processed, and stained with haematoxylin and eosin (HE). The obtained slides were inspected under an Olympus BHS microscope, and a proper paraffin block containing the thymus was chosen for the IHC examination. The identification of Hassall's corpuscle in the thymus was based on the description of Salkova and Flajshans (2016). Tissue sections with a thickness of 4 µm were used for the IHC.

Studied antibodies

The wide range cytokeratin (clone KL1), S-100 protein, vimentin, LCA (CD45), CD3 were used to map the components of the thymic microenvironment. The antibodies used in this study and their characteristics are summarised in Table 1. All the antibodies have been routinely used in human medicine, of mammalian origin and specifically designed to target antigens located on human cells.

Immunohistochemical examination

The immunohistochemical examination followed specific protocols developed by investigators and antibody suppliers, so that the heat-induced antigen retrieval procedure (LCA, CD3), or direct antibody application with endogenous enzymatic blockage (cytokeratin, vimentin, S-100 protein) were used. Each antibody dilution was performed individu-

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Table 1. The characteristics of the selected antibodies applied to the thymus of the sterlet (*Acipenser ruthenus*)

Antibody	Supplier	Clone	Antigen source	Dilution	Isotype control
Wide range cytokeratin	Immunotech	KL1	polyclonal	1 : 100	IgG_1
Vimentin	Roche	V9	monoclonal mouse	RTU	IgG_{2a}/K
S-100	Roche	4C4.9	monoclonal mouse	RTU	IgG/K
Leukocytes common antigen LCA (CD45)	Dako	2B11 + PD7/26	monoclonal mouse	1 : 400	IgG_1/K
CD3	Dako	F7.2.38	monoclonal mouse	1 : 100	IgG_1/K

RTU = ready to use

ally and was adjusted in accordance with the supplier guidelines and laboratory expertise (Table 1). The application of the primary antibody was followed by the overnight incubation in a humidity chamber at 4 °C. A detection system was used to bind with the primary antibody. The antigen-antibody complex was visualised using chromogen 3-3' diaminobenzidine tetrahydrochloride (DAB). Therefore, a positive result created a brown end-product. Harris' haematoxylin was used to perform the nuclear counterstaining.

Human tissues, (including the tonsil and appendix) that are known to be reactive for the investigated marker, were used as positive controls. The internal tissue control was conducted while evaluating the immunohistochemical slides. The results of the immunostaining were concluded as either negative or positive. The staining pattern (nuclear, cytoplasmic and membrane) was described for each marker. The intensity of the positive reaction was evaluated in accordance with the three-degree scale: weak (+), moderate (++), and strong (+++) positivity. Any other reactions (atypical or cross-reaction) were documented. The slides were evaluated independently by two observers (ES, CS).

RESULTS

The histological examination revealed a lobulated thymus, covered by a fine fibrous capsule on the surface, and consisted of an outer cortex and inner medulla.

Wide range cytokeratin

Wide range cytokeratin labelled the epithelial components: a fine network of epithelial cells and Hassall's corpuscles revealed moderate membranous positivity.

A positive internal control was approved on the flat/cuboidal epithelium covering the branchial cavity, on the squamous epithelium in the oral cavity and on the stratified squamous epithelium of the skin (Figure 1A).

Vimentin

Vimentin labelled the fine fibrous capsule on the thymic surface with strong cytoplasmic intensity (Figure 1B). A positive internal control was observed on the basement membrane of the skin.

S-100 protein

The S-100 protein revealed a strong cytoplasmic positivity on the stromal reticular cells within the cortex and medulla (Figure 1C). A positive internal control was observed on the adjacent peripheral nerve and the peripheral ganglion, i.e., the nerve fibres revealed a strong cytoplasmic positivity, whereas the neurons, composing the peripheral ganglion, were negative.

A positive internal control was found on the afferent nerve fibres accompanying the taste buds in the oral cavity.

LCA (CD45)

The LCA (CD45) was negative on the targeted structures, i.e., the thymic lymphoid elements were negative. Simultaneously, negativity was noticed on the leukocytes located within the connective tissue adjacent to the skin and leukocytes within the gills.

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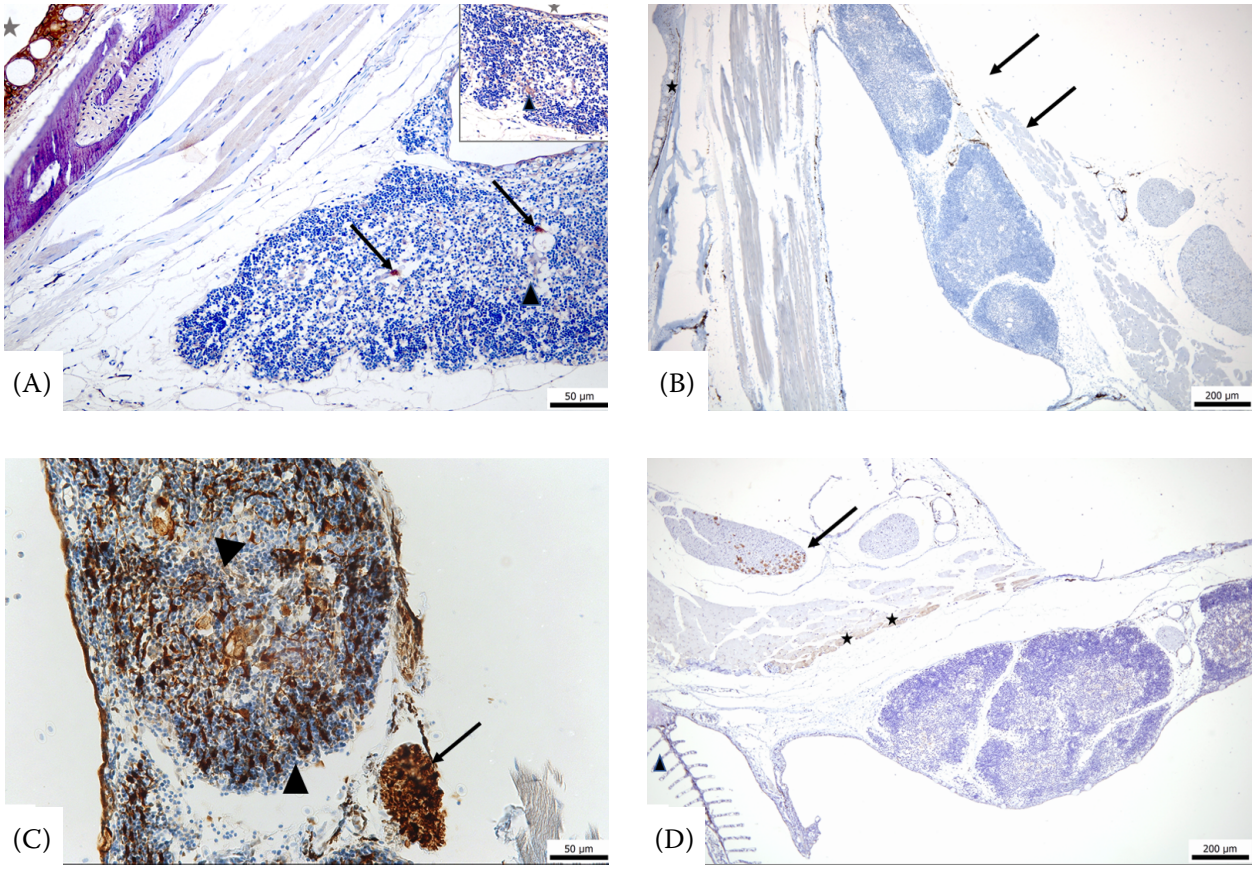

Figure 1. The immunohistochemical examination of the thymus in the sterlet *(Acipenser ruthenus*). (A) Wide range cytokeratin, clone KL 1 (magnification × 40; scale bar 50 µm). The fine network of epithelial cells revealed moderate membranous positivity (arrowhead); the fine network is presented as an inset in the figure in the upper right corner. Hassall's corpuscles reacted with strong positivity (arrows). A positive internal control was observed on the epidermis and on the flat/cuboidal epithelium covering the adjacent branchial cavity (star). (B) Vimentin (magnification × 20; scale bar 200 µm). The thin fibrous capsule on the thymic surface revealed strong cytoplasmic positivity (indicated by the arrows). A positive internal control was observed on thin basement membrane in the skin (star). (C) S-100 protein (magnification × 60; scale bar 50 µm). The stromal reticular cells within the cortex and medulla revealed strong cytoplasmic positivity (arrowheads). A positive internal control was presented on the adjacent peripheral nerve (positivity on nerve fibres) located in close proximity to the thymus (arrow). (D) CD3 (magnification × 20; scale bar 200 µm). The thymic lymphocytes were negative. A cross-reaction was noticed on the adjacent non-targeted structures: the stars pointed at the muscular fibres, the arrowhead at the supportive tissue in the gill lamellae, the arrow at the neurons within the peripheral ganglion

CD3

The CD3 was negative on the targeted thymic lymphocytes. The cross-reactivity with an altered staining pattern (cytoplasmic instead of membranous positivity) was revealed on the non-targeted tissues including the neurons in the peripheral ganglion, the striated muscle fibres and myocardium, the scattered developmental stages of granulocytes within the meningeal myeloid tissue (Figure 1D).

DISCUSSION

Cytokeratins are intermediate filament proteins that contribute to the cytoskeleton of epithelial cells. There are two large families of cytokeratins, acidic and basic cytokeratins, but all contain the same basic domain (Schaffeld et al. 2001). Cytokeratins are expressed in epithelial cells. According to the knowledge from human research, individual human cytokeratins are numbered 1 to 20. Anti-cytokeratin antibody applications in ich-

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thyology is well documented. Generally, commercially available antibodies originally produced for human medicine have been applied in fish tissues. Several studies are mapping the occurrence of cytokeratins in different types of epithelial cells under physiological conditions, i.e., Schmitz (1998) tested the polyclonal anti-keratin antibody in the notochord cells of shortnose sturgeon (*Acipenser brevirostrum*), West African lungfish (*Protopterus annectens*) and yellow perch (*Perca flavescens*). Garcia et al. (2005) performed a study to analyse the keratin occurrence in carp and goldfish (*Carassius auratus*) within different epithelial tissues (skin, fin tip, gill, liver) and compared the results with zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*). Ng et al. (2005) prepared a study concerning the formation of the digestive system in zebrafish using an anti-pan-cytokeratin antibody to highlight the intestinal epithelium morphogenesis. Bunton (1993) tested the anti-cytokeratin antibodies (cytokeratin AE, MAK-6 and CAM 5.2) in multiple fish tissues in striped bass (*Morone saxatilis*) and medaka (*Oryzias latipes*). Moreover, there are studies describing the anti-cytokeratin antibody application to perform a tumour identification and classification, i.e., Iaria et al. (2019) applied the anti-cytokeratin antibody in bream (*Abramis brama*) to confirm papilloma. Yasumoto et al. (2015) performed an immunohistochemical study in common carp (*Cyprinus carpio*) using a spectrum of antibodies including anti-pan-cytokeratin to perform the diagnosis of undifferentiated gonadal carcinoma. Only a small number of studies described the application of fish-specific antibodies designed and produced by the scientist themselves [Pan et al. (2000) in stomachless fishes]. The application of fish-specific antibodies is thought to avoid the negativity/non-reactivity or cross-reaction, respectively (Ramos-Vara et al. 2008).

In this study, we have shown that the fine network of thymic epithelial cell and juvenile and premature Hassall's corpuscles were cytokeratin positive. Our finding was, thus, in agreement with the immunohistochemical studies performed on lungfish thymi (Mohammad et al. 2007) and human thymi (Raica et al. 2006).

Vimentin is an intermediate-sized filament and helps to constitute the main part of the cellular cytoskeleton (Herrmann et al. 1996). Among mammals, birds, reptiles, amphibians and teleost fishes, vimentin shares an extensive amino acid sequence homology (Schaffeld et al. 2001). Moreover, despite the highly conserved primary structure, the expression pattern of vimentin in fish is different than in mammals (Herrmann et al. 1996). Reportedly, the environmental temperature is an important principle for the fine primary protein structure setting (Herrmann et al. 1996; Schaffeld et al. 2001). Vimentin reacts with cytoplasmic positivity and is reported to be expressed in most cells of mesenchymal origin, including fibroblasts, endothelial cells, melanocytes, smooth muscle cells and is regarded to be a sensitive and specific marker for mesenchymal derivation and differentiation (Schaffeld et al. 2001; Iaria et al. 2019). Vimentin reacts with cytoplasmic positivity. Iaria et al. (2019) applied an anti-vimentin antibody to identify tumours in fish: vimentin positivity obtained in sand steenbras helped to perform the diagnosis of a dermal fibroma. In contrast, vimentin negativity contributed to conclude the diagnosis of an undifferentiated carcinoma in carp (Yasumoto et al. 2015). In this study, the thin fibrous capsule on the thymic surface reacted positively, just as the other structures of mesenchymal origin (basement membrane of the skin).

The S-100 proteins play an important role in cell physiology involving various cellular activities such as signal transduction, cell differentiation, gene transcription, calcium homeostasis and cell cycle progression (Fonseca et al. 2011). The S-100 protein family is comprised of 20 members (primarily described in humans), they are highly similar in structure, but exhibit distinctive patterns of cell and tissue distribution (Fonseca et al. 2011). The S-100 protein results in cytoplasmic positivity and is reportedly expressed in cells of neuroectodermal origin (Sandulescu et al. 2011; Iaria et al. 2019), as well as on the antigen-presenting cells, such as stromal reticular cells within the thymus (Raica et al. 2006). Application of the antibody against the S-100 protein contributed to the diagnosis of melanoma in an *Xiphophorus* hybrid and schwannoma in a crucian carp (*Carassius carassius*) (Iaria et al. 2019). In our study, the thymic stromal reticular cells revealed strong cytoplasmic S-100 protein positivity. However, Raica et al. (2006) described S-100 protein positivity in human Hassall's corpuscles, while, in our study, no positivity was expressed in Hassall's corpuscles. The different antibody clone application, heat-induced

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epitope retrieval, incubation times or different developmental stages in Hassall's corpuscles might explain the conflicting results (Ramos-Vara et al. 2008). Moreover, no background cross-reactivity was observed in the abovementioned markers in the sturgeon tissue.

The leukocyte common antigen (LCA, CD45), a transmembrane protein tyrosine phosphatase, is expressed in haematopoietic cells. LCA plays an important role in the maturation and differentiation of B- and T-lymphocytes, T-cells without LCA cannot respond to the antigen. In mammals, LCA labels the thymic lymphocytes (Kurtin and Pinkus 1985). LCA can be expressed in different isoforms depending on the cell type, the state of activation and differentiation (Diaz del Pozo et al. 2000). The CD45 gene structure in fugu (*Takifugu rubripes*) seems to be similar to the human CD45 gene, i.e., similar exon/intron organisation. Some differences were noticed in the 5' end structure in the fugu, however, the CD45 gene in the fugu is smaller than the human gene due to the smaller size of the introns in the fugu (Diaz del Pozo et al. 2000). Holmes (2005) pointed out that the polymorphic variants result in a high number of variations in the coding region, which was evident in mammals and hagfish. When performing the LCA examination, a positive result is considered as membranous positivity.

CD3 represents a marker specific for T-lymphocytes, and in mammals, reacts positively with the early developmental stages of the thymocytes (Mason et al. 1989). CD3 is an essential component of the T-cell receptor (TCR) complex antigen. TCR is expressed as a multisubunit membrane complex and CD3 components are responsible for coupling the antigen ligation events with the intracellular signalling pathway (Alabyev et al. 2000). Most of the commercially available antibodies target the CD3 component. The CD3 component was analysed and sequenced in sterlet (Alabyev et al. 2000), in rainbow trout (Boardman et al. 2012) and Japanese pufferfish (*Takifugu rubripes*) (Araki et al. 2005). Some studies have suggested particular sequence conservation among fish species and some homology with mammalian CD3 (Boardman et al. 2012). Still, a high transcript heterogeneity is demonstrated in the sterlet CD3 (Alabyev et al. 2000). When performing the IHC in the CD3, the CD3 results in membranous positivity. In our study, no reactivity of the examined and targeted lymphoid markers (LCA, CD3) was detected. Additionally, a cross-reaction with different staining pattern (cytoplasmic instead of membranous positivity) was present while examining the CD3. The LCA and CD3, thus, proved ineffective at staining the cells of lymphoid origin in sterlet. The structural polymorphism and heterogeneity may represent the main reasons for the unsuccessful IHC examination of the lymphoid markers (LCA and CD3) in the sterlet. Consequently, and naturally, further research is required to design sturgeon specific LCA and CD3 antibodies (Ramos-Vara et al. 2008).

The immunohistochemical examination was performed using monoclonal antibodies (vimentin, S-100 protein, LCA, CD3) and a polyclonal antibody (cytokeratin). The applied antibodies were commercially available and originally designed for human medicine. Three of the five tested antibodies (cytokeratin, vimentin, S-100 protein) brought conclusive results with the expected staining pattern (references), and no cross-reactivity was detected in the background. Positive results were obtained regardless of the origin of the monoclonal or polyclonal antibody. Therefore, these markers (cytokeratin, vimentin, S-100 protein) can be recommended for application on sturgeon.

Negative results, or non-reactivity respectively, were obtained when examining the LCA. While testing the CD3, the obtained results were negative with a cross-reactivity at the adjacent tissues. The applied antibodies were monoclonal and of mouse origin. As it was as expected, the monoclonal antibodies bound to a single epitope on an antigen, and, thus, provided high specificity (Ramos-Vara et al. 2008). The high specificity of the monoclonal antibodies and its mammalian origin might explain why negative results (or non-reactivity) were obtained when testing the lymphoid markers (LCA and CD3). Consequently, these findings support the proposal of Ramos-Vara et al. (2008) for researchers to invent and produce fish specific antibodies.

This immunohistochemical study on the thymic microenvironment in sterlet supports the previous histological findings concerning the cellular components in the thymus (Gradil et al. 2014; Salkova and Flajshans 2016). To our knowledge, this is the first study to demonstrate the application of human antibodies on a sturgeon's thymus: most of the tested antibodies (cytokeratin, vimentin, S-100 protein) were suitable for usage in stur-

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geons. The non-reactivity observed in the lymphoid markers LCA and CD3 support the importance of fish specific antibody generation (Ramos-Vara et al. 2008).

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Conflict of interest

The authors declare no conflict of interest.

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