THESES OF DOCTORAL (PhD) DISSERTATION

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Investigation of neoplastic diseases in captive-bred Salmonids

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1. Background of the Research and Aims

Background of the research

Rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) is one of the most commonly bred fish species in Europe, which plays an important role in the gastronomy. The aquaculture will provide part of the human consumption in the future. The rainbow trout (Oncorhynchus mykiss, Walbaum 1792) is an anadromous fish species which is cultured in substantial quantities across Europe and whose consumption has been increasing in our diet each year. Healthy broodstocks are valuable assets to fish farms, since they ensure the continuity of production. Fish only reach maturity by the age of two to three years. A single broodfish may produce tens of thousands of eggs during its lifetime, thus providing spawn, both for the market as well as for the broodstock itself. The death of such a broodfish means a considerable financial loss for the farm, as the cost of cultivation, feeding, and possible treatment over the nearly 3-year-long period until the specimen reaches maturity is wasted. The role of the rainbow trout in the aquaculture is unquestionable. This is one of the reason, why this species and its health management is so important. The brood stock has a significant value on the farms, therefore the prevention of illnesses is crucial. The neoplasms are new risk for the broodstock near the viruses, bacteria, fungal and parasitic infections. In this aspect, death by tumours in the broodstock is a serious problem. The investigation of commonly-occurring trout tumours is a pioneering research area. The causes of the neoplasms are unknown. The fish tumours, their metastasis and the affected organs can give a new aspect of fish oncology. The investigation of the malformations can give more information on the veterinary and human oncology fields. The rainbow trout as model animal could have an important role in the future. So far this is the first deep research on rainbow trout neoplasms and their effects in Hungary.

The scope of the research is the salmonid fish, especially the tumors of the rainbow trout and diagnostic imaging, histopathological and immunohistochemical investigations, virology and electron microscopic examinations.

Aims of the research

- The aim of the study was the veterinary investigation the rainbow trout broodstock in the Hungarian aquaculture.
- During my research I used histology and immunohistochemistry methods to identify and differentiate the tumours.
- Our plan was to identify the viral origin of the neoplastic malformations.
- We tied to develop an optimal diagnostic imaging technique, which can help the breeders and the aquatic veterinarians to identify the tumours in the early stage.

2. Materials and Methods

Field Diagnostic and Preparation

An entire broodstock of approximately 800 rainbow trout and the younger fish (1-1,5 years) was examined externally by the authors on a Hungarian trout-breeding farm during the breeding season. According to the owner of the farm, some of the fish began to show clinical signs in the days leading up to the examination, such as lying separated at the bottom of the pool and a noticeable loss of appetite. In the following days, the fish began to swim on their sides, later stretching out in a state of agony. Physical examination was performed under anesthesia. The rainbow trouts were anesthetized with MS-222 with a concentration of 100 mg/l. During the physical examination of the fish, the condition of the gills was examined, and the body cavity was palpated. Portable ultrasound (Mindray M9Vet, C5-1s convex ultrasound transducer, 1.4-5.1 MHz bandwidth) was used to confirm the primary diagnosis. Where an abdominal mass was found, MS-222 was used at 250 mg/l to euthanize the animals and the entire body of the fish was fixed in 10% neutral buffered formalin. Fixative was injected into the body cavity too. During our study 40 tons of 1-15 years old fish were examined.

CT-MRI imaging

The CT imaging was performed by a Siemens Somatom Definition AS+ scanner (Siemens, Erlangen, Germany) using the following settings: tube voltage – 120 kV, exposure – 200 mAs, focal spots - 1.2, collimation - 128*0.6 mm, spiral data collection mode with pitch 0.6. Overlapping slices were reconstructed with an iterative convolution kernel (I49s) in 200 mm field of view (FOV) and 0.6 mm slice thickness by Syngo CT VA48A software (Siemens, Erlangen, Germany).

The MRI examination was carried out using a Siemens Biograph mMR scanner. T2 weighted images were reconstructed in the transversal plane. The acquisition settings of the turbo spin echo sequence were repetition time (TR) 8000 ms, echo time (TE) 100 ms, slice thickness 3 mm, spacing between slices 3.6 mm, averages 3, matrix 256x256, flip an-gle 160°, field of view 250 mm.

The scans were archived in DICOM (Digital Imaging and Communications in Medi-cine) file format. The 3D Slicer freeware software was used for visualization and image evaluation.

Dissection

After removing the abdominal wall with a single cut, the pericardial and abdominal cavities were able to be examined intact and in one piece. During the course of the research, only the macroscopic tumorous alterations and unaffected macroscopical tissue were sampled. Affected tissues (gastrointestinal system, liver, and gills) were removed and placed into a 10% buffered formaldehyde fixative. Pictures were made by Nikon D7000 about the macroscopic malformations.

Histopathology

Histological samples were kept in fixative at room temperature for 24 hours, and then processed with an automated tissue sample preparation system. From the paraffin-embedded tissue blocks, 3-4 μm-thick slices were prepared and stained with hematoxylin-eosin. The prepared slides were examined with a Nikon Optishot-2 light microscope.

Immunohistochemistry

For immunohistochemistry, soft tissue samples were fixed in 8% neutral-buffered formalin for 24 hours at room temperature, dehydrated in a series of ethanol and xylene, and embedded in paraffin. The 3-4μm-thick sections were routinely stained with hematoxylin and eosin (HE). Slides for the immunohistochemical reaction were deparaffinised in xylene and graded ethanol. After antigen retrieval (Target Retrieval Solution, DAKO, Glostrup, Denmark), at pH 6, in a microwave oven for 30 min, the deparaffinised sections were treated with primary antibodies against cytokeratin AE1-AE3 or pancytokeratin (diluted 1:100, mouse monoclonal, DAKO), E-cadherin (diluted 1:100, mouse monoclonal, DAKO), claudin-5 (diluted 1:100, mouse monoclonal, DAKO), vimentin (diluted 1:200, mouse monoclonal, DAKO), α-SMA (Smooth-muscle actin) (diluted 1:1200, mouse monoclonal, Sigma), S-100 protein (diluted 1:50, rabbit monoclonal, DAKO) and c-Kit or CD117 (diluted 1:100, rabbit polyclonal, DAKO) at room temperature for 60 min. Immunohistochemical staining was performed using the streptavidin-peroxidase procedure. The antigen-bound primary antibody was detected using a standard avidin-biotin immunoperoxidase complex (LSAB2 Kit, DAKO). The chromogen substrate was diaminobenzidine (DAB), and Mayer's haemalaun was used for counter-staining. For negative control, the slides were stained with the omission of primary antibody. The following external positive controls were implemented: tumour

cells from the simple infiltrating carcinoma of the canine mammary gland for pancytokeratin, intact epidermal layers of canine skin for E-cadherin, canine cutaneous haemangioma for claudin-5, canine vaginal fibroma for vimentin, canine vaginal leiomyoma for α-SMA, and canine peripheral nerve sheath tumor for S-100 protein. The slides were scanned with Panoramic MIDI II (3DHistech); photographs were made by CaseViewer (3DHistech).

Viral Metagenomics, Next-generation Sequencing and Bioinformatic Analyses

Three trout samples, an individual tissue from intestinal carcinoma (sample 17) and two pooled specimens (samples 18 and 19) which contained 3-3 intestinal carcinoma tissue samples were subjected to viral metagenomic analysis using random (RT-)PCR amplification of viral-particle protected nucleic acids as previously described. Briefly, phosphate-buffered saline-diluted (PBS) tissue homogenates were filtered through a 0.45-mm filter (Millipore) and then treated with a mixture of DNases (Turbo DNase from Ambion, Baseline-ZERO from Epicentre, and Benzonase from Novagen) and RNase (Fermentas) to digest unprotected nucleic acids. Nucleic acids were then extracted (RNA extraction without DNAase step) using the QIAamp spin-column technique (Qiagen, Hilden, Germany) and subjected to sequence independent random PCR/RT-PCR amplification. Viral cDNA library was constructed by ScriptSeqTM v2 RNA-Seq Library Preparation Kit (Epicentre) and PCR/RT-PCR amplicons sequenced using the MiSeq Illumina platform according to the manufacturer's instruction. Paired-end reads of 250 bp generated by MiSeq are debarcoded using Illumina vendor software. Using an in-house analysis pipeline running on a 36-nodes Linux cluster human and bacterial reads were subtracted by mapping to human reference genome hg38 and bacterial nucleotide sequences from nt using Bowtie 2. Adaptor and primer sequences were trimmed using the default parameters of VecScreen. The cleaned reads were then de-novo assembled using Ensemble Assembler. The assembled contigs greater than 100-bp, along with singlets, were aligned to an in-house viral proteome and nucleotide database using GenBank BLASTx and BLASTn using E-value cut-off of 10-10. The significant hits to virus were then aligned to an in-house non-virus-non-redundant universal proteome database using DIAMOND. A web-based graphical user interface was used to show the viral matches, along with taxonomy information and processing meta-information..

Electron-Microscopic Examination

Preparation for Immersion

During the examinations masses were found in fish. Samples from the animals were fixed in a mixture of 0.2% glutaraldehyde (stored in eppendorf) and 4% buffered formaldehyde (stored screw top vial). The usual size of the samples were 1—5 mm^3. Mixed immersion solution was used within 24h. After the labeling of the tubes the samples were placed into the fridge.

Samples were collected from each tumor (intestines, gills, liver) approximately 1—5 mm^3, from the tumor margin and peripheral live (non-necrotic) whole tumor tissue area of the mass. Multiple small sections were excised from each area to allow selection in later processing. The excised mass sections were placed in the vials. The vials were gently shaked to confirm samples are not stucked together. Samples were collected also from the healthy tissues.

Following Dissection

The glutaraldehyde and formaldehyde immersion solution was changed to formaldehyde (stored in screw top vial) after 3 days. Larger excised chunks stayed for a maximum of 5 days (after 5d the glutaraldehyde makes a hard cortical layer on samples). Plastic pipette was used to remove the glutaraldehyde solution from each vial. We prepared the 50 nm thick slides with ultramicrotome (Leica Microsystems, Reichert, model: Reichert Ultracut S) and was contrasted with 3% lead citrate solution. The slides were examined with transmission electron microscope, operating at 80 kV (e.g., JEOL, model: JEM-1011). The pictures were taken by digital camera incorporated with TEM (e.g., Mega-View-III digital camera and a Soft Imaging System (SIS, Münster, Germany).

Electron Microscope Image Acquisition

Image acquisition was done in pseudo-random manner for qualitative analysis.

3. Results

Aquatic veterinarian surveillance is a regular routine in trout farms. The veterinarian tests the water parameters, performs health checks on the animals and executes the post mortem examinations. During physical examination of the trout, clinical sigs as anorexia and abdominal distension became visible on the affected animals. We found 51 animals with neoplastic malformations during the investigation (2015–2022) in the rainbow trout broodstock.

External examination revealed marked signs of anorexia in some cases, and a loss of musculature was observed along the spine from the head to the tail. No signs of external trauma or parasites could be observed on the skin of the trout. In several cases, minor damage was detected on the upper part of the caudal fins, which was most-probably caused by biting during instances of rivalry. Every year, 100–150 kg commercial size fish are processing. We could not find any macroscopically visible neoplastic malformations in those animals.

Results of Diagnostic Imaging

The ultrasound image described a lobulated, heterogeneous globular mass in the first segment of the mid-intestine of the anorexic animal. The diameters of the growth were 7.69 cm and 7.4 cm (Figure 1a-b.). Normal images were observable about the internal organs near the abnormal malformation in the gastrointestinal system.

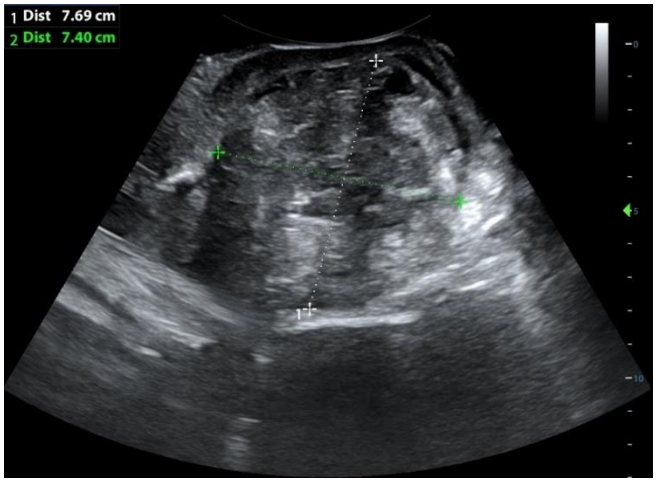


Figure 1.: Ultrasound image of the rainbow trout. The transverse ventral pictures of the mid-intestine shows the cross section of a large demarcated, heterogeneous, lobulated roundish mass in the gastrointestinal tract.

Further ultrasound examination showed tumourous malformation in the stomach, in the gastrointestinal tract and in the liver in other fish.

The reconstructed computed tomography and magnetic resonance imaging pictures confirmed the soft tissue malformation in the lower - mid part of the body cavity after the liver and in front of the spleen. Sagittal and transversal plane images and three-dimensional computed tomography pictures show the tumour-like soft tissue mass in the first segment of the mid-intestine (**Figure 2.**).

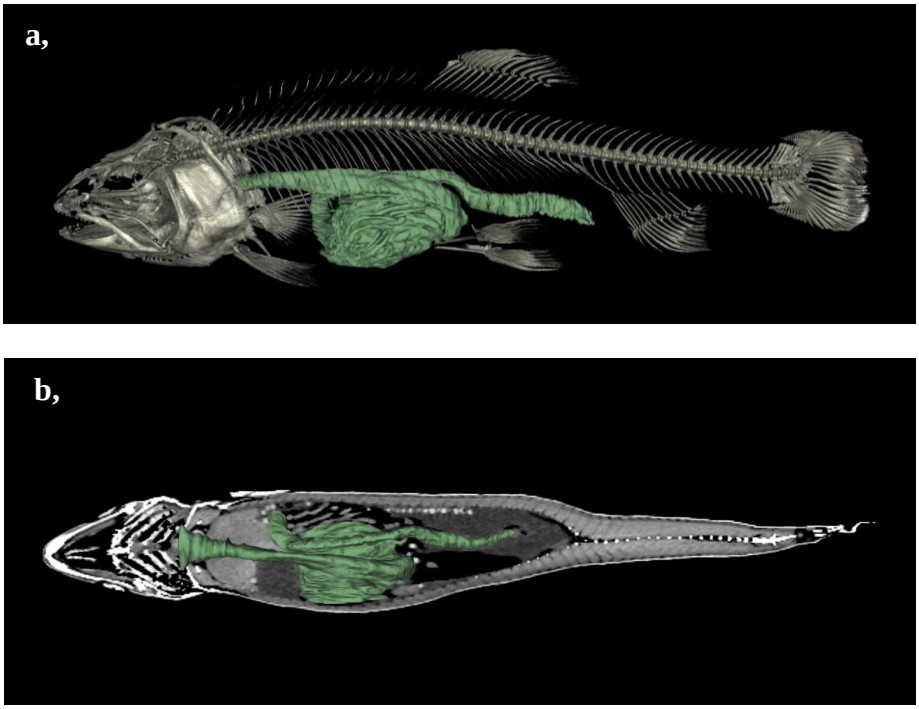


Figure 2. a-b.: Sagittal and transversal CT images about the malformation. Reconstructed 3D pictures shows the enlarged pyloric caeca area

Results of Necropsy

We only found tumours in the broodstock. Neoplasia was not observed in the younger population.

During necropsy, intussusceptions in the proximal part of the intestines became visible, where the proximal segment of the mid-intestine (*intussusceptum*) was found to be folded into the distal portion (*intussuscipiens*). At the site of the invagination, the veins became contracted and a congestion formed, which consequently led to the swelling of the intestines, and the formation of fibrinous exudate, anchoring the two segments together. The mid-intestine became distorted as the intestinal lumen narrowed, becoming

denser to the touch. Upon opening the intestinal lumens, tumour-like growths at the pylorus, from 1 cm^3 or bigger at the site of the intussusception, and lumps from 2 to 46 mm in diameter were found in the intestine (**Fig. 3**). All fish having invaginations had tumours at least in their intestinal tracts. The intestinal lumen of the fish was filled with mucus, and whitish, filamentary pseudofaeces could be observed at the anal region.

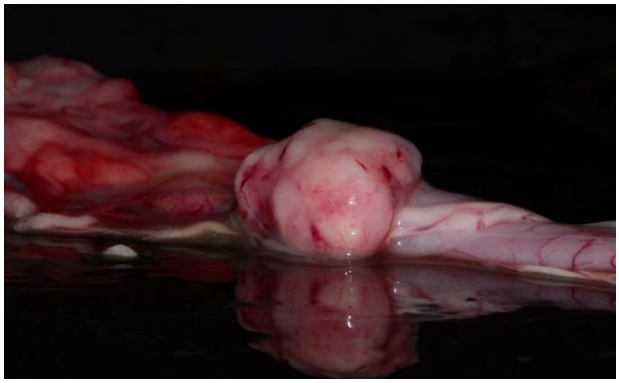


Figure 3.: Tumour inside the invagination

Following incision, macroscopic examination of the cross-section of the lumps revealed a whitish and dry surface, having a shine resembling that of lard, with a homogeneous structure. Within the bigger masses, red-coloured, irregular nodules, segmented by whitish trabeculae of connective tissue were found, bleeding areas were observed in the tissue of the lumps, and metastases were found in the livers. After elevating the healthy operculum, tumour-like, distinct, whitish nodules became apparent between the gill arches and the gill filaments. The data of the examined fish are shown in the following table **(Table 1.)**.

1. Table: Data of examined trouts with tumours

No.	Species	Gender	Weight (kg)	Date	Tumour location
1.	Rainbow trout	female	0,67	2015.08.23	intestine
2.	Golden trout	female	1,57	2015.08.04	intestine, gill
3.	Golden trout	female	1,2	2015.09.06	intestine
4.	Golden trout	female	1,2	2015.09.06	intestine
5.	Golden trout	female	1,1	2015.09.06	intestine
6.	Rainbow trout	female	1	2017.11.23	stomach
7.	Rainbow trout	female	2	2017.12.19	intestine
8.	Rainbow trout	female	1,9	2017.12.22	intestine
9.	Golden trout	female	1,5	2017.12.23	intestine, pylorus caeca, liver
10.	Golden trout	female	1,5	2017.12.23	intestine, pylorus, liver
11.	Rainbow trout	male	1,5	2017.12.25	intestine, invagination

12.	Rainbow trout	female	2,5	2017.12.25	intestine, kidney
13.	Golden trout	female	3,1	2018.01.07	stomach
14.	Rainbow trout	female	4,5	2018.02.10	intestine
15.	Golden trout	female	2	2018.02.11	intestine, liver
16.	Golden trout	female	2,5	2018.02.11	intestine, gill
17.	Golden trout	male	1,5	2018.03.14	intestine
18.	Rainbow trout	female	1,7	2018.03.19	intestine
19.	Golden trout	female	2	2018.04.13	intestine
20.	Rainbow trout	male	2,1	2018.04.19	intestine
21.	Golden trout	female	1,16	2018.04.21	intestine
22.	Golden trout	female	0,85	2018.04.21	intestine
23.	Palomino trout	female	0,8	2018.04.21	intestine, pyloric caeca
24.	Palomino trout	male	1,2	2018.05.02	intestine
25.	Palomino trout	female	1,3	2018.05.22	intestine, liver
26.	Palomino trout	male	1	2018.05.29	intestine
27.	Rainbow trout	female	1,7	2018.06.01	intestine, pylorus,
28.	Golden trout	female	3,1	2018.06.01	intestine, liver
29.	Rainbow trout	male	1,5	2018.06.03	intestine, liver
30.	Rainbow trout	female	1,3	2018.06.29	intestine, gill
31.	Palomino trout	male	1,2	2018.07.12	intestine
32.	Palomino trout	male	0,8	2018.07.23	intestine
33.	Rainbow trout	male	1,6	2018.08.19	pylorus, intestine
34.	Rainbow trout	female	2	2018.08.20	intestine
35.	Rainbow trout	female	3,1	2018.09.16	pylorus, kidney
36.	Rainbow trout	male	2,8	2018.09.24	intestine
37.	Rainbow trout	female	3,2	2018.10.08	stomach
38.	Golden trout	female	1,09	2019.08.20	intestine, stomach, liver
39.	Golden trout	female	0,68	2019.08.31	intestine
40.	Rainbow trout	male	1,5	2019.09.20	stomach, gill
41.	Golden trout	female	0,7	2019.10.06	intestine, kidney, pylorus, stomach
42.	Golden trout	female	2,25	2020.12.12	stomach. liver, kidney
43.	Golden trout	female	2,18	2020.12.12	intestine, kidney
44.	Golden trout	male	0,5	2020.12.12	intestine
45.	Rainbow trout	female	3	2021.01.12	intestine, pylorus, liver, gill
46.	Rainbow trout	female	1,9	2021.01.24	intestine
47.	Golden trout	female	3,5	2021.01.23	intestine
48.	Rainbow trout	male	0,99	2021.05.05	stomach
49.	Rainbow trout	male	1,9	2021.05.12	intestine
50.	Rainbow trout	female	3,06	2021.07.11	skin, intestine
51.	Golden trout	female	2,2	2022.05.08	intestine, liver

The prevalence of macroscopic tumours reached 6% in the rainbow trout broodstock.

Results of Histopathology and Immunohistochemistry

The histopathological examination revealed adenocarcinomas at different parts of the midgut, originating from the intestinal epithelium which broke through the basal lamina in multiple places **(Fig. 5)**. The tumour cells showed heterogeneous, glandular morphology which differed significantly from the healthy acinar structure of the intestinal epithelium with the cells arranged in nests and only a few segments enclosed with the basal lamina. The nuclei of the adenocarcinoma cells were heteromorph and euchromatic with marked hyperchromatosis around the nuclear membrane and prominent nucleoli. Cell division was frequently observed implying a high-level of malignity, and the number of goblet cells was also elevated.

Over the course of the examinations, distant metastases were found in the gills and the livers of the fish. A cellular structure identical to that of the mid-intestine tumours led us to conclude that the metastases had formed rapidly, allowing us to identify the neoplasm as Grade III adenocarcinoma **(Fig. 5)**.

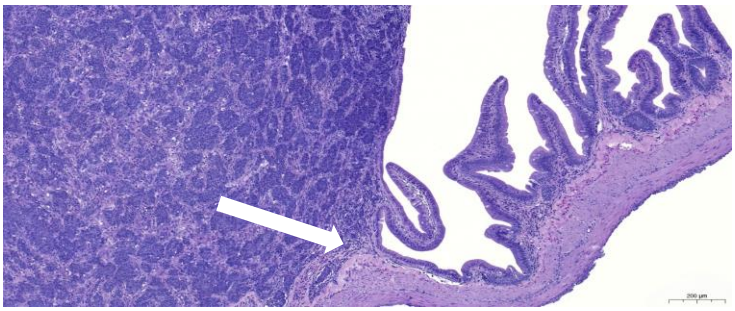


Figure 5.: Adenocarcinoma in the mid-intestine (arrow: border of normal and neoplastic tissue) Haematoxylin and eosin (61X) Bar=200 μm

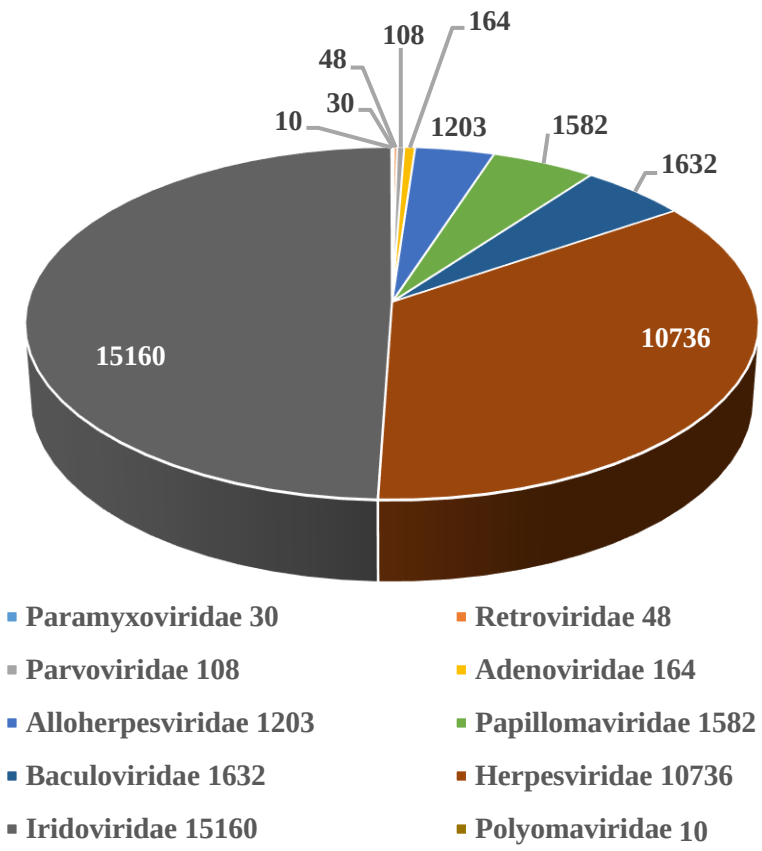
The immunohistochemical examination of the intestinal carcinoma revealed E-cadherin and pancytokeratin positivity (Fig. 8a-b). Additionally, the endothelial cells of the peritumoural vessels showed claudin-5 positivity. The following antibody tests produced negative results during the investigation: anti-vimentin, anti- α-SMA, c-Kit and the anti-S-100 protein.

Metastases were found in the gill, in the liver and as a new finding in the kidney.

Results of Virological Investigations

The result of the next-generation sequencing showed several virus families with oncogenic properties. The sequences of *Iridoviridae* (22,5%) and *Herpesviridae* (15,93%) were in the highest amount in the samples. A significant number of sequences *containing Baculoviridae* (2,24%), *Papillomaviridae* (2,34%), *Alloherpesviridae* (1,78%), *Adenoviridae* (0,24%), *Parvoviridae* (0,16%), *Retroviridae* (0,07%) and *Paramyxoviridae* (0,04%) families were also present correlatedto the total copy number **(Diagram 1.)**.

Diagram 1. Potential oncogenic virusis and coppy number



Results of the Electron-Microscopic Examination

During our research we did not find any viral forms or viral particles. During the qualitative analysis we investigated the primer tumour cells, the kidney metastasis cells and the healthy intestinal cells also. The epitheloid cells from the intestinal neoplasm showed abnormal cytoskeletal elements, high percentage of euchromatin (showing a higher degree of activity), thickened cell membrane, and an assortment of vacuoles of light (electrolucent) and dark (electrodense) densities indicating a high degree of activity and abnormal appearance. Tumourous cells showed an array of irregular cytoskeletal elements, different vacuole structures and an abundance of free ribosomes in the cytosol.

Control intestinal sample (from control fish) showing normal superficial luminal brushborder on left with prominent electrodense cellar junctions on apical intestinal epithelial cells. Goblet cells may also be seen interspersed between the superficial epithelial cells with prominent electrolucent vacuoles.

Comparison between intestinal tumor cell and surrounding tissue with control intestinal sample:

- Same magnification, approximately same superficial/luminal layer in frame.
- Tumor cell does not show differentiation in superficial mucosal and submucosal layers as well as complete loss of brushborder/microvilli.
- In tumor sample, the cells are irregular and loosely connected to each other in stroma.
- In tumor samples some cells are packed with ribosomes, others with vacuoles filled with electrolucent material and many cells have irregular (wavy and indistinct) cytoskeletal elements.

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4. Conclusions

We investigated gastrointestinal tumours and their metastasis in the rainbow trout broodstock with diagnostic imaging techniques, histological, immunohistochemistrical and virological methods. More and more information and research are available about fish tumours. Our findings, the gastrointestinal neoplasms in rainbow trout is not unique, Gombač et al (2021) reported the same malformations. Like our team, they also have found metastasis in the liver, they published metastasis in the heart for the first time.

The tumours are formed only in the 3-4 years old animals. One viable explanation could be, that, even in a species having a relatively short lifespan such as trout, the development of tumours requires time. The prevalence of tumours was 6% in the investigated broodstock. We used diagnostic imaging techniques on the fish which showed clinical symptoms. The portable and less expansive ultrasound examinations are promising, their use in the everyday procedures can be useful.

The MRI and CT investigation provided better image quality, perspicuity and processing. However the costs are higher, the length of the examinations and the immovability of the machines makes this method not usable on the field. MRI investigation provide good image quality about the soft tissues, but the length of the examination could be more than 20 minutes. Trouts need oxygen rich and crystal clear water, which could not be provided near the machines. For this method the anaesthesia is necessary. The CT provides quick examination time, which could be appropriate in case of fish. Iodine contrast material could provide better imaging, but the CT machines are not so easily portable, and the costs are almost the same as in the case of MRI. This technique is useful during post mortem investigations and for research.

The number of goblet cells was found to be elevated on the histological slides which were prepared from the tumours. This larger number of cells precipitates increased mucus secretion. This elevated secretion may also thereafter reduce the rate of absorption from the intestines, leading to a worsening of the fishes' overall condition. Tumours protruding to the intestinal lumen due to the peristalsis, initially cause partial, then complete, obstruction or intussusception.

Ulcers may form, which break the continuity of the epithelium. The damaged intestinal epithelium's capacity to function as a protective barrier against infections then decreases. At the site of these ulcers, bacteria is able to invade the deeper tissue layers, potentially leading to septicaemia. Feed material and foreign bodies may become stuck in the ulcers as well as in the tumours themselves.

Bacterial infection could cause invagination in Nile tilapia and in catfish, however tumour induced intussusception was not published previously.

Using immunohistochemical tests, we proved that the tumors found in the trout were adenocarcinomas. Tests with mouse and rabbit antibodies (pancytokeratin and E-cadherin) give positive results and also prove that neoplastic diseases in fish differ in almost nothing from the same examinations with these antibodies in mammals, such as dogs and cats. The conclusion can be drawn from this that adenocarcinoma could have been present already in the early stages of evolution. The degree of malignancy of the tumors were grade III in all cases. This means that tumor cells are completely different from normal, and their growth and spread are faster than those of tumors with a lower degree of differentiation (grade I-II) in fish. If the tumours were only grade I or II, the growth of the neoplasms would have been slower, they might not have formed metastases, and they might have caused a serious problem only after aging out of the "breeding age".

Nine virus species were found during the diagnostic and most of them have oncogenic properties. Sequences of virus families were also detected in the samples, which could have environmental or feed origin. Among them, we also found viruses from algae or invertebrate species. Several species of Polyomaviruses can cause neoplastic disease, as has been described in black bear and golden hamsters. Birds, amphibians and reptiles, and even fish have their own Polyomaviruses. Although it has been described in black sea bass, *Trematomus pennellii* (Regan, 1914), and in guitarfish, its exact role and course of disease are not yet clear. The Paramyxoviridae family also includes some species which can infect fish, as reported by numerous studies. *Atlantic salmon paramyxovirus* causes proliferative lesions in gills. Although the copy number of the Retroviridae family was relatively low, it is abund in oncogenic virus species. Sarcoma-causing retroviruses are also found in

salmonids, but their tumor-causing effects have already been described in many other fish species. The progress of disease in some species of the Parvoviridae family is well known from higher vertebrates. However, it is not well known that it has also been associated with tumor formation in slow loris.

Parvoviruses have already been described in fish, including perch and tilapia, which does not rule out their possible harmful role in our case either. Sequences specific tothe Adenoviridae family were also found in the samples in a higher proportion than the previous families. Their damage is well known in the animal world. It can cause disease in many species of fish (Acipenseridae, codfish, flounder, etc.), in some of these cause hyperplastic lesion. Tumours of viral origin affecting lower vertebrates have been reported in the Alloherpesviridae virus family. Amphibious *Ranid herpesvirus 1* induces renal adenocarcinoma in leopard frogs (*Rana pipiens*, Schreber, 1782). They also cause tumours in fish, as discussed earlier in the literature review. *Cyprinid herpesvirus 1*, causes pox lesions in carp and has a tumorigenic effect. *Salmonid herpesvirus 2* is associated with neoplastic changes in salmonids. The other viruses were present in about 2% of the samples. It can also be a potential cause of tumors in our case, even though intestinal neoplastic lesions have not been described so far in connection with fish infected with viruses. Compared to the previously mentioned viruses, the number of viral copies of the Papillomaviridae family in the sample continued to increase. Papillomas in fish can be caused by viruses in many cases, as mentioned by Roberts (2012). Fish from aquaculture and ornamental fish can also become infected with the virus and develop papillomas on the skin.

Insect viruses include members of the Baculoviridae family. So far, no pathogenic effect caused by them in fish, but their anti-apoptotic effect has been proven in insects. In the absence of natural cell death, not as many cells die as are produced. Due to the apoptosis being out of balance, more and more cells are created in the tissue or organ, thereby increasing the chance of developing tumors in susceptible species. *Herpesviridae* was one of the virus families producing the highest number of copies. Although it has been associated with cancer in humans (nasopharyngeal carcinoma, Kaposi's sarcoma caused by human herpes virus type

8, lymphoma induced by the Epstein-Barr virus, and tumors of epithelial origin), there are still few examples of their oncogenic effects in animals. One of these virus species is the *Gallid herpesvirus 2*, which causes Marek's disease, and which forms lymphoid tumors in birds. Based on these, even a herpesvirus species may be behind the investigated tumor lesions. The Iridoviridae virus family producing the highest copy number in the samples. They cause diseases in many species, including fish. Whether in freshwater or marine environments, iridoviruses cause the disease called lymphocystis, which affects the fibroblast cells of the skin. Cells can be up to 100,000 times larger than their normal size. The grouper iridovirus genome contains an antiapoptotic B-cell lymphoma-2-like gene. If this is expressed - interrupting the transience of cells - it can even lead to tumour formation. The assumption therefore arises that similar related viruses belonging to the aforementioned families can even cause neoplastic changes in rainbow trout.

Although viral forms were not detected in the tumour cells during electron microscopic examinations, this does not rule out their presence. Differences were observed when comparing healthy and tumour cells. Nowadays, qualitative electron microscopic diagnostic procedures are still used. Molecular biological methods, such as the metagenome method, can be faster and more accurate, but we can get a picture of viral pathogens with the previously mentioned test. In fact, among the viral diagnostic methods, both cell culture, electron microscopy, and molecular biological methods are used, often in parallel. One method can confirm and complement the other, thus making the diagnosis more precise.

The number of individuals may decrease because of the dead fish, and the risk of inbreeding in the stock may increase. The question arises whether we can talk about a hereditary tendency in intestinal adenocarcinoma in trout? If yes, then in the case of a successful reproduction, if the male or the female inherits this tendency, the frequency of tumour reversal in the heterozygous offspring may increase. The chance of this can be even higher in possible homozygous individuals.

Fish feed could play a major role in the development of tumors in many cases, as described by Dale et al. (2009). The question arises in the name of One Health, whether plant and animal feed can get into fish and possibly cause tumours

in the human body? Can substances be transferred into our body that have hidden effects? Currently it is prohibited to feed slaughterhouse waste and by-products of animal origin in any form to predatory fish intended for food in the European Union. It would be worthwhile to develop an alternative feeding method for certain fish species, which could lead to the feeding of pellets containing a large proportion of plant parts. There are already good results for the use of insect protein and other lower organisms in fish feeding. In our study the feeds (AquaGarant, Alltech Coppens) come from verified suppliers and undergo serious screening tests at several points of production. Both companies have certified that the feeds are free of mycotoxins.

The broodstock represents a high value in fishfarms. They ensure the continuity of production and the new generation. Animals reach sexual maturity at the age of 3-4 years. Thousands of eggs can be collected from one female fish during its lifetime, which will become the next generation and the next broodstock. The death of female broodstock represents a serious economic loss for fish farms. During the 3 years until sexual maturity, the amount spent on its upbringing, feeding and possible medical treatment is then all shown as a loss.

The research of neoplastic malformations in fish has recently begun to develop. Our research is a pioneering work not only in Hungary, but also internationally. Our studies have brought important, new results in the field of neoplastic diseases in trout, however further studies are needed to clarify the details.

5. New scientific results

The new scientific results are the following

1. A new tumor screening method was developed in domestic fish farms with the help of imaging diagnostic procedures. During the research, we identified the pathological changes using Mindray M9Vet ultrasound, Siemens Somatom Definition AS+ CT machine and Siemens Biograph mMR machine. During the comparison of the methods, with the help of CT and MRI equipment, we were the first to carry out this kind of examination on trout in Hungary. The ultrasound examination proved to be the most useful in field diagnostics.
2. Pathological examinations were performed, and the lesions were identified using histopathological and immunohistochemical methods. We were the first to publish intussusception caused by intestinal tumors in rainbow trout. This is the first time, that gastrointestinal adenocarcinoma metastases in the kidneys of rainbow trout were described.
3. Using histopathological and immunohistochemical methods, we proved the metastasis of the primary tumor in the gastrointestinal tract in the liver, gills and kidneys showed pancytokeratin and E-cadherin positivity.
4. Rainbow trout tumours were tested for viruses for the first time, for which we used new-generation sequencing methods in Hungary. During this study, we identified 9 potentially oncogenic virus families.
5. We examined the tumor tissue with the help of electron microscope, which has not been done by anyone else in Hungary in this species and this type of tumor. We described morphological differences between the cells of the intact and the tumor tissue.

6. Recommendations

Based on our research, fish suffering from tumour must be screened out as soon as possible from the affected stock. It is necessary to minimize costs of sick animals (feeding, labor, husbandry technology, veterinarian). This can only be achieved if regular monitoring tests are carried out in fish farms. An appropriate method for this is the ultrasound examination, during the application of the method it is not necessary to kill the fish. Physical examination under anesthesia as part of a routine check-up can give an adequate picture of the occurrence of tumors. In this way, the production can be made even more economical and safer.

7. Publications on the topic of the dissertation

- Hoitsy, Márton ; Hoitsy, György ; Jakab, Csaba ; Molnár, Tamás Gergely ; Baska, Ferenc,; Gastrointestinalis eredetű daganatok azonosítása szivárványos pisztrángban (*Oncorhynchus mykiss*, Walbaum, 1792) MAGYAR ÁLLATORVOSOK LAPJA 142 : 1 pp. 55-64. , 10 p. (2020)

- Márton Hoitsy, György Hoitsy, Csaba Jakab, Tamás Molnár, János Gál, Ferenc Baska, Intussusception caused by intestinal neoplasia in mature rainbow trout (*Oncorhynchus mykiss*, walbaum 1792) J Fish Dis, 2021, DOI: 10.1111/jfd.13347

- Hoitsy, Márton, Molnár, Tamás Gergely, Baska, Ferenc, János Gál, Halakat érintő daganatos megbetegedések, ACTA AGRARIA KAPOSVÁRIENSIS (Elfogadott, 2022)