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Full Length Research Paper

Susceptibility of rainbow trout (*Oncorhynchus mykiss)* and carp (*Cyprinus carpio*) to *Myxobolus cerebralis*, the agent of whirling disease

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We have previously investigated the resistance or the susceptibility of carp (*Cyprinus carpio*) to *Myxobolus cerebralis*-spores, the causative agent of whirling disease in salmonids, through histological and molecular methods. To further our study we conducted the same studies on the rainbow trout (*Oncorhynchus mykiss*), which is known to be susceptible to the causative agent. Polymerase chain reaction (PCR), nested-PCR and histological examination Hamatoxylin-Eosin (H&E) methods were compared for the detection of *M. cerebralis*. The histological investigations of 20 fish between 3 to 5 days post-exposure (*p.e)* revealed only 3 parasite stages (single, degenerate, agglomerated) in the tail fin of carp. The nested-PCR results showed that only *M. cerebralis* exists in 15% of the caudal fin samples in the rainbow trout 60 days *p.e*, although in the single-round PCR test, all the samples were tested negative. Our results indicated that the nested-PCR-method is more sensitive to detect early stages after 12 h in the rainbow trout and carps in comparison to the single-round PCR test and histological examination. The infection of rainbow trout with *M. cerebralis*-spores was confirmed by clinical symptoms and histological lesions in the cartilaginous areas of the skull, while in all the infected carps, it was not possible to divide the triactinomyxon-spores (TAM) in the epidermis at 12 h *p.e*; thus, it penetrates deep into the dermis in order to reach the brain.

Key words: Polymerase chain reaction, *Myxobolus cerebralis,* carps, salmonids.

INTRODUCTION

Whirling disease was first described in 1898 by Hofer in rainbow trout (Hofer, 1903). In the United States, the disease first appeared in 1958 in Pennsylvania, leading to a severe decline in stocks of rainbow trout (*Oncorhynchus mykiss*) (Nehring and Walker, 1996; Vincent, 1996, Hedrick et al., 1988; Baldwin et al., 2000). *M. cerebralis*, a Myxozoa parasite, is the causative agent of whirling disease in salmonids. Markiw (1984) was the first to confirm that the worm *Tubifex tubifex* is an alternate host of *M. cerebralis* (Myxosporea). There are three-stages of development, in the skin, nervous system and cartilage of the fish (El-Matbouli et al., 1999). After death of the fish, *M. cerebralis* spores are released from the cartilage into the water, where they are taken up by their final host *T. tubifex* to localize into their intestinal epithelium. Once released from the oligochaete, spores penetrate the epidermis of fish and as amoeboid cells. The circular swimming movements and black discoloration of the posterior third of the body are classic symptoms of the disease, and they first appear 35 to 80 days after infection (Schaeperclaus, 1931; Markiw, 1992a).

The developmental stages of *M. cerebralis* are both in the ossifying skull and in the bony portions of gills and gill arch (Mándok, 1993; EL-Matbouli et al., 1995). We have previously showed that after exposure of several fish species, including carp (*Cyprinus caprio*), Guppy (*Poecilia reticulata*), nose (*Chondrostoma nasus*) and

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Abbreviations: H&E, Hamatoxylin-Eosin, **p.e,** post-exposure; **TAM,** *Triactinomyxon*-spores; **DNA,** deoxyribonucleic acid.

goldfish (*Carassius auratus*), only rainbow trout and Guppy showed evidence of spore development (EL-Matbouli et al., 1999). For the precise detection of *M. cerebralis* infections, molecular biological methods, such as polymerase chain reaction (PCR) have been developed (Andree et al., 1997a). These techniques are more sensitive than histopathological methods and allow the determination of a small number of stages in the tissue (Lorz and Amandier, 1994; Baldwin et al., 2000; Andree et al., 2002). Indeed, the sensitivity of PCR (92% detection rate) is higher than histopathology and the trypsin/pepsin digest (PTD) (Lorz and Amandi, 1994; Baldwin et al., 2000; Andree et al., 2002). Andree et al. (1998) developed a nested-PCR assay to detect the genomic DNA of *M. cerebralis* which was found in all exposed rainbow trout at 2 h, 1, 2 and 3 wk post exposure (*p.e).* In sharp contrast, histological examination of stained tissue sections of the same group revealed only 2 positives out at 2 h *p.e.* Quantitative PCR (qPCR) test allowed the highest detection rate of *M. cerebralis* in the examined rainbow trout as reported by Andree et al. (1998). Also, this method showed that the staining procedure did not affect actinospore reactivity and the parasite stages were highly detected in carp than in trout gills (Kallert et al., 2009). Developmental stages of *M. cerebralis* associated with cartilage degeneration could only be found in the head cartilage of rainbow trout. Susceptibility of cyprinid species relative to the whirling disease is largely unknown. Accordingly, the aim of the current study was to investigate the possible susceptibility of common carp to whirling disease by using a histological approach in comparison with nested-PCR and PCR.

MATERIALS AND METHODS

Exposure experiments

In order to determine the nature of susceptibility to the parasite, exposure experiments with 2 to 3 cm long and 1 to 2 g hatchlings of carp and rainbow trout breeding were carried out. All fish were from our own Specific Pathogen Free breeding facility. Laboratory infected fish were exposed in a plastic container (500 ml) for 3 h to 5000 TAM-Spores/Fish. After three hours of exposure the fish were transferred into 70 L glass aquaria. The water temperature was 14°C for the rainbow trout and 22°C for the carp. After different times (Table 1) post-infection with TAM-spores, 20 fish from each group were removed, anesthetized with the same concentrations of MS-222 (0.007%) (tricaine methanesulfonate) (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) and dissected. As a control group, 20 other animals from each group were kept in a TAM-spore-free aquarium and killed with the anaesthetic MS 222 (0.007%) at various times post-exposure *(p.e)* for examination.

Histological studies

For the histological investigations 20 fish from each group have been sacrified. The tail fin, dorsal fin, skin of the spine and the head were fixed for 48 h in 5% buffered formalin and embedded in paraffin. 5 micron thick sections were stained with haematoxylin and eosin and examined microscopically for parasites in the skin and the cranial skeleton. To compare the intensity of the infection, the number of invading amoeboid cells in slices of skin from the lateral side of the body and fins was determined on an area of 7.502 mm^2.

Molecular biological studies

PCR and nested PCR assay for molecular diagnosis *of M. cerebralis*

To verify the results of the histological samples from skin biopsies, tail fins, caudal fin and head cartilage, 20 samples from each group were analysed with conventional PCR and nested-PCR according to the protocol by Andree et al. (1998). 20 to 30 mg skin tissue of the tail fin, dorsal fin, spine and the head were taken from each group and frozen at -80°C. The samples were then examined using PCR. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH) following the manufacturer protocol. The PCR was carried out in a thermocycler (Mastercycler Gradient, Eppendorf, Wesseling-Berzdorf). Thermal cycling parameters consisted of an initial denaturation step at 95°C for 5 min; 35 reaction cycles, denaturation at 95°C for 1 min, annealing at 65°C for 2.5 min, elongation at 72°C for 1.5 min and a final extension at 72°C for 10 min, then down through to cooling temperature of 4°C.

The PCR reaction mixture consisted of Reddy Mix, TM-PCR Master Mix (50 mM KCl, 75 mM Tris-HCl pH 8.3, 20 mM Mg (OAc) 2), 1.5 mM$MgCl_2$, 0.2mm dNTP, dCTP, dGTP, dTTP) (from ABgene, UK): The standard reaction volume was Master Mix 17.9 ul, primer Tr5-17: 0.8 ul, primers Tr3-17: 0.8 ul, sample DNA: 0.5 ul. The primers used for *M. cerebralis* specific one-step-PCR (MWG-Biotech, Martinsried) were designed to give an amplicon length of 1030 bp and they were: Tr5-16 (-GCATTGGTTTACGCTG ATGTAGCGA) and Tr3-16 (-GAATCGC CGAAACAATCATCGAGCTA) for First-Round, while for the second round were: Tr5-17(-GGCACACTACTCCAACACTGAATTTG) and Tr3-17 (-GCCCTATTAA CTAGTTGGTAGTATAGAAGC). In the second round the primer pairs were designed to produce amplicons 423 bp in length. The DNA extracted from Triactinomyxon-spores was used as a positive control and the negative control (with no DNA). Visualization of the amplification products of 20 samples were directly applied in a 1.5% agarose gel electrophoresis and stained by Ethidium bromide and detected under UV light. For the size determination of DNA standard markers (4 µ) were used (100 bp DNA-Ladder Reddy Run Super ladder, Fa. ABGene®, UK). In each case 5 µl per lane were used.

RESULTS

Histological examination of the skin

To get information about the entry of the TAM-spores, 20 fish from each group (rainbow trout and carp) were infected by 5000 TAM-spores/fish and samples from the caudal fin, skin tissue, tail fin and dorsal fin were collected at different times *p.e*. At 5 min *p.e*, 25 amoeboid cells were found in the trout while 8 amoeboid cells in the carp. Table 1 summarizes the findings in the epidermis of the tail fin. Invaded parasite stages were observed in about 1 cm^2 of the epidermis. The parasite was localised in the tail fin rather than the whole skin tissue (Figure 1a, b and c).

Thereafter, in the trout as well as in the carp, parasites

Table 1. Results of histological examination: Average number of stages of TAM spores on a surface of 1 cm ² of the tail fin.

Time	5 min	4 h	6 h	8 h	12 h	18 h	1 day	3 days	5 days
Rainbow trout	25.62	16.82	11.42	7.22	7.86	0.00	0.15	0.00	0.00
Carp	7.24	4.15	2.8	0.88	0.00	0.00	0.00	0.00	0.00

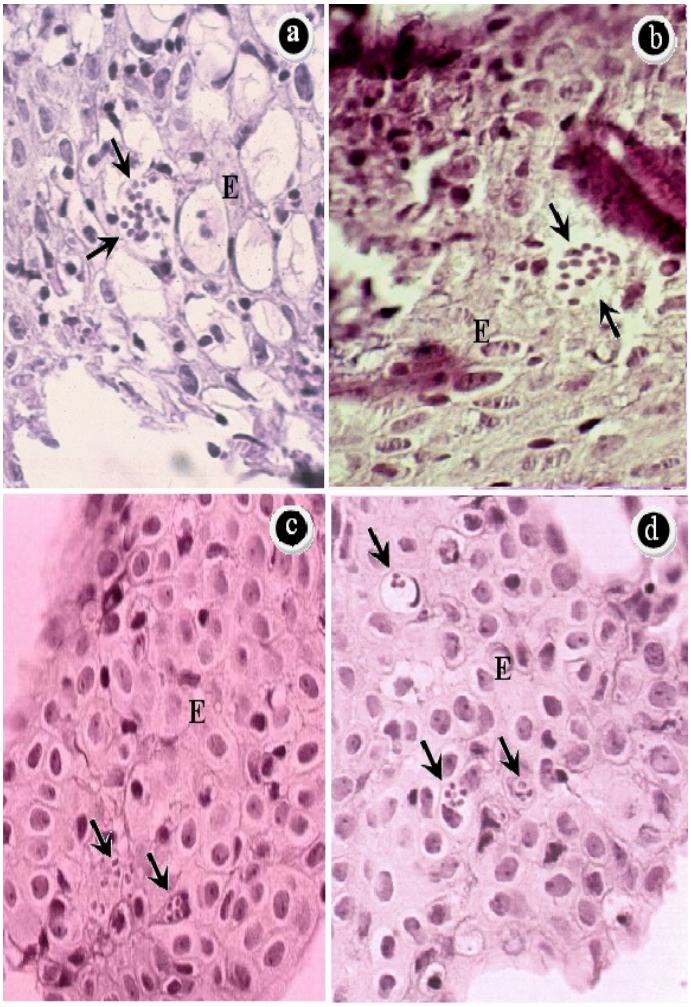

Figure 1 a. Intercellular parasite stages in the epidermis; five min *p.e* in carps; 400x. b. Intact Intercellular parasite stage in the tail epidermis; four hours *p.e* in carps; 400x. c. Two parasite stages in the skin epidermis, 4 h *p.e* in trout. d. Three parasite stages in the tail epidermis 8 hours *p.e* in rainbow trout; 400x. 5000 TAM spores/fish. ; HE-staining. (E) Cell epidermis; (➤) Spores.

were found in the tail fin until 8 h *p.e* (Figures 1d and 2e, respectively). In the first hours of infection in rainbow trout the intensity of infection was higher as compared to carp. Investigation of infected carp between 8 and 12 h *p.e* showed the presence of intact stages of non dividing amoeboid cells in the epidermis of the tail fin but not in the skin. The amoeboid cells were round to oval and had a diameter of 2 to 2.5 microns. In each rainbow trout 30 histological sections were prepared from each sample at 12 h (p.e), only dividing sporoplasmes (amoeboid cells) were detected between the epithelial cells in the fins (Figure 2, f and g). From 5 min to 1 day *p.e* a decrease of the infection intensity could be found in caudal fins of rainbow trout. The average spore density was highest in caudal fins. In histological examination 8 h *p.e* 7 parasite stages were counted rainbow trout in the skin tissues (the lateral side of the body) of rainbow trout while no TAM-spores were found in carp. At 18 h *p.e*, histological studies of the caudal fin epidermis of rainbow trout and carp showed no traces of intact or dividing amoeboid germ cells. At day 1 *p.e*, in our study of 30 slices, only five rainbow trout, had a very small number of intact amoeboid cells. Three to five days *p.e* parasite stages were found in the tail fin of carp (3 Stages), (single, degenerate, agglomerated) however no TAM-spores stages are detected in the epidermis of the skin In the rainbow trout (Figures 2h and 3i).

Histological examination of the skull cartilage 50 and 150 days post exposure

The head cartilage structures of the studied rainbow trout 50 days (p.e.) showed numerous foci with chondrolysis, because multiplication stages and plasmodia were visible (Figure 3j). Besides the remains of intense violet-coloured healthy cartilage, developmental stages of *M. cerebralis* (plasmodia) were observed. All 20 rainbow trout had moderate-to-severe degeneration of cartilage architecture in the skull. On histological examination 150 days (p.e.) there were mature spores of *M. cerebralis* in the cartilage tissue of the skull, often with invading leukocytes (Figure 3l). Conversely, in the examined carp no mature *M. cerebralis* or lesions were found in the head cartilage from 50 to 150 days (p.e.) (Figure 3m). The detection of the mature *M. cerebralis* within the degenerated head cartilage of rainbow trout between 50 and days 150 (p.e.) was associated with the circular swimming movements and black discoloration of the posterior third of the body.

Molecular biological results

Rainbow trout

In the present study, all skin samples, caudal fin and the tail fin samples in all infected fish samples at 1, 4 and 8 h *p.e* showed positive amplification for *M. cerebralis* DNA

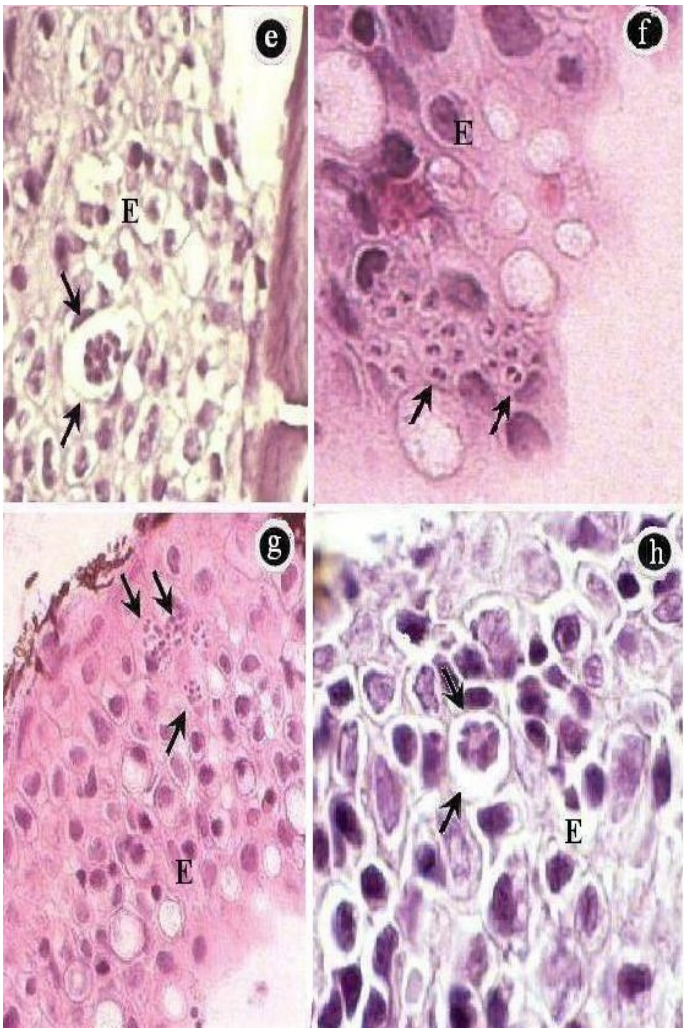

Figure 2 e. Intercellular parasite stages in the tail epidermis; 8 hours *p.e* in carps; 400x. f. Parasite stages during the division process (tail) 12 h *p.e*; trout; 1000x. g. Four parasite stages during the division process in the tail epidermis 12 h *p.e*; rainbow trout; 400x. h. One parasite stage clumps in the epidermis cells (tail) three days *p.e*; carps; 1000x. (5000 TAM spores/fish). HE-staining. (E) Cell epidermis; (➤) Spores.

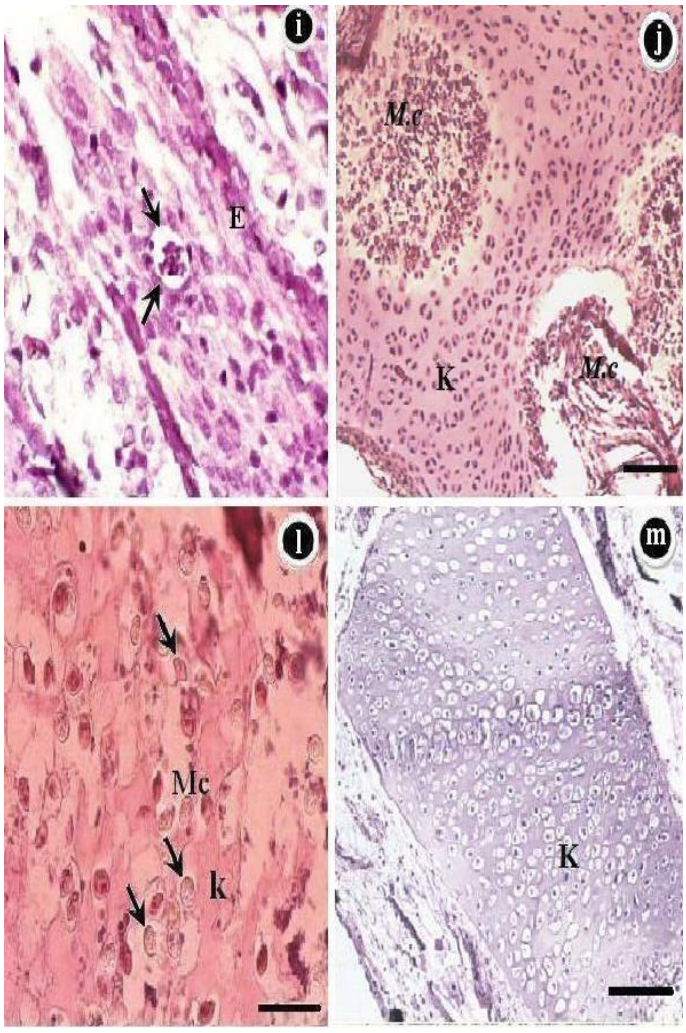

Figure 3 i. Lysis of parasite stage in the tail epidermis; 5 days *p.e*; carps; 400x. j. Destroyed cartilage cells and high number of development stages of *M. cerebralis*-spores (*M.c.*) 50 days (*p.e*; rainbow trout; 400x. l. Destroyed cartilage architecture (K) as well as high number of mature spores of *M. cerebralis* (M.c.)150 days *p.e*; rainbow trout; 400x. m. Overview of intact cartilage architecture of carps exposed to 5000 TAM-spores; 150 days *p.e*; 200x. HE-staining. (E) Cell epidermis; (➤) Spores.

by conventional PCR and nested-PCR (100%) (Figure 6). However, 95% of tail fins and 60% of skin samples tested positive for *M. cerebralis*- spores by nested-PCR after 16 h *p.e*. Among the 20 analysed skin samples after three days *p.e*, no sample showed positive amplification for *M. cerebralis* DNA by conventional PCR and 1 sample was positive (5%) by nested-PCR. From day 4 to 20 *p.e* no *M. cerebralis* DNA was found in the skin in any of the fish examined by nested-PCR. After that *M. cerebralis* was exclusively detected in the tail fins. The last positive detection of caudal fin samples occurred in the rainbow trout 60 days *p.e* (15%) (Figures 5 and 6). These 60-days samples were tested positive by nested-PCR and negative with conventional PCR. From 50, 60, 80,120 and 150 days *p.e*, Among 20 head samples form fish with clinical specimens analysed, *M. cerebalis* DNA was detected in all the samples by PCR and nested-PCR, confirming the positive histological findings.

Carp

Examination of the tail fins, caudal fin and skin samples using conventional PCR and nested-PCR after 1, 4 and 8 h *p.e* revealed *M. cerebralis spores* in 100% of the fish sampled (Figure 7). In the 16 h group, 14 out of 20 (70%) of the tail fin samples and 12 out of 20 (60%) of the skin samples tested positive. However, the DNA for *M. cerebralis* was found 1 day *p.e* only in 7 of 20 (35%) from the tail fin samples and 5 of 20 (25%) samples of skin

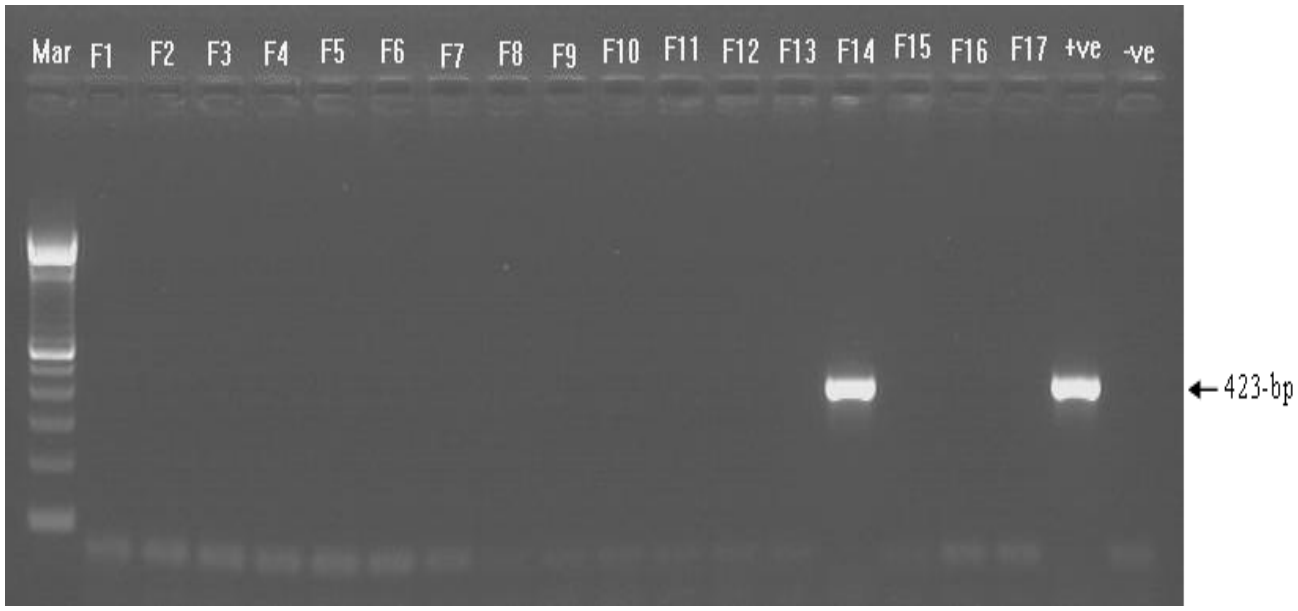

Figure 4. *M. cerebralis* DNA detection in samples amplifed by nested-PCR, submitted to 1.5% agarose gel electrophoresis, stained with ethidium bromide and detected under UV light. Lanes 1 (F1) - 1 (F17) samples 5 µl DNA tail fin. Only Lanes 14 (F14) the 423-bp fragment represent *M. cerebralis* DNA detection in the tail fin samples of carps 4 days *p.e*. Lane 19 (-ve) is the negative control (with no DNA) and lane 18 (+ve) DNA from Triactinomyxon-spores was used as a positive control. Lane M is the 100-bp ladder (molecular marker). Lanes 1 (F1) – 12 (F12) and lane 15 (F15) – 17 (F17) are negative.

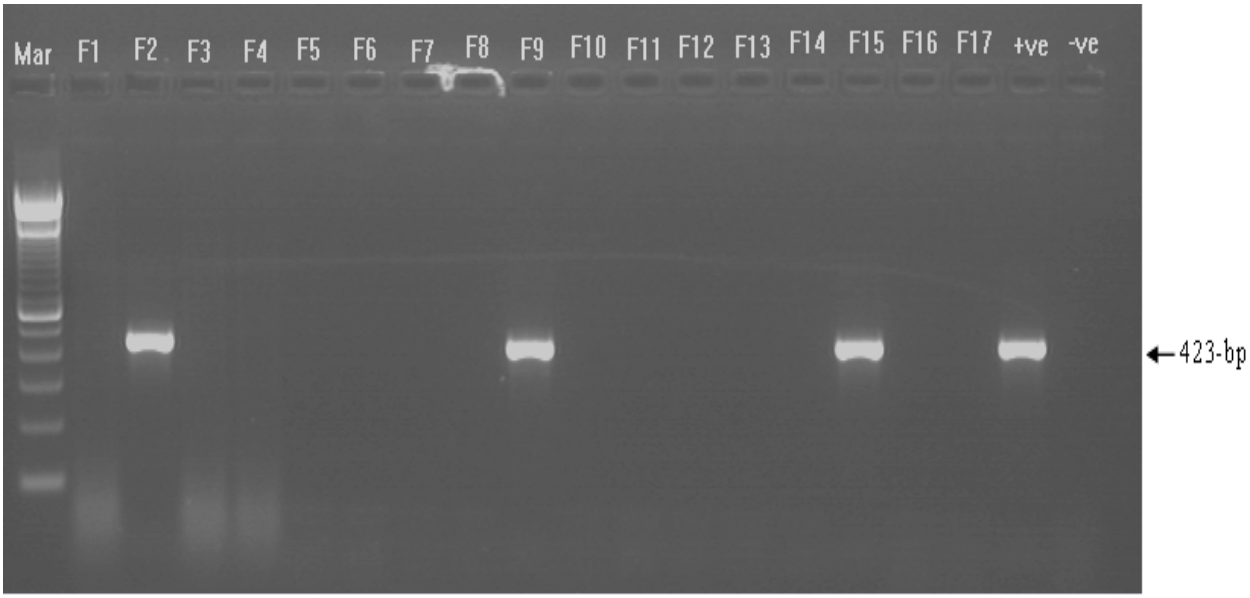

Figure 5. *M. cerebralis* DNA detection in samples amplifed by nested-PCR, submitted to 1.5% agarose gel electrophoresis, stained with ethidium bromide and detected under UV light. Lanes 1 (F1) -1 (F17) samples 5 µl DNA tail fin. Lane 2 (F2), lanes 9 (F9) and lane 15 (F15) the 423-bp fragment represent *M. cerebralis* DNA detection in the tail fin samples of rainbow trout 60 days *p.e*. Lane 19 (-ve) is the negative control (with no DNA) and lane 18 (+ve) DNA from Triactinomyxon spores was used as a positive control. Lane M is the 100-bp ladder (molecular marker). Lane 1 (F1), lanes 3 (F3) – 8 (F8), lanes 10 (F10) –14 (F14) and lanes 16 (F16) – 17 (F17) are negative.

using nested-PCR. At 2 days *p.e* samples tested negative with the conventional PCR. After investigation by nested-PCR, however, only 2 samples of the 20 tail fins (10%) were positive and only 1 was positive after 4 days (Figure 4), while no skin samples were positive after 3 and 4 days *p.e* using the nested-PCR. From 6 to 150

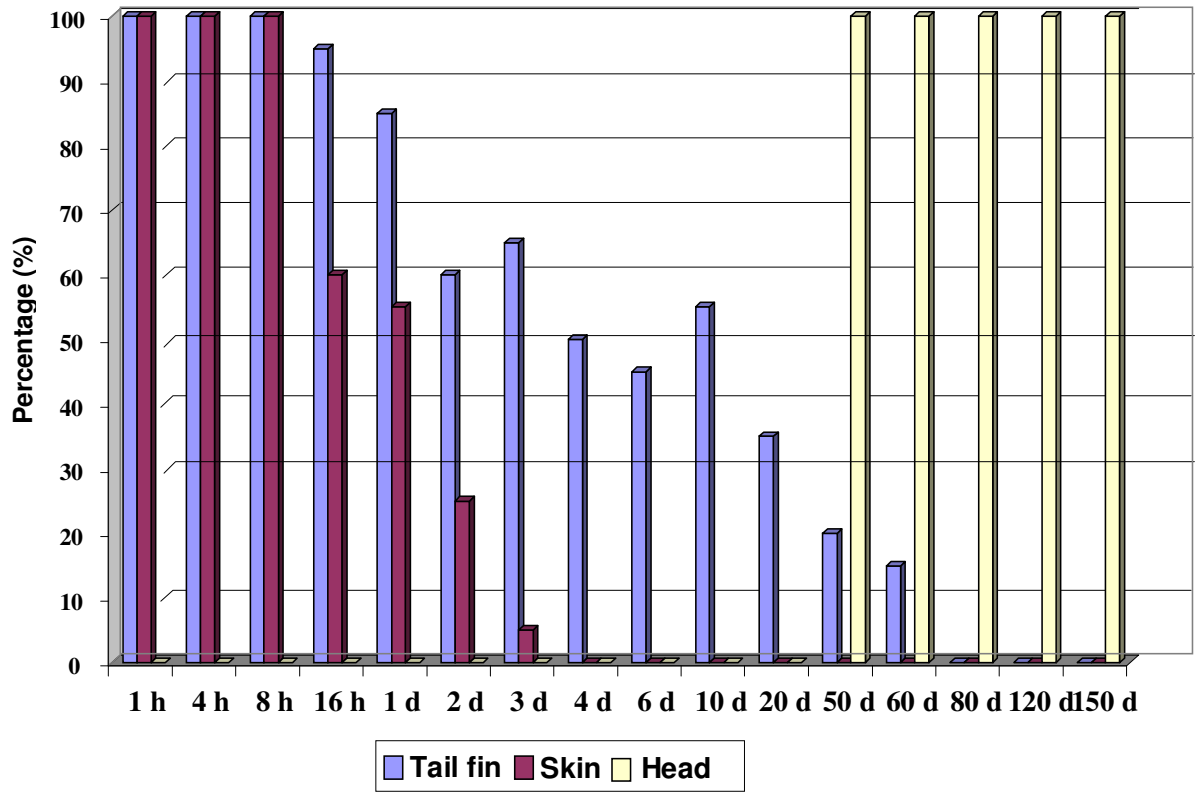

Figure 6. Results of nested-PCR- from head cartilage, skin and skin‘s tail of rainbow trout.

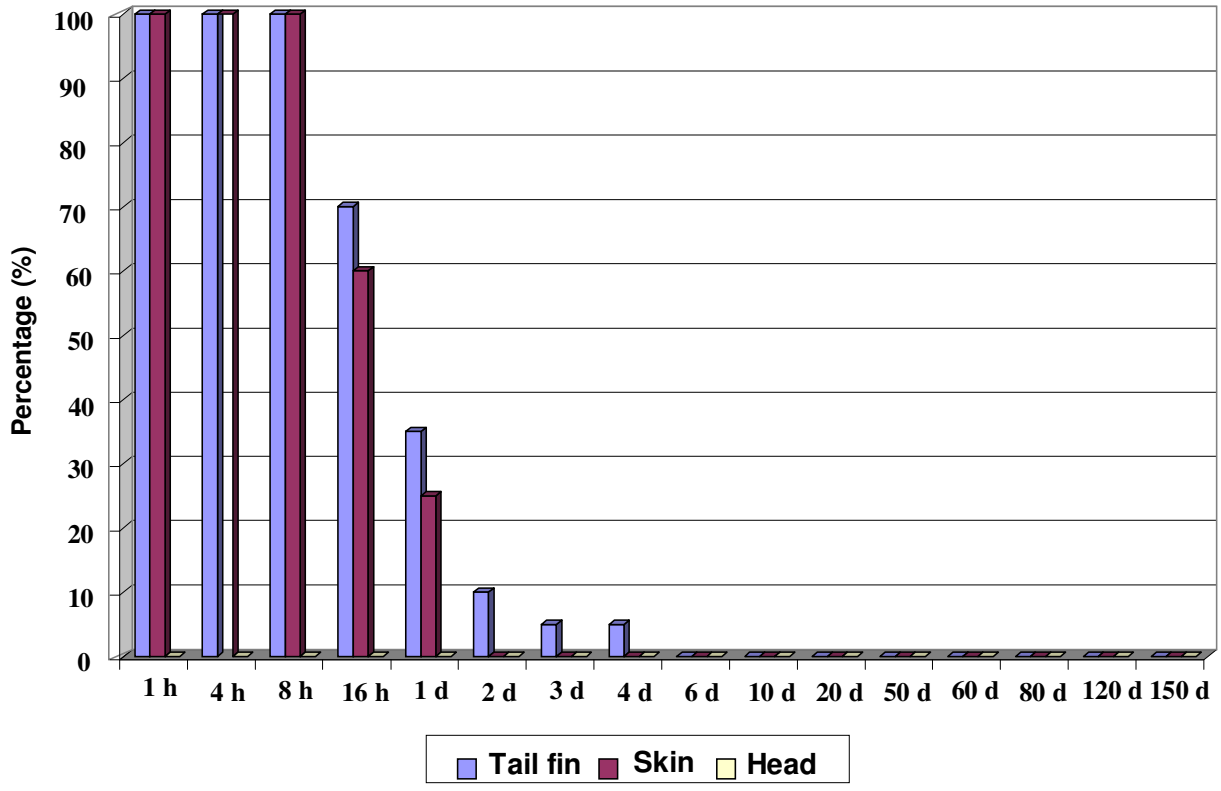

Figure 7. Results of nested-PCR- from head cartilage, skin and skin‘s tail of carps.

days *p.e* no parasite was found in the skin, tail fin and head cartilage of the examined fish (Figure 7).

DISCUSSION

The microscopic examination of stained tissue sections of infected carp and rainbow trout can yield valuable information about the entry and the development of the TAM-spores in the caudal fin, tail fin and the skin tissues side 5 min, 4, 6, 8, 10, 12, 18, 1, 2, 3, 4 days and 5 *p.e*. The histological study in the rainbow trout at five min *p.e* has showed the penetration of twenty five amoeboid cells in the caudal fin epidermis and the skin tissues. In the carp, five minutes after exposure an average of eight amoeboid cells penetrated into the epidermis of the tail fin and caudal fin but was rarely found in the epidermis of the skin tissue. Using fluorescence staining, Kallert et al. (2009) investigated the infection of actinospores sporoplasm after three minutes post-exposure. In the first phase post exposure rainbow trout and carp, the number of amoeboid cells collected in the tail fin was not significantly different from those found by Kallert et al. (2009). However our results showed that this number was higher; with fewer invading parasite stages in the carp tails fins.

Other studies, EL-Matbouli et al. (1999) investigated the vulnerability to *M. cerebralis* of goldfish (*C. auratus*), carp (*C. caprio*), nose (*C. nasus*) and guppy (*P. reticulata*) after exposing all fish groups to Triactinomyxon-spores showing that the fish have similar reactions to infection. However, sporoplasm (amoeboid cells) could only be detected histologically in the skin of guppy fish. No positive results were found in any samples of the nose and goldfish. All of the examined slices of rainbow trout four hours after exposure, an average of 16 intact amoeboid cells were found in the epidermis of the tail fins and skin as reported by EL-Matbouli et al. (1995). The microscopic examination of the caudal fin, skin tissue and the tail fin were collected from carp at 12 h (*p.e*) were counted intact stages of non dividing amoeboid cells in the epidermis of the tail fin. In comparison, the investigation of whirling disease susceptible rainbow at 12 h *p.e* revealed the presence of only dividing amoeboid cells between the epithelial cells in the fins. However, all of the examined slices of 20 rainbow trout, the amoeboid cell divisions occurred 8 to 12 h post exposure in the epidermis. According to the results of the present study, we could assume that the number of epidermal dividing amoeboid cells could be explained by the fact that, in salmonids, a genetic program was activated after a penetration of a parasite, which would either initiate a cell division of the amoeboid cells or stop it (own observations). The hypotheses about sporoplasm penetration and its inhibition or destruction by the different mechanisms in carp remain unsettled. This initial surprising result can be related to movements of the fish.

Indeed, trout always show more physical activity than cyprinids. Alternatively, a chemotactic active exploration of a host could be proposed. Trout and carp are not entirely comparable because of morphological findings from both groups of fish, which do not demonstrate such a chemotactic capacity (El-Matbouli et al., 1992). From 18 h to1 day *p.e*, in our study of 30 slices, only five rainbow trout, had a very small number of intact no dividing amoeboid cells. They were the last amoeboid cells demonstrated histologically in H&E prior to its migration into the dermis and subcutis (EL-Matbouli et al., 1995). This result agrees with the findings of less degenerated, lumpy or parasite cells on day 3 and 5 *p.e* in the tail fin of carp. These were apparently prevented from further development and migration, by the rainbow trout's host immune defences (EL-Matbouli et al., 1995).

The results of comparative experiments which improved the development of TAM-spores to *M. cerebralis*-spores in the carp was clearly not possible, because it could not be divided in the epidermis between 8 to 12 h *p.e*; thus, migrating at various stages through tissues and along nerves, in order to reach the cerebral cartilage. Also the skin of cyprinids does not represent an insurmountable barrier against infection by *M. cerebralis*. In each rainbow trout between 50 to 150 days *p.e* developmental stages of *M. cerebralis* associated with cartilage degeneration could only be found in the head cartilage of rainbow trout. This agrees with previously published studies (El-Matbouli et al., 1992), which prove that TAM-spores was fully capable of infection. A strong correlation between the detection of *M. cerebralis* in the cartilage head as well as the circular swimming movements and black discoloration of the posterior third part of the body of rainbow trout at 50 days *p.e* were obtained using a histological approach in comparison with nested-PCR and PCR (Oumouna, 2008). The infection of rainbow trout with *M. cerebralis* was proved by classic symptoms of the whirling disease, while all carp were infected, without showing any clinical signs. Our key findings are the cell division which occurs in the epidermis between 8 and 12 h *p.e* in the rainbow trout. This division is very important in the life cycle of TAM-spores to produce more amoeboid cells, which migrate deeper in the tissue layers to finally reach the brain where they develop into mature *M. cerebralis*- spores in the cartilage. Finally, we propose to use the light microscopy to describe the *M. cerebralis*-spores and sporogenesis.

Molecular analysis

The additional use of a molecular biological method was mainly due to the lack of sensitivity of histological examination to detect early stages after 12 h in the rainbow trout and carps. In the present study, carp were first tested for infection with *M. cerebralis* between 1 h

and 150 days *p.e* using single-round PCR and nested-PCR in comparison to histological examination. Recently, quantitative real-time PCR (qPCR) method has been shown to be useful to confirm the entry of TAM-spores of carp (*C. caprio*) (Kallert et al., 2009). The later reported that rainbow trout were very infected while the carp were not after three minutes of exposure. Our extensive experiments have been completed up to 150 days and they confirmed the results of the authors. When using conventional PCR and nested-PCR as the reference method, the extraction of DNA at 1, 4 and 8 h *p.e* identified *M. cerebralis* DNA in all 20 fish (100%). The sensitivity was compared with that of histological techniques and revealed an important number of penetrated amoeboid cells in the caudal fin epidermis, tail fin and the skin tissues. However, the conventional PCR enables positive results to be confirmed by great quantities of amoeboid cells. At 4 and 20 days *p.e* in rainbow trout skin tissue (the lateral side of the body) samples were histologically examined, all fish showed no traces of intact or dividing amoeboid germ cells. These samples were negative for *M. cerebralis* DNA by nested-PCR. This result corresponds to similar infection experiments carried out by Andree et al. (1998) in rainbow trout. It was clear that the sensitivity of microscopic detection is low to detect small numbers of TAM-spores were asymmetrically distributed in the tissue. The nested-PCR results showed that only *M. cerebralis* identified 3 of 20 (15%) of the caudal fin samples in the rainbow trout 60 days *p.e*.

Although, the single round PCR test and the microscopic examination samples tested negative. Our results in the present study confirm what was previously found also by Skirpstunas et al. (2006). Considering all the previous results, the nested-PCR was able to consistently detect very low number of penetrated amoeboid cell in the tissue carps and rainbow trout. The examination of rainbow trout specimens with classic symptoms using light microscopy at 50, 60, 80, 120 and 150 days *p.e* revealed important mature *M. cerebralis* in the head. All of the exposed fish collected at the same time were tested positive with the nested-PCR and PCR. Compared with results obtained by polymerase chain reaction (PCR), the sensitivity of the *M. cerebralis DN*A with clinical specimens was 20 of 20 (100%). The extraction of DNA from head tissues of infected rainbow trout with amplification products of a 423 bp DNA fragment corresponding to the presence or absence of *M. cerebralis*. We concluded that the use of amplification methods improved the sensitivity of detection of *M. cerebralis* DNA in clinical samples with classic symptoms of the whirling disease. According to the obtained results, the present study showed a higher sensitivity of molecular biology assays than histological examination and in particular of the nested-PCR compared to the single-round PCR. Finally, we propose the most sensitive technique (nested-PCR) according to the protocol by Andree et al. (1998) in order to improve the *M. cerebralis*- spores, the causative Agent of whirling disease in salmonids.

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