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DIVERSE *CHLAMYDIA*-LIKE AGENTS ASSOCIATED WITH EPITHELIOCYSTIS INFECTION IN TWO CYPRINID FISH SPECIES, THE COMMON CARP (*CYPRINUS CARPIO* L.) AND THE GIBEL CARP (*CARASSIUS AURATUS GIBELIO* L.)

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During a general annual fish health survey in natural waters and ponds, epitheliocystis infections were recorded in fingerlings of two cyprinid fish species, the cultured common carp and the wild gibel carp. Benign and heavy infections were equally observed without mortality. In addition to the general health inspection of fish, histopathological examinations of infected gills and molecular biological investigations of separated epitheliocysts were performed. Epitheliocysts were formed both in the interlamellar epithelial cells and in the lamella-free multilayered epithelium of the gill filaments. At the early stage of infection dark-staining inclusion bodies densely stuffed with some pathogenic agents were located at the centre of the cell, while in a progressive stage of the process inclusion bodies within the host cells were disseminated in the cytoplasm and stained pale. Molecular studies demonstrated three different agents related to *Neochlamydia*, *Protochlamydia* and *Piscichlamydia* based on sequence analysis of short regions of the 16S rRNA gene. Among them, *Piscichlamydia* is a primary fish pathogen, while *Neochlamydia* and *Protochlamydia* mostly infect free-living amoebae but have adapted thoroughly to fish.

Key words: Epitheliocystis, *Chlamydia*-like, common carp, gibel carp

Epitheliocystis is a disease characterised by cytoplasmic bacterial inclusions (cysts) in epithelial cells of the gills and rarely of the skin in fish. This infection has been reported from over 90 fish species all over the world (Nowak and LaPatra, 2006; Stride et al., 2014), including marine and freshwater fishes growing either in natural environments or cultured in aquaculture farms. Mortality rates range from 4 to 100% in larvae and fingerlings, although the condition is usually benign in other age groups (Nowak and Clark, 1999; Crespo et al., 2001). Plehn (1920), who first described the disease as 'mucophilosis', presumed that the pathogen was a unicellular alga or fungus. Hoffman et al. (1969) recognised the bacterial nature of the aetiological agent and named the disease epitheliocys-

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tis. Based on ultrastructural studies Paperna et al. (1978) as well as Paperna and Sabnai (1980) supposed that the causative organism was a *Chlamydia*- or *Rickettsia*-like bacterium. During their light and electron microscopic studies, Molnár and Boros (1981) observed different intracellularly developing stages of the epitheliocystis organism in common carp fry. Accurate identification of the causative agent has been achieved only relatively recently, primarily with the help of molecular techniques (Everett et al., 1999; Draghi et al., 2004; Meijer et al., 2006). Further studies confirmed that the mysterious obligate intracellular Gram-negative bacterium belonged to the order *Chlamydiales*. Molecular classification of pathogens from epitheliocystis infections of different marine and freshwater fish species revealed 11 *Chlamydia*-like organisms. The taxonomic status of these agents has been disputed: Pawlikowska-Warych and Deptuła (2016) classified nine of them into six families, designated as Parachlamydiaceae, Rhabdochlamydiaceae, *Candidatus* Parilichlamydiaceae, *Candidatus* Clavichlamydiaceae, *Candidatus* Actinochlamydiaceae, *Candidatus* Piscichlamydiaceae, while Gupta et al. (2015) delineated three clades within the phylum Chlamydiae, each of them involved the families concerned, like Parachlamydiaceae *(Para-, Proto-, Meso-, Meta-, Neochlamydia)* and *Candidatus* families as Clavichlamydiaceae, Rhabdochlamydiaceae *(Rhabdo-, Renichlamydia*), Parilichlamydiaceae and Piscichlamydiaceae. New data suggest that some non-chlamydial, γ- and β-proteobacteria could also be affiliated with the disease (Toenshoff et al., 2012; Contador et al., 2016). The majority of epitheliocystis cases have been reported in marine fish species, while relatively few infections (approx. 20 percent) are known in freshwater fishes (Stride et al., 2014). The affected freshwater fish species are the centrarchids bluegill *(Lepomis macrochirus)* and largemouth bass *(Micropterus salmoides),* the ictalurids brown bullhead *(Ictalurus nebulosus)* and channel catfish *(Ictalurus punctatus),* the clarid African catfish *(Clarias gariepinus),* the salmonid lake trout *(Salvelinus namaycush),* the acipenserid white sturgeon *(Acipenser transmontanus),* the latid barramundi *(Lates calcarifer),* the terapontid silver perch *(Bidyanus bidyanus),* the moronid white perch *(Morone americanus)* and striped bass *(Morone saxatilis),* the cichlid tilapias (*Tilapia aurea* × *nilotica* hybrid, *T. nilotica*, *T. mossambica*), and the caracid pacu *(Piaractus mesopotamicus)*. Moreover, epitheliocystis has also been detected in the cyprinid common carp *(Cyprinus carpio)* and grass carp *(Ctenopharyngodon idella)* (Hoffman et al., 1969; Wolke et al., 1970; Zachary and Paperna, 1977; Paperna and Baudin-Laurencin, 1979; Paperna and Sabnai, 1980; Paperna et al., 1981; Zimmer et al., 1984; Bradley et al., 1988; Desser et al., 1988; Anderson and Prior 1992; Groff et al., 1996; Frances et al., 1997; Szakolczai et al., 1999; Goodwin et al., 2005; Meijer et al., 2006; Steinum et al., 2010; Steigen et al., 2013). Although epitheliocystis infection had been first recorded in the common carp (Plehn, 1920) and the disease was observed in cyprinid fishes from time to time in several regions of the world (Hungary – Molnár

and Boros, 1981; Israel, Portugal – Paperna and Alves de Matos, 1984; Japan – Miyazaki et al., 1986; Russia – Voronin and Chernysheva, 1997; South Korea – Kim et al., 2005), the taxonomic status of the causative agents in these fishes has not been studied in sufficient detail. So far only a short *Chlamydia*-like sequence associated with epitheliocystis in the cyprinid grass carp *(Ctenopharyngodon idella)* has been deposited in GenBank (Kumar et al., 2013).

The objective of this study was to perform a phylogenetic comparison of the causative agents of epitheliocystis in common carp and gibel carp with those in other fish species, using sequence data published earlier.

Materials and methods

Fishes

In July and August of 2013–2015, during routine farm health monitoring and natural water surveillance altogether 240 common carp *(Cyprinus carpio)* and 27 gibel carp *(Carassius auratus gibelio)* specimens were studied for epitheliocystis infection. Common carp (2–4 months old and 4–10 cm in length) arrived from four fish farms in the eastern [farm 1 (sample EP2), 3 (sample EP5), and 4 (sample EP6)] and western [farm 2 (sample EP4)] parts of Hungary. From gibel carp (1–2 years old and 3–9 cm in length) 15 specimens were obtained from a fish farm and 12 specimens were collected from Lake Balaton and its tributaries. The fish were transported alive to the laboratory in plastic bags and kept in 40-litre aquaria.

Sampling

The fish were euthanised with 20 ppm clove oil (Javahery et al., 2012) added to the water and gill samples were collected. For further histological and molecular studies, specimens with a relatively high number of epitheliocysts were selected. Hemibranchia infected by various developmental stages of epitheliocystis were fixed in Bouin's solution, embedded in paraffin wax, cut to 4–5 μm thick sections, and stained with haematoxylin and eosin. The cysts were carefully separated from tissue debris under a stereomicroscope at high magnification and in each case about 10 cysts were put in a vial into 70% ethanol and preserved for molecular studies.

Molecular study

Genomic DNA was extracted from homogenates of epitheliocysts (TissueLyser LT, Qiagen, Hilden, Germany) using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. As control, pieces of gill filaments of non-infected fishes were also homogenised and

tested for chlamydial DNA. The presence of any *Chlamydia*-like organism was tested using the eight-primer combined universal PCR designed by Ossewaarde and Meijer (1999) with 'touchdown PCR' protocol. Briefly, fragments were amplified by PCR in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, Carlsbad, CA, USA). The 45 µl amplification reaction mixture contained 1 × Dream Taq Buffer, 200 nM dNTP-mix, 1 µM of each primer, 1.25 U Dream Taq (Thermo Fisher Scientific, Carlsbad, CA, USA), template DNA and water. The PCR program included 3 min of initial denaturation at 95 °C, and 7 min of the final elongation, then 35 cycles at 95 °C for 30 s, annealing for 30 s, and at 72 °C for 30 s. The annealing temperature of the reaction was decreased by 1 °C in every second cycle from 67 °C to 57 °C, at which temperature 10 cycles were carried out. In the last 25 cycles the annealing temperature was 59 °C. The purified approximately 270-bp-long PCR fragments obtained using the Geneaid PCR purification Kit (Geneaid Biotech, New Taipei, Taiwan) were sequenced using an ABI Prism BigDyeTerminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Perkin-Elmer) according to the producer's recommendations. Sequences were used for phylogenetic analysis; the alignment of 5 own and 43 16S rDNA sequences, including members of most families within the order Chlamydiales, especially data from cystic distortion of fish gills, was performed using MEGA6 software. The best-fit nucleotide substitution model for the dataset was GTR+G+I. Phylogenetic trees were built using a Maximum Composite Likelihood algorithm (with 1,000 bootstraps) and the Neighbour-Joining method of tree topology reconstruction (Tamura et al., 2011).

In a further molecular study only the sequences of *Piscichlamydia*-related samples were extended successfully using specific PCR with Pisci211F and Pisci1363R primers (Toenshoff et al., 2012).

Results

Epitheliocystis infection was detected by light microscopic examination in common carp fingerlings from samples of four farms in Hungary. Twenty-six percent of the sources, altogether 62 common carp specimens and only a single gibel carp from a natural water source of the Zala river flowing into Lake Balaton were found infected. The pathological examinations detected epitheliocysts in the epithelium between the secondary lamellae of the gills (Fig. 1) as well as in the non-lamellar portions, at the dorsal and ventral edges and tips of the gill filaments (Fig. 2). Under the light microscope, at the first stage of infection, a clump of chlamydiae arranged in honeycomb structure was observed as a membrane-bound small inclusion body in the host cells. The inclusion increased in size due to multiplication of the infectious agent and was positioned centrally. At the end of the developmental process, the expanded inclusion occupied the bulk

of the cytoplasmic compartment, enlarging the epithelial cell and pushing the nucleus into a corner (Fig. 3). Finally, the matured elementary bodies broke through the membrane, dispersed in the cytoplasm of the host cell and showed Brownian motion.

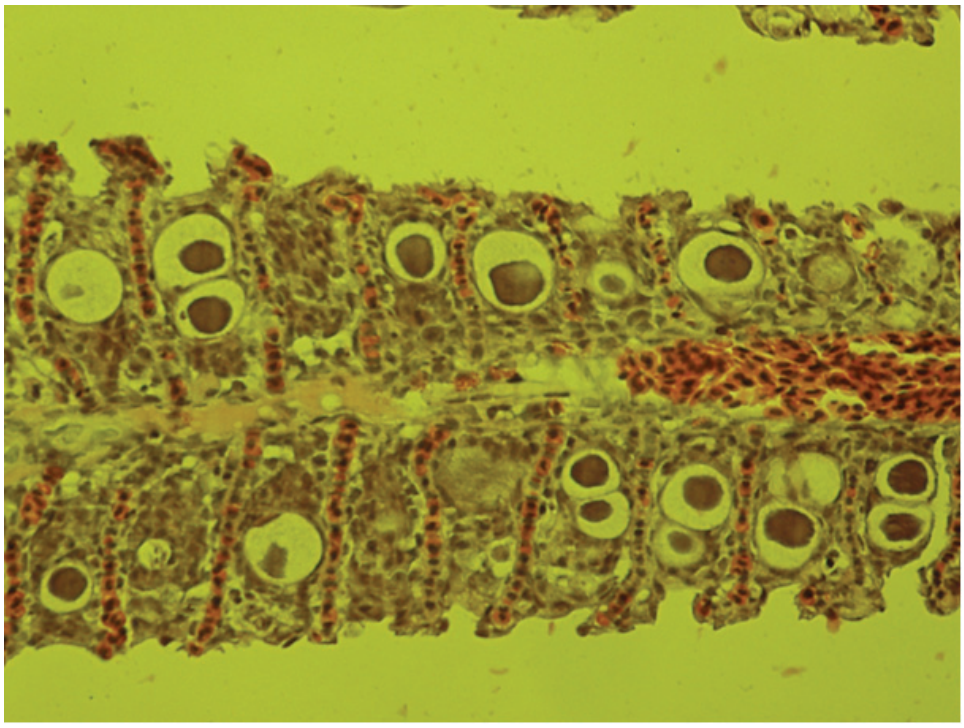

Fig. 1. Heavy epitheliocystis infection in the gill filament of a common carp fingerling. Cysts are located in the multilayered epithelium between the neighbouring gill lamellae. Inside the enlarged cells dark-staining bacteria accumulated in the centre of the degenerated mucous material of the host cell. Haematoxylin and eosin (HE) staining, × 500

In histological sections stained by haematoxylin and eosin the early-stage vacuoles stained dark, while in a more developed stage of infection the enlarged, centrally located inclusions stained pale.

Although in two out of the five cases severe epitheliocystis infections were observed, the fish did not show apparent clinical signs.

The molecular study did not provide congruent results. DNA samples from cultured common carp (EP5 and EP6) from farms 3 and 4, respectively, and wild gibel carp (EP) from the Zala River showed sequence similarity to *Piscichlamydia* spp. detected in grass carp (Kumar et al., 2013) and Atlantic salmon (Draghi et al., 2004), while further two sequences from common carp were related to *Protochlamydia* and *Neochlamydia* spp. The EP5 and EP6 sequences

showed 97.4% similarity to each other and 94.7% and 93% similarity, respectively, to the gibel carp sample. They differed from *Candidatus* Piscichlamydia cyprinis (JX470313) in 9.2%, 7.5% and 3.1%, and from *Candidatus* Piscichlamydia salmonis (AY462244) in 11.5%, 8.3% and 5.3%, respectively. Although longer fragments (> 1200 bp) from these samples were amplified and analysed successfully, a refreshed phylogenetic tree was not built, since the results did not shift their taxonomic status and a sufficiently long sequence of the putative closest-related sample from grass carp (*Candidatus* Piscichlamydia sp. E4, Kumar et al., 2013) is not available in the GenBank.

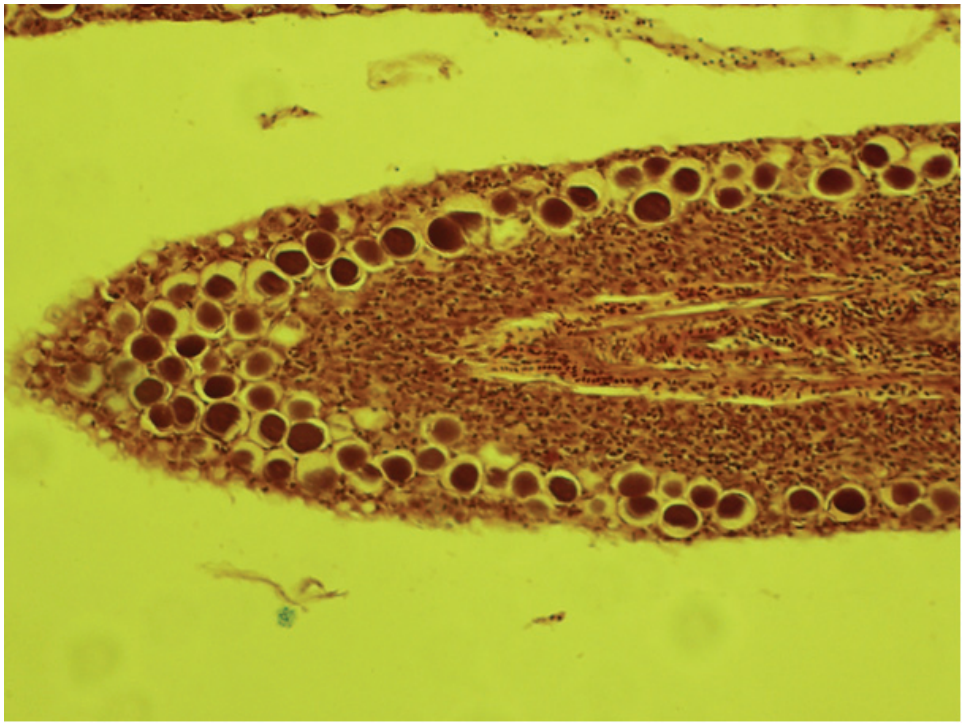

Fig. 2. Intensive epitheliocystis infection in the multilayered epithelium of the tip of a gill filament. HE staining, × 500

The sequence of the EP2 common carp sample from fish farm 1 was related to the endosymbiont of *Acanthamoeba* sp. (AF098330) and *Neochlamydia hartmannellae* (AF177275) with 85.1% and 85.8% similarity, respectively. The sequence of the EP4 common carp sample from fish farm 2 showed close similarity to *Protochlamydia naegleriophila* from different sources (DQ632609, FJ976103, EU384664, and FJ976101). The sample differed from them in 12.6%, 12.6%, 12% and 15.4%, respectively (Fig. 4). In the control tissue samples not

presenting visible chlamydial infection by light microscopy no sequences resembling known epitheliocystis agents were detected.

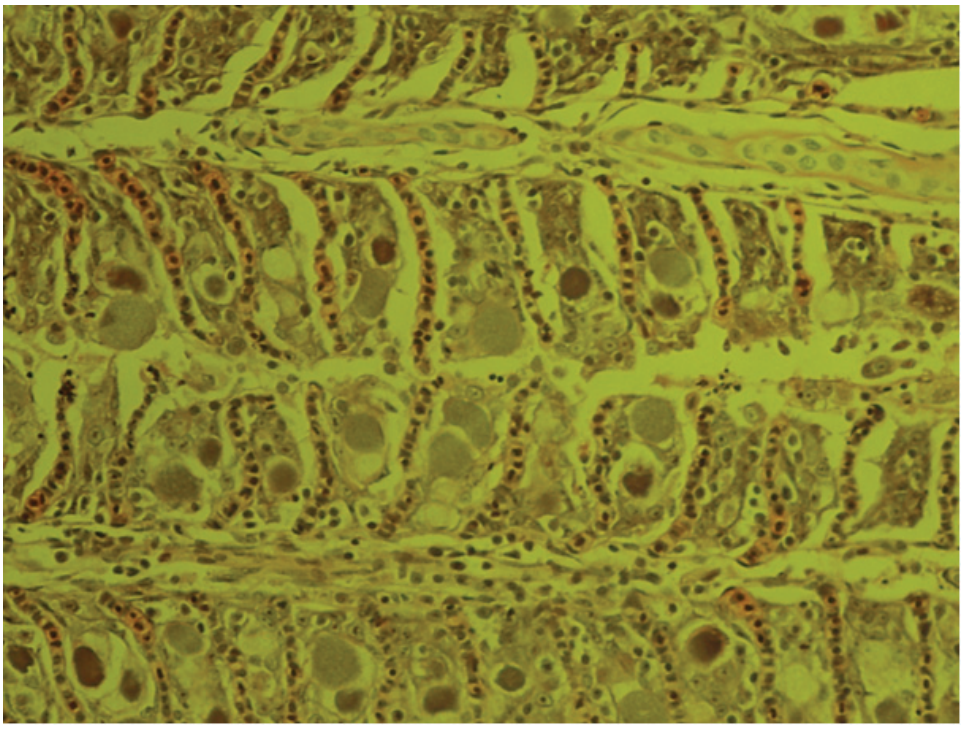

Fig. 3. Advanced epitheliocystis infection in the gills of a common carp fingerling. Some of the infected cells have compact, dark-staining, centrally located bacterial inclusions; in some pale-staining cells (arrows) bacteria have been dissolved in the mucous material of the degenerated cells. HE staining, × 500

Discussion

Epitheliocystis causes significant economic losses to aquaculture all over the world (Nowak and Clark, 1999; Crespo et al., 2001). This infectious gill disease, histologically characterised by cysts in the branchial epithelia of the hosts, has been investigated mainly in marine fish species, although freshwater fishes are also affected (Nowak and LaPatra, 2006). Recent studies have revealed that several, primarily *Chlamydia*-like pathogens may contribute to the pathological changes developing in the gills (Stride et al., 2014).

Our observations on fingerlings of cultured common carp and wild gibel carp indicated the presence of different *Chlamydia*-like bacteria (*Proto*-, *Neo*-, and *Piscichlamydia*) as causative agents in epithelial infections of cyprinids. Epi-

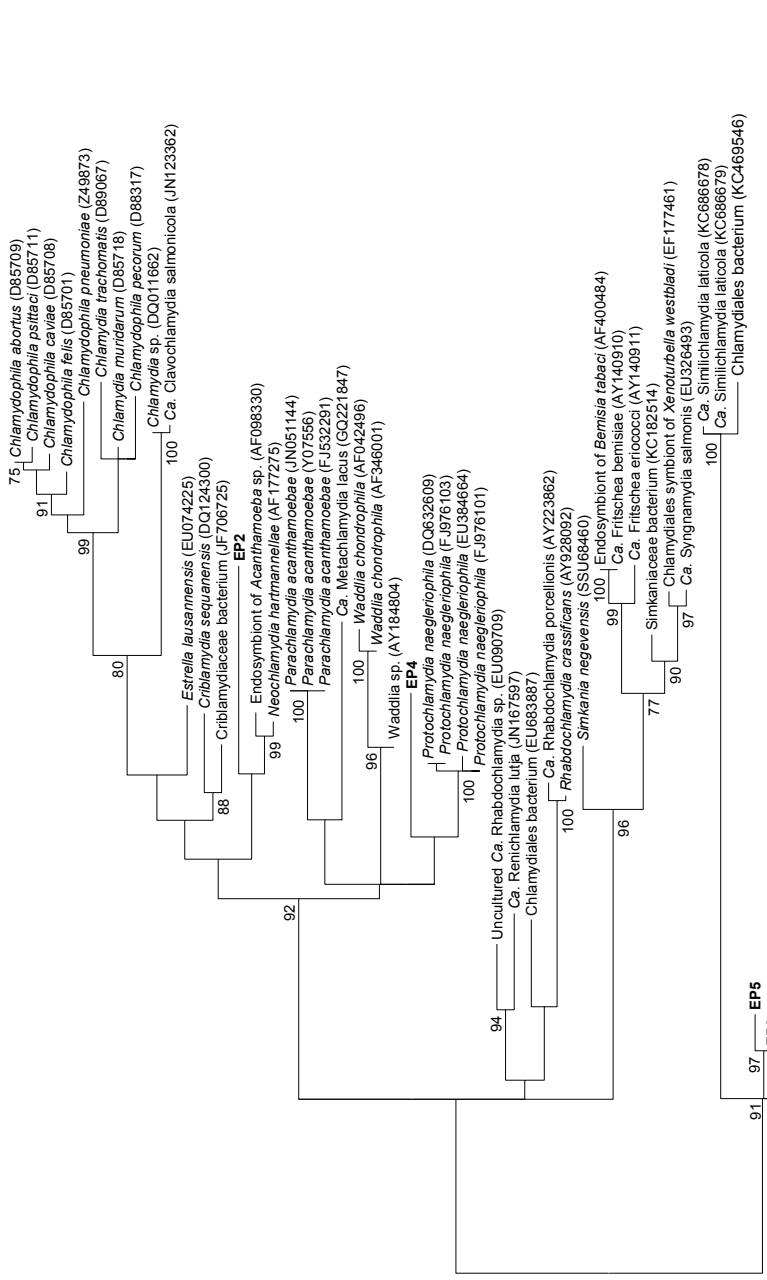

Fig. 4. Phylogenetic tree inferred using a Maximum Likelihood analysis (MEGA6) of a 200-bp region of the 16S rRNA gene sequence from members of the phylum Chlamydia, showing the positions of cyprinid samples (EP, EP2, EP4, EP5, and EP6). Bootstrap support value (> 75%) at the nodes

theliocystis induced by various, distantly related organisms (*Candidatus* Piscichlamydia salmonis, *Candidatus* Clavichlamydia salmonicola and *Candidatus* Branchiomonas cysticola) has already been detected in Atlantic salmon as well (Nylund et al., 2015). Our results demonstrate that within a given host species diverse causative agents may be in the background of epitheliocystis infections, even if the hosts are in identical or very similar environments.

The variability mentioned above may be associated with the manifestation of the disease. Epitheliocystis occurs as a benign or severe proliferative disease, according to the inflammatory host response. In benign infections apparently only a slight host response could be detected, even in the presence of large numbers of cysts surrounded by a thin epithelial envelope layer. While the intense, proliferative host response induces hyperplasia in the branchial epithelium, the cysts forming concentric layers can cause respiratory insufficiency and mortality by obstructing the capillary network of the gill filament (Arkush and Bartholomew, 2011).

In the cases studied by us mostly benign infections were observed, but proliferative changes were also recorded. These latter changes were restricted to the multi-layered epithelium and never affected the epithelium of the respiratory lamellae.

In our specimens three different putative epitheliocystis agents, related to *Neochlamydia*, *Protochlamydia* and *Piscichlamydia*, were detected by sequence analysis of short regions of the 16S rRNA gene. *Piscichlamydia* is a typical pathogenic agent of epitheliocystis, which was identified first (Draghi et al., 2004) in gill cysts of Atlantic salmon *(Salmo salar)*. The proliferative changes were associated with heavy mortality and reduced growth of survivors on salmonid farms in Ireland and Norway. *Neochlamydia hartmanellae* and *Protochlamydia naegleriophia* (Horn et al., 2000; Michel et al., 2000), the initial members of their families, were first discovered as parasites of free-living amoebae *(Hartmannella vermiformis, Naegleria lovaniensis)*. These bacteria could grow and survive in amoeboid hosts, which means that they had adapted to hidden intracellular life without killing their host. Occasionally they could be detected in both human and animal diseases (von Bomhard et al., 2003; Baud et al., 2008; Casson et al., 2008). Our observations suggest that the role of amoebae as the environmental reservoir of some epitheliocystis agents should be reconsidered.

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