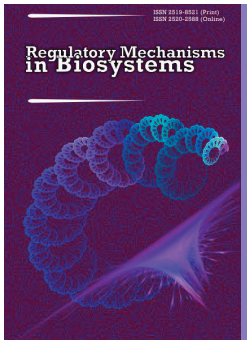

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Epitheliocystis: Development of PCR assay for the monitoring among the commercially important aquaculture species of Ukraine

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Epitheliocystis is an emerging disease of wild and cultured fish caused by a number of bacterial species, characterized by the presence of cytoplasmic bacterial inclusions in the epithelial cells of the gills, which contribute to the merging of the gill plates, and in some cases also spread to the skin of fish. This disease may manifest as hypertrophy and inflammation of the gills, white nodular lesions of epithelial tissue in the gills or skin, gasping on the surface of the water, lethargy, poor swimming and stunted growth. Among the commercially important aquaculture species of Ukraine, such as Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus*), Candidatus *Clavochlamydia salmonicola* and Candidatus *Piscichlamydia salmonis* are associated with epitheliocystis. There are currently no tools at the disposal of ichthyologists and veterinary laboratories in Ukraine to identify Ca. *C. salmonicola* and Ca. *Piscichlamydia salmonis*. Our basic concern was to develop a PCR assay of epitheliocystis diagnosis. We suggest the use of general primers for simultaneous detection of Ca. *C. salmonicola* and Ca. *Piscichlamydia salmonis*. The developed PCR assay for identification of Ca. *C. salmonicola* and Ca. *Piscichlamydia salmonis* has shown its suitability for amplifying control DNA. Confirmation of the amplification products identity was performed using selective recognition of the sequence by the TasI restriction endonuclease (Thermo Fisher Scientific, US). Analytical specificity verification of the PCR assay performed by amplifying the control DNA of 10 species of the Chlamydiales order showed the absence of PCR products, but observed in one. The designed PCR assay, after approbation on clinical material, can be used by researchers for extensive monitoring of epitheliocystis, doctors of veterinary medicine for diagnosis clarification, in addition to introduction into the practice of veterinary medicine laboratories and implementation in fish farm improvement programmes. The amplicon size of 197 base pairs theoretically permits application of this oligonucleotide primers pair for real-time PCR.

Keywords: Ca. *Piscichlamydia salmonis*; Ca. *Clavochlamydia salmonicola*; chlamydial infections in fish; Ukraine.

Introduction

Epitheliocystis is a common disease of fish affecting the gills and skin of fish caused by a number of bacteria species, characterized by the presence of "cysts" (cytoplasmic bacterial inclusions) in the epithelial cells of the gills, which leads to the fusion of gill lamellae and on rare occasions spreads to the fish skin. Epitheliocystis may be the cause of hypertrophy and inflammation of the gills, white nodular lesions of epithelial tissues of gills or skin, gasping at the water surface, lethargy, weak swimming behaviour and growth retardation (Lewiset al., 1992). Microscopically, infected epithelial cells progressively enlarge and contain spherical cysts filled with bacteria encapsulated by a membrane. There have been cases where inflammation extended to other tissues, causing severe respiratory disturbances and death of fish (Meijer et al., 2006; Draghi et al., 2007). Syasina et al. (2004) described the case of the disease in a population of fish with high mortality rate (up to 100%). In this case, in addition to the clinical manifestations listed above, the disease was manifested by exophthalmos, lens lesion, phacocele, corneal clouding and blindness, as well as skin ulcers (Syasina et al., 2004). The aforementioned disease has been first reported as "mucophilosis" in 1924 (Demollet al., 1924), the term "epitheliocystis" appeared later, in 1969 (Hoffman et al., 1969). To date, epitheliocystis has been described in more than 90 fish species around the world (Pawlikowska-Warych et al., 2016). Despite this, epitheliocystis remains one of the unexplored diseases of fish in Ukraine. Ukraine lost about 2/3 of its fish catch after the Crimea was annexed. The domestic fish industry shows signs of development in accordance with international standards: more and more fish farms are being built, which in the future will allow Ukraine to reduce the share of imports in the consumption structure (Conference Fish and Seafood, 2018).

On the territory of Transcarpathia region are the largest reserves of freshwater resources in Ukraine, and accordingly the largest concentration of aquatic biological resources, more than 75 percent of which are valuable as well for species that have a special conservation status. The Carpathian Biosphere Reserve is engaged in breeding brown trout (*Salmo trutta*) for release into numerous mountain rivers and streams to support natural populations. In addition, the number of private family-owned enterprises for brown trout breeding is growing because this fish is very expensive and valuable, and the natural conditions of Transcarpathia are favourable for the development of such mini-farms.

Atlantic salmon (*Salmo salar*) is one of the most intensively farmed marine fish all over the world (Naylor et al., 2005). Atlantic salmon fish farms have begun to develop in Ukraine, and the number of such farms will increase due to the high profitability of such enterprises.

According to the data of State Agency of Fisheries of Ukraine, growing marketable fish in aquaculture conditions amounted to 20.2

thousand tons, of which 15.9 thousand tons of aquaculture products were harvested, according to data for the last year. Carp and Far Eastern herbivorous fish accounted for 87% of total aquaculture production. The production of European catfish, pike, trout, and sturgeon totals 13%. Ukrainian fish and fish products are exported to Europe. Aquaculture has a significant amount of animal protein, and according to this index it takes the third place after growing livestock and poultry (Public report of the State Agency of Fisheries of Ukraine for 2018). All of the above shows the growth prospects of the fisheries sector of Ukraine.

Many species of *Chlamydia*-like bacteria are associated with epitheliocystis in commercially important aquaculture species, including Candidatus *Clavochlamydia salmonicola*, Candidatus *Piscichlamydia salmonis*, Candidatus *Parilichlamydia carangidicola*, Candidatus *Actinochlamydia clariae*, Candidatus *Similichlamydia laticola*, Candidatus *Similichlamydia labri*, Candidatus *Similichlamydia latridicola*, Candidatus *Renichlamydia lutjani*, *Syngnamydia venezia*, *Neochlamydia*-like.

Amongst all known species of *Chlamydia*-like bacteria associated with epitheliocystis only Ca. *Clavochlamydia salmonicola*, Ca. *Piscichlamydia salmonis* and *Syngnamydia venezia* can be distinguished in Ukraine, since their hosts are common in these parts (Shcherbukha, 1987; Zezekalo et al., 2019). And only Ca. *C. salmonicola* and Ca. *P. salmonis* are associated with epitheliocystis infection among the commercially important aquaculture species, including Atlantic salmon (*Salmo salar*) (Karlsen et al., 2008; Schmidt-Posthaus et al., 2012; Guevara et al., 2016) brown trout (*Salmo trutta*) (Blandford et al., 2018); grass carp (*Ctenopharyngodon idella*) (Kumaret al., 2013), common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus*) (Sellyei et al., 2017). Moreover, the problem of epitheliocystis has not been studied, there is no information about epitheliocystis in scientific papers in Ukrainian or Russian. However, there are many diseases of the gills and skin in fish, as well as diseases of fish juveniles, leading to their death described. Notwithstanding the significance of *Chlamydia*-like bacteria species associated with epitheliocystis, studies on their prevalence among wild and farmed fish in Ukraine have not been performed due to the lack of available diagnostic methods. Presently, there is no toolkit for identification of Ca. *C. salmonicola* and Ca. *P. salmonis* at the disposal of ichtyopathologists and veterinary laboratories in Ukraine. The objective of our work was developing a PCR assay, an inexpensive and simple method of epitheliocystis diagnosis.

Materials and methods

The research was performed applying the polymerase chain reaction (PCR). It was conducted in the laboratory of animal health and the laboratory of genetics of the Institute of Pig Breeding and Agro-Industrial Production, NAAS, which is certified for genetic analysis at the DNA level (Compliance certificate "state of the measurement system" number 021-19 from 01/31/2019).

Alignment of 111 nucleotide sequences of 36 bacteria of the order Chlamydiales was implemented by using the MEGA7 software, for detection of the presence of polymorphic hybridization site of both Ca. *C. salmonicola* and Ca. *P. salmonis*. NCBI Primer design tool www.ncbi.nlm.nih.gov/tools/primer-blast was used to design oligonucleotide primers. Online service Primer-BLAST was used to ensure a target specificity of the primers. Oligonucleotide primers for detection of Ca. *P. salmonis* and Ca. *C. salmonicola* in single PCR assay were synthesized (by Metabion international AG, Germany). The indicated pair of oligonucleotide primers flanking the 16S rRNA gene fragment sizing 197 bp. In addition to the oligonucleotide primers, the reaction mixture included reagents manufactured by Thermo Fisher Scientific, were used in PCR assays: deionized water, PCR buffer, $MgCl_2$, solution of deoxyribonucleoside triphosphates (dNTP) and Taq polymerase.

DNA size marker pUc19/MspI (Thermo Fisher Scientific, US) was used as DNA marker. DNA amplification was performed on a Tercyc-2 multichannel thermo cycler (DNA technology, Russia). The amplification products and restriction products were separated by using 2% agarose gel electrophoresis in an electrophoresis chamber (Cleaver Scientific Ltd, UK). On completion of the electrophoresis process, the gel was stained with a solution of ethidium bromide (10 mg/cm^3) and outcome of electrophoresis was registered by gel documentation system (Cleaver Scientific Ltd, UK). Confirmation of the identity of the amplification products was performed using selective recognition of the sequence by the TasI restriction endonuclease (Thermo Fisher Scientific, US).

The following control DNA samples were used: *Parachlamydia acanthamoebae* strains Berg17 and Bn9 were kindly provided by Dr. Michel Rolf (Central Military Hospital Koblenz, Germany), DNA of *Parachlamydia acanthamoebae*, strain Hall obtained from Prof. Gilbert Greub (l'Institut de Microbiologie Médecin chef des laboratoires de microbiologie diagnostique Institut de Microbiologie de l'Université de Lausanne, Switzerland), DNA samples of *Waddlia chrondophila*, *Chlamydia avium*, *Chlamydia pecorum*, *Chlamydia abortus*, *Chlamydia psittaci*, *Chlamydia suis*, *Chlamydia caviae*, *Chlamydia trachomatis*, *Chlamydia abortus*, *Chlamydia felis*, *Chlamydia muridarum*, *Chlamydia pneumoniae*, *Chlamydia gallinacea*, were obtained from Dr. Christiane Schnee (Institut für molekulare Pathogenese, Jena, Germany). Ca. *C. salmonicola* and Ca. *P. salmonis* samples were obtained from Dr. Heike Schmidt-Posthaus, (Center for Fish and Wildlife Health, Bern, Switzerland).

Analysis of the causative epitheliocystis agents is complicated by an inability to cultivate them for refined in vitro experimentation. Epitheliocystis has not been studied in Ukraine yet, therefore, for functional verification of developed PCR assay we have used DNA samples kindly provided by Dr. Heike Schmidt-Posthaus.

Results

Oligonucleotide primers were designed for simultaneous detection of two epitheliocystis agents (Ca. *P. salmonis* and Ca. *C. salmonicola*): forward – PCSALF: GCTAACGCGATAAGTGTGCC, and reverse – PCSALR: CCATGCAGCACCTGTGTAGT, with expected PCR product size of 197 base pairs.

Gel electrophoresis of PCR products of amplification of selected oligonucleotide primers with control DNAs of Ca. *P. salmonis* and Ca. *C. salmonicola* determined the size of the amplified DNA fragments as 197 bp. The fragments corresponded to the expected sizes of the DNA fragments of 16S rRNA gene Ca. *P. salmonis* and Ca. *C. salmonicola*, therefore functional verification of PCR assay was performed (Fig. 1).

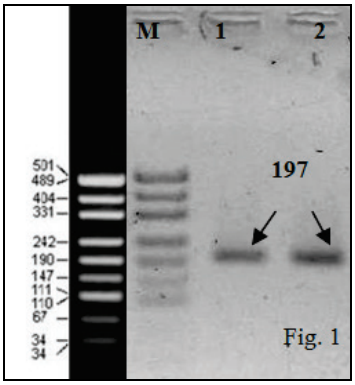

Fig. 1. Electrophoregram of amplification Ca. *P. salmonis* and Ca. *C. salmonicola* control DNAs with PCSALF/PCSALR primers: M – DNA size marker pUc19/MspI; *1* – PCSALF/ PCSALR primers with DNA of Ca. *P. salmonis* (197 bp), *2* – PCSALF/PCSALR primers with DNA of Ca. *C. salmonicola* (197 bp)

The restriction enzyme TasI was selected for PCR product digestion, the sizes of the fragments generated corresponds to the predicted sizes (40, 57, 100 bp, for Ca. *P. salmonis* and 13, 40, 44, 100 bp, for Ca. *C. salmonicola*). Hereby the amplification products identity was confirmed (Fig. 2).

Analytical specificity verification of the PCR assay performed by amplifying the control DNA of 10 species of Chlamydiales order showed the absence of PCR products, but observed in one. Consequently, the analytical specificity of the PCR product was confirmed (Fig. 3).

The developed PCR assay for the identification of Ca. *C. salmonicola* and Ca. *P. salmonis* has shown its suitability for amplifying control DNA

(Fig. 1). Primers used in this study were able to direct the amplification of DNA from both tested Ca. *C. salmonicola* and Ca. *P. salmonis*, giving a band of the expected size after gel electrophoresis. Restriction enzyme TasI gave fragments of the expected size, demonstrating that the amplified DNA was specific for Ca. *C. salmonicola* and Ca. *P. salmonis* (Fig. 2). The developed PCR assay demonstrated high analytical specificity – lack of amplification (except expected) on the control DNA matrix of 10 species belonging to the order of Chlamydiales (*Parachlamydia acanthamoebae*, *Waddlia chondrophila*, Ca. *Clavochlamydia salmonicola*, Ca. *P. salmonis*, *Chlamydia avium*, *Chlamydia pecorum*, *Chlamydia abortus*, *Chlamydia psittaci*, *Chlamydia suis*, *Chlamydia caviae*) (Fig. 3).

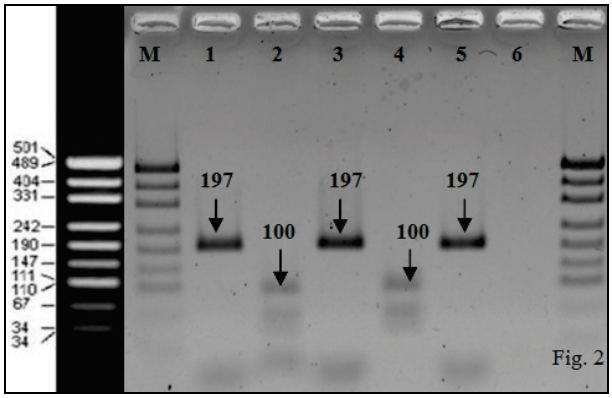

Fig. 2. Electrophoregram of restriction analysis of amplification fragments: M – DNA size marker pUc19/MspI; *1* – PCSALF/PCSALR primers with DNA of Ca. *P. salmonis* (197 bp) uncut control, *2* – PCSALF/PCSALR primers with DNA of Ca. *P. salmonis* digested with TasI restriction enzyme (40, 57, 100 bp); *3* – PCSALF/PCSALR primers with DNA of Ca. *C. salmonicola* (197 bp) uncut control, *4* – PCSALF/PCSALR primers with DNA of Ca. *C. salmonicola* digested with TasI restriction enzyme (13, 40, 44, 100 bp), *5* – K^+, PCSALF/PCSALR primers with DNA of Ca. *P. salmonis* and DNA of Ca. *Clavochlamydia salmonicola*, *6* –K^-, PCSALF/PCSALR primers with deionized water instead of DNA

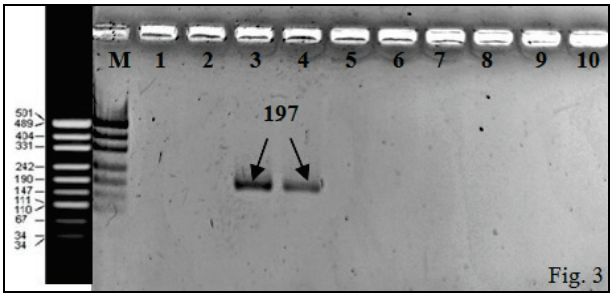

Fig. 3. Electrophoregram of PCR amplification with primers PCSALF/PCSALR DNA of 10 species of Chlamydiales: *M* – DNA size marker pUc19/MspI; *1 – Parachlamydia acanthamoebae*; *2 – Waddlia chondrophila*; *3* –Ca. *C. salmonicola* (197 bp); *4* –Ca. *P. salmonis* (197 bp); *5 – Chlamydi aavium*; *6 – Chlamydia pecorum*; *7 – Chlamydia abortus*; *8 – Chlamydia psittaci*; *9 – Chlamydia suis*; *10 – Chlamydia caviae*

Discussion

Despite the fact that epitheliocystis was first mentioned back in 1924 (Demoll et al., 1924) and that the term "epitheliocystis" appeared in 1969 (Hoffman et al., 1969), we still have an information gap about epitheliocystis in Ukraine. Moreover, little is known about this disease in the available Ukrainian and/or Russian scientific sources (Zezekalo et al., 2019). To date, only two bacteria (Ca. *P. salmonis* and Ca. *C. salmonicola*) from the Chlamydiales order, which cause pathogenic lesions in commercially important aquaculture species of Ukraine, have been described (Shcherbukha, 1987; Movchan, 2011; Pawlikowska-Warych et al., 2016; Zezekalo et al., 2019).

The diagnosis of epitheliocystis in fish is mainly based on the clinical signs of the disease, including visible damage of gills and skin, changes in fish behaviour with subsequent tissue microscopy to determine characteristic morphological changes. This is a time consuming and laborious process, not suitable for wide screening, as well as for screening for prophylactic purposes, as epitheliocystis can be occasionally asymptomatic (Pawlikowska-Warych et al., 2016). There are no culture methods for detection of the causative agent (Nowak et al., 2006). In this regard, molecular genetic techniques are mainly used to diagnose epitheliocystis in fish (Schmidt-Posthaus et al., 2012; Kumar et al., 2013).

There are foreign-made PCRs for identification Ca. *P. salmonis* (Draghi et al., 2004; Karlsen et al., 2007) and Ca. *C. salmonicola* (Schmidt-Posthaus et al., 2012; Toenshoff et al., 2012; Guevara Soto et al., 2016), which in the current economic situation are not available for mass use in Ukraine, due to the high price and/or the necessity for expensive equipment (for real-time PCR tests). Taking advantage of the sequence homology (revealed similarity 81%) between these epitheliocystis agent (Ca. *C. salmonicola* and Ca. *P. salmonis*), we propose the use of general primers for their simultaneous detection. This approach seems more convenient than using specific primers for Ca. *C. salmonicola* and Ca. *P. salmonis* or a mixture of such primers in a single tube. To our knowledge, this is the first report of PCR assay for detection both Ca. *C. salmonicola* and Ca. *P. salmonis* with a single test for the wide screening and/or confirmation of epitheliocystis diagnosis. This type of approach could be highly practical in the tough economic environment of modern Ukraine.

Conserved 16S rRNA gene was selected as the target gene. This would allow identifying both spices with a single test for epitheliocystis, since this conservative gene is the most universal among bacteria, and has hypervariable regions, which allows one to choose a specific region for both species. (Chakravorty et al., 2007). We used selective sequence recognition by endonucleases restriction. The restriction of the digestion profiles showed that the amplification bands visualised by ethidium bromide staining were specific for both Ca. *C. salmonicola* and Ca. *Piscichlamydia salmons*.

The developed PCR assay for identifying both types of *Chlamydia*-like organisms in one PCR test kit has an advantage over methods using separate tests to determine each type, since it is cheaper and suitable for testing a large number of samples in order to investigate the disease, since epitheliocystis treatment is alike regardless of the pathogen. For scientific purposes, it is possible to use a restriction analysis or individual PCR tests, to determine a specific agent.

Conclusion

Identification of epitheliocystis will promote the growth of profitability of aquaculture and will allow control of chlamydial infection in commercially important farmed fish species of Ukraine. The designed PCR assay, after approbation on clinical material can be used by scientists for extensive monitoring of epitheliocystis, by doctors of veterinary medicine for diagnosis clarification, in addition to introduction into the practice of veterinary medicine laboratories and implementation in improvement programmes for fish farms. The PCR assay for recognition of Ca. *C. salmonicola* and Ca. *P. salmonis* can be suggested for clinical trials in Ukraine. Our PCR assay is considerably simpler to use, faster, and carries lower cost than other diagnostic techniques which use separate tests to determine each of the species. The expected amplicon size 197 bp theoretically allows application of this pair of oligonucleotide primers for real-time PCR. Still, the clinical specificity and clinical sensitivity of the developed PCR technique should be evaluated in a statistically-valid amount of sampling.

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Directeur de l'Institut de Microbiologie Médecin chef des laboratoires de microbiologie diagnostique Institut de Microbiologie de l'Université de Lausanne, Switzerland), Dr. Christiane Schnee (Arbeitsgruppenleiterin, Institut für molekulare Pathogenese, Jena, Germany), Heike Schmidt-Posthaus, PD, Dr. med vet, Dipl. ECVP (Centre for Fish and Wildlife Health Bern) for the invaluable help you gave us in the form of control DNA samples, thanks to which it became possible to carry out our research.

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