Part 2. Biotechnology and genetics

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PCR TEST SYSTEMS FOR THE *CLAVICHLAMYDIA SALMONICOLA* AND *PISCICHLAMYDIA SALMONIS* DETECTION IN FISH

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Summary. The aim of our work was to develop PCR test systems for the identification and differentiation of the *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*, species, that are known epitheliocystis infection agents of gill and fish skin diseases, characterized by the presence of specific 'inclusions' in the epithelial cells of the gills. To date, the diseases of fish associated with chlamydial infections have been detected in more than 90 species of freshwater and marine fish worldwide. For now, there is no available information on the prevalence of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*, which can cause epitheliocystis of commercially important aquaculture species in Ukraine. Identification of these pathogens is possible only using molecular genetic methods. As a result of our research, we got PCR tests for the identification and species differentiation of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*. The use of diagnostics for the identification of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola* makes chlamydial infections monitoring among various fish species possible and it will increase the economic efficiency of fish farms.

Keywords: *Piscichlamydia salmonis*, *Clavochlamydia salmonicola*, chlamydial infections in fish, Ukraine, PCR, Atlantic salmon, *Salmo salar*, brown trout, *Salmo trutta*

Introduction. Bacteria of the order Chlamydiales are gram-negative, obligate, and intracellular with a special two-phase development cycle, associated with disease in a wide range of hosts (Ksonz, 2012). More than 400 host species are documented worldwide, most of which are wild animals (Burnard and Polkinghorne, 2016). To date, chlamydial infections have been described in more than 90 freshwater and marine fish species around the world (Pawlikowska-Warych and Deptuła, 2016; Taylor-Brown et al., 2015).

Bacteria of the order Chlamydiales are associated with epitheliocystis, a common condition of the fish gill and fish skin epithelium, characterized by the presence of cytoplasmic bacterial inclusions in the epithelial cells of the gills and on rare occasions spreads to the fish skin (Stride, Polkinghorne and Nowak, 2014; Sellyei, Molnár and Székely, 2017). For the first time, the aforementioned disease has been reported as 'mucophilosis' and was described in the common carp (*Cyprinus carpio*) in 1924 (Plehn, 1924), the term 'epitheliocystis' appeared later, in 1969 (Hoffman et al., 1969).

In most cases epitheliocystis in fish, especially free-living, has a chronic course. This is not a life-threatening disease. Epitheliocystis may cause hypertrophy and inflammation, white nodular lesions of epithelial tissues of gills or skin, gasping at the water surface, lethargy, weak swimming behavior and growth retardation (Lewis et al., 1992). There are known the cases when inflammation extended to other tissues, causing severe respiratory disturbances and death of fish (Meijer et al., 2006; Draghi II et al., 2007). Syasina, Park and Kim (2004) described the case of the disease in a population of fish with a high mortality rate (up to 100%). In addition to clinical manifestations mentioned above, the disease was manifested by exophthalmos, lens lesion, phacocele, corneal clouding and blindness, as well as skin ulcers (Syasina, Park and Kim, 2004).

Initially, it was thought that the same etiologic agent caused epitheliocystis in all fish species, but already in 1977 it was recognized that these bacteria demonstrated a high degree of host specificity (Zachary and Paperna, 1977).

As for *Chlamydia*-like bacteria associated with epitheliocystis, they include:

Candidatus Clavochlamydia salmonicola,
Candidatus Piscichlamydia salmonis,
Candidatus Parilichlamydia carangidicola,
Candidatus Actinochlamydia clariae,
Candidatus Similichlamydia laticola,
Candidatus Similichlamydia labri,
Candidatus Similichlamydia latridicola,
Candidatus Renichlamydia lutjani,
Candidatus Syngnamydia venezia,
and *Neochlamydia*-like bacteria.

Ca. P. salmonis is associated with chlamydial infection in the Atlantic salmon (*Salmo salar*) (Draghi II et al., 2004), and in the brown trout *(Salmo trutta)* (Schmidt-Posthaus et al., 2012). Other family members of Piscichlamydiaceae might be a causative agent of the epitheliocystis of the grass carp (*Ctenopharyngodon idella*) (Kumar et al., 2013), the common carp (*Cyprinus carpio*) and the Gibel carp (*Carassius gibelio*) (Sellyei, Molnár and Székely, 2017).

Ca. C. salmonicola is known to be an intracellular bacteria and infects salmonid fish. For example, the Atlantic salmon (*Salmo salar*) and the brown trout (*Salmo trutta*) are extremely valuable species bred in Ukraine (Schmidt-Posthaus et al., 2012; Guevara Soto et al., 2016; Karlsen et al., 2008; Blandford et al., 2018; Shcherbukha, 1987).

Other *Chlamydia*-like bacteria, such as *Ca.* P. carangidicola, are pathogens of the yellowtail kingfish (*Seriola lalandi)* (Stride et al., 2013a); *Ca.* S. laticola is a pathogen of the Australian barramundi (*Lates calcarifer*) (Stride et al., 2013c); *Ca.* S. labri causes epitheliocystis in the ballan wrasse *(Labrus bergylta),* native inhabitants of the northeastern Atlantic (Steigen et al., 2015)*; Ca.* S. latridicola was isolated from the striped trumpeter (*Latris lineate*), native to the temperate oceans of New Zealand and the eastern coast of Australia (Stride et al., 2013b); *Ca.* A. clariae causes epitheliocystis in the catfish (*Clarias gariepinus*) in Uganda (Steigen et al., 2013); *Ca.* S. venezia is associated with chlamydial infection in the broad nosed pipefish (*Syngnathus typhle*) (Fehr et al., 2013). There are reports of *Neochlamydia*-like bacteria that associated with epitheliocystis in the Arctic charr (*Salvelinus alpinus*) (Draghi II et al., 2007).

Among all these types of chlamydia-like bacteria only *Ca.* C. salmonicola*, Ca. P. salmonis*, and *Ca.* S. venezia can be distinguished in Ukraine, since their owners are common in our country, from which only *Ca.* C. salmonicola and *Ca.* P. salmonis are associated with epitheliocystis infection among the commercially important aquaculture species of Ukraine.

For today, there are no test systems in the arsenal of ichtyopathologists and laboratories of veterinary medicine of Ukraine, which allows detecting, identifying and differentiating *Ca.* C. salmonicola and *Ca.* P. salmonis.

The aim of our work was to develop PCR test systems for the identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis, epitheliocystis agents of commercially important aquaculture species of Ukraine.

Materials and methods. The studies were carried out in the Laboratory of animal health and the Laboratory of genetics of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Agrarian Sciences, which is certified for DNA genetic analyses (Compliance certificate 'state of the measurement system' number 021-19 from 01/31/2019).

Bioinformatics studies were focused on finding the specific region of various types of chlamydia pathogens. Analysis was performed by aligning 111 primary nucleotide sequences of the 16S rRNA gene using the MEGA7 software (Tamura et al., 2007). Species-specific oligonucleotide primers were designed and tested for absence of complementarity with the nucleotide sequences of other microorganisms using the online service Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi).

Molecular genetic analysis was performed using PCR. DNA samples of *Chlamydia*-like organisms pathogenic for fish: *Ca.* C. salmonicola and *Ca.* P. salmonis were used as the positive controls. DNAs were kindly provided by Dr. Heike Schmidt-Posthaus (Center for Fish and Wildlife Health, Bern, Switzerland). The control DNA samples of *Parachlamydia acanthamoebae* strains Berg17 and Bn9 were kindly provided by Dr. Michel Rolf (Central Military Hospital, Koblenz, Germany), DNA of *Parachlamydia acanthamoebae* strain Hall's coccus was received from Prof. Gilbert Greub (Institute of Microbiology of the University of Lausanne, Switzerland), DNA samples of *Waddlia chondrophila, Chlamydia avium, Ch. pecorum, Ch. abortus, Ch. psittaci, Ch. suis, Ch. caviae* were received from Dr. Christiane Schnee (Institute of Molecular Pathogenesis, Jena, Germany). DNA amplification was performed on the 'Tercyc-2' multichannel thermocycler (DNA-Technology LLC, Russia).

Oligonucleotide primers were synthesized (by Metabion International AG): to amplify a fragment of 16S rRNA gene *Ca.* P. salmonis which forward is PICHSF:

CTAGACTAGAGTTCAAGGGGG

and reverse is PICHSR:

GCTAGGGTTGAGACTAGCTAC,

and *Ca.* C. salmonicola forward is CLACHSF:

GAGTTCGTTAAAGCGGGGGA

and reverse is CLACHSR:

CAGGTCTTTCTTGTCCCCAAG.

Amplification was performed according to the manufacturer's protocol (Thermo Fisher Scientific). The identity of the PCR amplification product *of Ca.* C. salmonicola was confirmed by restriction analysis using *Alu* I endonuclease, according to the manufacturer's protocol (Thermo Fisher Scientific).

PCR and restriction products were separated using 2% agarose gel electrophoresis in 1 × TBE buffer for 2 hours at a current of 50 mA in an electrophoresis chamber (Cleaver Scientific Ltd). Plasmids *pUC*19 hydrolyzed with *Msp* I endonuclease (Thermo Fisher Scientific) were used as a molecular weight marker. After the end of the electrophoresis process, the gel was stained with a solution of ethidium bromide (10 mg/cm^3) and the results of

electrophoresis were documented on a transilluminator using a digital camera.

Results of the study. For primers design, the 111 16S rRNA gene primary sequences of 36 species from Chlamydiales order were analyzed. Using MEGA7 software, alignment of primary sequences obtained from the international databases GenBank and NCBI was carried out, as a result of which the common nucleotide sequence segments were found for both bacteria, and also specific regions of the nucleotide sequences of *Ca.* C. salmonicola and *Ca.* P. salmonis were found.

Amplification of control *DNA*s of *Ca.* C. salmonicola and *Ca.* P. salmonis with corresponding primers and the following gel electrophoresis of PCR products determined the size of the amplified DNA fragments as 207 base pairs (bp) and 276 bp respectively, the fragments corresponded to the expected sizes of the DNA fragments of 16S rRNA gene of *Ca.* C. salmonicola and *Ca.* P. salmonis (Fig. 1).

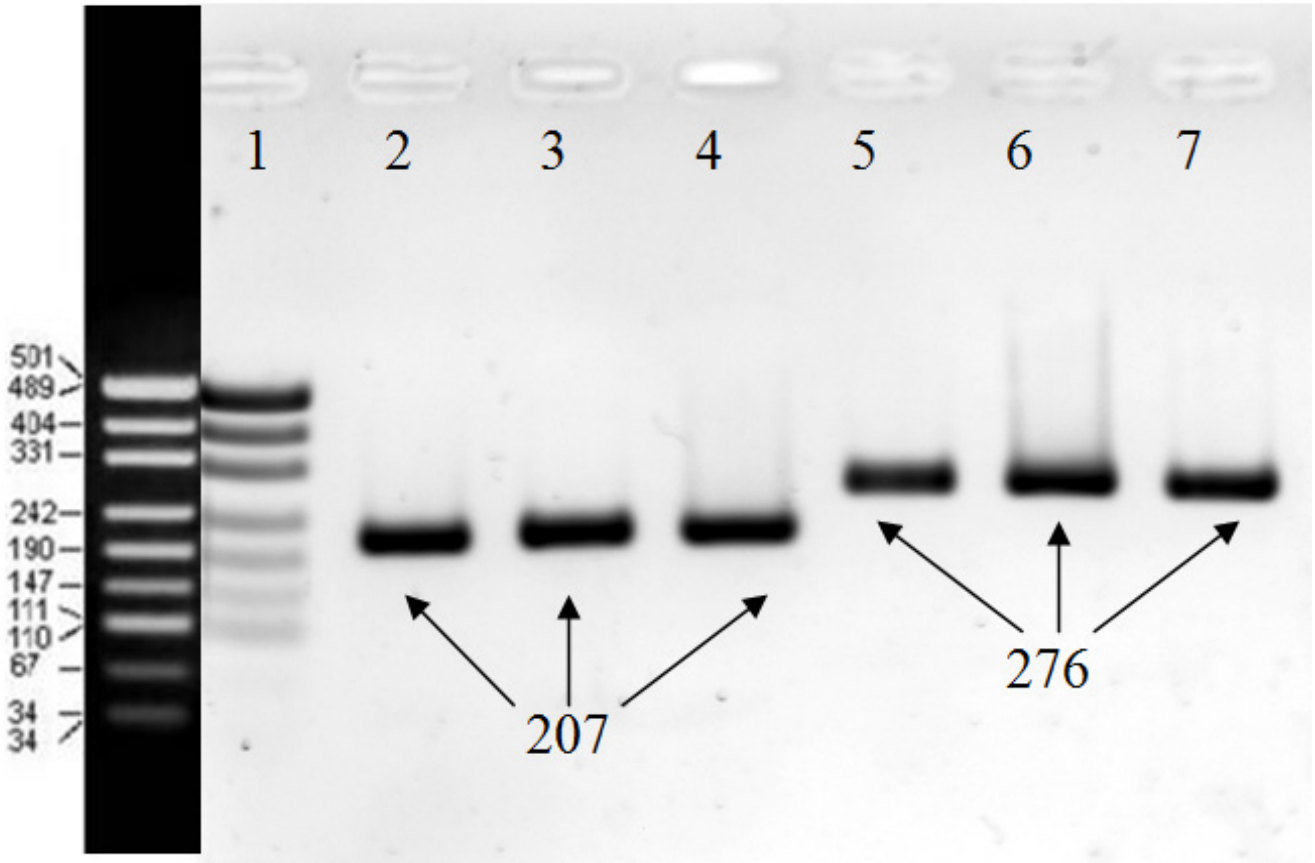

Figure 1. Electrophoregram of *Ca.* Piscichlamydia salmonis and *Ca.* Clavochlamydia salmonicola control DNAs amplification with PICHSF/PICHSR and CLACHSF/CLACHSR primers, using different annealing temperature: 1 — DNA size marker *pUc*19/*Msp*I; 2–4 — PCR detection of *Ca.* Piscichlamydia salmonis 58 °C, 60 °C, 63 °C respectively; 5–7 — PCR detection of *Ca.* Clavochlamydia salmonicola 58 °C, 60 °C, 63 °C respectively.

The restriction analysis with *Alu* I endonuclease demonstrated the formation of three DNA fragments of 61 bp, 19 bp, and 196 bp, corresponding to the expected with the primary nucleotide sequence of *Ca.* C. salmonicola (Fig. 2a). To verify the identity of the PCR product, for *Ca.* P. salmonis identification, was used a *Tas* I endonuclease that formed two fragments of 29 bp and 178 bp (Fig. 2b.).

Thus, the identity of the PCR amplification product has been proven. The verification of the PCR test analytical specificity performed by amplifying the positive control DNAs of 10 species from Chlamydiales order showed the absence of PCR products, but expected one (Fig. 4, 5).

Discussion. The diagnosis of epitheliocystis in fish is mainly based on the clinical signs evaluation, including visible damage to the gills and skin, changes in the behavior of the fish. It is only possible to make a preliminary diagnosis of epitheliocystis based on morphological changes that can be detected by microscopy, but the identification of the pathogen is possible only by using molecular genetic diagnostic methods (Blandford et al., 2018; Pawlikowska-Warych and Deptuła, 2016).

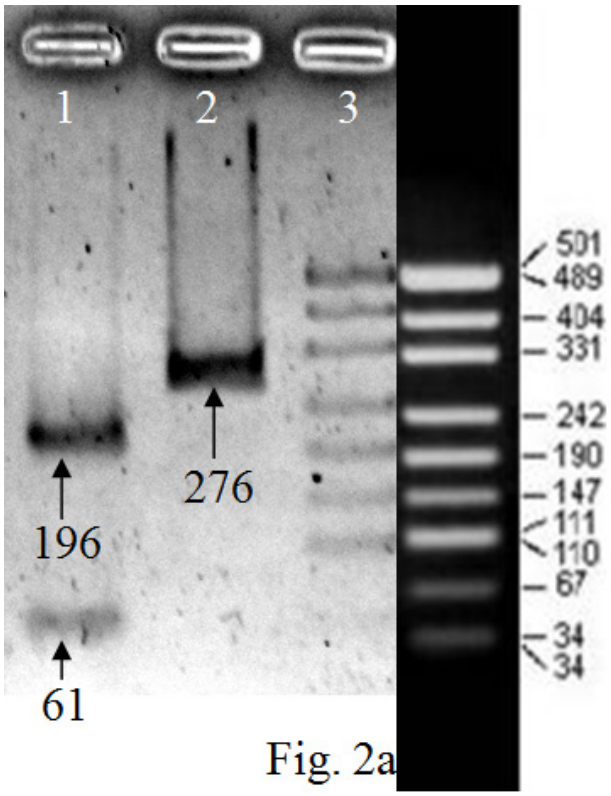

Figure 2a. Electrophoregram of *Ca.* Clavochlamydia salmonicola control DNA amplification with CLACHSF/CLACHSR primers and restriction of PCR product with *Alu* I endonuclease: 1 — restriction fragments of *Ca.* Clavochlamydia salmonicola PCR products (196 bp and 61 bp); 2 — absence of *Ca.* Clavochlamydia salmonicola DNA restricted PCR product (276 bp); 3 — DNA size marker *pUc*19 /*Msp*I.

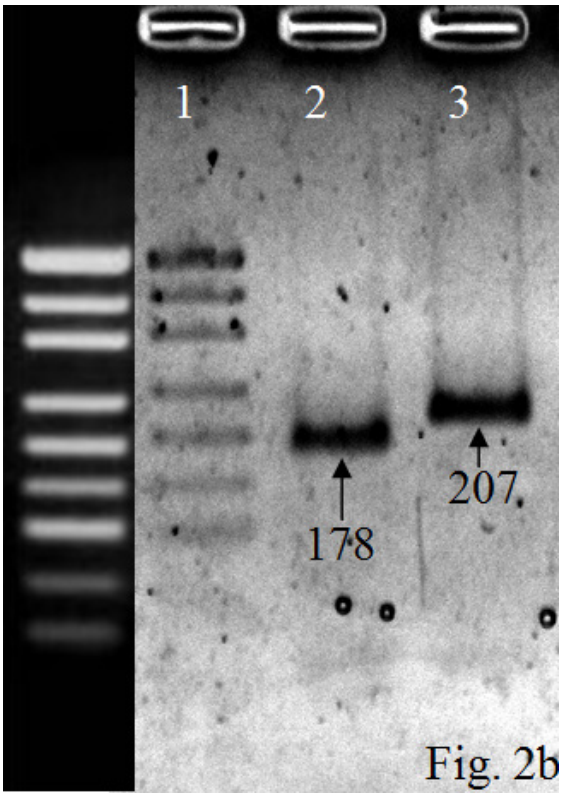

Figure 2b. Electrophoregram of *Ca.* Piscichlamydia salmonis control DNA amplification with PICHSF/PICHSR primers and restriction analysis with *Tas* I endonuclease: 1 — marker for DNA size *pUc*19/*Msp*I; 2 — restriction fragments of *Ca.* Piscichlamydia salmonis PCR product (178 bp and 29 bp are not shown in the picture); 3 — absence of *Ca.* Piscichlamydia salmonis DNA restricted PCR product (207 bp).

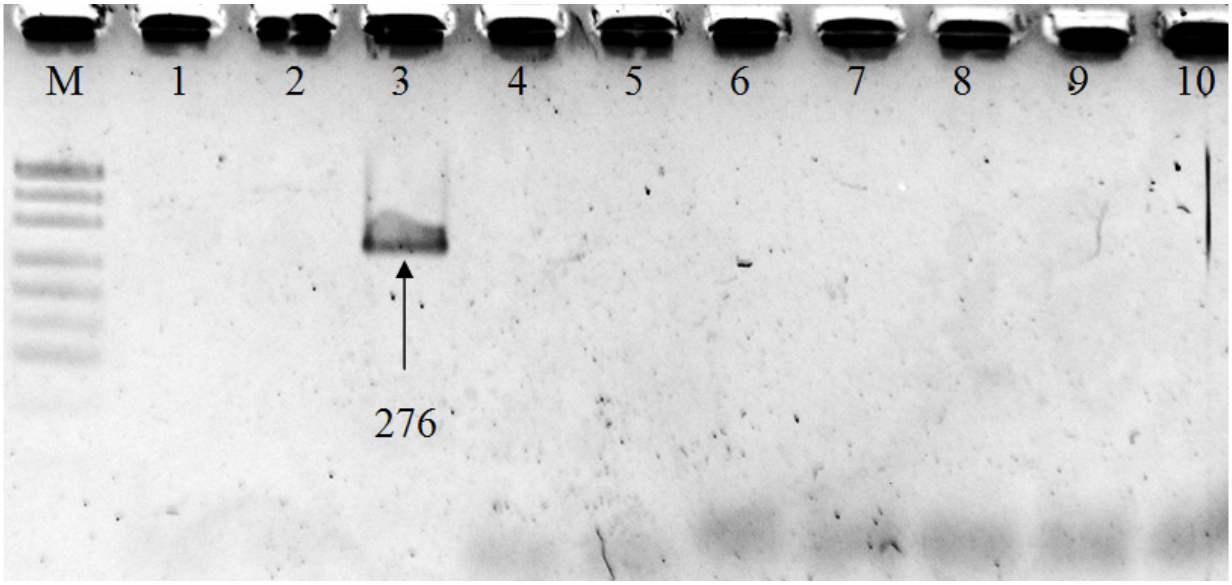

Figure 4. Electrophoregram of PCR products with primers CLACHSF/CLACHSR for *Ca.* Clavochlamydia salmonicola detection and DNA of 9 Chlamydiales species: M — DNA size marker *pUc*19/*Msp*I; 1 — *Parachlamydia acanthamoebae*; 2 — *Waddlia chondrophila*; 3 — *Ca.* Clavochlamydia salmonicola (267 bp); 4 — *Ca.* Piscichlamydia salmonis; 5 — *Chlamydia avium*; 6 — *Chlamydia pecorum*; 7 — *Chlamydia abortus*; 8 — *Chlamydia psittaci*; 9 — *Chlamydia suis*; 10 — *Chlamydia caviae.*

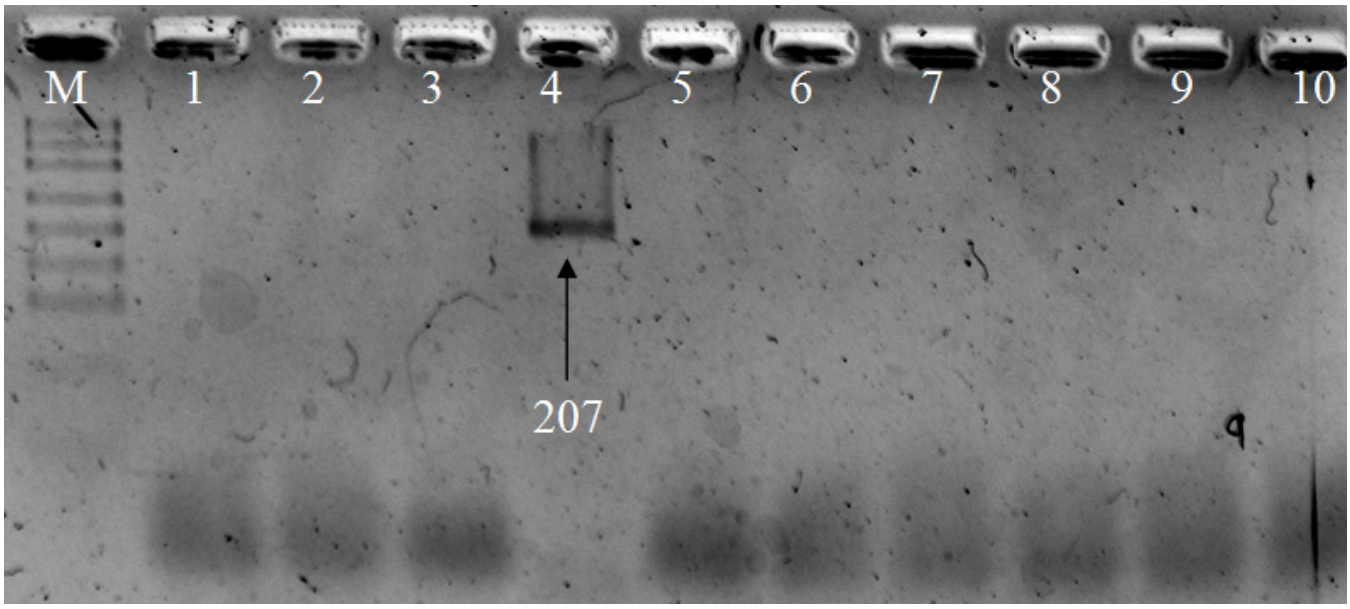

Figure 5. Electrophoregram of PCR products with primers PICHSF/PICHSR for *Ca.* Piscichlamydia salmonis detection and DNA of 9 Chlamydiales species: M — DNA size marker *pUc*19/*Msp*I; 1 — *Parachlamydia acanthamoebae*; 2 — *Waddlia chondrophila*; 3 — *Ca.* Clavochlamydia salmonicola; 4 — *Ca.* Piscichlamydia salmonis (207 bp); 5 — *Chlamydia avium*; 6 — *Chlamydia pecorum*; 7 — *Chlamydia abortus*; 8 — *Chlamydia psittaci*; 9 — *Chlamydia suis*; 10 — *Chlamydia caviae.*

Developed PCR tests for the identification and differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis have shown their suitability for amplifying positive control DNA. A restriction analysis with *Alu* I endonuclease enzyme confirmed that the amplification was performed on the DNA fragment of the 16S rRNA gene of the *Clavochlamydia salmonicola*.

There are foreign-made commercial PCR tests to identify *Ca.* C. salmonicola and *Ca.* P. salmonis, which are not available for mass use in Ukraine in the current economic situation, due to the high price and/or necessity of expensive equipment (especially, for qPCR providing). Thus, simple, practical, inexpensive methods were chosen for made it possible to put into practice the diagnosis of these bacterial infections.

PCR tests were also developed to identify and differentiate *Ca.* C. salmonicola and *Ca.* P. salmonis, they demonstrated high analytical specificity — lack of amplification (expected only one case) with the control DNA matrix of 10 species from Chlamydiales *(P. acanthamoebae*, *W. chondrophila*, *Ca.* C. salmonicola, *Ca.* P. salmonis, *Ch. avium*, *Ch. pecorum*, *Ch. abortus*, *Ch. psittaci, Ch. suis*, *Ch. caviae)*.

As a result, PCR tests for identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis can be recommended for clinical trials in Ukraine.

Conclusions. 1. As a result of our research, PCR tests for the identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis were developed.

2. The use of diagnostic kits for *Ca.* C. salmonicola and *Ca.* P. salmonis identification will help to increase the economic efficiency of fisheries and will allow to provide the monitoring of chlamydial infection among various fish species.

Perspectives for future research. The developed PCR tests for identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis after trials on clinical material can be used by scientists for extensive monitoring of epitheliocystis, veterinary medicine doctors to clarify the diagnosis, as well as introduced into the practice of veterinary medicine laboratories and used in fish farms improvement programs.

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