Real-time multiplex PCR method for detection of *A. veronii A. caviae A. salmonicida*

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Abstract. As a result of the experiments, the parameters for setting up a polymerase chain reaction were developed for the accelerated identification of *Aeromonas spp*. The design of species-specific primers was performed, the reaction parameters were selected, the specificity was checked, and the optimal amplification time was selected. As a result of this study, a multiplex real-time PCR test system was developed for the simultaneous detection of *A. salmonicida, A. caviae and A. veronii*. The sensitivity of the developed protocol was 5.56 pg/µl. The data obtained will allow for accelerated screening of these pathogens.

1 Introduction

The genus *Aeromonas* includes species of Gram-negative bacteria that primarily act as opportunistic pathogens. Aeromonads are ubiquitous in the microbial biosphere. These bacteria are naturally distributed in a variety of aquatic ecosystems, where they are easily isolated from animals such as fish and crustaceans [1;2]. The ability to adapt also makes Aeromonas able to colonize the terrestrial environment and its inhabitants, so these microorganisms can be identified from various sources such as soils, plants, fruits, vegetables, birds, reptiles, amphibians and others [2;3;4].

Aeromonas has a wide range of virulence factor elements that promote adhesion, colonization, and invasion into host cells [2;5;6;7]. The pathogenicity of *Aeromonas* in different hosts is mediated by several virulence factors such as endotoxins, cytotoxic enterotoxin, cytotoxins, hemolysins, adhesins, and extracellular enzymes such as proteases, amylases, lipases, ADP-ribosyltransferases, and DNases [8;9].

Bacteria of this genus can be conditionally divided into two groups. Aeromonads belonging to the psychrophilic group, represented mainly by *A. salmonicida*, are characterized as the main pathogens of fish and reptiles. Although bacteria belonging to the mesophilic group, such as A. *hydrophila, A. caviae* and *A. veronii*, are also considered pathogens in marine animals, they are more likely to cause infections and other diseases in humans, such as gastrointestinal diseases and septicemia [1;2;5;10].

To effectively detect outbreaks, diagnostic procedures for *Aeromonas* species require both sensitivity and specificity. The bacteriological approach of differentiation within the

genus does not always allow one to accurately identify widowhood due to a strong relationship [2;5].

2 Materials and methods

In our work, we used reference strains of aeromonads (A. *salmonicida ATCC 33568, A. caviae ATCC 15468, A. veronii ATCC 9071, A. hydrophila ATCC 49140*) and strains of aeromonads isolated from environmental objects and pathological material (A. *salmonicida 203 , A.salmonicida 204, A.salmonicida 205, A.salmonicida 206, A.salmonicida 207, A.salmonicida 208, A.salmonicida 2K, A.salmonicida 163, Aeromonas spp PR, Aeromonas spp M1, Aeromonas hydrophila A1, A. veronii bv.sobria 1, A. veronii bv.sobria 2 , A. veronii bv.sobria 3, A. veronii bv.sobria 4, A. veronii bv.sobria 5, A. veronii bv.sobria P1, A. veronii bv .sobria P1, A. veronii bv.sobria P2, A. veronii bv.veronii P3, Averonii 13A, Aeromonas hydrophila pA, Aeromonas spp 43*).

Materials. To set up PCR, we used 10x PCR buffer B (Synthol, Russia), Taq DNA polymerase, thermostable, highly processive (Diam, Russia), 2.5 mM dNTP mixture (Synthol, Russia), MgCl 25mM (Synthol, Russia), deionized water (New England Biolab, UK). To set up PCR with electrophoretic detection, we used the reaction mixture BioMaster HS-Taq PCR-Color (2×) (BioLambix, Russia) with the following composition: 100 mM Tris-HCl, pH 8.5, 100 mM KCl, 0.4 mM of each nucleoside triphosphate, 10 mM MgCl2, 0.1 units activity/μl HS-Taq DNA polymerase, 0.025% Tween 20, Taq DNA polymerase stabilizers. The polymerase chain reaction was carried out on DTprime amplifiers (DNA technology, Russia) and Bio-Rad T100 (Bio-Rad, USA). For electrophoresis, a 10x buffer of the following composition (per 1000 ml) was used: 60.5 g tris(hydroxymethyl)aminomethane (PanReac Applichem), 6.0 g Trilon B (LenReaktiv, Russia), 19.0 g boric acid (PanReac Applichem). Amplicons were separated by electrophoresis in 2% agarose gel. The agarose gel was stained with 1% ethidium bromide (AppliChem, USA), which was added at a concentration of 200 μl per 1 liter of gel. In this experiment, we used a PowerPac Basic power supply (BioRad, USA) and a Mini-Sub Cell GT (BioRad, USA). To visualize the result of electrophoresis was used Bio-print CX4 Edge gel-documenting system (Vilber, France).

Also used in the work: a centrifuge / vortex for test tubes (BioSan, Poland), a laminar box BMB-ii-"Laminar-s" -1 2 (LamSystem, Russia), a solid-state thermostat TDB-120 (BioSan, Poland), a centrifuge- medical shaker CM-50M series (ELMI, Poland), centrifuge/vortex for test tubes (BioSan, Poland), laminar flow hood BMB-ii-“Laminar-s”-1 2 (LamSystem, Russia), solid-state thermostat TDB-120 (BioSan , Poland), SM-50M series medical centrifuge shaker (ELMI, Poland), 0.2 ml PCR tubes (Russia), pipette tips 10, 20, 200, 500, 1000 μl (Thermo, Finland); single-channel dosing pipette 10, 20, 200, 500, 1000 μl (HLT, Poland). All primers used in this study were synthesized by OOO DNA-Synthesis.

To accumulate the bacterial mass, LB broth according to Miller (Diam, Russia) was used. *Aeromonas* strains were cultivated at 30^0C for 24 hours, except for the bacteria of the species *A. salmonicida*, which were incubated at 20^0C for the same period of time.

For DNA extraction, a set of reagents "M-SORB-OOM" was used to isolate DNA and RNA from clinical samples and environmental objects (on magnetic particles). To determine the sensitivity of the developed protocol, it was right to measure the concentration of NA reference strains (*A. salmonicida ATCC 33568, A. caviae ATCC 15468, A. veronii ATCC 9071*) that match the standards in this study, the concentration measurement was isolated on an EPPENDORF BioSpectrometer kinetic instrument (Eppendorf, Germany), the measurbecement was dried at λ = 260 nm, the concentration

was adjusted to 100 µg/ml with deionized water (New England Biolabs, United Kingdom). Measurement of the frequency of extraction of nucleic acids was performed at 260/280 nm, the average was 1.5-1.9.

This work was carried out on the basis of the State Budgetary Institution "Melekes Center for Veterinary Medicine and Food Safety named after S.G. Dyrchenkova" and the Department of Microbiology, Virology, Epizootology and Veterinary and Sanitary Expertise of the Federal State Budgetary Educational Institution of Higher Education "Ulyanovsk State Agrarian University".

3 Results

Before proceeding with the development of real-time species multiplex PCR, we selected primers for the accurate identification of bacteria of the genus Aeromonas. This was done in order to give the molecular genetic affiliation of all strains of microorganisms isolated by us to this genus. Previously, some of these species were assigned to this genus Aeromonas based on colony morphology on differential diagnostic media such as CIN agar (BD, United Kingdom), RYAN aeromonad medium (Conda, Spain) and BSIBG selective agar (Himedia, India), both the same and with the help of biochemical characteristics, including the results of the Voges-Proskauer test and lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase. Bacteria of the species *A. veronii* were previously typed with primers selected by us, the *ompA* gene was used as a site.

Based on the work of M A Yáñez· (2003), we chose the housekeeping gene for the DNA gyrase B subunit (*gyrB*) as the target gene. The selection of primers was performed based on the genomes of bacteria of the genus Aeromonas presented in the NCBI database (https://www.ncbi.nlm.nih.gov/), and on the website of the American Type Culture Collection (ATCC). The search and selection of a specific site was performed using the UGENE V 44.0 program (http://ugene.net) and the NCBI BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

We checked the stage performance of the examples on the Primer BlAST resource (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), as a result, the following primers were selected: forward primer (F) CCAGAACAAGACCCCGATCC reverse (R) GTCAGCGCGGTACGGAAAC. The amplification protocol was as follows: 1. pre-denaturation - 95^0C for 5 minutes, 1 cycle, 2. denaturation - 95^0C for 5 seconds, Annealing - 60^0C for 15 seconds, 40 cycles. Reference strains (A. *salmonicida ATCC 33568, A. caviae ATCC 15468, A. veronii ATCC 9071, A. hydrophila ATCC 49140*) served as positive controls. After performing amplification and electrophoresis, it was found that all strains in the collection belong to the genus *Aeromonas*.

The next stage of the work was the search for in-silico target regions of the genomes of *Aeromonas spp* to create primer systems for species identification in real time of the following species: *A.veronii, A.salmonicida, A. caviae*. These species were chosen, because they are one of the main representatives of the genus *Aeromonas*, which cause infectious processes in animals, in particular fish, and humans. After the genomes were presented, we analyzed the main genes used for species typing of bacteria of this genus: *atpD , cpn60 , dnaJ , dnaK , dnaX , gltA , gyrA , gyrB , mdh , metG , ppsA , radA , recA , rpoB , rpoD , tsf* and *zipA* (A. Navarro, A. Martinez-Murcia, 2018).. As target regions of the genomes for the identification of *A.caviae* and *A. veronii* bacteria, we used the *zipA* cell division protein gene, which stabilizes the *FtsZ* protofilaments by their cross-linking and serves as an anchor of the cytoplasmic membrane for the Z-ring, is also required for recruitment to the septal ring downstream proteins of cell division. This gene was chosen because it is a

conservative and constant structural element of bacterial genomes, and with the help of this gene it is possible to type closely related species of aeromonads from each other. The *vapA* gene was used to identify *A. salmonicida*. As a result of the work done, primers and hydrolysis probes were selected to identify the microorganisms of interest to us, the selection was carried out using the UGENE V 44.0 programs (http://ugene.net) and the NCBI BLAST website (https://blast.ncbi.nlm.nih.gov/ Blast.cgi). Primers are presented in Table 1.

Table 1. Primer systems for real-time identification of bacteria of the genus Aeromonas by PCR.

Bacterium	Target gene	Primers	Product length
A. veronii	*zipA*	Forward primer GCGACAGCCCCGACTTT	107 b.p.
		Reverse primer CGGGGCTGCGGCTGATA	
		Probe HEXCAGAGACAGTCCGTACCGCC BHQ2	
A. caviae	*zipA*	Forward primer TTGCCGCCCCTGTTGAA	246 b.p.
		Reverse primer CGTCATCGTAAGCGGGCTCT	
		Probe FAM GCACAGGTGATCCGCCGTA BHQ1	
A. salmonicida	*vapA*	Forward primer TTGCCGCCCCTGTTGAA	174 b.p.
		Reverse primer CGTCATCGTAAGCGGGCTCT	
		Probe ROX TCTGCTGGGTACCGTCGGAA BHQ2	

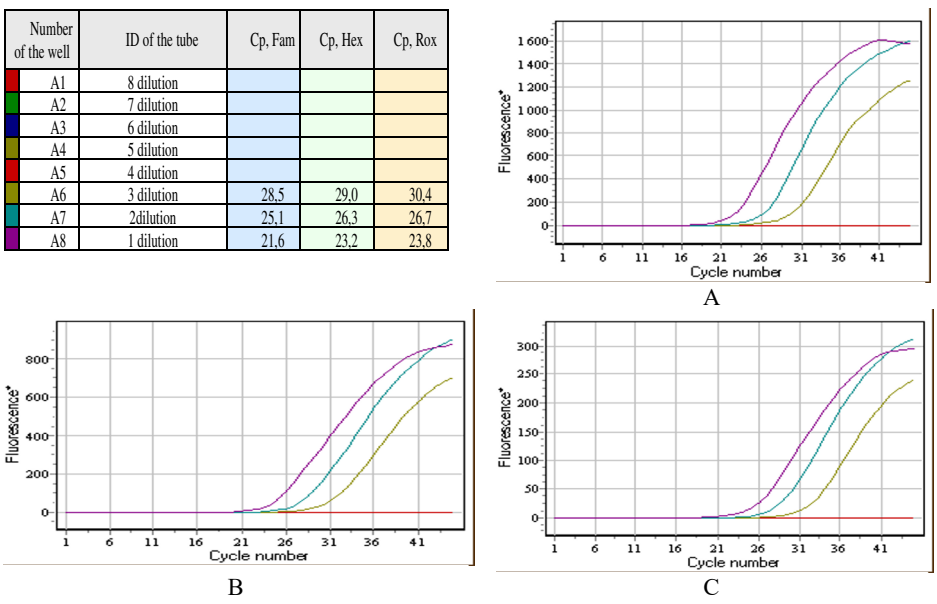

Figure 1. The result of amplification multiplex PCR test system in "real time" mode for the simultaneous detection of *A.caviae* (A), *A. veronii* (B), *A. salmonicida* (C).

After selecting the conditions for primer application, we developed a multiplex test system for the indication and detection of representatives of the genus Aeromonas with high sensitivity. According to the data obtained earlier, we decided to create a multiplex for the indication of A. salmonicida, A. caviae, A. veronii. To achieve this goal, nucleic acids of reference strains A. salmonicida ATCC 33568, A. caviae ATCC 15468, A. veronii ATCC 9071 were used, the amount of NA of each strain was 100 µg/ml. To determine the

sensitivity, a serial dilution of the mixture with the same concentration of NCs of selected microorganisms was performed. As a result of a series of experiments, we selected the composition of the reaction mixture (for 1 reaction): 10x PCR buffer B 2.5 µl, deoxynucleoside triphosphates (dNTP) 2.5 µl, MgCl2 (25 mM) 2.5 µl, SynTaq DNA polymerase (5E /µl) 0.2 µl, primer mix 1 µl, DNA sample 5 µl, ddH2O up to 25 µl volume. The primer mixture was prepared as follows: 100 pM of each forward and reverse primer and 40 pM of probes were mixed, the volume was adjusted to 100 µl with deionized water. The amplification protocol was as follows: 1. pre-denaturation - 95^{0}C for 5 minutes, 1 cycle, 2. denaturation - 95^{0}C for 5 seconds, Annealing - 60 ^{0}C for 15 seconds, 45 cycles.

As a result, a multiplex real-time PCR test system was developed for the simultaneous detection of *A. salmonicida, A. caviae, A. veronii*.. The detection of the application result was filmed using the following channels. The reaction of primers to *A.caviae* was detected through the Fam channel, and through the Hex channel - *A.veronii*, Rox - *A.salmonicida*. The sensitivity of the developed protocol was 5.56 pg/µl. The multiplex PCR test system was also tested on a collection of available strains, proving its effectiveness for the indication and typing of bacteria of the genus *Aeromonas*.

4 Discussion

With the growing demand for food and the global stagnation of marine fisheries production, the focus has shifted to aquaculture production to ensure food security for the world's population. The share of aquaculture in total world fish production increased rapidly from 7% in 1974 to 44.1% in 2014 (FAO, 2016). The constant focus on higher production targets has made aquaculture highly vulnerable to frequent disease outbreaks and consequent economic losses.

In many ways, the history of the genus Aeromonas reflects the chronicles of modern medical bacteriology, covering more than 100 years, from its inception as a recognized laboratory science in the late 19th and early 20th centuries to its evolution into the molecular postgenomic era.

The scientific community's perception of the genus Aeromonas also evolved over the same period. Aeromonads were originally thought to cause systemic disease only in poikilothermic animals. Today, the genus Aeromonas is regarded not only as an important pathogen causing disease in fish and other cold-blooded species, but also as an etiological agent responsible for many infectious complications in both immunocompetent and immunocompromised individuals.

In this regard, the use of molecular genetic methods, in particular PCR, is relevant. In this regard, the use of molecular genetic methods, in particular PCR, is relevant. Conventional uniplex PCR methods have been successfully used to identify individual virulence factors in clinical, nutritional and environmental strains of Aeromonas spp. Polymerase chain reaction (PCR) methods have been developed to detect the presence of Aeromonas species in a wide variety of samples. Özbaş Z. Y., et al. (2000) developed a PCR method for the detection of A. hydrophila in raw milk. In a recent study, waterborne isolates of Aeromonas were screened for different virulence genes in three multiplex PCR series targeting elastase (ahyB), lipase (pla/lip/lipH3/alp-1), flagella A, and flagella B (flaA and flaB) and enterotoxin genes act, alt, and ast.

Søren Persson (2014) presents a method for the identification of clinical Aeromonas species using a multiplex PCR method to detect Aeromonas hydrophila, A. caviae, A. veronii and A. media. However, this method does not allow identification of these bacteria in the “real time” mode. As a result of the analysis of the literature, it can be concluded that

at the moment it is relevant to develop species multiplex real-time PCR for the identification of common species of the genus Aeromonas, based on the genome regions of these bacteria.

As a result of the studies, species-specific primer systems were developed for the identification of bacterial strains of A. veronii A. caviae A. salmonicida. As a result of this study, a multiplex real-time PCR test system was developed for the simultaneous detection. The sensitivity of the developed protocol was 5.56 pg/μl. The proposed methods will speed up the identification and typification of bacteria of the genus Aeromonas.

5 Conclusion

Bacteria of the genus Aeromonas are ubiquitous aquatic organisms that can cause infections in humans and animals. We have developed polymerase chain reaction parameters for accelerated identification of Aeromonas bacteria. The design of species-specific primers was performed, the reaction parameters were selected, the specificity was checked and the optimal amplification time was selected, the sensitivity of the developed protocol for the identification of bacteria *A. veronii A. caviae A. salmonicida* was determined. The data obtained will allow for accelerated screening of this pathogen.

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