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DETECTION OF *AEROMONAS SALMONICIDA* IN FISH SAMPLES FROM LAKE OHRID BY CULTURE AND POLYMERASE CHAIN REACTION METHODS

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Abstract

Aeromonas salmonicida is a bacterial pathogen that causes infection mainly in Salmonidae family. The objective of this ongoing study was to isolate and identify the bacterial pathogen in Lake Ohrid, since the main fish species that populate this aquatic body belongs to the Salmonidae family. Salmo letnica and Salmo ohridanus are the main species where we have focused our analyses. These fish species not only are one of the main catches but also one of the main foods in the region around Lake Ohrid. Sampling was carried out during spring and autumn season in 2017. Material from liver and skin mucus from each sample was used for the inoculation of the TSA medium. The incubation period was between 5-7 days at 18°C which was followed biochemical test. PCR was performed by using MIY primer (MIY1 5'-AGCCTCCACGCGCTCACAGC-3' and MIY2 5'-AAGAGGCCCCATAGTGTGGG-3'). All the data were analyzed with Sigma Plot 12.5. A multiple Comparison Procedures (Tukey Test) between groups, water temperature, liver bacterial colonies and skin colonies ($p<0.05$) was performed.

Key words: *A. salmonicida, MIY primer, PCR, Sigma plot 12.5, TSA medium.*

INTRODUCTION

Lake Ohrid is a unique transboundary aquatic body due to the endemic fish species that populate it, which belongs to *Cyprinidae* and *Salmonidae* family. It has a significant importance in biological and economic aspects for the regions that surround this natural aquatic body. According to recent studies in Lake Ohrid were determined 21 native fish species, seven of them are considered endemic species (Talevski et al., 2009) such as *S. ohridanus* and *S.letnica* (Karaman, 1924). These two fish species belongs to Ohrid brown trout and represent one of the main catches and commercials fishes in the region (Mitchell et al., 2010). But over the last decade the stock of *Salmo letnica* has been constantly decreasing (Jordanova, 2016). Water pollution may be the main cause for such decline of this stock species although it's important to mention that there is a lack of published data for the wild stock situation through the years. As it's worth to mention that there are a few publications regarding fish pathology in Lake Ohrid, especially in reference with fish parasites (Dimovska et al., 2013). The study is focused on the detection of a bacterial pathogen, *Aeromonas salmonicida* that cause severe infection mainly on Salmonidae fish. In the last decade it has been shown that *A. salmonisada* can cause severe infection in other fish species that don't belong to Salmonidae family, including carp (*Cyprinus carpio*), eel (*Anguilla Anguilla*) (Rivera et al., 2014). Data shows that *A. salmonicida* infection can be transferred *via* water from infected fish to healthy fish (Skrodenyte-Arbaciauskiene et al., 2012). Furunculosis, the disease caused from *A. salmonicida*, represent an acute to chronic condition inflicted heavy losses in wild and cultured stocks fish (Sudheesh et al., 2012). Affected fish often show skin ulcerations, lethargy and in appetence (Wiklund and Dalsgaard, 1998). Hemorrhages may occur at the bases of fins and the abdominal walls, heart

and liver (Menanteau-Ledouble et al., 2016). Enlargement of the spleen and inflammation of the lower intestine are common features of chronic infections, but in acute outbreaks fish may die rapidly with few signs. The major route of transmission appears to be via infected fish and contaminated water (Hastings and Ellis, 1988). Although the disease causes mortality of all ages, the most serious losses occurs during spring-autumn in the sea water farms.

Clinical outbreaks and mortality appear to be triggered by stress factors such as crowding, poor water quality, fright, high temperature and physical trauma (Pekala-Safinska, 2018). By taking into consideration the information above, our research is mainly focused in isolation and detection of *A. salmonicida* in Salmonidae species (*S. ohridanus* and *S. letnica*) of Lake Ohrid by combining conventional methods and PCR technique.

MATERIALS AND METHODS

Materials: Samples that belongs to Ohrid trout (*Salmo letnica* and *Salmo ohridanus*) were collected in spring (April-May) and autumn (September-October) 2017. In total were collected 77 samples, mainly female and male adult individuals. The fish were capture from licensed fisherman in three sites as shown in Figure 1. These sites are mostly frequented from tourist near an urban community with multiply contaminations inputs. The first site was located in Lin village (n=21), the second in Udenisht (n=14) and the third site in Pogradec city (n=42). **Methods:** To evaluate the condition of fishes samples the Fulton condition factor (CF) was calculated according the formula: CF= 100 x BW (g) ÷ FL^3 (cm) (Jordanova et al., 2016; Nash et al., 2006). For each fish the total length (TL), fork length (FL) and body weight (BW) were measured. A visual evaluation for external and internal lesion after the dissection was made and recorded. For the isolation of bacterial pathogen, *A. salmonicida*, we used Trypton Soy Agar (TSA) medium which was inoculated with diluted mucus taken from each fish samples and from material taken from the liver. The plates were incubated for 4-7 days at the temperature of 18°C.

PCR analysis: DNA templates were prepared by lysing three to four bacteria from colonies, that grow in TSA medium after the incubation period, in 20µl lysis tampon (50 mM KCL, 10mM Tris pH 8.3, 2.5mM $MgCl_2$, 0.45% NP-40, 0.45% Tween 20 and milli-Q H_2O) (Tanaka et al., 2012; Rivera, 2015). The lysates were incubated at 95°C for 5 minutes and only 1 µl from the lysates is used for PCR mix volume. The PCR mixture contained 0.25 µl of Taq polymerase, 2.5µl of Buffer 10X, 2µl of $MgCl_2$, 0.25µl of dNTP 100mM, 0.5µl of each primer 200µM, 18µl of milliQ H_2O and 1µl of DNA template. The primer used for the amplification are MYI primer (MIY1 5'-AGCCTCCACGCGCTCACAGC-3' and MIY2 5'-AAGAGGCCCCATAGTGTGGG-3') (Byers et al., 2002). The mix volume was held for 2 min at 94°C, then amplified for 35 cycles (denaturation for 30s at 94°C and annealing and elongation for 1 min at 68°C) followed by a final extension for 3 min at 68°C. The PCR product size was 512bp. For the separation of the DNA fragment a 1% gel agarose was prepared and the DNA fragment runs at 100V for 45 min.

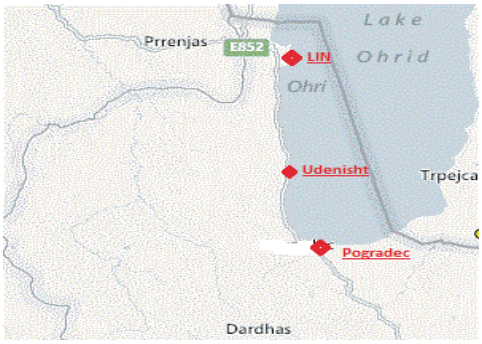

Figure 1: Google map where there are mark the three samples site: Lin village, Hudenisht and Pogradec

RESULTS AND DISCUSSION

For each fish samples we have recorded the morphometric data, external and internal symptoms if present. The Fulton condition factor has been calculated for each sample in order to evaluate the condition of the fishes that we have analyzed. For the Salmonidae species the Fulton factor values usually fall in the range 0.8 to 2. The result obtained show that 27% (n=21) of samples has CF value lower than

1.70% (n=54) of samples has a CF value between 1 and 2 and only 0.3% (n=2) has a CF value greater than 2. We have hypothesized that the health condition of the fish is related with the number of the bacterial colonies present in the liver, skin and with external/internal symptoms if observed. In the Table 1 it shows the output of the ANOVA analysis and whether there is a statistically significant difference between our group means (FC and liver bacterial colonies, bacterial skin colonies and fish symptoms present or not). A multiple comparisons analysis is performed to determine if there is a statistically difference within groups mentioned above. A Schefffe post hoc test revealed a statistically significance within Liver colonies and CF value lower than 1 (8.0476 ± 3.77460) and CF value between 1 and 2 (6.0556 ± 2.56549) (p=0.035) with a confidence level 95% (α=0.05). Also the test show a statistically significance within fish symptoms and CF value lower than 1 (4.7143 ± 0.46291) and CF value between 1 and 2 (4.3889 ± 0.49208) (p=0.037) with a confidence level 95% (α=0.05). There was no statistically significant difference within skin colonies and CF value lower than 1 and CF value between 1 and 2 (Figure 2). Also to determine if there is a correlation between the fish symptoms that we observed and the total number of the bacteria present in liver or skin we perform Person Correlation (2-tail) with statistical program SPSS 25.00. The data show that there is a positive correlation between fish symptoms and liver colonies, statistically significant (p=0.001) with a confident level 99% (α =0.01) and between fish symptoms and bacterial skin colonies statistically significant (p=0.002) with a confident level 99% (α =0.01). A Pearson Correlation (2-tailed) between fish symptoms and Fulton condition factor was also performed with SPSS 25.0 statistical program and show a negative correlation (R^2=-0.326) between the two variables, statistically significant (p=0.004) with a confident level 99% (α =0.01). Since the main objective of our research was to determine the presence of *A. salmonicida*, and data shows that the incidence of this bacterial pathogen is mainly in spring and autumn, we calculated with Sigma Plot 12.5 if there is a significance difference between water lake temperature in the time of sampling and the total number of bacterial colonies present in liver and skin (Figure 3). According to the literature there is a strong relation between the effect of temperature on pathogen multiplication and host immune mechanisms (Groberg et al., 1978).

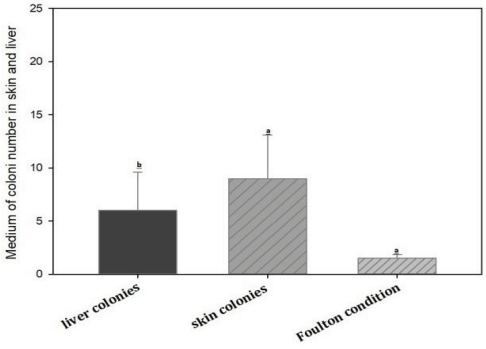

Figure 2. The differences in the median values among the treatment groups. The labels in different marks shows the difference in standart deviation between groups (Sigmaplot 12.5, Tukey test, statistically significant p<0.05)

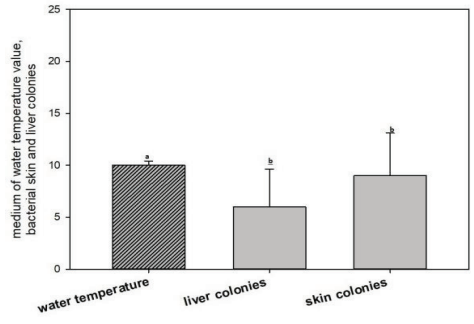

Figure 3. The differences in the median values among the treatment groups. The labels in different marks shows the difference in standard deviation between groups (Sigmaplot 12.5, Tukey test, statistically significant p<0.05)

The isolation of *A. salmonicida* in TSA medium resulted negative after morphological and biochemical tests. It doesn't observed dark brown bacterial colonies, typical of *A. salmonicida*. But instead we have isolated rod yellow, smoothly colonies that belongs Pseudomonas genus (Figure 4). We assume that the presence of these bacterial species in almost all the samples is related with the water quality of Ohrid Lake.

Table 1: ANOVA results perform with statistical program SPSS 25.0

		Sum of Squares	df	Mean Square	F	Sig.
Liver colonies	Between Groups	60.734	2	30.367	3.546	.034
	Within Groups	633.786	74	8.565		
	Total	694.519	76			
Skin colonies	Between Groups	81.526	2	40.763	3.140	.049
	Within Groups	960.786	74	12.984		
	Total	1042.312	76			
Fish symptoms	Between Groups	2.050	2	1.025	4.430	.015
	Within Groups	17.119	74	.231		
	Total	19.169	76			

* We can see that the significance value is 0.034 therefore, there is a statistically significant difference in the mean of liver colonies and Fulton condition factor (FC); skin colonies and Fulton condition Factor p=0.049; fish symptoms and Fulton condition factor p=0.015.

a)  b) 

Figure 4. a) *Salmo letnica* dissection, evaluation of internal organs.
b) Bacterial colonies after inoculation of TSA with liver material and skin mucus diluted material.

For each sample we have performed PCR amplification which resulted negative for the presence of *A. salmonicida*.

A. salmonicida represent a bacterial pathogen not only in fishes but also it is classified as a foodborne pathogen in human (Novotny et al., 2014). The salmonidae species (*Salmo letnica* and *Salmo orhidanus*) that we have selected for our research represent one of the main foods for the population that lives in the region around Ohrid, but also an important food attraction for tourist. Not only it's of a great importance having recorded data for the stock fish population of Ohrid brown trout but also knowing fish health situation. It is worth to mention that there are many publications about the water pollution of Lake Ohrid from chemical and biological hazards and their effect on fish health (Mali, 2014; Aliu et al., 2011; Lokovska et al., 2019).

Also after the fish dissection there are no sign, typical to the furunculosis such as a widespread hemorrhaging to a greater or less degree in the internal organs (liver, kidney etc.) (Bruno and Ellis, 1996).

CONCLUSIONS

In this article it has presented the first accumulated results, of an ongoing study, for the detection of *A. salmonicida* in fish samples from Ohrid Lake. We assume that the negative result obtained with culture medium and PCR are related with the relative small sample (n=77) that we have analyzed. The morphophysiological data that we have obtained from our fish samples (*S. letnica* and *S. ohridanus*) shows that we are dealing in general with healthy fish (n=54, 70% in total). No external signs that may lead to furunculosis disease such as darkening of the skin, reddening of the fin basis have been observed.

From the other hand we have observed sign in some fish samples (n=36 with external/internal

signs) that in general are not related with *A. salmonicida* infection, to mention a few white spot, weight drop, liver hyperplasia etc.

Since there are no publication (in our knowledge) about the isolation and identification of *A. salmonicida* in Salmonidae fish of Ohrid Lake, makes it difficult from our part to compare our data or to reach in a final conclusion. Since this is a ongoing study we are still analyzing fish samples from Ohrid Lake with the main objective detection of *A. salmonicida*.

REFERENCE

Aliu, S., Aliu, A., Mustafi, M., Selmani, L., & Ibrahimi, B. (2011). Physico-chemical paramethers in rivers and lakeshore of Lake Ohrid - law, economic and social aspects. *Science Direct*, 19, 499-503.

Bruno, E.D., Ellis, E.A. (1996). Salmonid disease management. *Developments in Aquaculture and Fish Science*, 29, 759-832.

Byers, K.H., Gudkovs, N., Crane, ST.J.M. (2002). PCR-based assays for the fish pathogen Aeromonas salmonicida.I. Evaluation of three PCR primers sets for detection and identification. *Diseases of Aquatic Organisms*, 49, 129-138.

Dimovska, B.D., Stojanovski, S., Hristovski, N. (2013). Parasite fauna of endemic fishes (Salmo letnica Karaman, 1924 and Salmo ohridanus Steindachner 1892) from lake Ohrid (Macedonia). *Natura Montenegrina Journal*, Podgorica, 12(3-4), 761-771.

Groberg, Jr.J.W, McCoy, H.R., Pilcher, S.K., Fryer, L.J. (1978). Relation of water temperature to infections Coho Salmon (*Oncorhynchus kisutch*), Chinook Salmon (*O. tshawytscha*), and Steelhead trout (*Salmo gairdneri*) with *Aeromonas salmonicida* and *A. hydrophila*. *Journal of the Fisheries Research Board of Canada*, 35 (1), 1-7.

Hastings, T.S., Ellis, A.E. (1988). The humeral immune response of rainbow trout, Salmo gairdneri, Richardson, and rabbits to *Aeromonas salmonicida* extracellular products. *Journal of Fish Diseases*, 11, 147-160.

Jordanova, M., Rebok, K., Rocha, E. (2016). Liver pathology of female Ohrid trout (*Salmo letnica* Kar.) from the Eastern coast of Lake Ohrid: Baseline data suggesting the presence of a Pollution gradient. *Turkish Journal of Fisheries and Aquaculture Science*, 16, 241-250.

Karaman, S. (1924). Pisces Macedoniae. Split (Hrvatska Stamparija). 1-90.

Lokovska, S.L., Veljanoska-Sarafiloska, M.E., Vasileska, M.A. (2109). Microbiological and Chemical Water Quality of Lake Ohrid and its tributaries in 2104. *ACTA Zoologica Bulgarica*, Sup.13, 19-24.

Mali, S. (2014). Evaluation of water quality of the Ohrid Lake (Albanian part) compared to the International standarts. International Journal of Engineering Science and Innovative Technology, Vol.2 (5). 568-573.

Menanteau-Ledouble, S., Kumar, G., Saleh, M., El-Matbouli, M. (2016). Aeromonas salmonicida: updates on an old acquaintance. *Disease of Aquatic Organisms*, 120, 49-68.

Mitchell, M., Vanberg, J., Sipponen, M. (2010). Commercial inland fishing in member countries of European Inland Fisheries Advisory Commission (EIFAC): Operational environments, property rights regimes and socio-economic indicators. Retrieved from http://www.fao.org/3/a-an222e.pdf.

Nash, R.D.M., Valencia, A.H., Geffen, A.J. (2006). The origin of Fulton's condition factor-Setting the rcord straight. *Fisheries*, 31(5), 236-238.

Novotny, L., Dvorska, L., Lorencova, A., Beran, V., & Pavlik, I. (2004). Fish a potential source of bacterial pathogens for human beings. *Vet. Med. Czech*., 49(9), 343-358.

Pekala-Safinska, A. (2018). Contemporary threats of bacterial infections in freshwater fish. *Journal of Veterinary Research*, 62, 261-267.

Rivera, L., Lopez-Patino, M.A., Milton, D.L., Nieto, T.P., Farto, R. (2014). Effective qPCR methodology to quantify the expression of virulence genes in Aeromonas salmonicida subsp. Salmonicida. *Journal of Applied Microbiology*, 118, 792-802.

Rivera, L. (2015). *Regulacion de factores de virulencia por el sistema de quorum sensing AsaI/R en Aeromonas salmonocida subsp. Salmonicida*. PhD Theses, University of Vigo, Spain.

Skrodenyte-Arbaciauskiene, V., Kazlauskiene, N., Vosyliene, Z.M., Virbickas, T. (2012). Aeromona salmonicida infected fish transfer disease to health fish via water. *Central European Journal of Biology*, 7 (5), 878-885.

Sudheesh, S.P., Al-Ghabshi, A., Al-Mazrooei, N. (2012). Comparative pathogenomics of bacteria causing infectious disease in fish. International *Journal of Evolutionary Biology*, doi:10.1155/2012/457264.

Talevski, T., Milosevic, D., Maric, D., Petrovic, D., Talevska, M., Talevska, A. (2009). Biodiveristy of ichthyofauna from Lake prespa, Lake Ohrid and Lake Skadar. *Journal of Biotechnology and Biotechnological Equipment*, 23, Sup.1, 400-404.

Tanaka, H.K., Dallaire-Dufresne, S., Daher., K.R., Frenette, M., Charette, J.S. (2012). An insertion sequence-dependent plasmid rearrangement in *Aeromonas salmonicida* causes the loss of the type three secretion systems. PLoS ONE 7(3): e33725. https://doi.org/10.1371/journal.pone.0033725

Wiklund, T., Dalsgaard, I. (1998). Ocuurence and significance of atypical *Aeromonas salmonicida* in non-salmonidae and salmonidae fish species; a review. *Disease of Aquatic Organisms*, 32, 49-69.