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Short Communication / *Kısa Bilimsel Çalışma*

Marine fish parasite, *Lernanthropus kroyeri* (Copepoda) is a repository of *Vibrio anguillarum* as evidenced by Loop-Mediated Isothermal Amplification method

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Abstract: Fish parasites act as a vector of other pathogenic diseases such as bacteria and virus in fish. Although it has been long known that fish parasites can act as a vector for bacterial pathogens, their role in the transmission of specific bacterial pathogens via particular parasites in the off-host stage has been neglected. The aim of the present study was to disclose that if the copepod parasite, *Lernanthropus kroyeri* in the off-host stage, can store up the aquatic bacterial pathogens: *Aeromonas hydrophila, Photobacterium damsela* subsp. *piscicida*, *Vibrio (Listonella) anguillarum*, *V. harveyi*, *V. vulnificus*, and *V. alginolyticus*. Our results using the Loop-mediated Isothermal Amplification (LAMP) method demonstrated that the bacteria, *V. anguillarum* is internally present in the off-host copepod parasites, *L. kroyeri*. It was also found that the parasite, *L. kroyeri* could survive in the off-host stage for a given time with its potential of vectoring ability in terms of transmission of the bacterium to the fish. This study demonstrates evidence that the copepod parasite in the off-host stage on the gills of sea bass is a reservoir of pathogen bacteria, *Vibrio anguillarum*.
Keywords: *Lernanthropus*, parasitic copepod, vector, *Vibrio anguillarum*.

Deniz balıkları paraziti, *Lernanthropus kroyeri* (Copepoda)'nın *Vibrio anguillarum* için bir depo olabileceğinin Loop-Mediated Isothermal Amplification metodu ile kanıtlanması

Özet: Balık parazitleri bakteri, virus gibi patojenler için vektör olarak hareket edebilmektedirler. Balık parazitlerinin bakteriyel patojenler için vektör olarak hareket ettiği uzun zamandır bilinmesine karşın bazı parazitlerin konakçı (balık) dışındaki dönemlerinde spesifik bakteriyel patojenleri bulaştırmasına ilişkin rolleri yeterince çalışılmamıştır. Bu çalışmadaki temel amacımız bir kopepod parazit olan *Lernanthropus kroyeri* 'nin konakçı dışında yaşadığı dönemde sucul bakteriyel patojenlerden: *Aeromonas hydrophila, Photobacterium damsela* subsp. *piscicida, Vibrio (Listonella) anguillarum, V. harveyi, V. vulnificus* ve *V. alginolyticus'*u bünyesinde tutup tutmadığının ortaya çıkarılmasıdır. Loop-mediated Isothermal Amplification (LAMP) metodu ile elde ettiğimiz sonuçlar, araştırılan bakterilerden, *V. anguillarum'*u kopepod parazit, *L. kroyeri'*nin taşıdığını göstermektedir. Ayrıca parazit *L. kroyeri* belli bir süre, balığa bakteri bulaştırma açısından potansiyel vektörel yeteneğini muhafaza ederek konakçı dışında hayatta kalabilmektedir. Bu çalışma levreklerin solungaçlarında bulunan kopepod parazitin konakçı dışında, patojen bakteri *V. anguillarum* için bir taşıyıcı olduğunu kanıtlamaktadır.
Anahtar sözcükler: *Lernanthropus*, parazitik kopepod, vektör, *Vibrio anguillarum.*

Intensive aquaculture is under the pressure of a huge diversity of the pathogens, evidenced by the propagations with a multiplication of disease outbreaks. Infections, particularly co-infections of parasites and bacteria, are common in aquaculture systems. The prevalence of copepod parasite, *Lernanthropus kroyeri* is high in sea bass, *Dicentrarchus labrax* culture, leading to economic losses due to impaired growth rate, feed conversion ratio and mortality associated with secondary pathogens (5, 11, 16). *Vibrio anguillarum*, also known as *Listonella anguillarum* is the aetiological agent of vibriosis, a systemic disease in fishes characterized by severe hemorrhagic septicemia in different fish from marine and fresh/brackish water. *V. anguillarum* has been one of the

challenging pathogens in mariculture for a long time as its serious effects on the economy in addition to the sustainability of aquaculture (7).

Loop-mediated Isothermal Amplification (LAMP) is a specific DNA-based detection method, providing technically superior to the others such as fast results on-site, a simplified amplification reaction, even more, specific than qPCR and immunoassays (1, 8). LAMP, with its high sensitivity and specificity, has been used for the detection of aquatic pathogens such as *Mycobacterium marinum* (12), infectious hematopoietic necrosis virus (IHNV) (6) and *Cyprinid herpesvirus* (8).

Synergistic interaction between parasites and bacterial infections in fish has been known as a result of the stress caused by parasites disrupting immune reactions and osmoregulatory mechanisms to other secondary bacterial infections (9). Parasite vectors in fish increase the transmission efficiency of bacterial pathogens by forming an entry portal (4). Hence, previous evidence of parasites acting as vectors is available. Parasitic crustaceans of fish are well-known vectors of a range of bacterial pathogens; however, the answers of the following questions are less studied: i) are the bacteria present in water or surface of the parasite and fish affected by mechanical damage of copepod parasite to fish? ii) Or is a copepod parasite a perpetual reservoir of the pathogenic bacteria? Theoretically, the simultaneous presence of Vibrio species and copepod parasites is known in cultured sea bass; however, the empirical evidence of the potential role of the parasite, *L. kroyeri* in transmitting pathogens does not exist. The purpose of this study is to ascertain that the copepod parasite, *L. kroyeri* can harbor the aquatic pathogen bacteria in the condition of off-host. We provide empirical evidence of the presence of *V. anguillarum* within the copepod parasite, *L. kroyeri* using the LAMP method.

Parasitized European sea bass *(Dicentrarchus labrax)* were obtained from a commercial marine aquaculture company located in Güllük Bay. The females of the copepod parasite *L. kroyeri* were carefully collected from the gills by using forceps in newly harvested fish. All collected females of the *L. kroyeri* were with egg-sacs. Parasites were checked for their species and sex, under the microscope. Collected female *L. kroyeri* individuals (N=20) were kept in distilled water for 15 days at 4 ºC and water exchanged every day. Parasites were washed with the antibiotic solution (1 mg/ml sulfadiazine/ trimethoprim) for one hour to remove the external bacteria on the surface of the parasites. After three washes with sterile distilled water, parasites were homogenized at Vortex for 1 minute for LAMP PCR. The LAMP assay was carried out in the instrument Genie II (Optigene, UK). Bacteria were purchased from DSMZ, Germany. Design of primers for the bacteria; *Aeromonas hydrophila* (ATCC 15338), *Photobacterium damsela* subsp. *piscicida* (ATCC 33539), *V. anguillarum* (ATCC 19264), *V. harveyi* (ATCC 27562), *V. vulnificus* (ATCC 27562) *and V. alginolyticus* (ATCC 17749) were carried out in Ak-YA Veterinary Laboratory, Muğla, Turkey. The gene region of 16s rRNA was selected as the target region. Extraction, amplification, and analysis of the results were carried out with LAMP PCR analyzer (Genie II, OptiGene, UK). All the procedures were completed within 90 minutes. Result graphics were automatically obtained from the LAMP PCR analyzer at the end of the procedures. Six primer sets: two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (loop F and loop B) were arranged by using software LAMP Designer. Recommendations of the manufacturer were followed in optimization: the reaction was performed in a 50 μL mixture containing 4 μL each of FIP and BIP; 1 μL each of F3 and B3; 2 μL loop F and loop B, respectively; 15 L 1× Isothermal Master Mix (OptiGene, UK) and 5x DNA template. The mixture was incubated at 75 ℃ for 15 min and at 95 ℃ for 5 min, respectively. At the stage of lamp and anneal, the incubation was at 65 ℃ for 60 min, and then termination was from 98 ℃ to 80 ℃ in 1 min. To assess off-host survival period, parasites following to detach from the gills of sea bass were transferred to a flask containing seawater at 22 ℃ and kept for 10 days with light aeration. They were daily checked for their death.

LAMP assay in the GenieII showed that the set of primers (*A. hydrophila, P. damsela* subsp. *piscicida, V. anguillarum, V. harveyi, V.vulnificus,* and *V. alginolyticus*) was only specific to *V. anguillarum.*

The optimization of LAMP reaction temperature for the primers is shown in Figure 1, and the obtained amplification rates in Figure 2. Figure 3 shows that none of the DNA samples of *A. hydrophila, P. damsela* subsp. *piscicida, V. harveyi, V. vulnificus,* and *V. alginolyticus* culminated with a positive response in the LAMP reactions. The set of primers was only specific to *V. anguillarum,* with a peak value of 85.9.

We have provided the empirical evidence that bacterial pathogen, *V. anguillarum,* could be found internally in the parasite, *L. kroyeri,* demonstrating that off-host copepod parasites exist as a repository in the aquaculture environment. The presence of bacteria within the parasitic copepod is a strong sign to their vectoring potential and the transmission efficiency for the bacterial pathogens. Vector-pathogen interactions in fish have been studied in different fish and parasites species, providing the findings in line with our results. Three pathogenic bacteria; *Tenacibaculum maritimum, Pseudomonas fluorescens and Vibrio* spp. isolated from sea lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae), and their salmon hosts had been isolated by Barker et al. (3), supporting the fact about potential role of parasitic diseases associated with bacterial infections in salmon aquaculture. Similarly, the presence of *Flavobacterium*

columnare in *Argulus coregoni* in *Oncorhynchus mykiss* has been reported by Bandilla et al. (2). Moreover, Xu et al. (15) provided scientific evidence to prove that a ciliate parasite, *Ichthyophthirius multifiliis* can act as vector of *Edwardsiella ictaluri* in channel catfish. Pylkkö et al. (10) also mentioned another example of bacterial interference occuring in infection by diplostomids, *Diplostomum spathaceum*, leading to increase in atypical *Aeromonas salmonicida* infections in fish.

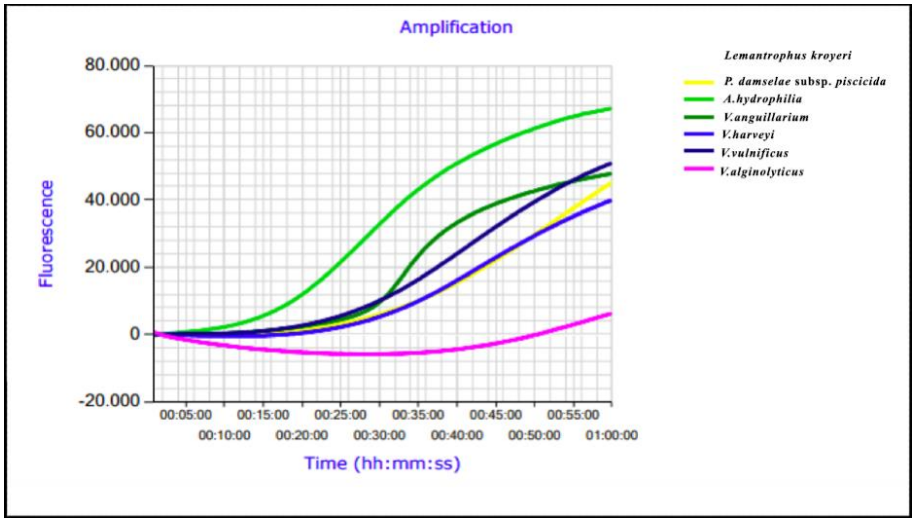

Figure 1. Optimization of LAMP reaction temperature for the primers.

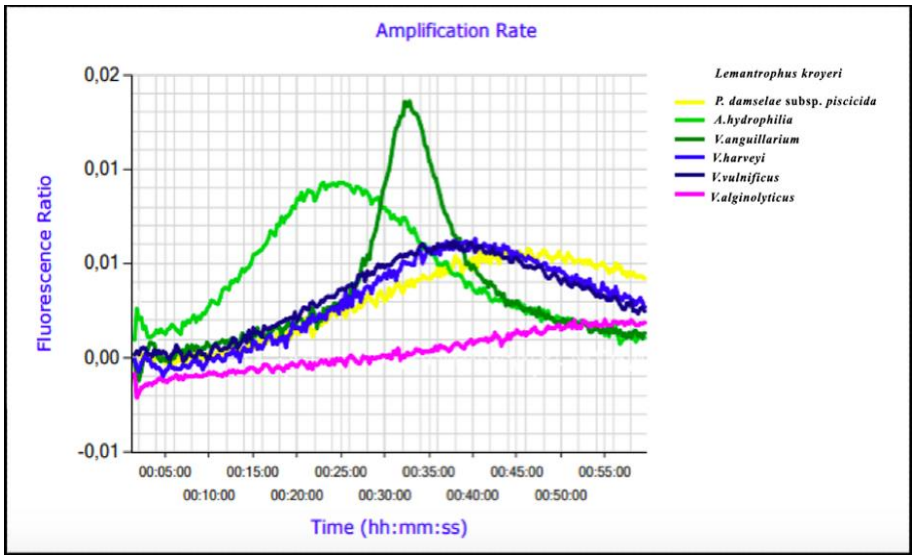

Figure 2. The obtained amplification rates for testing bacteria species.

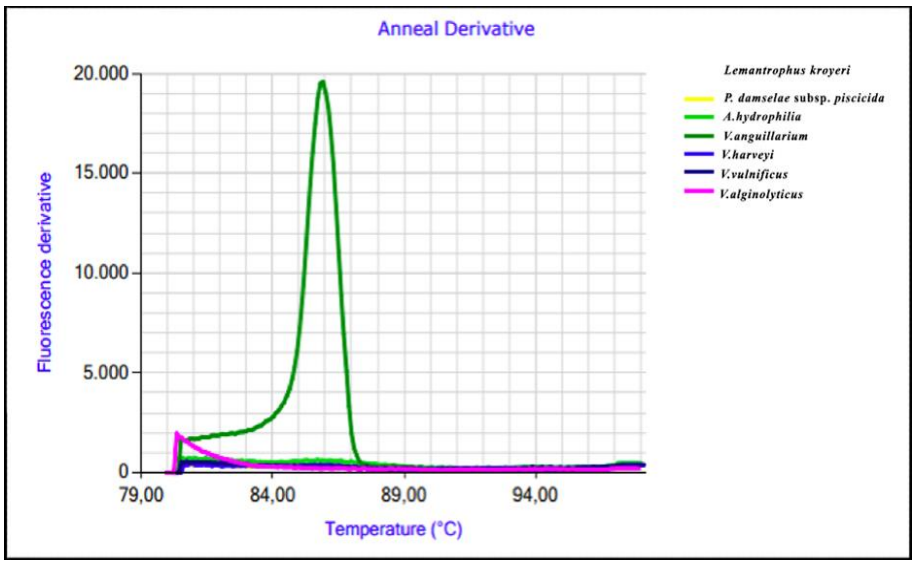

Figure 3. Specificity of the LAMP assay tested with DNA samples of different bacteria species.

On the other hand, crustacean parasites have the ability to survive off-host. Tully& Nolan (13) reported that pre-adult and adult lice *Lepeophtheirus salmonis* (Copepoda: Caligidae) can survive off-host for several days and can change their hosts. Similarly, adult Argulus species (Crustacea: Branchiura) can continue to live without any host up to two weeks (14). In our observations, *L. kroyeri* could survive more than seven days at 22 ^{o}C seawater temperature without any host and swim freely. It indicates a potential danger of dispersing *V. anguillarum* to various marine fish species as *L. kroyeri* may deliver pathogen bacteria to new hosts.

Surprisingly, the transmission dynamics or delivery models of vectors and their specificity have been neglected in parasitological studies in an aquatic environment, although co-infections of bacteria and parasites in fish is well-recognized in aquaculture. Thus, a more integrated approach to revealing the transmission dynamics of vector parasites in aquatic animals is needed to ensure the sustainability of the aquaculture. This research defined our study of LAMP for the detection of pathogen bacteria in a parasite in marine fish with high sensitivity and specificity for the first time. Conclusively, copepod parasite *L. kroyeri* on species of *D. labrax* is a potential reservoir and acts as a vector by perpetuating the pathogenic bacteria, *V. anguillarum*. In order to overcome the bacterial problems in aquaculture, the elimination strategy for parasites should take priority over all bacterial fish disease treatment methods.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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