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Molecular characterization and identification of *Shewanella putrefaciens* and isolation and morphological characterization of its lytic phage

Caracterización molecular e identificación de *Shewanella putrefaciens* y aislamiento y caracterización morfológica de su fagolítico

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ABSTRACT

The studies concerning bacteria isolated using traditional diagnostic methods, which have been employed for many years for the detection of pathogens in aquaculture, were restricted to 15-20 bacterial species until the 1990s. Conversely, the number of bacteria identified has reached 70 through the utilisation of 16S rDNA sequencing, which has been identified as the gold standard for identification in recent years. *Shewanella putrefaciens* is a pathogenic bacterium that has been isolated from both marine and freshwater fish. The number of reports concerning *Shewanella putrefaciens* has increased markedly with the application of this method, and it has been identified as an opportunistic pathogen in aquaculture. In this study, *Shewanella putrefaciens* bacteria were isolated from rainbow trout (*Oncorhynchus mykiss*) in cages in Karkamış Dam Lake and identified through the use of 16S rDNA sequencing. Furthermore, the lytic phages of this bacterium were isolated from the same dam lake and visualized by field emission electron microscopy. It was determined that the phages obtained exhibited an icosahedral head structure, lacked a tail, and were approximately 50-60 nm in length. One of the primary factors influencing the efficacy of phage therapy studies is the prevalence of opportunistic pathogens. This study demonstrated that the lytic phages of the opportunistic pathogen *Shewanella putrefaciens*, which has been increasingly reported in recent years, may have the potential to be utilized in phage therapy models.

Key words: Aquaculture; *Shewanella putrefaciens*; bacteriophage

RESUMEN

Hasta la década de 1990, los estudios sobre bacterias aisladas mediante los métodos de diagnóstico tradicionales, que se han empleado durante muchos años para la detección de patógenos en la acuicultura, se limitaban a 15-20 especies bacterianas. Por el contrario, el número de bacterias identificadas ha llegado a 70 mediante el uso de la secuenciación del 16S rDNA, que se ha identificado como el estándar de oro para la identificación en los últimos años. *Shewanella putrefaciens* es una bacteria patogénica que fue aislada de peces marinos en agua dulce. El número de informes sobre *Shewanella putrefaciens* ha aumentado notablemente con la aplicación de este método, y se ha identificado como un patógeno oportunista en la acuicultura. En este estudio, la bacteria *Shewanella putrefaciens* se aisló de la trucha arcoíris (*Oncorhynchus mykiss*) en jaulas en el lago de la presa Karkamış y se identificó mediante el uso de la secuenciación del rDNA 16S. Además, los fagos líticos de esta bacteria se aislaron del mismo lago de la presa y se visualizaron mediante microscopía electrónica de emisión de campo. Se determinó que los fagos obtenidos exhibían una estructura de cabeza icosaédrica, carecían de cola y tenían una longitud de aproximadamente 50-60 nm. Uno de los factores principales que influyen en la eficacia de los estudios de terapia con fagos es la prevalencia de patógenos oportunistas. Este estudio demostró que los fagos líticos del patógeno oportunista *Shewanella putrefaciens*, del que se ha informado cada vez más en los últimos años, pueden tener el potencial de ser utilizados en modelos de terapia con fagos.

Palabras clave: Acuicultura; *Shewanella putrefaciens*; bacteriófago

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INTRODUCTION

The genus of *Shewanella* bacteria encompasses more than 35 species, which are variously classified as *Pseudomonas*, *Achromobacter*, or *Alteromonas* [1]. *Shewanella* bacteria can be widely isolated in the environment, particularly in freshwater and seawater, and play an important role in the degradation of organic matter. Some species of this genus have been documented to cause infection in aquatic environments. Moreover, some have been documented to cause soft-tissue infections in humans [2].

Shewanella putrefaciens is a gram-negative and motile-positive bacterium with rod-shaped cells belonging to the Alteromonadaceae family. Despite the fact that this bacterium is particularly prevalent in the bacterial flora of marine fish intestines, studies examining its isolation in freshwater fish are scarce. Conversely, the role of *Shewanella putrefaciens* in fish pathology remains incompletely understood. To date, only a limited number of studies have been published on the subject of fish infections caused by this microorganism. These studies were conducted on seahorse species, including *Siganus rivulatus* and *Dicentrarchus labrax*, as well as two freshwater fish, namely carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) [2].

In recent years, there has been a notable increase in the reporting of these bacteria as an opportunistic pathogen in fish. Additionally, these bacteria have been identified as a novel etiological agent of a disease known as "Shewanellosis," which presents with symptoms such as numbness, skin discoloration, and lesion formation [2 ,3 ,4 ,5]. The first documented case of a disease outbreak in freshwater fish related to Shewanellosis was reported in Poland in 2004 [4]. Subsequently, it has been identified as a pathogen in a number of freshwater fish species [2]. The objective of this study was to isolate and characterize Shewanellosis, which has recently been identified as a pathogen in aquaculture, from sick rainbow trout samples sourced from aquaculture facilities operating in Karkamış Dam Lake. Additionally, the study aimed to visualize its lytic phages with potential prophylactic and therapeutic applications through electron microscopy following their isolation from the same dam lake using the double layer agar method.

MATERIALS AND METHODS

Bacterial isolation

The study encompasses 20 farms situated in the vicinity of the Karkamış Dam Lake. In the course of the field survey, the managers of each enterprise were interviewed to obtain the necessary anamnesis information. A total of 60 fish were collected, including three samples of fish that were observed to be stagnant in the cages, not taking feed, showing darkening of the skin colour and exophthalmus symptoms. A total of 420 fish were sampled seven times throughout the year and transported to the laboratory in ice-filled containers in a manner that ensured they did not come into contact with the ice. The fish were weighed in the laboratory. An autopsy was conducted on fish with an average weight of 250 grams, with a detailed examination of the internal organs. For the purposes of bacterial isolation, the external lesions, liver, spleen, kidneys and intestines of all samples were inoculated on Tryptic Soy Agar (TSA), Brain-Heart Infusion Agar (BHIA) and Nutrient Broth (NB) media. The inoculated media were incubated at 20-24°C (Lovibond BOD Incubator, TC 135 S, UK) for 48 hours. To obtain pure cultures of the bacteria, the inoculations were repeated three times, and the colonies were stored at -22°C with a mixture of Tryptic Soy Broth (TSB) and 20% glycerol for later use.

Bacterial identification

Bacterial samples extracted from the stock were isolated into pure culture after revival in appropriate media. Commercial DNA isolation kit (Vivantis GF-BA-100) was used for molecular identification of bacterial samples. For the identification of the isolated DNA samples, a universal bacterial primer called BAK2-F from the Internal Transcribed Spacer (ITS) gene regions: 5'AGTTTGATC(A/C)TGGCTCAG 3'BAK11-F: 5'GGACTAC(C/T/A) AGGGTATCTAAT 3'primer pairs were used. PCR mix protocol (10x PCR Buffer, $MgCl_2$, Primer F -Bak2, Primer R -Bak 11, DNA (Template), dNTP, Taq polymerase, ddH_2O) was applied for amplification of DNA samples under appropriate PCR conditions and amplification of DNA bands for DNA gene sequence analysis. After the preparation of the mix protocol, for the amplification of bacterial DNA (Thermo Scientific™, PCR Master Mix (2X), USA), denaturation was performed at 95°C for 5 min, followed by 30 s at 95°C, 45 s at 48°C, 1 min at 72°C, repeated as 35 x and the PCR (Thermo Scientific ARKTIK Thermal Cycler Type 5020 96 Well Lab, USA) study protocol was completed with 10 min at 72°C. After that, Exsosap purification for DNA gene sequence analysis was performed by mixing 2.5 µL of PCR product and 1 µL of Exsosap and the PCR protocol was applied at 37°C for 30 min and 80°C for 15 min. After Exso-SAP purification, the sequence PCR method was applied and nucleotides were fluorescently labeled with BigDye 3.1. All samples were then prepared with Cycle Sequencing PCR reaction mix and Cycle Sequencing PCR Protocol was applied. Before sequencing, all samples were Sephadex purified and loaded into AB3130XL16 Genetic Analyzer (Hitachi, Japan). For Sephadex purification; 1 g of Sephadex gel was dissolved in 14 mL of deionized ultrapure water and transferred into 700 µL receiver column and centrifuged at 1800 G for 2.5 min to remove the liquid part of the sephadex mixture. Sequence PCR products were added to the resulting cola and centrifuged (McKesson, 1015031, USA) at 2127.5 G for 2.5 min and the lower part after centrifugation was loaded into the sequencer and run. For the evaluation of sequence data, raw data were processed with Bioedit 7.2.5 software [6] and compared with reference databases using NCBI - Basic Local Alignment Search Tools (BLAST) program. All samples were blasted in duplicate after consensus was reached.

Phage isolation and identification

For phage isolation, water samples were taken with the help of a Nansen bottle from 3 different depths (surface-middle-bottom) into 250 mL sterile capped laboratory bottles from around the cage during the visits to the facilities where sick fish were sampled and transported to the laboratory under aseptic conditions [7]. The obtained samples were incubated at 15°C for 3 h after mixing for 10 min with the help of a sterile swab. During incubation, the bottles were frequently inverted and the water samples were taken into 20 mL sterile falcon centrifuge tubes and centrifuged at 1850 G, 4°C for 5 min. The supernatants were mixed with the same amount of liquid medium (NB) and incubated in Jeio-Tech brand shaking incubator (Jeio Tech, ISS-4075, Korea) at 64.75 G, 22°C for 24 h. After incubation, the samples were transferred to 15 mL falcon tubes and after adding 0.5 mL chloroform to each sample, they were inverted and mixed for 10 min. The prepared tubes were centrifuged at 1850 G for 15

min at +4°C and the supernatants were filtered with the help of a 0.20 µm pore diameter injector filter and the water sample obtained was considered as potential phage lysate and stored in separate sterile tubes at +4°C [8].

The determination of the presence of the phages in samples isolated and enriched was carried out using the double-layer agar plate evaluation method with minor differences. In the method carried out to determine the presence of phages in the samples collected, 2-stage casting was performed in the petri dish and the lower agar was hard and the upper agar was a mixture of water sample and bacteria. Tryptic Soy Agar (TSA) was used as the bottom agar forming the bottom of the petri dish and 15 mL was poured equally into a 90 mm sterile petri dish and allowed to dry. The top agar was prepared by adding 0.7% agar to Nutrient Agar (NA) and sterilized in autoclave and kept ready in a water bath at 30-40ºC. The previously stocked *Shewanella putrefaciens* was removed from the stock and each bacterium was used as a potential host for phage isolation. For each bacterial isolate, 200 µL of phage suspension was mixed in sterile vials with 200 µL of samples kept in liquid medium overnight. The mixture was incubated at room temperature for 5-10 min to allow the phages to adsorb the host cells and 5 mL of the liquid medium kept in the water bath was added to this mixture and the mixture was inverted several times. The mixture was poured onto the prepared sub-agar and the mixture was allowed to form a thin plate for 15 min. All prepared petri dishes were incubated at 15ºC for 24-48 h and the presence of plaques was analyzed. Formed plaques were evaluated as an indicator of the presence of lytic phages [9]. The plates were taken with a sterile pasteur pipette and 1 mL SM Buffer and 50 µL chloroform were added and the tubes were vortexed gently. The mixture was incubated in a Thermal Cycler (Thermo Scientific, ARKTIK Thermal Cycler Type 5020 96 Well Lab, USA) at 22ºC and 92.5 G for 2 h and the mixture formed at the end of incubation was tested again using the double layer agar inoculation method. The same process was repeated 3 times and the isolated phages were purified [10]. The resulting purified phage suspension was filtered with a 0.20 µm pore diameter injector filter; 10 µL of phage suspension was mixed with 10 mL of host bacterial culture grown in liquid medium and incubated at 64.75 G +22°C overnight. After incubation, it was filtered again and 50 % glycerol and 10 % SM Buffer were added to the filtrate and stocked as purified phage stock at - 22°C [11].

The examination of the phage by field emission scanning electron microscope

The isolated and purified bacteriophage extracts were diluted at 1:10 ratio with SM buffer (2.0 g/L $MgSO_4$, 5.8 g/L NaCl, 5 mL/L pre-sterilised 2% gelatin, pH 7.5, 50 mL/L of 1 M Tris) and filtered with a 0.20 µm pore diameter injector filter. The filtrates obtained were taken to Kayseri Erciyes University Technology Research and Application Center (TAUM) and imaged by a field emission scanning electron microscope (ZEISS, GEMINI 500, Germany). 20 µL of the samples were dropped onto carbon-coated 300 mesh grids on a parafilm surface and allowed to dry overnight. The grids were then washed twice with distilled water and observed and recorded with the field emission scanning electron microscope.

RESULTS AND DISCUSSION

Bacterial isolation and identification

For bacterial isolation, sick fish samples were grouped according to the facilities where they came from and their symptoms were recorded by taking photographs. As with the previously reported [12]. Symptoms of shewanellosis in rainbow trout, *Aeromonas sobria* and *Shewanella putrefaciens* were isolated from the samples with necrotic lesions and exophthalmus in the macroscopic examination (FIG. 1). After the external examination of the fish grouped in the laboratory, they were cleaned with 70% ethyl alcohol in a sterile cabinet and dissected. Additionally, as reported by Pekala *et al*. [4] in a study on the pathogenicity of *Shewanella putrefaciens*, intense haemorrhages in the peritoneum and internal organs and enlargement of the spleen were observed in fish (FIG. 2).

FIGURE 1. Fish samples showing disease symptoms for bacterial isolation

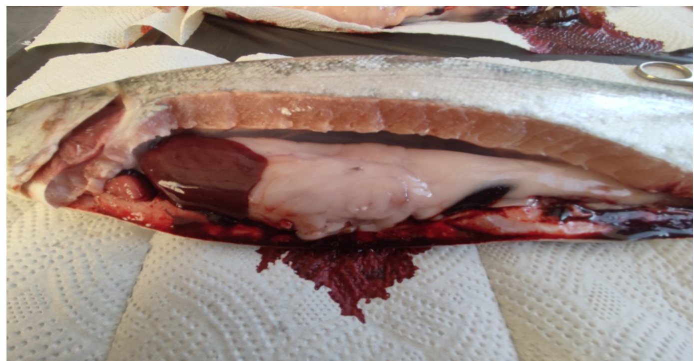

FIGURE 2. Necropsy for bacterial isolation

The specimens were collected from the liver, spleen, kidneys and intestines of the necropsied fish in a sterile cabinet with the help of a sterile core and cultured on previously prepared media (Tryptic Soy Agar, Brain-Heart Infusion Agar, Blood Agar and Nutrient Agar). After incubation in TSA and BHIA for 48 hours, smooth and rounded cream-colored bacterial colonies were obtained (FIG. 3). Bacteria detected in all samples.

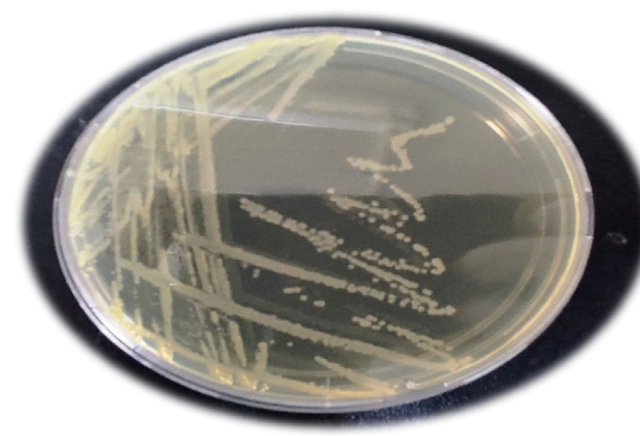

FIGURE 3. Bacterial isolation media

In similar studies, *Shewanella putrefaciens* identification was performed with API 20E and biochemical identification of collected *Shewanella putrefaciens* strains as well as enzymatic activities tested in API Zym were determined [13]. In this study, the identification of the target bacteria was carried out using the 16s rDNA gene sequencing method, which is accepted as the golden method in bacterial identification. After bacterial isolation, passaged and purified colonies were prepared for bacterial identification according to the Vivantis bacterial DNA isolation kit protocol and PCR protocols were applied to the isolated DNAs. The products obtained were visualized on agarose gel for control before sequencing.

The results obtained after bacterial gene sequencing were confirmed to be 97% similar to the strain with accession number 'CP028435.1' in NCBI and DNA sequence chromatograms consistent with *Shewanella putrefaciens* as shown in FIG. 4.

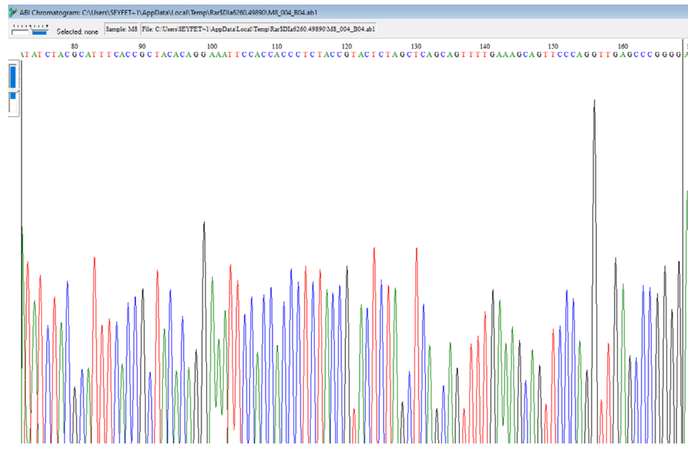

FIGURE 4. The chromatograms of Bacterial DNA Sequence

The sequence results were compared with reference strains of *Shewanella putrefaciens* in the National Center for Biotechnology Information (NCBI) database to ascertain the degree of similarity (FIG. 5). The most closely related species was identified as *Shewanella putrefaciens* WS13 (sequence ID: CP028435.1). The highest degree of similarity was observed with *Shewanella putrefaciens* WS13, with a similarity percentage of 661/680 (97%). There were two gaps in the alignment, representing 0% of the total number of gaps. The total score was calculated as 1.179 bits (638). The similarity values and alignment comparison are provided in FIG. 6.

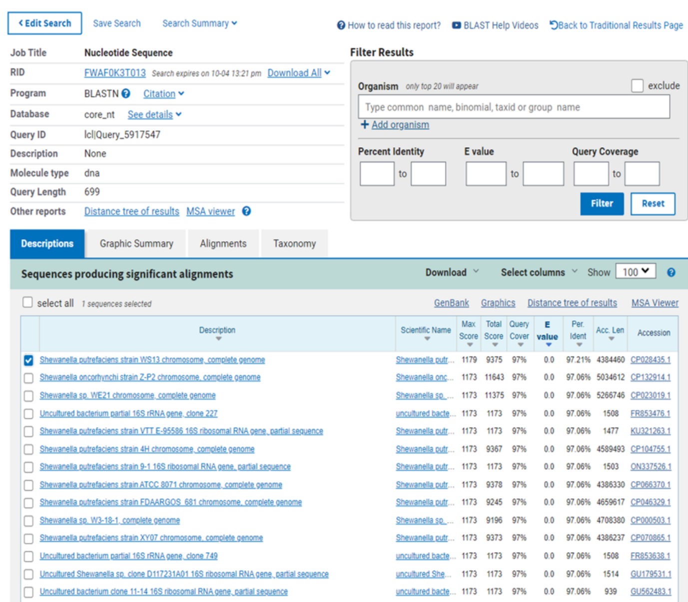

FIGURE 5. A comparison of the sequence of one *Shewanella putrefaciens* with the sequences of reference strains of *Shewanella putrefaciens* in the National Center for Biotechnology Information (NCBI) database was conducted to ascertain the level of similarity

Shewanella putrefaciens strain WS13 chromosome, complete genome

Sequence ID: CP028435.1 Length: 4384460 Number of Matches: 8

Range 1: 2355981 to 2356659 GenBank Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
1179 bits(638)	0.0	661/680(97%)	2/680(0%)	Plus/Plus

```
Query  18       TACGCATTTCACCGCTACACCTGGAATTCTACCCCCTCWACAAGACTCTAGTTCGCCAGT  77
Sbjct  2355981  TACGCATTTCACCGCTACACCTGGAATTCTACCCCCTCTACAAGACTCTAGTTCGCCAGT  2356040
Query  78       TCGAAATGCTATTCCTAGGTTGAGCCCAGGGCTTTCACATCTCGCTYMMCMAACCGCCTG  137
Sbjct  2356041  TCGAAATGCTATTCCTAGGTTGAGCCCAGGGCTTTCACATCTCGCTTAACAAACCGCCTG  2356100
Query  138      CGCACGCTTTACGCCCAGTAATTCCGATTAACGCTCGGACCCTCCGTATTACCGCGGCTG  197
Sbjct  2356101  CGCACGCTTTACGCCCAGTAATTCCGATTAACGCTCGGACCCTCCGTATTACCGCGGCTG  2356160
Query  198      CTGGCACGGAGTTAGCCGGTCCTTCTTCTGKAGGTAACGTCACAGATATARCGTATTAAR  257
Sbjct  2356161  CTGGCACGGAGTTAGCCGGTCCTTCTTCTGTAGGTAACGTCACAGATATAGCGTATTAAA  2356220
Query  258      YTACACCCTTTCCTCCCTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGC  317
Sbjct  2356221  CTACACCCTTTCCTCCCTACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGC  2356280
Query  318      GGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAG  377
Sbjct  2356281  GGCATGGCTGCATCAGGGTTTCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAG  2356340
Query  378      GAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGSTGaksrkssTCTCAGAACAGCTAGGGAT  437
Sbjct  2356341  GAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAGAACAGCTAGGGAT  2356400
Query  438      CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACCTAGMTTCATCCAATC  497
Sbjct  2356401  CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACCTAGGTTCATCCAATC  2356460
Query  498      GCGGAAGGCCCGAAGGTCCCCTCCTTTCCCCCGTAGGGCGTATGCGGTATTAGCAGTCGT  557
Sbjct  2356461  GCGGAAGGCCCGAAGGTCCCCTCCTTTCCCCCGTAGGGCGTATGCGGTATTAGCAGTCGT  2356520
Query  558      TTCCAACTGTTATCCCCCTCGACTGGGCAGATCCCTA-GCATTACTCACCCGTCCGCCGC  616
Sbjct  2356521  TTCCAACTGTTATCCCCCTCGACTGGGCAGATCCCTAGGCATTACTCACCCGTCCGCCGC  2356580
Query  617      TCGCCACCTCAGAAGTAAACTCCCTTGTGCTGCCGCTCGACTTGCATGTGTTAGGCCTGC  676
Sbjct  2356581  TCGCCACCTCAGAAGTAAACTCCCTTGTGCTGCCGCTCGACTTGCATGTGTTAGGCCTGC  2356640
Query  677      CGCCCAGCGTTCAATCTGAG  696
Sbjct  2356641  CG-CCAGCGTTCAATCTGAG  2356659
```

FIGURE 6. Alignment result for the taxon *Shewanella putrefaciens* (Query: Shewanella putrefaciens, Sbjct: *Shewanella putrefaciens* WS13 (Sequence ID: CP028435.1)

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There are a few publications on *Shewanella putrefaciens* bacterium in Turkey related to soft skin infection in human field; in aquaculture, it has been reported in eels (*Anguilla anguilla*) in Antalya Bay, carp (Cyprinidae) in Çoruh River, oysters in Marmara Sea and endemic fish species in Deriner Dam Lake [14 , 15]. The identification of this bacterium, which was reported for the first time in Karkamış Dam Lake, was carried out by 16s rDNA gene sequencing method in the present study unlike the studies in the literature.

The isolation and examination of the lytic phages of Shewanella putrefaciens

The supernatants obtained from the water samples collected from around the net cages of the enterprises operating in the reservoir were isolated by enriching with liquid medium at a ratio of 5X (NB). The isolation obtained was recorded with the double layer agar method and the presence of plaque was confirmed and recorded as shown in FIG. 7. In several studies on about lytic phage of *Shewanella putrefaciens,* it was isolated 5 from 6 *Shewanella putrefaciens* strains [16] and 1 from 3 *Shewanella putrefaciens* strains isolated from marine sources [17] by investigating their prophages.

FIGURE 7. The double layer agar plate image for plaques of *Shewanella putrefaciens*

The plates visualized in FIG. 7. were obtained with a sterile pasteur pipette and the phages were single dropped by using the double layer agar inoculation method (FIG. 8). Single fallen phage plates were collected with the help of a sterile glass pasteur pipette and filtered with 1mL SM buffer using a 0.20 µm pore diameter syringe filter and stocked into sterile eppendorf tubes at +40 °C.

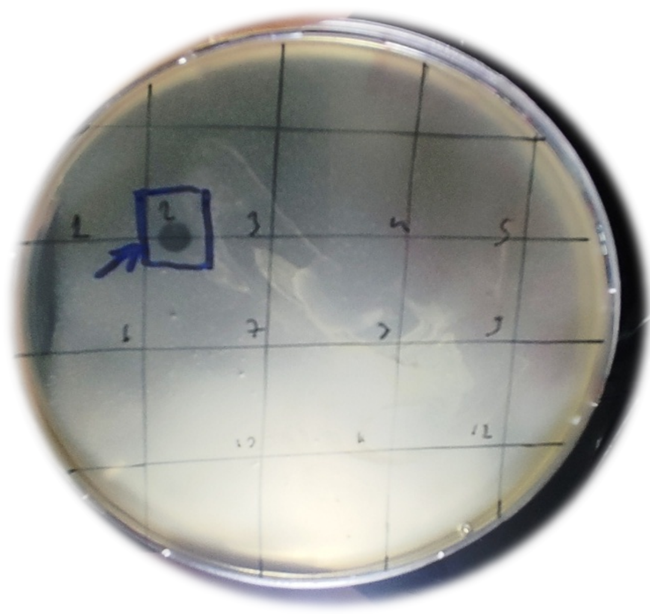

FIGURE 8. Diluted and single-dropped phage plaque images

The isolated and purified phage filtrates were diluted with SM Buffer and the grids prepared were observed by electron microscopy and phage particles were measured and recorded as shown in FIG. 8. The imaged phages were found to have icosahedral head structure, tailless and 50-60 nm in size (FIG. 9). In another study in which *Shewanella* lytic phages were imaged by TEM, it was reported that the SFCi1 phage isolated in the morphological analysis of the images obtained belonged to Myoviridae family with an icosahedral capsid diameter of approximately 70 nm [18].

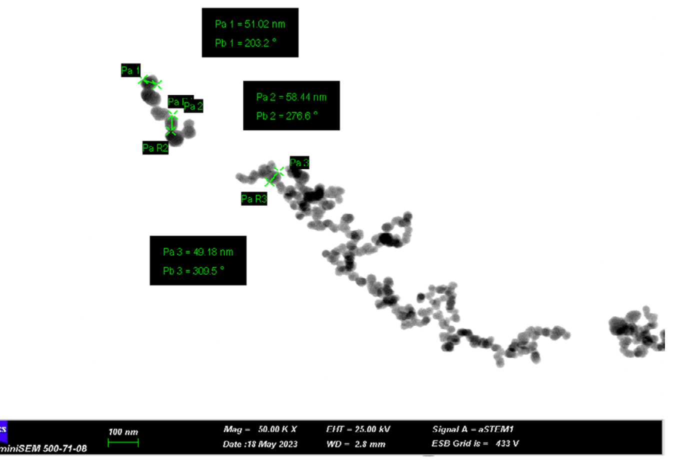

FIGURE 9. Size measurement of phages of *Shewanella putrefaciens* by electron microscopy

Shewanella putrefaciens has been reported as an opportunistic pathogen in aquaculture in recent years. Classical microbiological diagnostic methods used in the diagnosis of bacterial diseases in aquaculture are reliable but may not be fast and practical and may not give reliable results in the diagnosis of such bacteria. The 16S rDNA sequence analysis method, which was preferred for bacterial identification in this study, is considered to be the gold standard for the diagnosis of bacterial infections and for the identification of bacteria at genus and species level [19]. Bacteriophages, also the subject of this study, are a subgroup of prokaryotic viruses that specifically invade bacterial cells. Shortly after their discovery in 1917, they were tested as antibacterial agents against bacterial infections in animals and humans, but remained in the background for many years due to the discovery and widespread use of antibiotics [20 , 21].

CONCLUSION

The increasing incidence of infection due to antibiotic-resistant bacteria in recent years has led to a renewed interest in phages and phage therapy. Although different phage isolation and identification studies have been conducted on *Shewanella putrefaciens*, the number of studies evaluated in terms of aquaculture is relatively limited. In this study, *Shewanella putrefaciens* and its lytic phages, which have been reported as pathogens in aquaculture in recent years, were isolated and the phages were visualized by electron microscopy. Pathogenic bacteria causing many primary and secondary infections reported in aquaculture and, more phages should be isolated and more therapy models should be developed for prophylactic/therapeutic use.

ACKNOWLEDGEMENTS

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Ethical Approval

All animal studies were approved by the Animal Ethics Committee of KSÜZİRHADYEK and Research Institute (Protocol number: 2022/1).

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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