Culture and molecular characterization of phages isolated from rainbow trout farms and sewage treatment plants and investigation of their effects on *Yersinia ruckeri*

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Abstract

In the present study bacteriophages isolated from rainbow trout farms and sewage treatment plants were genetically identified and their effectiveness on *Yersinia ruckeri* isolates from clinical cases of red mouth disease was investigated. Fish samples suspected to red mouth disease were collected from rainbow trout farms located in west Azerbaijan. *Y. ruckeri*, the causative agent of red mouth disease was initially identified using biochemical tests. The biotypes of all *Y. ruckeri* isolates were determined and their identity was confirmed by employing genus specific primers. Antimicrobial resistance of *Y. ruckeri* isolates were examined using common antibiotics in use in aquaculture. In order to isolating lytic bacteriophages, environmental samples mainly from rainbow trout farms and sewage treatment plants were collected in a period of six months. Isolated bacteriophages were titrated using two-layer agar method and their bactericidal effects were examined. For molecular characterization of bacteriophages, genomic DNA was extracted. Extracted genomic DNA from bacteriophages was digested using *Msp*I endonuclease. The results revealed that 4.48% of examined fish were positive for *Y. ruckeri*. Bacteriophages isolated from urban sewage treatment plants were effective on *Y. ruckeri* isolates. Maragheh and Urmia sewage treatment plants had the maximum and minimum phage titers, respectively. The genomic DNA of all isolated phages were smaller than genomic DNA of *Lambda* phage and all examined phages showed similar genomic DNA digestion patterns. It was concluded that sewage treatment plants could be an important source for phages effective on *Y. ruckeri* and maybe other aquaculture bacterial pathogens.

Keyword: Bacteriophage, Rainbow trout, Redmouth disease, Wastewater, *Yersinia ruckeri*.

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Introduction

Yersinia ruckeri is a Gram-negative enterobacterium, causing enteric redmouth disease (ERM) which mainly affects farmed salmonid species (Davies and Frerichs 1989; Horne and Barnes 1999). Outbreaks of certain strains of *Y. ruckeri* have been reported in vaccinated trout farms (Fouz *et al.*, 2006; Arias *et al.*, 2007). ERM is characterized by reddening of the mouth and throat, which is caused by subcutaneous haemorrhaging. Inflammation and erosion of the jaws and palate, haemorrhaging around the base of the fins, bilateral exophthalmia are other external signs of disease (Austin and Austin, 2007). In many cases of yersiniosis outbreaks, antibiotic administration is the first therapeutic and preventive measure; however, significant disadvantages accompanied with the use of antibiotics in aquaculture such as developing antibiotic resistance, necessities the need for developing alternative disease control strategies (Ryckaert *et al.*, 2010).

The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages (Kutateladze and Adamia, 2010). Bacteriophages or phages are bacterial viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause the bacterium to lyse. The application of bacteriophages against bacterial infections could be an effective alternative approach for the control of bacterial infections, as the efficacy of bacteriophages against both Gram-positive and Gram-negative bacteria has been reported previously (Weber-Dabrowska *et al.*, 2000; Stone 2002; Inal, 2003). In addition, because of the lack of bacteriophage receptors on eukaryotic cells, they might be associated with fewer side effects compared to antibiotics (Sabouri Ghannad and Mohammadi, 2012). Human phage therapy has been practiced for the first time in France since 1919, when d'Hérelle successfully treated several children who were suffering from severe dysentery (Pirnay *et al.*, 2011). One of the best-known recent studies on the use of phages in veterinary medicine is using phages to treat experimental *E. coli* infections in mice. Soothill (1994) reported the utility of phages in preventing and treating experimental disease in mice and guinea pigs infected with *Pseudomonas aeruginosa* and *Acinetobacter*, and they suggested that phages might be efficacious in preventing infections of skin grafts used to treat burn patients (Sulakvelidze *et al.*, 2001).

Bacteriophages have several characteristics that make them potentially attractive therapeutic agents. They are (i) highly specific and very

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effective in lysing targeted pathogenic bacteria, (ii) safe, as underscored by their extensive clinical use in Eastern Europe and the former Soviet Union and commercial sale of phages in the 1940s in the United States, and (iii) rapidly modifiable to combat the emergence of newly arising bacterial threats.

The present study was undertaken in order to isolating bacteriophages which are effective on *Yersinia ruckeri*, isolated from clinical cases of ERM disease in rainbow trout and investigating the genetic variation of isolated bacteriophages.

Materials and methods

Examined fish for ERM

During August 2009 to June 2011, a total number of 223 farmed rainbow trout fish from 21 farms of west Azerbaijan, Iran, with clinical signs of inappetence, exophthalmia and haemorrhage base of fins referred to the microbiology laboratory of Aremia and Aquatic Animals Research Institute, Urmia University were examined. Liver, kidney and blood samples were aseptically collected from diseased fish and immediately used for routine bacteriological examination.

Isolation of Y. ruckeri

Fish tissues were cultured aseptically by streaking a loop on to brain heart infusion (BHI) agar and MacConkey agar plates and incubated at 25°C for 48 h. Colonies of grown bacteria were subcultured on trypticase soy agar (TSA) to check purity, then cultured in trypticase soy broth (TSB) for 48 h at 25°C and identified using conventional biochemical system (Austin and Austin, 2007). Obtained biochemical results were compared with the reported biochemical properties of this bacterium in the literature (Horne and Barnes, 1999; Romalde *et al*., 2003; Austin and Austin, 2007). Biotyping of isolated bacteria was also undertaken using motility test and fermentation of sorbitol, Tween 80 and Tween 20 hydrolysis (Akhlaghi and Sharif Yazdi, 2008).

Amplification of 16S rRNA

All the isolated bacteria identified as *Y. ruckeri* by biochemical procedure were also confirmed using a specific PCR assay for definitive identification of *Y. ruckeri*. A fragment of 575 bp in size was amplified targeting *16S rRNA* gene using a pair of primers YER8 (5'-GCGAGGAGGAAGGGTTAAGTG-3') and YER10 (5'-GAAGGCACCAAGGCATCTCTG-3') described by Gibello *et al*. (1999). Bacterial genomic DNA was extracted using boiling method (Kawasaki *et al*., 2005). The PCR amplification was carried out in a total volume reaction of 25 µL containing 50-100 ng of genomic bacterial as described previously (Gibello *et al*., 1999). The amplification reaction was carried out in a gradient Mastercycler (Eppendorf, Germany) using an initial denaturation at 94ºC for

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5 min and 35 cycles of denaturation for 1 min at 94 ºC, annealing at 58ºC for 1 min, and extension for 1 min at 72 ºC, following by a final extension step of 72ºC for 5 min. The resultant PCR products were separated on a 1.5% agarose gel.

Antimicrobial susceptibility test

Antimicrobial susceptibilities of *Y. ruckeri* isolates to Cefotaxim (30 µg/disk), Erythromycin (15 µg/disk) were tested by the disk diffusion method according to the National Committee for Clinical Laboratory Standards (2013). All isolates were tested on Mueller-Hinton agar (Merck, Germany).

Isolation of bacteriophage

A number of ten *Y. ruckeri* isolates from ERM cases were referred to the microbiology laboratory (Artemia and Aquatic Animals Research Institute, Urmia, Iran). They were differentiated using biochemical tests and PCR technique and used for enrichment of bacteriophages from environment.. For bacteriophage isolation water, samples from a number of 27 rainbow trout farms, three dams, three rivers and six wastewater plants were collected. An amount of 50 mL of water samples from above mentioned sources was centrifuged at 3,000×g for 30 min. The supernatant was filtered through 0.45 µm microbiological filter for removing particulates. An amount of 45 mL of filtrated wastewater was added to 8 h old *Y. ruckeri* culture grown in 5 mL TSB with 1% NaCl (TSBS), 5 mL double strength TSB and 70 µL $MgSO_4$ further incubated at ambient temperature (25°C) for 24 h. To the mixture, 3 mL chloroform was added then mixture vortexed for 15 min, incubated at room temperature for 2 h in order to suspending chloroform then biphasic solution centrifuged at 3,500 ×g for 15 min at 4°C. The supernatant was filtered through 0.45 µm filter to remove bacterial cells and cellular debris and the filtrate tested for the presence of phage.

Propagation of phage and determination of their titers

The isolated phages were propagated on their respective host grown as a lawn culture on TSAS in Roux bottles. The host culture was grown on TSBS for 12 h and 4 mL host culture and 1 mL phage suspension were mixed and spread on the surface of TSAS agar in Roux bottles. After 5–6 h incubation at room temperature (25°C), the phages were harvested in phage buffer (5.8 g/L NaCl, 2.0 g/L $MgSO_4$, 50 mL/L 1 M Tris, pH 7.5, 5 mL/L of pre-sterilized 2% gelatin). A soft agar overlay technique (Anderson *et al.*, 2011) was used for determining the phage titres. Soft agar (15 g/L Tryptone, 5 g/L Yeast extract, 10 g/L NaCl, 1 g/L MgCl, 1 M 10 mL/L $CaCl_2$, 3 g/L Agar) tubes were maintained at 48–50°C in a water bath. Phage suspensions were serially diluted in phage buffer. One hundred microliter of each phage dilution was then mixed with 1 mL of 8 h old host culture in

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TSBS and added to 5 mL of molten soft agar in tubes. The mixture was rolled between palms to enhance good mixing and overlaid on TSAS plates. After solidification the plates were incubated overnight at ambient (25±1°C) temperature and plaques were counted and expressed as plaque forming units/ milliliter (PFU/mL).

Evaluation of antibacterial activity of phages

The antibacterial effects of phages against *Y. ruckeri* strains were examined by the tube method and the double-layer agar plate method at 24 h of incubation at 25°C. Three different concentrations of phages were evaluated as low ($<10^4$ PFU/mL), medium (10^4–10^7 PFU/mL), and high ($>10^7$ PFU/mL). According to the intensity of growth inhibition, the results were reported as +++ (75–100% reduction of bacteria compared to control group), ++(50–75% reduction of bacteria compared to control), + (25–50% reduction of bacteria compared to control), and - (<25% reduction of bacteria compared to control).

Analysis of phage DNA

For phage propagation and extraction of nucleic acid from phage particles, the method described by Su *et al.* (1998) was followed. Phages were harvested from soft agar overlaid plates with confluent plaque formation. To each plate, 5 mL of phage buffer was added and gently shaken in a shaker for 2 h. Bacterial cells were separated by centrifugation at 14,000×g for 15 min at 4°C. The supernatant was filtered through 0.45 µm filter and purified by phage PEG precipitation/purification method.

Phage precipitation was carried out using sterile 7.5 mL 20 % PEG-8000/2.5 M NaCl PEG (for 100 mL add 20 g PEG-8000 and 14.6 g NaCl, filter sterilize) at a ratio of 1:50 for 24 h at 4°C and pelleted at 4000 ×g for 5 min. The pellet was resuspended in STE buffer (STE: for 100 mL add 1 mL 1 M Tris (pH=8), 0.2 mL 0.5 M EDTA (pH=8), 2 mL 5 M NaCl). Genomic DNA was extracted from isolated phages using high pure viral DNA extraction kit (Roche, Germany). Extracted DNA was electrophoresed on 0.7% agarose gel and the gel photographed using ultraviolet transillumination. Extracted genomic DNA from isolated bacteriophages was further characterized using restriction endonuclease *Msp*I. Digestion reaction was carried out in a volume of 15 µL including 10 U *Msp*I endonuclease, 1.5 µL of ×10 endonuclease buffers and 10 µL extracted genomic DNA. The total reaction volume was well mixed and incubated at 37°C for 2-3 h. Digested DNA was visualized on 1% agarose gel containing etidium bromide and photographed. Lambda DNA was used as a molecular marker for estimating bacteriophages DNA size.

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Results

Isolation of Y. ruckeri from diseased fish

After routine bacteriological examination for detecting *Y. ruckeri*, a number of 10 (4.48%) out of 223 examined fish were positive for this pathogen. Biochemical characteristics of isolated bacteria are shown in Table 1. The results of biotyping of *Y. ruckeri* isolates revealed that seven (70%) isolates were belonged to biotype 1 and three (30%) classified as biotype 2 (Table 2).

Antimicrobial susceptibility

Antimicrobial susceptibility test revealed that *Y. ruckeri* isolates were susceptible to a number of antibiotics. Quinolones were the most effective antibiotics against *Y. ruckeri* isolates while Lincomycines were not effective on these bacteria. The percentage of susceptibility of *Y. ruckeri* isolates to different antibiotics is shown in Table 3.

Table 1: Biochemical characteristics of isolated *Yersinia ruckeri*.

Test	*Yersinia ruckeri* isolates									
	112	**113**	**133**	**134**	**137**	**138**	**139**	**140**	**141**	**142**
Cellebiose	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	-	+	+	+	+	+	-	-
Catalase	+	+	+	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Production of H2S	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Terhalose	+	+	+	+	+	+	+	+	+	+
Mahnose	+	+	+	+	+	+	+	+	+	+
TSI	y/y*	y/y	y/y	y/y	y/y	y/y	y/y	y/y	y/y	y/y

*****y=yellow

Table 2: Biochemical test for biotyping *Yersinia ruckeri* isolates.

Isolates	motility	Sorbitol	Tween 80	Tween 20
112	+	+	+	+
113	-	-	-	-
133	+	+	+	+
134	+	+	+	+
137	+	+	+	+
138	+	+	+	+
139	+	+	+	+
140	+	+	+	+
141	-	-	-	-
142	-	-	-	-

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Table 3: Results of antibiogram test on *Yersinia ruckeri* isolates.

Antibiotic	Susceptibility (%)
Cephalosporins	92.85
Quinolones	97.5
Aminoglycosides	65
Penicillins	16.66
Tetracyclines	83.33
Macrolides	45
Lincomycines	0

PCR

All *Y. ruckeri* isolates detected by biochemical tests were yielded a fragment of 575 bp in size in PCR reaction using specific primers targeting *16s rRNA* gene of the bacteria for confirming the accuracy of the biochemical tests (Fig. 1).

Isolation and propagation of bacteriophages and determination of their titers

Bacteriophages were isolated only from wastewater samples collected from wastewater plants. Attempts for isolating of bacteriophages from fish farms, rivers and dams were unsuccessful. Table 4 shows the wastewater plants that bacteriophages were isolated, the titer of propagated bacteriophages and the diameter of bacterial growth inhibition zone. The growth inhibition zone generated by bacteriophages was clear and had a diameter of 0.5-1 mm (Fig. 2). Bacteriophages isolated from Maragheh wastewater plants had the highest titer which was 1.42×10^5 and the lowest titer of isolated bacteriophages belonged to Urmia wastewater plants which it was 3.8×10^2.

Molecular characterization of isolated bacteriophages

The molecular sizes of all extracted genomic DNA from bacteriophages were almost equal and under 48kb (Fig. 3). The restriction fragment length polymorphism (RFLP) patterns generated using *Msp*I endonuclease revealed that all examined bacteriophages DNA had similar patterns (Fig. 4).

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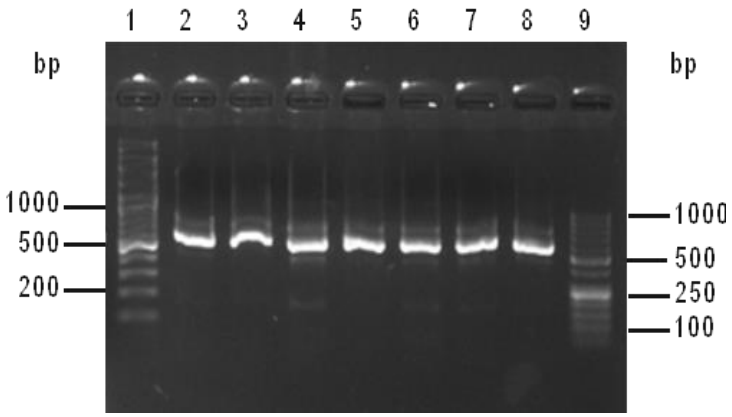

Figure1: Electerophoretic analysis (2% agarose gel) of DNA amplified fragments from different *Yersinia ruckeri* strains that isolated in this study compared with standard strain. Lane 1: Marker 100 base pair; Lane 2: Positive control (*Yersinia ruckeri* BCCG/LMG 3279); Lane 3-8: *Yersinia ruckeri* field isolates; Lane 9: Marker 50 base pair.

Table 4: Wastewater plants and the bacteriophages that were isolated and their titers.

	Wastewater plant	Bacteriophage code	Bacteriophage Titer	Bacteria Inhibition zone diameter (mm)
1	Urmia	YPU	3.8×10^{2}-1×10^{5}	0.5-1
2	Salmas	YPS	2×10^{3}-8×10^{3}	0.5-1
3	Khoy	YPKh	1.2×10^{3}-6.2×10^{3}	0.5-1
4	Miandoab	YPMb	1.12×10^{5}	0.5-1
5	Maragh	YPM	$2.5\times103\times1.42\times10^{5}$	0.5-1
6	Saghez	YOSg	1×10^{3}-7.7×10^{3}	0.5-1

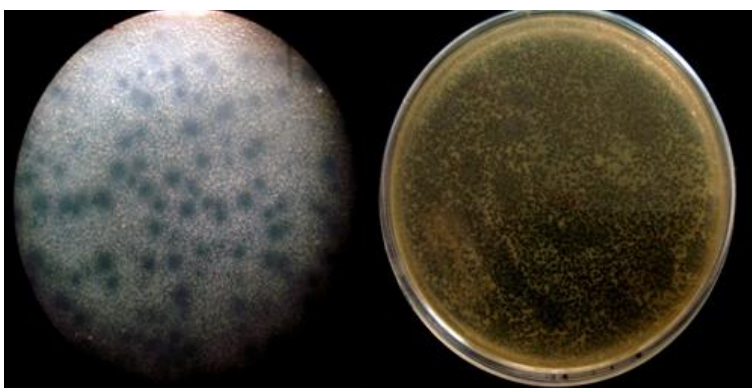

Figure 2: Plaques generated by lytic bacteriophages in *Yersinia ruckeri* grown plate.

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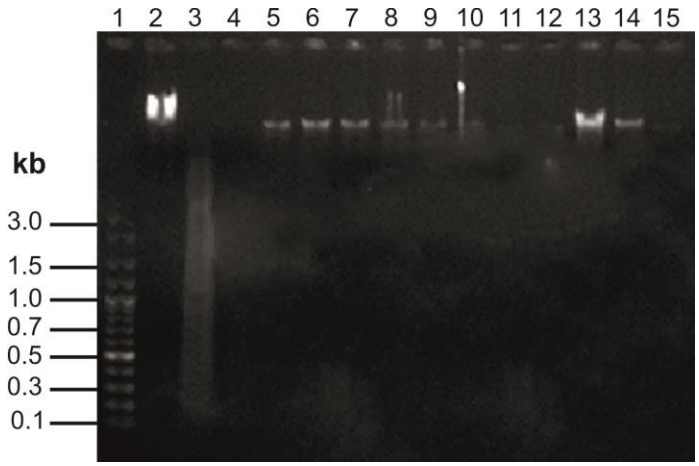

Figure 3: Extracted genomic DNA from isolated bacteriophages and lambda DNA on 1% agarose gel. Lane 1: Molecular marker 100bp (Fermentas, CA), Lane 2: Lambda DNA, Lanes 3: YPM3 DNA, Lane 4: Negative control, Lane 5-15: YPM1,YPS1, YPKh1, YPSg2, YPKh2, YPS4, YPU2, YPMb1, YPU1 and YPMb3 DNAs, respectively.

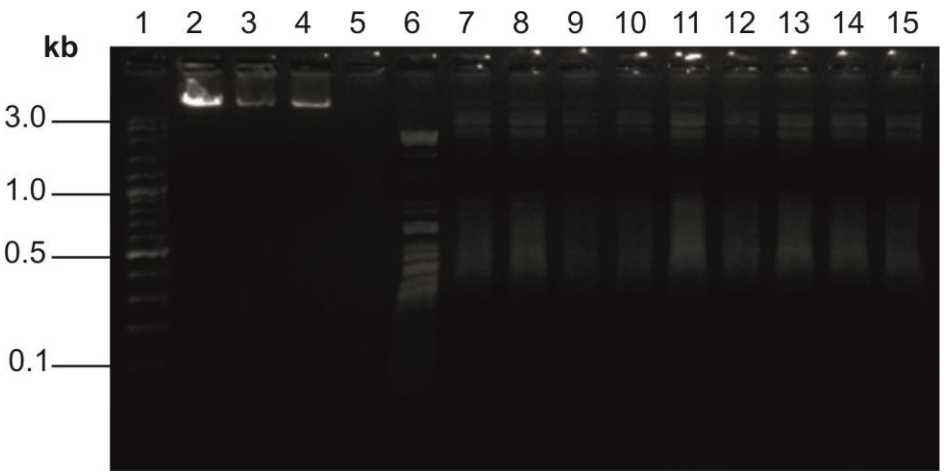

Figure 4: Undigested and digested extracted genomic DNA from bacteriophages of different wastewater plants. Lane 1: Molecular weight 100 bp, Lane 2: Lambda DNA, Lane 3: YPKh1 genomic DNA, Lane 4: YPU2 genomic DNA, Lane 6: *Msp*I RFLP pattern of Lambda DNA, Lanes 7-15: *Msp*I RFLP patterns of genomic DNAs of YPMb, YPM1, YPU1, YPS1, YPHh1, YPSg1, YPSg2, YPM3 and YPKh2.

Discussion

Y. ruckeri causing a significant economic loss in salmonid fish has been reported from many countries (Tobback *et al.*, 2007). In Iran, *Y. ruckeri* infections of rainbow trout has been reported in different regions (Soltani *et al.*, 1999; Akhlaghi and Sharifi Yazdi, 2008). In the present study, tissues from diseased fish were used for isolation of *Y. ruckeri* and the isolated bacteria were confirmed by PCR. It was found that 4.38% of examined fish were positive

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for *Y. ruckeri*, indicating the presence of this pathogen in Northwest of Iran.

Although *Y. ruckeri* is sensitive to many antibiotics, acquired resistance of *Y. ruckeri* strains to various antimicrobial agents has been reported. Frequently use of antimicrobial agents in fish culture has increased the resistant strains of many bacterial pathogens (Davies and Davies, 2010). Antimicrobial susceptibility of *Y. ruckeri* isolates showed that these bacteria were highly sensitive to Cephalosporins, Quinolones and Tetracyclines. However, isolated bacteria were less sensitive to the other antibiotics, showing an increase in antibiotic resistance to antimicrobial agents. Stock *et al*. (2002) reported that *Y. ruckeri* has natural susceptibility to Cephalosporins, Quinolones and Tetracyclines.

Bacterial diseases are main problem in aquaculture industry. The massive use of antibiotics to control infections in intensive and semi-intensive aquaculture systems has resulted in the development of resistant strains, which have made antibiotic treatments ineffective (Almeida *et al*., 2009). Therefore, there is a real need to find safe and practical alternatives in aquaculture production to both prevent and treat fish diseases. Nakai and Park (1965) reported the effectiveness of phage therapy on experimentally induced bacterial infections of cultured fish.

In the present study, isolation of phages and their application for treatment of ERM was attempted and there was promising results in our *in vitro* experiment. The bacterial inhibition zone diameter of 0.5-1 mm showed that isolated phages had the potential for biocontrol of ERM in aquaculture. In a study by Mishra *et al.* (2012) an aquatic phage characterized and it was showed that the phage was virulent to two strains of *Entrobacter aerogenes*. The bacteriolytic activity of isolated bacteriophage from the sediment samples of the rainbow trout culture in Korea was evaluated and the results showed the efficient effect of the bacteriophage for the reduction of bacterial growth (Kim *et al*., 2012).

The importance of bacteriophages as an effective biocontrol approach against different pathogenic bacteria draws attentions of many researchers to evaluate and characterize bacteriophages isolated from different sources (Lin *et al*., 2010; Machuca *et al*., 2010; Yang *et al*., 2010; Castillo-Ruiz *et al*., 2011; Parasion *et al*., 2012). In our study, isolated bacteriophages from wastewater plants was evaluated and characterized. It was found that all isolated bacteriophages effective against *Y. ruckeri* were in the same molecular weight and almost similar genotypic structure. The molecular characterization of bacteriophages effective against various bacteria has been reported previously (Garner *et al*., 2004; Ceyssens *et al*., 2010; Klumpp *et al*., 2010). However, to the best of our knowledge, the present study describes the molecular characteristics of

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bacteriophages effective against *Y. ruckeri* for the first time.

The increasing number of different aquaculture bacteria which develop resistance against antibiotics worldwide makes it essential to seek alternative strategies to combat antibiotic resistance. In the future the therapeutic use of bacteriophages alone or in combination with antibiotics against pathogenic bacteria may become an essential and inevitable approach. In the present study, the effectiveness of bacteriophages against *Y. ruckeri* was examined *in vitro* and their molecular characteristics were investigated. More studies including *in vivo* examination of the effectiveness of isolated bacteriophage against different biotypes of *Y. ruckeri* and also exploring their nucleotide sequences are needed.

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