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

Ahmadpour S.¹; Mardani K.¹; Tukmechi A.^{2*}

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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. 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 MspI 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*.

¹⁻ Division of Molecular Epidemiology, Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azarbaijan, Iran.

²⁻ Department of Microbiology, Faculty of Veterinary Medicine,, Artemia and Aquatic Animals Research Institute, Urmia University, Urmia, West Azarbaijan, Iran.

^{*} Corresponding author's Email: a.tukmachi@urmia.ac.ir

Introduction

Yersinia ruckeri is a Gram-negative enterobacterium. causing enteric redmouth disease (ERM) which mainly 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 versiniosis outbreaks. antibiotic administration is the first therapeutic and preventive measure; significant disadvantages however, accompanied with the use of antibiotics aquaculture such as developing antibiotic resistance, necessities need for developing alternative disease control strategies (Ryckaert et 2010).

The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents become critical problem in has a 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 bacteriophages against bacterial infections could be an effective alternative approach for the control of bacterial infections, as the efficacy of bacteriophages against both Grampositive and Gram-negative bacteria has previously (Weberbeen reported Dabrowska et al., 2000; Stone 2002; Inal, 2003). In addition, because of the lack of bacteriophage receptors eukaryotic cells, they might be associated with fewer side effects compared to antibiotics (Sabouri Ghannad and Mohammadi, 2012). therapy Human phage has been practiced for the first time in France since 1919, when d'Hérelle successfully several children who 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

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 immediately and 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, Obtained biochemical results 2007). 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 Tween 80 and Tween 20 sorbitol, 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 described bacterial as previously (Gibello et al., 1999). The amplification reaction was carried out in a gradient Mastercycler (Eppendorf, using an initial denaturation at 94°C for

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

Antimicrobial susceptibility test

Antimicrobial susceptibilities of *Y*. ruckeri Cefotaxim (30)isolates to μg/disk), Erythromycin (15 µg/disk) by tested the disk were diffusion method according the Clinical **National** Committee for (2013).Laboratory Standards 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 biochemical PCR using tests and 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 um 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 MgSO₄ 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 suspension were mixed and phage 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 MgSO₄, 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 CaCb, 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

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 Υ. ruckeri against 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 low $(<10^4$ PFU/mL), evaluated as medium (10⁴–10⁷ PFU/mL), and high (>10⁷ PFU/mL). According to 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 $14,000 \times g$ 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 $4000 \times g$ 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 extracted from isolated DNA was 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 MspI. Digestion reaction was carried out in a volume of 15 µL including 10 U MspI 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 photographed. Lambda DNA was used as a molecular marker for estimating bacteriophages DNA size.

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=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	-	-	-	-

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

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

PCR

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

Isolation and propagation of bacteriophages and determination of their titers

Bacteriophages were isolated only from samples collected from wastewater wastewater plants. Attempts for isolating of bacteriophages from fish farms, rivers and dams were Table 4 unsuccessful. shows the wastewater plants that bacteriophages were isolated, the titer of propagated bacteriophages and the diameter of bacterial growth inhibition

The growth inhibition zone. zone generated by bacteriophages was clear and had a diameter of 0.5-1 mm (Fig. Bacteriophages 2). isolated from Maragheh wastewater plants had the highest titer which was 1.42×10^5 and titer of isolated the lowest bacteriophages belonged Urmia wastewater plants which it was 3.8×10^2 .

Molecular characterization of isolated bacteriophages

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

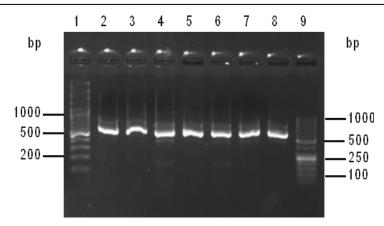


Figure 1: Electerophoretic analysis (2% agarose gel) of DNA amplified fragments from different *Yersinia ruckeri* strains that isolated in this study compared with standard strain. Lane 1: Marker 100 base pair; Lane 2: Positive control (*Yersinia ruckeri* BCCG/LMG 3279); Lane 3-8: *Yersinia ruckeri* 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	$3.8 \times 10^2 - 1 \times 10^5$	0.5-1
2	Salmas	YPS	$2 \times 10^3 - 8 \times 10^3$	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	$2.5 \times 103 \times 1.42 \times 10^{5}$	0.5-1
6	Saghez	YOSg	$1 \times 10^3 - 7.7 \times 10^3$	0.5-1



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

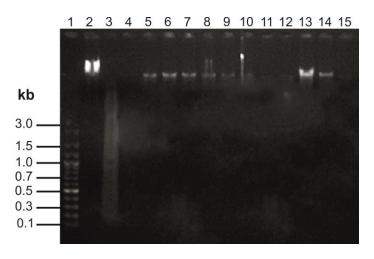


Figure 3: Extracted genomic DNA from isolated bacteriophages and lambda DNA on 1% agarose gel. Lane 1: Molecular marker 100bp (Fermentas, CA), Lane 2: Lambda DNA, Lanes 3: YPM3 DNA, Lane 4: Negative control, Lane 5-15: YPM1, YPS1, YPKh1, YPSg2, YPKh2, YPS4, YPU2, YPMb1, YPU1 and YPMb3 DNAs, respectively.

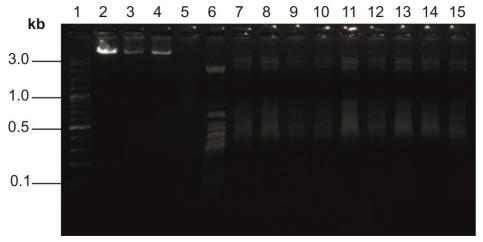


Figure 4: Undigested and digested extracted genomic DNA from bacteriophages of different wastewater plants. Lane 1: Molecular weight 100 bp, Lane 2: Lambda DNA, Lane 3: YPKh1 genomic DNA, Lane 4: YPU2 genomic DNA, Lane 6: *Msp*I RFLP pattern of Lambda DNA, Lanes 7-15: *Msp*I RFLP patterns of genomic 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

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

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

Bacterial diseases are main problem aquaculture industry. The massive use of antibiotics to control infections intensive and semi-intensive aquaculture systems has resulted in the development of resistant strains, which antibiotic have made treatments ineffective (Almeida 2009). et al..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 for bacteriophage the reduction 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 bacteriophages isolated effective against Y. ruckeri were in the same molecular weight and almost similar The genotypic structure. 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 molecular characteristics the of bacteriophages effective against *Y. ruckeri* for the first time.

The increasing number of different bacteria which develop aquaculture resistance against antibiotics worldwide makes it essential to seek alternative to combat antibiotic strategies resistance. In the future the therapeutic of bacteriophages alone or in combination with antibiotics against pathogenic bacteria may become essential and inevitable approach. In the present study, the effectiveness 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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References

Akhlaghi, M. and Sharifi Yazdi, H., 2008. Detection and identification of virulent *Yersinia ruckeri*: the causative agent of enteric redmouth disease in rainbow trout (*Oncorhynchus mykiss*) cultured in Fars Province, Iran. *Iranian Journal of Veterinary Research*, 9, 347-352.

Almeida, A., Cunha, A., Gomes, N.C., Alves, E., Costa, L., Faustino,M. A., 2009. Phage therapy and

photodynamic therapy: low environmental impact approaches to inactivate microorganisms in fish farming plants. *Marine Drugs*, 7, 268-313.

Anderson, B., Rashid, M.H., Carter, C., Pasternack, G., Rajanna, C., Revazishvili, T., Dean, T., Senecal, A., Sulakvelidze, A., 2011.

Enumeration of bacteriophage particles: Comparative analysis of the traditional plaque assay and real-time QPCR- and nanosight-based assays. *Bacteriophage*, 1(2), 86–93.

Arias, C. R., Olivares-Fuster, O., Hayden, K., Shoemaker, C. A., Grizzle, J. M., Klesius, P. H., 2007. First report of *Yersinia ruckeri* biotype 2 in the USA. *Journal of Aquatic Animal Health*, 19, 35-40.

Austin, B., Austin, D. A., 2007.

Bacterial fish pathogens: Disease of farmed and Wild Fish, 4th ed. Springer.

Austin, D. A., Robertson, P. A.,
Austin, B., 2003. Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum).

Systematic and Applied Microbiology, 26, 127-131.

Castillo-Ruiz, M., Vines, E. D., Montt, C., Fernandez, J., Delgado, J. M., Hormazabal, J.C., Bittner, M., 2011. Isolation of a novel Aggregatibacter actinomycetemcomitans serotype b bacteriophage capable of lysing bacteria within a biofilm. Applied

- and Environmental Microbiology, 77, 3157-3159.
- Ceyssens, P.J., Brabban, A., Rogge, L., Lewis, M. S., Pickard, D., Goulding, D., Dougan, G., Noben, J.P., Kropinski A, Kutter E, Lavigne R., 2010. Molecular and physiological analysis of three *Pseudomonas aeruginosa* phages belonging to the "N4-like viruses". *Virology*, 405,26-30.
- **Davies, J., Davies, D., 2010.** Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Review*, 74(3), 417–433.
- Davies, R. L., Frerichs, G. N., 1989.

 Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *Journal of Fish Disease*, 12, 357-365.
- Fouz, B., Zarza, C., Amaro, C., 2006. First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. *Journal of Fish Disease*, 29, 339-346.
- Garner, S. A., Everson, J.S., Lambden, P. R., Fane, B.A., Clarke, I. N., 2004. Isolation, molecular characterisation and genome sequence of a bacteriophage (Chp3) from Chlamydophila pecorum. Virus Genes, 28, 207-214.
- Gibello, A., Blanco, M. M., Moreno, M.A., Cutuli, M. T., Domenech, A., Dominguez, L., Fernandez-Garayzabal, J. F., 1999.

- Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. *Applied and Environmental Microbiology*, 65, 346-350.
- Horne, M. T., Barnes, A. C., 1999.

 Enteric redmouth disease (*Yersinia ruckeri*), p. 455-477. *In* Woo PTK,
 Bruno DW (ed.), Fish Diseases and
 Disorders: Viral, Bacterial and
 Fungal Infections, vol. 3. CABI
 Publishing.
- **Inal, J. M., 2003.** Phage therapy: a reappraisal of bacteriophages as antibiotics. *Archivum Immunologiae Therapia Experimentalis*, 51, 237-244.
- Kawasaki, S., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., Kawamoto, S., 2005. Multiplex PCR for simultaneous detection of Salmonella spp., Listeria monocytogenes, and Escherichia coli O157:H7 in meat samples. Journal of Food Protection, 68, 551-556.
- Kim, J. H., Son, J. S., Choi, Y. J., Choresca, C. H., Shin, S.P., Han, J. E., Jun, J. W., Kang, D. H., Oh, C., Heo, S. J., Park, S. C., 2012. Isolation and characterization of a lytic Myoviridae bacteriophage PAS-1 with broad infectivity in *Aeromonas salmonicida*. *Current Microbiology*, 64, 418-426.
- Klumpp, J., Calendar, R., Loessner, M. J., 2010. Complete nucleotide sequence and molecular characterization of *bacillus* phage TP21 and its relatedness to other

- phages with the same name. *Viruses*, 2, 961-971.
- Kutateladze, M., Adamia, R., 2010.

 Bacteriophages as potential new therapeutics to replace or supplement antibiotics.

 Trends in Biotechnology, 28(12), 591-595.
- Lin, N. T., Chiou, P. Y., Chang, K. C., Chen, L. K., Lai, M. J., 2010. Isolation and characterization of phi AB2: a novel bacteriophage of *Acinetobacter baumannii. Research in Microbiology*, 161, 308-314.
- Machuca, P., Daille, L., Vines, E., Berrocal, L., Bittner, M. 2010. Isolation of a novel bacteriophage specific for the periodontal pathogen Fusobacterium nucleatum. Applied and Environmental Microbiology, 76, 7243-7250.
- Mishra, C. K., Choi, T. J., Kang, S. C., 2012. Isolation and characterization of a bacteriophage F20 virulent to Enterobacter aerogenes. Journal of Genetic Virolology, 93, 2310-2314.
- Parasion, S., Kwiatek, M., Mizak, L., Gryko, R., Bartoszcze, M., Kocik, 2012. J., **Isolation** and characterization of novel a bacteriophage phi4D lytic against Enterococcus faecalis strains. Current Microbiology, 65, 284-289.
- Pirnay J.P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Van den Mooter, G., Buckling, A., Debarbieux, L., Pouillot, F.,

- Kutter, Azeredo, J., E., Dublanchet, A., Górski, A., Adamia. R., 2011. The phage therapy paradigm. **Pharmcological** Research, 28(4), 934-7.
- Romalde, J. L., Planas, E., Sotelo, J. M., Toranzo, A. E., 2003. First description of *Yersinia ruckeri* serotype O2 in Spain. *Bulletin of the European Association of Fish Pathologists*, 23, 135-138.
- Ryckaert, J., Pasmans, F., Tobback, E., Duchateau, L., Decostere, A., Haesebrouck, F., Sorgeloos, P., P., 2010. Bossier, Heat shock proteins protect platyfish (Xiphophorus maculatus) from Yersinia ruckeri induced mortality. Fish and Shellfish Immunology, 28, 228-231.
- Sabouri Ghannad, M., Mohammadi A., 2012. Bacteriophage: Time to reevaluate the potential of phage therapy as a promising agent to control multidrug-resistant bacteria. *Iran Journal of Basic Medical Science*, 15(2), 693–701.
- **Soltani, M., Fadaiifard, F., Mehrabi, M. R., 1999.** First report of a yersiniosis-like infection in farmed rainbow trout. *Bulletin of the European Association of Fish Pathologists*, 19, 173-176.
- **Soothill, J.S., 1994.** Bacteriophage prevents destruction of skin grafts by *Pseudomonas aeruginosa. Burns*, 20(3), 209-11.
- **Standards**, **NCCLS.**, **2013**. Methods for antimicrobial susceptibility tests

- for bacteria that grow aerobically, 2nd ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
- Stock. I., Henrichfreise, B., B., Wiedemann. 2002. Natural antibiotic susceptibility and biochemical profiles of Yersinia enterocolitica-like strains:Y. bercovieri, Y. mollaretii, Y. aldovae and Y. ruckeri. Journal of Medical Microbiology, 51, 56-69.
- **Stone, R. 2002.** Bacteriophage therapy: Stalin's forgotten cure. *Science*, 298, 728-731.
- Su, M. T., Venkatesh, T. V., Bodmer, R., 1998. Large- and small-scale preparation of bacteriophage lambda lysate and DNA. *Biotechnology*, 25, 44-46.
- Sulakvelidze, A., Alavidze, Z., Morris, G. 2001. Antimicrob agents chemother. *Journal of Bacteriophage Therapy*, 45(3), 649–659.

- Tobback, E., Decostere, A., Hermans, K., Haesebrouck, F., Chiers, K., 2007. *Yersinia ruckeri* infections in salmonid fish. *Journal of Fish Diseases* 30, 257-268.
- Weber-Dabrowska, B., Mulczyk, M., Gorski, A., 2000. Bacteriophage therapy of bacterial infections: an update of our Institute's experience. Archivum Immunologiae Therapia Experimentalis, 48, 547-551.
- Yang, H., Liang, L., Lin, S., Jia, S., 2010. Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii. BMC Microbiology*, 10, 131.