

ISSN 2602-2575 • EISSN 2618-6144

# EUROPEAN JOURNAL OF BIOLOGY

OFFICIAL JOURNAL OF ISTANBUL UNIVERSITY FACULTY OF SCIENCE

Volume: 77 • Issue: 1 • June 2018

[ejb.istanbul.edu.tr](http://ejb.istanbul.edu.tr)



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European Journal of Biology (Eur J Biol) is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official publication of Istanbul University Faculty of Science and it is published biannually on June and December. The publication language of the journal is English. European Journal of Biology has been previously published as IUFS Journal of Biology. It has been published in continuous publication since 1940.

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Review Article	5500	250	No limit	5	6
Letter to the Editor	500	No abstract	5	No tables	No media



Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

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Books with a Single Author: Sweetman SC. *Martindale the Complete Drug Reference*. 34<sup>th</sup> ed. London: Pharmaceutical Press; 2005.

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# *In Vitro* Characterization of Chitosan-Based Particles as Carrier of Influenza Viral Antigens

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**Please cite this article as:** Karaaslan M, Turan K. *In Vitro* Characterization of Chitosan-Based Particles as Carrier of Influenza Viral Antigens. Eur J Biol 2018; 77(1): 1-10.

## ABSTRACT

**Objective:** Chitosan is a natural polysaccharide derived from chitin by deacetylation. It is a non-toxic, biocompatible and biodegradable polymer, and has attracted considerable interest in a wide range of pharmaceutical applications including drug and vaccine delivery. The immune-stimulating activity of chitosan microparticles such as the activation of macrophages and polymorphonuclear leukocytes has been reported. In this work, we have investigated the releasing properties of influenza virus antigens from the chitosan microparticles and beads.

**Materials and Methods:** Chitosan microparticles and beads were prepared by coacervation and ionotropic gelation method, respectively. The microparticles and beads were loaded with the viral antigens by passive adsorption and/or entrapment into the microparticles. The titration of the viruses was defined by haemagglutination assay or by quantitation of viral proteins using the Bradford method.

**Results:** The results showed that the loading efficiency and the loading capacity of chitosan microparticles/beads with the viral antigens and the releasing profiles of the antigens from the particles changed depending on the type of chitosan, the pH of the loading buffer and the methods used to prepare the particles. The influenza viral antigens, passively adsorbed onto microparticles/beads, were released within 2 hours to 5 days. In contrast, the viral antigens entrapped into the chitosan microparticles were released more slowly and continued for up to 30 days.

**Conclusion:** It was concluded from the viral antigen releasing profiles of chitosan particles that the viral antigens entrapped into the microparticles are more suitable for *in vivo* applications as a potential mucosal vaccine.

**Keywords:** Chitosan, mucosal vaccines, influenza A viruses, microparticles, beads

## INTRODUCTION

Chitin, the main source of chitosan, is the second most abundant polysaccharide in nature (1). It is the principal component of the exoskeletons of crustaceans and insects as well as of cell walls of fungi and some bacteria (2-4). Chitosan [(1-4)-2-amino-2-deoxy- $\beta$ -D-glucan] is obtained by the alkaline deacetylation of chitin (1, 5). Chitosan is a positively charged biopolymer, which can form complexes with the negatively charged molecules (6-8). It is a safe and non-toxic linear molecule composed of randomly distributed  $\beta$ -(1 $\rightarrow$ 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. All these characteristics make the chitosan an ideal biopolymer for formulation and controlled release of some drugs and vaccines. A number of studies have been carried out with the aim of using chitosan-based microparticles as a

carrier of drugs, vaccines and even plasmid DNA (7-11). Some studies have shown that chitosan plays a role in the activation of macrophages and cytotoxic T lymphocytes (12,13). Due to these immune-stimulatory effects, chitosan is of great importance, especially in research into the development of mucosal vaccines (11,14). In this study, the potential of chitosan as a carrier for influenza A viruses, which pose a great risk for human health, was investigated. These viruses characteristically cause recurrent epidemics of diseases resulting in considerable mortality and morbidity worldwide each year, especially among high-risk groups such as the elderly and young children (15,16). This is largely due to the continual changes in the antigenic properties of influenza viruses via minor and major antigenic changes (17). In particular, the changes of the viral hemagglutinin proteins enable the virus to avoid the immunolog-



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Received: 10.04.2018 Accepted: 11.05.2018

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ical defense of the host organism. Consequently, the control of the influenza viruses by vaccination is not completely effective. However, vaccines are one of the most important tools to deal with influenza virus infections. Current commercially available influenza vaccines are the formulations of live attenuated viruses (attenuated vaccines), inactivated viruses (whole *inactivated* or *split vaccines*), or purified viral surface antigens (subunit vaccines) (18-19). However, different approaches to developing more effective vaccines against influenza virus infections, such as mucosal vaccines have been considered in several studies (20). In this context, the nasal mucosa is an attractive site for the delivery of vaccines, and it has certain advantages over the other application routes (21,22). Mucoadhesive particulate carrier systems such as chitosan, starch and hyaluronic acid offer significant potential for the development of mucosally administered vaccines (23,24). Particulate carriers based on chitosan, a biodegradable polymer, have great potential for the delivery of viral proteins for vaccination. The incorporation or adsorption of the viral antigens onto the chitosan particles was recently shown to have a good adjuvant effect (25-27). Chitosan could open the intercellular tight junctions and facilitate the paracellular transport of macromolecules; nano- and microparticles are suitable for controlled vaccine release (28,29). Thus, chitosan and its derivatives could induce strong systemic and mucosal immune responses against viral antigens (13). In the present study, we have investigated the potential of chitosan for the sustained release of influenza viral antigens. We have prepared chitosan microparticles/beads loaded with influenza viral antigens, and examined the releasing behavior of the viral antigens from microparticles/beads in *in vitro* conditions.

## MATERIAL AND METHODS

### Chitosan

Chitosan biopolymers were purchased from Fluka Co. Ltd, Switzerland. Two types of chitosan were used for the formation of chitosan microparticles and beads; low-viscous chitosan (L-chitosan / Cat. No. 50494 / <200 mPa.s; 1% solution in 1% acetic acid at 20°C) and middle-viscous chitosan (M-chitosan / Cat. No. 28191 / 200-400 mPa.s; 1% solution in 1% acetic acid at 20°C) with 150 kD and 400 kD average molecular weight, respectively. Chitosan solutions 0.5% (w/v) and 2% (w/v) were prepared by dissolving 0.5 g and 2 g of chitosan in 100 ml, 1% (v/v) acetic acid.

### Influenza Viruses, Growth, Purification and Quantitation

The Influenza virus A/Puerto Rico/8/34 (H1N1) was used in the present experiments. The viruses were grown in the allantoic cavity of 10 day-old embryonated eggs. Using a syringe with a 25-gauge needle, 0.1 ml of virus sample (100-200 pfu) diluted in phosphate buffer solution (PBS) supplemented with bovine serum albumin-fraction V (1%, w/v) was inoculated into the allantoic cavity of hen eggs. The inoculated eggs were incubated at 35.5 °C for 48 hours. The allantoic fluid collected from 25 eggs was harvested and clarified by centrifugation at 3,000g for 10 minutes. The viruses were concentrated with polyethylene glycol (PEG) precipitation. For this procedure, solid PEG-6000 was added to the virus containing fluid at a final concentration of

8% (w/v) and stirred on ice for 1 h. The virus precipitate was recovered by centrifugation at 10,000g for 30 min at 4°C and re-suspended in 1 ml of NTE (100 mM NaCl, 1 mM Tris HCL, pH 7.5, 1 mM EDTA) buffer. The viruses were purified by ultracentrifugation on a sucrose cushion. A two-layer sucrose solution was prepared by adding 1.5 ml 60% sucrose and 2 ml 15% sucrose solutions into the 4 ml ultracentrifuge tubes (Sorvall TST 60.4 Swing Out Rotor) and, 0.5 ml of the concentrated virus sample was layered over the 15% sucrose solution. The samples were centrifuged at 100,000g for 1 hour at 4°C, and the viruses concentrated in the interface region between 15% sucrose layer and 60% sucrose cushion was removed. The virus fraction was diluted to 4 ml with NTE buffer and re-centrifuged 100,000g for 30 min at 4°C. The virus precipitate was resuspended in 0.5 ml NTE buffer and stored as small aliquots at 80°C (30). The purified influenza viruses were titrated with haemagglutination assays. A doubling dilutions of virus sample in 100 µL of PBS was prepared in 96-well U-bottom plate. Equal volume of 1% red blood cell was added to diluted samples and allowed to stand undisturbed for 30 min. After incubation, the haemagglutination in end-point dilution was defined and, the virus titer was calculated as haemagglutination unit (HAU) with the following equation (30).

$$\text{Virus quantity (HAU/100 } \mu\text{L)} = (A + B / 2)$$

A, the end-point dilution factor giving positive haemagglutination; B, the first-point dilution factor giving negative haemagglutination.

### Testing of Influenza Virus Purity

The purity of the prepared virus sample was checked by both the analysis of viral structural proteins by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) technique and electron microscopic analysis. For SDS-PAGE analysis, the virus samples were mixed with x4 SDS sample loading buffer at a ratio of 3:1, then denatured for 5 minutes in boiling water and, loaded onto 7.5% polyacrylamide gel. The electrophoresis was carried out in the first 20 minutes at 10 mA/gel and then at 20 mA/gel for 2 hours. The proteins separated on gel were visualized by staining with *Coomassie brilliant blue R-250*. For electron microscopic analysis, virus samples were negatively stained (31) and electron micrographs of viruses were taken with a transmission electron microscope (Jeol T-100).

### Inactivation of the Influenza A Viruses

Formaldehyde and β-propiolactone are widely used for inactivation of viruses for the preparation of vaccines (32). In this work, formaldehyde was used for the inactivation of purified influenza viruses. Formaldehyde was added to the virus samples at 0.1% final concentration and incubated at 4°C for a week. The inactivated virus sample was used for entrapment in chitosan microparticles.

### Preparation of Chitosan Microparticles

Chitosan solution 0.5% (w/v) was prepared by dissolving 0.2 g of L- or M-chitosan in 20 ml, 1% (v/v) acetic acid. For the formation of chitosan microparticle, an equal volume of 20% sodium sulfate solution was added dropwise to the chitosan solutions

and mixed on a magnetic stirrer for 1 hour. To strengthen the microparticles, glutaraldehyde was added to the solution at 0.1% final concentration and stirring continued for 45 minutes. Then, the chitosan microparticles were recovered by centrifugation (10,000g, 4°C, 20 min), and repeatedly washed with deionized water. Chitosan microparticles were freeze-dried by using a freeze dryer, passed through a sieve with 53 µm mesh size and stored in a desiccator at 4°C. The average sizes of microparticles was determined using a light microscope with an ocular micrometer by measuring at least 100 particles.

### Preparation of Chitosan Beads

The chitosan beads were prepared by the ionotropic gelation method (33) using sodium tripolyphosphate (TPP) as an ionic agent. For this, a 2% chitosan solution prepared in 1% acetic acid was used. The chitosans solution was dropped into the 10% TPP solution (pH 6 or pH 9) stirred at 140 rpm using a syringe with a 22-gauge needle under a constant pressure applied on the syringe to get the beads in uniform sizes. The chitosans beads were cross-linked with 1% w/v of glutaraldehyde and repeatedly washed with deionized water. The beads were dried at room temperature or freeze-dried with a freeze-dryer. The average sizes of chitosan beads was determined using a light microscope with an ocular micrometer by measuring at least 25 beads.

### Scanning Electron Microscopic Examination of Chitosan Beads

The surface characteristics and porosity of the chitosan beads were examined with a scanning electron microscope (SEM). The beads were coated with 20 nm of gold and, images were taken with SEM (JSM 5200).

### Loading of the Chitosan Microspheres with Influenza Viruses

The chitosan microparticles were loaded with purified influenza viruses by following two methods. First, the chitosan microparticles, prepared as mentioned above, were loaded with viruses by passive adsorption. 7.5 mg of the chitosan microparticles were added to 1 ml PBS containing influenza viruses equivalent to the 1920 HAU virus and mixed with a tube mixer at 30 rpm for 5 hours at room temperature. Then the microparticles were precipitated by centrifugation at 10,000g for 10 min. The supernatants were removed and used for the titration of unabsorbed viruses to the chitosan microparticles. Virus titration in the supernatants were determined with HA assay. The microparticle precipitates were used in releasing experiments.

In the second method, the viruses were entrapped in the chitosan microparticles during the preparation processes. An inactivated influenza virus sample equivalent to 40000 HAU (~220 µg of viral protein) was added to 20 ml of 10% sodium sulfate and this solution was used for the preparation of microparticles as described above. Then the microparticles were precipitated by centrifugation at 10,000g for 10 min. The supernatants were removed and used for the titration of untrapped viruses by measuring viral proteins using the Bradford method. The precipitates were washed with deionized water, freeze-dried, passed through a sieve with 53 µm mesh size and stored in a

desiccator at 4°C. The microparticle sizes was determined using a light microscope with an ocular micrometer by measuring at least 100 particles.

The loading efficiency (i) and loading capacity (ii) of microparticles were calculated with the following equations as previously described (34,35).

- i. Loading (encapsulation) efficiency (%) =  $(A - B / A) \cdot 100$
- ii. Loading (encapsulation) capacity  
(HAU or µg viral protein / mg microparticles) =  $(A - B / C)$

A, the total amount of viruses (HAU or µg viral protein); B, unabsorbed or untrapped virus quantity (HAU or µg viral protein); C, the weight of chitosans microparticles or beads (mg).

### Loading of the Chitosan Beads with Influenza Viruses

The chitosan beads were loaded with the purified viruses by the passive adsorption at three different pH (pH 5, 7 and 9). 20 mg of the chitosan beads and purified influenza viruses equivalent to 1920 HAU were mixed in 1 ml PBS at different pH and rotated with a tube mixer at 30 rpm for 5 hours at room temperature. Then the liquid phase was completely removed and used for the titration of the unabsorbed virus with HA assay. The virus loaded chitosan beads were used in the releasing experiments.

### In vitro Release Studies

The influenza virus release from the loaded chitosan microparticles and beads was studied in PBS using the shaking water bath, 60 rpm at 37°C. The microparticle samples were centrifuged at 10,000g for 10 min at defined time intervals and, 200 µL of sample from the supernatant was removed for virus titration with HA assay or viral protein quantitation using the Bradford method. In release studies with chitosan beads, 200 µL samples from the liquid phase were directly removed without centrifugation and used for the titration of viruses with HA assay. After taking the samples, 200 µL of fresh PBS was added to the medium and the incubation continued. The cumulative release of the viruses as HAU or µg viral proteins was determined and plotted to time intervals.

### Statistical Analysis

Statistical methods used in this work include descriptive statistics (arithmetic mean and standard deviation) and the non-parametric Mann-Whitney test.  $p < 0.05$  defined statistical significance.

## RESULTS

### The Titer and Purity of Influenza A Viruses

The influenza A viruses obtained from virus infected allantoic fluids of 25 embryonated hen eggs were purified by centrifugation on sucrose cushion and concentrated in 500 µL of NTE buffer. The titer of the purified virus sample was detected by haemagglutination assay as 19200 HAU / 100 µL (12800 + 25600 / 2 = 19200) (Figure 1a). Inactivation with formalin and/or entrapment of influenza A viruses in chitosan microparticles inhibit the haemagglutination ability of the viruses (data not

shown). The virus titrations were determined by quantifying viral protein using the Bradford method in experiments carried out with virus-entrapped chitosan microparticles. Therefore, a standard plot showing the viral protein quantity in the samples with a defined virus titer (as HAU) was generated (Figure 1b).

The purity of influenza A viruses prepared from embryonated hen eggs were checked both by an analysis of viral structural proteins with SDS-PAGE (Figure 2a) and electron microscopic observation of the negatively stained virus particles (Figure 2b). As shown in Figure 2a, the virus sample prepared by centrifugation at sucrose chosen was found to be very pure compared to infected crude allantoic fluid. The transmission electron microscopic observation (Jeol T-100) of the negative stained virus samples supported the purity of the viruses and gave information about the morphology of purified virus particles (Figure 2b). Hereby, it has been shown that viruses are highly purified from impurities such as cellular debris, and they have typical influenza virus morphology.

### The Sizes of the Chitosan Microparticles

The sizes of virus-free (unloaded) and virus-entrapped microparticles prepared with two different types of chitosan (L- and M-chitosan) were determined using a light microscope with an ocular micrometer. The size of a minimum of 100 independent microparticles were measured from each formulation and the average sizes are given in Table 1. It was observed that the size of chitosan microparticles varied depending on the type of chitosan used. The chitosan microparticles prepared with M-chitosan were found to be larger than those of L-chitosan but not

statistically significant. Furthermore, the addition of viruses during microparticle preparation caused the formation of larger microparticles in size ( $p < 0.05$ ).

### The Sizes and Morphological Analysis of Chitosan Beads

The sizes of the beads prepared with 2% L-chitosan and M-chitosan solutions at two different pH were determined using a light microscope with an ocular micrometer in the way as the

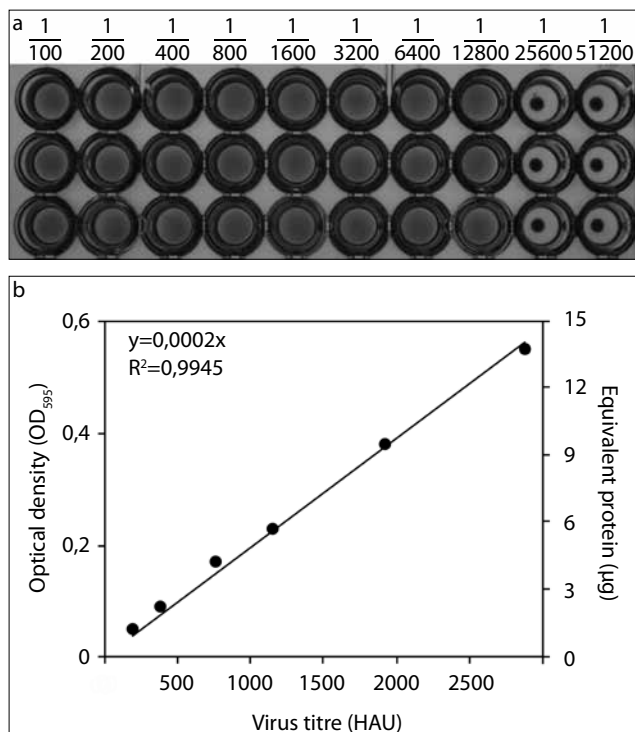


Figure 1. a, b. a) Titration of influenza A viruses by haemagglutination assay; and b) Bradford method.

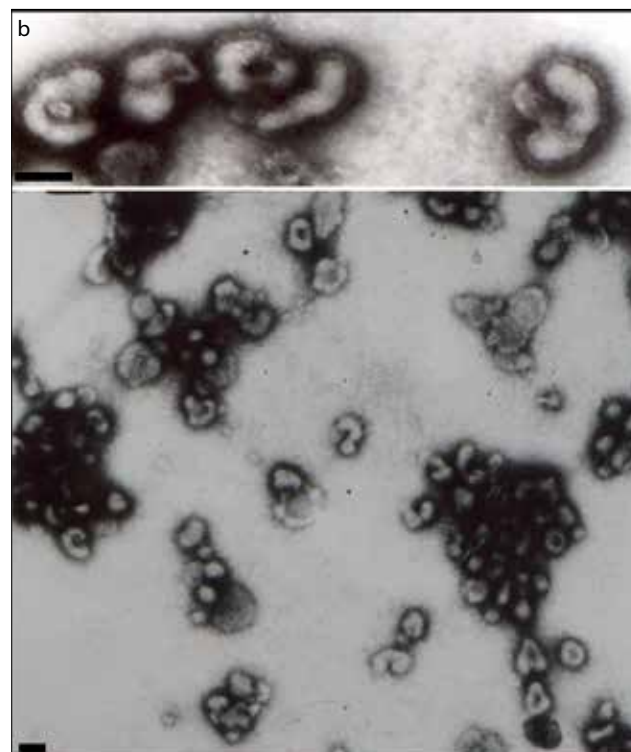
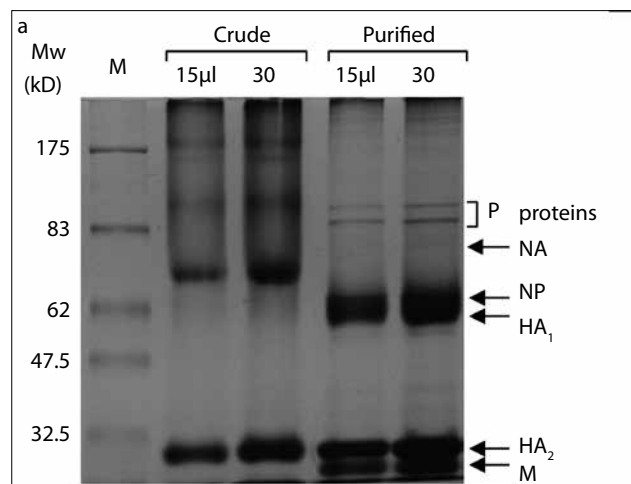


Figure 2. a, b. a) Checking of the purity of influenza A viruses produced in embryonated hen eggs by analysis of viral proteins with polyacrylamide gel electrophoresis; and b) electron microscopic observations. M, prestained protein marker (NEB, broad range). For electron microscopic observation, the purified viruses were negatively stained with 1% ammonium molybdate. The bar represents 100 nm.

**Table 1.** The sizes of chitosan microparticles prepared with L-chitosan (LM) and M chitosan (MM). LMv and MMv samples were loaded with inactivated influenza virus particles during microparticle preparation. n≥100.

formulation codes	LM	MM	LMv	MMv
Size (µm)	1.57± 0.1	1.68±0.12	1.78± 0.15	1.9±0.16

**Table 2.** The sizes of chitosan beads prepared with L-chitosan and M-chitosan. LBA and MBA beads were prepared at pH.6; LBB and MBB beads were prepared at pH 9. n≥25.

formulation codes	LBA	LBB	MBA	MBB	
Size (mm)	(lyophilized)	1.40± 0.04	1.53± 0.05	1.47± 0.04	1.62± 0.06
	(dried at r. t.)	0.62± 0.02	0.70± 0.03	0.66± 0.02	0.74± 0.03

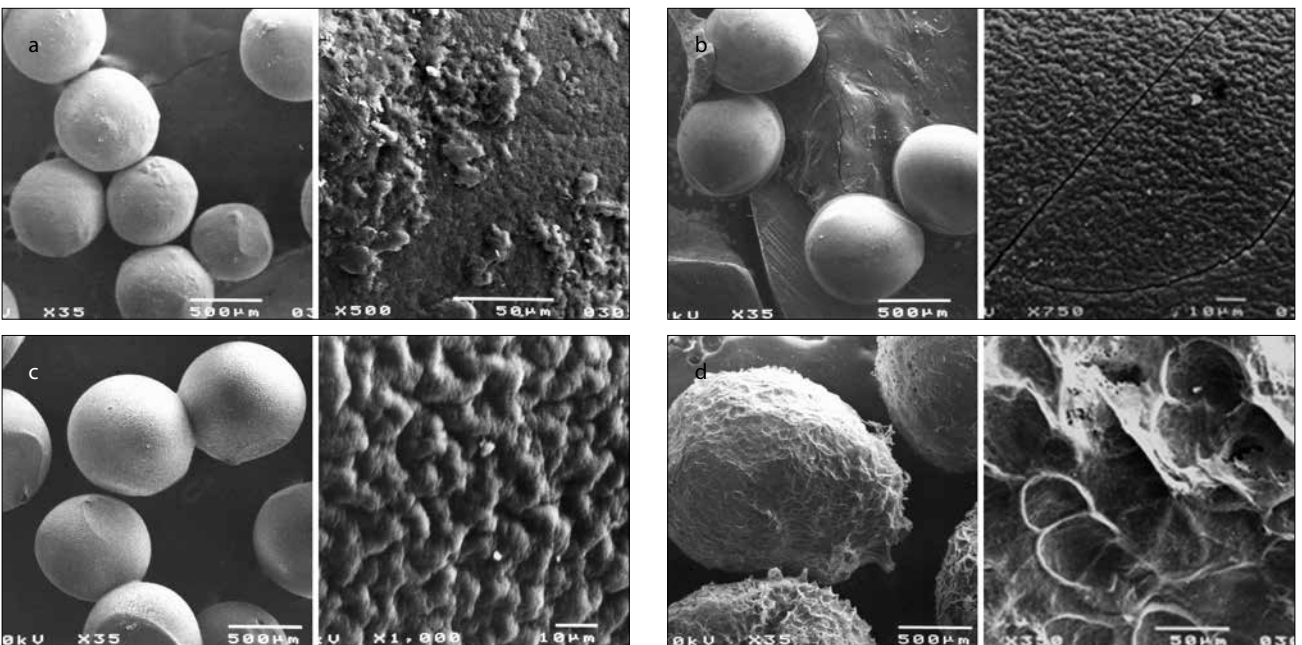


Figure 3. a-d. SEM images of chitosan beads prepared under different conditions. The samples dried at room temperature: a) LBA, b) MBA, c) MBB; d) freeze-dried sample: LBB.

microparticles. The sizes of beads varied depending on the pH of the TPP solution used for the bead formation and drying method rather than the type of chitosan as shown in Table 2. It was found that the beads prepared at pH 6 (LBA and MBA) were smaller than the beads prepared at pH 9 (LBB and MBB) ( $p>0.05$ ), and lyophilized beads were 2-3 times larger than the beads dried at room temperature ( $p<0.05$ ). The surface morphologies of chitosan beads prepared under different conditions were also examined by SEM (Figure 3). The dimensions of the chitosan beads observed with SEM were close to the values obtained with light microscopy. In addition, it was observed that the chitosan beads prepared with TPP at basic pH have more porous surface comparing to those prepared at acidic pH (Figures 3a, 3b and 3c); the lyophilized beads were larger than the beads dried at room temperature and had irregular spongy shapes (Figure 3d).

### The Virus Loading Efficiency and Loading Capacity of Microparticles

Chitosan microparticles were loaded with influenza viral antigens by passive adsorption or entrapment of inactivated virus particles during the preparation of microparticles. The antigen loading efficiency of microparticles loaded by passive adsorption was determined by measuring the titer of unabsorbed viruses with HA assay. It was found that microparticles prepared using L-chitosan had higher loading efficiency than those prepared with M-chitosan. Furthermore, the pH of the loading medium affected the loading efficiency of microparticles. The loading efficiency of microparticles was found to be higher at pH-7 than that at pH-5 and pH-9 of the medium ( $pH: 7>5>9$ ) ( $p<0.05$ ). The loading capacity of microparticles were found in correlation with loading efficiency (Table 3).



**Table 3.** Virus loading efficiency and loading capacity of microparticles. Microparticles (7.5 mg) were loaded with purified influenza A viruses (1920 HAU) by passive adsorption

formulation codes	loading pH	loading efficiency (%)	loading capacity (HAU/mg microparticles)
LM	5	82.5±4.1	224.0±10.7
	7	86.6±2.6	228.0±6.8
	9	22.3±1.3	61.3±3.5
MM	5	49.2±2.9	134.4±7.4
	7	69.3±1.8	192.0±4.8
	9	19.3±1.2	52.3±3.2

**Table 4.** Loading efficiency and loading capacity of microparticles loaded with inactivated influenza A viruses during microparticle preparation

formulation codes	loading efficiency (%)	loading capacity (µg viral prot./mg microparticles)
LMv	59.3±2.1	1.3±0.05
MMv	93.7±1.2	2.3±0.03

**Table 5.** Virus loading efficiency and loading capacity of chitosan beads. Beads (20 mg) were loaded with purified influenza A viruses (1920 HAU) by passive adsorption

formulation codes	loading pH	Chitosan Beads			
		freeze-dried		dried at r.t.	
		loading efficiency (%)	loading capacity (HAU/mg beads)	loading efficiency (%)	loading capacity (HAU/mg beads)
LBA	5	17.1±0.8	18.0±0.7	7.5±0.4	7.9±0.4
	7	9.5±0.3	9.9±0.3	4.1±0.2	4.3±0.1
	9	7.2±0.2	7.5±0.2	3.1±0.2	3.3±0.1
MBA	5	10.0±0.6	10.3±0.6	5.4±0.4	5.6±0.4
	7	6.6±0.3	6.7±0.3	3.2±0.2	3.4±0.2
	9	5.4±0.2	5.5±0.2	2.7±0.1	2.8±0.1
LBB	5	26.2±1.3	27.9±1.2	10.8±0.4	11.2±0.4
	7	14.3±0.4	15.0±0.4	6.3±0.2	6.3±0.2
	9	10.4±0.2	10.8±0.2	5.2±0.1	5.4±0.1
MBB	5	17.5±0.8	16.8±0.7	7.1±0.4	7.2±0.4
	7	10.6±0.5	27.0±0.4	4.7±0.2	4.8±0.2
	9	8.9±0.3	34.5±0.3	4.2±0.2	4.2±0.2

The antigen loading efficiency and loading capacity of the virus entrapped microparticle samples were indirectly determined by measuring viral proteins. After the formation of microparticles, the amount of viruses remaining in the liquid phase was determined using the Bradford method by using the standard plot given in Figure 1b, and loading efficiency and loading capacity of the samples were calculated as mentioned in methods (Table 4). The loading capacity and efficiency of microparticles prepared with M-chitosan were found to be significantly higher than that of L-chitosan ( $p < 0.05$ ).

#### The Virus Loading Efficiency and Loading Capacity of Chitosan Beads

The chitosan beads were only loaded with viruses by the passive adsorption method. The antigen loading efficiency and capacity of beads were determined by the titration of unadsorbed viruses with HA assay. Loading efficiency and capacity of chitosan beads were found to be lower than those of microparticles as shown in Table 5. Similar to the microparticles, the L-chitosan beads have higher loading capacity than the M-chitosan beads. In contrast, the loading efficiency and capacity of beads was higher at pH-5 than that of pH-7 and pH-9 of the medium (pH: 5>7>9) ( $p < 0.05$ ). The chitosan beads, prepared by coagulation in an aqueous TPP solution at pH-9 (LBB and MBB), were loaded more efficiently compared to the solution at pH-6 (LBA and MBA). In addition, it has been shown that the drying method used for beads has a significant effect on the loading efficiency and capacity of

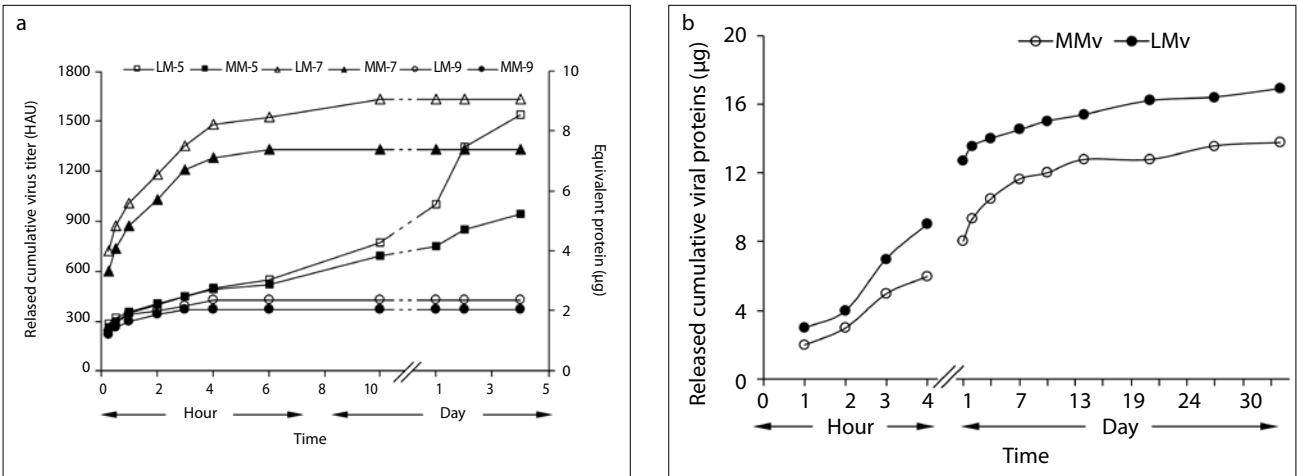


Figure 4. a, b) a) The release of viral antigens from chitosan microparticles that were loaded at different pH by passive adsorption; and b) microparticles loaded with viruses by encapsulation. The numbers added to the formulation codes of chitosan microparticles show the pH values of loading buffer.

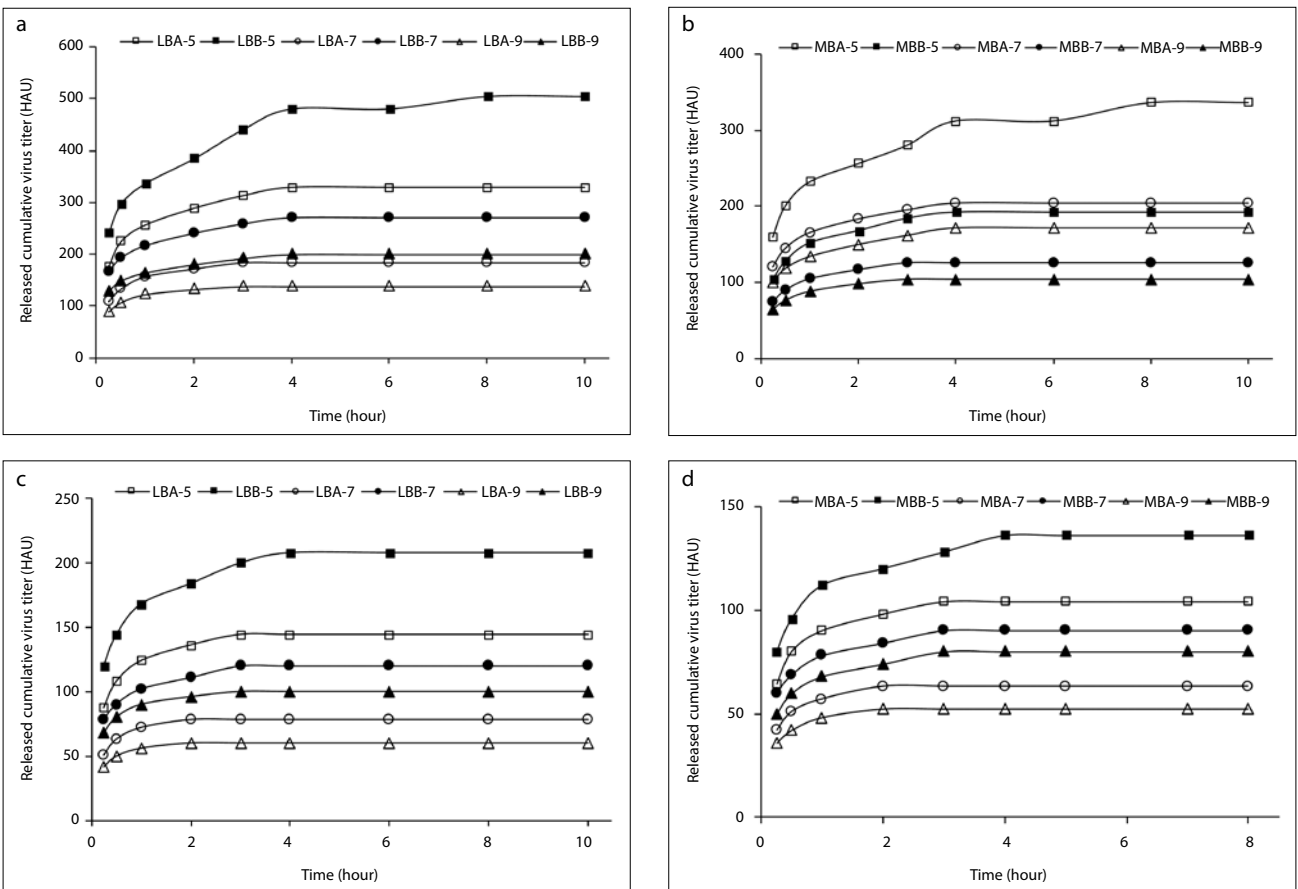


Figure 5. a-d) a) The viral antigen release from chitosan beads loaded at different pH. b) The beads prepared with L-chitosan and freeze-dried. c) The beads prepared with M-chitosan and freeze-dried. d) The beads prepared with L-chitosan and dried at r.t. The beads prepared with M-chitosan and dried at room temperature. The numbers added to the formulation codes of chitosan beads show the pH values of loading buffer.

beads. The beads dried by lyophilization have at least 2-fold higher loading efficiency and capacity than that of samples dried at room temperature.

### The Release of Viruses from Chitosan Microparticles

Cumulative virus releases from chitosan microparticles loaded with passive adsorption or entrapment with viruses are given



in Figure 4. The viruses adsorbed microparticles were released within 10 hours to 5 days depending on the pH of the loading medium. The viral antigens, which were loaded onto the microparticles at pH 5, were released very slowly and continued for up to 5 days (Figure 4a). In evaluations based on the loading efficiency of microparticles; the cumulative ratios of released viruses from chitosan microparticles were calculated as 26-42% for the microparticles loaded at pH 5 (LM5 and MM5), 71-78% for the microparticles loaded at pH 7 (LM7 and MM7) and 85-92% for the microparticles loaded at pH 9 (LM9 and MM9) at the second hour of the releasing experiment. After 6 hours, the cumulative releasing ratios were found to be 35-55%, 92-95% and  $\geq 95\%$  for microparticles loaded at pH 5, 7 and 9, respectively. The releasing of viruses from L-chitosan microspheres was slightly higher than that of M-chitosan microspheres. These results show that the loading pH is one of the most important factors affecting the rate of release of viruses from chitosan microparticles.

The releasing of viral antigens entrapped in chitosan microparticles was monitored by quantification of viral protein using the Bradford method as mentioned above. The cumulative releasing of viral proteins is given in Figure 4b. The viruses which were entrapped in microparticles were released very slowly and continued for up to 30 days. After 6 hours, the cumulative releasing ratios were found to be 27% and 31% for MMv microparticles and LMv microparticles, respectively. At the end of the 30-day period, the approximate cumulative release values for MMv and LMv had reached 63% and 57%.

#### The Release of Viruses from Chitosan Beads

The loading of chitosan beads with viruses was carried out at different pHs (at pH 5, 7 and 9) such as microparticle samples. Then, the beads were suspended in PBS and left at 37°C in a shaking water bath to release the viruses. The quantity of viruses in samples taken from the liquid phase at defined intervals was determined by HA assays. The cumulative release of viruses from the chitosan beads is given in Figure 5. Although the loading efficiency and loading capacities were different, the release profiles of the virus antigens from the chitosan beads prepared in different conditions were relatively similar. The releasing of viral antigens from beads occurred more rapidly and about 90% of adsorbed antigens were released within 2-4 hours.

#### DISCUSSION

According to the World Health Organization report, infectious diseases constitute over 10% of the top 10 causes of deaths from 2000 to 2015 (36). Viral diseases have an important ratio among the infectious diseases in humans (37). However, the success rate of the prevention of viral infections is very low compared to bacterial diseases. The most effective way to prevent viral infections is viral vaccines, but they are not completely effective for a number of reasons. Therefore, a variety of new approaches are required to enhance the immune response and to ensure the long-term efficacy of the vaccines. The carrier polymers, which are biodegradable, non-toxic and allow the

controlled release of antigens, are of great importance both in increasing the efficacy of the vaccines and in their ability to be used in the formulation of mucosal vaccines (38-40). Intensive studies have been conducted on the use of chitosan polymers in the formulation of vaccines as well as many other pharmaceuticals (27,41,42). In this study, the potential of microparticles and beads prepared with chitosan polymers as a carrier of the virus antigen, were evaluated in *in vitro* conditions.

Influenza A viruses were produced in the allantoic cavity of embryonated hen eggs and purified by sucrose gradient centrifugation technique. A pure virus sample equivalent to 19200 HAU/100  $\mu$ l (totally 500  $\mu$ l) was obtained from 25 hen eggs (Figure 1). The purity of viruses was demonstrated both by polyacrylamide gel electrophoresis of viral structural proteins and by electron microscopic analysis of the virus particles (Figure 2). The viruses were used for incorporation and/or adsorption into the chitosan microparticles and beads.

Physical properties such as the sizes and surface structure of the chitosan microparticles and beads vary depending on the molecular weight of the chitosan, and the conditions applied for formulations (43). In the present study, two types of chitosan with different molecular weights were used for the formation of microparticles and beads. Although there was no statistically significant difference, it was found that the average sizes of the microparticles formed with L-chitosan were smaller than those formed with M-chitosan (Table 1). However, the entrapment of influenza viruses to the chitosan microparticles during the formulation resulted in significant increases in particle sizes ( $p > 0.05$ ). Similarly, the average sizes of chitosan beads changed depending on the molecular weights of the chitosan and the conditions applied to form beads. It was determined that the beads formed with both L- and M-chitosan in acidic pH were smaller than the beads formed in basic pH (Table 3). The beads prepared with L-chitosan were found to be smaller than those of M-chitosan. The most important factor affecting the sizes of the chitosan beads was the drying condition. The beads which were freeze-dried were 2-3 times larger than that of beads dried at room temperature. The drying process of chitosan beads also affected the porosity of the beads. Drying with a freeze-dryer resulted in more a porous and sponge-like structure of beads (Figure 3). The pH of the TPP used for ionic gelation was another factor affecting the pore structure of the beads. It was observed that the beads prepared in TPP at basic pH have a more porous surface structure.

Methods such as passive adsorption or encapsulation are utilized for the formulation of low molecular weight drugs, pharmaceutical peptides or vaccine antigens with different polymers to improve the bioavailability (23,44-46). The characteristics of the molecules to be formulated are considered for the selection of the formulation method. The passive adsorption process is simple and just involves adding the solution of the molecules or the antigens onto the inert carriers such as microparticles and mixing in appropriate conditions (47). In some cases, the molecules can be adsorbed at high levels (up to 70% w/w)

onto suitable carriers (48). However, the burst release in adsorption-loaded microparticles is usually very high and, releasing is very fast. In the present study, it was found that the chitosan microparticles can adsorb up to 86% of the viral antigens added in the medium depending on the conditions. It was also found that the loading efficiency of chitosan microparticles significantly depends on the pH of the medium. The loading efficiency of microparticles was found to be around 20% at pH 9. The highest loading efficiency was achieved at pH 7 (Table 3). The viral antigen loading capacities of chitosan microparticles prepared by encapsulation was also reasonably high (Table 4). About 80% of the viral antigens added to the medium were entrapped in microparticles prepared with M-chitosan. The entrapment ratio of viral antigens in L-chitosan microparticles was found to be 58.5%.

The viral antigens adsorbed microparticles were released within 4 hours to 5 days depending on the chitosan type and the pH of the loading medium (Figure 4a). The viral antigens which were loaded onto the microparticles at pH 9 were released very quickly and reached the maximum within 4 hours. In contrast, the viral antigens loaded onto the microparticles at pH 5 were released very slowly and continued for up to 5-6 days. In the microparticles loaded with viral antigens at pH 7, the release was faster than expected and reached the maximum value in 10 hours. It was concluded that the reason for the different release profiles of microparticles loaded at pH 5 and pH 7, despite their close loading efficiency, is the increase in the swelling capacity of the chitosan microparticles (49,50) and changes in the charges of chitosan polymers and the viral antigens in the low pH buffer (51). Studies into increasing the efficacy of the vaccines have shown that the slow release of antigens formulated in microspheres enhances antibody production (52). Therefore, a slow, controlled release of antigen is preferred in cases of vaccine formulations. In this study, the viral antigens especially entrapped in the microparticles were released very slowly (Figure 4b). A very low initial burst release and a longer controlled release of the viral antigen from the chitosan microparticles was observed. The viral antigen release from both L- and M-chitosan microparticles continued for up to 30 days. Due to these properties, influenza A viral antigen-loaded chitosan microparticles especially prepared by entrapment are considered to be suitable for mucosal vaccine preparation.

The use of chitosan beads loaded with viral antigens may not be suitable for mucosal administration in *in vivo*. However, it can be considered as a model for monitoring the releasing characteristics of viral antigens from beads in *in vitro*. In this study, the chitosan beads were loaded with viral antigens with passive adsorption such as loading of the microparticles at different pH. The highest loading efficiency of beads was defined at pH 5 (Table 5). Both the loading efficiency and the loading capacity of beads were much lower than that of microparticles. The porous chitosan beads freeze-dried had approximately a 2 times higher loading efficiency and capacity than the beads dried at room temperature. Moreover, the releasing of viral an-

tigens from beads occurred more rapidly. Approximately 90% of adsorbed antigens from all types of chitosan beads released within 8 hours.

As a conclusion, the chitosan polymers have great potential for the development of long-acting viral vaccines due to their unique properties such as biocompatibility, non-toxicity, bio-degradability and their immune stimulatory effects. The loading efficiency and capacity of the chitosan microparticles and beads for viral antigens varies depending on the type of chitosan polymers and the conditions applied for formulations. The results gave us useful information about the potential of chitosan microparticles as a carrier of viral antigens, which might be employed in *in vivo* studies as a mucosal vaccine.

**Acknowledgements:** The authors thank Dr. Seyhun Solakoğlu and Biologist Funda Durmaz (Istanbul University, Istanbul Faculty of Medicine) for their kind assistance for obtaining electron microscopic images.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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# Bacteria Recovered from Cultured Gilt-Head Seabream (*Sparus aurata*) and their Antimicrobial Susceptibilities

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**Please cite this article as:** Canak O, Akayli T. Bacteria Recovered from Cultured Gilt-Head Seabream (*Sparus aurata*) and their Antimicrobial Susceptibilities. Eur J Biol 2018; 77(1): 11-7.

## ABSTRACT

**Objective:** Gilt-head seabream (*Sparus aurata*) is an important species for Turkish aquaculture and bacterial diseases are one of the limiting factors for the production of this species. The aim of this study is the identification of the bacterial disease agents in cultured gilt-head seabream in Turkey and the determination of their antibacterial susceptibilities.

**Materials and Methods:** In this study, 27 diseased gilt-head seabream samples between 3-130 g, showing various clinical symptoms, were examined bacteriologically. Bacterial isolates recovered from moribund fish samples were identified by using standard bacteriological methods and API 20E rapid identification strips. Antibiotic susceptibility tests of 10 different substances against these isolates were determined by using Kirby-Bauer disc diffusion method.

**Results:** *Vibrio anguillarum*, *V. scophthalmi*, *V. logei*, *V. harveyi*, *Pseudomonas anguilliseptica*, *P. stutzeri*, *Staphylococcus sp.* and *Micrococcus luteus* isolates were isolated from diseased fish samples as pure and mixed infection. It was detected that API 20E may give erroneous results for fish pathogenic bacteria since they are not included in the database of the kit. Generally, enrofloxacin, florphenicol and oxytetracycline were found to be the most effective antibiotics against bacterial isolates.

**Conclusion:** This study is the first report for *V. scophthalmi*, *V. logei*, *V. harveyi*, *P. stutzeri* and *M. luteus* infections of gilt-head seabream in Turkey.

**Keywords:** Gilt-head seabream, fish diseases, vibriosis, antibiogram susceptibility

## INTRODUCTION

Gilt-head seabream (*Sparus aurata*) is one of the most common species cultured in the Mediterranean basin and Turkey is among the leading producer countries with a production of 58.254 tons in 2016 (1,2). Despite the fact that Turkey has suitable environmental conditions and a sufficient number of large-scale hatcheries for the production of this fish species, bacterial diseases are the main limiting factor of production. A great majority of the infectious diseases of gilt-head seabream are caused by Gram-negative bacteria, especially *Vibrio* representatives (3,4).

Previously, various Gram-negative bacteria such as *Vibrio anguillarum* (also known as *Listonella anguillarum*), *V. alginolyticus*, *V. ordalii*, *Aeromonas hydrophila*, *Photobacterium damsela* subsp. *piscicida*, *Ph. damsela* subsp. *damsela*, *Pseudomonas fluorescens* and *Flavobacterium sp.* were isolated from infected gilt-head seabream cultured in Turkey (5-13). Gram-

positive bacteria also caused diseases in this species in Turkey and *Staphylococcus epidermidis* and *S. hominis* infections were reported (14,15).

Antibacterial substances have been used in the therapy of bacterial fish diseases for more than 60 years. As the production amount and the number of cultured fish species has increased, the frequency and diversity of the fish diseases has also shown an increase and a greater amount of antibiotics were used in this period (16). This increase in the use of antibacterial substances has brought about a number of problems. Various factors such as the incorrect use or dose of antibacterial substances causes the development of resistance in bacteria. Furthermore, the residue of antibacterial substances in cultured fish is a threat for human health. Hence, the use of some antibacterial substances in aquaculture, such as chloramphenicol, have been banned or limited (16,17).



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Received: 24.10.2017 Accepted: 19.03.2018

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The aim of this study is the identification of the bacterial disease agents in cultured gilt-head seabream in Turkey and the determination of their antibacterial susceptibilities.

## MATERIALS AND METHODS

In this study, 27 cultured diseased gilt-head seabream specimens between 3-130 g showing some clinical symptoms of bacterial diseases supplied from 5 different fish farms (will be mentioned as Farm No. 1, 2, 3, 4 and 5 in the rest of the article) located in the Aegean Sea coasts of Turkey were examined bacteriologically between April 2009 and July 2010 (17, 18). After anesthesia with 2-phenoxyethanol, bacteriological inoculations from the liver, kidney and spleen of the fish samples were made onto TSA (Tryptic Soy Agar) containing 1.5% NaCl, TCBS and Marine Agar. Inoculated media were incubated at 22 °C for 72 hours. Bacterial isolate growths from moribund fish samples were identified based on their biochemical profiles by using standard laboratory methods (17-19).

API 20E rapid identification strips (Biomerieux, France) were used as an additional method. Since it is not discriminative for Gram-positive bacteria, this kit was not used for these isolates and they were identified by using the results of the standard laboratory methods only (17). API 20E test strips were used according to the instructions of the manufacturer. Briefly, fresh cultures of bacteria were suspended in sterile distilled water containing 0.85% NaCl and well emulsified. Bacterial suspension was added into the wells of the test strip and incubated at 24 °C for 24-48 hours.

Antibacterial susceptibilities of these isolates against 10 different substances were determined by using the Kirby-Bauer disc diffusion method, which was replicated three times and evaluated according to the CLSI standards (20). Briefly, fresh cultures of bacterial isolates were suspended in sterile phosphate buffered saline (PBS) and diluted to a turbidity equivalent to the McFarland tube No. 0.5 standard solution. The bacterial suspension (0.1 ml) was spread onto Mueller-Hinton agar and antibiotic disks were placed (21). Petri dishes were incubated at 22 °C for 18-24 hours and the sensitivity zones were measured with a ruler and their arithmetic mean was calculated.

## RESULTS

During the field sampling studies, slow swimming behavior near the surface of the seawater and loss of appetite was detected in all moribund fish samples. In the 20 g fish samples examined in farm no 1, hemorrhages at the fin bases and anus, erosion in the lower jaw and anemia in the internal organs were observed (Figures 1a and 1b). In the 80 g fish samples examined in farm no 2, pale skin color, loss of scales, ulcers and lesions on the skin, hemorrhages at the base of the fins, anemia in the gills and internal organs and in some samples the accumulation of a translucent fluid were observed (Figures 1c and 1d). In the 10 g fish samples examined in farm no 3, loss of scales, erosion in the lower jaw, fin base hemorrhages, shallow skin lesions and ane-

mia in the internal organs were observed (Figures 1e and 1f). In 3-6 g fish samples examined in farm no 4, distended abdomen and pale skin color, distended liver with petechial hemorrhages, splenomegaly and liquefaction in the kidney were observed (Figures 1g and 1h). In 130 g fish samples examined in farm no 5, distended abdomen, pale skin color, loss of scales, hemorrhages on the internal organs, splenomegaly and accumulation of a bloody fluid in the intestines and peritoneal cavity were observed (Figures 1i and 1j).

A total of 15 bacterial isolates were recovered from the visceral organs of moribund fish samples. Depending on their morphological and biochemical properties that are shown in Table 1, these isolates were identified as *V. anguillarum* (n=6), *V. scophthalmi* (n=3), *V. logei* (n=1), *V. harveyi* (n=1), *P. anguilliseptica* (n=1), *P. stutzeri* (n=1), *Staphylococcus sp.* (n=1) and *Micrococcus luteus* (n=1).

As a result of bacterial identification studies carried out after the bacterial examination of the fish samples, *V. anguillarum* was recovered as a pure infection in fish samples obtained from fish farm no 1. Mixed infections of *V. anguillarum* with *V. scophthalmi* in one fish and *V. harveyi* in another fish sample were detected in fish farm no 2. In fish farm no 3, mixed infections of *V. anguillarum* and *V. scophthalmi* in the first fish sample; *V. anguillarum* and *V. logei* in the second fish sample and *V. anguillarum* and *P. stutzeri* in the third fish sample were detected. Moreover, a pure *V. anguillarum* infection in one fish sample, mixed infections of *V. anguillarum* and *Staphylococcus sp.* in the second fish sample and *V. anguillarum* and *M. luteus* in the third fish sample were detected in fish farm no 4. A pure infection of *P. anguilliseptica* was detected in fish farm no 5.

As a result of the antimicrobial susceptibility tests, generally, enrofloxacin, oxytetracycline and ciprofloxacin were found to be the most effective antibiotics against all bacterial isolates. Florphenicol and sulphametaxazole-trimetoprim were found to be the most effective antibiotics against *Vibrio* spp. isolates. Despite being recovered from different fish farms, isolates of the same species gave similar results in the antimicrobial susceptibility testing. Most of the isolates showed a complete resistance to ampicillin and streptomycin. Antimicrobial susceptibility test results of these isolates are shown in Table 2.

## DISCUSSION

Gilt-head seabream are commonly cultured in the Mediterranean basin and Turkey is one of leading countries in the production of this species (1,2). Bacterial diseases are one of the most important limiting factors in fish production (17). Identification of the pathogens involved in the disease conditions of the gilt-head seabream and taking proper precautions for the treatment of disease is the key to success in the culture of this species. This study was carried out for the detection of bacterial pathogens affecting gilt-head seabream cultured in the Aegean Sea coasts of Turkey and determination of their antimicrobial susceptibilities.



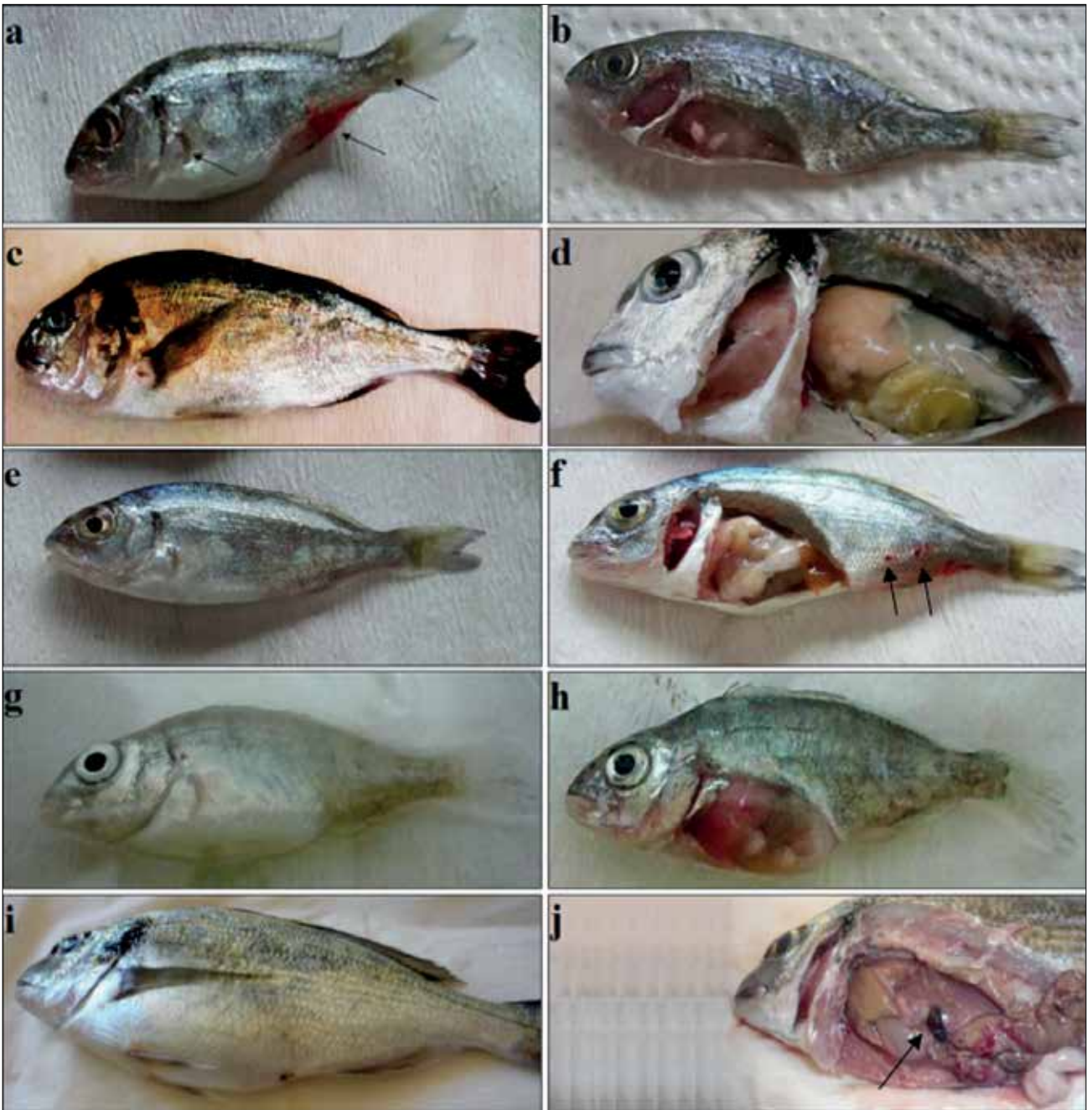


Figure 1. General clinical external and internal symptoms observed in fish samples a & b: Farm no 1 (ulcers on the body are indicated by arrows), c & d: Farm no 2 (loss of scales and anemia in the internal organs), e & f: Farm no 3 (hemorrhages and ulcers on the body [indicated by arrows], anemia in the liver), g & h: Farm no 4 (distended abdomen, erosion of the caudal fin and distended liver), i & j: Farm no 5 (distended abdomen, splenomegaly [indicated by arrows] and fluid accumulation in the intestines).

In the field studies, general clinical symptoms such as pale skin color, loss of scales, hemorrhages and ulcers on the body surface and anemia in the internal organs were observed and showed similarities with previous reports for each particular bacterial fish pathogen (7,17,18).

Previously, various researchers conducted long-term disease monitoring studies on gilthead seabream and they all reported

that they have recovered Gram-negative bacteria in a great majority (more than 90%), particularly the *Vibrio* species (70%) (3,4,12). Similarly, in this study, it was determined that the members of the genus *Vibrio* are the main pathogens of this fish species and some other bacteria are involved in cases of mixed infections.

Vibriosis is a common bacterial fish disease worldwide that is caused by various *Vibrio* species and *V. anguillarum* is among

**Table 1.** Phenotypic and biochemical properties of bacterial isolates

	<i>V. anguillarum</i> n=6	<i>V. logei</i> n=1	<i>V. harveyi</i> 6 n=1	<i>V. scophthalmi</i> n=3	<i>P. stutzeri</i> n=1	<i>P. anguillispetica</i> n=1	<i>Staphylococcus sp.</i> n=1	<i>Micrococcus luteus</i> n=1
Gram	-	-	-	-	-	-	+	+
Morphology	B	B	P	B	B	B	GLC	T
Motility	+	+	+	+	+	+	-	-
O/F	F	F	F	F	O	O	F	O
Oxidase	+	+	+	+	+	+	-	-
Catalase	+	+	+	+	+	+	+	+
O/129	S	S	S	S	R	R	R	R
TCBS	Y	G	Y	Y	-	-	-	-
VAM	Y	Y	-	-	-	-	-	-
Arginine	+	-	-	+	-	-	-	-
Lysine	-	+	+	-	-	-	-	-
Ornithin	-	+	+	-	-	-	+	+
Indole	+	+	+	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-
VP	+	-	-	-	-	+	+	-
β-galactosidase	+	+	-	-	-	-	-	-
Gelatinase	+	-	-	-	+	-	-	-
Amilase	+	-	-	-	-	-	-	-
Aesculine	-	+	W	+	-	-	-	-
Haemolysis	β	β	β	γ	α	β	α	α
Urease	-	-	-	-	-	-	+	-
Citrate	-	+	-	-	+	-	-	-
Nitrate reduction	+	+	-	+	+	+	+	-
H2S	-	-	-	-	-	-	-	+
Acid production from								
Fructose	+	+	-	+	-	+	-	-
Arabinose	+	-	-	-	-	-	-	-
Mannitol	+	-	+	+	-	-	-	-
Mannose	-	+	-	+	-	-	-	-
Xylose	-	+	+	+	-	-	-	-
Lactose	-	+	+	+	-	-	-	-
Growth at								
4 °C	W	-	-	-	W	+	-	-
37 °C	+	-	+	+	+	-	+	+
40 °C	W	-	+	-	-	-	+	+
0% NaCl	-	-	-	-	-	+	+	+
3% NaCl	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+
8% NaCl	-	-	-	-	-	W	-	W
API 20E Profile(s)	3247526 3047127 3267126	5764105	4356525	2000024 2000124	0200004	2200004	N/A	N/A

+: Positive reaction, -: Negative reaction, B: Bacilli, P: Pleomorphic, GLC: Grape-like clusters, T: Tetrad, O: Oxidative, F: Fermentative, S: Sensitive, R: Resistant, Y: yellow, G: green, W: Weak reaction, VAM: *Vibrio anguillarum* medium, α: Alpha-hemolysis, β: Beta-hemolysis, γ: Gamma-hemolysis



**Table 2.** Antibiotic susceptibilities of bacterial isolates recovered from moribund gilt-head seabream samples (mean zone diameter in cm)

	<i>V. anguillarum</i>	<i>V. scophthalmi</i>	<i>V. logei</i>	<i>V. harveyi</i>	<i>P. stutzeri</i>	<i>P. anguilliseptica</i>	<i>Staphylococcus sp.</i>	<i>M. luteus</i>
Erythromycin (15 µg)	R (1.4)	R (1.5)	R (1.1)	I (1.9)	R (1.3)	R (0)	I (1.7)	S (4)
Enrofloxacin (5 µg)	I (2.5)	S (3)	I (2)	I (2)	S (3.3)	I (2.2)	S (2.7)	I (1.6)
Oxytetracycline (30 µg)	I (2)	S (2.9)	I (2)	I (1.8)	I (2.4)	R (1.5)	I (2.5)	S (2.8)
Ampicillin (10 µg)	R (0)	I (2.2)	R (0)	R (0)	I (2)	R (0)	R (1.2)	S (4.1)
Kanamycin (30 µg)	R (1.6)	I (2)	R (1.3)	R (1.5)	I (2)	I (1.7)	R (1.5)	I (2.3)
Ciprofloxacin (5 µg)	I (2.5)	S (2.9)	I (1.8)	I (1.9)	S (3.1)	I (2.3)	I (2)	R (1.4)
Flumequine (30 µg)	I (2.4)	S (3.2)	S (2.6)	I (2.2)	S (3.7)	I (1.6)	I (2.2)	R (0)
Florphenicol (30 µg)	S (3.6)	S (3.1)	S (2.8)	S (3)	I (2.3)	R (0)	R (0)	S (4)
Streptomycin (10 µg)	R (0)	R (0)	R (0.9)	R (1.1)	R (1.3)	R (0)	R (0)	S (2.6)
Trimethoprim sulfamethoxazole (25 µg)	S (2.8)	S (3)	S (3)	I (2)	S (3.3)	R (0)	S (3.2)	R (0)

R: Resistant, I: Intermediately Sensitive, S: Sensitive

the oldest of known fish pathogens and is reported to affect more than 48 aquatic species world-wide (17-21). It has been reported to be involved in the diseases of various cultured marine fish species such as gilt-head seabream, Atlantic salmon, European sea bass, red porgy and also rainbow trout cultured in marine and freshwater environment in Turkey (5,6,22-32).

*V. harveyi* was previously recovered from moribund gilthead seabream in Spain by Balebona et al. (3) and Zorrilla et al. (4). This bacterium was also recovered from moribund European seabass in Turkey by Korun and Akayli (33) and Korun and Timur (25). *V. scophthalmi* was previously recovered from the tank water in turbot culture (34) and turbot intestinal flora (35), but it was reported as a non-pathogenic species by Cerda-Cuellar et al. (36). *V. logei* was previously recovered from moribund cultured Atlantic salmon in Iceland (37). As stated above among the *Vibrio* species isolated from gilt-head seabream in this study, *V. anguillarum* was previously reported from this fish species in Turkey, but *V. harveyi*, *V. scophthalmi* and *V. logei*, were recovered for the first time in mixed infections of gilt-head seabream cultured in Turkey.

*P. anguilliseptica*, which causes winter disease, was previously recovered from moribund cultured gilthead seabream in France and this bacterium is rarely recovered from gilt-head seabream in Turkey (38,39). It was also previously reported from cultured European sea bass in Turkey (11). In this study, this bacterium was recovered from a chronically infected fish sample in summer, where a great majority of the batch was lost due to this infection in winter.

*P. stutzeri*, a bacterium that is found in freshwater, marine and soil environments and in wastewater (40,41) was reported among the most abundant bacteria in Iskenderun Bay, Turkey

by Matyar et al. (42). This bacterium also caused disease in rainbow trout in freshwater environments (43). In this study, this organism was recovered from the moribund cultured gilthead seabream in Turkey for the first time.

As previously reported in long-term disease monitoring studies of gilt-head seabream (3,4,12), Gram-positive bacteria were also recovered in this study too. One isolate could only be identified at the genus level as *Staphylococcus sp.* Previously, Kubilay and Ulukoy (14) reported a *Staphylococcus epidermidis* infection and Korun et al. (15) reported a *S. hominis* infection in gilthead seabream cultured in Turkey. Another Gram-positive isolate was identified as *Micrococcus luteus*. This organism was reported to be a pathogen of gilt-head seabream in Spain (3), and in this study, it was recovered from moribund gilt-head seabream cultured in Turkey for the first time.

API 20E bacterial identification kits were first developed for the rapid identification of medically important bacteria and later they were used in the field of fish disease (44), but in many cases, erroneous identification results have been achieved. Since *V. anguillarum* is not included in the API database, Santos et al. (45) reported that 35 of their 53 *V. anguillarum* strains were misidentified as *Aeromonas hydrophila* and, furthermore, they have faced similar problems with other important fish pathogens such as *A. sobria*, *A. caviae* and *Y. ruckeri*. Austin et al. (46) recorded 117 different API 20E profiles with their 260 *V. anguillarum* isolates from various parts of the world. Altun et al. (30) and Balta and Balta (32) reported that their *V. anguillarum* isolates were misidentified as *V. vulnificus* by this kit.

Similarly, in this study 3 different API 20E profiles (3247526, 3047127 and 3267126) were recorded with *V. anguillarum* isolates including a very similar profile as the dominant profile ob-

tained by Austin et al. (46); 3247527. However, in the API 20E database, our isolates were all misidentified as *A. hydrophila*. A very similar profile (3207526), with only one difference in the indole test result, was also reported by Balta (26) with *V. anguillarum* strains isolated from moribund European sea bass cultured in the Black Sea region of Turkey. When the previously reported API 20E profiles and the results of this study were evaluated, it was detected that generally citrate, sorbitol and indole test results can be variable in this kit for *V. anguillarum* (46). This variability in the API 20E test results of *V. anguillarum* strains isolated from various regions of Turkey was also previously reported (24, 26).

Previously, Soffientino et al. (47) reported an API 20E profile of 4356525 with a *V. carchariae* (which is another name for *V. harveyi*) strain isolated from cultured summer flounder in the USA. The same API 20E profile was recorded in this study with the *V. harveyi* isolates and it was misidentified in the API database as *V. alginolyticus*. Also, other fish pathogenic bacteria, *Pseudomonas stutzeri* and *P. anguilliseptica* isolated from moribund gilt-head seabream in this study were both misidentified as *Pseudomonas fluorescens/putida* with this kit. Hence, when using biochemical tests, more reliable results to identify *Vibrios* and other fish pathogenic bacteria can be obtained with systemic bacterial identification books (17,48) or specially designed identification keys (19). Besides biochemical methods, for a more precise identification of the fish pathogenic bacteria, the use of species-specific molecular or serologic methods would be useful (15,17,18,24,26,30).

Despite the common use of antibiotics in disease treatment, these substances are only a small part of a comprehensive management plan and should not be relied upon exclusively to solve all health problems in aquaculture (16). Proper culturing methods should be applied and prophylactic precautions such as the use of probiotics or vaccination should be taken for the prevention of disease outbreaks (17).

Generally, enrofloxacin, oxytetracycline and ciprofloxacin were found to be the most effective antibiotics against all bacterial isolates. In particular, florphenicol and sulphametaxazole-trime-toprim were found to be the most effective antibiotics against 11 *Vibrio* spp. isolates.

Florphenicol, flumequin, furanase, nutrafurazon, oxolinic acid, oxytetracycline and sulphamerazin were reported to be used successfully in the treatment of fish vibriosis (17). Also, Korun (6) reported that oxytetracycline treatment for 7 days has been successful against the *V. anguillarum* infection in gilthead seabream. But in this study, it was determined that *V. anguillarum* isolates showed a slight resistance to oxytetracycline and more successful results were achieved against this bacterium with other substances in *in vitro* tests. Altun et al. (30) also reported a resistance to oxytetracycline in *V. anguillarum*. Balta and Balta (32) applied a treatment with florphenicol during a *V. anguillarum* infection in rainbow trout and achieved good results. Similarly, in this study, florphenicol was found to be effective on *V. anguillarum* isolates. Also, Balta (26) reported that all *V. anguillarum* strains isolated from cultured European seabass

showed ampicillin-resistance and florphenicol was found to be effective against this bacterium. Similar results were obtained in this study too. In contrast, Altun et al. (30) reported that some of their *V. anguillarum* strains recovered from rainbow trout developed a resistance to many antibiotics including florphenicol and sulphametaxazol.

Ciprofloxacin and enrofloxacin were found to be effective against Pseudomonads and moreover, it was detected that *P. anguilliseptica* is resistant to 6 of the 10 antimicrobials tested. The results showed that, enrofloxacin, oxytetracycline and sulphametaxazole-trime-toprim were the most effective antibiotics against *Staphylococcus sp.* while erythromycin, ampicillin and florphenicol were effective against *Micrococcus luteus*.

## CONCLUSION

In conclusion, as the main purpose of aquaculture is to produce fish for human consumption, it is recommended that less dangerous methods such as probiotics, immunostimulants and vaccines should be used for health management and prophylaxis in aquaculture instead of antimicrobials. If it is obligatory, in each epizootic observed in aquaculture, the required tests should be performed on the bacterial isolates and the most suitable antibacterial substance should be used while bearing in mind the banned substances list. Hence, healthier food for human consumption will be produced and bacterial resistance will be prevented.

**Acknowledgement:** This work was supported by Istanbul University Scientific Research Projects Fund (Project No. 5087).

**Conflict of Interest:** The authors have no conflict of interest to declare.

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## Fishery of Sinop Coasts in the Black Sea Surveys

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**Please cite this article as:** Bat L, Sahin F, Sezgin M, Gonener S, Erdem E, Ozsandikci U. Fishery of Sinop Coasts in the Black Sea Surveys. Eur J Biol 2018; 77(1): 18-25.

### ABSTRACT

**Objective:** The current work was carried out between 2013-2014 during the fishing periods to evaluate certain fish agglomerations within 3 miles of the coastal zone. Therefore, some basic characteristics of fish populations along the Inceburun coast of the Sinop province of the Black Sea were examined.

**Materials and Methods:** Data was collected from 5 trawl operations in the Sinop-Inceburun Region of the Black Sea at depths of 20 to 39 meters at 5 different locations. Biometric measurements of each species was made. A literature search was performed to gather information about the status of threatened fish species found in the region. The length-weight parameters of 4 commercial fish species were estimated.

**Results:** During the fishing season between 1<sup>st</sup> September 2013 and 15<sup>th</sup> April 2014 different fish species were captured depending on the season and climate. In these surveys, 16 teleost species from 15 families, 2 elasmobranch species from 2 families and 2 species of invertebrates were recorded from 5 different stations. In these surveys random samples of red mullet, bluefish, horse mackerel, turbot etc. were collected from 5 different stations. The total mean C<sub>PUA</sub> was estimated at 425.30 kg/km<sup>2</sup>.

**Conclusion:** The Black Sea areas including those around the Sinop coast have an important effect on the entire production of sea fish of the Turkish fishery. In addition, Sinop fisheries generate significant fishery production supporting logistics and employment. In the Sinop peninsula, there is an increasing trend towards the production of commercial species of the Black Sea.

**Keywords:** Black Sea, Inceburun, Sinop, fishery, bottom trawl

### INTRODUCTION

The Black Sea contains the most isolated of ecosystems of all natural inland seas, connected to the Mediterranean by a very thin waterway through the Turkish Straits. The Black Sea contains a comparatively large variety of marine fauna (1), in spite of its brackish waters (17‰) and anoxic conditions below a depth of 180 m (with high levels of H<sub>2</sub>S). Approximately 3,800 species have been reported in the Black Sea. 42.9% of them belong to fungi, algae and higher plants, 52.5% are invertebrates, 4.5% fishes and 0.1% mammals (2).

Fish are one of the top aquatic organisms which serve as a source of protein (3,4). The fishery industry pays a

major role in Turkey's economy as a source of employment and revenue from exports. Turkey has many commercially important marine fishery resources which include demersal and small pelagic fish species. Fish production decreased from 589 129 tonnes in 2007 to 301 463,6 tonnes in 2016 (5). One of the problems associated with the fishing industry is the over-fishing of commercial fish species throughout the year. Being the most important fishing area, the Black Sea is being developed by the government of Turkey as a fishing center. The Black Sea is heavily influenced by various types of contaminants (6-9). Household and industrial waste are causing great harm to the fishing industry in Turkey. The City of Sinop is one of the most important fishing cities on the Black Sea coast (10). Besides com-



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Received: 05.02.2018

Accepted: 01.04.2018

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mercial fishing, recreational fishing is also common in Sinop. Being surrounded by sea on three sides, Sinop is an important area for the fishing industry (10). The fresh catch from marine fisheries is supplied to local fish markets. The national per capita consumption was only 6.2 kg p.a. in 2015 (5). This value is higher in Turkey's coastal cities including Sinop. Sinop is the center of the fishing industry with many trawlers and fishing vessels using Sinop Fish Harbour. A recent review has pointed out that numerous contaminants, several of which are carcinogenic, and toxic materials such as heavy metals have been found in the marine ecosystem (6). These hazardous pollutants are also moving into the diet of human beings consuming the seafood including fish. However, many recent studies indicate that heavy metals in commercial fish species caught near the Sinop coast are well below the acceptable levels set for EU Commission Regulations and TFC (11-21). In this respect, the fish obtained from Sinop shores are among the better species for consumption.

Although fish constitute only a small portion of the biodiversity of the Black Sea, the main species include anchovy (*Engraulis encrasicolus*), sprat (*Sprattus sprattus*), whiting (*Merlangius merlangus*) and Atlantic bonito (*Sarda sarda*) which have always been important in the area because of their great commercial value. The current study was carried out between the fishing periods of 2013 and 2014, and several fundamental features of the fish population along the Inceburun coast of the Sinop province of the Black Sea were examined.

**MATERIALS AND METHODS**

Samples of fish were taken from waters along the Inceburun coast of the Sinop province in the Black Sea. Samples were collected at depths of between 20-39 m within 3 miles of the coastal zone at 5 different locations using bottom trawling hauls by a fishing vessel during the fishing season of 2013-2014 (Figure 1).

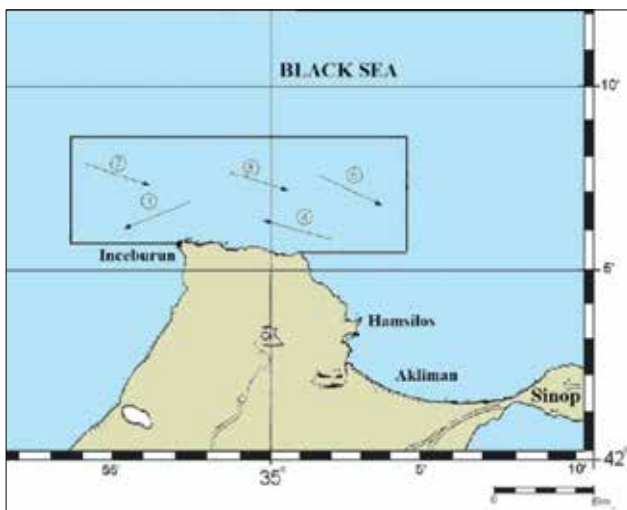


Figure 1. Trawl locations in the Sinop-Inceburun area (Black Sea)

The sizes of the trawl net used were as follows: head rope length – 10 m; mesh size – 44 mm. Haulings were carried out during daylight hours with 30-45 min hauling duration at a vessel speed of 3.66 km/h on the ground (Table 1). After each haul, the total catch was sorted and placed into boxes by species. After this process, biometric measurements of each species were taken.

**Length-Weight Relationship**

Equation 1 was used to determine length-weight relationship (22):

$$W = a * L^b \quad (1)$$

where;

W : Weight (g)

L : Total length (cm)

a and b : regression coefficients

**Fulton's Condition Factor (K)**

$$K = \frac{W}{L^3} * 100 \quad (23)$$

**Biomass Estimation**

Biomass was estimated using the swept area method. The swept area, a, can be calculated from equation 2 (24)

$$a = D * hr * X2 \quad (2)$$

$$D = V * t$$

where:

a : Swept area

V : Velocity of the trawl over the ground

X2 : Fraction of the head – rope length

hr : Headrope length

t : Duration of haul

D : Distance covered

Equation 3 (24) was used for the estimation of relative biomass, the catch per unit of area (CPUA)

$$CPUA = \frac{Cw}{a} \quad (3)$$

Where;

a : Swept area (km<sup>2</sup>)

Cw : Catch (kg)

Once caught, species were weighed and measured then stored in 4% formalin for laboratory analysis, excluding bigger fish. Specimens were classified using the identification keys of



**Table 1.** Information on trawl operations in the period of 2013-2014 in the Sinop-Inceburun region (Black Sea)

Hauls	Coordinate		Time		Total Duration (min)	Average Depth (m)
	Start	Finish	Start	Finish		
1	42° 7'48.47"N	42° 7'23.67"N	09:45	10:15	30	39
	34°57'42.84"E	34°56'36.23"E				
2	42°10'44.32"N	42°10'9.83"N	11:10	11:40	30	20
	34°53'46.37"E	34°54'42.27"E				
3	42° 9'41.56"N	42° 9'4.59"N	12:35	13:05	30	29
	34°57'44.13"E	34°58'36.74"E				
4	42° 6'15.19"N	42° 6'48.04"N	14:35	15:20	45	34
	35° 2'39.06"E	35° 1'42.31"E				
5	42° 7'56.96"N	42° 7'16.19"N	16:10	16:55	45	35
	35° 2'38.75"E	35° 3'21.51"E				

**Table 2.** Fish of Sinop coasts during the trawl operations in the period of 2013-2014 in the Sinop-Inceburun region (Black Sea)

Class	Ordo	Familia	Species	Common name	
Chondrichtyes	Myliobatiformes	Dasyatidae	<i>Dasyatis pastinaca</i>	Common stingray	
	Rajiformes	Rajidae	<i>Raja clavata</i>	Thornback ray	
Osteichtyes	Perciformes	Mullidae	<i>Mullus barbatus</i>	Red Mullet	
		Pomatomidae	<i>Pomatomus saltatrix</i>	Bluefish	
		Carangidae	<i>Trachurus mediterraneus</i>	Horse mackarel	
		Centracanthidae	<i>Spicara maena</i>	Blotched picarel	
		Trachinidae	<i>Trachinus draco</i>	Greater weever	
		Gobiidae	<i>Neogobius melanostomus</i>	Round goby	
			<i>Mesogobius batrachocephalus</i>	Toad goby	
		Uranoscopidae	<i>Uranoscopus scaber</i>	Atlantic stargazer	
		Sciaenidae	<i>Umbrina cirrosa</i>	Shi drum	
		Pleuronectiformes	Scophthalmidae	<i>Scophthalmus maximus</i>	Turbot
			Bothidae	<i>Arnoglossus laterna</i>	Mediterranean scaldfish
Soleidae	<i>Pegusa lascaris</i>		Sand sole		
Gadiformes	Lotidae	<i>Gaidropsarus mediterraneus</i>	Shore rocking		
Clupeiformes	Clupeidae	<i>Alosa agone</i>	Twait shad		
Scorpaeniformes	Scorpaenidae	<i>Scorpaena porcus</i>	Black scorpionfish		
Syngnathiformes	Syngnathidae	<i>Hippocampus hippocampus</i>	Sea horse		

Whitehead et al. (25), Fischer et al. (26) and Bat et al. (27). The specimens caught were classified to the smallest taxon according to the identification key. A Literature study was carried out to gather information on the status of threatened fish species.

In order to gain insight into regional fishing activities and current issues in the region, local fishermen were interviewed to gather information concerning the condition of commercially utilized fish stocks and threatened fish species. Available pub-

lished papers were examined, particularly for species formerly present in the Sinop coasts of the Black Sea, and additional species were included into the inventory of native ichthyofauna.

**Table 3.** Calculated Fulton's Condition Factors for 9 fish species from trawl operations in the Sinop-Inceburun region (Black Sea)

Species	K	N
<i>Mullus barbatus</i>	1.15±0.14	188
<i>Pomatomus saltatrix</i>	0.91±0.01	100
<i>Trachurus mediterraneus</i>	0.86±0.02	56
<i>Uranoscopus scaber</i>	1.61±0.03	29
<i>Raja clavata</i>	1.64±0.01	28
<i>Scorpaena porcus</i>	1.93±0.08	22
<i>Trachinus draco</i>	0.20±0.07	8
<i>Gobius melanostomus</i>	1.27±0.05	20
<i>Gobius batrachocephalus</i>	0.84±0.04	4

## RESULTS

In the survey; 16 teleost species from 15 families, 2 elasmobranch species from 2 families and 2 species of invertebrates were sampled (Table 2). Fulton's Condition Factor has been calculated and is illustrated in Table 3. The catch per unit area (CPUA) values of sampled fish of trawl operations in the Sinop-Inceburun region are shown in Table 4. The total mean CPUA

is estimated at 425.30 kg/km<sup>2</sup>. The minimum, maximum and average length and weight values of each species are shown in Table 5. As tunicates, crabs and seahorses were released back to sea immediately after hauling, only the numbers of those samples were recorded (Table 6).

The main target species of bottom-trawl are whiting (*Merlangius merlangus*) and red mullet (*Mullus barbatus*) in the region. Whiting was not recorded in the hauls. Moreover, approximately 90% of total catch consisted of bycatch. 63% of the red mullet caught were under the legal catch size which is 13 cm. Furthermore, red mullet was the most abundant species in terms of individual numbers.

**Table 4.** Estimated CPUA values of fish samples in the Sinop-Inceburun region during the surveys in the fishing period 2013-2014.

Species	CPUA (kg/km <sup>2</sup> )					
	Stations					
	I.	II.	III.	IV.	V.	Average
<i>Mullus barbatus</i>	69.02	55.30	24.54	2.33	33.92	37.02±11.68
<i>Pomatomus saltatrix</i>	70.93	2.62	0	23.13	110.05	41.35±21.37
<i>Trachurus mediterraneus</i>	18.25	5.41	2.35	7.98	1.09	7.02±3.05
<i>Uranoscopus scaber</i>	17.54	12.46	20.38	5.94	13.37	13.94±2.46
<i>Dasyatis pastinaca</i>	8.96	470.93	50.00	59.89	182.26	154.41±84.25
<i>Scorpaena porcus</i>	0	0	0	25.17	0	5.03
<i>Neogobius melanostomus</i>	36.12	0.00	0.00	12.20	6.19	10.90
<i>Trachinus draco</i>	0.00	7.27	1.91	0	1.46	2.13
<i>Mesogobius batrachocephalus</i>	17.87	0	0	0	0	3.58
<i>Scophthalmus maximus</i>	0	2.62	12.57	3.39	0	3.71
<i>Umbrina cirrosa</i>	0	44.64	17.49	0	0	12.43
<i>Spicara maena</i>	0	0	0	0.40	1.09	0.30
<i>Raja clavata</i>	493.83	0	0	128.93	39.78	132.51
<i>Pegusa lascaris</i>	0	1.75	0	0	0	0.35
<i>Arnoglossus laterna</i>	0	0	0	0.55	0	0.11
<i>Gaidropsarus mediterraneus</i>	2.62	0	0	0	0	0.52
Total	735.14	603.01	129.23	269.91	389.22	425.30±109.69



**Table 5.** Total length and weight values (means  $\pm$ SE) of fish species obtained from trawl operations in the Sinop-Inceburun region (Black Sea)

Species	N	Min. Length (cm)	Max. Length (cm)	Mean $\pm$ SE	Min. Weight (gr)	Max. Weight (gr)	Mean $\pm$ SE
<i>Mullus barbatus</i>	188	3.8	18.5	12.07 $\pm$ 0.18	2	64	19.78 $\pm$ 0.84
<i>Pomatomus saltatrix</i>	100	12	22.9	17.43 $\pm$ 0.21	12	110	50.02 $\pm$ 1.81
<i>Trachurus mediterraneus</i>	56	8	18	11.18 $\pm$ 0.26	3	58	12.95 $\pm$ 1.07
<i>Uranoscopus scaber</i>	29	9.5	20.4	14.1 $\pm$ 0.46	12	150	50.07 $\pm$ 5.72
<i>Raja clavata</i>	28	20	46	31.64 $\pm$ 1.11	121.83	1699.58	583.76 $\pm$ 67.02
<i>Scorpaena porcus</i>	22	8.5	17	11.67 $\pm$ 0.40	15	62	31.41 $\pm$ 2.64
<i>Neogobius melanostomus</i>	20	11.4	22.8	15.96 $\pm$ 0.57	20	170	58.3 $\pm$ 7.03
<i>Trachinus draco</i>	8	11	18	15.86 $\pm$ 0.41	15	35	26 $\pm$ 1.23
<i>Mesogobius batrachocephalus</i>	4	20.5	22.2	21.3 $\pm$ 0.13	70	105	81.75 $\pm$ 2.93
<i>Scophthalmus maximus</i>	3	15	24	19.3 $\pm$ 0.84	48	93	70.5 $\pm$ 5.91
<i>Umbrina cirrosa</i>	2	20	42	31 $\pm$ 2.89	200	817	508.5 $\pm$ 81.02
<i>Spicara maena</i>	2	9.5	15	12.25 $\pm$ 0.72	11	30	20.5 $\pm$ 2.49
<i>Dasyatis pastinaca</i>	3	40	78	58.67 $\pm$ 3 .53	63.79	9036.68	4555.94 $\pm$ 757.54
<i>Pegusa lascaris</i>	1	-	-	16.5	-	-	32
<i>Arnoglossus laterna</i>	1	-	-	12.5	-	-	15
<i>Gaidropsarus mediterraneus</i>	1	-	-	19	-	-	48

**Table 6.** Numbers of Tunicates, Sea horses and Crabs by Stations.

Species	I. Station	II. Station	III. Station	IV. Station	V. Station	Total
<i>Ascidia aspersa</i> (Tunicate)	320	18	-	-	-	338
<i>Hippocampus hippocampus</i> (Seahorse)	11	2	1	-	-	14
<i>Liocarcinus depurator</i> (Crab)	63	124	80	250	95	612

Length–Weight relationships of sampled fish which were of sufficient sample size are illustrated in Figure 2. Red mullet, horse mackerel (*Trachurus mediterraneus*) and black scorpionfish (*Scorpaena porcus*) showed negative allometric growth while bluefish showed isometric growth. The average length values of red mullet which is the target species of demersal trawl were as follows; 12.01 cm, 12.06 cm, 10.75 cm, 10.15 cm, 13.89 cm respective to station number. The average length of red mullet specimens caught in Station V was significantly higher than those derived from other stations according to the One-Way Anova statistical test ( $p < 0.05$ ).

## DISCUSSION AND CONCLUSION

In the present study bottom trawler fishing in the region of the Sinop coast reveals very small numbers of the target species. The majority of commercial fish species were Red mullet

followed by Turbot (*Scophthalmus maximus*) and Thornback Ray (*Raja clavata*) (see Table 4).

Rays and sharks are usually seen as unwanted fish species which are discarded due to the fact that they are not consumed in the domestic market. Several pelagic fish species such as Bluefish (*Pomatomus saltatrix*) and Twaite shad (*Alosa agone*), are also accidentally caught in bottom trawl fisheries but they are generally discarded excluding those large enough to be commercially marketed.

Sinop which is located on the Boztepe Peninsula is the northernmost point of the Turkish Black Sea coast and is referred to as the midpoint of the Black Sea. The fact that three sides of the city of Sinop are surrounded by the Black Sea has made fishing an important industry. Gerze, Ayancik and Türkeli are seaside districts of Sinop provincial (10).

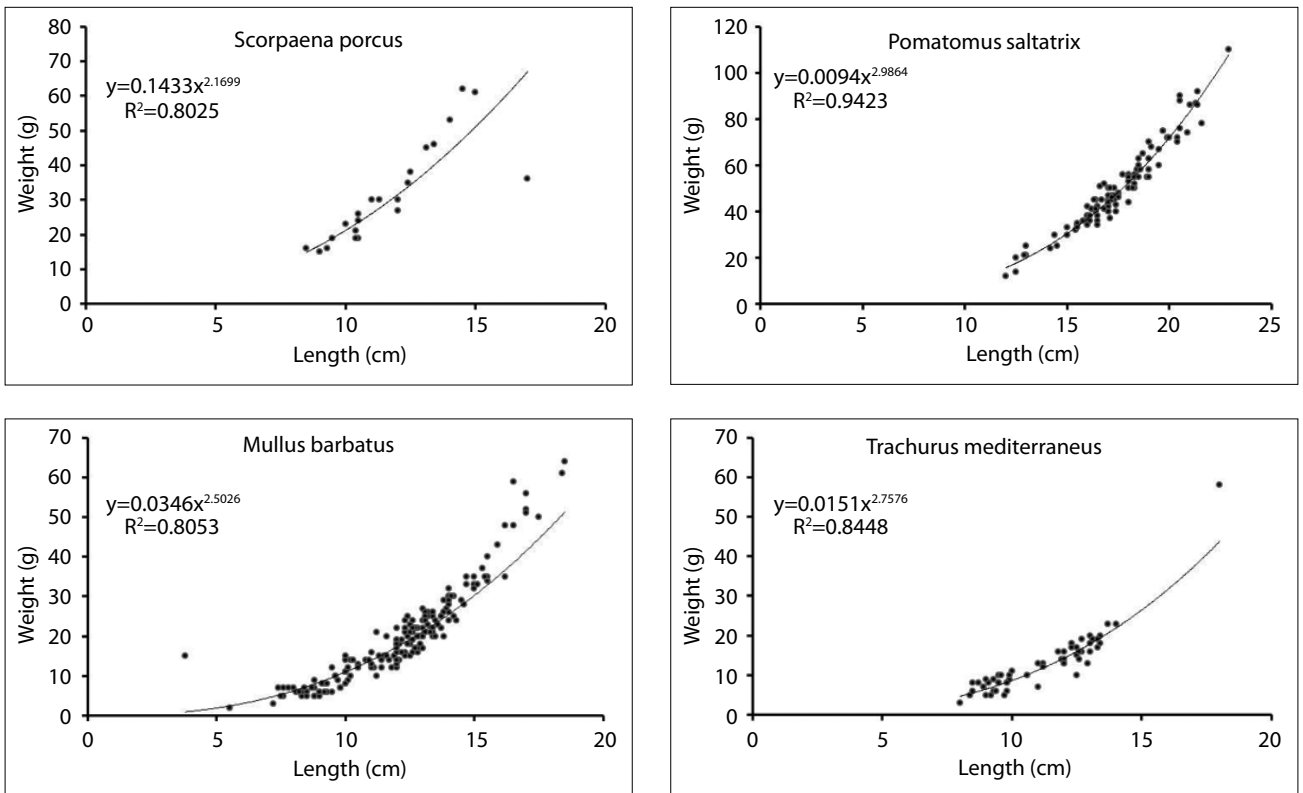


Figure 2. Length–weight relationships for *S. porcus*, *P. saltatrix*, *M. barbatus* and *T. mediterraneus* from the Sinop-Inceburun (Black Sea) during the trawl surveys in the fishing period 2013-2014.

Commercial fishing is carried out in two ways; large-scale (purse seine and trawl fisheries) and small-scale (fishing boats smaller than 12 m which operate gillnet, fishing line etc.). A considerable amount of the anchovy captured in the Black Sea is along the Sinop coast.

The length-weight relationship is widely used in the analysis of fishery data (28, 29), mostly because of the difficulty and time required to record weight in the field (30). The relationship is often used to convert growth-in-length equations for prediction of weight-at-age and used in stock assessment models (31), to calculate condition indices (32) and for lifetime and morphological comparison of populations from various regions (33). In the equation;  $b = 3$  refers to isometric growth,  $b < 3$  or  $b > 3$  refers to allometric growth.

Data concerning the marine fish of the Sinop coast is limited. Information on local fish faunas is scarce. A total of 94 species of 44 families along the Sinop – Samsun coastline have been identified (34). Gönener and Bilgin (35) reported that whiting, horse mackerel, red mullet, turbot, black scorpion fish, Thwaite shad, picarel (*Spicara maena*), picked dogfish (*Squalus achantias*), thornback ray and goby (*Gobius* sp.) were sampled with bottom-trawl in the Sinop-Inceburun area during the 2007 and 2008 fishing seasons. Similarly, Gönener and Özdemir (36) studied annual, monthly and daily catch amounts from the Sinop-Inceburun region during the 2008 and 2009 fishery seasons. They found whiting, red mullet, turbot, shad, picarel, horse

mackerel, shark and goby (*Gobius* sp.) in the same region of the Black Sea. Sampled species are similar to the current study except for whiting. Whiting could not be caught in our study, which may be due to the fact that the hauling was performed in shallow waters.

A sum of 3455 authorized fishermen and 536 fishing boats are present in the Sinop region with ports like Gerze and Ayancık (37). Fishing operations continue throughout the year depending on with meteorologic conditions and the fishing season lasts between 60 and 270 days. The quantity of catch varies. The areas in which seaside fishing operations take place are Akliman, Sarkum, Inceburun and Gerze (Çakıroğlu) (37).

As a consequence of eutrophication caused by a rise in the influx of nutrients from large northwest rivers over the past several decades, the Black Sea environment has changed considerably. The effects of the change in nutrient levels can be seen in the quality of biota with the inclusion of ichthyofaunal (34). The Black Sea has a fairly straight continental shelf and very weak oxygenated surface thickness and a eutrophic structure which provides a habitat for pelagic fishes. There is an increase in the fish composition caught at depths in which small pelagic fish constitute more than 90% of the total amount (38). Bottom trawls are an extremely effective fishing technique in demersal fishing with a catch rate of 60% (39). The most profitable sea yields off the Black sea coasts include pelagic species such as anchovy, horse mackerel, sprat, bluefish, bonito, benthic fish such as red mul-

let, whiting, turbot as well as and sea snails and mussels, (40). The fish caught in the Sinop area are generally of similar species. Moreover, the Black Sea, particularly around the Sinop shores is one of the best spawning fields for whole commercial fish species, including predator species, which migrated for spawning or feeding (34). It is indicated that marine fishery accounts for 76% of the total production of Turkish fisheries in 2007 (41). With respect to TUIK (5) anchovy accounts for 67% of the Black Sea fishing catch and 49% of fisheries in Turkey. Thus, variations in the distribution of anchovy and the amount of catch in any given fishing season have a serious influence on overall yearly fishing quantity (40). To support the fishing industry, the Turkish Government have implemented 1) a tax relief scheme for the diesel oil used in fishing vessels and 2) a subsidized credit scheme for fishermen (35). Gücü (38) claimed that reducing the expense of fishing has, to some extent, eased the situation for the fisheries, however, on the other hand, it has had a harmful effect on fish stocks. In general, fishing in Turkey, including in the Sinop region, operates on a diurnal basis, fishing in deep waters during the day and then returning to harbor (42). Moreover, it is rather apparent that the Black Sea area including the Sinop coast has a substantial effect on total production of marine fish of Turkish fisheries and Sinop fisheries constitute an important fishery industry and contribution to employment. There is an improvement in the availability of catch and biological data for economic species caught from the Sinop coast of the Black Sea. It should be noted that bio-ecologic information on capture and working statistics for all commercial fishing is still extremely limited. Therefore, it is suggested that further research to be carried out before any outcome is drawn.

**Acknowledgement:** This study was made possible by the contribution of European Community's Seventh Framework Programme (FP7/2007–2013) under Grant Agreement No. 287844 for the project "Towards COast to COast NETworks of marine protected areas (from the shore to the high and deep sea), coupled with sea-based wind energy potential" (COCONET).

**Conflict of Interest:** The authors have no conflict of interest to declare.

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## Diagnosis of *Vibrio anguillarum* in Cultured Rainbow Trout (*Oncorhynchus mykiss*) by Different Methods

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**Please cite this article as:** Akayli T, Aydin B, Urku C, Kayalar O. Diagnosis of *Vibrio anguillarum* in Cultured Rainbow Trout (*Oncorhynchus mykiss*) by Different Methods. Eur J Biol 2018; 77(1): 26-31.

### ABSTRACT

**Objective:** In this study, the diagnosis of infection caused by *Vibrio anguillarum* in cultured rainbow trout (*Oncorhynchus mykiss*), in Turkey using bacteriological, histopathological and immunohistochemical methods was attempted.

**Materials and Methods:** 15 rainbow trout samples showing signs of disease were investigated using bacteriological and histopathological methods.

**Results:** Because of physiological and biochemical characteristics of bacteria growing on the medium, these bacteria were isolated and identified as *V. anguillarum*. As a result of the histopathologic examination of the tissues of diseased rainbow trout, hyperemia in the liver, degeneration of the epithelium of kidney tubules and necrosis in the interstitial area, enlargement in the gill filaments, cells having picnotic nucleus and several necrotic areas in the spleen were observed. It was also detected in the streptavidin-biotin staining method that the causative agent accumulates in the liver, spleen, kidney and also in the gills especially in the areas near around the blood vessels depending on the development stage of the disease.

**Conclusion:** As a result, an immunoenzymatic method, streptavidin-biotin staining method was used in this study for the detection of the presence of *V. anguillarum* in the infected rainbow trout tissues.

**Keywords:** Rainbow trout, *Vibrio anguillarum*, histopathology, streptavidin-biotin

### INTRODUCTION

Bacterial pathogens are among the leading causes of economic loss in fish farming (1,2). One of the most important bacterial diseases leading to hemorrhagic septicemia in fish both in the natural and culture environment is vibriosis (1,3). It was reported that *Vibrio anguillarum*, known as the most common pathogen of vibriosis, causes mortality in many cultured fish species such as rainbow trout (4,5), sea bass (*Dicentrarchus labrax*) (6,7), and sea bream (*Sparus aurata*) (8,9). In general, it was reported that the primary clinical findings in fish infected with *V. anguillarum* are erythema around the fins and mouth, as well as lethargy, loss of appetite, change in skin color, ulcers on the body surface, formation of red necrotic lesions in the abdominal muscles and accumulation of bloody liquid in the intestine (1,5). It was reported that histopathological changes such as hemorrhage in the liver, gills, spleen, kidney and muscle focal necrosis in the

spleen and skeletal muscles and severe degeneration in the intestinal mucosa epithelium were observed in fish infected with *V. anguillarum* (2,5,10).

The identification and localization of the presence of the pathogen in tissues by different immunohistochemical methods used in studies conducted on *V. anguillarum* by different researchers were used for various purposes such as detecting its density in tissues, understanding how the pathogen infects healthy fish, and investigating how bacteria entering through the portal tracts spread to the fish tissues and organs (7,11). Bacterial methods have frequently been used for the identification of the disease agent. However, since this method is somewhat time-consuming, various alternative methods have recently come into use. Immunohistochemical methods are among these specific methods that are used for the identification of the agent since they have a high specificity and provide more efficient results in a shorter time (1).



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Received: 12.03.2018

Accepted: 29.05.2018

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In this study, the diagnosis of infections caused by *V. anguillarum* in cultured rainbow trout in Turkey using bacteriological, histopathological and immunohistochemical methods was attempted.

**MATERIALS AND METHODS**

**Fish Sampling**

In this study, the 15 moribund rainbow trout (*Oncorhynchus mykiss*) used as material were obtained from 4 different trout fish farms in the Aegean Region. The three healthy rainbow trout used as the control in immunohistochemical investigations was obtained from Istanbul University, Sapanca Inland Waters Fish Culture Research and Application Unit. Table 1 shows data on the diseased fish examined in this study.

**Bacteriological Examination**

Bacteriological samples of liver, kidney, spleen and blood were streaked onto Tryptic Soy Agar (TSA) and the plates were incubated at 22-23°C for 48-72 hours. Conventional bacteriological methods were applied to pure cultures of bacteria grown in the media after incubation, and the isolated bacteria were identified (1). In the application of conventional bacteriological methods, the RV22 strain of *V. anguillarum* O2 serotype obtained from Santiago de Compostela University in Spain was used as the control.

**Histopathological Examination**

After the necropsy examinations tissue samples such as kidney, spleen, liver and gills were fixed in 10 % formalin, dehydrated in ethanol and embedded in paraffin wax, section at 5 µm and stained by routine methods with haemotoxylin and eosin (H&E) (12,13).

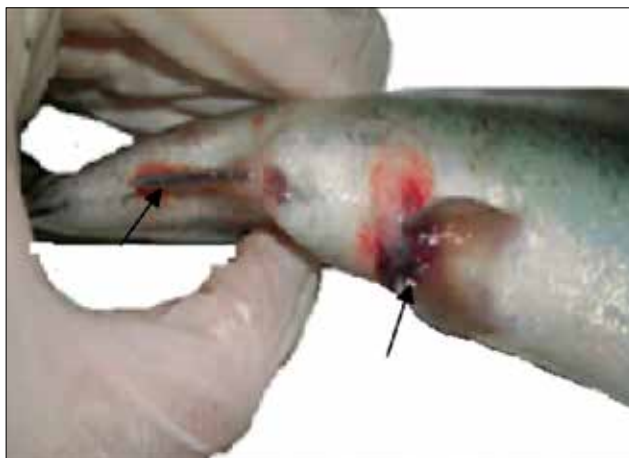


Figure 1. Large hemorrhagic foci and lesions around the pelvic and anal fin of the moribund rainbow trout (arrowed).

**Immunohistochemical Staining (Streptavidin-Biotin Method)**

The identification of *V. anguillarum* and its localization in the gill, kidney, spleen and liver were determined by Strep-ABC staining method (14). Organs such as gills, kidney, spleen and liver collected from healthy and diseased fishes were fixed in 10% neutral buffered formalin for 24 h. They were subjected to a routine paraffin embedding method. Tissue sections of 4-µm from paraffin-embedded organs were deparaffinized and rehydrated. The sections were treated with 10 mM citrate buffer (pH 6.0) in a microwave oven. The endogenous peroxidase activity in tissues was eliminated by 3% hydrogen peroxide for 30 min. After washing, the sections were treated with a normal goat blocking serum for 15 min at room temperature. And then, the sections were incubated with mouse anti-*Vibrio anguillarum* primary antibody (Ibt FM-040AX-5) at a 1:1000 dilution (the best dilution rate) for 2 h at room temperature. They were labelled by secondary antibodies (goat IgG) according to the manufacturer’s instructions for 15 min at room temperature (Invitrogen, Histostain Plus-peroxidase kit 859043). The peroxidase activity was revealed by a 3-amino-9-ethylcarbazole substrate kit (Invitrogen 002007). Sections were counterstained with Mayer’s hematoxylin. No immunolabelling was detected when the primary antibody was replaced with either PBS instead of primary antiserum. Sections were investigated under a light microscope.

**RESULTS**

**Clinical and Macroscopical Findings**

Externally all moribund rainbow samples showed external signs of disease, hemorrhages on the body surface, in the pectoral, pelvic, and anal fins (Figure 1), darkening in color internally, anemia in the gill, exfoliation at the tip of the gill filaments, petechial hemorrhage on the viscera and in the visceral adipose tissue, splenomegaly and paleness in the liver color (Figure 2).

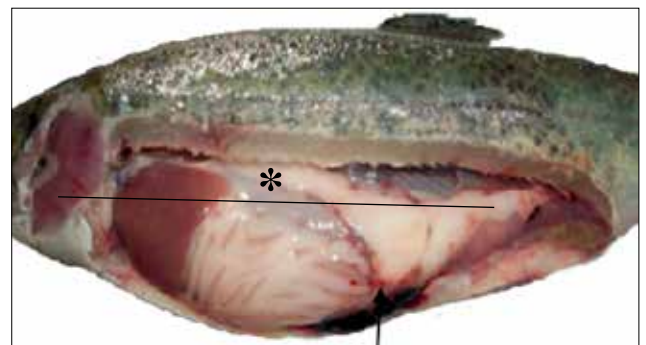


Figure 2. Anemia in the gills of diseased rainbow trout and exfoliation at the ends of gill filaments (\*), splenomegaly, hemorrhage in the visceral adipose tissue (arrowed).

**Table 1.** The rainbow trout used in this study and obtained from different fish farm and its weight

Fish Species	Fish Farm (A)	Fish Farm (B)	Fish Farm (C)	Fish Farm (D)	Control
Rainbow trout	4 fishes (150-200 g)	4 fishes (150-200 g)	4 fishes (170-250 g)	3 fishes (100-150 g)	3 fishes (100-150 g)

### Bacteriological Findings

After the incubation of the bacteriological inoculations from the spleen, kidney and liver, bacteria produced round, convex, slightly fluffy, bright, and cream-colored colonies onto

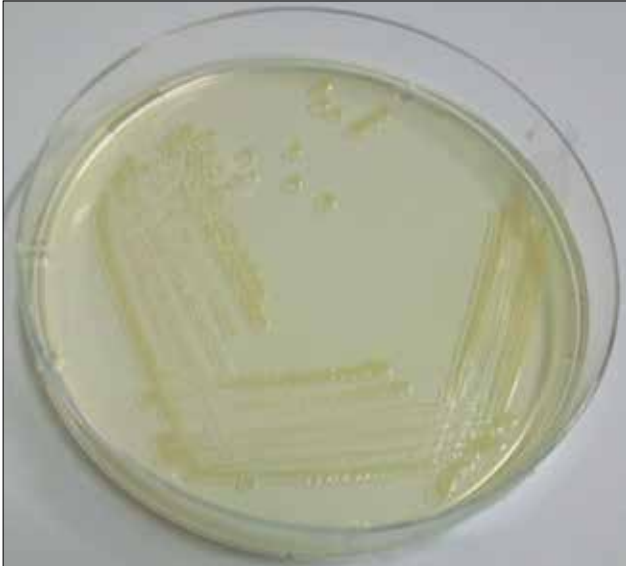


Figure 3. The appearance of *Vibrio anguillarum* colonies on TSA.

TSA (Figure 3). It was determined that gram-negative, motile, bacilli bacteria isolates (15 isolates) isolated from moribund rainbow trout belonged to *Vibrio* genus since they exhibited a fermentative characteristic, were sensitive to O/129 vibriostat test, and positive reaction in cytochrome oxidase and catalase tests. The isolated bacteria were identified as *V. anguillarum* since indole, beta-galactosidase (ONPG), Voges-Proskauer (VP), and arginine dihydrolase tests of the isolates gave a positive reaction, and H<sub>2</sub>S, Methyl Red, lysine, and ornithine dihydrolase tests were negative, they did not produce gas from glucose, used citrate in a citrate medium, the urease enzyme production was negative and gelatinase and amylase productions were positive, and also, they were similar to the reference bacteria (RV22).

### Histopathological Findings

Histopathologically, mild hyperemia in the liver (Figure 4a), degeneration of the epithelium of kidney tubules and very severe necrosis in the interstitial area (Figure 4b), the enlargement in the gill filaments (Figure 4c), cells having picnotic nucleus (Figure 4d) and several necrotic areas in the spleen were observed.

### Streptavidin-Biotin Method Findings

As a result of examining the visceral organs (liver, kidney, spleen) and the gills, of moribund rainbow trout infected

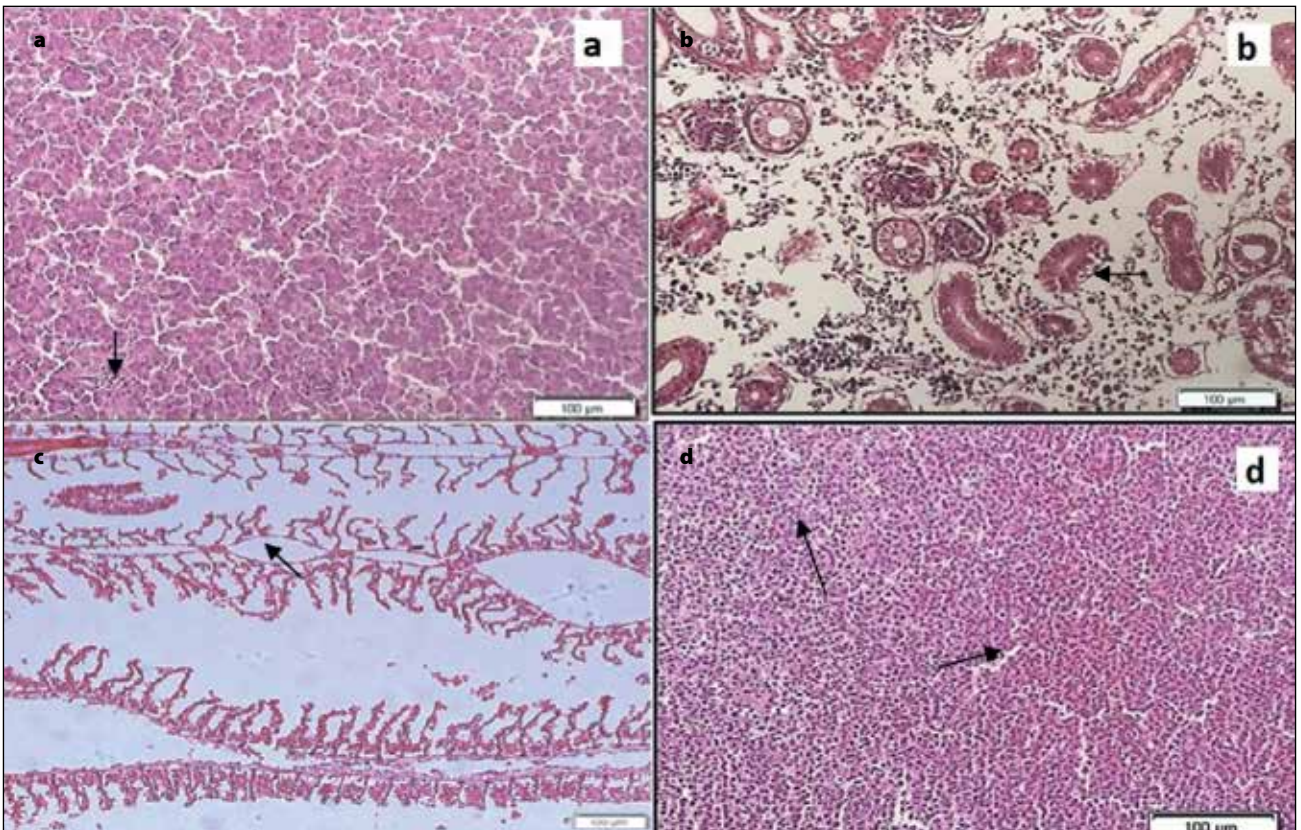


Figure 4. a-d. (a) Moribund rainbow trout infected with *V. anguillarum*. Mild hyperemia (arrowed) in the liver, (b) degeneration of the epithelium of kidney tubules (arrowed) and very severe necrosis in the interstitial area, (c) the enlargement (arrowed) in the gill filaments, (d) cells having picnotic nucleus in the spleen (arrowed) tissue of moribund rainbow trout samples.



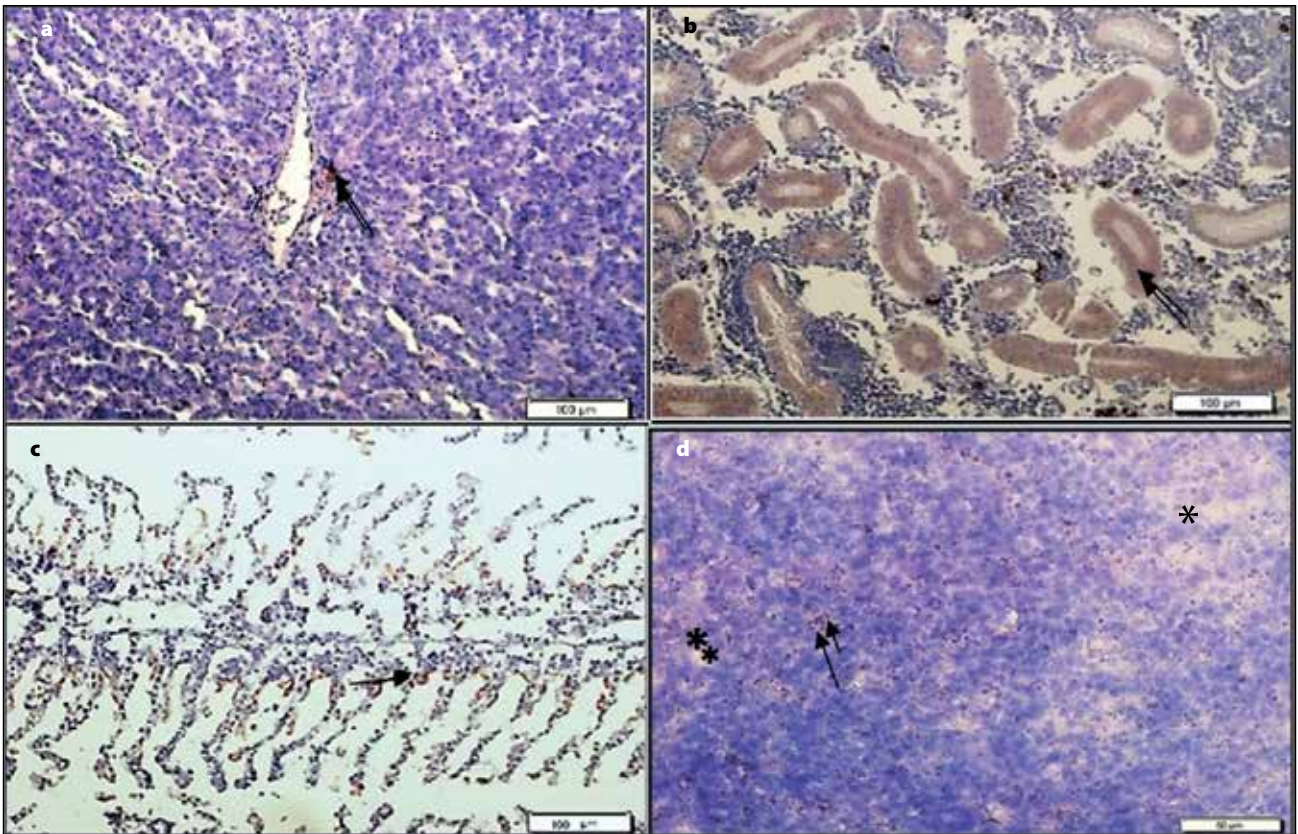


Figure 5. a-d. (a) Immunopositive reactions in the liver sinusoids, necrotic areas and at the vein center (arrowed), (b) in the kidney degenerative tubules (arrowed), (c) in the hyperplastic cells of the gills (arrowed), (d) in the necrotic area cells (arrowed) and several necrotic areas (\*) in the spleen.

with *V. anguillarum* by the immunohistochemical staining method, immunopositive reaction was not determined in the cells of the tissue sections such as the gills of the fish in the control group. The immunopositive reaction detected sinusoids in the necrotic areas and at the vein center in the liver (5a), the kidney tubules (Figure 5b), the gill filaments and cells in the hyperplastic areas (Figure 5c) and the necrotic of the spleen tissue (Figure 5d). Upon evaluation of the tissue samples obtained from infected fish according to the result of immunostaining, it was determined that the tissues with the most intense positive staining were in the cells of gills and kidney. Furthermore, this method provided a species-specific identification of *V. anguillarum* in a shorter time when compared with the routine bacteriological methods.

## DISCUSSION

*V. anguillarum* has been isolated and identified from diseased rainbow trout, in previous studies conducted in Turkey (5,15). In this study, the diagnosis of this bacterium in cultured fish species was made and the presence of this organism was detected in the tissues of rainbow trout samples showing clinical symptoms of vibriosis using bacteriological, histopathological and immunohistochemical methods.

The bacteria isolated from moribund fish samples were identified as *V. anguillarum* due to the fact that 15 isolates isolated from diseased trout formed yellow-colored colonies in the TCBS media, exhibited a fermentative characteristic and were sensitive to O/129 vibriostat test. Moreover, their physiological and biochemical characteristics were similar to the characteristics reported by other researchers (1,15,16). Our clinical findings about moribund rainbow trout are also similar to the clinical findings reported in fish infected with *V. anguillarum* (1,5,15).

As indicated by different researchers conducting studies on rainbow trout infected with *V. anguillarum*, hyperemia observed in the liver and degeneration of the epithelium of kidney tubules were observed in this study (5). Unlike this research, melanomacrophage activity and hemosiderosis in the center of the melanomacrophages were not observed in the present study.

Scientists working on fish health have determined the effect of *V. anguillarum* bacterium on the tissues of different fish species using mostly peroxidase-antiperoxidase and avidine-biotin-alkaline phosphatase methods among immunohistochemical methods (17,18). The streptavidin-biotin technique used in this study was used by Planas et al. (19) in the

treatment of the disease caused by *Roseobacter* sp. with a probiotic characteristic in the larvae of turbot (*Scophthalmus maximus* L.). The Strep-ABC method is known to be around 5 to 10 times more sensitive than the avidin-biotin complex method when compared to the peroxidase-antiperoxidase and avidin-biotin-peroxidase complex methods (20). Thus, in contrast to other research, an immunochemical method, streptavidin-biotin staining was used for the determination of the presence and identification of the fish pathogen *V. anguillarum* in the moribund rainbow trout samples with this study.

In spite of the fact that the observation of immunopositive reactions in the liver tissues of all infected rainbow trout in sinusoids and necrotic areas in this study is similar to the findings obtained by other researchers in moribund rainbow trout and turbot infected with *V. anguillarum*, widespread immunopositive reaction was observed in liver tissues, especially in the cells around the artery (19,21). Furthermore, it was noted that immunopositive reactions in the tissues of trout infected with *V. anguillarum* was intense only in kidney tubules (19), similar to the results of previous studies, and that melanomacrophage centers received this stain as indicated by Mutoloki et al. (22). Immunopositive reactions in the examined spleen tissue sections was determined in sinus and necrotic areas, similar to the result of the study conducted by Avcı et al. (17). However, immunopositive reactions were also found in areas near the vein in spleen tissues, in contrast to previous studies.

In this study, the presence of immunopositive reactions in cells near the veins in the spleen and liver tissues is an indication that, as in the turbot, these bacteria are transported from the lamina propria of the intestine to the liver and other viscera by the blood (21), it may be similar in the rainbow trout. However, the intensive presence of the factor in the gill cells confirms that *V. anguillarum* infects the fish not only through the intestinal tract but also through the gills, as indicated by Laurencin and Germon (21).

As a conclusion, in this study, *V. anguillarum* was isolated from and identified in vibriosis-suspected moribund rainbow trout obtained from different fish farms. By using the streptavidin-biotin staining method, which is among immunohistochemical methods, the presence of this pathogen in the moribund rainbow trout was determined for the first time. It was found out that the factor concentrates in gills, especially in the area near the blood vessels, as well as in the liver, spleen, kidney, and heart, depending on the development of the disease. Due to the high immunosensitivity of this method, it was observed that this method can be used in the diagnosis of the bacterium and in the determination of the entry routes of the pathogen in infected tissues. This method provided a species-specific identification of the agent in a shorter time when compared with the routine microbiological methods. Thus, it is thought that this method can be used in fish-health laboratories as a routine method.

## Acknowledgement

Istanbul University Research Projects Fund supported this study (Project numbers: 19534 and 41630).

**Conflict of Interest:** The authors have no conflict of interest to declare.

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# The Combination Effect of Ferulic Acid and Gemcitabine on Expression of Genes Related Apoptosis and Metastasis in PC-3 Prostate Cancer Cells

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**Please cite this article as:** Eroglu C, Avci E, Secme M, Dodurga Y, Vural H, Kurar E. The Combination Effect of Ferulic Acid and Gemcitabine on Expression of Genes Related Apoptosis and Metastasis in PC-3 Prostate Cancer Cells. Eur J Biol 2018; 77(1): 32-7.

## ABSTRACT

**Objective:** Prostate cancer is the second most common cause of cancer-related deaths in men. Nowadays, new treatment approaches have been tested for cancer therapy including natural compounds with low toxicity. Ferulic acid (FA) is known as an abundant phenolic compound found in various fruits and vegetables. As a potent antioxidant, the anticarcinogenic effect of FA has been demonstrated in various cancer cell lines. The objective of this study was to investigate the combined effect of FA and gemcitabine on apoptosis and metastasis in PC-3 human prostate cancer cell lines.

**Materials and Methods:** Cell viability was determined using the XTT method after the cells were treated with gemcitabine or FA and gemcitabine. According to the results of cytotoxicity assays, PC-3 cells were treated with <math>IC\_{50}</math> doses of combination (200  $\mu$ M FA and 35  $\mu$ M gemcitabine) and  $IC_{50}$  dose of gemcitabine. Expressions of genes that are important in apoptosis and metastasis pathways were evaluated in dose and control groups by qPCR.

**Results:** According to the results, the combination of FA and gemcitabine affected the expression of more genes in apoptosis and metastasis with a higher fold change compared with the single treatment of gemcitabine in PC-3 human prostate cancer cell lines.

**Conclusion:** Our study indicates that FA can be an effective part of the combination treatments.

**Keywords:** Ferulic acid, gemcitabine, PC-3 cell line

## INTRODUCTION

Prostate cancer is one of the most prevalent types of cancer in men with the highest mortality rate together with lung and colorectal cancer, especially in industrialized countries (1). When prostate cancer is diagnosed at an early stage, stage 1 and 2, the 5-year survival rate can reach 90%, while the likelihood of successful treatment at advanced stages such as stage 3 and 4 is low (2). Digital rectal examination (DRE) and measurements of prostate-specific antigen (PSA) level are often used for the diagnosis of early-stage prostate cancer, but these methods are inadequate because of low specificity (3). Androgen is one of the most important risk factors for the development of prostate cancer, so androgen deprivation therapy (ADT) is widely used as the main treatment method as well as surgery and radiation therapy (4,5). When patients don't respond to the

ADT for a long time, chemotherapeutic approaches are applied in treatment (6). Unfortunately, resistance to treatments is eventually seen in most of the patients, and, for this reason, new therapeutic strategies are needed for the treatment of prostate cancer.

It is known that more than 60% of anticancer drugs currently used are derived from natural sources (7). Therefore, the development of more effective chemotherapeutic agents from natural products and the investigation of the abilities of inducing apoptosis or cancer prevention by different mechanisms are the most important focus points in cancer studies. The findings of research illustrated that various natural products had protective effects and could inhibit carcinogenesis by regulating the expressions of genes in apoptosis, invasion, angiogenesis and metastasis pathways (8).



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Received: 28.05.2018

Accepted: 04.06.2018

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Ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid), is a caffeic acid derivative found in vegetables, fruit, some beverages (for example, coffee and beer) and is an effective component of some Chinese medicinal herbs, for instance, *Angelica sinensis*, *Cimicifuga racemosa* and *Ligusticum chuangxiang* (9). It has been reported that FA has various pharmacological effects including antioxidant, antimicrobial, antiinflammatory, antithrombotic and antihypercholesterolemic effects (10-14). Moreover, the anticancer effect of FA has been demonstrated through studies involving various cancer cell lines and its anticancer activity is attributed to an antioxidant property which is associated with its phenolic nucleus and unsaturated side chain (15). It has been shown that FA has cytotoxic effects on cancer cells and affects important processes such as apoptosis, cell cycle and metastasis in the studies conducted with human breast cancer (MCF-7), human pancreatic cancer, human prostate cancer (PC-3 and LNCAP), human lung cancer (H1299) and human osteosarcoma (143B and MG63) (16-20). However, little is known about its effectiveness as a part of the combination therapies in cancer treatment (21).

In studies involving the investigation of anticancer properties of natural products, demonstration of their apoptotic and anti-metastatic effects is a priority. The apoptosis process, which is an important part of the mechanism that regulates the death or survival of the cell, is controlled by various signaling pathways (22). The apoptosis pathway is divided into three categories (i) the pathway of death receptor or extrinsic induced by death receptors, (ii) the intrinsic or mitochondrial pathway, and (iii) the perforin/granzyme pathway which is induced by granzyme A and granzyme B from cytotoxic T cells (23). It is important that chemotherapeutic agents have inhibiting capabilities to prevent invasion and metastasis in prostate cancer cells in addition to inducing apoptosis because bone metastasis occurs in 80% of advanced prostate cancer patients and is one of the main causes of prostate cancer related deaths (24,25). Tumor metastasis is a multistep process that includes the development of new blood vessels, detachment of metastatic cells from the primary tumor, invasion to stroma, intravasation to the blood and lymphatic vessels and extravasation to the target organ and growth of secondary tumor (26). All these steps are mediated by different factors and, especially, molecules involved in cell-cell and cell-matrix interaction, and the proteases responsible for the degradation of extracellular matrix components which are the most important participants in this process (27).

Gemcitabine is a deoxycytidine analog that exhibits anticancer activity against various solid tumors such as pancreatic cancer, lung cancer and prostate cancer (28,29). It is also thought that gemcitabine can be part of combination therapies based on the phase studies involving combinations with various chemotherapeutic agents in prostate cancer (30,31). The aim of this study is to investigate the combined effect of FA and gemcitabine on apoptosis and metastasis by evaluating the expression levels of genes important in apoptosis and metastasis in PC-3 prostate cancer cell lines.

## MATERIAL AND METHODS

### Cell Culture

A PC-3 (ATCC® CRL-1435™) human prostate cancer cell line, obtained from the ATCC (Manassas, VA, USA), was cultured in RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. FA and gemcitabine were purchased from Sigma-Aldrich Chemical Company (USA).

### Cytotoxicity Assay

The cytotoxic effects of gemcitabine and its combination with FA on PC-3 cells were determined by XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide) assay. The cells (1×10<sup>3</sup> cells/well) were seeded into 96-well plates and incubated for 24 h. The cells were treated with 0-70 μM gemcitabine for 48 h after the incubation. And then XTT solution was added to each well and incubated at 37°C for 4 h. The absorbance was read at 450 nm (reference wavelength 630 nm) in a microplate reader. The concentration of gemcitabine which inhibited 50% of cell viability (IC<sub>50</sub>) was determined. The combination doses were detected according to IC<sub>50</sub> doses of gemcitabine and FA. IC<sub>50</sub> dose of FA was previously indicated by Eroglu et al. (18). Then cells were treated with gemcitabine and FA at various concentration (0-150 μM doses and 0-900 μM doses, respectively). According to the combined effect of gemcitabine and FA on cell viability, two groups were formed for subsequent experiments. Therefore, PC-3 cells were treated with 200 μM FA and 35 μM gemcitabine (<IC<sub>50</sub> doses), and 50 μM gemcitabine (IC<sub>50</sub> dose) for 48 h.

### RNA Isolation and qPCR Analysis

Total RNAs were extracted from PC-3 cells using TRIzol Reagent and reverse transcription was performed using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

The primer sequences of target and reference genes were designed using IDT PrimerQuest (<https://eu.idtdna.com/site>). Primers used in the qPCR reaction are presented in Table 1. The effects of FA and, gemcitabine on apoptosis and metastasis were evaluated using qPCR (Biorad CFX Connect). Each qPCR mix was set up in 20 μl final volume containing 10 μl 2X SYBR Green Master Mix, 5 pMol of each primer and 1 μl cDNA. The following PCR profile was used: denaturation at 95°C for 10 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

### Statistical Analysis

The gene expressions analysis of the groups was determined by using the 2<sup>-ΔΔCT</sup> method. The volcano plot analysis, from RT<sup>2</sup> Profiles™ PCR Array Data Analysis, which is assessed statistically using Student's t test, was used in the comparison of the groups.

## RESULTS

### Anti-proliferative Effects of Gemcitabine and Combination of Gemcitabine and FA in PC-3 Cells

The cytotoxic effects of gemcitabine and combination of gemcitabine and FA were determined using the XTT assay. Gemcitabine inhibited the cell viability of PC-3 cells in a dose depen-

**Table 1.** Primers sequences used qPCR analysis

Gene	Primer sequence
GAPDH	F:5-TGAACGGGAAGCTCACTGG-3
	R:5-TCCACCACCTGTTGCTGTA-3
CASP3	F:5-GAGCCATGGTGAAGAAGGAATA-3
	R:5-TCAATGCCACAGTCCAGTTC-3
CASP7	F:5-CGAAACGGAACAGACAAAGATG-3
	R:5-TTAAGAGGATGCAGGCGAAG-3
CASP8	F:5-GCCCAAATTCACAGCATTAG-3
	R:5-GTGGTCCATGAGTTGGTAGATT-3
CASP9	F:5-CGACCTGACTGCCAAGAAA-3
	R:5-CATCCATCTGTGCCGTAGAC-3
BCL2	F:5- GTGGATGACTGAGTACCTGAAC-3
	R:5- GAGACAGCCAGGAGAAATCAA-3
BAX	F:5- GGAGCTGCAGAGGATGATTG-3
	R:5- GGCCTTGAGCACCAAGTTT-3
FAS	F:5- GTGATGAAGGACATGGCTTAGA-3
	R:5- GCCCAAATTCACAGCATTAG-3
CYCS	F:5- GGAGAGGATACTGATGGAGTA-3
	R:5- GTCTGCCCTTCTTCCTTCTT-3
TNF	F:5- CCTCTCTCTGCCATCAAG-3
	R:5- CCAGATAGATGGGCTCATACC-3
PPARG	F:5- TGGGTGAAACTCTGGGAGAT-3
	R:5- AAGTTGGTGGGCCAGAATG-3
MMP-2	F:5-TGGCAGTGCAATACCTGAA-3
	R:5-GCATGGTCTCGATGGTATTCT-3
MMP-9	F:5-GCAGACATCGTCATCCAGTT-3
	R:5-ACAACCTGTCATCGTCAAAT-3
TIMP-1	F:5-GCGTTATGAGATCAAGATGACCA-3
	R:5-AACTCCTCGCTGCGGTT-3
TIMP-2	F:5-GCTGCGAGTGCAAGATCA-3
	R:5-CTCTTGATGCAGGCGAAGAA-3
CDH1	F:5-GAGAGCGGTGGTCAAAGAG-3
	R:5-AGCTGGCTCAAGTCAAAGT-3
CDH2	F:5-GCTGACCAGCCTCCAAC-3
	R:5-CATGTGCCCTCAAATGAAACC-3
COL4A2	F:5-AAGTACAGCTTCTGGCTGAC-3
	R:5-AGCGGCTGATGTGTGTG-3
VEGFA	F:5-CGCAGACAGTGCTCCAG-3
	R:5-CACCCAAGACAGCAGAAAGT-3
HIF1A	F:5-ACCCTCTGATTAGCATGTAGAC-3
	R:5-TTACCCTGCAGTAGGTTTC-3

dent manner as shown in Figure 1. The IC<sub>50</sub> dose of gemcitabine was found to be 50 μM in the PC-3 cell line for 48 h and the IC<sub>50</sub> dose of FA have been determined as 300 μM in the PC-3 cell line for 48 h in a previous study (18).

For determining the cytotoxic effect of combinations, cells were treated with gemcitabine and FA simultaneously for 48 h at various concentrations. The combination of gemcitabine and FA inhibited the cell viability of PC-3 cells in a dose dependent manner as shown in Figure 2. Considering these results, combination doses used for subsequent experiments were determined as 35 μM gemcitabine and 200 μM FA. The PC-3 cells were treated to IC<sub>50</sub> doses of gemcitabine and combination <IC<sub>50</sub> doses of both for 48 h.

### Effects of Gemcitabine and FA on Expressions of Genes Associated with Apoptosis

The effects of gemcitabine and combination of FA and gemcitabine on expressions of genes are important in apoptosis including *CASP3*, *CASP7*, *CASP8*, *CASP9*, *BCL2*, *BAX*, *FAS*, *CYCS*, *TNF* and *PPARG*, and were determined using qPCR analysis, after total RNA isolation and cDNA synthesis from control and dose group cells.

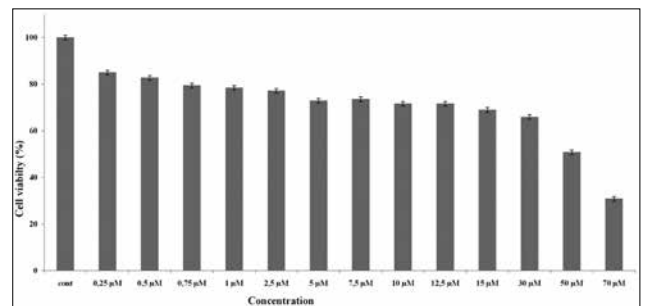


Figure 1. Effect of gemcitabine on the viability of PC-3 cells for 48 h. The cells were treated with gemcitabine with different concentrations for 48 h and cell proliferation was determined by XTT assay. The IC<sub>50</sub> dose of gemcitabine was found to be 50 μM for 48 h in the PC-3 cell line. The data is the average results of three independent experiments.

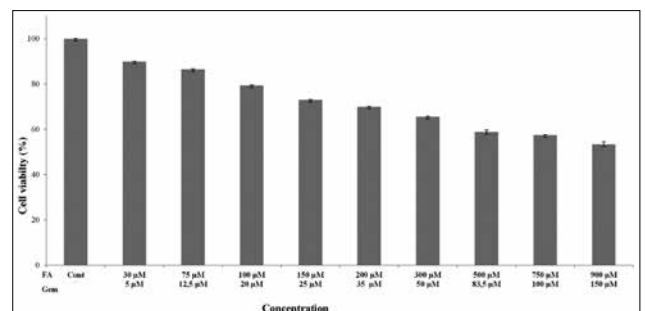


Figure 2. The combined effect of gemcitabine and FA on the viability in PC-3 cells for 48 h. The cells were treated with gemcitabine and FA with different concentrations for 48 h and the cytotoxic effect of combination doses was determined by XTT assay. The data is the average results of three independent experiments.



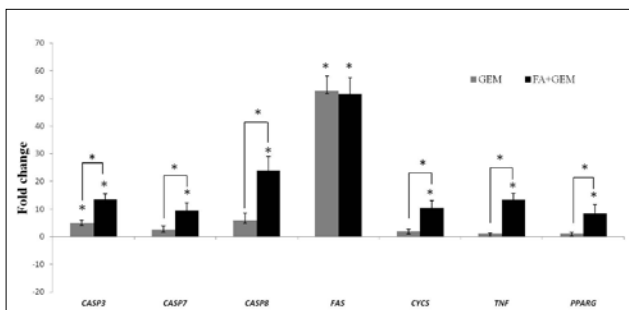


Figure 3. The effect of gemcitabine and FA on the expression of important genes in apoptosis for 48 h. Values represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ .

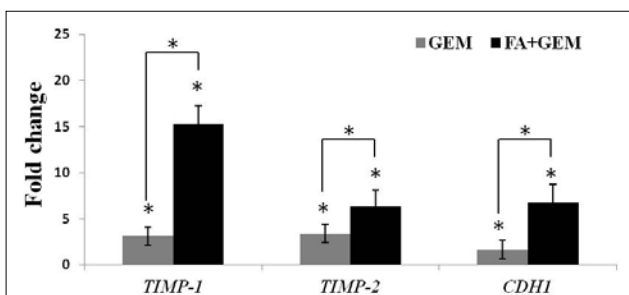


Figure 4. The effect of gemcitabine and FA on the expression of important genes in metastasis for 48 h. Values represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ .

According to the qPCR results, when the cells were treated with the  $IC_{50}$  dose of gemcitabine, the expression of *CASP3* (5.01 fold) and *FAS* (52.8 fold) genes were significantly upregulated. After the treatment with a combination of FA and gemcitabine, significant increases in the expression of *CASP3*, *CASP7*, *CASP8*, *FAS*, *CYCS*, *TNF* and *PPARG* were observed as 13.55, 9.48, 23.83, 51.63, 10.34, 17.86 and 14.63 folds, respectively compared with the control group ( $p < 0.05$ ). On the other hand, *CASP3*, *CASP7*, *CASP8*, *CYCS*, *TNF* and *PPARG* expressions were significantly elevated in combination treatment when compared with the gemcitabine treatment ( $p < 0.05$ ) (Figure 3).

#### Effects of Gemcitabine and FA on Expressions of Genes Associated with Metastasis

After the treatment with gemcitabine or combination of FA and gemcitabine, the expression levels of important genes associated with metastasis including *MMP-2*, *MMP-9*, *TIMP-1*, *TIMP-2*, *CDH1*, *CDH2*, *COL4A2*, *VEGF* and *HIF1A* were determined using qPCR analysis.

As shown in Figure 4, after the treatment with gemcitabine, the expressions of *TIMP-1* (3.13 fold), *TIMP-2* (3.41 fold) and *CDH1* (1.66 fold) genes were significantly elevated compared with the control group. On the other hand, a significant increase in the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes was observed when a combination of gemcitabine and FA was used as 14.24, 6.30 and 6.73 folds, respectively ( $p < 0.05$ ). Furthermore, significant changes in expressions of *TIMP-1*, *TIMP-2* and *CDH1* genes

were determined in combination treatment compared with the gemcitabine treatment ( $p < 0.05$ ). No significant change was observed in the other genes.

#### DISCUSSION

It is known that the consumption of vegetables, fruits, whole grains reduces the incidence of chronic diseases and various cancers especially stomach, esophagus, lung, oral cavity, pancreatic and colon (32,33). The anticancer activities of these foods are associated with the phenolic compounds which are secondary metabolites with a common aromatic ring possessing one or more hydroxyl group (34,35).

Various studies, including those on prostate cancer, have shown that phenolic compounds can induce apoptosis by affecting important signaling pathways or can inhibit invasion and metastasis. Furthermore, some of these compounds have been found to have a synergistic effect with chemotherapeutic agents used in standard therapy (36-38). According to these results, the investigation of the anticancer properties of natural compounds by cell culture and animal studies has become an important focus for the development of new therapeutic strategies against cancer.

FA, a natural phytochemical which is found in rice, wheat, barley, orange, coffee, apple and peanuts, was investigated for its possible synergistic effect with gemcitabine on prostate cancer cells in present study (39,40). Although there is only a limited number of studies investigating the synergistic effect of FA with various agents in cancer, previous literature illustrated that FA can be part of the combination therapies in various situations. For example, Pan et al. reported that FA and *Z-ligustilide* which is another major component of *Angelica sinensis*, have a synergistic effect on cold-induced vasospasm by regulating cold-sensing proteins TRPM8 and TPRA1 (41). Another study indicated that low doses of FA combined with a subthreshold dose of piperine, a bioavailability enhancer, have synergistic antidepressant-like effect on depression-like behaviors in mice (42). Canturk has shown that FA exhibits a synergistic anticandidal and apoptotic effects in combination with caspofungin against *C. albicans* (43). The potential protective effect of FA on splenic toxicity was investigated and it has been reported that its combination with ascorbic acid has a significant recuperative effect on aniline induced spleen toxicity in rats (44). The effectiveness of FA in combination treatments has also been demonstrated in studies with cancer cells. The combination of FA and  $\delta$ -tocotrienol ( $\delta$ -T3), another important component of rice bran, significantly reduces the proliferation of human prostate carcinoma, human breast adenocarcinoma and human pancreatic carcinoma cells as compared to single treatment (45). Likewise, in another study, the same researchers demonstrated that the combination of FA and  $\delta$ -T3 significantly decreases cellular telomerase activity in colorectal adenocarcinoma cells. Moreover, it was thought that FA increases the bioavailability of  $\delta$ -T3 (46). In a study conducted with breast cancer cells, it was shown that FA renders cancer cells more hypersensitive to ABT-888, poly

(ADP-ribose) polymerase (PARP) inhibitors, compared to stand alone treatment of ABT-888 (21).

In this study, to demonstrate the efficacy of FA in combination therapy, a combination dose was primarily determined considering the  $IC_{50}$  doses of FA and gemcitabine. The cytotoxic effect of gemcitabine in PC-3 cell lines was detected in a time- and dose-dependent manner using the XTT method. The  $IC_{50}$  dose of gemcitabine in PC-3 was found to be 50  $\mu$ M for 48 h.  $IC_{50}$  doses of FA have been determined as 300  $\mu$ M for the same period in a previous study. Then, combination doses were determined at lower concentrations to demonstrate the possible additive or synergistic effect of FA and gemcitabine in PC-3 cells. After the treatment of the prescribed doses to the cells, expressions of genes, which are important in apoptosis and metastasis, were analyzed in dose and control groups.

Apoptosis, described as a physiological process, plays an important role in the maintenance of hemostasis and the control of the cell proliferation in normal tissue. It is known that defects that occur in this mechanism cause cancer development (47). Molecules involved in this process are important for demonstrating the efficacy of newly developed agents. In the present study, the expressions of genes coding of molecules in both intrinsic and extrinsic pathways of apoptosis have been analyzed. According to the results, in the group treated with gemcitabine, the expressions of *CASP3* and *FAS* genes significantly increased. After the treatment with a combination of FA and gemcitabine, a significant increase was observed in the expressions of *CASP3*, *CASP7*, *CASP8*, *FAS*, *CYCS*, *TNF* and *PPARG* genes compared with the control group ( $p < 0.05$ ). In addition, the increases in the expressions of *CASP3*, *CASP7*, *CASP8*, *CYCS*, *TNF* and *PPARG* genes are significant compared with the gemcitabine treatment. According to the results, it can be concluded that the combination treatment affected expression of more genes in apoptosis compared with the single treatments in PC-3 human prostate cancer cell line.

Epithelial to mesenchymal transition (EMT), a biological process in which polarized epithelial cells undergo various biochemical changes resulting in increased cell migration, invasiveness and resistance to apoptosis, is the most important stage of metastasis (48). At this stage, there is a decrease in the expressions of various epithelial junction proteins such as E-cadherin,  $\alpha$ -catenin, and  $\gamma$ -catenin; while, there is an increase in nonepithelial cadherins such as N-cadherin (49). In addition, various enzyme groups, especially matrix metalloproteases (MMP), contribute to this process by destroying the extracellular matrix components (50). In this study, the anti-metastatic effect of FA and gemcitabine was investigated by analyzing the expression levels of gene encoding molecules that are important in the EMT process. According to the results, the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes significantly increased after the gemcitabine treatment compared with the control group. Likewise, after the treatment with a combination of FA and gemcitabine, a significant increase in the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes was found compared with the control group ( $p < 0.05$ ).

However, the increases in the expression of genes were higher than single treatments of gemcitabine. Moreover, the increases in the expressions of *TIMP-1*, *TIMP-2* and *CDH1* genes are significant compared with the gemcitabine treatment.

Chemotherapeutic agents are known to be toxic and have serious side effects. Natural products may exhibit a synergistic effect with the agents used in the treatment as well as reducing these side effects. In the present study, we demonstrated for the first time that a combination of FA with gemcitabine, an agent used in standard therapy, synergistically inhibited apoptosis and metastasis by regulating genes associated with these processes in prostate cancer cells. These results show that FA can promote efficacy of gemcitabine in prostate cancer cells and FA can be an effective part of the combination treatments.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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## Endemism in Istanbul Plants

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**Please cite this article as:** Tuncay HO, Akalin Urusak E. Endemism in Istanbul Plants. Eur J Biol 2018; 77(1): 38-41.

### ABSTRACT

**Objective:** Istanbul has exceptional plant diversity with 2500 species, (many of) which are under threat due to rapid urbanization. The aim of this study is to update the endemic plants lists of Istanbul to show how many of these endemic plants are only found in Istanbul, which might be helpful in preparing development plans.

**Materials and Methods:** A list of Istanbul's endemic plants is created according to "Flora of Turkey and East Aegean Islands" and related articles, books, herbarium records.

**Results:** Ten of the 60 endemic plant species of Turkey's flora are endemic only to Istanbul. Half of the remaining species are endemic to Istanbul and its surrounding areas (neighboring cities) and the other half have other distribution areas throughout Anatolia.

**Conclusion:** Not only the ten species endemic only to Istanbul, but also the other 50 species found in the area, should be conserved because of their high biological value stemming from their limited distributions.

**Keywords:** Istanbul, endemism, endemic, rare

### INTRODUCTION

Istanbul, the most populated city in Turkey, with a population of over 15 million in a provincial land area of 5461 km<sup>2</sup>, is the largest urban agglomeration in Europe. Its rapid urbanization, a 10 times increase in population in six decades, creates pressure on biodiversity, which is under threat worldwide due to global change (1,2). Istanbul has an exceptional plant diversity with 2500 species due to its diversity of soil, geographical position between two seas and two continents, climate, topography and land use, and its long history as a major city (3). There are seven important plant areas in Istanbul, which are natural areas with extraordinary richness in Flora and have been determined by international criteria that includes endemic, rare or threatened plants and rare habitats (Figure 1); Terkos-Kasatura Coastline (Terkos-Kasatur Kıyıları), Ağaçlı Dunes (Ağaçlı Kumulları), Kilyos Dunes (Kilyos Kumulları), Western Istanbul Heathlands (Batı İstanbul Meraları), Northern Bosphorus (Kuzey Boğaziçi), Sahilköy-Şile Coastline (Sahilköy-Şile Kıyıları), Ömerli Basin (Ömerli Havzası) (4). The aim of this study is to update the current list of endemic plants in Istanbul, which

is necessary to prepare conservation plan and increase awareness among the general public and policy makers because endemic plants, especially those with narrower distribution, should be conserved in their native lands.

### MATERIALS AND METHODS

Firstly, a list of Istanbul's endemic plants is created according to "Flora of Turkey and East Aegean Islands" and related articles, books (5-9). The list is then compared with previously created lists (3,10-12). The Herbarium of the Faculty of Pharmacy of Istanbul University (ISTE) records and literature are searched to determine the localities of endemic plants other than Istanbul. The plants collected from outside of Istanbul are identified so that a list of plants found only in Istanbul can be created.

Endemic plants of Istanbul are classified into three zones: The core zone represents the Istanbul Province, the second zone is composed of Istanbul and the surrounding areas (neighboring cities), and the third zone represents Turkey's endemic plants that are also found in Istanbul (Figure 2).



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**Received:** 21.02.2018 **Accepted:** 11.05.2018

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## RESULTS AND DISCUSSION

The ISTE herbarium is a rich resource for this study in terms of the number of species it contains. There are 10, 459 specimens of 1,988 species collected in Istanbul in the ISTE herbarium, which holds samples of about 80% of all species recorded in Istanbul.

Some species that have been mentioned in earlier literature as endemic only to Istanbul have since been found to be endemic to other areas of Turkey, in addition to Istanbul. These records and their sources are as follows:

*Allium rhodopeum* subsp. *turcicum*, *Cirsium polycephalum*, *Galanthus plicatus* subsp. *byzantinus*, *Lathyrus undulatus*, *Taraxacum aznavourii*, *Taraxacum pseudobrachyglossum*, *Isatis*

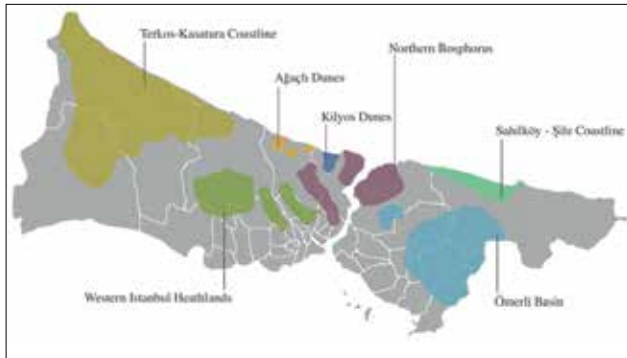


Figure 1. Seven important plant areas of Istanbul (3).

**Table 1.** Endemic plants only found in the Istanbul Province and the IUCN Red List categories of these plants

Plant Species	IUCN Red List Category
<i>Allium istanbulense</i> Özhatay, Koçyigit, Brullo & Salmeri	-
<i>Atriplex tatarica</i> L. var. <i>constantinopolitana</i> Aellen	Critically Endangered (CR)
<i>Cephalaria tuteliana</i> S.Kuş & Göktürk	-
<i>Colchicum micranthum</i> Boiss.	Endangered (EN)
<i>Crocus olivieri</i> Gay subsp. <i>istanbulensis</i> Mathew	Endangered (EN)
<i>Erysimum aznavourii</i> Polatschek	-
<i>Erysimum sorgerae</i> Polatschek	-
<i>Euphorbia belgradica</i> Forssk.*	-
<i>Polygonum istanbulicum</i> M.Keskin	-
<i>Trifolium pachycalyx</i> Zohary	Data Deficient (DD)

\*The existence of *Euphorbia belgradica* is doubtful.

*areneria*, *Linum tauricum* subsp. *bosphori*, *Thymus aznavouri*, *Onosma proponticum*, *Silene sangaria*, *Hypericum trachphyllum* (Source: ISTE Herbarium).

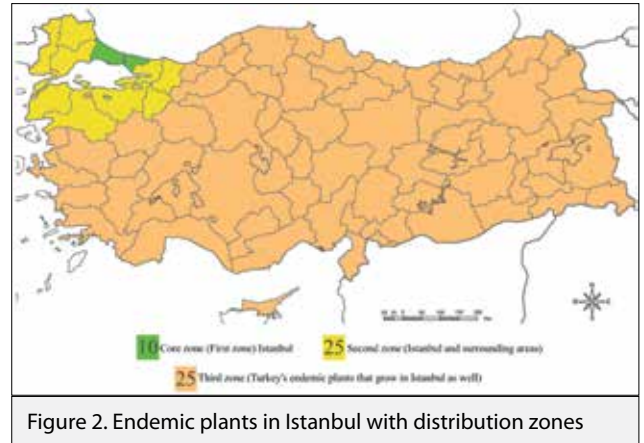


Figure 2. Endemic plants in Istanbul with distribution zones

**Table 2.** Endemic plants recorded in Istanbul and surrounding areas (neighboring cities)

<i>Allium rhodopeum</i> Velen. subsp. <i>turcicum</i> Brullo, Guglielmo & Terrasi	<i>Jurinea kilaea</i> Azn.
<i>Ballota nigra</i> L. subsp. <i>anatolica</i> P. H. Davis	<i>Lamium purpureum</i> L. var. <i>aznavourii</i> Gand. Ex Aznav.
<i>Bupleurum pendikum</i> Snogerup	<i>Lathyrus undulatus</i> Boiss.
<i>Centaurea hermannii</i> F. Hermann	<i>Linum tauricum</i> Willd. subsp. <i>bosphori</i> Davis
<i>Centaurea kilaea</i> Boiss.	<i>Onosma proponticum</i> Aznav.
<i>Cirsium byzantinum</i> Steud.	<i>Ophrys sphegodes</i> subsp. <i>catalcana</i> Kreutz
<i>Crocus pestalozzae</i> Boiss.	<i>Silene sangaria</i> Coode & Cullen
<i>Dianthus cibrarius</i> Clem.	<i>Symphytum pseudobulbosum</i> Aznav.
<i>Erysimum degenianum</i> Aznav.	<i>Taraxacum aznavourii</i> Van Soest
<i>Euphorbia amygdaloides</i> subsp. <i>robbiae</i> (Turill) Stace	<i>Taraxacum pseudobrachyglossum</i> Van Soest
<i>Galanthus plicatus</i> Bieb. subsp. <i>byzantinus</i> (Baker) D. A. Webb	<i>Thymus aznavouri</i> Velen.
<i>Hieracium noeanum</i> Zahn.	<i>Verbascum degenii</i> Hal.
<i>Hypericum aviculariifolium</i> subsp. <i>byzantinum</i> (Azn.) N.Robson	



**Table 3.** Endemic plants found in Istanbul and Anatolia

<i>Allium peroninianum</i> Aznav.	<i>Onopordum anatolicum</i> (Boiss.) Boiss. & Heldr. ex Eig
<i>Anthemis aciphylla</i> Boiss. var. <i>discoidea</i> Boiss.	<i>Onosma bornmuelleri</i> Hausskn.
<i>Asperula littoralis</i> SM.	<i>Onosma bracteosum</i> Hausskn. Et Bornm.
<i>Astragalus vulnerariae</i> DC.	<i>Pilosella hoppeana</i> (Schultes) C. H. & F.W. Schultz subsp. <i>lydia</i> (Bornm. & Zahn.) Sell & West
<i>Bellevalia clusiana</i> Griseb.	<i>Scrophularia cryptophila</i> Boiss. & Heldr.
<i>Campanula lyrata</i> Lam. subsp. <i>lyrata</i> Lam.	<i>Senecio castagneanus</i> DC.
<i>Carduus nutans</i> L. subsp. <i>falcato-incurvus</i> P. H. Davis	<i>Taraxacum turcicum</i> Van Soest
<i>Carduus nutans</i> L. subsp. <i>trojanus</i> P. H. Davis	<i>Trifolium apertum</i> Bobrov var. <i>kilaeum</i> Zoh. & Lern.
<i>Centaurea consanguinea</i> DC.	<i>Trifolium pannonicum</i> Jacq. subsp. <i>elongatom</i> (Willd.) Zoh.
<i>Ferulago thirkeana</i> Boiss.	<i>Tripleurospermum conoclinium</i> (Boiss. & Bal.) Hayek
<i>Isatis arenaria</i> Azn.	<i>Verbascum bithynicum</i> Boiss.
<i>Knautia byzantina</i> Fritsch	<i>Vincetoxicum fuscatum</i> (Hornem.) Reichb. <i>FIL.</i> subsp. <i>boissieri</i> (Kusn.) Browicz
<i>Knautia degenii</i> Borbas Ex Formanek	



Figure 4. *Allium istanbulense* Özhatay, Koçyigit, Brullo & Salmeri. Photo taken by T.Avcı.



Figure 5. *Crocus olivieri* GAY subsp. *istanbulensis* Mathew. Photo taken by S.Yüzbaşıoğlu.



Figure 6. *Cephalaria tuteliana* S.Kuş & Gökürk. Photo taken by S.Yüzbaşıoğlu.

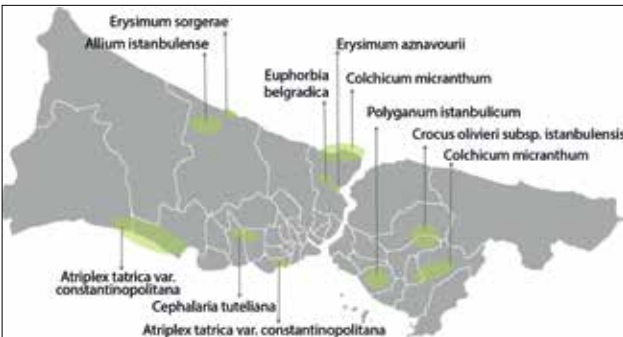


Figure 3. Distribution of Istanbul's endemic plants in Istanbul.

*Lamium purpureum* L. var. *aznavourii* (DUOF No:3185) from A3 Bolu (13) [Source: Düzce University Faculty of Forestry Herbarium (DUOF)].

*Bupleurum pendikum*, *Euphorbia amygdaloides* var. *robbiae* (5) (Source: Flora of Turkey and East Aegean Islands)

As a result of these findings, 60 plants endemic to Turkey are found in Istanbul. Only 10 of these plants are endemic only to Istanbul (Table 1) (Figure 3). Half of the remaining 50 plants are found only in Istanbul and its surrounding areas (neighboring cities) (Table 2) and the remaining 25 are more commonly found in Anatolia (Table 3). The International Union for Conser-

vation of Nature (IUCN) Red List categories of endemic plants in Istanbul are shown in Table 1(14).

There have been doubts about the existence of some plants which are endemic to Istanbul. According to Boissier, *Euphorbia belgradica* from A2(E) Istanbul (Belgrad forest) is likely assignable to either *E. platyphyllos* or *E. pubescens* Vahl, but it is not possible to be sure because of the imperfect material (15). There is information on the distribution of *Trifolium pachycalyx* outside of Istanbul. However, information on its existence in Izmir could not be verified. In addition, there is a recently discovered endemic plant species: *Allium istanbulense*, a new species of *Allium* section *Codonoprasum*, in areas surrounding Istanbul (European Turkey) (Figure 4) (16).

There are some species of plants endemic to Istanbul's that carry the epithet "Istanbul" or various names of Istanbul: *Allium istanbulense*, *Crocus olivieri* subsp. *istanbulensis* (Figure 5), *Polygonum istanbulicum*, *Atriplex tatarica* L. var. *constantinopolitana*, *Euphorbia belgradica*, *Bupleurum pendikum*, *Centaurea kilaea*, *Cirsium byzantinum*, *Galanthus plicatus* subsp. *byzantinus*, *Hypericum aviculariifolium* subsp. *byzantinum*, *Jurinea kilaea*, *Linum tauricum* subsp. *bosphori*, *Ophrys sphegodes* subsp. *catalcana*, *Knautia byzantina*, *Trifolium apertum* var. *kilaeum*. Some of Istanbul's plants are named after botanists' to honour them: Georges Vincent Aznavour, Betül Tutel, Friederike Sorger, Arpád von Degen (17). For instance, *Cephalaria tuteliana* is named after Prof. Dr. Betül TUTEL, the Faculty of Science Botany Institute at Istanbul University.

## CONCLUSION

This study shows that among the 60 Turkish endemic plants that are found in Istanbul, ten of them are endemic only to Istanbul. These 10 species are in danger because of their limited distribution area. Rapidly growing urbanization poses a risk to plants and many face the threat of extinction. New urbanization projects pose a particular threat to the Black Sea coast of European side of Istanbul and Çekmeköy on the Anatolian side (18). It is very important to identify endemic plants of Istanbul immediately and protect them from the harmful effects of urbanization. There is an ongoing conservation plan for *Cephalaria tuteliana* (Figure 6) by the Turkish Republic Ministry of Forestry and Water Affairs. The required work for the project has been completed and the report is now in its preparation stage. In addition, the IUCN Red List categories of endemic plants in Istanbul are shown in Table 1. However, the IUCN Red List categories of these plants need to be updated.

**Acknowledgements:** Thanks to Prof. Dr. Neriman Özhatay for her contributions. Thanks to Dr. Sırrı Yüzbaşıoğlu and Tamer Avcı for plants photos.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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# First Report of *Lernaea cyprinacea* (Copepoda: Lernaeidae) on Rainbow Trout (*Oncorhynchus mykiss*) from the Sari Mehmet Dam Lake in Van-Turkey

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**Please cite this article as:** Urku C, Onalan S. First Report of *Lernaea cyprinacea* (Copepoda: Lernaeidae) on Rainbow Trout (*Oncorhynchus mykiss*) from the Sari Mehmet Dam Lake in Van-Turkey. Eur J Biol 2018; 77(1): 42-5.

## ABSTRACT

**Objective:** This report focuses on the diagnosis of rainbow trout mortality in the Sari Mehmet Dam Lake.

**Materials and Methods:** The ten rainbow trout (*Oncorhynchus mykiss*) (300 - 350 g) examined were infested with numerous copepod parasites which were attached to body muscles especially in the caudal and abdominal area.

**Results:** According to morphological results, this parasite was identified as *L. cyprinacea*. Histopathological examination of lesions revealed inflammatory response in the dermis and skeletal muscles and infectious granuloma.

**Conclusion:** This paper is the first to describe cases of lernaecosis on the rainbow trout (*O. mykiss*) from the Sari Mehmet Dam Lake in Van-Turkey.

**Keywords:** Anchor worm, *Lernaea cyprinacea*, Rainbow trout, histopathology

## INTRODUCTION

*Lernaea cyprinacea*, is a common parasite and appears almost all over the world in freshwater fish (1-3), and has been isolated from 46 species of *Cyprinidae*. *Lernaea* (*Cyclopoida*: *Lernaeidae*), or anchor worms, cause lernaecosis and are parasitic copepod found on the skin and gills of freshwater fish (3,4). There are various *Lernaea* species but the most common species, *Lernaea cyprinacea* is known as an opportunistic ectoparasite of fresh water fish species, especially crucian carp (*Carassius carassius*) and occasionally on tadpoles of amphibians (4-6). According to Grabda (7), the rainbow trout (*O. mykiss*) is very sensitive to *L. cyprinacea*. This parasite is known to parasitize more than 100 hosts (8). It is thought to have spread with cyprinid hosts (9). During a recent survey, *L. cyprinacea* was recorded on various native fish species (3,10,11).

The parasite attaches itself to fish with an anchor, feeding on fish blood and tissue debris in this way, and macroscopically, causing the loss of scales, oedema on the skin, haemorrhages and deep ulcers and, thus, the parasite forms routes of entry for other pathogen such as fungi and bacteria causing secondary infection

(8,12). This parasite can cause the mass death of infected fish but Abbas et al. (13) reported that young fish are more sensitive to infestation. Specifically, in salmonids, *L. cyprinacea* was observed in stocked rainbow trout (*O. mykiss*) (12) and *Salmo* spp. (8).

Histopathologically, significant pathological lesions were reported in the skin, gills and eyes but pathological lesions have been observed in the visceral organs of the small fish such as the liver and kidney (14). Generally, this parasite causes granulomatous reactions, degeneration and necrosis in the skeletal muscle; inflammatory reactions in the subcutaneous tissue; hyperplasia and necrosis of gill epithelium; oedema and congestion and haemorrhages in the affected areas such as skeletal muscle and skin (11,12,14-16).

There are many studies about lernaecosis in Turkey (10,17-23). However, there is no report about lernaecosis on the rainbow trout from the Sari Mehmet Dam Lake-Van. The aim of the study was the diagnosis of *L. cyprinacea* infestation in rainbow trout and describes the histopathology of infected tissue such as skin, eyes, gills and other tissues.



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Received: 25.02.2018

Accepted: 29.05.2018

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## MATERIAL AND METHODS

### Fish Sampling and Parasitological Analysis

The fish samples were collected from the Sarı Mehmet Dam Lake in the province of Özalp / Van (38°48'15 "N and 43°48'37" E in October (Figure 1). Ten moribund rainbow trout (300-350 g) were examined micro- and macroscopically for the detection of the parasite. For this purpose, the parasites were carefully removed from the skin (24). Anchor worms were examined under a light microscope for diagnosis. The parasite was identified following the Bauer Method (25).

### Histopathology

All infected fish were subjected to necropsy and preserved in 10% buffered formalin solution for histopathological examination. Fixed tissue samples were embedded in paraffin, dehydrated, sectioned at 4-5 µm, stained with haematoxylin and eosin (H&E) and examined with a light microscope (26).

## RESULTS

### Clinical Findings

At the time of the fish sampling, the water temperature was measured as 23°C, pH 8.6. During sampling, ten rainbow trout were collected. All fish clearly showed haemorrhagic and ulcerative skin lesions (Figure 2a), loss of scales and also oedema (Figure 2b) especially on the attachment areas of the parasites. The parasites attached to infected fish with anchors were determined macroscopically on various parts of the body. The attachment area of the parasite was mainly in the caudal and abdominal regions.



Figure 1: Sampling area in the Sarı Mehmet Dam Lake-Van

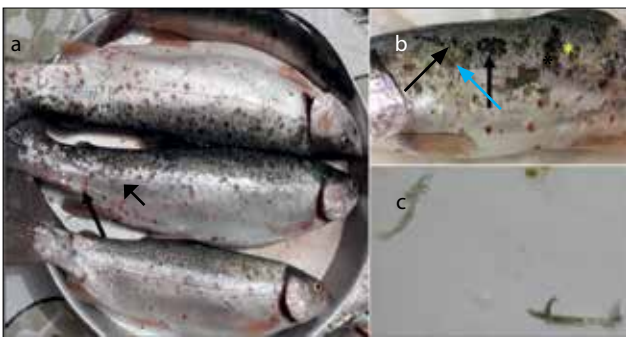


Figure 2. Rainbow trout infected with *L. cyprinacea*. Haemorrhages and ulcerative skin lesions on the attachment areas of the parasites (arrowed) (a), loss of scales (arrowed) and oedema (\*) (b), macroscopically two female parasites (c).

### Parasitological Findings

In the parasitological examination, a large number of female parasites were found macroscopically and microscopically, the parasite was identified as female *L. cyprinacea*, with two egg sacs and a length range of 15-17mm (Figure 2c).

### Histopathological Findings

Histopathologically, significant pathological lesions were reported in the skin, gills, eyes, and, most surprisingly, in the intestine. In the histopathologic examination of the tissues of rainbow trout infected with *L. cyprinacea*, hyperplasia, an increase in mucous cells in the distal end of primary lamella, vacuolization in secondary lamella, necrosis of gill epithelium, distortion in cartilaginous tissue (Figure 3a), haemorrhages and hyper-

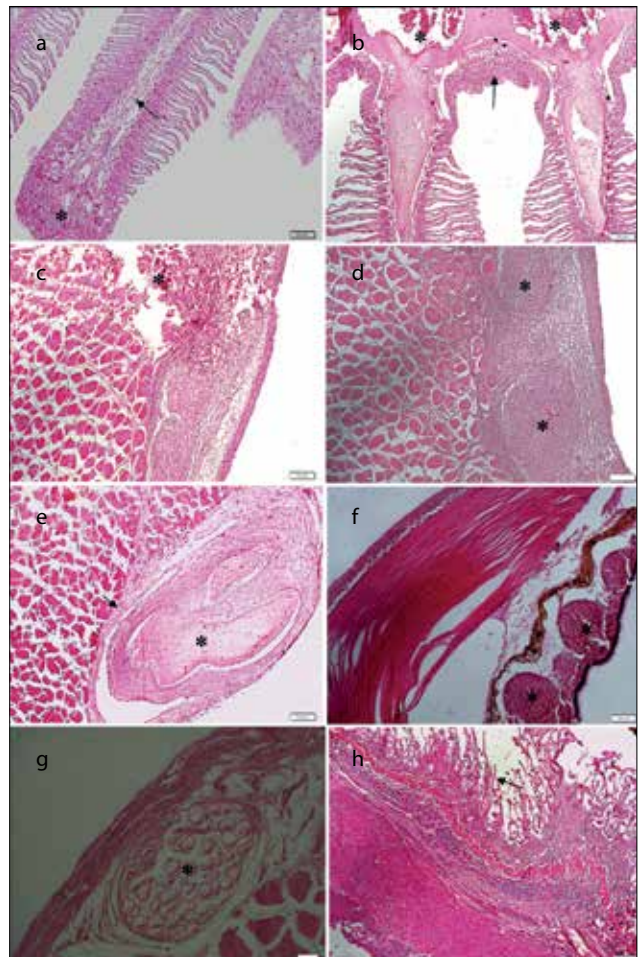


Figure 3. a-h. In the histopathologic examination of the tissues of rainbow trout infected with *L. cyprinacea*. Hyperplasia (\*), distortion in cartilaginous tissue (arrowed) (a); haemorrhages (\*) and hyperplasia at the base of the gills (arrowed) (b); degeneration and necrosis in the skeletal muscle, infiltration of inflammatory cells in the dermis (\*) (c); skeletal muscle granuloma like growths formed by collagen fibrils of dermis (\*) (d); parasitic sections (\*) surrounded by fibrous connective tissue (arrowed) in the necrotic muscle tissue (e); haemorrhages (\*) in the eyes (f); the cystic structure (\*) in the skeletal muscle (g); desquamation of mucosal epithelium in the intestine (arrowed) (h).

plasia at the base of the gills (Figure 3b); thickening of dermis, degeneration and necrosis in the skeletal muscle, infiltration of a massive number of inflammatory cells in the dermis and skeletal muscle (Figure 3c); granuloma like growths formed by collagen fibrils of dermis (Figure 3d) and also parasitic sections surrounded by fibrous connective tissue in the muscle tissue were observed (Figure 3e). Despite the fact that, macroscopically, *L. cyprinacea* was not detected in the eyes; haemorrhages and mild inflammatory infiltrations in the eyes were observed (Figure 3f). Surprisingly, in the skeletal muscle the cystic structure surrounded by fibrous connective tissue (Figure 3g) and desquamation of mucosa epithelium in the intestine was observed (Figure 3h).

## DISCUSSION

Sari Mehmet Dam Lake is the largest dam in the province covering an area of 1020 hectares and the pearl mullet is present (27). In recent years, cages have been placed on the dam reservoir for trout farming. For this reason, fish diseases in this region are important.

*L. cyprinacea* is an extremely harmful copepod ectoparasite found on the skin and gills of fresh water fish species and is known to be a causative agent of lernaecosis. This parasite is known as an opportunistic ectoparasite of crucian carp (6). In Turkey, many studies have been conducted on lernaecosis (10,17-22) but none were based in the Sari Mehmet Dam Lake/Van. Koyun and Atıcı (23) reported detection of *L. cyprinacea* on siraz fish (*Capoeta capoeta*) in Karasu Creek/Van. The study found that carp fish carry this parasite because the Karasu Creek feeds from the Sari Mehmet Dam Lake. As reported by Grabda (7) this study shows that rainbow trout is extremely sensitive to *L. cyprinacea*.

The gross pathology observed in our findings bears similarities to lernaecosis in various freshwater fish species (5,10,12,15,22). Especially, haemorrhagic and ulcerative skin lesions, loss of scales and oedema were similar to those in the findings of Berry et al. (12) in rainbow trout infected with *L. cyprinacea*.

The most significant factor in the spread of the disease is water temperature and water supply. From this point of view, the water source to which the event reported by Koyun and Atıcı (23) occurred is the water passage to the region where this work is carried out. Therefore, this parasitic infestation, which is more common in carp species, was transmitted by the water source and caused the same infestation in the trout. The water temperature measured during the collection of samples was calculated as 23 C°. The optimum water temperature for infestation is 24-25 C°.

Although mass mortalities have been reported in small fish, in this study mass deaths were detected because of the intense prevalence of rainbow trout (300-350 g) (13). Attachment was most commonly found in the caudal and abdominal regions. In this study, the parasite was not detected microscopically and histopathologically in the fish gills and head region. However,

studies carried out by other researchers have shown that parasites have also been found in the gills (11,12,23). It is worth noting that in this study, histopathologically, hyperplasia, an increase in mucous cells at the distal end of primary lamella, vacuolization in secondary lamella, necrosis of gill epithelium and distortion in cartilaginous tissue were observed.

Histopathologically, in this study significant pathological lesions were reported in the skin and skeletal muscle. Although Mirzaei (14) reported pathological lesions in the liver and kidney of infected fish, in this study desquamation of mucosa epithelium was observed in the intestine. This result may be due to secondary infection as reported by other researchers (8,12). Degeneration and necrosis in the skeletal muscle, infiltration of an extremely large number of inflammatory cells in the dermis and skeletal muscle, thickening of dermis, granuloma like growths formed by collagen fibrils of dermis and also parasitic sections surrounded by fibrous connective tissue in the muscle tissue were observed in the present study as in the other described reports (11,12,15,16). In addition to these findings, distinct cystic structures surrounded by fibrous connective tissue were observed in the skeletal muscle.

## CONCLUSION

In conclusion, our paper is the first to describe cases of lernaecosis on rainbow trout from the Sari Mehmet Dam Lake in Van-Turkey. Especially in parasitic infestations, prevention of disease is more important than treatment. For this reason, water temperature change times and stock intensity is of great significance as a precaution in aquaculture. In the case report, slow water flow and relatively high water temperature provide a suitable environment for *L. cyprinacea*. Exposure to these factors together with heavy parasitosis caused mass mortality on rainbow trout from the Sari Mehmet Dam Lake/Van.

**Conflict of Interest:** The authors have no conflict of interest to declare.

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## ERRATUM

**DOI: 10.26650/EURJBIOL.2018.12068**

In article by Morgil et al., entitled "Investigation of the Mechanism of Physiological Tolerance in Lentil (*Lens culinaris* Medik.) Cultivars under Drought Stress Conditions" (Eur J Biol 2017; 76(1): 31-5 DOI: 10.5152/EurJBiol.2017.1706) that was published in the June 2017 issue of European Journal of Biology, the Financial Disclosure information was not included due to an author error. This error has since been corrected;

**Financial Disclosure:** This work was supported by the Research Fund of Istanbul University (Project Number: 21207).