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INVESTIGATION OF ANTIMICROBIAL RESISTANCE OF *PHOTOBACTERIUM DAMSELAE SUB SP.PISCICIDA* STRAINS IN SEA BASS (*DICENTRARHUSLABRAX)* AND GILTHEAD SEA BREAM (*SPARUSAURATA*)

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ABSTRACT

The scope of this study is to establish the resistance of antimicrobial agents of *Photobacteriumdamselae* sub sp. *piscicida* strains isolated from 100 juvenile sea bass (*Dicentrarhuslabrax*) and 100 gilthead sea bream (*Sparusaurata*). A total of 21 *P. damselae*subsp.*piscicida* strains were used in this study. These strains were identified from 12 juvenile sea bass (*Dicentrarhuslabrax*) samples and 9 gilthead sea bream (*Sparusaurata*) samples. In this research, antimicrobial resistance was tested by the microdilution method. Oxytetracycline (30 µg), cloxacillin (5 µg), enrofloxacin (5 µg), florfenicol (30 µg), cefoperaz one (5 µg) and ampicillin (10 µg) were used in the study for antimicrobial susceptibility tests. In conclusion, it isrevealed that *P. damselae* sub sp. *piscicida* isolates were resistant to cloxacillin, cefoperazone and ampicillin.

INTRODUCTION

Photobacteriosis is one of the most threatening bacterial diseaseof aquaculture in Japan and the Mediterranean regions, causing severe losses in farmed fish with a high economic impact. The bacterial species *Photobacteriumdamselae* subsp. *piscicida* has been recognized as the causative agent since the first epizootic outbreak of 1963 in Chesapeake Bay (Snieszko *et al*., 1964). However, its taxonomic position remained controversial until DNA–DNA hybridization studies (Gauthier *et al*., 1995) provided evidence for its definitive reclassification in the genus *Photobacterium*, as *P. damselae*subsp.*piscicida* closely related to the subspecies *damselae*.

*P. damselae*subsp.*piscicida* is a facultative intracellular, halophile Gram-negative bacterium and is the etiological agent of pasteurellosisin the fish. This disease is a bacterial septicemia reported in a wide variety of marine fish, notably the yellowtail (*Seriolaquinqueradiata*) in Japan, gilthead seabream (*Sparusaurata*) and sea bass (*Dicentrarchuslabrax*) in Europe, striped bass (*Moronesaxatilis*) and white perch (*Moroneamericana*) in the USA and hybrid striped bass [*Moronesaxatilis* (*Moronechrysops*)] and cobia (*Rachycentroncanadum*) in Taiwan (Ho *et al*., 2011; Sano *et al*., 1998).

This bacterial species comprises strains that produce skin ulcers and haemorrhagic septicemia in a wide range of fish species, and, in addition, it may be a primary pathogen for mammals, including humans (Fouz *et al*., 1992; Shin *et al*.,1996).

Fish pasteurellosis is considered one of the most threatening diseases in world aquaculture due to high mortality, broad host range and ubiquitous distribution (Barnes *et al*., 2005). Effective prophylactic strategies based on vaccination programs exist for farmed fish such as gilthead seabream (*Sparusaurata*) (Magarin˜os *et al*., 1994), and sea bass (*Dicentrarchuslabrax*) (Bakopoulos *et al*., 2003). The vaccination strategies need to be optimized and for this reason, chemotherapy remains as a useful weapon against this pathogen. Evaluation of the susceptibility and resistance of the pathogen to antibacterial agents must be carried out to achieve successful treatment, and pharmacokinetic data in target fish species are also necessary for successful treatment together with challenge tests.

The aim of this study was to established to the resistance of antimicrobial agents of *P. damselae*subsp.*piscicida* strains

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isolated from juvenile sea bass (*Dicentrarhuslabrax*) and gilthead sea bream (*Sparusaurata*).

MATERIALS AND METHODS

A total of 100 juvenile sea bass (*Dicentrarhuslabrax*) and 100 gilthead sea bream (*Sparusaurata*) samples (weighing 50-200 g) indicating clinical symptoms of extensive hemorrhages in eyes, mouth and jawswere collected from various commercial fisheries in the Aegean Region of Turkey in the year of 2013.This research was carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS) of 1985 and in accordance with local laws and regulations. Samples taken from liver, spleen, kidney and heart were inoculated ontoblood agar [Blood Agar Base No 2 CM 271; Oxoid®, supplemented with 5% (v/v) sheep blood] and incubated at 20 ºC. All isolates were evaluated on their properties with regard to cell and colony morphology, odor, catalase and oxidase reaction, Gram staining properties, hemolysis, pigment production (Gauthier *et al*. 1995) and supplemented with biochemical tests using API 20E (bioMe´rieux) according to test reaction interpretations given by Austin and Austin (1999) and Grisez, Ceustersand Ollevier (1991). A total of 21 *P. damselae* subsp. *piscicida* strains were isolated in this study. These strains were identified from 12 juvenile sea bass (*Dicentrarhuslabrax*) samples and 9 gilthead sea bream (*Sparusaurata*) samples.

Antimicrobial sensitivity testing

Antimicrobial resistance was tested by the micro dilution method (Aldermanand Smith, 2001). The antibacterial agents were supplied by Sigma-Aldrich® (St. Louis, MO, USA), and two-fold dilutions of the stock solutions of the antibacterial agents were prepared in cation supplemented Mueller Hinton Broth (CSMHB). *P. damselae*subsp. *piscicida* strains were cultured on Mueller Hinton Agar plates and incubated at 22 ºC for 24-48 h. The inoculums prepared from cultures of the *P. damselae*subsp. *piscicida* strains streaked for single colonies on an agar plate. The inoculums standardized to have a concentration of $1x10^7$ CFU/ml. Dilution of the initial inoculum suspension was performed in caution supplemented Mueller Hinton Broth (CSMHB). Microdilution assays were examined in sterilized micro titer plates with 96 U-shaped wells containing 100 µl of CSMHB.

All antibiotics were prepared in volume of 100 µl of a twofold dilution series of an antibiotic solution in CSMHB with different concentrations of the antibacterial agents (ranging from 0.0625 to 256 mg/l) and added in the micro plates wells. The 5 µl bacterial inoculums containing $1x10^7$ CFU/ml bacteria were added in micro plates wells. The micro plates were incubated at 22 ºC for 24-48 h. After incubation, *P. damselae* subsp.*piscicida* strains end point titers were determined and recorded. These concentrations were reported as a MIC values at the *P. damselae*subsp.*piscicida* strains (Aldermanand Smith, 2001).

In this research, the antibiotics (Oxoid®) for the antimicrobial susceptibility tests areoxytetracycline (30 µg), cloxacillin (5 µg), enrofloxacin (5 µg), florfenicol (30 µg), cefoperazone (5 µg) and ampicillin (10 µg).

RESULTS

In this study, 21 (10.5%) *P. damselae* subsp.*piscicida* strains were identified from 200 samples. Of 21 *P. damselae* subsp. *piscicida* strains were identified 12 (57, 1%) juvenile sea bass (*Dicentrarhuslabrax*) samples and 9 (42,9%) gilthead sea bream (*Sparusaurata*) samples which were brought to our department in year of 2013. All strains were confirmed with API 20E (bioMe´rieux) and identified as *P. damselae* subsp.*piscicida.*

Antibiotic susceptibility tests of *P. damselae* subsp.*piscicida* strains were performed by micro dilution method. MIC interpretive standards (µg/ml) of antimicrobial agents used in this studyaregiven on Table 1. MIC ranges, MIC50, MIC90 and resistance rates are given in Table 2.

Table 1.MIC interpretive standarts (µg/ml)a ccording to CLSI (2011).

Antimicrobial Agent	MIC Interpretive Standarts (µg/ml)		
	S	I	R
Oxytetracycline	≤4	8	≥16
Cloxacillin	≤2	-	≥4
Enrofloxacin	≤0,5	-	≥4
Florfenicol	≤2	4	≥16
Cefoperazone	≤16	32	≥64
Ampicillin	≤8	16	≥32

Table 2 The MIC ranges, MIC50, MIC90 values and resistance rates for the *P. damselae* subsp. *piscicida* strains.

Bacterial Strains	Antimicrobial Agent	MIC Range (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Resistance (%)
P. damselae subsp. *piscicida* (n=21)	Oxytetracycline	0.0625-16	1	4	9,5
	Cloxacillin	1-256	16	64	**85.7**
	Enrofloxacin	0.625-16	0.5	2	9.5
	Florfenicol	0.625-16	0.25	8	14.2
	Cefoperazone	0.0625-64	2	64	**23.8**
	Ampicillin	0.5-64	4	32	**23.8**

According to the results, MIC50 value of*P. damselae* subsp. *piscicida* strains was found the least as level of0,25 µg/ml for florfenicoland the most as in the level of 16 µg/ml for cloxacillin. The results of MIC90 values indicate that the levels were 2 µg/ml for enrofloxacin and in 64 µg/ml forcloxacillin and cefoperazone.*P. damselae*subsp.*piscicida* strains were detected resistant to cloxacillin (85,7%), cefoperazone (23,8%) and ampicillin (23.8%), respectively.

DISCUSSION

The number of fish hatcheries is increasing globally. Turkey has great potential for fisheries industry in the basis of geographical location. Having access to an excellent quality aquatic resource improves fish farming hatchery operations. Hatching high numbers of fish together may sometimes cause outbreaks and economic losses.

Bacterial diseases are regarded as the most significant cause of economic losses in the aquaculture industry. Application of antibiotic treatment for bacterial diseases in fish hatcheries has been applied for many years. Various amounts of antibiotic residues may still be present in the effluent following antibiotic therapy. Numerous studies suggest a correlation between findings of increased bacterial resistance levels on and around

inland fisheries and the use of antimicrobial agents at the hatcheries (Kirkan *et al*., 2006). However, the occurrence of antibiotic resistance in pathogenic bacteria and the limited number of treatment options in certain countries are confining the availability of these substances (Uhlandand Higgins, 2006).

*P. damselae*subsp.*piscicida*, which may infect different species of fish and the causative agent of pasteurellosis, was formerly named as *Pasteurellapiscicida* and *Vibrio damsela*. It was first described in 1963, in wild populations of *Moroneamericanus* and *Moronesaxatilis* in Chesapeake Bay, USA. At the end of the 1960s and the beginning of the 1970s, the bacterium became economically important, causing severe problems for the first time: high mortalities were reported (Kusudaand Miura, 1972; Liu *et al*., 2003; Snieszko *et al*., 1964; Toranzo *et al*., 1991a; Toranzo *et al*., 1991b; Wang *et al*., 2007). Some fish species, such as *Cynoglossussemilaevis*, when infected by *P. damselae*subsp.*piscicida* exhibited hemorrhages of the basal fin and other organs, pale kidneys, and ulcerative lesions of the skin (Fouz *et al*., 1992; Wang *et al*., 2007), but many other species, such as cobia (Liu *et al*., 2003), yellowtail and gilthead bream (Romalde, 2002) show lesions similar to those of the golden pompano, with nodules scattered on the spleen and kidney.

*P. damselae*subsp.*piscicida* is a fish pathogen responsible for important losses in fish aquaculture worldwide. The importance of extracellular products, the presence of iron uptake mechanisms, enzymatic activities such as catalase and superoxide dismutase, and the capsular material as virulence factors in *P. damselae*subsp.*piscicida* are well documented (Arijo *et al*., 1998; Bakopoulos *et al*., 2004; Di´az-Rosales *et al*., 2006; Magarin˜os *et al*., 1992).

According to recent studies, the main virulence factors of *P. damselae*subsp.*piscicida* are extracellular protease, hemolysin and cytolysin (Liu *et al*., 2003; Romalde, 2002).

Effective prophylactic strategies based on vaccination schedules exist for some species such as gilthead seabream (*Sparusaurata*) (Magarin˜os *et al*., 1994), and sea bass (*Dicentrarchuslabrax*) (Bakopoulos *et al*., 2003). But, vaccination strategies need to be optimized and for this reason, chemotherapy remains as a useful weapon against this pathogen. Evaluation of the susceptibility and resistance of the pathogen to antibacterial agents should be carried out to achieve successful treatment, and pharmacokinetic data in target fish species are also necessary for successful treatment.

Based on the in vitro antimicrobial susceptibility tests, the *P. damselae*subsp. *piscicida*isolates could be distinguished from one another. In general, the Japanese isolates are resistant to a wider range of antibiotics than European isolates, which might reflect the effect of long-term use of the drugs for treatment of photobacteriosis (Bakopoulos *et al*., 1995). In Japan, a wide variety of antimicrobial agents are being used for the treatment of photobacteriosis (Sano, 1998). The application of chemotherapeutics has caused an increase in the incidence of drug-resistant bacteria, which have become a major problem in the treatment of bacterial diseases, such as photobacteriosis (AokiandTakahashi, 1987; Aoki, 1992). In the Mediterranean region, the range of antimicrobial compounds varies considerably from one country to another, depending on the legal requirements of each country (Gue´rin-Fauble´e *et al*., 1996). In Portugal and Spain, chloramphenicol, tetracyclines, ampicillin and nitrofurantoin are usually applied (Baptista *et al*., 1996; Toranzo *et al*., 1991a), while in Greece the disease is treated with quinolones (e.g. oxolinic acid and flumequine), potentiated sulphonamides (sulphadiazin/trimethoprim) and/or tetracyclines (Bakopoulos *et al*., 1995). The difference in availability of drugs reflects the differences in drug-resistant isolates between the Mediterranean countries. In general, the antibiotic sensitivity and resistance patterns of the European isolates are analogous to those previously reported (Bakopoulos *et al*., 1995; Balebona *et al*., 1992; Baptista *et al*., 1996; Toranzo *et al*., 1991a; Zorrilla*et al*., 1999). Both Greek and Italian strains were resistant to erythromycin and sensitive to most of the other antibiotics tested. The Spanish strains were resistant to erythromycin and sensitive to ampicillin and sulphomethoxazole-trimethoprime. In contrast with previous studies, resistance to a wide variety of chemotherapeutic agents, such as chloramphenicol, gentamycin, flumequine, oxolinic acid, oxytetracycline and tetracycline were detected (Balebona *et al*., 1992, Toranzo *et al*., 1991a; Zorrilla *et al*., 1999).

CONCLUSION

In this study, it is shown that MIC50 value found to be between 0.25-16 (mg/l) and MIC90 value were found between levels of 2-64 (mg/l). The resistance rates implied that *P. damselae*subsp. *piscicida* isolates were detected resistant to cloxacillin, cefoperazone and ampicillin, susceptible to enrofloxacin, florfenicoland oxytetracycline.

Our findings indicate that enrofloxacin and florfenicol have become effective for therapy of *P.damselae* subsp. *piscicida* in recent time. Besides, it is notable that *P.damselae* subsp. *piscicida* has got high incidence of resistant strains.

In conclusion, the findings show that *P. damselae* subsp. *piscicida* strains are able to develop resistance to the antimicrobial agents. This could imply that successful and efficient treatment with these antimicrobial agents could become more and more difficult. Therefore, the isolation of causative agent and referral of the therapy with regard to antimicrobial resistance tests are recommended.

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