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A study on the antimicrobial resistance of *Lactococcus garvieae*

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Abstract

The aim of this study is to determine resistance of *Lactococcus garvieae* isolated from sick rainbow trout (*Oncorhynchus mykiss*, W.) in farms in Antalya, the Mediterranean Region of Turkey to various antibiotics. Field samples studies started in July 2018 and finished in September 2018. Fish sampling studies were carried out in commercial trout farms in Kemer, Korkuteli, Serik and Manavgat districts in Antalya. Ninety fish were examined during the study period. The body weights of the fish ranged from 110 grams to 350 grams. For bacteriological studies, inoculations were prepared from the eye and internal organs of the moribund fish and inoculated onto BHIA. The inoculated petri dishes were incubated at 25 ± 2°C for 72 hours. After incubation period, morphology and color of the bacterial colonies were determined. A variety of morphological, physiological and biochemical tests were applied to the isolates. The PCR technique was used in confirmation of *L. garvieae*. Antibiotic resistance tests of the isolates were performed *in vitro* conditions. The Multiple Antibiotic Resistance (MAR) index values of *L. garvieae* isolates were also calculated. Similar clinical findings were detected in all the affected fish. Skin color darkening, unilateral and/or bilateral exophthalmia with periocular hemorrhages, abdominal dropsy, darkening of the spleen color and splenomegaly, hemorrhages in the internal organs and ascites were detected in fish. According to the phenotypic and PCR results, the isolates were identified as *Lactococcus garvieae*. It was determined all of the isolates were resistant against ampicillin, nalidixic acid, nitrofurantoin, oxalinic acid and sulfamethoxazole and were susceptible to amoxicillin, enrofloxacin, florfenicol and chloramphenicol. The MAR index values of the isolates ranged from 0.3 to 0.6.

Keywords: rainbow trout, Lactococcus garvieae, antimicrobial resistance, MAR

1. Introduction

Lactococcosis is an important bacterial infection that seriously affects the rainbow trout culture (Kurtoğlu and Korun, 2018). The disease was first described from a trout farm in Spain in 1988. Later, it was reported from South Africa, France, Israel, Greece, Iran, Korea, Taiwan and the USA (Timur *et al.*, 2011). The first coccal infection was detected in Turkey in 1997, and lactococcosis epizootics have been observed throughout the country since 1997 (Kurtoğlu and Korun, 2018). The development of the disease depends on the water temperature and the microbiological quality of the water; however, lactococcosis is often characterized as hyper acute haemorrhagic septicemia (Vendrell et al., 2006; Fukushima et al., 2017). Intense pathology of lactococcosis begins with rapid and general anorexia, melanosis, lethargy, loss of orientation and irregular swimming behaviour. External pathological sings of the disease are unilateral or bilateral exophthalmia, haemorrhages in the periorbital and intraocular parts of the eye, haemorrhages at the base of the fins and in the opercular region, distended abdomen, resulting from ascites and anal prolapses. Internally, infected fish show septicaemia and splenomegaly, necrosis in the spleen and kidney, bloody fluid in the intestine and can develop yellowish exudate on the brain surface (Vendrell *et al.*, 2006; Fukushima *et al.*, 2017). *L. garvieae* is a member of the genus *Lactococcus* in the Streptococcaceae family (Avsever *et al.*, 2014). The bacterium is Gram-positive, coccus shaped, facultative anaerobic and catalase negative (Rubiã and Mesquita, 2018). *L. garvieae* is a bacterial species which does not have host specificity. It is isolated from various fish species including rainbow trout, yellow tail, tilapia, mullet, catfish and japan eel fish. Among the cultured fish species, rainbow trout is the most susceptible to *L. garvieae*, while carp is the most resistant to this bacterial species (Vendrell *et al.*, 2006; *Aguado-Urda* et al., 2010). Although focusing on the use of antibiotics such as erythromycin and doxycycline in the treatment of lactococcosis in general (Brunt and Austin, 2005), amoxicillin, erythromycin, oxytetracycline and low dose of doxycycline have been as common antibiotics which are used in the treatment and control of lactococcosis in rainbow trout (Vendrell *et al.*, 2006).

The discovery and development of antimicrobial agents for the treatment of systemic bacterial infections is undoubtedly among the most exciting events in the history of microbiology (Rodgers, 2001). However, the increase in antibiotic resistance is becoming increasingly important in both human medicine and veterinary medicine. As a result, serious restrictions are imposed on the use of these agents (Rodgers,

2001; Soltani *et al*., 2009). Resistance to antibiotics in bacterial species develops either by exposure to non-lethal levels of antibiotics in the environment or directly by DNA transfer mechanisms. Pathogens which show resistance against antibiotics can cause infections that are difficult to control in humans and/or animals (Lekshimi *et al*., 2017). Similar strategies as in other animal production sectors are used in aquaculture such as vaccination and the use of antimicrobial agents in the control of bacterial diseases (Heuver *et al*., 2009). The most common usages of antimicrobial agents in aquaculture are to add them to feed and to treat fish by adding the agents directly to water (dipping method). However, antimicrobial agents can be used intensely as a result of these application methods and strong selective pressure can be seen not only in fish but also in aquatic environments exposed to these agents (Heuver *et al*., 2009). The Multiple antibiotic resistance (MAR) index is calculated by the ratio of the number of antibiotics that the organism tested is resistant to the total number of antibiotics tested. The MAR index shows the spread of bacterial resistance in population, and the calculated MAR index reveals the presence of environmental derived bacterial strains using several antibiotics if the MAR index is greater than 0.2 (Krumperman, 1985; Ehinmidu, 2003). For this reason, it has been tried to be determined by investigating the resistance and/or susceptible of *L. garvieae* strains isolated from diseased rainbow trout (*O. mykiss*) against various antibiotics and the multi antibiotic resistance (MAR) in the bacterial strains with the current study.

2. Materials and Methods

2.1. Sampling and isolation of bacteria

Between June and September 2018, samples of 90 moribund rainbow trout (110 g to 350 g) were taken from different commercial farms in Kemer ($36^0 36'3''$N-$30^0 34'21''$E), Korkuteli ($37^0 4'1''$N-$30^0 12'0''$E), Serik ($36^0 55'2''$N-$31^0 6'16''$E) and Manavgat ($36^0 46'59''$N-$31^0 25'59''$E) in Antalya, the Mediterranean Region (MR) of Turkey (Fig. 1) and the samples were examined for lactococcosis. Water temperature in fish ponds was measured as the highest 21 °C and the lowest 15 °C. Ten fish from each farm were selected and necropsies of fish were performed under sterile conditions at the farm. Samples of eye, liver, spleen and kidney from fish were inoculated onto Brain Heart Infusion Agar (Merck), for bacteriological analysis. All plates were incubated at 25 °C ± 2 °C for 72 h (Austin and Austin, 2012). The research approval was obtained from Akdeniz University Experimental Local Ethics Committee (Number: 2018.01.015).

2.2. Phenotypical characterization of isolates

After the incubation period at 25 °C ± 2 °C for 72 h, the morphology and color of the bacterial colonies on BHIA plates were examined and then subcultures of the isolates were made. A series of physiological and biochemical tests including cytochrome oxidase, catalase, O/F (Leifson) fermentation, H_2S production, growth at different temperature degrees, growth in different salt concentrations, methyl red, Voges-Proskauer (VP), indole production, gelatinase production, citrate utilization, starch hydrolysis, NO_2 production, ONPG (β-galactosidase) and hemolysis of sheep erythrocytes were used to determine the phenotypic properties of *L. garvieae* (Vendrell *et al*., 2006; Sharifiyazdi *et al*., 2010; Austin and Austin, 2012).

2.3. Molecular identification of L. garvieae isolates

2.3.1. DNA isolation

DNA isolation from *L. garvieae* strains was performed in accordance with the manufacturer's recommendations using commercial DNA isolation kit. DNA samples were stored at -20 °C until used in PCR studies (Temizkan and Arda, 2004).

2.3.2. Polymerase Chain Reaction (PCR)

PCR technique was used in the confirmation of *L. garvieae* strains isolated in the study. Each assay contained 20 µg primers, 10 µg PCR buffer, 1 µg taq, 32 µg ddH$_2$O and 5 µl DNA template within a 50 µl reaction. The further identification of the bacterium, the forward primer PLG-1 (5'-CATAACAATGACAATCGC-3') and reverse primer PLG-2 (5'-GCACCCTCGCGGGTTG-3') prepared by targeting the 16S rRNA gene sequence (Zlotkin *et al*., 1998) were synthesized and used. PCR reactions were carried out using a commercial kit and the PCR cycles were set according to Ravelo *et al*., (2003).

2.3.3. Agarose gel electrophoresis

PCR amplification products were run in 1% agarose gel for 45 minutes at 100 V. Later, the bands were visualized on the transilmulator under UV lamp.

2.4. Antibiotic susceptibility tests

Antibiotic susceptibility tests of *L. garvieae* strains were performed in two replicates according to disc agar diffusion technique *in vitro* conditions (CLSI, 2006). After the turbidity of the bacterial suspension was adjusted to McFarland no: 0.5 (1.5 x 10^8 CFU/mL), the suspension was spread onto the surface of petri dishes containing Mueller-Hinton (Condalab). The commercial antibiotic discs were placed on the surface of the medium. The petri dishes were incubated under aerobic conditions for 24 to 28 h at 28 ± 2 °C. Then, the diffusion zone diameters observed around the discs were measured at the end of the incubation period and the arithmetic averages were taken. The evaluation of the zone diameters was made according to NCCLS (2003), EUCAST (2011), CLSI (2016) and EUCAST (2019). The strains were determined as sensitive, intermediate resistance and resistance. Commercial antibiotic discs Bioanalyse in the study are given as follows: amoxicillin (25 µg), ampicillin (10 µg), bacitracin (0.04 µg), enrofloxacin (25 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg), furazolidone (15 µg), kanamycin (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), oxytetracycline (30 µg), sulfamethoxazole (25 µg), streptomycin (10 µg) and trimethoprim (5 µg).

2.5. Multi Antibiotic Resistance (MAR)

The MAR index of *L. garvieae* strains isolated from moribund fish in the farms was calculated according to the formula a/b. A represents the number of antibiotics that the isolated bacteria show resistance, while b represents the number of antibiotics that the isolate is exposed to (Krumperman, 1985).

3. Results

3.1. Clinical findings

3.1.1. Behavioral symptoms of the fish

Similar clinical symptoms were observed in fish affected by lactococcosis in all farms which were visited for

sampling. The affected fish appeared lethargy and were in appetent.

3.1.2. External clinical signs of the fish

All affected fish showed darkening of skin, unilateral and/or bilateral exophthalmia with periocular hemorrhages, pallor of the gills, opacification in one eye (Fig. 2) and loss of the eye in some cases, eroded fins and they also had abdominal dropsy and anal prolapsed.

3.1.3. Internal clinical signs of the fish

Internally, clinical signs consisted of splenomegaly and darkening of the spleen (Fig. 2), hemorrhages in the liver, muscle (Fig. 2), gonads, Internal clinical sings of the fish pyloric caeca, accumulation of ascetic fluid in the intestinal and peritoneal cavity and darkening of the kidney.

3.2. Phenotypic properties of L. garvieae strains

3.2.1. Morphological characterization of the strains

Eighty-two *L. garvieae* strains were isolated in the present study. After incubation period for 72 h on BHIA, bacterial colonies were white color, circular and convex (Fig. 4) when examined in a stereo microscope. The isolates of *L. garvieae* did not exhibit active or passive movement in the wet mount preparations. The bacterial cells were Gram-positive and oval-coccus shaped under the light microscope (x40) and the cells occurred in short chains and/or single.

3.2.2. Physiological characterization of the strains

All 82 isolates of *L. garvieae* were able to develop at 4 °C, 25 °C and 37 °C. When the salinity tolerances of the isolates were investigated, the isolates were able to develop at different salt concentrations including 0%, 2%, 4%, and 6.5% NaCl. The results are presented in Table 1.

3.2.3. Biochemical characterization of the strains

The all isolates were fermentative and negative for the cytochrome oxidase and catalase tests. They produced α (alpha)-hemolytic colonies on the blood agar. The isolates were methyl red (MR) positive but did not produce indole, H_2S, amylase, gelatinase and Voges-Proskauer (VP). All biochemical traits of the isolates are presented in Table 1.

3.3. Results of PCR study

Further identification of 82 strains identified as *L. garvieae* according to the bacteriological study results and isolated from all fish samples was done using by PCR technique. All the strains gave 1100 bp amplicons in the agarose gel (Fig. 5).

3.4. Antibiotic susceptibility tests results

According to the antimicrobial susceptibility test results of 82 *L. garvieae* strains isolated in the study, all strains (100%) were found to be resistant to ampicillin, nalidixic acid, nitrofurantoin, oxolinic acid and sulfamethoxazole. All strains (100%) were susceptible to amoxicillin, enrofloxacin, florfenicol and chloramphenicol. While 47 of the 82 strains (57.32%) were sensitive against bacitracin, 35 strains were found to show resistance against the same antibiotic. 49 of 82 strains (59.76%) were susceptible to erythromycin, the remaining 43 strains showed resistance. While 71 strains (86.59%) were resistant to flumequine, 11 strains were found to be sensitive. While 61 (74.39%) of 82 strains were sensitive to furazolidone, 21 strains were resistant, 36 strains were moderately resistant

to kanamycin, 41 strains were resistant and 7 strains (8.54%) were susceptible to the same antibiotic. 78 strains (95.12%) were sensitive to oxytetracycline, 4 strains showed resistance. Although 75 (91.46%) of 82 strains were resistant against to streptomycin, 7 strains were sensitive to the same antibiotic and 75 strains were resistant to trimethoprim while 7 strains (8.54%) were found to be resistant against this antibiotic. The reference ranges given in Table 2 were used to determine the antibiotic susceptibilities of the strains.

3.5. Multi Antibiotic Resistance (MAR) results

In the study, the MAR index values of *L. garvieae* strains isolated from moribund fish in trout farms in Kemer, Korkuteli, Serik and Manavgat were found to be 0.5, 0.3, 0.6 and 0.4, respectively. It was determined that the MAR index result was greater than 0.2 in all strains.

4. Discussion

4.1. Behavioral symptoms and clinical signs of the fish

Karami *et al*., (2019) isolated *L. garvieae* strains from sick rainbow trout samples in farms located in Kohkilooieh and Boyerahmad provinces in Iran. The researchers reported that they observed behavioral findings such as lethargy, lack of feeding, gathering in water inlets and outlets, irregular swimming in the affected fish. As clinical findings in these fish, bilateral exophthalmia and hemorrhages in the eyes, darkening in the skin color, skin ulcers in some fish, enlargement and color change in the spleen, hemorrhages in the liver and visceral fats were detected (Durmaz and Kılıçoğlu, 2015; Karami et al., 2019). Durmaz and Kılıçoğlu (2015) informed behavioral symptoms such as anorexia, lethargy and irregular swimming in the fish affected by lactococcosis in the trout farms in the Black Sea Region of Turkey. At necropsy, hemorrhages in the liver, spleen and kidneys, splenomegaly and accumulations of yellowish fluids in abdominal cavity were seen. The main clinically symptoms in this study were similar to the symptoms noticed in other works other parts of the world such as Brazil, Iran and in Turkey (Durmaz and Kılıçoğlu, 2015; Fukushima *et al*., 2017; Karami *et al*., 2019).

4.2. Phenotypical characterization of the L. garvieae isolates

Identification of *L. garvieae* may be done by a series of phenotypic diagnostic tests. However, some biochemical properties of the bacteria vary depending on the strain (Vendrell *et al*., 2006). Sharifiyazdi *et al.,* (2010) reported that *L. garvieae* strains isolated from 32 rainbow trout affected in Iran by lactococcosis were non-motile, Gram-positive, coccus-shaped, catalase and oxidase negative and caused alpha hemolysis in the blood agar. These 32 strains gave negative reactions for H_2S, VP and indole. Timur *et al*., (2011) isolated and identified *L. garvieae* from rainbow trout farms which located in the Marmara Region of Turkey. It was noted that the strains were non-motile, Gram-positive and formed short chains. The bacteria were cytochrome oxidase and catalase negative and produced alpha hemolytic colonies on the blood agar. The strains gave positive reaction for MR test. The citrate reaction of the strains was negative. The authors informed that the strains were able to growth at 6.5%NaCl. In the present study, 82 *L. garvieae* strains isolated and identified from moribund fish samples. It was determined that *L. garvieae* strains formed small whitish colonies on BHIA. They were Gram-positive, oval-cocci shaped, non-motile, fermentative, cytochrome oxidase and catalase negative. These findings

agree with those of Sharifiyazdi *et al.*, (2010) and Timur *et al.*, (2011).

4.3. Results of PCR identification studies

The PCR amplification of genomic DNA from *L. garvieae* isolates using the PLG-1 and PLG-2 primers from the region of the 16S rRNA defined by Zlotkin *et al.*, (1998) produced a single amplicon product with a fragment of 1100 bp in length. Haghighi Karsidani *et al.*, (2009) used these primers to identify cases of streptococcosis/ lactococcosis in culture rainbow trout in Iran. According to the results of the study, 37 (35.2%) of 108 Gram-positive cocci were identified as *L. garvieae*. The isolates produced 1100 bp amplification as a result of the PCR study.

4.4. Antibiotic susceptibility tests results

Lactococcosis is a major disease problem affecting rainbow trout culture in the Southern Europe countries such as Italy, Spain, France, Portugal, Greece and Turkey and in the Middle East (Savvidis *et al.*, 2007; Soltani *et al.*, 2008). Erythromycin, oxytetracycline and enrofloxacin are commonly used antibiotics in the treatment of sick fish in farms (Savvidis *et al.*, 2007). Despite the *in vitro* antimicrobial test results of *L. garvieae*, the use of erythromycin, oxytetracycline and enrofloxacin for therapeutic purposes in those farms is not satisfactory (Savvidis *et al.*, 2007). Kurtoğlu and Korun (2018) reported that *L. garvieae* strains isolated from sick fish in trout farms which were located in Fethiye, the Mediterranean Region of Turkey were susceptible to ampicillin, erythromycin, chloramphenicol, sulphametoxazole, oxytetracycline and trimethoprim. Karami *et al.*, (2019) stated that *L. garvieae* strains isolated from rainbow trout in Iran were resistant against tetracycline, streptomycin and enrofloxacin; however, tetracycline and enrofloxacin have been mostly used in fish farms in the country. Fukushima et al. (2017) stated that only oxytetracycline is allowed against *L. garvieae* in Brazil. However, the widespread use of antibiotics is not effective in the treatment of lactococcosis due to the development and spread of antibiotic resistance (Fukushima *et al.*, 2017; Karami *et al.*, 2019). In this study, the high MAR index values of *L. garvieae* strains isolated from sick rainbow trout supported the resistance status in the isolates as stated above.

The resistance and sensitive patterns of the *L. garvieae* isolates in the present study were mostly similar and all were resistant against ampicillin, nalidixic acid, nitrofurantoin, oxolinic acid and sulfametoxazole, whereas all of the isolates were sensitive to amoxicillin, enrofloxacin, florfenicol and chloramphenicol. All *L. garvieae* isolates were highly sensitive to furazolidone and oxytetracycline. Kurtoğlu and Korun (2018) reported that *L. garvieae* strains isolated from sick fish in the trout farms in Fethiye (the Mediterranean Region of Turkey, MR) contained plasmids and noticed that the strains containing plasmids showed resistance against bacitracin, flumequine, furazolidone, kanamycin, nalidixic acid and streptomycin. In this study, the *L. garvieae* strains isolated from sick fish in fish farms located in Antalya (MR) were completely resistant against ampicillin unlike *L. garvieae* strains isolated from sick fish in trout farms located in Fethiye (MR). Erythromycin was mostly used in the treatment of sick fish in farms where lactococcosis was detected. *In vitro* antibiotic test studies, 56.76% of the strains were found to be sensitive against erythromycin. This situation revealed that *L. garvieae* was sensitive against erythromycin both in in field and laboratory conditions; however, when these results were evaluated, it was concluded that as a result of intensive use of erythromycin for control of lactococcosis in the farms conditions, it might be caused by a decrease in susceptibility to this antibiotic in the *L. garvieae* strains. In conclusion, the use of erythromycin in the treatment of lactococcosis may be difficult in later times and for this reason, alternative protection and control methods are needed.

Acknowledgements

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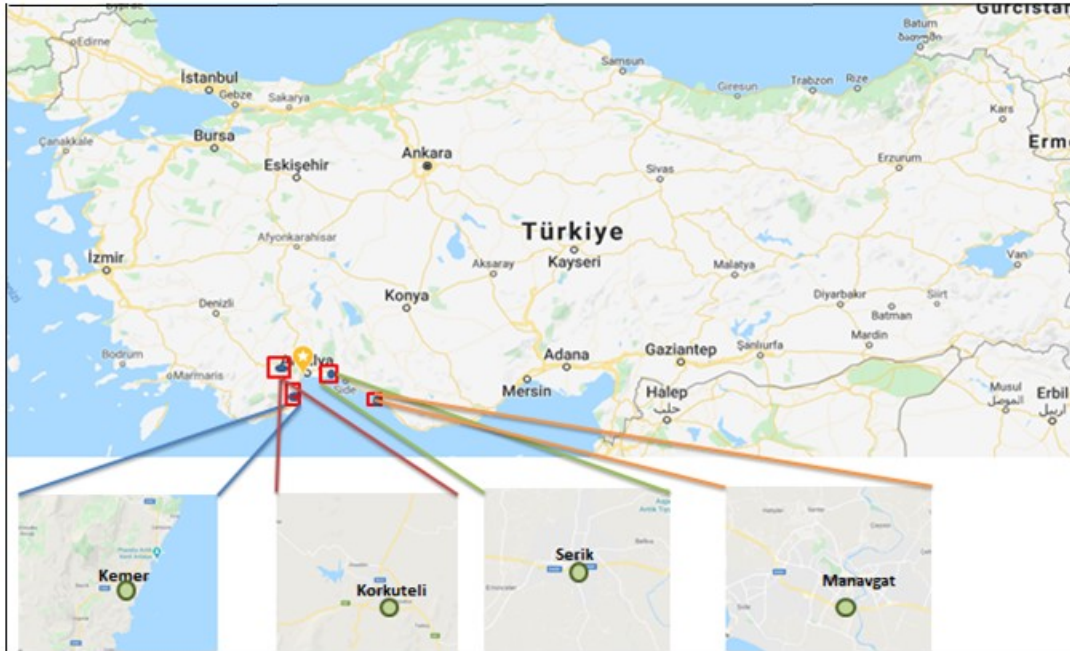
Captions for figures:

Fig. 1. Location of sampling areas in Antalya at the Mediterranean Region of Turkey (taken from google maps)

Fig. 2. Opacification in eye of the moribund fish affected by lactococcosis

Fig. 3. Hemorrhages in the muscle pyloric caeca, splenomegaly and darkening of the spleen in the moribund fish.

Fig. 4. Bacterial colonies of *L. garvieae* isolated from moribund fish

Table 1. Phenotypic characteristics of *L. garvieae* strains isolated from moribund rainbow trout in the study

Characteristics	Kemer (43 strains)	Korkuteli (9 strains)	Serik (10 strains)	Manavgat (20 strains)	*L. garvieae** (ATCC 43921)
Gram-stain	+	+	+	+	+
Motility	-	-	-	-	-
Catalase	-	-	-	-	-
Oxidase	-	-	-	-	-
O/F	F	F	F	F	F
H_2S	-	-	-	-	-
VP	-	-	-	-	+
MR	+	+	+	+	+
Hemolysis	α	α	α	α	α
Citrate	-	-	-	-	-
Indole	-	-	-	-	a
Gelatinase	-	-	-	-	-
Amylase	-	-	-	-	-
ONPG	-	-	-	-	-
McConkey	-	-	-	-	-
Growth in:					
0-2%NaCl	+	+	+	+	
4-6.5%NaCl	+	+	+	+	+
Growth at:					
4 °C	+	+	+	+	-
25 °C	+	+	+	+	a
37 °C	+	+	+	+	+

+: positive reaction, -: negative reaction, F: Fermentative, α: alpha, a: not stated, * Korun *et al.*, (2018).
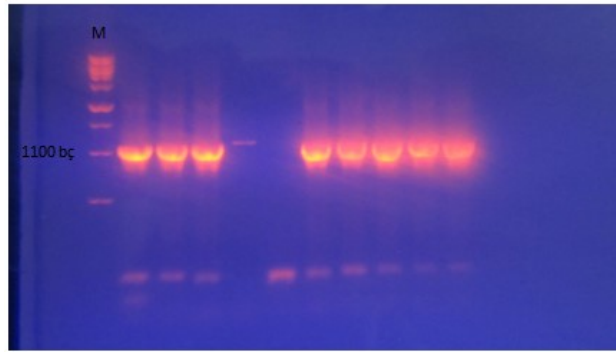
Fig. 5. Results of PCR study of *L. garvieae* strains in the agarose gel. M: Marker.

Table 2. Reference ranges used in antibiotic susceptibility detection of *L. garvieae* strains*

Antibiotic	Resistance	Intermediate Resistance	Sensitive
Ampicillin	≤11	12-13	≥14
Bacitracin	<14	-	-
Erythromycin	≤13	14-17	≥18
Florfenicol	≤14	15-18	≥19
Flumequine	<15	16-18	>21
Furazolidone	≤9	-	-
Kanamycin	≤13	14-17	≥18
Oxalinic acid	≤10	11-13	≥14
Oxytetracycline	≤18	19-22	≥23
Streptomycin	≤11	12-14	≥15
Trimethoprim	-	-	20-28

*: NCCLS (2003), EUCAST (2011), CLSI (2016), EUCAST (2019)