JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Preliminary studies on the detection and presence of lymphocystis disease virus (LCDV) in sea breams (*Sparus aurata*) raised in the Aegean Sea

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Research Article

Volume: 8, Issue: 2
August, 2024
Pages: 166-171

ABSTRACT

Lymphocystis disease (LCD) is the most frequently reported viral infection in sea bream farms in the South Atlantic and Mediterranean regions. Therefore, in this study, the presence of lymphocystis disease virus (LCVD) which is the causative agent of LCD was investigated in sea bream (*Sparus aurata*) farm in the Aegean region. The 78 fish samples, 40 of them showing fin/skin lesions characteristic to LCD and 38 fishes without skin lesions were collected. Samples from skin lesions and spleen and livers were taken from the fishes without skin lesions. The samples pooled were analyzed for the presence of LCDV by SYBR-Green real time PCR. All samples were found to be positive by real time PCR, but an amplification was seen only in 1 sample by conventional PCR. Sequence analysis has indicated that nucleotide sequences were belong to capsid gene of LCDV. In conclusion, this study shows that LCDV is present in Türkiye and causes serious health problems in sea bream in Izmir, Türkiye. Screening of fishes for LCDV by real time PCR is very crucial especially in fishes without skin lesions. Sequence analysis helps to determine circulating strains and variants of the virus in Türkiye.

Article History

Received: 10.06.2024
Accepted: 28.06.2024
Available online: 28.06.2024

Keywords: aquaculture, PCR, LCDV, sea bream, Türkiye

This study (PhD thesis) was funded by the Istanbul University-Cerrahpaşa (BAP-Project No: 35503).

DOI: https://doi.org/10.30704/http-www-jivs-net.1498518

To cite this article: Yardibi, M. E., Tali, H. E., Yılmaz, S. G., Aysun Yılmaz, A., Yılmaz, H., & Turan, N. (2024). Preliminary studies on the detection and presence of lymphocystis disease virus (LCDV) in sea breams (*Sparus aurata*) raised in the Aegean Sea . ***Journal of Istanbul Veterinary Sciences, 8*(2), 166-171. Abbreviated Title: J. İstanbul vet. sci.**

Introduction

Increase in World population and demand for animal proteins have the pressure on production of animals including fishery in the World as well as in Türkiye. Therefore, there is need to produce more but healthy food for human and animal consumption with minimized bacterial and viral infections in the populations. For these hygienic facilities and preventive measurements are necessary like vaccination and monitoring for infectious agents in aquaculture (Benkaroun et al., 2022).

There are two production methods in fishery industry; one of them is hunting and the other one is breeding which is called Aquaculture. Aquaculture is a fast-growing industry which has increased almost 12-fold in the last 30 years, with an average annual increase of 8.8 % worldwide (Ozrenk, 2023). Turkish aquaculture is rapidly growing in recent years, ranking among major producers in the World and the largest producer among the non-EU and EU member countries, together with Norway, UK, and Russia. In 2021, sea bream aquaculture has an important place in the income from fisheries in Türkiye with a 31 %

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share and a contribution of $ 637,187 to a total finance. Sea basses and sea breams are the most widely farmed species in marine waters in Türkiye (Yiğit et al., 2024).

Gilthead sea bream (*Sparus aurata*), a member of the Sparidae family, is naturally found in the Mediterranean region and from the British Isles to south Senegal. Türkiye, Greece and Spain are the main producers, accounting for more than 70 % of the sea bream production in the Mediterranean. (Borrego et al., 2017b). Sea bream production reached 152 thousand tons in 2022. Muğla and İzmir are at the top of the list in marine farming and sea bream production in Türkiye (WWF, 2021). Among other Sparidae species, Gilthead Sea bream is the most important fish species in Mediterranean aquaculture, providing good growth performances and regular production increases. However, health management remains one of the most important problems in sea bream culture, as diseases particularly viral infections can cause major losses in commercial production (Borrego et al., 2017a).

There are number of infectious agents which affect sea bream health and production. Lymphocystis disease (LCD) is the most frequently reported viral infection in sea bream farms in the South Atlantic and Mediterranean regions (Valverde et al., 2017). Lymphocystis disease virus (LCDV) a member of the Iridoviridae family, is the causative agent of lymphocystis disease (LCD). The aetiological agent is LCDV, a double-stranded DNA virus of cytoplasmic replication with complex icosahedral particles ranging from 130 to 300 nm in diameter, belonging to the genus Lymphocystivirus. (Chinchar et al., 2011).

At present, complete genome sequences are only available for 3 distinct isolates, LCDV-1, isolated from European flounder Platichthys flesus (Tidona & Darai 1997), LCDV-China, collected from Japanese flounder Paralichthys olivaceus (Zhang et al., 2004), and LCDV-Sa, from gilthead seabream *Sparus aurata* (López-Bueno et al., 2016). According to the sequences of the conserved viral major capsid protein (MCP), 9 different genotypes have been proposed, with clustering related to host species rather than geographic location (Kitamura et al., 2006; Hossain et al., 2008; Cano et al., 2010; Palmer et al., 2012; Labella et al., 2019)

LCD is a self-limiting disease causing hypertrophy of fibroblastic cells in the connective tissue of fishes characterised by the occurrence of whitish, reddish, or grayish nodules of hypertrophic fibroblastic cells in the dermis and sometimes in the viscera. These hypertrophied cells, referred to as lymphocysts or lymphocystis cells, are usually observed in the skin and fins, but they have also been described in several internal organs (such as the stomach, spleen, liver, kidney, and heart). In sea bream, LCD-associated lesions have been observed only in the skin and fins of affected fish and usually disappear after 20–45 days, depending on water temperature (Colorni & Diamant, 1995).

Diagnosis of LCDV is generally made by histopathology of skin lesions and detection of virus in clinical or subclinical samples. Several studies have shown that viral antigens can be detected in a number of organs and skin lesions of infected fish (Valverde et al., 2017). Molecular methods such as real time PCR and PCR frequently have been used. LCDV infects more than 150 marine and freshwater fish species belonging to 42 families causing great economical losses worldwide (López-Bueno et al., 2016). Lymphocystis disease was first described in gilthead seabream in Israel in 1982 (Paperna et al., 1982), and since then it has been frequently reported in several countries from the same geographic area (Labella et al., 2019). Outbreaks of LCD have been reported worldwide, but little is known about the spread and frequency of the virus in Türkiye (Pekmez et al., 2022). Therefore, this study was performed to investigate the presence of LCDV by PCR on clinical and subclinical samples reported in a sea bream farm in İzmir province, Türkiye.

Materials and Methods

Fish farm and sampling

A sea bream production farm located in Aegean sea having suspected cases of LCD was visited. The fishes showing clinical signs of LCD mainly skin lesions were examined. History and clinical signs of the fishes were recorded. LCDV-suspected fishes (n=78) were collected from the sea-cages of a commercial sea bream farm. They were then transported to the laboratory, Department of Virology, Veterinary Faculty, Istanbul University-Cerrahpaşa in a cold chain (4-8 °C). The samples were either processed directly or stored at -20 °C until required.

The samples were pooled and analysed for initial screening of LCDV by SYBR-Green real time PCR. Pool samples were prepared for DNA extraction. For pooling, the samples were taken from the fin/skin lesions (n = 40) and from the liver and spleen of the fishes (n = 38) without fin/skin lesions after necropsy. A total of 12 pools were formed.

DNA Extraction

All pooled samples were first homogenised separately using the tissue disrupter (Bullet Blender, Next

Table 1. Primers, reaction mixtures and PCR conditions for PCR analyses of the LCDV

Test	Target Genes	Primers (5'-3')	Product size	Reaction mixture	PCR Conditions	References
Real-Time PCR	MCP	qPCR-F1 AATGAAATAAGATTAACGTTTCA	151	MM:12,5 µl	95 °C- 10m	Ciulli et al., 2015
				Primer F: 1 µl		
		qPCR-R2 CGTTAGTGATCCATACTTG		Primer R: 1 µl	45 cycles of 95 °C- 15s 50 °C-30s 72 °C-30s	
				SYBR Green : 0,5 µl		
				Water: 8 µl		
				DNA: 2 µl		
Conventional PCR	Capsit Gene	LF7-F CGCGCTGCCTTATAATGA	789	MM:12,5 µl	95 °C- 3m	Ciulli et al., 2015
				Primer F: 1 µl		
		LC1-R GGAAAACCCATTGATCCGT		Primer R: 1 µl	35 cycles of 94 °C- 1m 55 °C-2m 72 °C-1m	
				Water: 7,5 µl		
				DNA: 3 µl		
					Last ext. 72 °C-3m	

Advance). Viral DNA was extracted from the homogenised tissue by using a commercial DNA extraction kit (The PureLink™ Genomic DNA Mini Kit, Invitrogen™, Cat No: K1820-02, Carlsbad, CA 92008) as described by the manufacturer. The amount of DNA in the extracts was measured by nanodrop device (NanoDrop, Thermo Scientific, Waltham, USA). They were then stored at -20 °C until required.

SYBR-Green real time PCR

SYBR Green based real time PCR and primers were used to detect LCDV in samples as described previously (Table 1) (Ciulli et al., 2015). In an optimized PCR reaction, a 25 ml PCR mixture composed of 2 ml template DNA, 12.5 ml mastermix (Maxima hotstart mastermix, Thermoscientific, USA), 1 ml forward and reverse primers, 0.5 ml of SYBR-Green and 8 ml nuclease-free water (Table 1). The PCR reaction was placed in a thermal cycler (StepOnePlus™ Real Time PCR System, Applied Biosystems™) using the cycling conditions as follows: after 10 minutes of initial incubation at 95 °C, 45 cycles of denaturation steps at 95 °C for 15 seconds, annealing at 50 °C degrees for 30 seconds, and extension at 72 °C for 30 seconds (Table 1). For all PCR reactions, negative and positive controls were always used. Positive controls were from the samples that were previously found to be positive in the Department of Virology, Veterinary Faculty of Istanbul University-Cerrahpasa. As negative control, nuclease free water was added in place of template DNA.

Results

Clinical signs and necropsy

It was observed that all the fishes analysed in this study were clinically ill. At necropsy, amongst 78 fishes, 40 showed significant skin and fin lesions (Figures 1 and 2). Among the fishes (38) that did not show lesions at necropsy examination, there were no obvious gross lesions in spleen and liver samples (Figure 3).

Figure 1. Fin/skin lesions on the fishes on clinical observation.

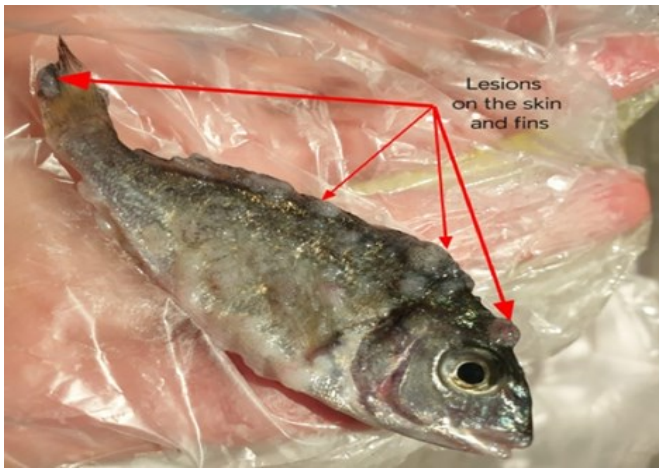

Figure 2. Skin and fin lesions on fish found to be positive for LCDV by both real time PCR and conventional PCR. Arrows indicate fin and skin lesions seen in PCR positive fishes.

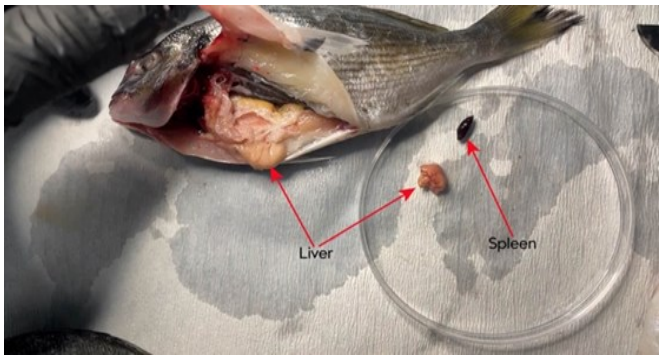

Figure 3. Spleen and liver samples taken from fish without skin and fin lesions.

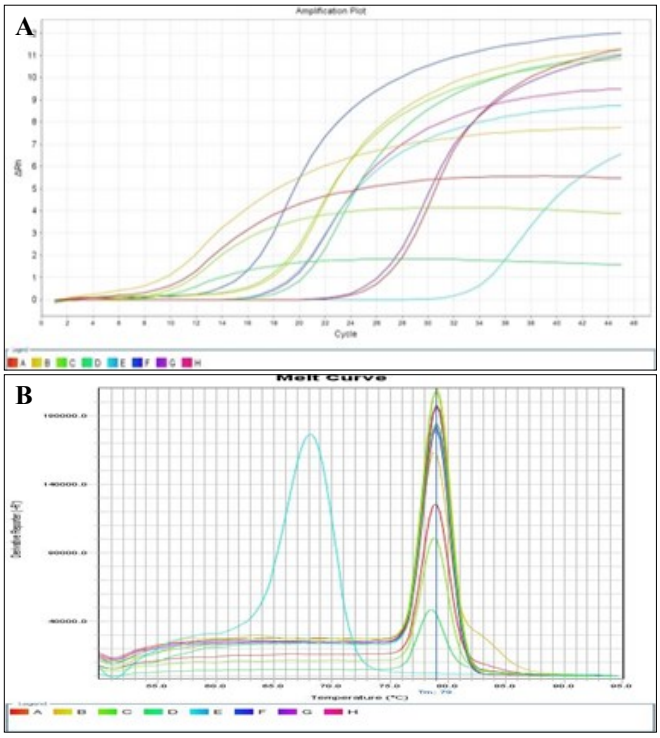

Figure 4. Ct values (A) and melting curves (B) of samples and controls analysed by SYBR-Green real time PCR

SYBR-Green real time PCR

Amplification was seen in all pool samples (n = 12) and positive control analysed by SYBR-Green real time PCR. Ct values were detected between 5.87-25.08 (Figure 4-A). No Ct was detected in negative control. Melting curves of positive control and positive samples were determined between 78.55-79 °C (Figure 4-B). Whereas melting curve of negative control was 68 °C (Figure 4-B).

Conventional PCR

When real time PCR positive samples were analysed by conventional PCR, an amplification product (789 bp) was seen on agarose gel electrophoresis. Sequencing data has indicated that the nucleotide sequences (data not shown) were part of capsid gene of LCDV.

Discussion

Lymphocystis disease (LCD) is a well-known iridoviral infection that affects both wild and cultured fish species living in freshwater and marine life worldwide. The clinical signs of the disease are characterized by the development of small pearl-like macroscopic nodules (0.3–2.0 mm) located mostly on the fins and skin, although internal organs may also be affected. The disease occurs in a wide range of salinities and water temperatures, and it has been reported in at least 150 fish species from 42 families. LCD has been reported in many countries but the knowledge about LCD in Türkiye is limited (Palmer et al., 2012; Ciulli et al., 2015; Labella et al., 2019; Pekmez et al., 2022). Therefore, this study was performed to investigate presence of LCDV in sea breams in Izmir, Turkey.

LCDV has caused significant economic losses in aquaculture in many parts of the world, especially in the South Atlantic and Aegean sea. Therefore, this study was performed in Izmir located in Agean sea. Our neighbours, especially Greece, have reported serious health problems in sea breams due to LCD (Colorni et al., 2011). Apart from Greece in the Aegean sea, LCDV has also been reported in countries such as Italy, Spain and Portugal, as well as in our neighbour Iran (Labella et al., 2019; Pekmez et al., 2022; Rahmati-Holasoo et al., 2023). It has been proposed that LCDV was spread through the Atlantic coasts of Europe and the Mediterranean along with the international trade of gilthead seabream (Chinchar et al., 2017). LCDV has an incidence rate as high as 70 % meaning it causes significant economic losses in the aquaculture sector, as external lesions appear and samples with disease symptoms are difficult to commercialize (Masoero et al., 1986).

Diagnosis of LCD is mainly based on pathological and molecular techniques. Presence of small pearl-like

macroscopic nodules on the fins and skin of the fish is characteristic appearance of LCD as reported in previous studies (Samalecos, 1986). In this study, serious pearl-like nodular lesions were observed in 40 of 78 sea bream fish produced in a seafood farm in Agean sea in Izmir. LCD was detected in all samples taken from these lesions. However, the problem in diagnosis of LCD is the cases without fin or skin lesions. Therefore, in the present study, liver and spleen samples were taken from the fishes without skin lesions and LCDV DNA was detected. These results and the results of other study (Ciulli et al., 2015) indicate that visceral organs can be used to investigate presence of LCDV in fishes without skin lesions. For this, the primers targeting to MCP gene of LCDV is highly conserved region of iridoviruses and were used in the present study and they are capable of detecting all LCDV strains as indicated previously (Tidona et al., 1998; Ciulli et al., 2015). Similarly, the primers and real time PCR detected LCDV in all pooled samples analysed in this study.

Incidence of LCD is very high and can be up to 70 % as reported previously (Masoero et al., 1986). In the surveillance studies carried out in Spain, the incidence in juvenile seabream samples from asymptomatic farms was 87.5-100 %, whereas it was detected in 30-100 % of symptomatic farms (Valverde et al., 2017) In this study, LCDV was detected by real-time PCR in samples taken from sea breams in 100 % of pooled samples with lesions and in 100 % of pooled samples formed from organs of fishes without lesions .

Conclusion

This study shows that LCDV is present in Türkiye and causes serious health problems in Izmir, Turkey. Screening of fishes for LCDV by real time PCR is very crucial especially in fishes without skin lesions. Conventional PCR helps for sequencing the virus to determine circulating strains and variants of the virus. This will input data in vaccine preparation and vaccination strategies.

Acknowledgement

We would like to thank to Istanbul University-Cerrahpasa (BAP, Project No: 35503-PhD Thesis) for funding this study.

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