

Exemplifying 'pathobiome' concept through case study: Co-infection with *Vibrio harveyi*, *Photobacterium damsela* and *Cryptocaryon irritans* in Salema (*Sarpa salpa*)

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ABSTRACT

A pathobiome approach has been revealed as a set of organisms (i.e. prokaryotic, eukaryotic, and virus associations) in the aquatic environment that interact with the host (fish) to cause disease. The approach of the one pathogen–one disease is not wholly satisfactory to comment on the impairment of health status and disease process in fish. To exemplify the pathobiome concept, we present a fish disease in which more than one pathogen and possible synergistic interaction of inadequate water quality. In this case, the heavy mortalities were observed in Salema (*Sarpa salpa*) from an exhibition aquaria. Bacterial identification with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) showed the pathogen bacteria species of *Vibrio* and *Photobacterium*. In the parasitic examination, the invasion of marine ciliata, *Cryptocaryon irritans* was observed in the diseased fish. Moreover, poor water quality was considered as contributing factor to disease emergence in this case. The co-existence of pathogen *Vibrio* species (*Vibrio harveyi*) and *Photobacterium damsela* with its subspecies (subsp. *damsela* and subsp. *piscicida*) as well as marine ciliata, *C. irritans* for a description of a disease picture in a marine fish species are reported for the first time, providing an example of a pathobiome paradigm.

Keywords: pathobiome, aquatic bacteria, ciliate, salema, *Sarpa salpa*

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Introduction

In the aquaculture systems, aquatic organisms, including fish and aquatic microbiome, interact closely with each other, leading to the complexity and dynamism of the marine environment (Tsang et al., 2021). Microbial communities associated with water and fish (host) have been studied for decades. Fish living contiguous to a potpourri of microbes continually interact with the surrounding environment. A single microliter of marine water can contain several microbial communities; thus, marine

water has been expressed as a "microbial soup" in terms of a wide variety of microbiome diversity (Bass et al., 2019). Recently, the concept of 'pathobiome' has been very much in the foreground. Basically, the term 'pathobiome' refers to the host-associated pathogens and impaired health status (Stentiford et al., 2017; Bass et al., 2019). Vayssier-Taussat et al. (2014) have put more emphasis on environmental factors in describing the pathobiome concept. The interactions of the various microorganisms are

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remarkable within the context of pathobiome. Increasing scientific facts about the pathobiome phenomenon, integrating the pathogen communities, the aquatic organism, and the water quality in a new understanding of the cause of the disease condition. The one pathogen–one disease approach is insufficient to express how the disease develops particularly in the aquatic environment; therefore, the approach in the aquatic environment has moved away from one pathogen-one disease to the direction of the pathobiome concept.

Bacteria belonging to the *Vibrio* genus have been known to be abundant and the most diverse group in marine environments. The *Vibrio* species are critical in marine aquaculture because several species such as *Vibrio harveyi*, *V. anguillarum*, *V. alginolyticus* and *V. parahaemolyticus* can cause serious diseases in fish and shrimp, resulting in increased mortality rates (Mougin et al., 2020). *V. scophthalmi* and *V. ichthyoenteri* are other opportunistic pathogens in the marine environment, leading to disease or death in fish when the environmental conditions are impaired.

Pathogenic ciliate, *Cryptocaryon irritans* is an obligate parasite, causing serious diseases known as cryptocaryonosis or the marine white spot disease in commercial marine farms and ornamental fish culture systems (Vendramin et al., 2016; Munisea et al., 2020; Cascarano et al., 2021). Some characteristics of this parasite are the ability to infect the fish with low host specificity and high virulence levels (Li et al., 2022). The place of fish ciliata and associated bacterial microbiome in the pathobiome concept has been well documented by Jahangiri et al. (2021); hence, symbiosis with *V. harveyi* was evidenced for *C. irritans* by Qiao et al. (2017).

Identification of pathogens, particularly diverse types of bacteria are more substantial in pathobiome concept. The developed techniques in identifying various bacteria species have benefits to pathobiome study. Advanced tools such as matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) enable easier, correct, and rapid discrimination of bacteria species. MALDI-TOF MS has been considered suitable for species identification of the pathogens found in the aquatic environments, particularly for *Vibrio* spp. (Jansson et al., 2020; Mougin et al., 2020). MALDI-TOF MS analysis based on the mass distribution of bacterial proteins has paved the way for the identification of the diverse bacteria in the present study.

In this case study, the multi-species causal pathogens and possible interaction with the water quality in the development of the disease in salema have been studied in the pathobiome concept.

Materials and Methods

Ethical approval: Fish were handled by the international animal ethical rules. Fish examined in this study were moribund.

Case definition: The disease outbreak in salema (*S. salpa*) occurred in an exhibition aquarium of Ankara, Turkey. Clinical signs of the diseased salema (N=30) were skin lesions with ulcerations, scale loss, and extensive hemorrhaging on the body surface (Figure 1). Among the behavioural signs of the outbreak the erratic swimming before death was indicated as an important sign by the aquarium managers. The mortality reached 100 % in several days. Other various fish species such as sharks and rays were living in the aquarium. These species had no disease signs. Water quality parameters in the aquarium were water temperature: 23.5°C; the salinity: 26-27 ppt; the dissolved oxygen concentration: 7.5 mg/L; un-ionized ammonia 0.1 mg/L. Fish were fed *ad libitum* with commercial feed containing approximately 35% raw protein. The model of aquarium made the routine aquarium husbandry, water or fish sampling difficult, resulting in problems in monitoring of fish and water quality.

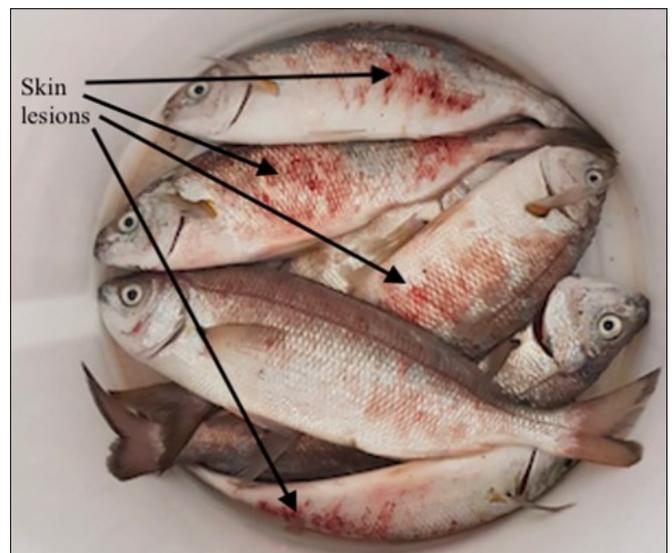


Figure 1. Salema (*S. salpa*) with lesions on the skin

Fish samples: Moribund salema (*S. salpa*) with disease symptoms were obtained from the exhibition aquarium. Fish weight was around 80 to 100 grams.

Isolation of bacteria from diseased *S. salpa*: The collected fish samples were processed in the Fish Health Laboratory, Department of Fisheries&Aquaculture, Ankara University, by following the standard microbiological methods. The samples for bacterial isolation were taken from the kidney and liver of 10 moribund salema and streaked onto Marine Agar 2216 (Difco). The plates were incubated at 22°C

Agar 2216 (Difco). The plates were incubated at 22°C for 48-72 h. The subculture from the fresh bacterial culture was done to obtain a single colony for the MALDI-TOF MS analysis.

Identification of bacteria using MALDI-TOF MS profiling: Bacteria species were identified through Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) by following the workflow for the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) and previous work (Dieckmann et al., 2010; Sogawa et al., 2011). Briefly, the “ethanol formic acid extraction” protocol was applied for the protein isolation of bacteria. The mass spectrum of bacteria grown on the marine agar, was performed by Microflex LT and analyzed using the MALDI Biotyper software package (version 3.0). Mass spectrum profile (MSP) was compared with the strains in the Bruker reference library (Version 9.0.0.0).

In evaluating the results, log-score values were classified by the MALDI Biotyper. A log score between 2.300 and 3.000 indicated a highly probable species identification. A log-score between 2.000 and 2.299 indicated secure genus identification, and probable species identification. A log-score between 1.700 and 1.999 indicated a probable identification at the genus level only. A log-score below 1.699 indicated no reliable identification.

Parasite examination: All moribund salema were examined under a microscope for the ectoparasites. The gill filaments of moribund salema invaded by *C. irritans* trophonts. To confirm the parasite was *C. irritans* the stages of tomont and theront were also observed through the propagation in seawater-adapted guppies. Briefly, guppies were acclimated to salt water gradually in three days. First day guppies were directly transferred to the water at 10 ppt salinity then, salinity increased to 18 ppt (2nd day) and 25-26 ppt (3rd day). The prevalence (%) and the mean intensity of the parasite in salema were determined as explained by Bush et al. (1997).

Results

MALDI-TOF MS results for culturable bacteria: MALDI-TOF MS results for culturable bacteria from the internal organs of diseased salema showed the log scores in the range of 2.300 and 3.000, corresponding to ‘highly probable species identification’ (Table 1). The log scores below 2.300 were excluded from the results. MALDI-TOF MS identified the profile for *Photobacterium damsela* with scores 2.25 to 2.009 in kidney samples of salema. The identification index of the MALDI BioTyper software log scores values for the liver sample was 2.24 for *V. harveyi*.

Mass spectrum of culturable bacteria species from the kidney and liver of diseased salema showed the *P. damsela* subs. *piscicida* and subs. *damselae* as well as *V. harveyi* based on the Bruker database (Figure 2).

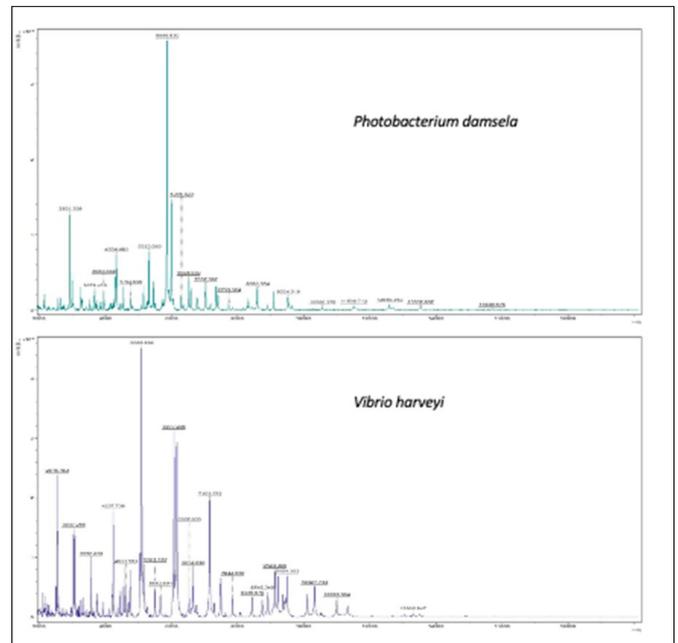


Figure 2. Representative mass spectra for *Photobacterium* and *Vibrio* species.

Table 1. MALDI-TOF MS profile based identification of bacteria species in infected salema, *S. salpa*

| Sampling organ | Identification with MALDI-TOF | Log score value* | NCBI Identifier | Identifier isolate by Bruker database |
|----------------|--|------------------|-----------------|---------------------------------------|
| Kidney | <i>Photobacterium damsela</i> subs. <i>piscicida</i> | 2.25 | txid38294 | DSM 22834T DSM |
| | <i>Photobacterium damsela</i> subs. <i>damselae</i> | 2.009 | txid85581 | DSM 7482T HAM |
| Liver | <i>Vibrio harveyi</i> | 2.248 | txid669 | DSM 19623T DSM |

*A log score bigger than 2.3: highly probable identification at the species level; between 2.000 and 2.299: secure genus identification, probable species identification; between 1.700 and 1.999: a probable identification at the genus level; below 1.699 no significant similarity with Bruker reference library 9.0.0.0.

Basic quantitative results for parasite, *C. irritans*: Of the quantitative parameters of parasite, *C. irritans* in the gills of salema, the prevalence was found to be 100% and mean intensity 57.73 ± 1.35 . Tomonts passaged from the sea water adapted guppies were about in 450 μm in size (Figure 3).

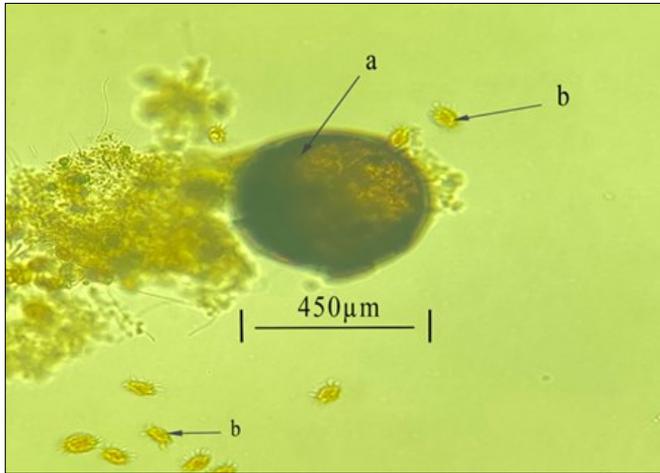


Figure 3. *Cryptocaryon irritans* tomont (a) and theront (b)

Discussion

The heterogenous pathogens involved in a disease case in salema in this study were evaluated in the frame of pathobiome concept. In this study, pathogens found on the moribund salema, *S. sarpa* were both bacteria and parasite; several Vibrionacea family members and marine ciliata, *C. irritans*. The evidence for the inclusion of various bacteria; *Vibrio* and *Photobacterium* in one disease picture as provided here help us to understand the factors in a disease occurrence. It is clear that the one-pathogen-one disease is not realistic in aquatic environment as exemplified in this study.

The culturable bacteria profiling with MALDI-TOF MS showed *V. harveyi* and *P. damsela* in internal organs of diseased salema. MALDI-TOF MS, which is mainly based on the profiling of bacterial ribosomal proteins is very efficient in distinguishing *Vibrio* species (Cheng et al., 2015; Osthoff et al., 2017; Mougin et al., 2020). There are difficulties in identifying *Vibrios* at the species level, even with some molecular techniques used (Moussa et al., 2021). The advantage of MALDI-TOF MS was better utilized in the identification of *Vibrio* species in this study. MALDI-TOF MS as an advanced tool in bacterial identification has improved our success in the determination of the various bacteria involved in the disease of the salema, contributing to pathobiome approach.

Vibriosis is one of the most common diseases in marine organisms (Ina Salwany et al., 2019). *Vibrio* spp. is prevailing in seawater aquaria, constituting 60%

of the total heterotrophic bacteria (Nagasawa & Cruz-Lacierda, 2004); Various *Vibrio* species in different fish species have been reported previously (Fabbro et al., 2012; Turgay et al., 2018; Mohamad et al., 2019). *Photobacterium damsela* has been recorded in cultivated marine fish such as meagre, yellowtail, sea bass, and sea bream (Rivas et al., 2013). It should be noted that consists of two subspecies: *P. damsela* subsp. *damsela* and *P. damsela* subsp. *piscicida*. *Photobacterium damsela* subsp. *damsela* has been known as an opportunistic pathogen for fish whilst *P. damsela* subsp. *piscicida* causes pseudotuberculosis in numerous marine fishes (Ina-Salwany et al., 2019). Both subspecies of *P. damsela* were identified in infected salema in this study.

Although the pathobiome approach is relatively new to fish pathology, previous studies have reported the occurrence of diseases due to mixed infections which were referred to as "co-infection" (Kotob et al., 2016). The examples from previous studies on more than one bacterial species were involved in the disease process, such as co-infection with the synergistic interaction of *Aeromonas veronii* and *V. cholera* in koi carp (Han et al., 2021), the concurrent presence of *V. harveyi* and *V. alginolyticus* in hybrid groupers, *Epinephelus polyphekadion* × *E. fuscoguttatus* (Mohamad et al., 2019) and the mix infection with *V. anguillarum* and *Lactococcus garvieae* in rainbow trout (Tanrikul & Gultepe, 2011).

The parasite, *C. irritans*, at the trophont stage was observed in the all moribund salema, showing the prevalence of 100% in the case presented here. Parasitic invasions that coincide with bacterial infections have been documented in aquatic animals (Kotob et al., 2016; Wise et al., 2021). Pathogen bacteria *V. harveyi* was described as an endosymbiont of the marine Ciliate parasite, *C. irritans*, representing an important example of the relationship between the bacterial microbiome and parasitic ciliates (Qiao et al., 2017). In our study, the co-occurrence of *C. irritans* and *V. harveyi* in salema may be related to this symbiosis. Ciliata parasite and its associated bacteria may render one pathogen-one disease approach pointless; however, it potentially contributes to the pathobiome concept's convenience.

Although the water quality parameters have not been regularly followed in our case unsuitable abiotic factors such as the un-ionized ammonia (0.1 mg/L) in this case is above the acceptable level (<0.05 mg/L) in the water. The increased susceptibility to various pathogens due to adverse water quality in the process of disease development has been documented by Svobodova et al. (2017). For example, the occurrence of *Edwardsiella tarda* has been linked to higher levels

ammonia in water (Karasu Benli & Yavuzcan Yildiz, 2004). In another example, the synergistic effects of *A. hydrophila* and *V. parahaemolyticus* in striped mullet, *Mugil cephalus* have been attributed to adverse water quality including higher levels of nitrogenous products (El-Son et al., 2021). Suboptimal water conditions can facilitate the reproduction of ciliata in warm-water aquaculture systems (Jahangiri et al., 2021). Thus our findings can contribute to the knowledge that poor water quality closes the disease emergence cycle, which can be one of the elements of the pathobiome in aquatic environment.

Conclusion

The one pathogen–one disease paradigm is moving to the pathobiome concept to characterize disease dynamics. In this concept, the combination of multiple pathogens, host, and environment is working together to disease or health of the organism (Vayssier-Taussat et al., 2014). Transitioning from the classic disease triangle comprising the one-pathogen, host, and water environment to a more realistic pathobiome concept would be more useful in solving the disease crisis in aquaculture. Our study contributes to understanding the pathobiome concept, providing the types of pathogens involved, the fish as a host, and the possible effect of impaired water quality holistically.

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