Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(7): 119 – 131 (2020)
www.ejabf.journals.ekb.eg

Janthinobacterium lividum an opportunistic psychrophilic bacteria associated with mortalities of the infected Red Sea Broomtail wrasse (*Cheilinus lunulatus*).

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ARTICLE INFO

Article History:
Received: Aug. 27, 2020
Accepted: Oct. 7, 2020
Online: Oct. 12, 2020

Keywords:
Broomtail wrasse,
Cheilinus lunulatus
janthinobacterium,
Red Sea,
aquarium

ABSTRACT

A bacterial infection was spread in the Broomtail wrasse (*Cheilinus lunulatus*) during winter 2019 in a marine aquarium without heating system while salinity diluted with tap water. The infected fish became lethargic, pale gills,change to excessive black colouration. Internally, the kidney was swollen and had granulomatous lesions, that showed the presence of bacterial colonies and necrotic phagocytes and accumulations of macrophages. The isolated strain was gram-negative rods with rounded ends bacteria, psychrophilic and non-pigmented bacteria in BHI, colored yellow in TCBS. Partial 16S rRNA sequences demonstrated high sequence homology with *Janthinobacterium* sp. and to *J. lividum*. The strain showed a high resistance to all the studied antibiotics and low sensitivity to chloramphenicol.

INTRODUCTION

Janthinobacterium consists is Gram-negative rod with rounded ends bacteria, sometimes slightly curved. No resting stages are formed. They are usually motile by a single polar flagellum and one to four lateral flagella. They are strict aerobes **(Sneath, 1984)**. The most common characteristic of this genus is psychrophilic bacteria which produce violet pigment. However, also reported as partly pigmented and non-pigmented bacteria within this genus reported as discoloring a variety of natural materials and occasionally as the causative agent of septicemia in humans and animals **(Gillis and De Ley, 2006 ; Kim *et al.*, 2012)**.

This genus has been reported as a component of the gut microbiota of marine fish **(Egerton *et al.*, 2018)**, the normal microflora of freshwater fish and soil fish **(Austin *et***

***al*., 1992, Hansen ; Olafsen, 1999)**, associated with adults of striped mullet, pinfish, and sand and spotted sea trout **(Larsen *et al*., 2013)** larvae of halibut **(Jensen *et al*., 2004)** present only in February on the skin of Gulf Killifish (*Fundulus grandis*) Microbiome **Larsen *et al.* (2013)**. Therefore, there would be a ready inoculum of the pathogen in the environment around. However, depending on the fish's immune status and the presence of other pathogens that affect fish health status, such as the organisms *Aeromonas*, *Vibrio*, and *Pseudomonas*, Janthinobacterium spp. might have pathogenic functions in water environments **(Oh *et al*., 2019)**.

Janthinobacterium lividum is the most common species in the genus were associated with mortalities at two fish farms of rainbow trout in Scotland and Northern Ireland the first site diagnosed with rainbow trout fry syndrome, in the second site the rise in mortalities coincided with a change from the use of spring to river water **(Austin *et al*., 1992)** *Janthinobacterium lividum* was first isolated in Korea as a pathogenic bacterium that infects rainbow trout. Mass mortality was observed in rainbow trout hatchery, and dead fish were necropsied **(Oh *et al*,. 2019)**.

The genus *Janthinobacterium* has been demonstrated to develop various pigments with antibiotic activity, such as violacein **(Pantanella *et al*., 2007; Becker *et al*., 2009)**, Violet purple pigment **(Mojib *et al*., 2010)** and purple-bluish pigments **(Shirata *et al*., 2000)**. Others did not exhibit violet pigmentation **(Dainty *et al*., 1978).**

Broomtail wrasse (*Cheilinus lunulatus*) adults occupy coral reefs and associated environments of rocks, sand and seaweed, typically along the coral-rich slopes of the fringing reef. Feeding mainly on hard-shelled invertebrates, particularly mollusks **(Gomon and Randall, 1984)**. Oviparous, distinct pairing during breeding **(Breder and Rosen, 1966)**. Human uses include aquarium minor commercial. In the present study, we recorded the first isolation of *J. lividum* as a pathogenic bacterium that infects Red Sea marine fish.

MATERIALS AND METHODS

1. Fish sampling:

A bacterial infection spread in the National Institute of Oceanography and fisheries marine aquarium during winter 2019. Broomtail wrasse (*Cheilinus lunulatus*) was one of the common species in five tanks with a mean volume of 6 tons, 26 fish were examend of total number of 42 fish. water temperature ranging from 17 to 14°C, salinity 30 -23‰ and pH 7.2 - 8.5. Fish became lethargic, pale gills, enhanced skin pigmentation with skin ulceration. Internally, the kidney was swollen. History and clinical pictures were reported.

2. Bacterial isolation:

Samples from skin ulcers, liver, and kidney of freshly dead fish were cultured on Brain Heart Infusion (BHI) Agar and Thiosulphate citrate bile salt sucrose agar (TCBS, Difco). With 50% seawater, (**Chen *et al.*, 1995)**. The plates were incubated at 20 - 25°C for up to 48 h. The dominant bacteria were sub-cultured and pure cultured bacterial colonies were gram stained to confirm purity and selected for identification, the isolates were preserved at -20°C in glycerol. Bacterial glycerol stocks also were grown on TSA for 18 h under the same conditions, to confirm the identification.

3. Bacterial identification

3.1. Morphological and Biochemical identification:

The isolated strains were streaked on BHI and incubated at 5, 10 and 20°C for 72 hrs. and the growth observed. Gram's staining was performed to detrmined shape and Gram's reaction as described **(Madigan *et al.*, 2004)**. Catalase enzyme. Oxidase and motility tests were also performed **(Pickett and Greenwood., 1986)**.

3.2. 16S rRNA Gene Sequencing

DNA was extracted from a pure cultured colony using the Gene JET genomic DNA purification kit (Thermo Scientific, EU) according to the manufacturer recommendations. Genomic DNA, amplified using the polymerase chain reaction (PCR) technique with the universal primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3') (Martin and Collen 1998). Using a size nucleotide marker (100 base pairs), the purified PCR products (amplicons) were reconfirmed with electrophoresis on 1% agarose gel. Bands were eluted and sequenced with the incorporation of dideoxynucleotides into the reaction mixture. The sample was sequenced using 27F and 1492R primers in the sense and antisense directions. The results of the sequencing were analyzed using the Basic Local Alignment Search Tool (BLAST) and Geneious prime 2020.1.2. to identify the specific bacteria. The sequences were aligned by CLC Sequence Viewer 7 from QIAGEN Bioinformatics. Partial sequences of the 16S rRNA gene were used to construct a phylogenetic tree, with the consensus sequences being imported into the Geneious prime 2020.1 software. Briefly, a bootstrap analysis was performed with 1,000 replications, and the neighbor-joining approach was used to construct the phylogenetic tree.

4. Antimicrobial susceptibility testing:

The susceptibility of isolates were tested for 15 antimicrobials by using the disk diffusion method **(Acar and Goldstein., 1991)**,using Mueller-Hinton agar (Lab M Limited) supplemented by 1.5% (w/v) sodium chloride incubated for 16 to 18 h at 25°C. The diameters of the inhibition zones surrounding the antimicrobial disks were interpreted as described in guideline M100 of the Clinical & Laboratory Standards Institute **(Pfaller *et***

***al*., 2010)**. Susceptibility pattern was assessed using ampicillin,10mg/disk; cephalothin,30 mg/disk; amikacin, 30 mg/disk; streptomycin, 10 mg/disk; neomycin, 30 mg/disk; oxytetracycline, 30 mg/disk; tetracycline, 30 mg/disk; chloramphenicol, 30 mg/disk; erythromycin, 15 mg/ disk; norfloxacin, 10 mg/disk ciprofloxacin, 5 mg/disk. Ofloxacin, 5 mg/disk; cefotaxime, 30 mg/disk; oxalic acid, 2 mg/disk, and gentamicin, 10 mg/disk; Antimicrobial disks were purchased from Bioanalyse, Turkey.

5. Pathological and histopathological analysis:

Samples, including skin ,subcutenious muscels, gills, liver, and spleen were collected from 6 moribund fishes and fixed in 10% phosphate-buffered formalin. Sections were obtained by using a rotatory microtome and stained with hematoxylin and eosin stain.

RESULTS AND DISCUSSION

1. Clinical signs

The Broomtail wrasse *(Cheilinus lunulatus*) was one of the common species that can survive in the National Institute of Oceanography and fisheries during winter 2019. Twenty six infected Broomtail wrasse fish became lethargic, pale gills, enhanced skin pigmentation and skin ulcers, the kidney was swollen **Figure (1)**. In the current study infected fish became lethargic, pale gills, enhanced excessive black colouration. Rainbow trout infection recorded at two sites, Frist site moribund fish displayed exophthalmia, pale gills, enhanced skin pigmentation, swollen abdomen and sometimes skin lesions. In the second site, the fry became very lethargic, developed enhanced skin pigmentation, and displayed severe gill hyperplasia as mucus-secreting cells increase **(Austin *et al*., 1992)**.

Figure 1. Broomtail wrasse (*Cheilinus lunulatus*) showed excessive black colouration, congestion in the tail, pectoral, dorsal and pelvic fins and skin ulcers and fried tail fin.

2. Bacterial isolation

Seven bacterial isolates were isolated from skin ulcers, liver and kidney of freshly dead fishes.

3. Identification of the Bacteria:

3.1. Morphological and Biochemical identification:

The colonies were low convex creamy to pale-yellow. They grew on TCBS agar and gave yellow colonies. Based on the biochemical and morphological characteristics, the seven isolates were identified as *Janthinobacterium sp*. All isolates were rod-shaped, Gram-negative, Motile, catalase and oxidase positive psychrophilic and non-pigmented bacteria in BHI and nutrient agar.

3.2. 16S rRNA Gene Sequencing

Partial sequencing targeting 16S rDNA yielded 1397bp from the cultured isolate. The phylogenetic tree constructed using sequence data, compared with NCBI database identified the etiological bacterium as *J. lividum*, the 16S rRNA gene of the isolate demonstrated high sequence homology with *Janthinobacterium* sp. 99.57% similar to the *Janthinobacterium* sp. FJ 812373.1 and 97.9 to *J. lividum* NR 164625, *J. lividum* NR026365 type strain (**Figure 2)**. Few studies considered janthiobacterium spp non pathogenic for fish and human **(Haack *et al.*, 2016)**.

4. Pathological and histopathologic Analysis:

The larger rainbow trout associated with surface lesions, the skin was sloughed off all over the fish flank from the operculum to tail, exposing the underlying necrotic muscle **(Austin and Austin., 2016)**. Mass mortality was observed at one rainbow trout hatchery **(Oh *et al.*, 2019)**.

In the present study chronic lesions in internal organs are characterized by the presence of white tubercles of about 0.5 to 3.5 mm in diameter. Granuloma production is a pathognomonic lesion of *Janthinoacterium lividum* infection in Broomtail wrasse (*Cheilinus lunulatus*) in spleen, skin, muscles, gills, and peritoneum. Histopathological analysis of granulomatous lesion revealed the presence of bacterial colonies and necrotic phagocytes and macrophage accumulations; many of these were necrotic, containing various bacteria. Moreover, the gills also showed granulomata composed of degenerated macrophages containing bacteria and necrosis of primary and secondary lamellae **Figure (3, 4)**. Rainbow Trout (*Oncorhynchus mykiss*) signed of tubular degeneration, bacteria were present near the tubules. Increase in immunocyte numbers, including melanomacrophages, lymphocytes, and neutrophils. The infiltration cells were observed along with the renal tubular structure **(Oh *et al*., 2019).**

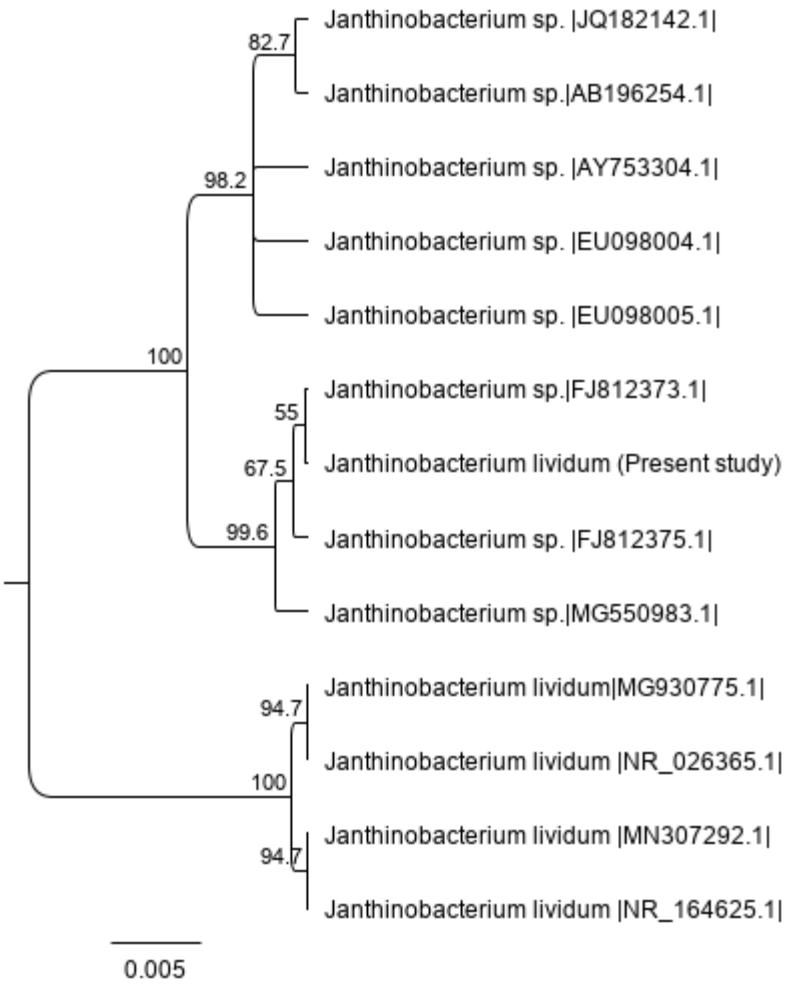

Figure 2: Molecular Phylogenetic analysis by Maximum Likelihood-based on the Tamura-Nei model for Janthinobacterium lividum with twelve other most closely related Janthinobacterium species based on 16S rDNA sequencing. A total of 1397 positions in the final dataset. Evolutionary analyses were conducted in Geneious prime 2020.1.2.

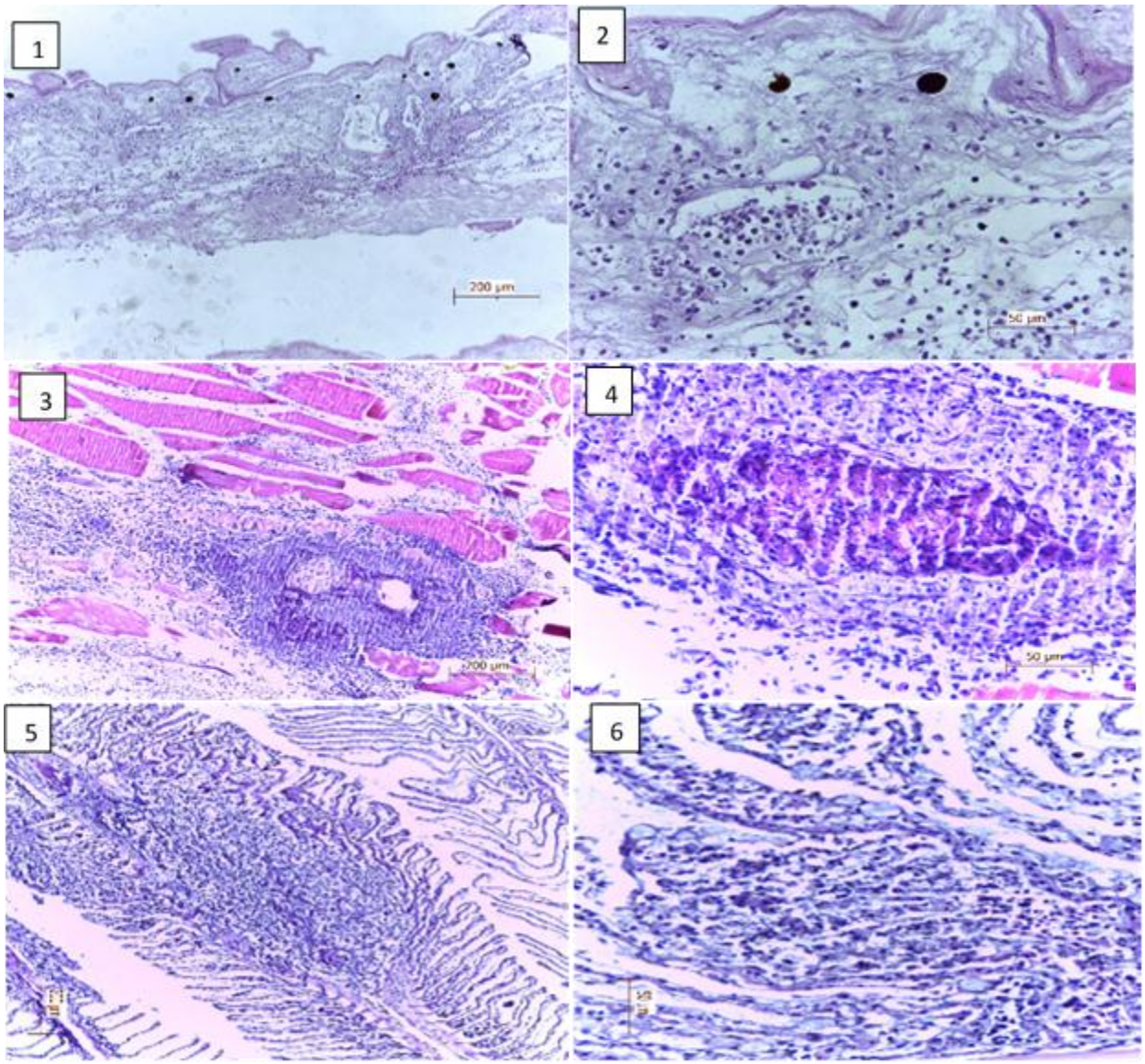

Figure 3. (1, 2) Skin infected with Janthinobacterium lividum revealed edema of skin with heavy heterophilic, macrophage and mononuclear cell infiltration, H&E, Bar= 200 µm & 50 µm. (3) The muscle of Broomtail wrasse Infected with J. lividum revealed diffuse mononuclear cell infiltration between muscle fibers and multiple granulomata associated with destruction and necrosis of muscular tissue, H&E, Bar= 200 µm. (4) Granuloma in skeletal muscle composed of central coagulative necrosis with bacterial colonies and surrounded with macrophages and heterophils. (5, 6) Gills revealed multiple granulomas in primary and secondary filaments composed of necrotic tissue, heterophils, and macrophages, H&E, Bar= 200 & 50 µm.

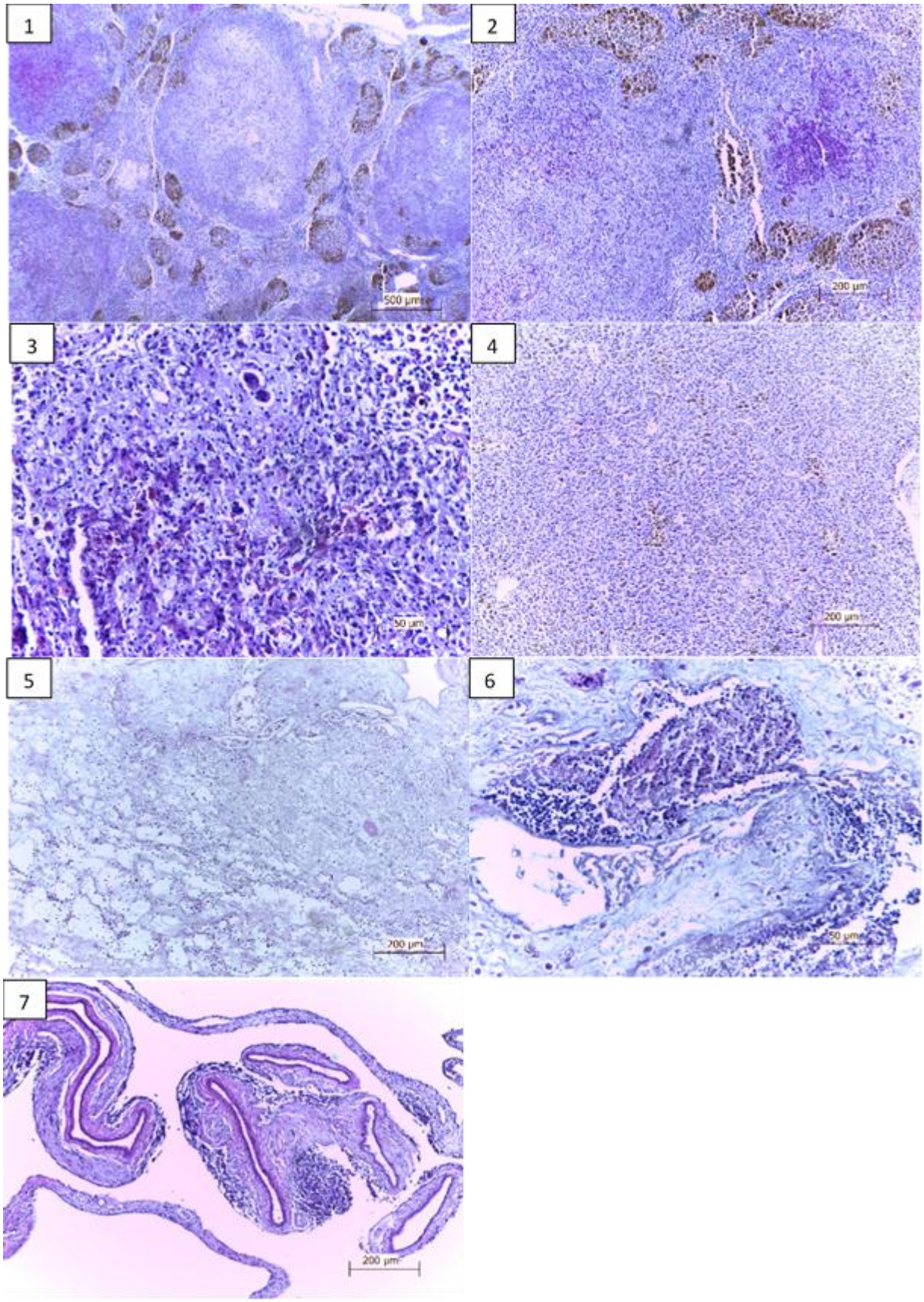
1
500 µm
2
200 µm
3
50 µm
4
200 µm
5
200 µm
6
50 µm
7
200 µm

Figure 4. (1,2) The spleen of Broomtail wrasse (Cheilinus lunulatus) infected with Janthinobacterium lividum revealed multiple granulomata composed of central coagulative necrosis and surrounded with macrophages. The granulomas were rich in heterophils, H&E, a. Bar= 500 µm, b. Bar= 200 µm. (3) The splenic granuloma in Broomtail wrasse infected with J. lividum composed of central coagulative necrosis rich in heterophils, surrounded with macrophages and giant cells, H&E, Bar= 50 µm. (4) Infected liver of revealed hepatic degeneration with bile accumulation and depletion of melanomacrophage centers, H&E, Bar= 200 µm. (5, 6) Subcutaneous fat revealed edema associated with diffuse infiltration of heterophils and macrophages. The focal granulomatous lesion was detected around vessels, H&E, Bar= 200 & 50 µm. (7) Microgranulomata in peritoneal membranes composed of chronic inflammatory cells, H&E, Bar= 200 µm.

5. Antibiotic Susceptibility Test: tested

The antibiotic susceptibility test for this study strain showed high resistance to all the tested antibiotics except chloramphenicol showed low sensitivity **(Table 1)**. This strain was more sensitive than MK757609 strain for the same antibiotics, as there no antibiotic treatment. *Janthinobacterium* spp. are known to normally be highly resistant to multiple antibiotics, especially β-lactam antibiotics in clinical studies **(Patijanasoontorn *et al.*, 1992 ; Schloss *et al.*, 2010)**, J. lividum Isolated from a non-healing diabetic foot wound showed failure in antibiotic treatment **(Redkar *et al.*, 2000)**, however, within 20 *J. lividum* strains only one has the resistance gene to Penicillin but all not have resistance gene to Gentamycin **(Shinkafi *et al.*, 2019)**. Sensitivity test for another species *J. psychrotolerans* a strain was resistant to penicillin and ampicillin but susceptible to streptomycin and tetracycline and the other was resistant to rifamycin SV, lincomycin and vancomycin **(Gong *et al.*, 2017)**. In general, there are no clinical trials have been published that determine if a particular treatment for *Janthinobacterium* infection **(Noga, 2010)**.

There has been a comprehensive analysis of the effectiveness inhibitory behavior of Janthinobacterium and other applications in different fields. Regarding pathogenic functions further work is required as well as investigations into possible therapies for these bacteria.

Table 1. Antibiotic susceptibility test results for J. lividum strain by the disk diffusion method.

Antibiotics	Drug amt. (mg)/disk	Diffusion zone breakpoint (mm)		Zone of Inhibition (mm)
		S	R	
Ampicillin (AM)	10	≥ 17	≤13	10
Cephalothin (KF)	30	≥ 18	≤ 14	10
Amikacin (AM)	30	≥ 17	≤ 14	5
Ofloxacin (OFX)	5	≥ 16	≤ 12	14
Streptomycin (S)	10	≥ 15	≤ 11	10
Neomycin (N)	30	-	-	12
Oxytetracycline (T)	30	-	-	10
Chloramphenicol (C)	30	≥ 18	≤ 12	19
Cefotaxime (CTX)	30	≥ 26	≤ 22	6
Erythromycin (E)	15	≥ 23	≤ 13	20
Norfloxacin (NOR)	10	≥ 17	≤ 12	12
Tetracycline (TE)	30	≥ 15	≤ 11	10
Ciprofloxacin (CIP)	5	≥21	≤ 15	15
Gentamicin (CN)	10	≥15	≤ 12	12
Oxalic acid (OA)	2	-	-	15

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