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# ISOLATION AND CHARACTERIZATION OF BACTERIAL PATHOGENIC *Bacillus* sp. FROM THE *Lethrinus lentjanin* COASTAL AREA OF INDIA

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**AUTHORS' CONTRIBUTIONS**

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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## ABSTRACT

India stands at the sixth-largest position in the sector of marine and freshwater fisheries and the second-largest in the production of aquaculture. In view of the economic benefits, this highlights the importance of controlling infectious fish diseases that pose threat to the production of aquaculture. It was hypothesized lesions found on the dorsal scales in the *Lenthrinus lentjan*, could be the reason for the massive morbidity and mortality. Live surface floating fish samples with pathological signs were collected from offshore areas of the Pallam fishing village, located in the Kanyakumari District of Tamil Nadu, India in August 2013. Detailed histological studies confirmed the presence of similar bacterial infections in the tissues adjacent to the lesion sites. The pathogen was isolated and it was subjected to a series of biochemical and 16S RNA sequencing and it was identified the causative bacterial pathogen as *Bacillus* sp. The pathogen possessed sub-terminal endospore-forming nature and also it has strong hemolytic activities. The organism was named as Bacillus sp. MSU1400. Furtherly, the study was complemented with the Pasteur method, performed on white carp fishes, in vitro, for the confirmation of the pathogen. The pathogen was sensitive to ampicillin, cephalexin, and gentamycin but it was resistant to tetracycline.

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**Keywords:** Histochemistry; *Lethrinus lentjan*; *Bacillus* sp.; 16S rRNA sequencing.

## 1. INTRODUCTION

Fish of genus *Lethrinus* have been utilized as significant edible resources along the areas from the western Pacific Ocean through the Indian Ocean because of their dominant population [1]. *Lethrinus lentjan* inhabits coastal and sandy areas, coral reefs, and deep lagoons of approximate depths of 50 m [2]. In the southwest of peninsular India - especially in the coastal areas of the Kanyakumari district - *Lethrinus lentjan* is the most widely and frequently spotted species [3]. Globally, marine fishes are often exposed to numerous environmental stressors: they include chemical and biological invaders. Such stressors are the potential predisposition factors that chronically suppress the immunity of marine aquatic animals in the affected marine habitats [4]. Severe problems that occur during climate change-linked environmental extremes might favour the pathogen and the additive effects of the rise in temperature and salinity, associated with climatic change are also of concern in this regard [5]. Stress associated with poor environmental conditions make them immunocompromised and, subsequently, more susceptible to pathogen infection [6]. Some fish species suffer from continuous depletion due to devastating environmental changes in their native aquatic environment [7]. The mode of transmission and virulence of pathogens can also be influenced by climatic change. Environmental perturbations associated with climatic change can significantly influence the disease process and thus lead to an increased disease outbreak and the spread of diseases to new geographical areas [5].

As the aquatic environment is also a habitat for many bacterial pathogens, such as obligatory and opportunistic pathogens including *Vibrio, Aeromonas, Pseudomonas, Photobacteria, Streptococci, and Staphylococci*, studies have shown their presence in several fingerlings, juveniles, adults and brood stocks of some marine fish species [8,9]. Fluctuating climatic changes also exacerbate bacterial invasion of marine species [10]. Incidentally, as a matter of fact, a rise in global warming also influences a rise in global atmospheric temperature that, in a long run, progressively causes the warming of land and water, making the aquatic environment hospitable for the thriving and proliferation of indigenous pathogens [11]. Infected *L. lentjan*, captured from Hurghada City coastline zone, Egypt, had diverse pathogenic infections, namely *Vibrio* sp. encompassing *V. cholerae, V. anguillarum, V. fluvialis, V. harveyi* and *V. alginolyticus*, etc.; this was attributed to the exposure of fish to variety of stresses like anthropogenic activities, sewage pollution, hypersaline water rejection of desalination plants, shipping operations, and landfilling [12]. Further in August 2009, the mass incursion of infected *L. lentjan* with polymicrobial skin lesions was reported along the coastal regions of Kanyakumari, India [13]. In the present study, we have identified and characterized the pathogen from *L. lentjan*, as a dominant, virulent *Bacillus* sp. MSU1400 causes dorsal scale-lesion and the massive incursion of the fish species.

## 2. MATERIALS AND METHODS

**A sampling of infected fishes:** Infected dead fishes of *L. lentjan* were found floating over the marine water surface and were collected from the offshore of Pallam (8’05’51’N 77°25’56’E) fishing village at Kanyakumari District of Tamil Nadu, India in August 2013. The samples were aseptically transported to the microbiology facility at the Centre for Marine Science and Technology (CMST), Rajakkamangalam within 30 minutes before the subsequent experimental analysis. Healthy *L. lentjan* were collected from the same fishing location for the control experiments.

**Identification of pathogen**

***Isolation of bacterial microbiota from infected fishes*:** Superficial areas with telltale signs of infection were wiped with 70 % ethanol and the tissue was dissected out aseptically, homogenized in Phosphate Buffered Saline (PBS), and serially diluted in the same buffer up to $10^{-7}$ dilutions. Triplicate samples were plated on Zobell Marine Agar 2216 (HiMedia Laboratories, Mumbai, India) for specific bacterial counts. The inoculated plates were incubated at 37 °C until the appearance of colonies (approximately 24 h).

***Histology:*** Infected and normal fish were dissected out, longitudinally, for the status of internal organs. The internal organs such as gills, intestine, and heart were dissected out in the control and infected *L. lentjan* fish and were fixed in 10% buffered formalin for 24 h. After 24 h, the fixed tissues were processed in graded levels of alcohol, cleared in xylene, impregnated, and embedded with paraffin wax. Trimmed blocks of the embedded tissues were treated, overnight, with 0.5% trichloroacetic acid (TCA) for the decalcification process. Decalcified tissue was thoroughly washed in running water and later sectioned (5-6 µm). De-paraffinized sections were rehydrated in graded alcohol, stained with hematoxylin and eosin, and were parallelly Gram-

stained. Further, the sections were dehydrated, cleared of xylene, and mounted using DPX. Stained sections were observed under a compound microscope and the observations were recorded and photographed at various dimensions [14].

***Phenotypic identification and characterization:*** Four dominant colonies from the Zobell Marine Agar plate passages were made into individual cultures. Basic microbiological and biochemical confirmations were performed as per Bergey's Manual of Systematic Bacteriology [15] and the Bacteriological Analytical Manual [16]. The identifications include Gram staining, motility, sub-terminal endospore-forming, hemolytic activity, indole production, methyl red, Voges-Proskauer, citrate utilization, glucose, adonitol, lactose, sorbitol, arabinose, mannitol, rhamnose, sucrose utilization, citrate test, ornithine test, urease test, phenylalanine test, deamination test, nitrate Reduction, and $H_2S$ production, etc.

***Genomic level identification by 16S rRNA sequencing:*** The dominant bacterial microbiota (*Bacillus* sp. MSU1400) was identified by 16S rRNA sequencing. Genomic DNA was extracted from the *Bacillus* sp. MSU1400 by CTAP DNA extraction protocol. One hundred nanograms of DNA template was amplified by standard PCR protocol using 16S rRNA universal primers (Forward: 5' CAGGCCTAACACATGCAAGTC 3'; Reverse: 5' GGGCGGWGTGTACAAGGC 3'). The PCR products were purified by a gel extraction kit (Medox Biotech India Pvt. Ltd) and sequenced (Amnion Biosciences, Bengaluru, India). The nucleotides of the 16SrRNA sequence were blasted in the NCBI database using the BLAST program. The construction of the phylogenetic tree was carried out using the Multiple Sequence Alignment by CLUSTALW software and the genetic distance was calculated.

***Pasteur method for confirmation of pathogen:*** Around twenty Carp fish, weighing an average of 20 gm and having a mean length of 15 to 20 cm, were introduced into a 0.04 $m^3$ glass tank, containing about 20 L tap water, maintained at a temperature of 20 ºC. High protein fish feed was added at a rate of about 1% of body weight per day. $LC_{50}$ value of MSU1400 was determined as described. A measure of 1ml ($9\times10^3 CFUml^{-1}$) of the isolate MSU1400 was added consecutively for five days to 20L of water where marine carp was kept. The Control tank contained healthy white carp, with no infection introduced. The experimental tank was monitored, for a period of two weeks, for the development of symptoms. Affected and dead fishes were subjected to the confirmation of the pathogen via performing histology and preliminary microbiological tests as mentioned above. Healthy carp fish, with no infection introduced, was used as a negative control.

***Antibiotic susceptibility*:** An antibiotic susceptibility test was performed against *Bacillus* sp. MSU1400 by single disk diffusion method [17]. Commercial discs such as ampicillin, kanamycin, cephalexin, tetracycline, gentamycin, and rifamycin were employed. *Bacillus* sp. MSU1400 was adjusted to a viable count of $1 \times 10^8$ $CFUml^{-1}$and the inoculum was spread uniformly over the entire surface of the Mueller-Hinton agar plate (Hi-Media). After 24 hours of incubation at 37°C, the diameters of the inhibition zone produced around the discs were measured and compared to those in an interpretive table.

## 3. RESULTS

***Anatomical changes of infected L. lentjan:*** Anatomical analysis of healthy versus diseased/infected fish revealed the presence of severe hemorrhages in muscles, with intense discoloration, throughout the body. We could not notice any deformations in any internal organs. The longitudinal section of the infected fish showed the presence of hemorrhages in most of the organs and caudal muscle region (Figs. 1A to G).

***Gram staining:*** Microbiological analysis revealed the presence of four different colonies from lesion sites. Histology with gram staining conducted on lesion sites revealed the presence of single-type rod-shaped bacteria (Figs. 2A to C). Surprisingly, there were no other bacteria observed in any other internal organs. The morphology of three of the four isolates did not match the one from the histology comparative analysis.

***Pathogen identification and confirmation:*** The phenotypic identification protocol revealed that the suspected bacterial microbiota was Gram-negative rod-shaped. The reducing sugars were found in the sub-terminal endospores at stressed conditions and utilized arabinose, mannitol, rhamnose, sucrose, galactose, and lactose while negative with indole, methyl red, Voges-Proskauer and were unable to utilize citrate and exert a β-hemolytic property in blood agar (Table 1; Figs. 3A and B). PCR amplification of the 16S rRNA gene from the genomic DNA of dominant rod-shaped bacterial samples gave a product of around 1,500 bp in size (Fig. 4A). It matched with a Gram-negative Bacillus species, and further confirmatory tests - through Biochemical tests. Then, the genome of the organism was extracted and the 16SrRNA was sequenced Sanger method as described in the materials and

methods. Phylogenetic and evolutionary analysis of the 16S rRNA sequence revealed that the organism is a *Bacillus* sp. and it was named *Bacillus* sp. MSU1400, the pathogenic *Bacillus sp.* shared the sequence homology with the *Bacillus pumilus, and Bacillus zhangzhouensis* as shown in Fig. 4B.

The suspected pathogen caused the same symptoms like scale weathering and hemorrhages on lesion sites as in *L. lentjan*. A minimum concentration of $2\times10^3$ inoculums was successful in developing skin lesions and death of white carp fish under laboratory conditions. Test carps and control healthy carps, when subjected to histological analysis, have revealed the presence of the suspected pathogen in the muscle tissues of the former whereas absent in the latter (Fig. 5).

***Antibiotic resistance*:** An antibiotic sensitivity test was tabulated in Table 2. The results revealed that, *Bacillus* sp. MSU1400 isolated from *L. lentjan* lesion was highly sensitive to ampicillin, cephalexin, and gentamycin and moderately sensitive to kanamycin and rifamycin. Also, they were resistant to tetracycline (Fig. 6).

**Table 1. Phenotypic identification of suspected bacterial microbiota isolated from the lesion of infected muscle tissue of *L. lentjan***

| Sl. No | Bio-Chemical Tests | Suspected bacterial micro biota isolated |
|---|---|---|
| 1 | Indole Test | - |
| 2 | Methyl Red | - |
| 3 | Voges-Proskauer's | - |
| 4 | Citrate Utilization | - |
| 5 | Glucose | + |
| 6 | Adonitol | - |
| 7 | Lactose | + |
| 8 | Sorbitol | - |
| 9 | Arabinose | + |
| 10 | Mannitol | + |
| 11 | Rhamnose | - |
| 12 | Sucrose | + |
| 13 | Citrate Test | + |
| 14 | Lysine Test | - |
| 15 | Ornithine Test | - |
| 16 | Urease | - |
| 17 | Phenylamine Deamination | - |
| 18 | Nitrate Reduction | - |
| 19 | $H_2S$ production | + |
| 20 | Glucose | + |
| 21 | Lactose | + |
| 22 | Arabinose | - |
| 23 | Adonitol | - |
| 24 | Sorbitol | - |

**Table 2. Antibiotic sensitivity/ resistance of Bacillus sp. MSU1400 isolated from the lesion of infected *L. lentjan***

| Sl. No | Antibiotics used | Activity | |
|---|---|---|---|
| | | Sensitive | Resistance |
| 1 | Ampicillin | Highly sensitive | |
| 2 | Cephalexin | Highly sensitive | |
| 3 | Kanamycin | Moderately sensitive | |
| 4 | Tetracycline | | Resistant |
| 5 | Gentamycin | Highly sensitive | |
| 6 | Rifamycin | Moderately sensitive | |

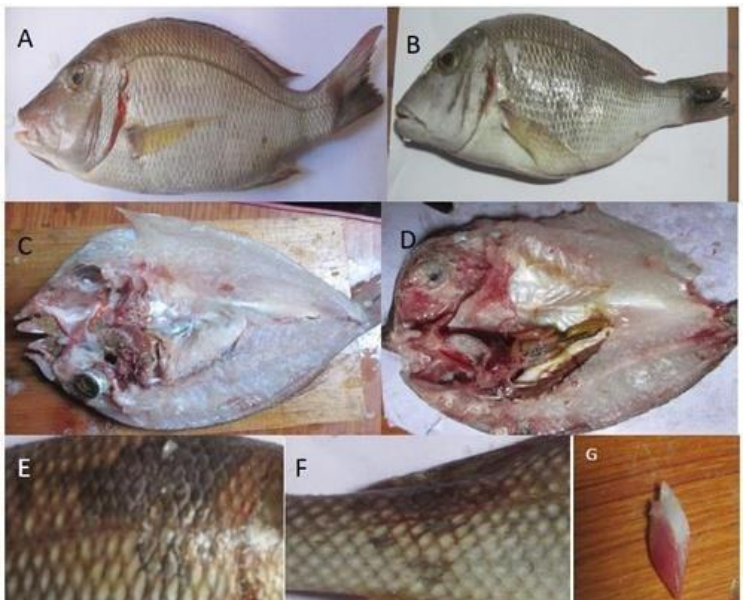


**Fig. 1. Longitudinal sectional view of Healthy and Infected *L. lentjan*. Normal *L. lentjan*, negative control (A), Infected *L. lentjan* with scale lesions and tart on dorsum area (B), Anatomy of healthy *L. lentjan* with no haemolysis or discolouration of muscles (C), Infected *L. lentjan* with haemolysis on major portions and pale colouration of muscles (D), Enlarged view of black tart due to haemorrhage in muscles at dorsal region (E), at tail region (F), haemorrhage in muscles (G)**

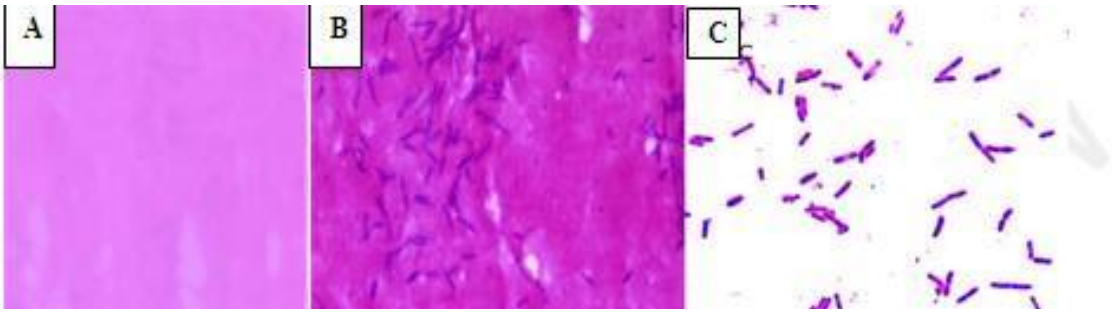


**Fig. 2. Gram staining through histopathological studies of the control fish muscle (A) and infected *L. lentjan* (B) at 40X magnification. Rod-shaped bacteria are present in the infected samples. Gram stained image of pathogen isolated sharing similarity in morphological view (C)**

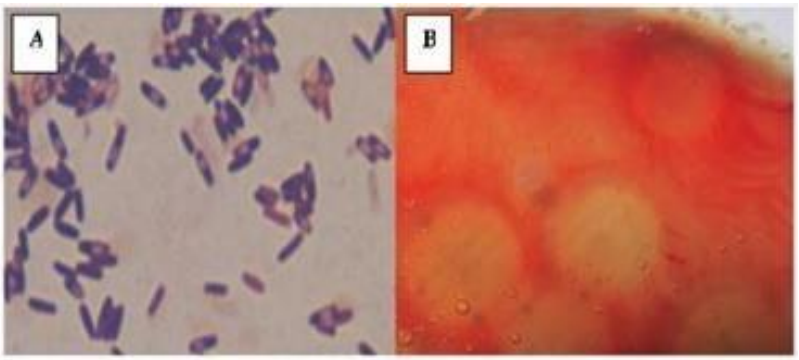


**Fig. 3. Preliminary characteristics of pathogen through microbiological assay. Sub terminal endospore forming nature of the suspected pathogen (a) and β hemolytic activity of the suspected pathogen in blood agar (b)**

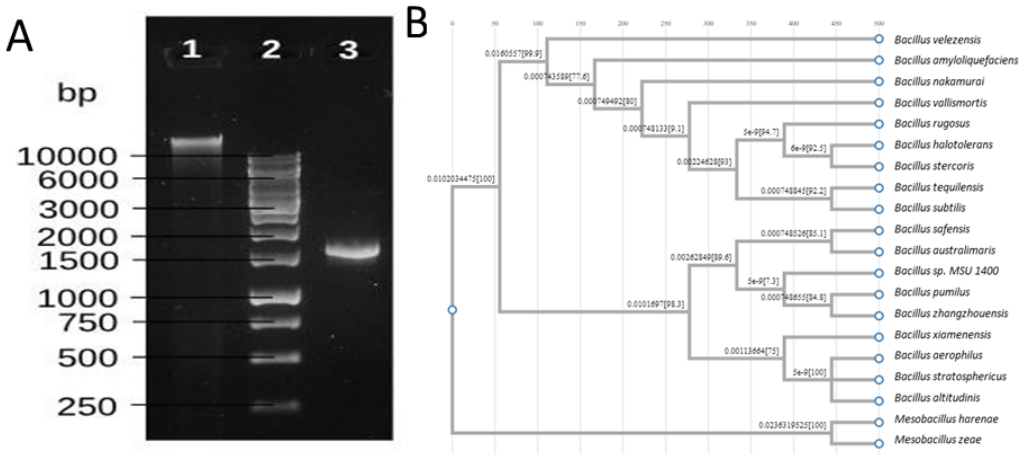


**Fig. 4. PCR amplification of 16S rRNA from genomic DNA of *Bacillus* sp. MSU1400 isolated from the lesion of infected *L. lentjan* using universal primers. Lanes1: Genomic DNA; Lane 2: Marker and Lane 3: 16S rRNA amplicon (A), Phylogenetic tree developed using CLUSTALW software where MSU1400 shares identity with *Bacillus* sp. (B)**

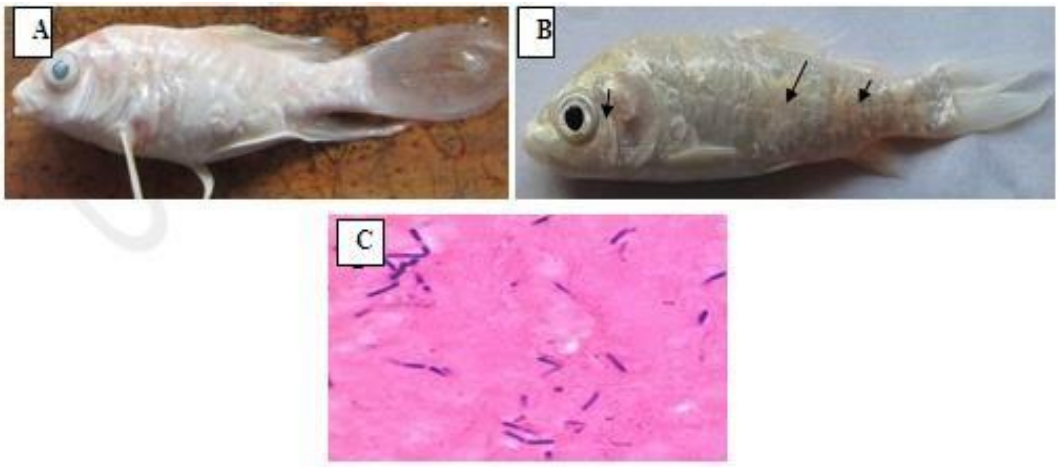


**Fig. 5. Pasteur method for confirming pathogen. Control healthy milky white carp (A), White carp infected with *L. lentjan* with scale loss, discoloration, and hemorrhage at eyes indicated with black arrows (B) Histology with Gram staining indicating presence of pathogen inside muscle tissues (C)**

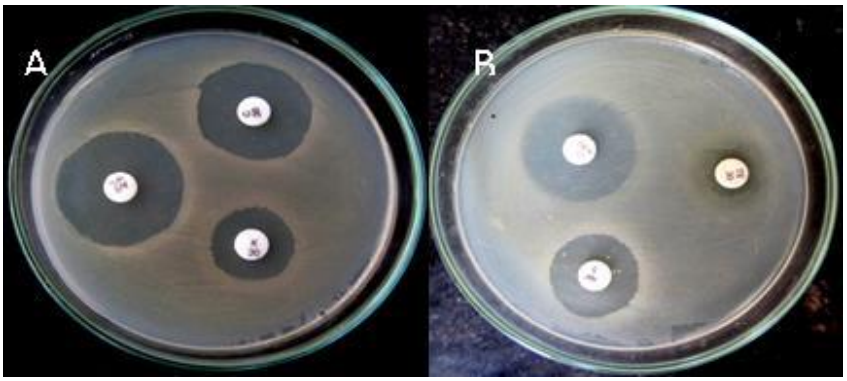


**Fig. 6. Kirby Bauer method of inhibition assay. Highly sensitive to ampicillin, cephalexin and moderate sensitive to kanamycin (A), and highly sensitive to gentamycin and rifamycin. Resistant to tetracycline with less zone formation (B)**

## 4. DISCUSSION

Environmental changes are inducing stress, at varying levels, to the aquatic animals and the fish population is more susceptible to microbial infections. Increases in temperature and salinity due to climatic variations are causing particular concern in this regard. The modes of transmission and virulence of pathogens can also be influenced by climatic change [5]. Devastating fluctuations in environmental variables have facilitated the depletion of different fish species like *Epinephelus tuna, Siganus rivulatus*, and *Dedlechilus labiosus* in the Suez Gulf at the Suez governorate. The representative fish samples had a broad range of bacterial pathogens including *Vibrio anguillarum, V. alginolyticus, Pfiesteria piscicida, Pseudomonas fluorescens, Aeromonas hydrophila,* and *A. sobria* [7]. Water temperatures have a significant impact on the concentrations of waterborne pathogens in surface waters. Growth patterns of pathogens are highly dependent on water temperature amongst some of the other environmental conditions mentioned. Some bacteria, algae, and amoebas can grow and proliferate in the aquatic environment independent of a host for replication. These species encompass *Acanthamoeba, Aeromonas, cyanobacteria, mycobacteria, Naegleria fowleri, and Pseudomonas aeruginosa* [11].

The present study was aimed at understanding the causative factor behind the mass mortality of the fish population along the coastal regions in the Kanyakumari district, and it could be attributed to the higher atmospheric temperature (~ 33 ºC) or other environmental changes in the sea. The environmental changes induce stress, leading to secondary microbial infection and ultimately to death [13]. A mass incursion of infected *L. lentjan* with polymicrobial skin lesions was reported on the Kanyakumari coast of India in August 2009 [18].

However, the present study was sensitized by the mass death of *L. lentjan* along the coastal side while some of them were incapacitated in a morbid state. They were sampled from the Pallam area of Kanyakumari with signs of hemorrhage and a visible pale coloration in muscles. Also, we found withering of scales and black tarts with lesions over the dorsum area of the skin. Stresses like the presence of heavy metals and oil pollution significantly affect marine fish populations and induce abnormalities in internal organs [19]. For example, histological alterations in the liver, gills, intestine, testis, heart, and muscle of *Oreochromis niloticus* and *Lates niloticus* by the exposure of different heavy metals like Fe, Zn, Cu, Pb, Cd, and Co, etc. The pollutants induce hemorrhage and hemolysis in the hepatocytes and dilation and intravascular hemolysis in hepato-portal blood vessels of the liver. Neotropical fish *Astyanax altiparanae,* collected from a polluted water stream had histological alterations in kidney tissues [20]. Due to the pollution or climatic and physicochemical changes, bacterial secondary infections induced damages like hemorrhage in muscles at the dorsal region of *L. lentjan.* Vibrio pathogens, including *Vibrio cholera, V. anguillarum, V. fluvialis, V. harveyi,* and *V. alginolyticus* were isolated from stressed fish species like *Siganus rivulatus*, *Mulloidichthys vanicolensis*, and *Lethrinus lentjan*, captured from the Red Sea along Hurghada City coastline zone, Egypt, due to Hurghada coastline being exposed to a variety of stresses as a result of anthropogenic activities, sewage pollution, hypersaline water, shipping operations, and landfilling [12]. The initial stage of microbiological identification revealed the presence of Gram-staining; phenotypic and genomic level identification have confirmed the lesion on scales at the dorsum area is responsible for the pathogenic *Bacillus* sp. MSU1400. Gram staining through histopathological studies is one of the effective methods for the identification of true bacterial pathogens responsible for the infection. Figs. 2a and b indicate the rod-shaped bacteria present in the tissue section. This supports the presence of rod-shaped Gram-positive *Bacillus cereus* WPD found inside the tissue sections of the abdominal muscles of the white patch infected *Litopenaeus vannamei* detected by histopathology with Gram staining [21].

The phenotypic identification confirmed that the suspected bacterial pathogen belongs to *Bacillus* sp. They also confirmed the endospore-forming nature and strong hemolytic activity. Some *Bacillus* sp. is highly virulent (proteolytic and hemolytic properties) and they cause secondary infections in fishes and shrimps. *Bacillus subtilis* is the causative bacterial pathogen causing bacterial white spot syndrome (BWSS) in *Penaeus monodon* in the commercial shrimp farms of Peninsular Malaysia [22]. *Bacillus cereus* WPD (white patch disease) caused high mortality in semi-intense cultured *Litopenaeus vannamei* because of their virulence factors like lipolytic and hemolytic activity during the environmental changes in the farms [21]. *B. subtilis* has been reported to excrete enzymes, mainly protease, amylase, glucanase, and lipase [23,24]. *B. cereus* is an important enterotoxigenic food-borne pathogen isolated from fish samples and had the *hbla* gene responsible for pathogenicity. [25,26] demonstrated that the abundance of *B. cereus* in the soil sediments carries a potentially functional *hlyII* gene and expresses the virulence factors which are optimal at the temperature of their natural host. The present study indicates that the higher hemolytic activity of the *Bacillus* sp. MSU1400 is responsible

for the infection with hemorrhage in the muscles and thereby causing weathering, black tart, and lesions in the scales of *L. lentjan.* The absence of pathogens in the rest of the vital organs arise an open-end question regarding the death of the fish. Possibly could be the hemorrhages that lead to fatal bleeding and deprivation of essential nutrients and oxygen. The presence and absence of any toxin were not confirmed in this study. Moreover, the genomic level identification reinforced the identity of the pathogen as being a *Bacillus* sp. and shared high similarity with several *Bacillus* species. The histopathological analysis also confirmed that the pathogenicity in Milky White Carps, with symptoms of scale loss, discoloration, and hemorrhage in eyes, is due to *Bacillus* sp. Aboyadak, I., isolated *Staphylococcus epidermidis*, *Bacillus cereus*, and *Pseudomonas stutzeri* from the infected fish called European bass [27]. Also Ali NGM, 2019 found a way to control the Gram-positive bacteria in aquaculture [28].

*Bacillus* sp. MSU1400 isolated from *L. lentjan* lesion was found to be highly sensitive to ampicillin, cephalexin, and gentamycin and moderately sensitive to kanamycin and rifamycin. *Bacillus* sp. including *B. megaterium, B. polymyxa, B. pumilus, B. subtilis, B. circulans, B. amyloliquefaciens* and *B. licheniformis* were susceptible to imipenem, vancomycin, chloramphenicol, gentamicin, and ciprofloxacin [28, 29]. Also, there are reports that tetracycline, chloramphenicol, kanamycin, and gentamicin inhibited almost all Bacillus strains.

Findings of the study concluded that, due to the environmental changes in the particular season, stress leads to secondary infection in *L. lentjan* by pathogenic Bacillus sp. MSU1400.

## 5. CONCLUSION

Animals evolve acquiring defensive strategies against infections, and concomitantly, microbes acquire adaptive strategies that make them able to invade the animals. Aquatic animals are no different in this aspect of animal-microbial interaction. The current study has been successful in attributing mass mortality of marine fish to the grave infection by a strain of the bacterial infection, which the current study has successfully unraveled and identified as a new strain – MSU1400. The study has shown that MSU1400 has wreaked havoc on the marine life of *L. lentjan*, and resulted in its severe morbidity and mortality. MSU1400 has been identified as a Gram-negative, rod-shaped bacterium, based on experiments. Reducing sugars were found at the sub-terminal endospores, and they utilized arabinose, mannitol, rhamnose, sucrose, and galactose for metabolic purposes. Cross-validation by inoculating the white carp fish with MSU1400 has reproduced the same pathogenic outcome(s), comprising morbid features such as weathering of scales and hemorrhages. The antimicrobial test has confirmed MSU1400 is more susceptible to antibiotics such as ampicillin, cephalexin, and gentamycin, and resistant to tetracycline. Based on PCR tests for 16SrRNA sequencing of the gene product, the pathogen has been identified as *Bacillus sp*, which is closely related to *B. pumilus.*

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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