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Isolation and Molecular Characterization of β- hemolytic *Carnobacteriummaltaromaticum* from Naturally Infected Longnose Parrot Fish (*Hipposcarusharid*) in Hurghada Red Sea, Egypt

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Abstract: *This study focused on the isolation of bacteria causing hemorrhagic septicemia to longnose parrot fish in the Fishery Port at Hurghada Red Sea, Egypt. The examined fish showed hemorrhagic skin ulcers in the head, fins and body. Water sample was taken to determine the water physico-chemical properties and also to identic their relationship with the incidence of the bacterial hemorrhagic septicemia..Full bacteriological, biochemical, and molecular characterization of the isolated bacterium were done. The isolated bacterial isolate was identified as Carnobacteriummaltaromaticum. It is Gram positive, non-spore forming rods, salt requiring, chitinolytic, β-hemolytic bacteria. The antibiotic sensitivity test indicated that the isolate was sensitive to ofloxacin, gentamycin, ampicillin, streptomycin, erythromycin, nalidixicacid, amikacin, ciprofloxacin, colistin, chloramphinacol, neomycin, doxycycline, aminocidin, sulfisoxazole, penicillin G, bacitracin, and cefaclor. The incidence of infection with C.maltaromaticum among fish in the Fishery port was about 13%. In this context, this is the first report of Carnobacteriummaltaromaticum from longnose parrot fish in warm water.*

Keywords: *Carnobacteriummaltaromaticum, longnose parrotfis, Red Sea.*

1. INTRODUCTION

Fish resource sector with its natural fisheries and fish farming is seen as one of the vehicles for attaining food security and bridging the widening food gap, and is a major part of the Egyptian economy. It is one of the important traditional components of Egyptian citizen's meal, for its comparative cheap fresh protein (Mohamed *et al*., 2010). Fish are susceptible to a wide variety of bacterial pathogens. Many of these bacteria are capable of causing disease. These bacteria only become pathogenic when fishes are physiologically unbalanced, nutritionally deficient, or when exposed to other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to proceed. Most common pathogens of fish are Gram-negative rods such as, *Aeromonas* and *Pseudomonas* spp, which are more common in freshwater and *Vibrio* spp, more commonly isolated from marine environments (Petty and Floyd, 2015). On the other hand infections by Gram +v rods are caused by streptococcus *Lactococcus*, *Enterococcus*, *Vagococcus*. *Carnobacterium*spp. appear to inhabit both the temperate and polar aquatic environments and was isolated from fish, marine sponges (Li and Liu, 2006), Antarctic lakes (Franzmann *et al.*, 1991;Bratina *et al.*, 1998), Arctic and Antarctic sea water as well as the deep sea (Galkin *et al.*, 1999; Groudieva *et al.*, 2004; Newberry *et al.*, 2004; Toffin *et al.*, 2004; Lauro *et al.*, 2007), and rivers in the northwest region of Spain (González *et al.*, 1999). *C. maltaromaticum* was reported to be a fish pathogen "by" Cone, 1982; Hiu et al., 1984; Herman et al., 1985; Michel et al., 1986; Baya et al., 1991; Starliper et al., 1992; Toranzo et al., 1993;Leinser*et al*., 2007; Thomas *et al*. 2008; Thomas *et al*. 2011; Casaburi*et al*.2011; Schaffer et al. 2013.*C.maltaromaticum*doesn't producecytochromeoxidase or catalase or H_2S. The temperature and salinity tolerance ranges from10 to 37°C and 0 to 6% NaCl, respectively (Baya*et al*., 1991). Other physical/ chemical parameters such as saltcontent, atmosphere and pH affect the survival and growth of these organisms in the natural

environment. The presence of the *C. maltaromaticum* -like bacterium was associated with splenomegaly, renal and splenic congestion, and thickening of the swim bladder wall with accumulation of mucoid exudates (Thomas *et al*., 2008). External reddening and hemorrhage in the peritoneum, body wall, and viscera are symptoms characteristics to a disease known as hemorrhagic septicemia (Petty and Floyd, 2015; Moeller, R.B., 2016). The present study was conducted to identify the bacteria causing hemorrhage in long nose parrot fish in warm water, Hurghada, Red Sea and investigate the symptoms of infected fish.

2. MATERIALS AND METHODS

Fish

Thirty (30) clinically diseased and moribund longnose parrot fish (*Hipposcarusharid*) were collected from Fishery port at Hurghada during summere 2014, they were subjected to clinical examination (Francis, 1999) and bacterial isolation.

Water Samples

Water samples were taken from the investigated Fishery port (control samples) in sterile bottles. Water temperature, pH, salinity and dissolved oxygen were measured using a multi-probe sensor of Hydro lab Instrument "according to" Barreto *et al*., 2007. Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were determined by 5-Day BOD technique and open reflux method respectively, total ammonia was determined using Pye-Unicam Spectrophotometer (Model PU-8600).

Bacterial Isolation

Samples for bacterial isolation were taken from liver and spleen of moribund and clinically diseased longnose parrot fish(*Hipposcarusharid*) and cultured on plates of Tryptone Soya Agar (TSA) (Oxoid) supplemented with 2% (w/v) NaCl. Plates were incubated at 30 °C for 2-5 days (Farmer &Hickman-Brenner, 1992).

Bacterial Characterization

The distinct colonies were sub cultured on blood agar to select the hemolytic bacteria as described by Buxton (2013), colonies showing β- hemolysis were characterized using the standard biochemical method as described by Alsina and Blanch (1994).Genomic DNA was extracted according to the manufacturer's instructions by boiling of 3 colonies in 100 µl of highly purified water.

PCR amplification of partial 16S rRNA gene was performed using the primer pair PA (5'-GAGTTTGATCCTGGCTCAG-3')and PH (5'-ACGGCTACCTTGTTACGACT-3').The PCR reaction mix was made up to 50 µl with 100 ng DNA, 30 nM each primer and 25µl of Dream *Taq* master mix from Fermentas. The amplification was performed using Applied Bio system therclermocy. The protocol for PCR amplification was a 5 min denaturing step followed by 35 cycles of 1 min at 95°C, 45 sec at 60°C and 45 sec at 72°C followed by a final extension step for 7 min at 72°C. The amplified 16S rRNA gene was purified by Qiaquik gel extraction kit from (Qiagen). The product was sequenced using ABI 370xl DNA sequencer using the forward primer. The nucleotide sequence was assembled in Bio Edit software (Hall, 1999). The nucleotide sequence was compared with similar sequences found in the NCBI database through BLAST program (www.ncbi.nlm.nih.gov/blast)). The nucleotide sequence was submitted to Gen Bank.

Antibiotic Sensitivity Assay

Sensitivity test was performed according to the manufacturer's instructions. TSA was supplemented with 2 %(w/v) NaCl, plates were evenly inoculated with the colony under test. The following chemotherapeutic agents were employed: ofloxacin 5µg, gentamycin10 µg, ampicillin 10 µg, streptomycin10 µg, erythromycin 15 µg, nalidixic acid 30µg, amikacin 30µg,ciprofloxacin 5µg,colistin 10µg, chloramphinaco l30µg,neomycin 30µg, doxycycline 30µg, aminocidin 60µg, sulfisoxazole 300µg, penicillin G10U, bacitracin10U,and cefaclor 30µg. The susceptibility to antibiotics were determined according to the size of clear zones (Miranda and Zemelman, 2001).

Results Clinical Signs

The clinical signs of the diseased *Hipposcarusharid* fish were skin darkness, scales detachment, ulcers, small and large areas of hemorrhages distributed over many parts of the body, particularly at

fin bases, mouth region and abdomen area which varied in its severity from fish to another, representative sample is "shown in"Fig.1. The main post mortem lesions varied among fish samples; some fish showed varied degrees of congestion, enlargement of internal organs especially liver, spleen, kidney and distention of gall bladder. Additionally, congestion of intestines and accumulation of body fluids in the abdominal cavities are "shown in" Fig.2.

Fig 1. *(Hipposcarusharid) with skin darkness and ulcers of varying sizes*

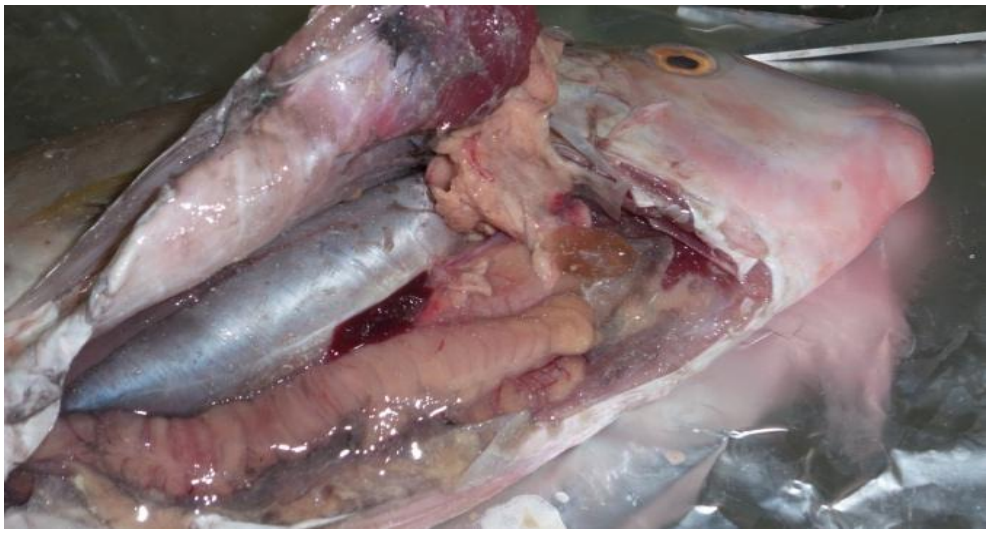


Fig 2. *(Hipposcarusharid) with congested & enlarged liver, spleen, kidney, and gall bladder distention*

Water Quality

The results revealed that the average(taken from 5 readings)of water quality parameters during June 2014were as follow: dissolved oxygen (DO), surface water temperature, pH, biological oxygen demand (BOD),chemical oxygen demand (COD) and ammonia recorded 3.49 mg/l, 31.66°C, 8.01, 1.95 mg/l, 23.52 mg/l and 8.24mg/l respectively.

Bacterial Characterization

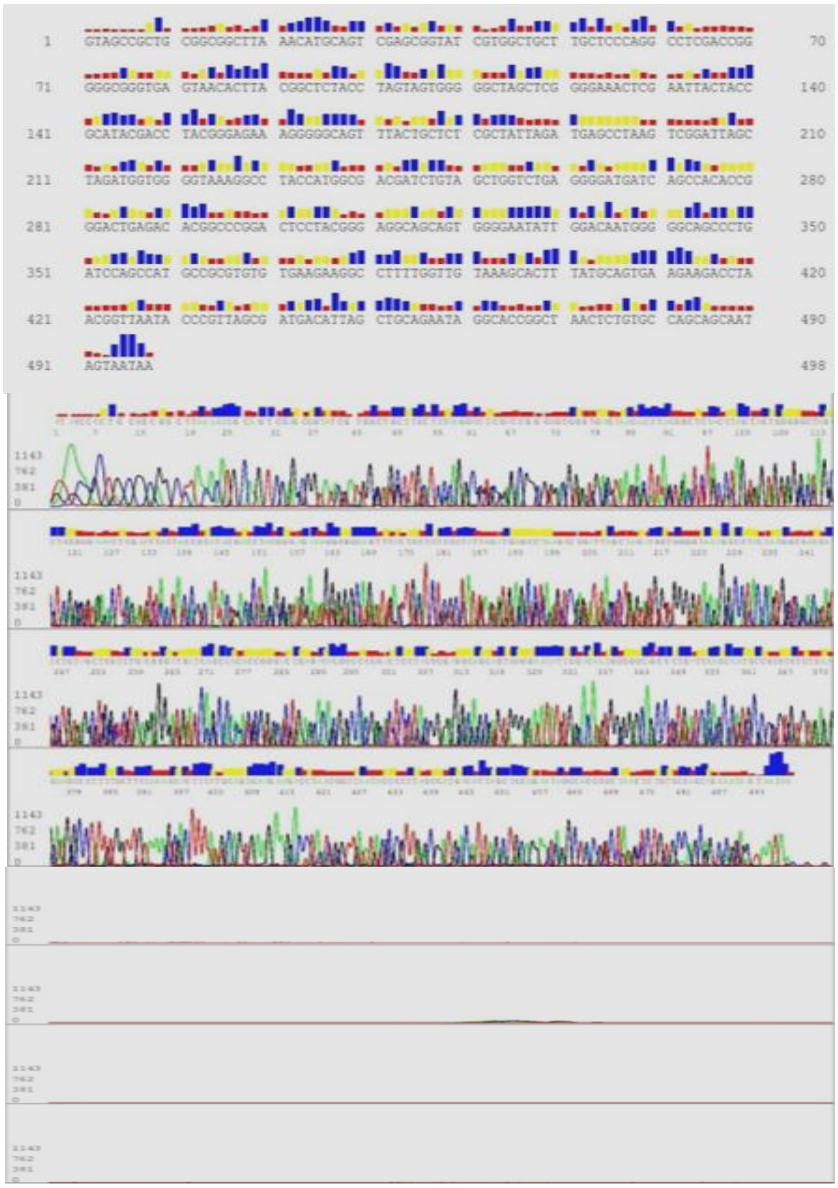
The β- hemolytic bacteria (Fig.3) were selected and subjected to biochemical and molecular characterization. Colonies produced on TSA were smooth, circular, buff-to-cream-colored and 10-15 mm in diameter with entire margins. The bacterial isolate was Gram positive rods, non motile, It didn't produce catalase, cytochrome oxidase or H_2S; moreover, the isolate was negative in Citrate and indole production tests. They were positive to lysine decarboxylase, ornithine decarboxylase, arginine dehydrolase and β-galactosidase. The bacterial isolate produced acid from carbohydrates fermentation test (Glucose, Manitol, Arbinose). However, no acid was produced from other carbohydrates such as inositol, sorbitol, sucrose, melebiose, amygdalinose or rhaminose .

Fig 3. *β Hemolysis* of *C. maltaromaticum*

Molecular Characterization

Sequencing of PCR product and comparison with NCBI data base resulted in the identification of bacterial isolate as *C. maltaromaticum* which was deposited in GenBank and "given the accession number of KY285267"(Fig.4).

F=Forward, R= Reverse **6F**



6R

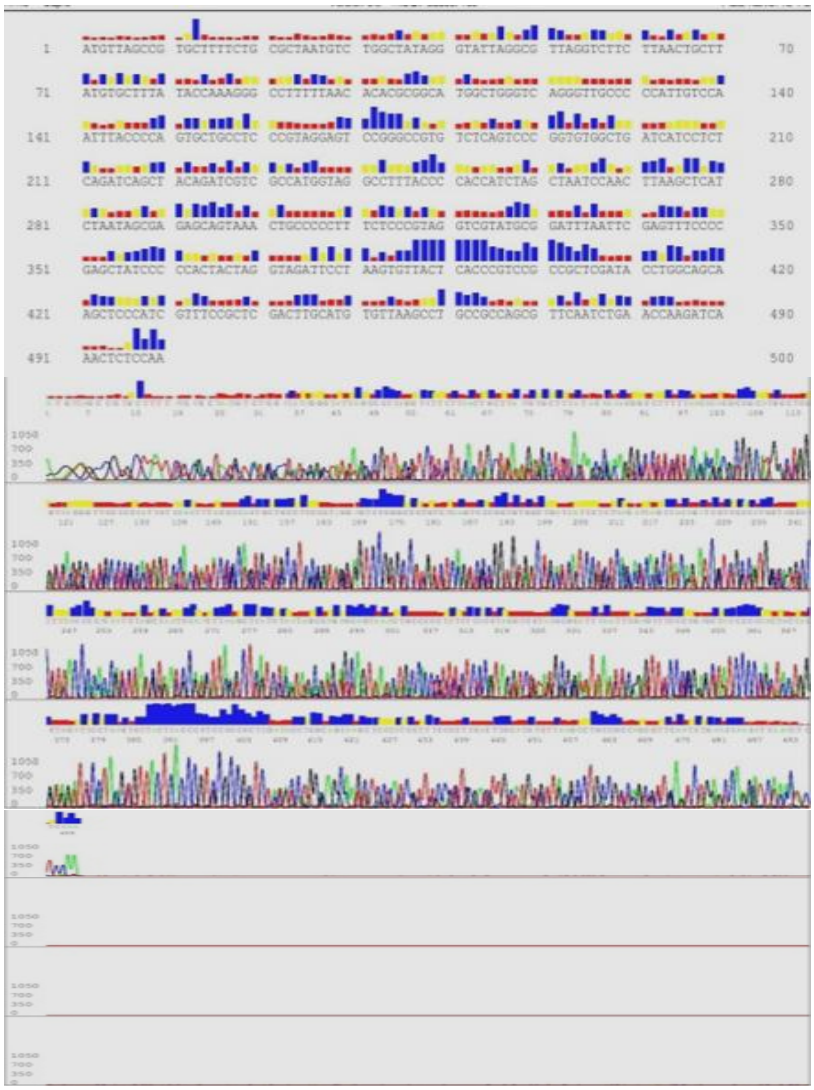


Fig 4. *Nucleatide sequencing of C. maltaromaticum*

Antibiotic Sensitivity Assay

The sensitivity test revealed that the isolated *C. maltaromaticum* was sensitive tostreptomycine, ofloxacin, ampicillin, nalidixic acid, amikacin erythromycin**,** chloramphinacol, neomycin, doxycycline, aminocidin, sulfisoxazole**,** ciprofloxacin, colistin, gentamycine, bacitracin, and cefaclor. as "shown in" Fig.5.

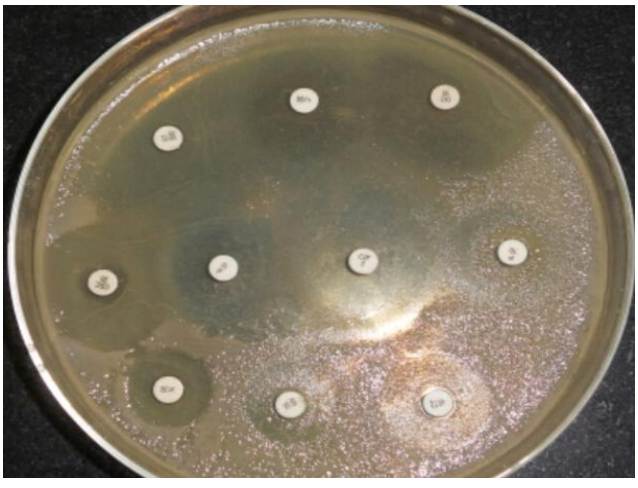


Fig 5. *Antibiotic sensitivity test with disc diffusion* of *C. maltaromaticum*

3. DISCUSSION

Although Carnobacteriummaltaromaticum was reported to be a fish pathogen by many authors (Cone, 1982; Hiu et al., 1984; Herman et al., 1985; Michel et al., 1986;Baya et al., 1991; Starliper et al., 1992; Toranzo et al., 1993;Leinser et al., 2007; Thomas *et al*. 2008; Thomas *et al*. 2011; Casaburi*et al*.2011; Schaffer et al. 2013), this is the first report in Egypt for the infection of Longnose parrot fish by *C.maltaromaticum*. The clinical signs of *C. maltaromaticum* infection in longnose parrot (*Hipposcarusharid*) fish are similar to other bacterial infections such as vibriosis, that were skin darkness, scales detachment, ulcers, small and large areas of hemorrhages distributed over many parts of the body. The post mortem lesions revealed congestion in liver, spleen, intestine, stomach (gastro enteritis) and kidney and enlargement of gall bladder. Similar observations were found in red mouth disease in fish (Kumar et al., 2015), and lake white fish (Thomas et al., 2008). The onset of the disease may be attributed to the suppression of the fish immune system due to decreased dissolved oxygen and increased ammonia values due to the increase in numbers of tourists and/or visitors in the recreational areas, and thus increasing the microbial pollution. The recorded dissolved oxygen of the Fishery port was 3.49 mg/l which is relatively lower than the average DO of the specified criteria according to Egyptian Environmental Lawglnesuid (Not less than 4 mg/l), BOD and recorded 1.95 mg/l and 23.52 mg/l respectively that were lower than the criteria of Egyptian Environmental Law, on the other hand ammonia recorded8.24 mg/l that was higher than the limits assigned by criteria of Egyptian Environmental law (3mg/l). Suomalainenet al. (2005) reported that the sharp increase in the ammonia level, water pH, physical contact and the sharp decrease in the dissolved oxygen are the most possible triggering factors for the initiation, establishment and spread of infection because these factors might jeopardizing the fish immune system. The β- hemolytic organism was suspected to be responsible for hemorrhagic symptoms; it was identified as *C. maltaromaticum* by the colony characters, cell morphology, Gram stain reaction, biochemical reactions and molecular identification using 16SrRNA. *C. maltaromaticum*was differentiated from other species by hemolysis test. To the best of our knowledge this is the first record of β- hemolytic activity in *C. maltaromaticum*, where previous reports (Baya et al., 1991; Leinser et al., 2007 Thomas et al., 2008;; Afzal et al., 2010 and Thomas et al., 2011) indicated α hemolytic activity of *C. maltaromaticum*. The antibiotic sensitivity test of *C. maltaromaticum*revealed that the isolated strain was sensitive tostreptomycine, ofloxacin, ampicillin, nalidixicacid, amikacin erythromycin, chloramphinacol, neomycin, doxycycline, aminocidin, sulfisoxazole, ciprofloxacin, colistin, gentamycine, bacitracin, and cefaclor. It is to be noted that Leinser *et al*., 2007described the positive and negative effects using Carnobacteria as probiotis in the environment and in food. This study highlights the importance of taking precautions before applying bacteria on large scale because of possible negative impact on human health. The extensive use of antibiotics in aquaculture to prevent bacterial infection may result in the emerging of antibiotics resistant strains, therefore It is a matter of importance in the field of fish disease control to find out other means of controlling the infection in aquaculture rather than use of antibiotics.

In conclusion the morphological, biochemical, and molecular assays performed in this study confirm that bacteria isolated from longnose parrot fish was *C. maltaromaticum*, this is the first report for isolation of *Carnobacteriummaltaromaticum*from longnose parrot fish in warm water of Hurghada, Red sea, Egypt. This finding illustrates that *Carnobacterium spp*. can potentially infect large portions of resident longnose parrot fish populations if conditions favorable for an epizootic occur. The implications of these outbreaks of disease are currently unknown and warrant further investigation

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