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PHOTOBACTERIUM DAMSELAE AS HISTAMINE PRODUCING BACTERIA IN DIFFERENT FISH SPECIES AND CANNED TUNA

By

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ABSTRACT

Histamine fish poisoning is a significant cause of food borne disease related to fish consumption. *Photobacterium damselae* are strong histamine producers, recently, it considered as an emerging, food borne pathogen of marine fish with a notable increase worldwide. So, a total of 20 fish samples (6 mackerel and 14 sardines) and 50 canned tuna were collected from different supermarkets and retail stores in Giza Governorate. The overall occurrence of histamine-producing bacteria (HPB) by plating the fish homogenates on modified Niven agar plates was 34.3%, the highest percentage was recorded in fresh sardine (85.7%), while the lowest one was detected in canned tuna (14%). The screening of the suspected colonies with PCR assay for the histidine decarboxylase encoding genes (*hdc*) evidenced that only 25% of fresh sardine HPB isolates were positive. Using of thiosulfate-citrate-bile salts agar (TCBS), evidenced that out of 70 fish homogenates, only 18 (6 mackerel and 12 sardines) isolates were identified as *Photobacterium spp*. The identification of these isolates using API 20NE evidenced that only 2 isolates obtained from fresh sardine isolates were *Photobacterium damselae* as well as harbored *hdc* gene. These results identify fresh sardine as a potential source of HPB, especially *Photobacterium damselae*. Therefore, proper handling of fish is mandatory in order to prevent this potentially serious public health threat.

Keywords:

Histamine, fish, TCBS, *hdc* gene and public health.

INTRODUCTION

Histamine fish poisoning (HFP) is the most common cause of ichythyotoxicosis worldwide and results from the ingestion of fish containing high levels of histamine in the Scombroidae and Scomberesocidae families, including mackerel, bonito, albacore, and skipjack. Other non-scombroid fish species are also implicated in HFP, as mahi-mahi, sardines, anchovies and herring **(Feng *et al.*, 2016)**.These fish contain characteristically high levels of free histidine in

their muscle tissues **(Lukton and Olcott, 1958)**. HFP commonly occurs because of inadequate time / temperature control during the transport, storage, or manufacturing of food products, which allows HPB to multiply and release histamine **(Etkind *et al.*, 1987)**. The histamine-producing bacterial (HPB) enzyme, histidine decarboxylase (HDC), converts large amounts of the free histidine present in these fish into histamine **(Takahashi *et al.*, 2008)**. Whereas, histamine is produced by a wide range of bacteria but the major in fish are Gram negative, mesophilic enteric bacteria, such as *Morganella morganii, Enterobacter aerogenes, Raoultella planticola,* and*Photobacteriumdamselae***(Kim *et al.*,2003, Hungerford, 2010)**. *Photobacterium damselae* is the most prevalent HPB in scombrotoxin-forming fish **(Bjornsdottir-Butler *et al.*, 2015)**; it belongs to the family *Vibrionaceae* that causes infections in a variety of marine animals and also in humans.This bacterium was detected by **Perez-Tirse *et al.* (1993)** in a 70 - year old man after suffering a knife cut while filleting bluefish (Pomatomus saltatrix). Moreover, it can cause opportunistic infections in humans that may evolve into necrotizing fasciitis with fatal outcome **(Rivas *et al.*, 2013)**.Although HPB are inactivated, HDC continue to produce histamine. Furthermore, the enzyme remains stable while frozen and may be reactivated very rapidly after thawing. Once histamine formed, not degraded by cooking, smoking or canning of fish, therefore, both raw and cooked fish might cause HFP **(Hungerfood, 2010)**. Histamine levels of ≥50 ppm in fish muscle tissues are indicative of decomposition, whereas levels of ≥500 ppm indicate a human health hazard **(FDA, 1995)**. The symptoms of HFP generally resemble with the symptoms from food allergies which are nausea, vomiting, diarrhea, oral burning sensation, hives, itching, rash, and hypotension **(Taylor *et al.*, 1989)**. Outbreaks are worldwide and are found in places where potentially spoiled, improperly handled scombroid-like fish species are eaten. In many incidents of HFP, mishandling of fish led to the formation of toxicological levels of histamine in the product by supporting the growth of HPB **(CDC, 2000).** Whereas, histamine in canned fish is due to use of poor quality raw material in which the amine has already formed. Moreover, histamine accumulation can occur also when frozen fish are thawed and kept for long periods at room temperature before further processing **(Prester, 2011)**. The fish products caused sporadic cases of HFP although it is mostly under strictly controlled temperatures during processing **(Takahashi *et al.*, 2015)**. Therefore, the purpose of the present study was to investigate the role of fish as potential sources of histamine producing bacteria (HPB), through detection of the occurrence of HPB in canned tuna and detect *Ph. damselae subsp. damselae* in scombroid (mackerel) and non-scombroid (sardine) fish samples.

MATERIAL AND METHODS

Samples collection:

A total of 50 canned tuna were purchased from different supermarkets, grocery and retail stores in Giza Governorate and 20 apparently healthy fish samples (frozen mackerel and fresh sardine) were collected. Six mackerel fish samples and 14 sardine fish samples were mainly derived from local markets or street fish vendors. All fish samples were put in sterile polyethylene bags, labeled, placed in ice box and immediately transferred to the laboratory of Zoonoses Department-Faculty of Veterinary Medicine- Cairo University, for further examination.

Samples preparation for bacteriological examination:

Ten grams of canned tuna as well as fish muscle tissues (obtained from back, abdomen and tail of fish) added into sterile poly-ethylene bag containing 90 ml of sterile 0.1% peptone water solution. The samples were homogenized for 2 min using stomacher (Lab blender 400, Seward lab Model No AB 6021) and centrifuged at 4000 rpm for 10 min. **(Fletcher *et al.*, 1998)**.

Isolation of HPB from fish muscles samples and canned tuna (Mavromatis and Quantick, 2002):

One ml from the previously prepared fish homogenate was plated on modified Niven's medium (MNM) that obtained from LAB M, Heywood, UK. Media components are tryptone (0.5%), yeast extract (0.5%), NaCl (0.5%), glucose (0.1%), Tween 80 (0.05%), $MgSO_4$. $7H_2o$ (0.02%), $CaCO_3$ (0.01%), bromocresol purple (0.006 %), $MnSO_4$. $4H_2o$ (0.005%), $FeSO_4.7H_2o$ (0.004%), agar (3%), and L-histidine monohydrochloride (2%). The plates incubated at 37 ºC for 48-72 h after sterilization at 121°C for 10 min, then examined every 24 h. Typical colonies of HPB are purple in color surrounded by purple halo on a yellowish background. The suspected colonies were picked up and sub-cultured for further examination on Tryptic Soya Agar.

Isolation of *Photobacterium damselae subsp. damselae* on thiosulfate-citrate-bile salts agar (TCBS):

One ml from fish homogenate plated on TCBS agar plates supplemented with 2% Nacl. The plates incubated at 25°C for up to 72 h, where typical *Photobacterium damselae* determined as colonies with green centers, **(Trevisani *et al.*, 2017).**

Identification of *Photobacterium damselae subsp. damselae*:

The morphological and biochemical identification of isolated colonies were applied using Gram staining, oxidase and catalase tests.

Detection of histidine decarboxylase gene (*hdc*) using convential PCR:

The simple boiling procedure developed by **Reischl *et al.* (1994)** involves thermal lysis has been used for the extraction of DNA from bacterial isolates. Two pairs of specific primers supplied from Metabion (Germany) targeting histidine decarboxylase gene (*hdc*) were used; Hdc_2F (5′-TGG-GGT-TAT-GTS-ACC-AAT-GG-3′) and Hdc_2R (5′-GTR-TGG-CCG-TTA-CGY-GAR-CC-3′) for amplifying *hdc* gene. According to **Wongsariya *et al.*, (2016)** in a PCR tube a mixture contained 12.5 µl of 2X PCR master mix, 1 µl of 10 Pico mole of each primer, and 3 µl of the template DNA in a 25µl reaction volume were added. A total of 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. were performed. The initial denaturation and final extension temperatures were 94 °C for 4 min and 72 °C for 4 min, respectively in a thermal cycler. PCR products (571bp) were visualized by agarose gel electrophoresis.

RESULTS

Table (1): Occurrence of histamine producing bacteria (HPB) on MNM and *hdc* gene by PCR in fish samples and canned tuna.

Samples type	Total examined No.	No. of HPB isolates		*hdc* gene	
		No	%	No	%
Canned Tuna	50	7	14.0	0	0.0
Fresh sardine	14	12	85.7	3	25.0
Frozen mackerel	6	5	83.3	0	0.0
Total	70	24	34.3	3	12.5

Table (2): Occurrence of *ph. damsealae* and *hdc* gene by PCR in fish samples and canned tuna.

Samples type	Total examined No.	No. of Photobacterium spp. Isolates on TCBS		Identification of *ph. damsealae* by API20 NE		*hdc* gene	
		No	%	NO	%	NO	%
Canned Tuna	50	0.0	0.0	0.0	0.0	0	0.0
Fresh sardine	14	12	85.7	2	16.7	2	100.0
Frozen mackerel	6	6	100.0	0.0	0.0	0	0.0
Total	70	18	25.7	2	2.9	2	100

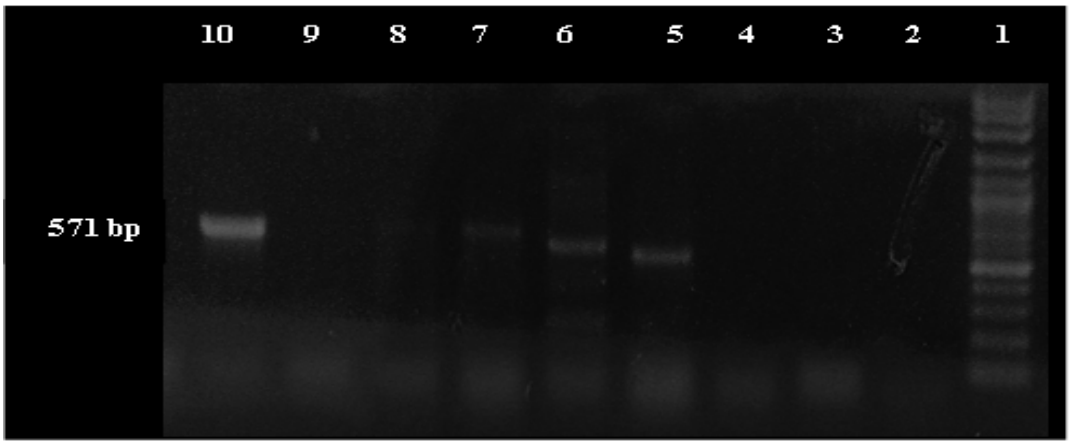

Photo (1): Detection of *hdc* gene of *bacterial isolates* obtained from fish**.**

Lane1: 100 bp DNA ladder.

Lane 5, 6, 7 and 10: *hdc* gene (571 bp).

DISCUSSION

Niven's medium was the superior medium for early detection of prolific histamine producing bacteria **(Chin *et al.,* 1989)**. The overall occurrence of HPB in muscles of fish samples on MNM was 34.3 % as shown in (Table 1), this finding is lower than that recorded by **Elsenduony *et al.* (2016) and Ibrahim *et al.* (2017)** they recorded that, the occurrence of HPB was 72.5 and 68 % in the examined frozen fish and fresh samples of carp fish muscles in Alexandria and Iraq, respectively. It worthy to mention that, the highest percentage of positive HPB was detected from fresh sardine (85.7%) and frozen mackerel (83.3%). The high recorded percentage of HPB in the examined fresh sardine and frozen mackerel may be attributed to some of these bacteria are present in the normal microbial flora of live fish, most seem to be derived from post-catching contamination on board fishing vessels, in the distribution system, or in markets, **Lehane and Olley (2000)**. On the other hand the lowest percentage was recorded in the examined canned tuna (14 %). Although this percentage is lower than those recorded in other examined fish sample, but it evidenced that contamination of cans occurred after heat processing. Our obtained results were lower than that reported by **Kim *et al.* (2004)** they isolated 40 % (12/ 30) of HPB from canned anchovies. Concerning the low number of isolates of HPB in canned tuna during the present study, this may be due to unfavorable growth condition, packing of the products with oil in cans; in addition, salt content in the canned products plays a critical role in inhibiting the growth of spoilage bacteria. Since Niven's medium is not selective, producing false positive isolates that must be

subsequently discriminated by PCR assays for the hdc gene **(De Las Rivas *et al.,* 2005)**. As cleared in Table and photo (1) the using of PCR with primer targeting to *hdc* gene in bacterial isolates revealed that 25% of fresh sardine HPB isolates were positive. While no *hdc* gene detected in the examined mackerel and canned tuna. This is evidence that not all positive isolates on Niven's medium gave PCR products when targeting the *hdc* gene, that nearly similar with that mentioned by **(Björnsdóttir *et al.,* 2009)**. This finding is clarified with **(Lopez-Sabater *et al.*, 1996; Fletcher *et al.*, 1998)**, who reported that detection of histamine-producing bacteria using Niven's agar resulted in false - positive rates. In addition, false-positive results are likely due to the production of one or more basic compound (s) capable of increasing the pH of the medium, resulting in the characteristic color change also created by histamine- producing strains **(Actis *et al.,* 1999)**. *Photobacterium damselae* has been recognized as a pathogen for a wide variety of aquatic animals and humans for this reason, it may be considered as an agent of zoonoses **(Austin, 2010)**. Meanwhile, NM was the superior medium for early detection of prolific histamine producing bacteria, many studies have demonstrated that among HPB there are some species that are not able to grow on the Niven medium a consequently false negative results occur as halophilic Photobacterium spp., are sensitive to its low pH **(Landete *et al.,* 2008; Costanza *et al.,* 2013)**. Therefore, to isolate the halophilic Photobacterium spp., TCBS specific culture medium is needed **(Trevisani *et al.,* 2017)**. As shown in (Table 2) out of the 70 cultured samples only 18 (25.7%) Photobacterium spp. colonies were detected by cultural method on TCBS. Whereas, Photobacterium spp. colonies were detected in all the examined frozen mackerel as well as it was detected in 85.7% (12/14) of the examined fresh sardine. On the other side, there is no Photobacterium spp. was isolated from canned tuna. The further identification of these isolates using API 20NE evidenced that out of 12 fresh sardine fish samples only 2 (16.7%) isolates were identified as *Photobacterium damselae,* however the all *Photobacterium spp.* isolates of frozen mackerel not identified as *Photobacterium damselae*. These findings are lower than that recorded by **Azwai *et al.* (2016)** who identified 3 (37.5%) *Photobacterium damselae* in 8 sardine fish samples in Libya. *Photobacterium damselae* was not identified in frozen mackerel harbored *Photobacterium spp*., this may be attributed to this bacterium is mesophilic bacteria can't grow at freezing temperature. Moreover, **Fujii *et al.,* (1994)** mentioned that *Photobacterium spp*. is low frozen-resistant species were scarcely detected in frozen-thawed fish and its products. For discrimination of *hdc* gene in *Photobacterium damselae* detected in fresh

sardine, PCR assays was applied and evidenced that two isolates of *Photobacterium damselae* from sardine was positive *hdc* gene. This result is similar to those of **Bjornsdottir *et al.*, (2009)** who recorded that all *Photobacterium damselae* isolates give positive reaction with *hdc* gene.

CONCLUSION

Despite, Niven's medium was the superior medium for early detection of prolific HPB, but it produces false positive isolates that must be subsequently discriminated by PCR assays for the *hdc* gene. Fresh sardine poses public health threat, whereas, the occurrence of HPB as well as ph. *Damselae* (high histamine producing bacteria) was high among the examined fresh sardine.

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