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INCIDENCE OF PATHOGENIC VIBRIOS AND OTHER RELATED SPECIES IN FRESH SHAOOR FISH FROM THE EGYPTIAN RED SEA FISHERIES.

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ABSTRACT

The occurrence and distribution of pathogenic vibrios and other related species in skin, gut and gills of shaoor fish *(Lethrinus nebulosus)* from the east coast of Egypt were studied. Microorganisms were enumerated using the most probable number (MPN) technique. Isolated species were identified using API 20E and BIOLOG biotyping systems. In skin samples, the highest incidence was observed for *Aeromonas media*-like DNA group 5 (70% of the samples). *Vibrio damsela* and *V. anguillarum* were the most frequently isolated species from gut (70%) and gills (50%), respectively. *Vibrio cholera* was detected in both skin and gills at the frequency of 5%, each. *Vibrio carchariae* was only present in 5% of the gills samples, whereas *V. alginolyticus* was only detected in skin samples at the same frequency. The high incidence of vibrios highlights potential risks of food borne diseases associated with the consumption of raw or undercooked fish.

Keywords : Pathogenic vibrios, *Lethrinus nebulosus,* API 20E, BIOLOG system.

INTRODUCTION

Vibrios are natural inhabitants of aquatic environments and marine species are transmitted to humans via consumption of raw or undercooked seafoods (Matte *et al.*, 1994). Vibrio is a large genus of the vibrionaceae family and containing 28 species as well as numerous biotypes and chemovars (Oliver, 1985). At least 11 species are known as pathogenic to humans (Hackney and Dicharry, 1988). These species include: *Vibrio alginolyticus, V. cholera, V. cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus, V. parahaemolyticus,* and *V. vulnificus.* Vibrios cause a variety of diseases including gastroenteritis, wound infection, ear infection and primary and secondary septicemia (Morris and Black, 1985; Wong *et al.*, 1995). Out of the 11 formentioned *Virbio* species, only 6 are associated with food borne diseases: *V. parahaemolyticus, V. vulnificus, V. mimicus, V. hollisae,* and *V. furnissii* (Hackney and Dicharry, 1988). Several studies on vibrios in seafoods have been reported (Kaysner *et al.*, 1990; Matte *et al.*, 1994; Wong *et al.*, 1995; Shih *et al.*, 1996; Buck, 1998; Sanjeev *et al.*, 2000; and Cavallo and Stabili, 2002).

Since, seafoods are becoming important commodities in the Egyptian diet and since, the risk-linked to *Vibrio* species, through the consumption of seafoods, was poorly evaluated. Therefore, this investigation was mainly initiated to study the incidence of pathogenic vibrios and other related species in one of the most popular fish type, Shaoor *(Lethrinus nebulosus)* from the Egyptian Red Sea fisheries.

MATERIALS AND METHODS

Fish samples

Twenty samples of whole fresh shaoor fish (*Lethrinus nebulosus*) from the Egyptian Red Sea fisheries were collected from different retail outlets in Cairo city. Each sample consisted of 2 fish and the average weight of individual fish was 1.0 kg. Samples were transported to the laboratory under aseptic conditions in crushed ice and analyzed immediately.

Reference microorganisms

Cultures of *Vibrio alginolyticus* (17749), *V. fluvialis* (33809), *V. furnissii* (35016), *V. hollisae* (33564), *V. mimicus* (33653), *V. parahaemolyticus* (17802), and *V. vulnificus* (27562) were originaly obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA. Stock cultures were maintained on Tryptic Soy Agar, TSA (Difco Laboratories, Detroit, Michigan, USA) containing 3% NaCl. Cultures were aerobically grown at 37°C (except *V. hollisae* and *V. vulnificus* at 30°C) for 24 h and refrigerated (4°C) until used. Cultures were transferred each two weeks for activation.

Bacteriological analysis

All bacteriological analysis were preformed on fish skin, gut and gills. Duplicate samples (ca. 50 g each) were homogenized for 2 min. in Waring Blender with sterile saline (0.85% NaCl) to give final dilution of 1:10.

Psychrotrophic microbial count

After homogenization, samples were then serially diluted using the same sterile diluent. From the appropriate dilutions, 0.1ml was spread plated on TSA. Plates were incubated at 7°C for 10 days (Cousin *et al.*, 1992) and colonies were counted and expressed as $\log_{10}$ psychrotrophic colony forming units (Log_{10} CFU/g).

Vibrio spp isolation and enumeration

Isolation and enumeration of vibrios were conducted in accordance with the Bacteriological Analytical Manual (Twedt and Stelma, 1984). Three 10 ml portions of the 1 : 10 dilution of fish samples were inoculated into three tubes containing, 10 ml double strength alkaline peptone water (APW). Three 1 ml portions of 1 :10, 1 : 100 and 1 : 1000 or higher dilutions were inoculated into single strength APW. Tubes were incubated overnight (18-24 h) at 35°C. After incubation, tubes showing growth were streaked onto thiosulfate citrate bile salts sucrose agar, TCBS (LAB-M, England) and incubated overnight at 35°C. Representatives from each colony type were selected according to color, shape and size. Isolates were purified by streaking on TSA plates supplemented with NaCl to a final concentration of 3%, and incubated at 35°C for 24 h. The isolated pure strains were then tested for oxidase reaction and identified using the API 20E identification system (API, BioMerieux, France) and the BIOLOG microstation identification system (Biolog, Hayward, CA, USA). Supplemental tests such as growth in Tryptic Soy Broth, TSB (Difco Labs, Detroit, Michigan, USA) containing 0 or 6% NaCl, and production of gas from glucose using phenol red glucose broth (Difco labs, Detroit,

(Difco Labs, Detroit, Michigan, USA) containing 0 or 6% NaCl, and production of gas from glucose using phenol red glucose broth (Difco labs, Detroit, Michigan, USA) were used when necessary. When the isolated colonies were finally identified, they were referred to the original positive dilutions in the APW enrichment broth and the 3-tube most probable number (MPN) tables (Barnard and McClure, 1984) were used for final enumeration of organisms.

Identification systems

API 20E system. Inocula were prepared from young (18-24 h) cultures grown on TSA supplemented with 2.5% (W/V) NaCl and cell suspensions, in 0.85% NaCl, were prepared according to the manufacture's instructions. Again identification steps were carried out as indicated by the manufacturer.

BIOLOG system. Cultures were grown for 18 h at 30°C (for *V. hollisae* and *V. vulnificus)* and at 37°C (for other vibrios) on TSA supplemented with 2.5% (W/V) NaCl. Inocula were prepared in a prewarmed (34°C) saline, and the density of cells was adjusted to give a transmittance level of 53% to 59% using the Biolog turbidimeter (Biolog Inc, Hayward, CA, USA). The Biolog-GN microplates (96 preloaded wells) were then directly inoculated with 150 µl / well of cell suspension using 8-channel repeating pipetter. Microplates were incubated at 34°C for 4 to 6 h or 24 h and readings were measured by the Biolog microplate reader using Microlog software for automated readings.

pH measurement

Duplicate samples of fish flesh (10 g) from each fish were homogenized in 20 ml distilled water for 2 min. The pH of the resultant slurry was then measured using Orion model 301 pH meter (Orion Research, USA).

RESULTS AND DISCUSSION

The fresh fish used in this study was of acceptable quality since its pH measurements indicated a level of 6.36 as an average. The maximum obtained reading was 6.78, while the lowest was 6.18. Frazier (1958) stated that the pH of fish flesh plays an important role on its perishability because of its influence on bacterial growth. He also indicated that the lower the pH of fish flesh, the slower the fish spoilage.

Psychrotrophic microbial count.

Healthy fish flesh is considered bacteriologically sterile and the highest contaminations of microorganisms in fish are found in intestine, gills, and surface slime (Nickelson II and Finee, 1992). It was also stated that the numbers and types of microorganisms found on freshly caught fish are influenced by the geographical location of the catch, the season and the method of harvest. Populations of psychrotrophic bacterial count (Log CFU/g) in fish skins, guts and gills are presented in Table 1. A mean value of 8.35 Log CFU /g skin was obtained. The maximum and the minimum values were

microorganisms associated with the slime coat of living fish. These numbers may range up to thousands per gram or cm^2 of skin surface. For gut samples, obtained results (Table 1) indicate an average bacterial load of 6.77 log CFU/g gut. The maximum and the minimum values were 7.72 and 5.63 log CFU/g, respectively. Ayres *et al.*, (1980) reported that intestinal microorganisms may reach up to 64 million/g. Finally, data in Table 1 show a high bacterial load in gills (8.29 log CFU/g gills). Much lower values were reported by Ayres *et al.*, (1980) with a range of 1.6×10^3 to 2.2×10^6 CFU/g. For almost all tested fish samples, much higher viable cell counts were obtained in the present study compared to those reported by Ayres *et al.*, (1980). Their data were obtained based on living fish (immediately after fish catch), whereas in the present study there was a significant time elapse between fish catch and sample collection and analysis. Certainly, such time allowed an extra growth of microorganisms originally contaminating fish. In addition, a possible microbial contamination and growth during fish handing and storage, delay in refrigerated storage and fluctuations in storage temperature could be considered to explain the above mentioned differences in viable cell counts.

Table (1): Total psychrotrophic count (Log_{10} CFU/g) of fresh fish (n= 20).

	Log_{10} CFU/g		
	Skin	**Gut**	**Gills**
Maximum	9.51	7.72	9.52
Minimum	7.34	5.63	7.11
Mean ± SD	8.35 ± 0.51	6.77 ± 0.52	8.29 ± 0.56

n = number of samples.

Identification of vibrios

Typing of bacteria involves characterization below the species level to enable subdivision into subgroups or types. A wide variety of typing methods exist, based on characterization of different properties of the bacteria. Such methods include : ribotyping (Austin *et al.*, 1997), serotyping (Larsen *et al.*, 1994), determination of outer membrane proteins (Lambert, 1986 and Austin *et al.*, 1997), determination of lipopolysaccharides (Kittelberger and Hilbink, 1993), plasmid typing (Rochelle *et al.*, 1985 and McCarthy *et al.*, 2000), biotyping with API 20E system (Austin *et al.*, 1995 and 1997), biotyping with BIOLOG-GN fingerprints (Kuhn *et al.*, 1996) and the PHENEPLATE system (Möllby *et al.*, 1993).

In this research, biotyping with API 20E as well as BIOLOG systems were examined using seven reference vibrio strains (Table 2). Close identification results were obtained for both systems, with the exception that API 20E system identified *Vibrio fluvialis* as *Aeromonas hydrophila / caviae* instead. It was also found that *Vibrio furnissii* was not listed in the API 20E system data base. *Vibrio metschnikovii* differs from all other vibrios in its lack of cytochrome oxidase (Lee *et al.*, 1978). Since BIOLOG system doesn't consider such test, therefore, isolates identified by BIOLOG system as

of cytochrome oxidase (Lee *et al.*, 1978). Since BIOLOG system doesn't consider such test, therefore, isolates identified by BIOLOG system as *V. metschnikovii were examined for their oxidase* activities. It was found that all *V. metschnikovii* isolates were oxidase positive and were reidentified as *V. cholera* by API 20E system. Both API 20E and BIOLOG are identification systems based on biochemical reactions of tested isolates. The obtained differences between such methods were partly due to the fact that the data were differently analysed.

Table (2): Accuracy verification of the used identification systems.

Ref. strain (ATTC #)	System	
	API 20 E	BIOLOG
V. mimicus (33653)	*V. mimicus*	*V. mimicus*
V. parahaemolyticus (17802)	*V. parahaemolticus*	*V. parahaemolyticus*
V. fluvialis (33809)	*A. hydr./ caviae*	*V. fluvialis* I
V. hollisae (33564)	*V. hollisae*	*V. hollisae*
V. alginolyticus (17749)	*V. alginolyticus*	*V. alginolyticus*
V. furnissii (35016)	Not listed	*V. furnissi*
V. vulnificus (27562)	*V. vulnificus*	*V. vulnificus*

Distribution of *Vibrio* species in shaoor fish.

Distribution of vibrios and related microorganisms was evaluated in skin, gut and gills of shaoor fish. In skin, the highest incidence was observed for *Aeromonas media*-like DNA group 5 (70% positive samples), followed by *V. anguillarum* (15%), *V. damsela* (10%), *A. trota* DNA group 13 (10%), *V. alginolyticus* (5%), *V. cholera* (5%) and *V. fluvialis* I (5%) (Table 3).

Table (3): Most probable number (MPN) and frequency of virbrios and related species isolated from skin of shaoor fish from the Egyptian Red Sea fisheries. (n = 20)

Species	MPN index /g		Frequency (%)
	Max.	Min.	
V. anguillarum	3.9×10^5	4.0×10^2	15
V. damsela	4.0×10^3	4.0×10^3	10
V. alginolyticus	4.0×10^4	4.0×10^4	5
V. cholera	4.0×10^3	4.0×10^3	5
V. fluvialis I	4.0×10^3	4.0×10^3	5
A. media-like DNA group 5B	$>1.1 \times 10^6$	4.0×10^2	70
A. trota DNA group 13	3.0×10^4	3.0×10^3	10

n = number of samples

Table 4 shows the MPN and the distribution frequency of vibrios and related species in shaoor's gut. While, *A. media* – like DNA group 5 was the major bacterium contaminating fish skin, *V. damsela* was the predominant in gut (70% positive samples). *Vibrio anguillarum, A. media*-like DNA group 5 and *A. trota* DNA group 13 were also recovered at 35, 10 and 5%, respectively, in tested samples.

Table (4): Most probable number (MPN) and frequency of vibrios and related species isolated from gut of shaoor fish from the Egyptian Red Sea fisheries. (n=20).

Species	MPN index /g		Frequency (%)
	Max.	Min.	
V. damsela	1.7×10^7	4.0×10^2	70
V. anguillarum	1.5×10^6	3.0×10^2	35
A. media- like DNA group 5B	9.3×10^4	9.3×10^3	10
A. trota DNA group 13	4.0×10^4	4.0×10^4	5

n = number of samples

In gills (Table 5), *V. anguillarum* exhibited the highest distribution (50%). *Vibrio damsela* and *A. media* –like DNA group 5 were the following most frequently isolated species (35%, each), although *A. trota* DNA group 13, *V. cholera, V. fluvialis* I and *V. carchariae* were also recovered at 10, 5, 5 and 5%, respectively.

Table (5): Most probable number (MPN) and frequency of vibrios and related species isolated from gills of shaoor fish from the Egyptian Red Sea fisheries. (n=20)

Species	MPN index /g		Frequency (%)
	Max.	Min.	
V. anguillarum	1.5×10^5	4.0×10^3	50
V. damsela	1.1×10^6	4.0×10^2	35
V. cholera	1.5×10^3	1.5×10^3	5
V. fluvialis I	7.0×10^3	7.0×10^3	5
V. carchariae	3.0×10^4	3.0×10^4	5
A. media-like DNA group 5B	1.1×10^7	4.0×10^2	35
A. trota DNA group 13	4.0×10^4	3.0×10^4	10

n = number of samples

The population of each species, determined as MPN / g, varied widely according to the tested part of the fish as well as to the retail outlet, the fish were purchased from (Tables 3,4,5). For example, A. media-like DNA group 5 ranged from (4.0 x 102 to > 1.1 x 106), (9.3 x 103 to 9.3 x 104) and (4.0 x 102 to 1.1 x 107) MPN/g) in skin, gut and gills, respectively. Vibrio damsela ranged from (4.0 x 102 to 1.7 x 107 MPN/g) within gut samples and from (4.0 x 102 to 1.1 x 106 MPN/g) within gills samples. On the other hand, the 2 positive skin samples, for the presence of such organism, carried equal number of cells (4.0 x 103 MPN/g). In gills, V. anguillarum the predominant organism (50% positive samples) ranged from (4.0 x 103 to 1.5 x 105 MPN/g). Such range increased in skin and gut samples reaching (4.0 x 102 to 3.9 x 105) and (3.0 x 102 to 1.5 x 106 MPN/g), respectively.

CONCLUSIONS

Both API 20E and BIOLOG systems were proven to be reliable mean for the identification of very close species of bacteria. The high incidence of

virbios and other related species indicates the potential risks of food borne diseases associated with the consumption of raw or undercooked fish. Good handling of fish after harvesting, good refrigerated transporation and storage will greatly reduce such risks. More studies are needed to evaluate the presence of such hazardous microorganisms in other types of fish and seafood from the Egyptian Red Sea fisheries.

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مدى تواجد ميكروبات الفبريو المرضية والأنواع الشبيهه فـــى أســماك الشــعور الطازجة من مصائد البحر الأحمر لجمهورية مصر العربية

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فى هذا البحث تم دراسة مدى تواجد ميكروبات الفبريو المرضية والأنواع الشـبيهة فـى جلود وأمعاء وخياشيم اسماك الشعور الطازجة من الساحل الشرقى لجمهورية مصر العربية. وقـد تم عد هذه الميكروبات باستخدام طريقة العد الأكثر احتمالا. كما تم التعـرف علـى الميكروبـات المعزولة باستخدام نظامى API 20E والـ BIOLOG. أظهرت النتـائج أن ميكـروبDNA *Aeromonas media*-like group 5B هو السائد في عينات الجلد حيث تواجد فـي ٧٠% من العينات المختبره. بينما كان ميكروبـى *Vibrio damsela* و *V. anguillarum* همـا السائدان فى عينات الأمعاء (٧٠%) والخياشيم (٥٠%) على التوالى. تم الكشـف عـن تواجـد ميكروب *V. cholera* فى عينات الجلد والخياشيم بنسبة ٥% لكل منهما. دلت النتائج عن وجـود *V. carchariae* فى الخياشيم فقط وبنسبة ٥% مـن العينـات بينمـا عـزل ميكـروب *V. alginolyticus* من عينات الجلد فقط وبنفس النسبة السابقة. التواجد الكبير لميكروبات الفـبريو يلقى الضوء على الخطورة المحتملة لحدوث الأمراض الغذائية والمتعلقة باستهلاك الأسماك النيئـة والغير جيدة الطبخ.