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Subclinical vibriosis in red swamp crayfish, *Procambarus clarkii*

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Abstract
Vibriosis is one of the major disease problems in shellfish. The infection is induced by several species of the genus *Vibrio*. The aim of the current study was to investigate infection with *Vibrio* spp. in red swamp crayfish, *Procambarus clarkii*, in Assiut, Egypt. Isolation and identification of different *Vibrio* spp. from the hemolymph and hepatopancreas of the red swamp crayfish in the River Nile and its tributaries were done. The isolates were characterized both biophysically and biochemically. Further characterization was achieved using PCR targeting 16S-23S rRNA intergenic spacer (IGS) regions of the genome. Results revealed that red swamp crayfish collected were infected with 10 different *Vibrio* spp. including *V. vulnificus* as the predominant species. The pathogenicity of a selected *V. vulnificus* isolate to red swamp crayfish was investigated through an experimental challenge via injection into the hemolymph. Characteristic signs and lesions appeared two weeks after challenge and included melanized spots on the dorsal and ventral surfaces of the body, melanization of both gills and hepatopancreas, and subsequently shell deformities. Eventually, challenged crayfish recovered by day 36 post challenge.

Keywords: Vibrio spp., red swamp crayfish, *Procambarus clarkii*, Egypt

1. Introduction
Red swamp crayfish, *Procambarus clarkii* production is a rapidly growing industry. In the United States of America, the annual income from this industry in Louisiana state ranging between US$30 and $50 million [1]. *P. clarkii* was introduced to Egypt in 1980's. Since then research has been conducted regarding their role as a carrier of many pathogens [2].
Many species of the genus *Vibrio* including *V. alginolyticus, V. harveyi, V. cholerae, V. mimicus, V. parahaemolyticus and V. vulnificus* can infect aquatic animals. Vibriosis has been verified as a disease of marine and freshwater fish, mollusks and crustaceans [3]. The infection in crustaceans is known as "shell disease" that is one of the most common diseases and characterized by typical signs of bacteremia [4]. Several cases of mortalities associated with *Vibrio* spp. in crayfish have been reported [5].
Systemic infections with *V. mimicus* and *V. cholerae* were recorded in red swamp crawfish, *P. clarkii* [6]. Also, *V.* mimicus was isolated from two commercially cultured species of Australian freshwater crayfish, the yabbie *Cherax albidus* and the red claw *Cherax quadricarinatus* [7].
The present study was conducted to isolate different *Vibrio* species from red swamp crayfish, and investigate the subclinical infection in such crayfish inhabiting the River Nile and its tributaries. The subclinically-infected crayfish may act as a reservoir for the pathogen and may cause overt infection under certain stress conditions or act as a potential source for infection to other aquatic animals or even human.

2. Materials and methods
2.1. Red swamp crayfish
All samples were caught from the River Nile and its tributaries, Assiut governorate, Egypt. Fifty crayfish were used for bacteriological isolation. Thirty four crayfish were collected from June to July 2011 with mean body weight of 25.7 ± 14.7 g. Because of their hibernation habit, there were no crayfish samples available after July 2011 to May 2012. The other sixteen crayfish were collected from June to July 2012, with mean body weight of 37.5 ± 11.1 g.

2.2. Isolation and identification of *Vibrio* spp.

Bacteria were isolated from the hemolymph as was previously described [8], and from the hepatopancreas (digestive gland) with a sterile loop after aseptic autopsy and disinfection of the surface.

Samples were cultured on Trypticase Soy Agar (TSA; Lab M Limited, United Kingdom) supplemented with 1.5% sodium chloride and on a *Vibrio* spp. enrichment medium; alkaline peptone water (peptone 10 g/L, sodium chloride 20 g/L with pH adjusted to 8.6 ± 0.2). After incubation at 28 °C for 48 h, the bacterial growth from either medium was subcultured on the *Vibrio* spp. selective medium; thiosulfate citrate bile salt sucrose agar (TCBS; Lab M Limited, United Kingdom) and incubated at 28 °C for 48 h as was previously described [9].

Purified bacterial isolates were identified both biophysically and biochemically based on colony morphology, microscopic examination and biochemical tests (Table 1) according to Farmer and Janda [10].

2.3. DNA fingerprinting

A polymerase chain reaction (PCR) targeting 16S-23S rRNA intergenic spacer (IGS) region was conducted that resulted in amplicons with variable lengths and sequences from one species to another. The resulted amplicons were analyzed by gel electrophoresis giving a unique species-specific pattern for each species [11].

The primers sequences used were: 5′-ACTGGGGTGAAGTCGTAACA-3′ for the 16S rRNA and 5′-CTTCATCGCCTCTGACTGC-3′ for 23S rRNA.

2.4. Experimental infection

Apparently healthy crayfish were acclimated to the lab conditions for 4 weeks and divided into 4 groups with 12 crayfish each. A selected *V. vulnificus* isolate in the current study was used for experimental challenge. Bacteria from a fresh culture on TSA (containing 1.5% sodium chloride) were suspended in sterile crayfish saline as was described by Goins [12]. Crayfish of three groups were injected in the hemolymph with 0.1 ml of the bacterial suspension (approximately 2.5×10^{10} CFU/ml of the bacterial suspension). The fourth (control) group was injected with the same volume of sterile crayfish saline without bacteria. Crayfish challenged were sacrificed at 3, 6, 14 and 36 days post injection where clinical signs were recorded, autopsy was performed and bacteria were re-isolated from either hemolymph or hepatopancreas.

3. Results

3.1. Isolation and identification of *Vibrio* spp.

A total of 41 isolates suspected to be *Vibrio* spp. were isolated from the hemolymph and hepatopancreas of examined crayfish. Thereafter, the biophysical and biochemical characteristics of these isolates revealed different species of the genus *Vibrio* (Table 1). Isolates recovered from hemolymph and/or hepatopancreas were identified as *V. vulnificus* (11 isolates, 26.9%), *V. parahaemolyticus* (8 isolates, 19.5%), *V. fischeri* (5 isolates, 12.2%), *V. penaeicida* (5 isolates, 12.2%), *V. harveyi* (3 isolates, 7.3%), *V. scophthalmi* (3 isolates, 7.3%), *V. mimicus* (2 isolates, 4.9%), *V. cholerae* (2 isolates, 4.9%), *V. alginolyticus* (1 isolates, 2.4%), and *V. mytili* (1 isolate, 2.4%). Thus, *V. vulnificus* had the highest isolation rate among the isolated *Vibrio* spp. (Fig. 1). Various isolates showed different colonies colors on TCBS (yellow or green) and different biochemical characters (Table 1).

Table 1: Biophysical and biochemical characteristics of the isolated bacteria (Number of positive/ total number of isolates)

Test	*V. vulnificus* n=11	*V. parahaemolyticus* n=8	*V. fischeri* n=5	*V. penaeicida* n=5	*V. harveyi* n=3	*V. scophthalmi* n=3	*V. mimicus* n=2	*V. cholerae* n=2	*V. alginolyticus* n=1	*V. mytili* n=1
Colony color[1]	G[2]	3Y[3]/5G	Y	G	Y	Y	G	Y	Y	Y
Gram stain	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Oxidase	7/11	8/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
Motility	11/11	8/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
OF test (F[4])	7/11	8/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
Catalase	7/11	8/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
H_2S	0/11	0/8	3/5	0/5	0/3	0/3	0/2	0/2	0/1	0/1
Indole	5/11	3/8	0/5	0/5	3/3	0/3	2/2	2/2	0/1	0/1
MR test	7/11	3/8	3/5	5/5	3/3	3/3	0/2	2/2	1/1	0/1
VP test	0/11	0/8	0/5	0/5	0/3	0/3	0/2	2/2	1/1	0/1
TSI	10K[5]/A[6]-1A/A	7K/A-1A/A	3K/A-2A/A	4K/A-1A/A	3K/A	A/A, gas	1K/A-1A/A	K/A	K/A, gas	K/A
Sensitivity to O/129:										
10 μg	11/11	0/8	5/5	5/5	0/3	3/3	2/2	2/2	1/1	0/1
150 μg	11/11	8/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
NaCl tolerance:										
0%	0/11	0/8	0/5	0/5	0/3	0/3	2/2	2/2	0/1	0/1
1.5%	11/11	8/8	0/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
3%	7/11	7/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1
6%	5/11	7/8	3/5	0/5	3/3	0/3	1/2	0/2	1/1	1/1
8%	0/11	5/8	0/5	0/5	0/3	0/3	0/2	0/2	1/1	1/1
Citrate (Simmons)	9/11	7/8	1/5	0/5	1/3	0/3	2/2	2/2	0/1	1/1
String test	11/11	5/8	5/5	5/5	3/3	3/3	2/2	2/2	1/1	1/1

[1]Bacteria cultured on TCBS, [2]Green, [3]Yellow, [4] Fermentative, [5]Alkali reaction, [6]Acid reaction
1.5% NaCl was added to each medium used for biochemical test

3.2. DNA fingerprinting

Performing PCR with 16S-23S rRNA primer for a representative of *V. vulnificus* isolates yielded two major bands (600 and 650 bp) and two minor bands (300 and 400bp) characteristic to *V. vulnificus* (Fig. 2).

3.3. Experimental infection

Three days post-infection, external and internal examination revealed no typical clinical signs of vibriosis except slight reddish coloration of the hemolymph. On the 6th day post-infection, slight congestion of gills and tail muscle has been

noticed. Thereafter, characteristic black spot lesions of the vibriosis on the dorsal and ventral surfaces of the shell were found 14 days post-infection. Also, discoloration and fissures in the chitin, shell deformities, blister formation, erosions, ulcerations and necrosis in uropod and telson have been recorded. Internally, there was congestion in the brain. In one case, gastrolith was severely congested. Gills and hepatopancreas were dark brown (melanization). Muscle varied from translucent to opaque in color and was severely congested in one case. However, after 36 days, no typical external clinical signs were recorded except in one case, where the shell was deformed. Gills ranged from normal to slightly brown in color. Hepatopancreas was brown, greenish or pale yellow. Fig. 3 shows the clinical signs appeared after the experimental infection. Bacteria were successfully re-isolated from either hemolymph or hepatopancreas.

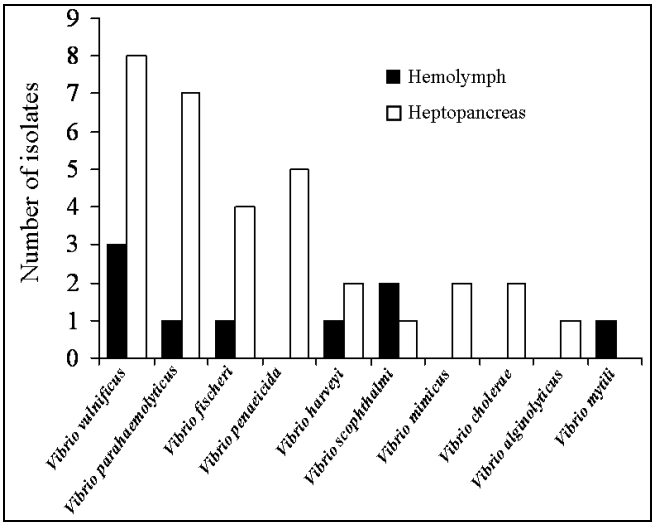

Fig 1: *Vibrio* spp. (n=41) isolated from hemolymph and/or hepatopancreas (digestive gland) of *Procambarus clarkii.*

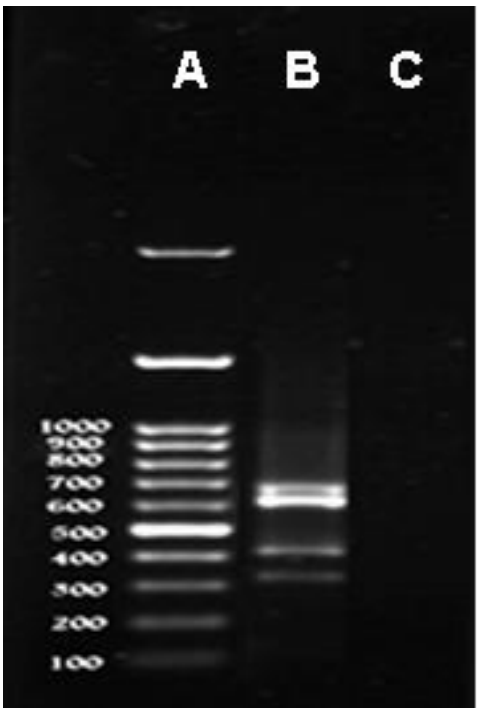

Fig 2: Electrophoretic analysis of PCR-amplified 16S-23S rRNA intergenic spacer (IGS) regions of *Vibrio vulnificus*. Lane A: Molecular weight marker (100-bp DNA ladder); Lane B: *V. vulnificus*; Lane C: negative control.

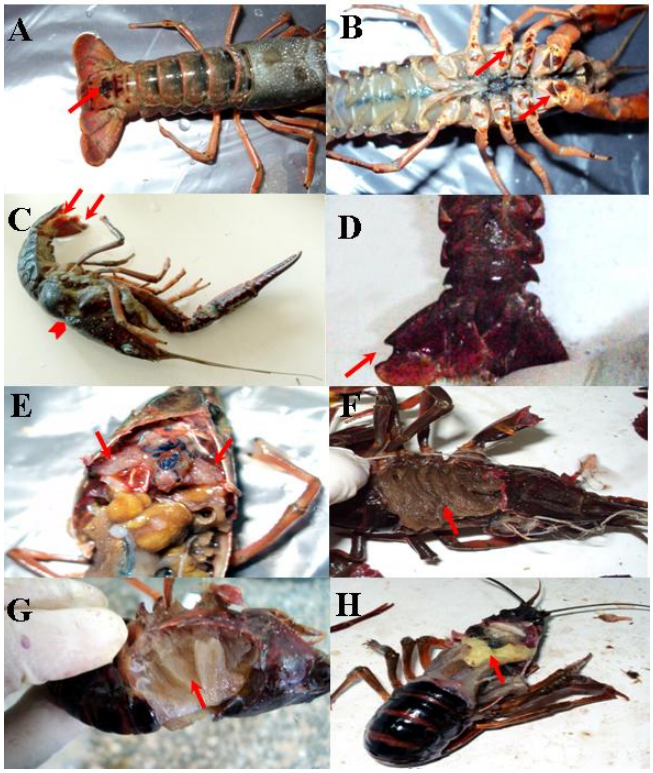

Fig 3: *Procambarus clarkii*, experimentally infected with *Vibrio vulnificus* (2.5×10^9 CFU/individual). Fourteen days post-infection: the dorsal surface (A) and ventral surface (B) displayed the characteristic black spot (melanization) sign of vibriosis (arrows), erosions in tail fan (C; arrows) and deformities in the shell (C; arrowhead), ulceration in the uropod (D; arrow), congestion in the brain (E; arrows), and dark brown gills (F; arrow). Thirty six days post-infection: gills became light brown (G; arrow), and hepatopancreas showed pale color (H; arrow).

4. Discussion

Aquatic crustaceans can tolerate bacteria in the hemolymph with no apparent pathology, and existence of bacteria is not necessarily an indication of disease [13]. However, the presence of hemolymph flora especially known pathogens does provide a source of potential threats of diseases. In the current study, 41 isolates of *Vibrio* spp. have been isolated from the hemolymph and hepatopancreas of red swamp crayfish exhibiting no specific clinical signs.

Due to winter-hibernation habit of the red swamp crayfish, all the samples were collected in summer. In this season, the high temperature may induce lowering of the crayfish immunity by thermal stress, while that temperature may be favorable for pathogens growth and multiplication. Scott and Thune [14] and Edgerton *et al*. [15] reported that the prevalence of bacteremia in healthy crayfish increased during periods of high temperature and low dissolved oxygen.

The present results revealed higher number of bacteria recovered from the hepatopancreas than from the hemolymph as shown in Fig. 1. This may be attributed to the eating habit of crayfish. They feed on aquatic plants and living organisms as earthworms and small fishes [2]. Pathogens enter the hemocoel of the body by oral route or through wounds, multiply in the hemolymph then distribute to body tissues [15, 16]. In the present study, 10 different *Vibrio* spp. have been isolated and characterized biochemically, as mentioned above. Previous studies [6, 17, 18] reported isolation of *V. mimicus*, *V. cholerae*, *V. alginolyticus* and *V. parahaemolyticus* from hemolymph and hepatopancreas of *P. clarkii*. The different biophysical and biochemical characteristics displayed by the

different isolates in the current work has been stated previously [10]. Of the isolated *Vibrio* spp. in the current study, *V. vulnificus* represented the most frequent isolated species (26.9%) with 11 isolates. Raissy *et al*. [19] isolated different *Vibrio* spp. from narrow-clawed crayfish, *Astacus leptodactylus* with highest isolation rate exhibited by *V. vulnificus*.

The detection and identification of *Vibrio* species is traditionally reliant on culture morphology on selective medium (TCBS) and biochemical characters [20]. However, *Vibrio* species and other closely related species show similar phenotypic features and are not easily distinguished biochemically [21]. Identification systems based on molecular techniques were proved to supply more accurate taxonomical information than the traditional biochemical methods [22, 23]. The differences in the length and sequences of the 16S–23S intergenic spacer regions (IGSs) of rRNA are used for bacterial identification [24, 25] to the species level depending on the special DNA pattern "fingerprint". A typing system based on PCR amplifying the intergenic spacer (IGS) for *Vibrios* proved to be rapid, reliable and efficient and has the capacity to identify *Vibrio*s at the species and the sub-species levels [11]. In the current study, the IGS typing system was used to confirm the conventionally identified *V. vulnificus* strain before being used in the experimental challenge.

Chitinolytic bacteria as *Vibrio*s are capable of degrading carapace chitin. This results in the typical erosion of the shell accompanied by pigmentation (melanization) that is a host defense mechanism against infection [26]. The most characteristic clinical sign of the diseases is the pigmentation on the exoskeleton in form of black spots. The bacteria can penetrate the cuticle and infect the hemolymph causing host tissue damage [4]. Severity of shell disease is associated with increase in hemocoelic bacterial infections. Pigmentation may be an external marker for internal health problem [27].

Experimental challenge of crayfish in the present work was carried out using a high dose of *V. vulnificus* (2.5×10^9 CFU/crayfish) to induce signs of disease later on. It is not uncommon to use such high challenge doses in cases of experimentally-induced vibriosis in crustaceans. A previous challenge study was reported in shrimp where an inoculum as high as 3.5×10^9 CFU/ml was used to induce the disease and to re-isolate the inoculated bacteria from the experimentally infected shrimp (reviewed by Saulnier *et al*. [28]).

In the current study, only slight lesions could be detected in the shell during the first 7 days post-infection suggesting that the disease was still in early stage. Smolowitz *et al*. [29] mentioned that in the mild form of the disease, the lesions were small and most likely found on the dorsal carapace just behind the rostrum or along the midline of the carapace. The disease was suggested to be in the medium stage on day 14 post-infection as black spots appeared on the dorsal and ventral surfaces of the shell and then progressed as the chitin became discolored as was previously reported [30]. In the more severe cases many erosions, dark brown and black spots (0.5 to 5 cm) often with softening and ulceration in the center were present on walking legs, chelae and carapace. The spots sometimes fused to each other leading to expansion of the lesion.

Also, 14 days post-infection, some cases revealed muscle with translucent color. This may be due to elastolytic protease, a proposed virulence factor that *V. vulnificus* possess [31]. In one case, the muscle was opaque. The opacity of the tail muscle may be due to necrotic changes. In shrimp exposed to stress conditions, the muscles lose its normal transparency and become blotched with whitish areas throughout and this may progress until the entire tail area become whitish [32].

Gills pathology may refer to the role of gills in bacterial accumulation and clearance as was previously suggested [33]. The consistency and color of the hepatopancreas changed from creamy "healthy" to yellow or dark brown "diseased" at day 14 and exhibited various colors ranged from dark to pale at day 36 indicating various degrees of recovery. Similar signs were recorded by Ryazanova [30].

Additional to directly causing losses or disease, *Vibrio* spp. infections in crayfish can lead to serious health problems in humans through ingestion of raw or undercooked crayfish [34-37]. Thus, the isolation of various *Vibrio* spp. from red swamp crayfish may represent a potential disease source not only to crayfish and other aquatic animals living in their vicinity, but also to the human consuming these subclinically-infected crayfish.

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