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First Record of *Vibrio vulnificus*/ *Anisakis pegreffi* Concurrent Infection in Black scorpionfish (*Scorpaena porcus*)from the South Mediterranean Basin.

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ABSTRACT

Vibrio vulnificus, a zoonotic bacterial pathogen was isolated for the first time from kidneys of black scorpionfish *Scorpaena porcus* collected from the south Mediterranean basin (western coast of Tripoli, Libya) during the early summer of 2013. Fish exhibited typical signs of Vibriosis with furuncle like lesions, skin hemorrhages, fin rot and congested spleen. The abdominal cavity, liver, intestinal wall, and gonads were suffering from mild larval nematode infection. Interestingly, a huge portion of the kidney tissue was replaced with the anisakids nematode *Anisakis pegreffi*. This is considered the first report of*V. vulnificus* / *A. pegreffi* in the Mediterranean black scorpionfish. The identities of the retrieved *V. vulnificus* isolates were confirmed using morpho-chemical and molecular tools. The research lead us to conclude that the deteriorated nature of the sewage polluted seawater at the western coast of Tripoli could have played a detrimental role in facilitating the invasion with the two diverse etiological agents (*V. vulnificus* and *A.pegreffi*) . We also hypothesize that *V. vulnificus* was lodged within nematode worms or at one of its life stages before invading fish intestine with consequent spread into other internal organs during its visceral larval migration stage.

Keywords: Zoonotic Diseases, Fish Nematodes, Vibriosis, Mediterranean Sea

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INTRODUCTION

Black scorpionfish is one of the most abundant and commercially important species of Scorpaenidae in Libya and other Mediterranean countries [1, 2]. *S. porcus* is a benthic, sedentary medium sized' fish that is commonly exists between rocks and sea bed, [3, 4].*S. porcus* is considered as amacrophagic carnivore due to the relatively larger size of its preferred prey [5]. Black scorpionfish might also feed on small fish, crustaceans and other invertebrates [6].

The zoonotic helminthes - bacterial pathogens interaction models are vulnerably increasing at the south Mediterranean basin due to the continuous direct dumping of municipal sewage into the sea coasts[7]. Several records of zoonotic nematodes such as *Anisakis* species have been reported in marine fish species other than black scorpionfish throughout the Mediterranean coasts of North African countries [8, 9]. Moreover, several bacterial infections of public health concerns such as Vibriosis, streptococcosis and Mycobacteriosis have been reported in several Mediterranean fishes but not the black scorpionfish[10 - 13]. Interestingly, records about parasitic and bacterial health issues of black scorpionfish are very scanty. Larvae of *A. pegreffi*, *A. typica* and *A. simplex* represent a major problematic health issue for several commercial fish species at the Mediterranean Sea [8, 9]. Using molecular typing *A. pegreffi* and *A. typica* were reported at marine fishes from the Moroccan , Tunisian and Libyan coasts [8]. Their visceral larval migration of *Anisakis* species within the human body represents a potential health threat. Further, they might represent food-borne allergens through consumption of raw or insufficiently processed fish (paratenic host) [8].

Health-wise, the potentially pathogenic *V. vulnificus* is a critically serious pathogen, since it is associated with fatal diseases in humans and aquatic animals [14]. Recent molecular typing studies have demonstrated that *V. Vulnificus* biotype II is a polyphyletic group [15]. It is also considered as a pathovar specifically pathogenic to fish with apparent signs of haemorrhages and skin ulcers [16].DNA-based methods are specific and sensitive approaches for the detection of bacterial pathogens in seafood [17].Parvathi*et al*.[18] suggested that *gyr*Bgene sequencing was a better tool for defining taxonomic and phylogenetic relationships at the species level among *V. vulnificus*.

The current study was designed to discuss the impact of concurrent occurrence of *A. pegreffi and V. vulnificus* on the health status of Black scorpionfish (*S. porcus*)collected from the western coast of Tripoli, Libya during the early summer of 2013.

MATERIALS AND METHODS

Fish sample

This study was a part of regular survey to investigate the most common diseases affecting commercial fishes at the western coasts of Tripoli. In the early summer of 2013, fifteen black scorpionfish *S. porcus* were collected from the western Mediterranean coast of Tripoli, Libya. Fish were collected using a trammel net at a mean depth of 15 m, according to the traditional local small-scale fishery techniques. Water temperature and salinity were determined and the mean values were calculated. Fishes were kept on ice in isothermal boxes till transferred to the laboratory of the Department of Poultry and Fish Diseases at the Faculty of Veterinary Medicine(DPFD), Tripoli University within 2 hours after collection at maximum. At the DPFD, fish body weight, standard and total length were measuredaccording to [19].Clinically, all fish were visually inspected for any possible lesions before laboratory examinationsare adopted[20].

Bacterial isolation

Fish were dissected under complete aseptic conditions using the three line technique accordingto [20]. Bacteriological swabs from skin lesions, liver and kidney of moribund*S. porcus* were streaked onto thiosulphate-citrate-bile-salt agar (TCBS, Oxoid, UK), brain heart infusion agar (BHI, Difco, USA), tryptic soy agar (TSA) and tryptic soy broth (TSB) (Difco). Each medium was supplemented with 1% NaCl (w/v). All plates were incubated at 25°C for up to 72 hours. Using sterile bacteriological needle, single colonies from plates with dense, virtually pure culture growth were re-streaked onto the aforementioned media plates to obtain pure isolates.

Morpho-chemical characterization

Morpho-chemical characterization was based on cultural characteristics, Gram staining and conventional biochemical tests. Further biochemical testing was performed using API20E tests (BioMerieux, NC), with isolates incubated at 25 °C. Results were interpreted at 24–48 hours according to the manufacturer's instructions. Isolates were presumptively identified as *V. vulnificus* according to the standard criteria described by [21] and [22].

Motility was assayed on TSA (1%NaCl) motility tubes containing 0.3% (w/v) Bactoagar (Difco) [23]. NaCl tolerance was tested in 1% peptone water amended with given concentrations ofNaCl (O-3-6% NaCl). Growth at 5°C, 37°C, and 42°C was tested in 1% peptone water containing 1% NaCl. O/129 sensitivity (10 and 150 mg per disc)were also performed for all retrieved isolates.

DNA extraction and PCR detection of *gyr*B gene

The retrieved isolates of *V. vulnificus* were stored in Luria-Bertani (LB) broth with 20% (vol/vol) glycerol (LB; Difco, MD, USA) at –80°C. All isolates were aerobically grown on TSA supplemented with 1% NaCl, and then incubated at 25°C for 24 h. Genomic DNA was extracted from cultured strains using a DNAzol® reagent (Invitrogen, Carlsbad, USA) as per manufacturer's instructions. The final washed DNA pellet was re-suspended in 200 ul sterile de-ionized water and stored at the fridge until used. To amplify a 680-bp fragment of gyrB; the specific primer pairs of *gyr*Bf1: 5'- TCGATCAAACTGTTTGAT -3', and *gyr*Br2: 5'-TCACCCTCCACTATGTAAAG -3' weredesigned based on DNA gyrase beta subunit (*gyr*B) gene of *V. vulnificus* (GenBank accession number EU118199). The following amplification protocol was used to amplify the target DNA: initial hold at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30min, primer annealing at 50 °C for 45 s, elongation at 72 °C for 50 s, and a final cycle at 72 °C for 10 min.

Cloning and sequencing of *gyrB*

The *gyr*B gene was sequenced according to Abdelsalam *et al.*[24]. The amplified products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), and the recombinant plasmid was introduced into Escherichia coli DH5α. The QIAprep Spin Miniprep kit (Qiagen, Germantown, MD, USA) was used to purify the plasmid DNA. Sequencing reactions were performed using the oligonucleotide primers SP6 (5-ATTTAGGTGACACTATAGAA-3) and T7 (5-TAATACGACTCACTATAGGG-3) with the Genome Lab DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA). The samples were then loaded into the CEQ 8000 Genetic Analysis System (Beckman Coulter) and the nucleotide sequence were determined. The nucleotide sequences were analyzed using the BioEdit version 7.0 [25]. The phylogenetic analysis was carried out by the neighbor joining method using MEGA version 5 [26]. The nucleotide sequences of the *gyr*B gene were submitted to the DNA Data Bank of Japan (DDBJ) nucleotide sequence database. The accession numbers of sequenced *gyrB* genes are AB971238, AB971239, AB971240 and AB971241.

Parasitology

The body cavity was opened using three line technique , and internal organs were placed on Petri dishes and examined for the presence of any possible internal parasites *Anisakis*. Also, squash preparations from internal organs were made and examined under both low power (10 X) and medium power (40 X) light microscope for possible entrapment of helminthes with special reference to nematodes. Being highly zoonotic to human, *Anisakis* larvae were the main target of the parasitological examination during the entire survey. Larvae were examined using both stereo (X4) and compound (X10) microscopes (Olympus, USA) for morphological identification.). The suspect *Anisakis* larvae were collected using either sharp needle or forceps then moved to vials containing hot 4% formaldehyde solution in physiological saline for morphological studies. For light microscopical examination, they were cleared with glycerin according to [27]. Identification of the *Anisakis* larvae was based on the morphology of digestive tract, the position of the boring tooth in relation to the excretory pore, and the morphology of the post-anal tail with a typical terminal mucron[28, 29].

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Histopathology

Tissue specimens from kidneys, spleen and liver were fixed in 10% neutral buffered formalin for routine histopathological examinations. The fixed specimens were washed in tap water overnight and exposed to ascending concentrations of ethanol (70, 80, 90 and 100%), cleared in xylene and embedded in paraffin. Tissue slides of 5 µm thick sections were prepared and stained with hematoxylin and eosin (H&E). The histological alterations of H&E stained tissue sections were microscopically assessed under low (10 X) / high microscopic (40 X) powers. Thehistopathologicalprocedures were performed according to [30].

Statistical analysis

This experiment was designed by Randomized Complete Block Design (RCBD). Data were analyzed using Statistical Analysis System [30] utilizing the analysis of variance (ANOVA).

RESULTS

The mean values of water temperature were25 °C, and 32 ppt for salinity. The fish total length ranged from 8.5 to 22.5 cm (15.5 cm at average) and average weight was 250 gm.

Clinical findings

External clinical examination of the collected black scorpionfish has revealed the presence of variable degrees of fin rot, skin hemorrhages and skin ulcers (Fig. 1). Internally, the dissected fishes were suffering from splenic congestion, and certain degrees of fibrinous inflammation / adhesions of the internal organs. Further, most of the abdominal cavity, liver, intestinal wall, and gonads were suffering from mild nematode infections which were further identified as *A. pegreffi* larvae. Interestingly, huge portion of the kidney tissue was completely replaced with the anisakid nematode that remarkably changed the color / texture of the kidney tissue into to pale whitish brown color / rubbery texture (Fig. 2).

Figure 1: Black Scorpionfish with generalized fin rot and hemorrhage.

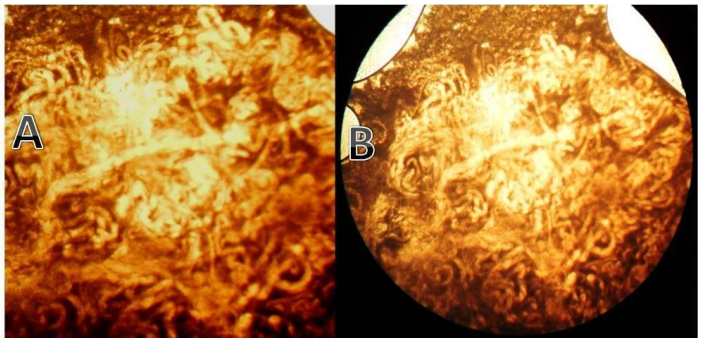

Figure 2:An ultra-magnification of the renal tissue showing an extreme case of severe infestation of the kidney tissue with *Anisakis pegreffi* larvae.

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Parasitology

Macroscopical as well as microscopical examinations of fish tissues have revealed the presence of anisakid larvae with relative distribution within the internal organs as well as peritoneal cavity. The general morphology of all retrieved larval nematodes examined by light microscope consorted with that of *A. pegreffi* based upon morphological criteria described by [29, 32] (Table 1).To date, this finding represents thefirst record of such anisakid species in *S. porcus* collected from the Libyan cost of the Mediterranean Sea. On visual examination, some worms look wondering through the surface of internal organs and abdominal cavity.Larvae were usually found encapsulated in tight flat coil on /in the viscera of fish. The total number of anisakids larvae collected from all infected fish was 1284 third-stage larvae. Examination indicated that 92.2% of the total retrieved aniskaid larvae were lodged in the kidney (Table 2). Intensity of anisakid worms in most of the internal organs and abdominal cavity were ranging from 3-5 worms per organ, while fairly large portions of kidneys werelargelyoccupied with huge number of *A. pegreffi* larvae. The average intensity of *Anisakis* infection in different internal organs of examined black scorpionfish were 92.4; 4.8; 1.6; 0.7 and 0.19 in kidney, abdominal cavity, liver, spleen and gonads, respectively (Table 3).

Table 1: Morphological characteristics of the retrieved *Anisakis pegreffi* third larval stage

Morphological criteria	Measurement in µm
Total body length	11.10-26.7
Maximum body length	0.38-0.60
Distance of nerve ring to anterior end	0.20-0.30
Length of esophagus	1.04-2.11
Ventriculus length	0.50-0.78
Ventriculus width	0.12-0.26
Tail length	0.05-0.12
Mucor Length	0.02-0.03

Table 2: Intensity of *Anisakis* infection in different internal organs of examined black scorpionfish

# fish	Total number of retrieved Anisakis larvae	Intensity of Anisakis larvae in black scorpionfish organs									
		Abdominal cavity		Liver		Spleen		Gonads		Kidney	
		Number	%	Number	%	Number	%	Number	%	Number	%
1	109	5	4.587	2	1.835	1	0.917	1	0.917	100	91.743
2	100	3	3	1	1	1	1	0	0	95	95
3	100	5	5	1	1	1	1	0	0	93	93
4	98	4	4.082	2	2.041	0	0	0	0	92	93.878
5	90	3	3.333	2	2.222	1	1.111	0	0	84	93.333
6	69	7	10.145	1	1.450	1	1.450	0	0	60	86.957
7	70	3	4.286	2	2.857	0	0	0	0	65	92.857
8	100	6	6	2	2	1	1	1	1	90	90
9	88	5	5.682	1	1. 136	1	1.136	0	0	81	92.045
10	80	4	5	1	1.25	0	0	0	0	75	93.75
11	70	5	7.143	2	2.857	2	2.857	0	0	61	87.143
12	65	0	0	0	0	0	0	0	0	65	100
13	60	0	0	0	0	0	0	0	0	60	100
14	110	9	8.19	3	2.73	1	0.91	1	0.91	96	87.273
15	75	5	6.667	2	2.667	0	0	0	0	68	90.667
Total	1284	64	4.984	22	1.713	10	0.779	3	0.234	1185	92.290

Table 3: Average Intensity of *Anisakis* infection ± S.E. in different internal organs of examined black scorpionfish.

Internal organ	Intensity of Anisakis infection (%)
Abdominal cavity	04.8812 ± 0.656 b
Liver	01.6724 ± 0.228 c
Spleen	00.7600 ± 0.191 cd
Gonads	00.1913 ± 0.094d
Kidney	92.4960 ± 0.958 a

a,b,c: values don't have the same letter are significantly different (Pr ≥ 0.05).

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Histopathology

Microscopical examination of H & E stained sections from kidneys of black scorpionfish has revealed the presence of severe lymphocytic infiltrations of the interstitial renal tissue, focal interstitial fibrous tissue proliferation together with moderate necrotic tissue and severe degeneration of glomerular tufts with severe lymphocytic infiltration. At the level of nephrons, severe renal tubular degeneration and necrosis of renal tubular as well as glomerular tissues were recorded (Fig. 3). In respect to spleen , examination of H&E stained splenic tissue sections have presented severe melanomacrophage centers (MMC) activation , severe lymphocytic infiltration within the splenic tissue together with splenic disorganization of splenic parenchyma. In liver, focal necrosis of hepatic tissue with marked interstitial lymphocytic infiltration, severe vacuolar degeneration and disorganization of hepatic cords were obvious through all examined liver sections (Fig. 4).

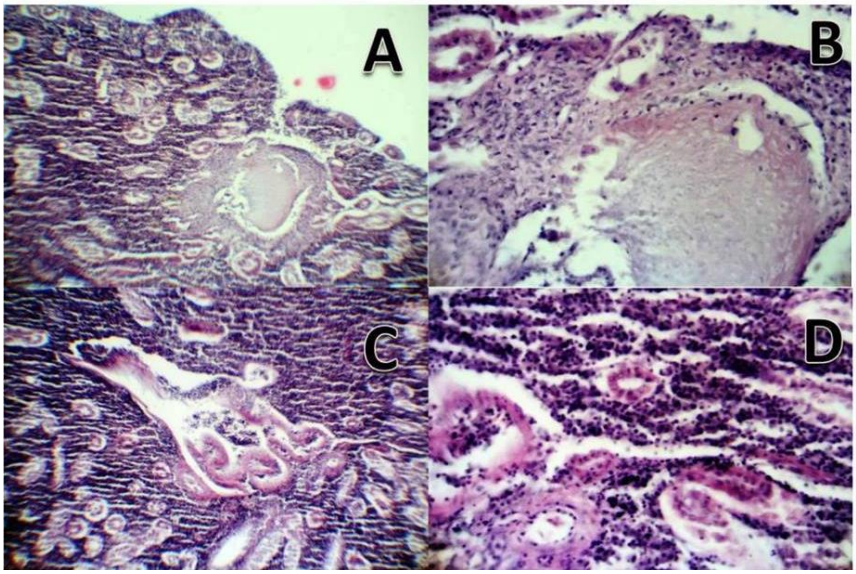

Figure3:A. Photomicrograph of black scorpionfish showing severe lymphocytic infiltrations of the interstitial renal tissue (H & E, 10X); B. Focal interstitial fibrous tissue proliferation together with moderate necrotic tissue (H & E, 40X); C. Severe degeneration of glomerular tufts with severe lymphocytic infiltration (H&E, 10 X). D. Severe renal tubular degeneration and necrosis of renal tubular as well as glomerular tissues (H&E, 40 X).

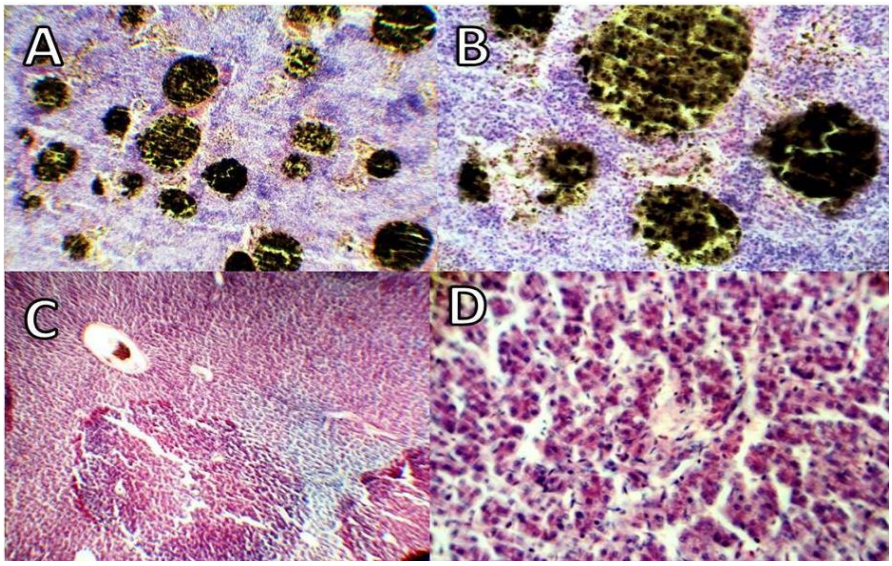

Figure 4: A. Photomicrograph of black scorpionfish splenic tissue showing severe melanomacrophage centers (MMC) activation(H & E, 10X); B. Severe lymphocytic infiltration within the splenic tissue together with splenic disorganization of splenic parenchyma (H & E, 40X); C. Focal necrosis of hepatic tissue with marked interstitial lymphocytic infiltration (H&E, 10 X). D. Severe vacuolar degeneration and disorganization of hepatic cords (H&E, 40 X).

Morpho-chemical characteristics of retrieved isolates

Vibrio vulnificus isolates were recovered from all examined *S. porcus* kidney samples. The typical greenish colonies on TCBS were observed. All isolates from the examined clinically affected *S. porcus* exhibited the typical morphological / biochemical characteristics of *V.vulnificus* reported by [33]. Bacteria were short Gram-negative, motile, oxidase, catalase positive, and O/F positive without gas production. All isolates were sensitive to the Vibrio static agent O/129 (10 and 150 mg /disc).The morpho-chemical characteristics of the retrieved *V. vulnificus* isolates were summarized at Table (4).

Table 4: Selected morpho-chemical charcteristics of the retreived *Vibrio vulnificus* isolates

Morpho-chemical characteristics	Results
Colony morphology on TCBS	Green colonies
Gram staining	Gram negative slightly curved short rods
Motility	+
Growth in 0 % NaCl	-
Growth in 3 % NaCl	+
Growth in 6 % NaCl	+
Growth at 42 °C	-
Oxidation / Fermentation	+ / +
Oxidase	+
Catalase	+
Gelatinase	+
H_2S production	-
Indole	-
Nitrate reduction	+
Arginine dihyrolase	-
Lysine decarboxylase	+
Ornithine decarboxylase	-
Esculin hydrolysis	-
Urease	-
Voges-Proskauer	-
Sensitivity to O/129: 10µg , 150 µg	+

***Gyr*B sequence analysis of retrieved isolates**

All *V. vulnificus* isolates reacted positively to the *gyr*B gene primers that were designed from DNA gyrase beta subunit (*gyr*B) gene of *V. vulnificus* (GenBank accession number EU118199). A single amplification product with the expected size of 680-bp was detected for all the examined isolates (Fig.5). The *gyrB*gene sequences of four isolates retrieved from the examined black scorpionfish were submitted to the GenBank sequence database. The sequences derived from the four retrieved isolates were identified as *gyrB* sequence of *V. vulnificus* based on the standard nucleotide–nucleotide BLAST results. All retrieved isolates were identical (100% sequence identity). The phylogenetic tree generated on the basis of the *gyrB*gene sequences of *V. vulnificus* isolates retrieved from scorpionfish and other related *V. vulnificus* isolated from eels, oyster and human were shown in Figure (6). The phylogram has obviously exhibited that all sequenced *V. vulnificus* isolates from scorpionfish/ eels belonged to only one cluster, and they were separated from other related *V. vulnificus* isolated from oyster and human.

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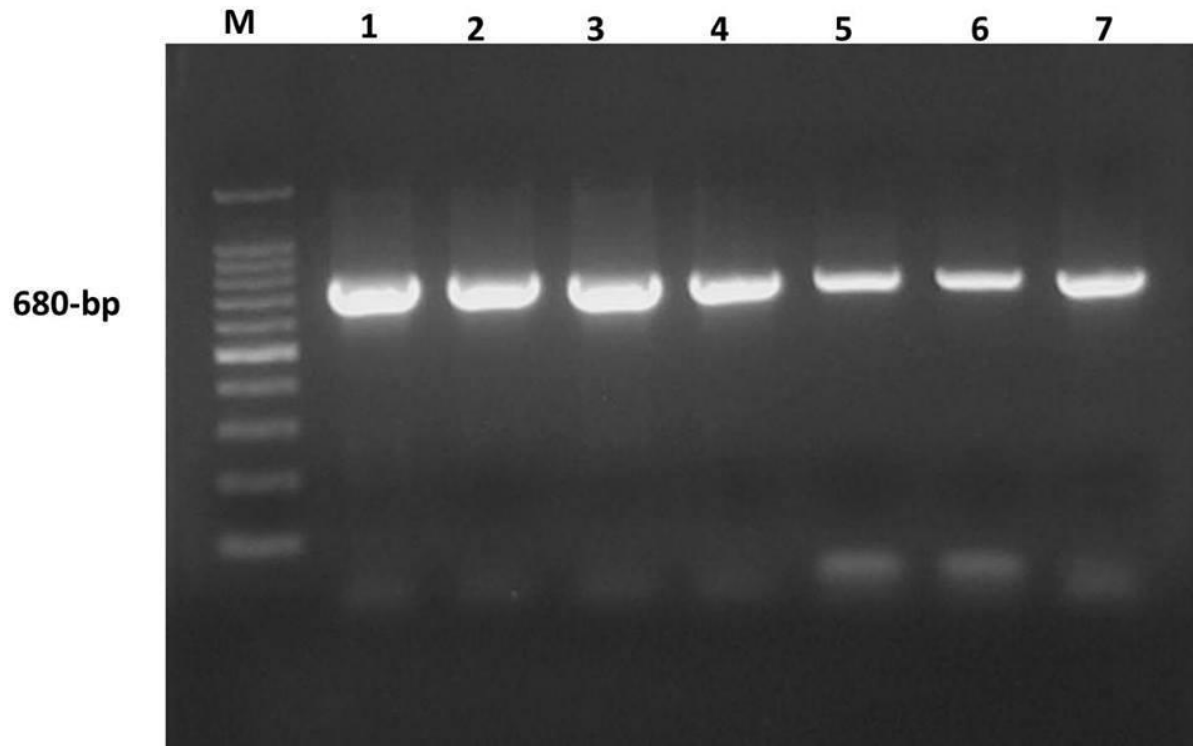

Figure 5:Amplification of the gyrB locus extracted from scorpionfish *V. vulnificus* isolates and *V. vulnificus* ATCC33149 yielded 680-bpwhen the primer pairs gyrB f1 and gyrB r2 were used. Lane M, marker; lane 1, *V. vulnificus* ATCC33149;lanes 2, 3 and 4black scorpionfish isolates *V. vulnificus*; lanes 5, 6 and 7, red scorpionfish isolates *V. vulnificus*.

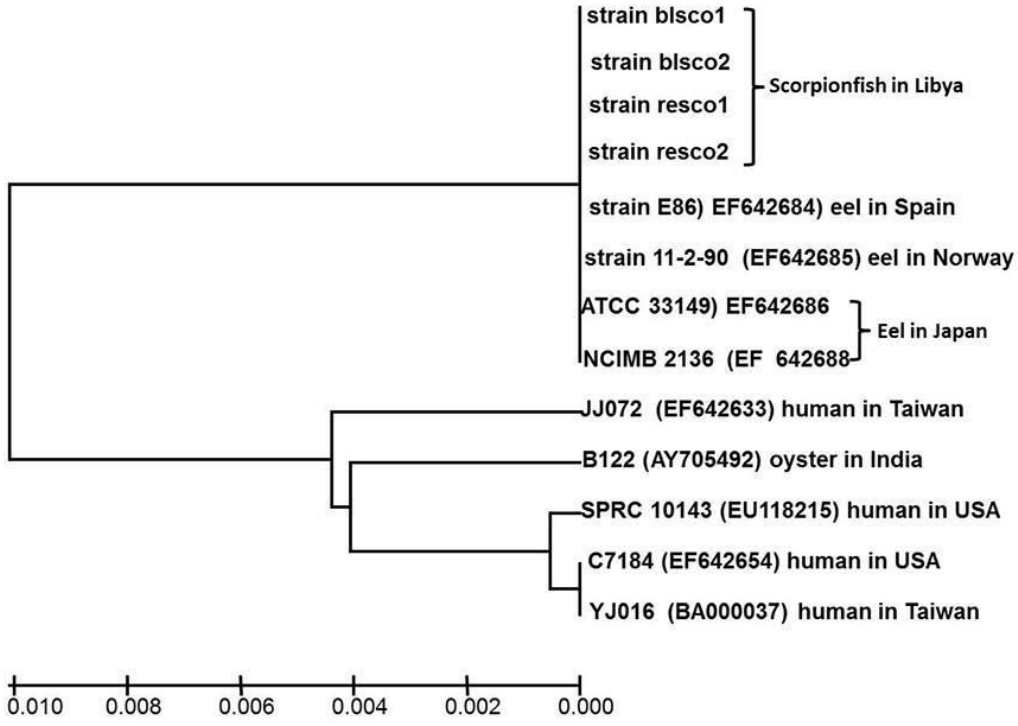

Figure 6:Phylogenetic tree generated based on the comparative analysis of the gyrB gene sequences, showing the relationship among the scorpionfish strains of V. vulnificus and related isolates of V. vulnificus of human, eels and oyster.

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DISCUSSION

Disease is an interaction between three dynamic factors, host-environment-pathogen in a definite period of time [34; 35].Environmental change, especially habitat degradation by anthropogenic pollutants and oceanographic alterations induced by climatic, can influence parasitic-host interaction [36]. The direct dumping of municipal sewage into the Mediterranean shallow basin is a classical regime that is adopted by many countries like Egypt, Libya, Tunisia and Morocco and many other bordering countries[37]. Such irresponsible anti-environment behavior is representing a continuous evolving source of biological/chemical sorts of pollution with consequent degrading effects on the natural marine environments as well as its biodiversity[35, 38]. Municipal sewage may possibly transfer vulnerable zoonotic biological agents from land to the sea[39]. Communicable pathogens such as Vibrios (*V. cholera, V. parahemolyticus* and *V. vulnificus*) and zoonotic Anisakid nematodes (*A. simplex , A. typica* and *A. pegreffi*) are among those biological agents . These nematodes or any of their life stages will be dispersed into sea water and eventually settling down to the filter feeder shellfish/bivalves which will then be eaten by the benthic fishes like *S. porcus*.

In the current study, the taxonomical measures of the retrieved anisakid nematode perfectly coincided with the standard morphological criteria of *A. Pegreffi* described by [29,32].Briefly, total body length, esophageal length and length/ width of the ventriculus are much shorter than those reported for *A. simplex and A. typica*. Although*A. pegreffi* were among the most prevalent *Anisakis* species reported in European hake (*Merluccius merluccius*) from Tripoli coast, Libya [8], yet we firstly report it in the black scorpionfish from Tripoli.

Also, the identity of retrieved *V. vulnificus* isolates was presumptively confirmed by the semi-automated API 20 E test and affirmatively confirmed by a panel of highly specific molecular assays. The fact that all *V. vulnificus* isolates have reacted positively to the *gyr*B gene primers that were designed from DNA gyrase beta subunit (*gyr*B) gene of *V. vulnificus* (GenBank accession number EU118199) accurately confirm their identity . The sequences derived from the four strains were identified as *gyrB* sequence of *V. vulnificus* based on standard nucleotide–nucleotide BLAST results. Accurately, the four isolates, collected from scorpionfish, were identical (100% sequence identity). The subsequently made phylogram have obviously revealed that all sequenced scorpionfish/ eels *V. vulnificus* isolates belonged to only one cluster, and they were separated from other related *V. vulnificus* isolates from oyster and human. These molecular data were not previously reported at any study through Libya, or any country at the North African Mediterranean coast. Thus, a succeeding trail of molecular studies should be further done on scorpionfish collected from other geographical locations through the Mediterranean coast and other cohabitating fish species as well.

At the aquatic environment, the concurrent parasitic / bacterial infections are the real scenarios for infections in the natural environments where water bodies are naturally inhabited by a wide spectrum of ubiquitous bacteria and parasites[39; 40]. Sun *etal*. [41] explained an endosymbiotic relationship between *F. columnare* and the parasitic ciliate *Ichthyophthirius multifiliis* in which the bacteria adheres to the parasite through association with the cilia. The case is not only restricted to the endosymbiontrelationship between the parasite and bacteria, but also extended to a synergistic type of relationship in which the parasite enhances the bacterial invasion into the fish skin with consequent disease development [39,40, 42,43].

From public health perspective, Saha*et al*. [44] have indicated that heavy nematode infection in intestinal loops of Indian diarrheal patients has drastically immune-suppressed the local intestinal mucosal immunity in such way that the prevalence of the ubiquitous *V. Cholera* pathogenic bacteria was too high concurrently with the intestinal nematode infection. In a very similar way, we would assume an aquatic model of nematode/vibrio concurrent infection in the benthic teleost scorpionfish. The voracious night eating behavior of black scorpionfish allow them to apprehend as much as they can from the marine mussels and mollusks [6]that contain early larval stages of the highly zoonotic *Anisakis* species .Once get lodged into the gastrointestinal tract of the fish, the early *Anisakis* larvae starts a unique stage of development into 3rd larval stage which is capable of invading intestinal wall to peritoneal cavity , blood circulation and then to different internal organs with special preference to hematogenous organs like kidney and spleen. The larval migration of the 3rd larval stages through the intestine would demolish or at least corrupt the local mucosal resistance of intestinal epithelia of this night eating fish. There is a well-established fact that *Vibrios* are among the symbiotic bacteria living inside the intestine of fishes and other aquatic species with a very facultative way[45]. However, the apparent suppression of the intestinal mucosal immunity have synergized together with the

continuously changing intestinal environment pH to favor an acute invasion into the intestine then to the blood circulation. After arriving the circulation, typical case of septicemia is on the way through which the bacterial toxins where secreted and pathogen lodged into internal organs like kidney, spleen and liver[46; 47]. Vibrio possesses a wide array of virulence factors, including acid neutralization, capsular polysaccharide expression, iron acquisition, cytotoxicity, motility, and expression of proteins involved in attachment and adhesion[45, 46,48]. These factors likely require concerted expression for pathogenesis to take place. Conclusively, *V. vulnificus* is a complex microorganism with physiological characteristics that contribute to its survival in the marine environment and in the human host[47-49].The microscopic wounds induced by the *Anisakis* larval migration through intestinal wall and other internal organ with consequent microscopical hemorrhages at these organs would produce a reliable amount of iron due to blood cell damage in a very similar way to the action of *Ascaris* in the human intestine [44]. The sequestered iron represents an eminent enhancer for *V. vulnificus* pathogenicity within the fish body as the iron acquisition is one of the main pathogenic mechanisms of such pathogen[50].This could describe how *V. vulnificus* together with *Anisakis* larvae were synergistically working in a very aggressive way to induce a clinical disease in the target fish.

It is well documented that temperature rise above 22°C enhances *Vibrio*s pathogenicity and virulence by the following mechanisms: increases the bacterial growth rate by an average of 30%; increases the adhesion capacity of the bacterium to the fish tissues[49,51]. The sharp decrease in dissolved oxygen due to temperature rise above 22-25°C jeopardizes the immune system of fish by increasing the potentials of the ubiquitous bacterial invasion[40]. Surveys combining information on bacteriological and parasitological burden under natural conditions have shown that mixed infections are very common, and hence the etiology of some disease outbreaks or clinical conditions is complex and difficult to ascribe to a single pathogen[40,41,52]. In the current study, black scorpionfish collected from western coast of Tripoli, Libya havepresented signs of severe*V. vulnificus* infection concurrently with a heavy infestation of *A. pegreffi* larval anisakid nematode. It was evident that the high rate of larval nematode entrapment by the hematopoietic kidney tissue has resulted in relatively low prevalence of the same larvae in other internal organs within the same fish. This can be explained by the whitish discoloration and elastic texture of the infested kidneys which were explained by the huge number of larvae occupying fairly large portions or even the entire area of the kidney. The necrotic foci, tubular degeneration, lymphocytic infiltration and fibrous tissue proliferation are all sequels of the larval nematode entrapment within the kidney tissue. We inferentially hypothesized that the damaging effect of *Anisakis* larvae on intestinal mucosal immunity and renal humoral / cellular immunity would have presented the main triggering factor in swift invasion of *V. vulnificus* to fish tissues and the subsequent enhancement of the pathogenic mechanism of the pathogen.

Several studies have been performed on the molecular identification of the genus *Vibrio* using the sequencing method targeting some housekeeping genes [53]. They have reported that the rRNA genes which are usually used for studying the phylogenetic relationship show very much interspecies homology in the case of *Vibrio spp*, hence they are not suitable for phylogenetic analysis of *Vibrio spp*. The *gyrB* gene, which encodes the subunit B protein of DNA gyrase and type II DNA topoisomeraseis distributed universally among bacterial species. The *gyrB* sequences of *V.vulnificus* usually provide additional discrimination power than 23 S rDNA sequences and may enable identification at the species level of even most closely related *V. vulnificus* of different biotype; therefore it is ideally fitted to phylogenetic studies [18].

Herein, the sequencing of the *gyrB* gene was performed to match different isolates collected from different scorpionfish. A 100% sequence identity was determined among *V. vulnificus* isolates irrespective of fish origin. Thus, the phylogenetic analysis demonstrated that fish *V. vulnificus* isolates belonged to one cluster with that isolated from eels and distinct from other *V. vulnificus* recovered from human and oyster. Interestingly, *V. vulnificus* of fish origin appeared to be more related to *V. vulnificus* biotype 2that is responsible of eel mortalities. Our results suggested that *gyrB*gene analysis also could be a valid tool for inferring relationships among intra-species bacterial species (e.g. as a typing tool at the strain level).

Fish parasites play a major role in marine biodiversity, causing serious damage in feral fish populations in few reports [54]. The location of *Anisakis* larvae in the kidney of fish might impair the immunity of fish, as kidney is the main organ of immunity. Bacterial pathogens vectored by nematodes pose serious aquaculture, economic and human health threats. However, little is known of the ecological and evolutionary aspects of pathogen transmission by nematodes.

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CONCLUSION

In conclusion the detection of the two diverse etiological agents *V. vulnificus* and *A. pegreffi* in kidneys of the affected fish is considered the first record of such complex concurrent infection in black scorpionfish (*S. porcus*) across the Mediterranean Sea. The voracious feeding habits of such benthic fish together with their wide spatial distribution closer to some of the marine mammals (final host for the *Anisakis*) as well as municipal sewage pollution could have synergized together to induce the discussed systemic pathology. We also hypothesize that *V. vulnificus* was lodged within nematode worms or at one of its life stages before invading fish intestine with consequent spread into other internal organs during its visceral larval migration stage.

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