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## Bacteriological Studies on Vibriosis in Egyptian Sole (*Solea aegyptiaca*) Fish

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### Abstract

The main objectives of this work:

- 1- Seasonally collect fish from Mediterranean-sea and studying the clinical picture in the examined fish.
- 2- Isolation and identification the pathogenic bacteria affecting Solea fish.
- 3- Registering the percentage of total and seasonal prevalence in such fish.
- 4- Make trials for diagnosis using recent techniques.
- 5- Application of biosafety in the lab.
- 6- Commitment of the basis and principles of scientific research ethics in all study stages.
- 7- Studying histopathological changes caused by these bacterial diseases from naturally infected fish.

In the present study, a total of 120 Egyptian sole (*Solea aegyptiaca*) with various body weights (50-70 g) and lengths (13-19 cm) were collected seasonally and randomly from costal area of Mediterranean-sea in Port Said Governorate, Egypt from September-2019 till August-2020. Moribund or freshly dead specimens collected in aerated airbags and/or iceboxes were transferred to Food Hygiene and Bacteriology Unit, Animal Health Research Institute, Port Said for further bacteriological, and molecular examinations in particular for *Vibrio* infection. Fish were found to be infected with *Vibrio alginolyticus* based on the characteristic phenotypic and biochemical profiles with a total prevalence of 29.16%. During the study, the highest incidence of vibriosis among the naturally infected sole fish was observed in the summer season (31.43%), with the prevalence in the spring and winter seasons being equal (25.72% each). In contrast, the lowest incidence of infection was observed during autumn (17.14%). Molecular investigation of pure isolates using specific set

of primers targeting the *16S rRNA* conserved gene of *Vibrio* species gave expected amplicons size of 663 bp.

**Keywords:** Egyptian sole (*Solea aegyptiaca*), *Vibrio alginolyticus*, molecular diagnosis

### Introduction

Fish is an essential component of the human diet due to its excellent nutritional value. It is a rich source of polyunsaturated fatty acids (PUFAs), especially omega-3 and omega-6, that can help prevent thrombosis and atherosclerosis. These fatty acids possess protective qualities against autoimmune disorders, arrhythmias, high blood pressure, and coronary heart disease. Nearly every mineral found in fish is necessary for human bodies. Iron, zinc, phosphorus, calcium, selenium, iodine and fluorine are the minerals found in fish. Certain minerals have a high bioavailability, making it simple for the body to absorb them. (Pal et al., 2018). The expanding need for aquatic products was the primary driver of the aquaculture industry' rapid rise (Su et al., 2020). By 2030, the population of the entire world would consume 30 million tons of fish, according to the Food and Agriculture Organization of the United Nations (FAO, 2018). Aquaculture is the only method that may enhance fish production in Egypt.

The Mediterranean mariculture sector is developing, with production capable of meeting both local needs and export to other

countries (Eissa et al., 2017). The most frequently raised marine species in the Mediterranean basin include Gilthead Seabream, Mulletts, Seabass and Meagre (FAO, 2018). A number of marine fish species are being considered for aquaculture production. The most significant and valuable commercial flatfishes in Egypt are *Solea* species, which are also highly regarded by consumers of seafood (Gabr et al., 2003). Because of its superior meat, the common sole (*Solea Solea*) is a highly regarded fish in Egypt, especially in coastal towns, and it contributes up to 90 million LE to the country's annual economic output (Mehanna, 2014). The most prevalent species of soles is the Egyptian sole (*Solea aegyptiaca*), which made up by nearly 6.5% of the entire trawl fisheries catch and 13% of its gross profits (Mehanna, 2007). This fish is prized as a food fish and is utilized for human consumption. It is primarily caught using trawls on the seabed (Hureau, 2016).

In the Mediterranean countries, the culture of the Senegalese sole is a promising industry (Conceição et al., 2007). Unfortunately, these fish species' intense culture has contributed to the rise of multiple epidemics with varying mortality

rates. According to (Toranzo *et al.*, 2005; Austin and Austin, 2012), Vibriosis is a bacterial infection that has a significant impact on Mediterranean fish species raised on farms, as well as marine fish species kept in captivity. (Gudding and Goodrich, 2014). In summer, cultured marine fish often suffered from vibriosis outbreaks caused by the deterioration of crucial water parameters such as pH, temperature, dissolved oxygen, and salinity. (Albert and Ransangan, 2013).

However, only a few bacteriological surveys have included disease outbreaks in marine species. The presence of bacterial pathogens has been widely reported in both cultivated and wild freshwater fish species (Alicia, *et al* 2005). the aim of this study is to investigate into the prevalence, molecular typing associated with natural vibrio infection among marine fish species, *Solea aegyptica*.

### Material and Methods

Approval ethics:

The Ethical Committee of the Faculty of Veterinary Medicine, Suez Canal University approved all fish handling and experimental procedures (No. 201842).

### Clinical and postmortem examination:

In this study, total of 120 Egyptian sole (*Solea aegyptiaca*) with body weights (50-70 g) and average lengths (13-19 cm) were collected randomly and seasonally from fishermen on the Mediterranean-sea

in Port Said Governorate, Egypt from September-2019 to August-2020. Moribund and freshly dead fish were kept in large, insulated iceboxes and transferred immediately to Food Hygiene and Bacteriology Unit, animal health research institute, Port Said for further clinical and bacteriological examinations. To detect any gross lesions or abnormalities, the clinical investigation followed Amlacher's (1970) protocol, while the postmortem examination of freshly dead and moribund fish following the standard methods of Noga (1996).

### Isolation and identification of *V. alginolyticus*:

Aseptic samples of gills, liver, kidneys, and spleen were collected and streaked directly onto tryptic soy agar (TSA), which were supplied with varying concentrations of sodium chloride (1.5-8%) and thiosulfate-citrate-bile-sucrose (TCBS) agar. After incubating the cultured plates at 37°C for 48 hours, suspected colonies were harvested and re-streaked onto new plates of their original culture media before being incubated under the same conditions. The purified colonies were then inoculated into a semi-solid medium (a nutrient slop) by deep stabbing and incubated for 24 hours at 37 °C. The resulting cultures were stored frozen at -20°C for further analysis. The morphology, culture, and biochemical features of isolates were

evaluated based on Bergey's Manual of Determinative Bacteriology criteria. (**Baumann and Furniss 1994**).

#### **Molecular characterization of *V. alginolyticus*:**

Genomic DNA templates were recovered from pure bacterial cultures isolated from infected fish, and previously identified morphologically and biochemically. The gDNA of the retrieved isolates was obtained using a QIAamp DNA mini kit according to the manufacturer's protocol. Before being used as a PCR template, the DNA templates were adjusted to 100 ng/μl and stored at -80 °C. The recovered isolates were tested for *Vibrio* species using specific primers that target the *16S rRNA* conserved gene of *Vibrio* according to **Tarr et al. (2007)**. The primers sequence and PCR conditions are given in (**Table 1 & 2**). 25 μl reaction mixtures were used for all PCR amplifications. A T100TM gradient thermocycler (BIO-RAD) and a Mastercycler personal (Eppendorf) apparatus were used for the PCR amplification conducting. PCR products were identified using 1.5% (w/v) agarose electrophoresis stained with 5 μl RedSafe TM

(INTRON BIOTECH) for 30 minutes at 100 V, and then they were seen by a documentation system (INGENIUS 3, SYNGENE). A 100 bp molecular ladder (NZYDNA Ladder V; nzy tech) was used.

#### **Gel purification and sequence analysis of *V. alginolyticus*:**

A purified PCR product was sequenced in the forward and/or reverse orientations on an Applied Biosystems 3130 automated DNA Sequencer using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA), Cat. No. 4336817. (ABI, 3130, USA). The sequences were deposited in the GenBank under the entry number OP324632. A BLAST® analysis (Basic Local Alignment Search Tool) (Altschul et al., 1990) was performed to identify sequence identity to GenBank accessions. Then multiple sequence alignment was performed using CLUSTAL W tools in the MegAlign module of Lasergene DNASTar software Pairwise version 1.83(**Thompson et al., 1994**). The phylogenetic tree was drawn using neighbor joining and maximum parsimony in MEGA6 (**Tamura et al., 2013**).

**Table (1):** List of Oligonucleotide primers in the present study.

Target gene	Primers sequences 5'-3'	Amplicon size (bp)	References
<i>Vibrio 16S rRNA</i>	F: CGGTGAAATGCGTAGAGAT	663	<b>Tarr et al., 2007</b>
	R: TTACTAGCGATTCCGAGTTC		

**Table (2): PCR Cycle conditions**

Target	Initial denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
<i>Vibrio</i> <i>16S</i> <i>rRNA</i>	94°C 5 min.	94°C 30 s.	56°C 40 s.	72°C 45 s.	35	72°C 10 min.

## Results

### Clinical and postmortem examination:

Most of Egyptian sole (*Solea aegyptiaca*) naturally infected, displayed skin detachment, abdominal distention (ascites with serohemorrhagic fluids), pale and friable liver and pale gills (**Figs 1 & 2**).

### Bacteriological assay:

Out of 60 bacterial isolates, 35 were identified as *V. alginolyticus* based on their morphology. These isolates had colonies that were 2-3 mm in diameter and creamy whitish in colour on TSA, but yellow on TCBS. All tested isolates showed biochemical profiles identical to *V. alginolyticus* (**Table 3**). The highest rate of *V. alginolyticus* infection was recorded in the summer, whereas the highest bacterial intensity was recorded in the gills, followed by the liver and kidneys (**Table 4 & 5**).

### Molecular assay and sequence analysis:

In this study, the representative tested isolates were positive to *16S rRNA*, a conserved gene of *Vibrio* spp. and gave expected amplicons

size of 663 bp (**Fig. 3**). The tested isolate with accession No. OP324632 shared a striking genetic similarity with other *V. alginolyticus* isolates from various sources. The phylogenetic tree was built after multiple alignment of the *16S rRNA* sequence of the isolate strain with other homologous sequences accessible in the GenBank database (**Fig. 4**).

Prevalence and intensity of *V. alginolyticus* among naturally infected Egyptian sole (*Solea aegyptiaca*)

The results revealed that 35 out of 120 examined fish were infected with *V. alginolyticus*. The summer season had the highest prevalence of *V. alginolyticus* infections (31.43%), followed by winter and spring (25.72% each), then autumn (17.14%) (**Fig. 5**). The intensity of *V. alginolyticus* isolates among the internal organs was illustrated in **Figure (6)**. As shown, the prevalence of infection was highest in the gills (35.95%), followed by the liver (27.12%), kidneys (20.34%), and spleen (16.95%).

**Table (3):** Biochemical characteristics of *Vibrio alginolyticus* isolated from naturally infected Egyptian sole (*Solea aegyptiaca*).

In vitro test	Reaction
Cytochrome oxidase (OX)	+
Catalase	+
Indole production (IND)	+
Citrate utilization (CIT)	-
Methyl red (MR)	+
Acetoin production (VP)	+
Sucrose	+
Lactose	-
Arabinose	-
Mannitol	+
Inositol	-
Gelatinase production (GEL)	+
H <sub>2</sub> S production (H <sub>2</sub> S)	-
ONPG	-
Urease production (URE)	-
Arginine dihydrolase(ADH)	-
Lysine decarboxylase (LDC)	+
Ornithine decarboxylase (ODC)	+
Gram stain	-
0% NaCl	-
3% NaCl	+
6% NaCl	v
8% NaCl	v
10% NaCl	-
Motility	Motile

+, positive; - negative; v, variable

**Table (4):** Seasonal prevalence of *V. alginolyticus* among naturally infected Egyptian sole (*Solea aegyptiaca*).

	No of collected fish	No of infected fish	Winter		Spring		Summer		Autumn	
			No	%	No	%	No	%	No	%
<i>V. alginolyticus</i>	120	35	9	25.72	9	25.72	11	31.43	6	17.14

**Table (5)** Intensities of *V. alginolyticus* in different internal organs of naturally infected Egyptian sole (*Solea aegyptiaca*).

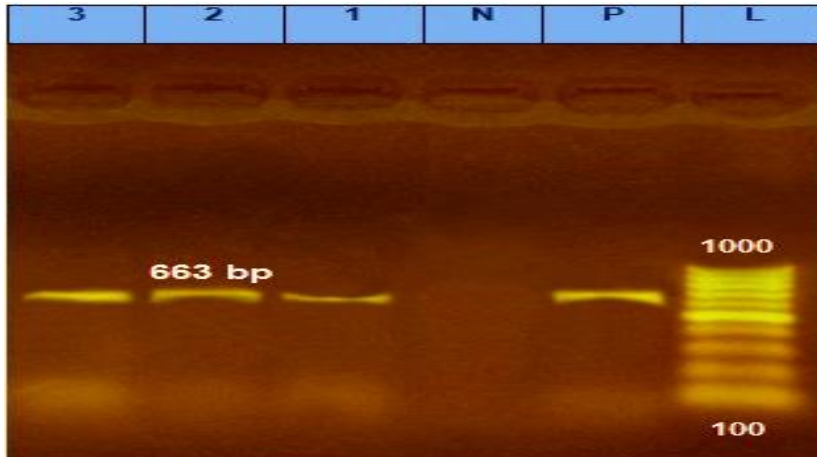
Bacterial species	No of isolates	Gills		Liver		Kidney		Spleen	
		No	%	No	%	No	%	No	%
<i>V. alginolyticus</i>	59	21	35.59	16	27.12	12	20.34	10	16.95



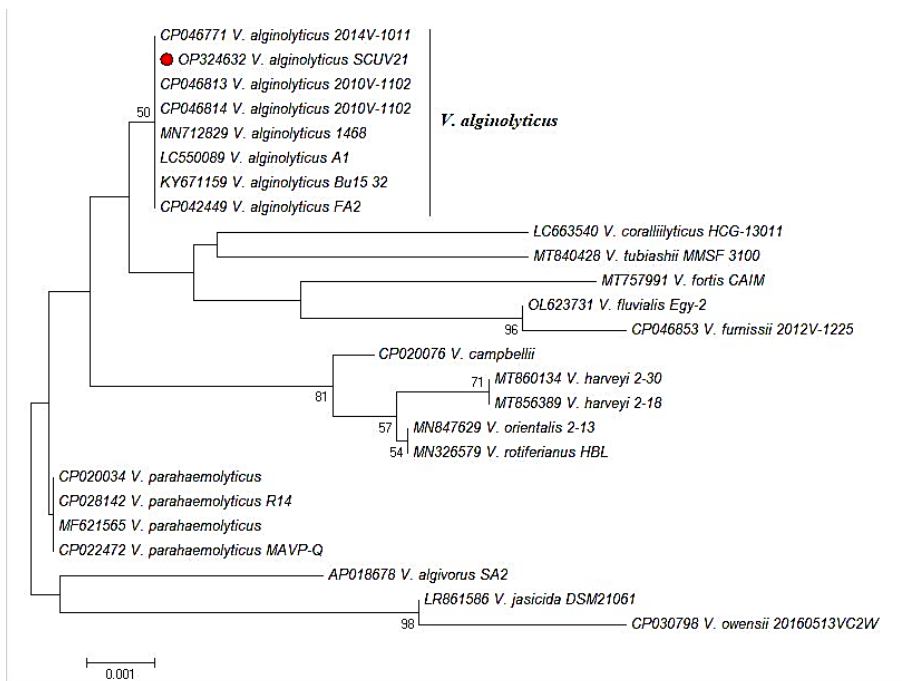
**Fig. 1:** Naturally infected Egyptian sole (*Solea aegyptiaca*) showing skin detachment and abdominal distention.



**Fig. 2:** Naturally infected Egyptian sole (*Solea aegyptiaca*) showing pale friable liver and pale gills.

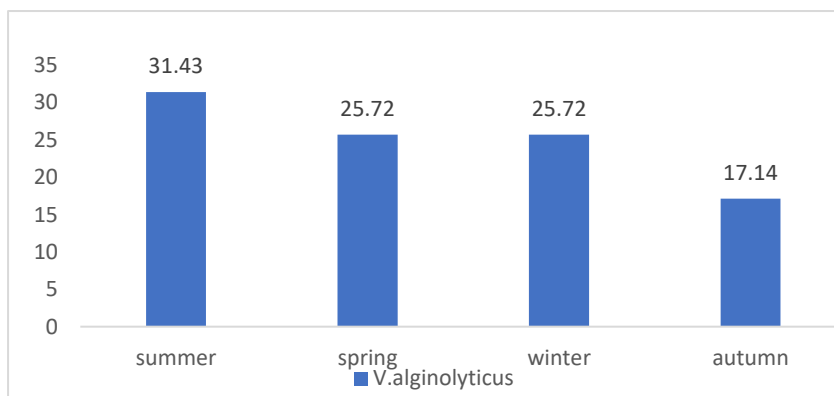


**Fig. 3:** Electrophoresis pattern of *16S rRNA* gene of *V. alginolyticus* strains isolated from naturally infected *Solea aegyptiaca*. Lane L, molecular weight ladder; Lane P, a positive control; Lane N, a negative control; Lanes 1–3, the specific PCR amplified products of the tested isolates with fragments size of 663 bp.

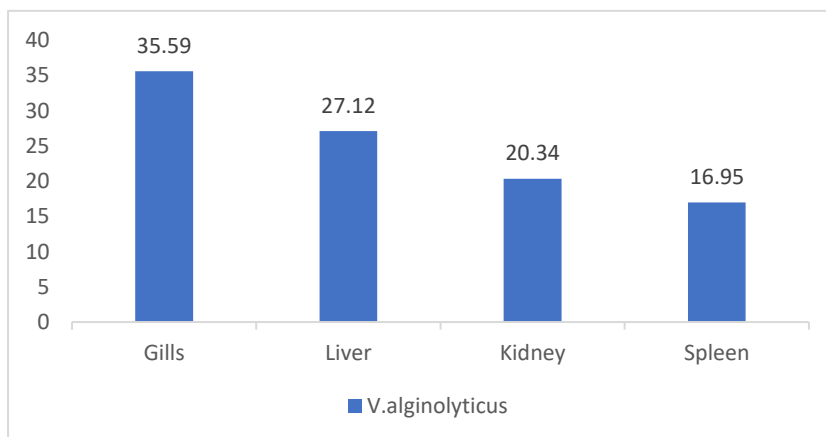


**Fig. 4.** Phylogenetic analysis of *Vibrio alginolyticus* 16S ribosomal RNA gene sequencing. The tree explains the genetic relationship between the tested *V. alginolyticus* strain and other homologues from different sources retrieved from the GenBank database. The tested strain is denoted by a red circle.





**Fig. 5:** Seasonal prevalence of *Vibrio alginolyticus* among naturally infected Egyptian sole (*Solea aegyptiaca*).



**Fig. 6:** Intensities of *Vibrio alginolyticus* in different internal organs of naturally infected Egyptian sole (*Solea aegyptiaca*)

### Discussion

Vibriosis (*Vibrio* spp.) is a prevalent bacterial disease that affects a variety of marine fish species, including Senegalese sole (*Solea senegalensis*) (Menanteau-Ledouble et al., 2016). The clinical picture of the most infected fish showed skin detachment. Some fish revealed slight abdominal distention (ascites due to serohemorrhagic fluids). These results resemble those

that were noted by (Zhang et al., 2010; Austin and Austin, 2012; Abdelaziz et al., 2013 and Abdelaziz et al., 2017). The postmortem findings of naturally infected fish showed pale gills and friable livers. The results were nearly similar to those obtained by (Rico et al., 2008; Abdelaziz et al., 2013; Hanna et al., 2014; Salah et al., 2019 and Hassan et al., 2021). All signs of anemia and hemorrhagic septicemia which were

observed in some infected fish were due to bacterial hemolysins that cause lysis of erythrocytes in the host (Zhang and Austin, 2005). *V. alginolyticus* produces multiple virulent extracellular products including proteases, hemolysin, and siderophores which are responsible for the signs of hemorrhagic septicemia (El sayed et al., 2019). *V. alginolyticus*' morphological characteristics and biochemical activities fulfilled the standard criteria that were also described by (Zorrilla et al., 2003; Kahla-Nakbi et al., 2006; Tang et al., 2008; Younes et al., 2016; Abdelaziz et al., 2017 and El sayed et al., 2019). All of the examined *V. alginolyticus* isolates tested positive for the 16S rRNA gene of *Vibrio* spp. and gave amplicons size of 618 bp, according to the molecular characterization of the recovered isolates. Also, the examined *Vibrio* isolate (Accession No. OP324632) showed a genetic identity to other *Vibrio alginolyticus* strains from other origins, according to the sequencing analysis of that gene. Using the partial 16S ribosomal RNA gene sequence and 100% nucleotide identity, the isolate was also identified as *Vibrio alginolyticus*.

The seasonal prevalence of *V. alginolyticus* was of the highest value in the summer (31.43%), followed by spring and winter (25.72% each), while the lowest prevalence was recorded in the autumn (17.14%). These results were roughly in agreement with

(Winfield, 2018; Salah et al., 2019 and Hassan et al., 2021), while contradicting the results of (Golomazou et al., 2006) who found that *V. alginolyticus* were not associated with a particular season. Accordingly, the ability of *Vibrios* to use skin mucus significantly increases during the summer as a chemoattractant was credited, which improved the pathogen's entry into the host (Otoole et al., 1999). The validity of this claim was supported by (Austin and Austin, 2007) who noted that vibrio outbreaks frequently take place when water temperatures exceed 15 °C. Furthermore, (Su and Liu, 2007) noted that vibriosis might not be detected during the winter, as water temperatures are unfavorable. Similarly, (Roberts, 2001) reported significant wild fish mortality rates due to *Vibrios* infection in the late summer, when the temperatures are high.

Regarding the intensities of *V. alginolyticus* in the various organs of naturally infected solea fish, the gills were found to have the highest prevalence (35.95%) this may be due to the gills are constantly exposed to a wide range of microorganisms in aquatic environment, including bacteria and also gills have a large surface area, which increases the likelihood of bacterial attachment and colonization, followed by the liver (27.12%), kidney (20.34%), and skin lesions (16.95%). These results were consistent with Austin and Austin (2016) who mentioned

the high prevalence of vibrio spp. in gills of infected fish, *Moustafa et al.*, (2010) and *El-Bassiony* (2001) who reported a high intensity of vibrio in infected liver followed by kidneys and spleen. In fact, liver is the main organ that is involved in the process of detoxification and biological transformation and its function can be greatly affected by water pollutants (*Camargo and Martinez*, 2007). Thus, the present findings proved that the liver and kidney are the main disease target organs, and that this tissue preference was mostly due to some of the virulence tools inherited by the invading pathogens, which favoured their presence in the detoxifying organ (liver) and the major immunological warrior (kidney) (Qin et al., 2014).

### Conclusion

In conclusion, our study confirmed that wild Egyptian sole (*Solea aegyptica*) as any marine fish may be affected with vibriosis due to *Vibrio alginolyticus* especially in seasons with high temperature. The most affected organs were gills and liver. Moreover, molecular approach is a sensitive, rapid and specific tool for proper recognition of *Vibrio alginolyticus* isolated from naturally infected *Solea aegyptica*.

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دراسات بكتيرية على الفيبريو في اسماك موسى المصرية  
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### الملخص العربي

في هذه الدراسة، تم تجميع عدد 120 من أسماك موسى المصرية بأوزان (50-70 جم) وأطوال (13-19 سم) بشكل عشوائي وموسمي من البحر الأبيض المتوسط في محافظة بورسعيد من (سبتمبر 2019 الى أغسطس 2020). جمعت عينات معقمة من الخياشيم والكبد والكلية والطحال للفحص البكتريولوجي. تم زرع العينات على اطباق من وسط محدد للفيبريو. تم تقييم جميع العزلات النقية باستخدام بادئات عالمية ومحددة خاصة بالفيبريو.

1. أظهر الفحص الاكلينيكي للأسماك المصابة عن انفصال الجلد. أظهرت بعض الأسماك انتفاخ طفيفا في البطن.

2. تم رصد الصفة التشريحية للأسماك المصابة وقد وجد في التجويف البطني سائل أبيض الى أصفر اللون مع تفتت في الكبد والخياشيم باهتة اللون.

3. كانت أعلى نسبة إصابة بالفيبريو ألبينوليتكس سجلت في فصل الصيف بنسبة (31.43%) يليه فصل الشتاء والربيع بنسبة (25.72%) لكل منهما بينما سجلت أقل نسبة إصابة في فصل الخريف بنسبة (17.14%).

4. كانت نسبة الإصابة بالفيبريو ألبينوليتكس في الأسماك المصابة في الخياشيم بنسبة (35.95%) يليه الكبد بنسبة (27.12%) ثم الكلية بنسبة (20.34%) بينما سجلت أقل نسبة إصابة في الطحال بنسبة (16.95%).

5. التحقيق الجزيئي للعزلات النقية باستخدام برايمرز محدهه كان إيجابي 16 اس ريبوزومال ار ان ايه لجينات فيبريو واعطي 663 بي بي.