Occurrence of *vibrio spp.* in marked shellfish

Esraa M. Zaghlal

Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Zagazig, 44511, Egypt.

Abstract

Seventy- five samples of the common marketed shellfish Shrimp, Crab, Oyster, lobster and squilla mantis (15 of each) were collected from the fish market in Sharkia Governorate, Egypt. All shellfish samples were subjected to bacteriological examination for isolation and identification. The overall prevalence of vibrio in examined shellfish was 72%. The identified species *Vibrio. fluvialis* were detected in 1(7%), 4(33%) and 3(25%) of examined crab, squilla mantis and oyster, respectively. *Vibrio mimicus* was detected in 2(13%), 2 (17%) and 3(50%) of examined *crab, mantis and lobster,* respectively. *Vibrio alginolyticus* was detected in 3(20%), 3 (33%), 4(33%) and 3(25%) in examined *crab, shrimp, Oyster and squilla mantis,* respectively . *Vibrio vulnificus* was detected in 3(20%), 1(11%) and 3 (25%) of examined *crab, shrimp* and *oyster,* respectively. *Vibrio cholera* was detected in 3(20%), 2(22%), 1(8%) and 1 (17%) of examined *crab, shrimp, Oyster and Lobster,* respectively. *Vibrio parahaemolyticus* was detected in 3(20%), 3 (33%), 2(17%), 2(17%) and 2 (33%) of examined *crab, shrimp, Oyster, squilla mantis and Lobster,* respectively.

1- Introduction

shellfish considered as important food element for broad section of world population and considered the main protein of animal source . Recently, more people exchange red meat to fish **(Adebayo-Tayo *et al.*, 2012).**

shellfish is a main dietary Supply of proteins of rich biological value, vitamins, minerals and omega-3 which are essential for optimum neural development, and is low in saturated fatty acid. They are very healthy foods.**(National Public Health Institute of Finland, 2004)**

Although Seafood is a nutritious food that constitutes one of the desirable components of a healthy diet. One of the main risks involves the consumption of uncooked or improperly cooked seafood that may be naturally contaminated by foodborne pathogens present in the marine environment. Such risk is further increased if the food is mishandled during processing where pathogens could multiply exponentially under favorable conditions**.(Food and Environment Hygiene Department, 2005).**

The main microorganism identified in foodborne diseases of marine shellfishes are *Vibrio anguillarum, V. alginolyticus, V. fluvialis, V. furnissii, V. harveyi, V. parahaemolyticus and V. Vulnificus* as reported by **(Buck 1990 and Liao et al., 2004).**

The public health hazard of consumption of raw, inadequately cooked or insufficiently processed fish, and fish products include nausea, vomiting, diarrhea, abdominal cramps, headache as well as muscle and joint pain. Good manufacturing practices should be in place to minimize the risk of food poisoning associated with the consumption of fish and seafood products.**(Pal *et al.*, 2016)**

This study was aimed to investigate the prevalence and distribution vibrio spp. in different marked shellfish.

2- Materials and Methods

2.1. Collection of shellfish samples

A total of 75 random samples of shellfish (Shrimp, Crab, Oyster, lobster, squilla mantis) 15 of each were collected from Sharkia governorate fish markets and from different retail seafood stores during winter of 2018 . The collected samples were packaged separately and transferred with a minimum of delay to the laboratory in a sterile ice box. All samples were subjected to bacteriological examination for enumeration, isolation and identification.

2.2. Preparation of samples:

The samples were rapidly washed with tap water Then the surfaces were swapped with 70% ethyl alcohol for surface disinfection. The shells were removed, Then the muscles were removed with a sterile forceps and a scalpel and placed into sterile Petri dishes. Five grams were taken under aseptic conditions to sterile homogenizer containing 45ml of sterile alkaline peptone water (3% NaCl and pH 8).

Screening of Vibrio spp.:

It was done according to **FDA (2004) as follows:**

Enrichment broth:

The previous prepared tubes were incubated over night at 37ºC.

Isolation:

Loopfuls from each previous cultured tube were separately streaked onto Thiosulfate citrate bile and sucrose agar (TCBS) then incubated at 37ºC for 24hrs.

2.3. Identification of total Vibrio specie:

The suspected isolates were further identified according to the protocol recommended by **ISO/ TS 21872-1 (2007) and ISO/ TS 21872-2 (2007).**

1. Morphological examination:

Films were prepared from a pure culture of the isolated microorganism stained with Gram's stain and then examined microscopically. Vibrio species were short, rigid, Gram –ve, single flagellum and comma – shape.

2. Biochemical identification:

2.1. Oxidase test:

Oxidase test was done by streaking of the pure culture onto filter paper moistened with oxidase reagent. The test is positive if the colour turns to mauve, violet or deep purple within 10 seconds. All vibrio species are oxidase positive.

2.2. TSI agar test:

The agar slope was inoculated by stabbing to the bottom of the agar butt and streaking longitudinally along the slope. Then it was incubated at 37°C for 24hrs.

2.3. Detection of Ornithine decarboxylase (ODC):

Suspected colonies were inoculated into ornithine decarboxylase medium just bellow the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hrs. Turbidity and violet color after incubation indicate a positive ODC.

2.4. Detection of L- lysine decarboxylase (LDC):

Suspected colonies were inoculated into L- lysine decarboxylase medium just bellow the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hrs. Turbidity and violet color after incubation indicate a positive LDC.

2.5. Detection of Arginine decarboxylase (ADH):

Suspected colonies were inoculated into arginine decarboxylase medium just bellow the surface. One ml of sterile mineral oil was added to the top of the medium and incubated at 37°C for 24 hrs. Turbidity and violet color after incubation indicate a positive ADH.

2.6. Detection of β- galactosidase (ONPG):

The suspect colonies were inoculated into a sterile tube containing 2.5% Nacl solution and mixed. Drop of toluene was added and the tube was shaken. The tube was placed in the water bath adjusted at 37°C and left to stand for approximately 5 min. About 0.25 ml of the reagent was added for detection of β- galactosidase (2- ortho-Nitrophenyl-β-D-galactopyranoside) "ONPG" and mixed. The tube was replaced in the water bath set at 37°C and left to stand for 24 hrs then examined from time to time. A yellow color indicates positive β- galactosidase.

2.7. Indole test:

A tube containing 5 ml of the tryptone-tryptophan saline medium was inoculated with the suspected colony; Incubated at 37°C/for 24 hrs, One ml of the Kovacs' reagent was added. Formation of red ring indicates a positive production of indol.

2.8.Halotolerancetest:

Series of sterile peptone water was produced with increasing salt (Nacl) concentration: 0 %, 2 %, 4 %, 6 %, 8 % and 10 %. Suspension with the suspected colony was prepared to be identified and each of the tubes with loopful was lightly inoculated and incubated at 37°C for 24 hrs. The turbidity indicates that the suspected colony could grow at a concentration of sodium chloride present in the tube of sterile saline peptone water.

Polymerase Chain Reaction (PCR)

1. Materials used for PCR:

1.1. Reagents used for agrarose gel electrophoresis:

1.1.1. Agarose powder, Biotechnology grade (Bioshop^{R}, Canda inc. lot No: OE16323).

It prepared in concentration 2% in 1× TAE buffer.

1.1.2. Tris acetate EDTA (TAE) electrophoresis buffer (50×liquid concentration) (Bioshop^{R} , Canda inc. lot No: 9E11854).

The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. Water to be used in the preparation of the gel or as a running buffer.

1.1.3. Ethedium bromide solution (stock solution) biotechnology grade (Bioshop ® Canda Inc, Lot No: 0A14667):

The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4ºC. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

1.2. Gel loading buffer (6×stock solution) (Fermentas, lot No: ooo56239).

The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

1.3. DNA ladder (molecular marker):

100 bp **(Fermentas, lot No: 00052518).**

1.4. 5X Taq master (Fermentas):

Containing polymerase enzyme, Magnesium chloride (Mg Cl_2), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

1.5. Primer sequences of *V. parahaemolyticus* for virulence factors:

Molecular characterization of virulence factors including **thermostable direct hemolysin (tdh) and thermolabile hemolysin (tlh) genes of** *V. parahaemolyticus*.

2. DNA Extraction using QIA amp kit (Shah et al., 2009):

After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100ºC for 20 minutes. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and the manufacturer's instructions (Qiagen, Hilden, Germany) were applied according to the following steps:

1. Equal volume from the lysate (50-200 µl) was added, addition of 20-50µl of proteinase K, then incubation at 56 ºC for 20-30 min. After incubation, 200 µl of 100% ethanol was added to the lysate.
2. The solution was added to the column and centrifuged at 8000 rpm for 1 min. then the filtrate was discarded.
3. The sediment was washed using AW1 buffer (200 µl), the column was centrifuged at 8000 rpm / 1 min, and the filtrate was discarded.
4. Washing was applied by using the AW2 buffer (200µl), the column was centrifuged at 8000 rpm / 1 min. and the filtrate was discarded.
5. The column was placed in a new clean tube and 25-50 µl from the elution buffer was added, centrifuged at 8000 rpm/1min.
6. The column was discarded. The filtrate was put in clean tube containing the pure genomic DNA. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

3. DNA amplification:

3.1. Amplification of *V.parahaemolyticus* virulent genes (Khalil et al., 2014):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). The final volume of the reaction mixture was adjusted to 50 µL with sterile deionised distilled water. Amplification of DNA segment was performed) with the following temperature cycling parameters; initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 57°C for 30 sec, primer extension at 72 °C for 1 min and a final extension at 72 °C for 2 min. Ten µL of each amplified

product was loaded in 1.5% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089M Trisbase, 0.089M boric acid and 0.002 M EDTA (pH 8.0)] at 100 volts for 40 minutes. After electrophoresis, amplification products were captured and visualized on UV trans illuminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

3- Results and Discussion

From the results clarified in Table (1) , the incidence of vibrio species in table (4) and figure (4) were 100% ,60% ,80%,80%,40% in the studied crab, shrimp, oyster, mantis and lobster, respectively The average percentage was 72% overall examined shellfish.

These results nearly agreed with those reported by **Waffa *et al.*(2011),**who found that the total rate of isolation of Vibrio spp. isolated from shellfish was 88%. The low incidences of Vibrio in shellfish obtained by **Ripabelli et al. (1999)** they stated that Vibrio spp.

were isolated from shellfish with percentage of 48.4%.

The shellfish Contaminated by Vibrio spp because it is naturally present in aquatic environments. It is the primarily marine bacterial pathogens associated with illness. It is responsible for the third-highest number of shellfish-related illnesses, after non cholera Vibrio spp. and Norwalk viruses. Toxigenic O_1 (epidemic biotype) of Vibrio spp. infections are associated with profuse, watery diarrhea whereas nontoxigenic, non-Ol biotype (except O139) infections result in septicaemia and mild gastroenteritis **(Wittman and Flick,1995 ; Gnanambal and Patterson, 2005; DePaola et al.,2010 ; CDC,2010)**.

Table 1:): Incidence of vibrio species in examined shellfish samples (No = 15 for each)

Type	Crab	Shrimp	S.mantis	Oyster	Lobster	Total
No. of positive sample	15	9	12	12	6	54
%	100	60	80	80	40	72

In the present study, The obtained results in table (2) and figure (1) showed that *Vibrio. fluvialis* were detected in 1(7%), 4(33%) and 3(25%) of examined crab, squilla mantis and oyster, respectively . The incidence of V. fluvialis was recorded by **Yücel and Balci (2010)** in blue crabs (>10%).

Vibrio mimicus was detected in 2(13%), 2 (17%) and 3(50%) of examined crab, mantis and lobster, respectively. A lower prevalence of finding obtained by ***Wafaa et al. (2011)*** who found 11% *in some seafood.*

Vibrio alginolyticus was detected in 3(20%), 3 (33%), 4(33%) and 3(25%) in examined crab, shrimp, Oyster and squilla mantis, respectively . A higher prevalence of *V. alginolyticus* had been reported by **Gomathi *et al.* (2013)** who found 80% in sea food.

Vibrio vulnificus was detected in 3(20%), 1(11%) and 3 (25%) of examined *crab*, *shrimp* and *oyster*, respectively. Nearly similar finding obtained by **El Far *et al.* (2015)** who found 16.7% in Indian prawn. A higher prevalence of V. *vulnificus* was recorded by **Ripabelli et al. (1999)** in shellfish (32%).

Vibrio cholera was detected in 3(20%), 2(22%), 1(8%) and 1 (17%) of examined crab, shrimp, Oyster and Lobster, respectively.

Vibrio were the most commonly reported cause of seafood-associated outbreaks where toxigenic V. cholerae caused 3 outbreaks and 10 illnesses without deaths and non-O1,

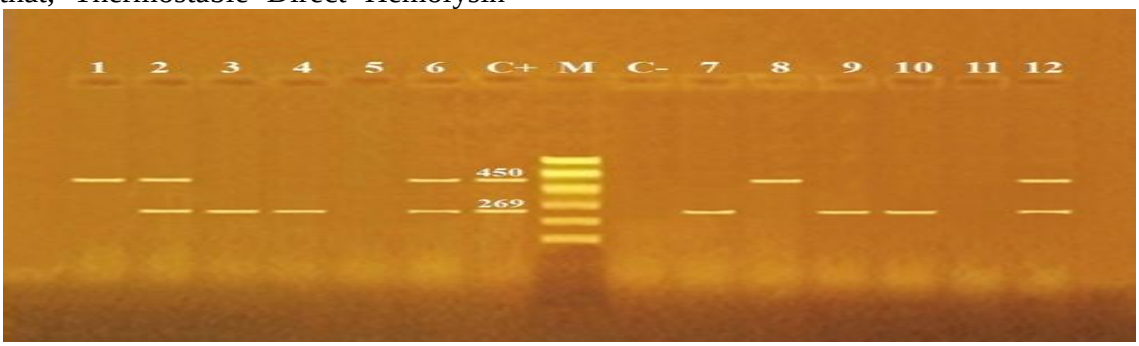
non-O139 *V. cholerae* caused 4 outbreaks and 12 illnesses without deaths **Iwamoto et al.,(2010).**

Table 2. Frequency distribution of isolated vibrio organisms recovered from examined shellfish sample:

Isolate	Crab		Shrimp		S.mantis		Oyster		Lobster	
	No.	%	No.	%	No.	%	No.	%	No.	%
V.cholera	3	20	2	22	0	0	1	8	1	17
V.alginolyticus	3	20	3	33	4	33	3	25	0	0
V.fluvialis	1	7	0	0	4	33	3	25	0	0
V.parahaeolyticus	3	20	3	33	2	17	2	17	2	33
V.vulnificus	3	20	1	11	0	0	3	25	0	0
V.mimicus	2	13	0	0	2	17	0	0	3	50

In the present study in photograph (1) showed that: Lane M:100 bp ladder as molecular size DNA marker. Lane C+: Control positive V. parahaemolyticus for tdh and tlh genes. Lane C-: Control negative.Lanes 3, 4, 7, 9 & 10: Positive V. parahaemolyticus strains for tdh gene.Lanes 1 & 8: Positive V.parahaemolyticus strains for tlh gene. Lanes 2, 6 & 12: Positive V. parahaemolyticus strains for tdh and tlh genes. Lanes 5 & 11: Negative V. parahaemolyticus strains for tdh and tlh genes. The obtained results of agarose gel electrophoresis virulence genes showed Positive *V. parahaemolyticus* strains for tdh gene and tlh gene. Our results supported the results obtained by **Cook et al.(2002)** and **Okuda et. al.(1997a)** who stated that, Thermostable Direct Hemolysin (TDH) was the first recognized virulenc factor for *V. parahaemolyticus* and has been used as an important marker for identifying virulent strains. In addition, The TDH encoding gene (tdh) is able to induce intestinal chloride secretion resulting secretory diarrhea **(Raimondi et. al.,1995).**

Simillary, **Bej et. al.,(1999)** reported that out of 111 isolates of V. parahaemolyticus 60 (54%), showed amplification of tdh gene by using similar primer pair study which yielded desired amplified product of approximately 269 bp similar to the reference strain of V. parahemolyticus using the primer pair for tdh gene. Therefore proper chilling and handling of seafood's of marine water origin is essential.



Photograph (1): Agarose gel electrophoresis of multiplex PCR of *tdh* (269 bp) and *tlh* (450 bp) virulence genes for characterization of *V. parahaemolyticus*. Lane M: 100 bp ladder as molecular size DNA marker.**Lane C+:** Control positive *V. parahaemolyticus* for *tdh* and *tlh* genes.**Lane C-:** Control negative. **Lanes 3, 4, 7, 9 & 10:** Positive *V. parahaemolyticus* strains for *tdh* gene. **Lanes 1 & 8:** Positive *V. parahaemolyticus* strains for *tlh* gene. **Lanes 2, 6 & 12:** Positive *V. parahaemolyticus* strains for *tdh* and *tlh* genes. **Lanes 5 & 11:** Negative *V. parahaemolyticus* strains for *tdh* and *tlh* genes.

4- Conclusion

It was concluded that examined shellfish samples were contaminated with Vibrio spp. which considered one of the main food poising microorganisms.

5- References

Adebayo-Tayo, B. C., Onilude, A. A., & Etuk, F. I. (2011). Studies on microbiological, proximate mineral and heavy metal composition of freshwater snails from Niger Delta Creek in Nigeria. *AU Journal of Technology*, *14*(4).

Bej, A.K.; Donald P.; Pattersona, Cynthia W.; Brashera, Michael C.L.; Vickerya,Daniel D.; Jonesa and Charles A. Kaysnerb (1999). Detection of total and hemolysin-producing Vibrio parahaemolyticusin shellfish using multiplex PCR amplification of tl, tdhand trh J. of Microbiol Meth. 36(3): 215-225.

Buck, J. D. (1990): *Vibrio species* in sea water and marine animals in the Sarasota, Florida area. Journal of Coast. Rev. 6: 943-948.

Centers for Disease Control and Prevention (CDC), "Preliminary foodnet data on the incidence of infection with pathogens transmitted commonly through food," Morbidity and Mortality Weekly Report, vol. 59, no. 14, pp. 418–422, 2010.

Cook, D.W.; Leary, P.; Hunsucker, J.C.; Sloan, E.M., Bowers, J.C.; Blodgett, R.J. and Depaola, A. (2002). Vibrio vulnificusand Vibrio parahaemolyticusin U.S.retail shell oysters: A National Survey from June 1998 to July 1999. J. Food Prot., 65 (1): 79-87.

DePaola, A., Jones, J. L., Woods, J., Burkhardt, W., Calci, K. R., Krantz, J. A., ... & Williams-Hill, D. (2010). Bacterial and viral pathogens in live oysters: 2007 United States market survey. Applied and Environmental Microbiology, 76(9), 2754-2768.

El Far, S. A., Khalil, R. H., Saad, T. T., El-Tanekhy, M., & Abdel-Latif, H. M. (2015). Occurrence, characterization and antibiotic resistance patterns of bacterial communities encountered in mass kills of pond cultured Indian prawn (Fenneropenaeus indicus) at Damietta governorate, Egypt. International Journal of Fisheries and Aquatic Studies, 2(4), 271-276

FDA (Food and Drug Administration) (2004): Bacteriological Analytical Manual, *vibrio* Chapter-9 (BAM).

Food and Environment Hygiene Department (2005): Vibrio species in seafood. Risk Assessment Studies, Report No. 20.The Government of the Hong Kong Special Administrative Region.

Gnanambal K. and J. Patterson, "Biochemical and microbiological quality of frozen fishes available in Tuticorin supermarkets," Fishery Technology, vol. 42, no. 1, pp. 83–84, 2005.

Gomathi, R.S.; Vinothkumar R and Arunagiri K. (2013): Isolation and Identification Vibrios from marine Seafood Samples. Int. J. Curr. Microbiol. App. Sci., 2 (2):36-43.

International Organization for Standardization/ technical specification "ISO/TS 21872-1" (2007): Specifies a horizontal method for the detection of the two main pathogenic *Vibrio* species causing intestinal illness in humans: *V. parahaemolyticus* and *V. cholerae.*

International Organization for Standardization/ technical specification "ISO/TS 21872-2" (2007): Specifies a horizontal method for detection of the enteropathogenic Vibrio species, causing illness in or via the intestinal tract, other than *Vibrio parahaemolyticus* and Vibrio cholerae. Include *Vibrio fluvialis, Vibrio mimicus* and *Vibrio vulnificus.*

Iwamoto, M., Ayers, T., Mahon, B. E., & Swerdlow, D. L. (2010). Epidemiology of seafood-associated infections in the United States. *Clinical microbiology reviews*, *23*(2), 399-411.

Khalil, S., Abou-Akkada, A. and El-Hoshy, S. (2014): Molecular studies on Vibrio species isolated from imported frozen fish. Global Veterinaria 12 (6): 782-789.

Liao, I. C., Huang, T. S., Tsai, W. S., Hsueh, C. M., Chang, S. L., & Leaño, E. M. (2004). Cobia culture in Taiwan: current status and problems. Aquaculture, 237(1-4), 155-165.

National Public Health Institute of Finland. (2004) Finnish Food Composition Database. Available at: http:// www.fineli.fi. Accessed 15th July 2006

Okuda, J.; Ishibashi, M.; Abbott, S.L.; Janda, J.M. and Nishibichi, M. (1997a). Analysis of the thermosatble direct hemolysin (tdh) gene and tdh-related hemolysin (trh)genes in urease-positive strains of Vibrio Parahaemolyticus isolated on the west coast of the United States. J. Clin. Microbl., 35: 1965-1971.

Pal M., Ketema A., Anberber M., Mulu S. and Dutta Y (2016). Microbial quality of Fish and Fish Products. BEVERAGE & FOOD WORLD - Vol. 43 - No. 2

Raimondi, F.; Kao, J.P.Y.; Kaper, J.B.; Guandalini, S. and Fasano, A. (1995). Calcium dependent intestinal chloride

secretion by Vibrio parahaemolyticus thermostable direct hemolysin in a rabbit model. Gastroenterology., 109:381-386.

Ripabelli, G., Sammarco, M. L., Grasso, G. M., Fanelli, I., Caprioli, A., & Luzzi, I. (1999). Occurrence of Vibrio and other pathogenic bacteria in Mytilus galloprovincialis (mussels) harvested from Adriatic Sea, Italy. *International Journal of Food Microbiology*, *49*(1-2), 43-48.

Shah, D., Shringi, S., Besser, T. and Call, D. (2009): Molecular detection of foodborne pathogens, Boca Raton: CRC Press, In Liu, D. (Ed). Taylor & Francis group, Florida, USA, Pp. 369-389.

Wafaa MK, Hanssah-Walaa A and Abaza AF. (2011): Detection of Salmonella and Vibrio species in some seafood in Alexandria, J. Amer. Sei, 7(9): 663-668.

Wittman, R. J., & Flick, G. J. (1995). Microbial contamination of shellfish: prevalence, risk to human health, and control strategies. *Annual Review of Public Health*, *16*(1), 123-140.

Yücel, N., & BALCI, Ş. (2010). Prevalence of Listeria, Aeromonas, and Vibrio species in fish used for human consumption in Turkey. *Journal of food protection*, *73*(2), 380-384.

مدى تواجد ميكروب الفيبريو في قشريات الاسماك المسوقه.

اسراء مصطفي عطيه زغلل

قسم الأمراض المشتركة - كلية الطب البيطرى –جامعة الزقازيق- الزقازيق- 44511-مصر

الملخص العربى

تم تجميع عدد 75 عينة عشوائية من قشريات الاسماك المسوقه في محافظة الشرقية بواقع 15 عينة لكلا من (الجمبري ، الكابوريا ، الجاندوفلي ، الشيكال ،الاستاكوزا) خلال شتاء 2018 وتم ارسال هذه العينات تحت ظروف صحيه الي المعمل لفحصها بكتيريولوجيا وقد دلت النتائج علي الاتي :

نسبه تواجد ميكروب الفيبريو كان 100 % فى الكابوريا و 60% فى الجمبري و80% فى الشيكال و80 % فى الجندوفلي و40% فى الاستاكوزا.

وتم تصنيف ميكروب الفيبريو فلافيالس بنسب (7% ،33 % ، 25%) فى الكابوريا والشيكال الجندوفلي على التوالي و فيبريو يميكس بنسب (13% ، 17% ، 50%) فى الكابوريا والشيكال والاستاكوزا على التوالي وفيبريو الجينوليتيكس بنسب (20% ، 33% ، 33% ، 25%) فى الكابوريا والجمبري والجندوفلي والشيكال على التوالي وفيبريو فالينيفيكس بنسب (20% ، 11% ، 25%)فى الكابوريا والجمبري و الجندوفلي على التوالي وفيبريو كوليرا بنسب (20% ، 22% ، 8% ، 17%) فى الكابوريا والجمبري والجاندوفلي والاستاكوزا. الفيبريو باراهيموليتيكس بنسب (20% ، 33% ، 17% ، 17% ، 33%)فى الكابوريا والجمبري والشيكال و الجندوفلي والاستاكوزا على التوالى.