# CLINICAL AND EXPERIMENTAL STUDY ON VIBRIOSIS IN ORNAMENTAL FISH

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## ABSTRACT

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The aim of this study was to investigate vibrio infections among some species of ornamental fishes in Assiut, Egypt. A total number of 100 ornamental fishes showing signs of septicaemia were collected from private ornamental fish shops in Assiut Governorate. Dropsy, exophthalmia, detachment scale, and haemorrhage on the body surface were the main clinical signs observed on the fish collected. According to conventional identification, 59 isolates suspected to be vibrio species were recovered from 40 fish. Cultural, morphological and biochemical characteristics of these isolates identified them as *V. vulnificus* (38.98 %), *V.parahaemolyticus* (28.8), *V.harveyi* (11.86%), *Vibrio alginolyticus* (5.08%), *V.mimicus (*5.08%), *V. ordalii* (6.78%). and *V. fisheri* (3.39%). A molecular typing system based on amplification of the intergenic spacer (IGS) region was used to confirm the identity of a *Vibrio vulnificus* isolate to investigate its pathogenicity in fantail fish through an experimental challenge. *Vibrio cholerae* is also molecularly identified and discussed but not isolated. The prominent signs seen on experimentally infected fish included hemorrhages in the peritoneum and visceral organs. The intestine were filled with bloody fluid, while gelatinous exudates were covering the gas bladder. The *V. vulnificus* strain used for experimental challenge was sensitive to Oxytetracyclin, Neomycin, and Erythromycin, but was resistant to Ampicillin and Tobramycin.

***Key words:*** *Vibriosis, ornamental fish, PCR intergenic spacer (IGS).*

## INTRODUCTION

Vibriosis is one of the most important bacterial diseases in ornamental fishes that cause economic losses. Septicemia induced by vibriosis is characterized by haemorrhages on the fin base, exophthalmia, edematous lesions on the body surface and loss of appetite (Toranzo *et al.*, 2005). Relatively scanty data on the microbial communities associated with these ornamental fishes or the aquarium water in which they are transported and housed in Egypt.

The aim of this study is to investigate the infections with vibriosis among some species of ornamental fishes. Also, the pathogenicity induced by a selected *Vibrio vulnificus* isolate in fantail fish (*Carassius auratus*) was studied. In addition, the sensitivity of *V. vulnificus* to commonly used antibiotics was determined.

## MATERIALS and METHODS

**Fish**:

1-1 naturally infected fish:

A total number of 100 moribund ornamental fish showing signs of septicaemia were collected from ornamental fish shops in Assiut Governorate. Seventy three Moribund fantail (*Carassius auratus auratus*), 17 black molly (*Poecillia latipinna*), and 10 koi carp (*cyprinus carpio*), with body weight ranged from 10 to 25g were transported to the Aquatic Animals Diagnostic Laboratory, Faculty of Veterinary Medicine, Assiut University.

1-2 Experimental fish:

A total number of 100 apparently healthy fantail with an average weight of 15-20g were collected from pet shops. Fish were maintained in glasses aquaria and acclimated for two weeks according to Ellsaesser and Clem (1986) before being used in experimental challenge. Aquaria were supplied with aeration, heater (28°C) and filter. Fish were fed daily with commercial feed at 3-5% body weight. The fish were randomly examined to ensure that they were disease-free prior to using in the experimental challenge.

**Clinical and post mortem examination:**

Fished collected were subjected to clinical (Stoskoph, 1993).

**Isolation and characterization of bacteria**:
Bacteria were isolated directly from the liver and kidney of fantail fish on brain heart infusion agar (BHI) supplement with 3, 6, or 8% NaCl, and thiosulphate citrate bile salt sucrose agar (TCBS). Plates were incubated at 28°C for 48h. Colonies were picked up on the basis of morphological features, purified by sub-culturing, and stored on BHI slants at 4°C for further investigations. Isolates were identified according to the diagnostic schemes described by Austin and Austin (2007).

**Molecular identification:**
**PCR-based method for targeting 16s-23s rRNA intergenic spacer (IGS) regions among vibrio species:**
The technique depends on amplifying the IGS region with genus-specific primers results in amplicons with variable lengths and sequences giving a unique pattern for each species. The resulted amplicons are analyzed by rapid separation technique (gel electrophoresis) result on a strain-specific pattern (Hoffmann *et al.,* 2010).

Vibrio isolates were cultured on trypticase soy agar (TSA) containing 1.5% NaCl for 24 hours conventionally identified as *V. vulnificus.* DNA extraction was performed. Intergenic spacer PCR primers for 16s.6 (5´-ACTGGGGT-GAAGTCGTAACA-3´) and 23S.1 (5´-CTTCATCGC-CTCTGACTGC-3´) were used (Hoffmann *et al.,* 2010).

PCR was performed in a 50 µl volume containing 300 µM dNTP, 5U of Hot Start Taq Polymerase, 1×Taq polymerase buffer, 1.5mM Mgcl2 and a 300nM concentration of each primer with 100 ng of DNA template. The amplification program was 95°c for 15 min, 10 cycles at 95°c for 30 sec., 73°c-64°c (decreasing 1°c/cycle) for 10 sec and 72 °c for 45 sec. Afterwards, complete amplification was achieved with 34 cycles of 95°c for 30 sec, 64°c for 10 sec and 72°c for45 sec., finished with a single cycle at 72°c for 1 min and stored at 4°c.

Heteroduplex formation was resolved with an additional amplification cycle. PCR products were diluted 1:5 in a 30 µl volume. Then were subjected to a single amplification cycle of 95°c for 15 min, 64°c for 1 min and 72°c for 10 min in a similar reaction mixture containing 600 nM primer concentrations (Hoffmann *et al.,* 2010).

**3-1 Bacterial strain:**
Bacterial strains were kept in BHI broth with 15% glycerol (El-Gomhurrhia, Cairo, Egypt) at -20ºC. The *V. vulnificus* selected strain was passed three times in fantail fish through intraperitoneal injection before using for experimental challenge.

**3-2 Bacterial challenge suspension and counts:**
Colony forming units (cfu) counts in bacterial suspensions were determined using spectrophometery optical density values at wavelength of 600 nm and standard-plate-count method with ten-fold serial dilution (Elkamel and Thune, 2003).

**3-3: Experimental challenge**
A preliminary challenge with immersion for 90 minutes indicated that $1X10^7$ cfu/ml of *V. vulnificus* can induce infections and mortalities in fish. Thirty acclimated fantail ornamental fish were subdivided into six equal groups with five fish each. Fish from three groups were subjected to an immersion bath in a solution containing $1X10^7$CFU/ml of *V. vulnificus* for 90 minutes. The other three groups were subjected to the same procedures and immersed in solution without bacteria. All groups were kept in 110 L glass aquarium at water temperature 28 °C with constant aeration and observed for 15 days. The clinical signs and numbers of dead fish were recorded during the observation time. Re-isolation and identification of the inoculated organism from freshly dead and moribund fish were carried out as described above.

**Antibiotic sensitivity assay:**
Due to the high frequent isolation of *V. vulnificus*, antibiotic sensitivity test was carried out as described by Carter and Cole (1990) using different members of antibiotics.

## RESULTS

**Clinical and bacteriological examinations:-**
The clinical signs associated with natural infections fish were dropsy, exophthalmia, detachment scales, and haemorrhages on the body surface. Internally, ascetic fluid was seen in the abdominal cavity. Liver was congested with enlarged gall bladder, while kidneys were congested.

A hundred ornamental fish were used for bacteriological examination. A total of 92 bacterial isolates of different morphological characteristics were isolated from liver and kidney of the infected fish. Fifty nine fishes were suspected to be Vibrio species based on morphological and biochemical characteristics. Suspected Vibrio isolates were recovered from 40 out of the examined fish. All isolates were Gram negative, motile rod shape curved bacilli, produced cytochrome oxidase, oxidation fermentation test, and exhibited catalase activity. All suspected isolates tolerated BHI media supplemented with 3 % sodium chloride. Biochemical characterization distinguished 7 species as: *V. vulnificus* (23, 38.98%), *Vibrio parahaemolyticus* (17, 28.81%), *V. harveyi* (7, 11.86%), *V. 0rdalii* (4, 6.78%), *Vibrio alginolyticus* (3,5.08%), *Vibrio mimicus* (3, 5.08%), and *V. fisheri* (2,3.39%). Results

of the biochemical characters and enzyme activities of suspected isolates are shown in Table (1).

**DNA fingerprinting:**
Performing PCR with 16s-23s rDNA primer on suspected *V. vulnificus* isolates yielded the typical pattern for the *V.vulnificus* with two major bands of 600 and 700 bp (Lane, B), although the strain c biochemically reveal *V. vulnificus*, in 16s-23s IGS technique, the strain showing the three major bands of 600- 700- 750 (Lane C) characteristic to *V. cholerae* probably non-O1/non-O139 (Fig.2).

**Experimental infection:**
Moribund fantail ornamental fish in the challenged group showed lesions similar to those of naturally infected fish. These clinical signs were lethargic, erratic swimming, sluggish movement, detachment scales, increase mucus secretion covering body surface and gills and ascitis, while lesions were superficial ulcer in the body surface, bilateral exophthalmia and petechial haemorrhage on the body surface. Postmortem lesions observed due to vibriosis were hemorrhagic peritoneum and visceral organs. Intestine were filled with bloody fluid (Fig. 1). Gelatinous exudates (material) were covering gas bladder. By the end of observation time the cumulative mortality of the experimentally infected fish reached 53.33%. Re- isolation of the bacteria in pure culture was done from freshly dead and moribund fish. There was no mortality or clinical signs of infection in both of the control groups.

**Antibiotic sensitivity assay:**
The antibiotic sensitivity test revealed that the isolated *V. vulnificus* was sensitive to Oxtetracycline, Neomycin, and Erythromycin. Controversially, it was resistant Ampicillin and Tobramycin.

**Table 1:** Cultural and biochemical characters of isolated bacteria (n=59)

| Test | | *V.vulnificus* n=23 | *V. parahaemolyticus* n=17 | *V. harveyi* n=7 | *V. alginolyticus* n=3 | *V. mimicusus* n=3 | *V. ordalii* n=4 | *V. fisheri* n=2 |
|---|---|---|---|---|---|---|---|---|
| Growth on TCBS | | G | G | Y/G | Y | G | G | Y |
| Oxidase | | 23/23 | 17/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| Catalase | | 23/23 | 17/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| Simmon citrate | | 11/23 | 14/17 | 4/7 | 0/3 | 3/3 | 0/4 | 1/2 |
| o/f test | | 23/23 | 17/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| SIM media | Sulphide | 0/23 | 0/17 | 0/7 | 0/3 | 0/3 | 0/4 | 0/2 |
| | Indole | 14/23 | 11/17 | 7/7 | 3/3 | 3/3 | 0/4 | 0/2 |
| | Motility | 23/23 | 17/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| Methyl red | | 23/23 | 15/17 | 7/7 | 3/3 | 0/3 | 0/4 | 2/2 |
| Voges proskauer | | 0/23 | 0/17 | 0/7 | 3/3 | 0/3 | 0/4 | 0/2 |
| String test | | 20/23 | 15/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| Salt tolerance | NaCl 3% | 23/23 | 17/17 | 7/7 | 3/3 | 3/3 | 4/4 | 2/2 |
| | NaCl 6% | 9/23 | 13/17 | 4/7 | 1/3 | 1/3 | 1/4 | 0/2 |
| | NaCl 8% | 0/23 | 10/17 | 0/7 | 0/3 | 0/3 | 0/4 | 0/2 |
| Lactose | | 0/23 | 1/17 | 0/7 | 0/3 | 0/3 | 0/4 | 1/2 |
| Sucrose | | 6/23 | 0/17 | 4/7 | 2/3 | 0/3 | 3/4 | 0/2 |
| Mannose | | 12/23 | 14/17 | 4/7 | 0/3 | 2/3 | 0/4 | 2/2 |

*Y=yellow, G=green
O/F test=oxidation fermentation test
SIM= sulfide indole motility test

**Table 2:** Frequent isolation of vibrio species from the examined fish

| Species | No. of examined fish | Isolation and identification* |
|---|---|---|
| **Fantail** | 73 | 33 (45.20%) |
| **Black molly** | 17 | 4 (23.53%) |
| **Koi carp** | 10 | 3 (30.00%) |
| **Total** | 100 | 40 (40%) |

*Fish yielded vibrio species bacteriologically

**Fig.1:** Fantail *(Carassius auratus auratus)* challenged with immersion route of *Vibrio vulnificus* showing bloody fluid filling the intestine.

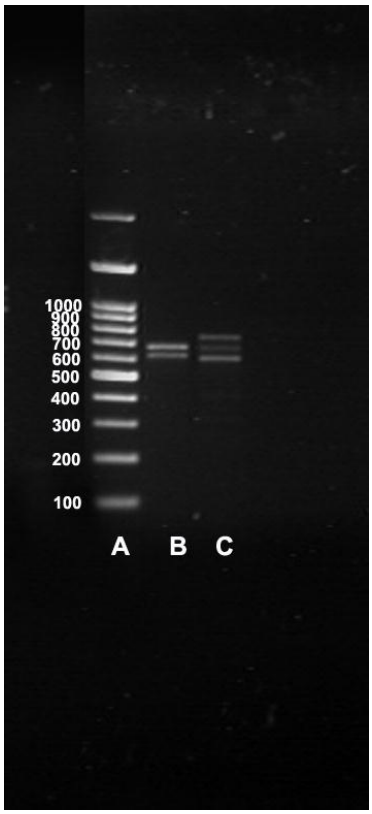


**Fig. 2:** Electrophoretic analysis of PCR- amplified IGSs of Vibrio species using vibrio species specific primer. Lane A-100-bp DNA ladder (size marker), Lane B, *V. vulnificus*, Lane C, *V. cholera.*

## DISCUSSION

Seven species of vibrio known to be pathogenic to ornamental fishes were isolated from the examined fishes in Assiut. Vibrio infections are common in ornamental fish settings (Barker, 2001). *Vibrio* species are inhabitants of healthy fishes and aquatic systems that can become pathogenic and cause substantial mortality when conditions are stressful (smith *et al.,* 2012). These results are supported by Katherine *et al.* (2012) who stated that freshwater fish tanks in pet/ aquarium shops are generally filled using treated tap water, where aquarium water samples had several species of bacteria. Also, the use of infected marine fish in the feeds of healthy fish has also caused epizootics (Bullock, 1977).

The Vibrio strains isolated in the present study showed the typical morphological, cultural, and biochemical characteristics of vibrio species. Using such characters, isolates were identified as *V. vulnificus, V. parahaemolyticus, V. harveyi, Vibrio alginolyticus, V. mimicus, V. ordalii,* and *V. fischeri* as guided by Austin and Austin, (2007). Interestingly, the same species of *Vibrio* were previously isolated from ornamental fish from different localities (Sonia and Lipton 2012, Toranzo *et al.,* 2005 and Hashem and El-Barbary 2013). This may suggest the susceptibility of such ornamental fishes to vibrio infections caused by the same species.

Results of the current study indicated that 40 % out of the collected ornamental fish were naturally infected with vibrios. This percentage is higher than those reported by Hewiarachchi and Cheong (1994) and Sonia and Lipton (2012). The high percent of infection may be due to the accumulation of unconsumed feed and bad water quality influence the growth of pathogenic forms of the *Vibrio* sp., and the higher temperature in Assiut that may favor the growth and multiplication of bacteria. In addition, the high percent of infections was also noticed by Musa *et al*. (2008) who suggested that many stress factors could contribute to bacterial infection in ornamental fish, namely, poor water quality, crowding, transportation and inadequate nutrition.

Vibrio species and other closely related species usually show similar phenotypic features and are not easy distinguished biochemically (Gomez –Gil *et al.,* 2004). Consequently identification systems based on molecular techniques were proved to lead to a conclusive identification unlike the traditional conventional biochemical methods (Thompson *et al.,* 2005). The differences in the length and sequence of the 16S-23S intergenic spacer regions (IGSs) of rRNA persons were used to develop IGS–typing system for vibrio species (Hoffmann *et al.,* 2010). Intergenic spacer typing system was used to confirm the conventionally identified *Vibrio vulnificus* strains before their use in the experimental challenge.

Conventionally identified isolate as *Vibrio vulnificus* strains, was re-identified as *V. cholera* using the IGS-typing system. Senderovich *et al.* (2010) suggested that fish of various species and habitats contain *V. cholera* in their digestive tract. They demonstrated that fish serves as intermediate reservoirs of *V. cholerae* in various aquatic ecosystems (non potential pathogen). *V. cholerae* isolated from ayu fish in Japan (Kiiyukia *et al.,* 1992) and from aquarium water from fish imported from Thailand and Sri Lanka to Czechoslovakia (Plesnik and Prochazkova 2006). Rehulka *et al.* (2015) isolated *Vibrio cholerae* non-O1/non-O139 from the fry of the Cardinal tetra, Paracheirodon axelrodi and in adult Raphael catfish, Platydoras costatus in the Czech Republic.

Results of experimental challenge in the present study proved that *V vulnificus* isolated from naturally infected ornamental fish is pathogenic to fantail fish challenged. The bacteria were re-isolated from moribund fish after bacterial challenge. The clinical and necropsy findings of the naturally and experimentally fish were support those of Stoskoph, (1993), Ransangan and Mustafa (2009) and Sonia and Lipton (2012). The postmortem lesions were similar to those previously reported by Gauger *et al.,* 2006, Lee *et al.,* 2002, Liu *et al.,* 2004 and Hashem and El-Barbary 2013.

The pathogenicity of *V. vulnificus* to fish may attribute to extracellular products (ECPs) which were harmful to fish. Anemia, which commonly accompanies vibriosis, may be caused by the destruction of red blood cells by hemolysins or to blood loss from hemorrhaging. Umbreit and Tripp (1975) showed that *V.anguilarum* produced a toxic substance for goldfish *(Carassius auratus)* and that heating to 100 C increased the potency of the extracellular toxin.

The antibiotic sensitivity test indicated that *Vibrio vulinificus* was sensitive to Oxytetracycline, Neomycin and Erythromycin. These drugs can be used to control bacterial disease of ornamental fish. Sonia and Lipton (2012) reported *Vibrio vulnificus* was moderately sensitive to Neomycin. This result may be due to extensive use of antibiotics and other chemotherapeutic agents has resulted in an increase in drug-resistant bacteria in aquatic environments.

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# دراسة اكلينيكية وتجريبية عن مرض الضمات فى أسماك الزينة

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الهدف من هذه الدراسة التحقيق من أنتشار وضراوة مرض الفيبريو في بعض الأنواع من أسماك الزينة فى محافظة أسيوط.تم تجميع عدد ١٠٠ سمكة من محلات أسماك الزينة بمحافظة أسيوط تظهر عليها الاعراض المرضية مثل الأستسقاء، جحوظ العينين وبقع نزفية على سطح الجسم. وقد تم عزل ٥٩ من عترات ميكروبات الفيبريو من الاعضاء الداخلية ل ٤٠ سمكة. وقد تم تصنيف العترات المعزولة باستخدام الخصائص المورفولوجية والكيميائية الحيوية لهذة العترات كانوا كالتالى: 23 (39.98%) فيبريو فلنيفيكس ،17( 28.85%) فيبريو باراهيموليتيكس،7 (11.86%) فيبريوهارفى،3 (5.08%) فيبرو الجينوا ليتيكس، 3 (5.08%) فيبريو ميميكس، 4 (6.78% ) قيبرو اوردالى، 2 (3.39% ) قيبرو فيشارى. تم استخدام طباعة مناطق الفاصل الوراثى البينى لتصنيف سلالات الفيبريو فلنيفيكس. وباجراء تفاعل البلمرة المتسلسل ببادى 16S-23S للحامض النووى الريبوزى للريبوسوم اسفر عن نطاقات لسلالات الفيبرو فلنيفيكس مطابق يحتوى على نطاقين رئيسيين عند 600 و 700 زوج من القواعد. تم عمل العدوى الصناعية بنجاح باستخدام بكتيريا الفيبيرو فلنيفيكس المعزولة فى الأسماك الفانتيل عن طريق الغمر وكانت العلامات المرضية المميزة هى أحتقان فى الغشاء البرتيونى والأعضاء الداخلية وقد أمتلات الأمعاء بسائل دموى وظهرت المثانة الهوائية يغطيها طبقة جلاتينية. كما أظهر أختبار الحساسية للمضادات الحيوية أن ميكروب الفيبرو فلنيفيكس شديد الحساسية لكل من الأوكسيتتراسيكلين والنيومايسين والاريسروميسين بينما كان مقاوم ل الأمبيسلين وتوبراميسين.