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CITROBACTER SEPTICEMIA IN SHARPTOOTH CATFISH, AN EMERGING INFECTION

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ABSTRACT

Bacterial septicemia are responsible for severe losses in fish farms over the world. This study aimed to investigate the *Enterobacteriaceae* infections in sharptooth catfish *Clarias gariepinus*. A total of 120 catfish were collected from small canal in Farshout, Qena Governorate, and were subjected to clinical and bacteriological examination. Catfish sampled showed signs of typical hemorrhagic septicemia on the skin and internal organs. Bacteriological investigations resulted in isolation of 240 isolates that belong to *Enterobacteriaceae*. Interestingly, conventional biochemical identification showed that 16 isolates are suspected to be *Citrobacter* spp. Based on genetic sequence analysis of the 16S rDNA, only 8 (50%) of the suspected isolates were identified as *Citrobacter freundii*. To investigate the pathogenicity of isolated *C. freundii* strains to catfish, an experimental challenge experiment was conducted where challenged fish showed signs and lesions of acute septicemia. The current study is the first to report *Citrobacter* infections in catfish in Upper Egypt.

Key words: *Citrobacter freundii, Clarias gariepinus,* 16S rDNA sequence analysis, bacterial septicemia.

INTRODUCTION

A comprehensive range of enteric bacteria have been associated with fish diseases; some of these pathogens are responsible for drastic mortalities in fish (Austin and Austin, 2016). These bacteria include *Edwardsiella tarda*, *Edwardsiella ictaluri* and *Yersinia ruckeri*. Although *Citrobacter freundii* is an opportunistic bacterium with considerable importance in medical and veterinary science (Chuang *et al.*, 2006), its pathogenicity to fish is not well documented. Sato *et al.* (1982), however, recorded the first isolation of *C. freundii* from infected sunfish *Molamola* with description of hemorrhagic gastroenteritis and other pathological lesions. Thereafter, *Citrobacter* came to the attention of fish disease diagnostician and were isolated from various fish species, such as Atlantic salmon and rainbow trout (Baya *et al.*, 1990a; Sanz, 1991; Jeremic *et al.*, 2003), Carp, *Cyprinuscarpio* (Karunasagar *et al.*, 1992), doctor fish *Garrarufa obtuse* (Baeck *et al.*, 2009), and *Pseudoplatystoma reticulatum* (Santiago *et al.*, 2014). In a study on septicemia-causing bacterial agents of fresh water ornamental fish in Sri Lanka, *Citrobacter* spp. came second to *Aeromonas* spp. (Jagoda *et al.*, 2014). Also *C. freundii* were implicated in infections of crustaceans (Shen *et al.*, 2005).

Little is known about*C. freundii*as an emerging fish pathogen in aquaculture. To our knowledge, there is no published data about the pathogenicity of *C. freundii* in catfish in Egypt, however, Elsherief *et al.* (2014) isolated *C. freundii* and *C. diversus* from *Tilapia nilotica* and *Mugil cephalus*. Thus, the present study was conducted to investigate *C. freundii* pathogenicity to sharptooth catfish.

MATERIALS AND METHODS

Catfish

A total of 120 African Sharptooth catfish, *Clarias gariepinus*, with 250- 300g weight and total length of 30-40cm were collected either a live or moribund fromsmall canal in Farshoot, Qena Governorate, Egypt. They were rapidly transported on ice to the Aquatic Diagnostic Laboratory, Faculty of Veterinary Medicine, South Valley University, where clinical and bacteriological examination were conducted.

Clinical Examination of catfish

Catfish were clinically examined to record any apparent clinical signs or postmortem lesions according to (Austin and Austin, 2007).

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Bacteriological examination of catfish

Tissue samples were collected from the kidneys, liver and spleen, inoculated on Salmonella Shigella agar (SS agar, Biolife, Italy), and incubated at 25°C for 2 days. Suspected individual colonies were purified on trypticase soya agar (TSA, Biolife) and preserved with glycerol at -80 °C.

Conventional biochemical characters were conducted as previously described by (Brenner, 1984) and included colony morphology, microscopic examination of Gram stained film, oxidase, catalase, indole (Kovac´s method), Voges-Proskauer, methyl red, H_2S production and motility using semisolid agar.

Molecular identification of suspected isolates

Sequence analysis-based differential identification assay was implemented to accurately identify the suspected isolates using the 16S rDNA gene sequence. This included extraction of the genomic DNA from suspected colonies, amplification of specific 16S rDNA target, sequencing of the amplified targets, and finally BLAST analysis of the amplified targets.

DNA was extracted using the Gene JET genomic DNA purification kit (Thermo Scientific, EU) according to the manufacturer recommendations and then kept at -20°C until use. Polymerase chain reaction (PCR) was conducted to amplify the hypervariable 1500 base pair (bp) segment of the 16S rDNA using the universal primers 27F and 1492R (Martin and Collen, 1998). The PCR reactions were carried out in a total 50µl volume which consisted of 25µl MyTaq red mix (Bioline, UK), 2µl of each primer, 4µl template DNA and 17µlH_2O (RANase /DNase free).

Polymerase chain reaction amplification was performed in the Veritithermal cycler (Applied Biosystems, USA) starting with an initial denaturation step at 95°C for 5min and followed by 35 cycles consisted of denaturation at 94°C for 1min, annealing at 55°C for 1min, an extension step at 72°C for 1.5min, and a final extension step at 72°C for 10min.

The amplification products were analyzed by gel electrophoresis of 1.5 % agarose in Tris-acetate EDTA (TAE) buffer, stained with ethidium bromide (50µl/L) and visualized on an UV transilluminator, (UVP, MultiDoc-It, Digital Imaging System). A 100-bp DNA ladder was used to determine the size of the PCR products.

Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) was used to purify PCR products from gel for sequencing. Purified PCR products were sequenced using the same amplification primers and analyzed by the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

Pathogenicity of *C. freundii* to catfish

Catfish

Apparently healthy catfish, with average body weight of 120±5g were obtained from a private fish farm and transported to the aquatic laboratory at Faculty of Veterinary Medicine, South Valley University, Qena, and acclimated to laboratory conditions for 2weeks according to the recommendations for the maintenance of bioassay fish described by (Ellsaesser and Clem, 1986).

Bacterial strains

The *C. freundii* strain isolated from the fish showing most severe signs was passed three times in healthy catfish through intraperitoneal injection before using for experimental challenge. The isolate was grown on trypticase soya broth (TSB, Biolife) and suspended in sterile saline to be used for experimental infection.

Bacterial counts and dilutions

Bacterial Colony forming units (cfu) counts were determined using spectrophotometery optical density at 600nm and standard plate count method (Elkamel and Thune, 2003). Bacterial suspension of *C. freundii* isolate was diluted in sterile saline to $6x10^7$cfu/m1 final concentration for challenge.

Experimental infection

Acclimated catfish were divided into three groups with 5 catfish each. One group remained un-injected as a control, while the other two groups were intraperitoneally injected with either 0.5 ml of sterile saline (sham control) or diluted bacterial suspension $6x10^7$cfu/m1. The whole experiment was done in 3 replicates.

Clinical signs and mortalities were recorded daily for 21 days. Moribund catfish were bacteriologically examined to re-isolate the causative *C. freundii* strain from the internal organs. At the end of the experiment, all remained catfish were sacrificed and examined as described above.

RESULTS

Clinical and post-mortem examination of naturally infected catfish:

Examined catfish revealed signs of generalized septicemia, as ulceration and sloughing of the tips of fins with fin rot (Fig. 1. A), several ulcerative lesions on body surface, and congestion of pectoral and anal fins. Petechial hemorrhages were widely spread in

many parts of the body with hyperemia of the anal region (Fig. 1. B) and abdominal distension (Fig. 1. C).

Necropsy examination showed congestion and enlargement of the internal organs which were also friable. Abdominal blood vessels were congested with enlargement of the gall bladder (Fig. 1. D). There was accumulation for bloody ascetic fluid (Fig. 1. E) and congestion of the liver (Fig. 1. F).

Bacteriological examination:
Bacteriological examination resulted in recovery of 240 isolates from 120 catfish examined. Isolates identified according to cultural and morphological characters, gram stain, motility and cytochrome oxidase characters as *Enterobacteriaceae* were kept for further investigations.

Colonies on SS agar were colorless with or without dark central spot, but on TSA were smooth, opaque and gray with a shiny surface after incubation at 25°C for 48 hours. All isolates were Gram-negative, motile short bacilli. Based on the biochemical characters, only 16 isolates were suspected to be *Citrobacter* where they showed similar characters except in indole and TSI tests (Table 1). However, the phenotypic characteristics were insufficient to provide a definite identification of all isolates at the species level.

Molecular identification of suspected isolates:
BLAST-based sequence analysis of the PCR amplified hypervariable target of 16S rDNA gene, identified only 8 out of 16 suspected isolates as *C. freundii*. The other bacteria were *Klebsiella*, *Enterobacter* and other species of *Citrobacter*.

Experimental infection
Catfish challenged with *C. freundii* strain showed clinical signs similar to those recorded in naturally infected catfish with generalized hemorrhagic septicemia. Mortalities reached 66.6%, where dead fish showed several ulcerations with hemorrhages at the base of the dorsal fin with fin rot (Fig. 2. A). Petechial hemorrhages at the abdominal area with hyperemia of the anal opening (Fig. 2. B). Hemorrhages scattered all over the body surface with congestion of fins (Fig. 2. C). Superficial ulcerations with emaciation were also seen (Fig. 2. D). Necropsy examinations of dead catfish revealed accumulations of bloody ascetic fluids in the body cavity (Fig.2. E), with clotted blood (Fig. 2. F) and enlargement of the posterior kidney, gall bladder (Fig. 2. G) and spleen (Fig. 2. H).

Table 1: Biochemical characters of 16 *Citrobacter* spp. strains isolated from naturally infected sharptooth catfish, *Clarias gariepinus*, Qena, Egypt.

Test		Number of strains (n)=16
Indole		13/16
Methyl red		16/16
Vogus Proskauer		0/16
TSI	H_2S	16/16
	Gas	16/16
	K/A	13/16
	A/A	3/16

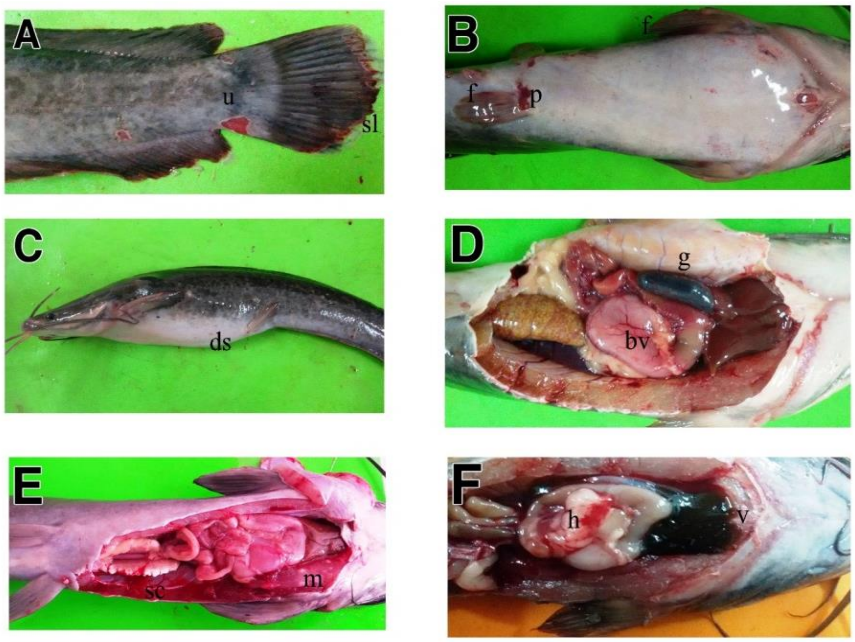

Figure 1: Sharptooth catfish, *Clarias gariepinus*, naturally infected with *Citrobacter freundii* showing: (**A**) ulceration (u), sloughing of the tips of fins and fin rot (sl), (**B**) congestion of pectoral and anal fins (f) and hyperemia of the anal region (p), (**C**) abdominal distension (ds), (**D**) congestion of abdominal blood vessels (bv) and enlargement of gall bladder (g), (**E**) hemorrhages on the muscles (m) and accumulation of bloody ascetic fluid (sc), (**F**) congestion of liver (v) and hemorrhages in abdominal cavity (h).

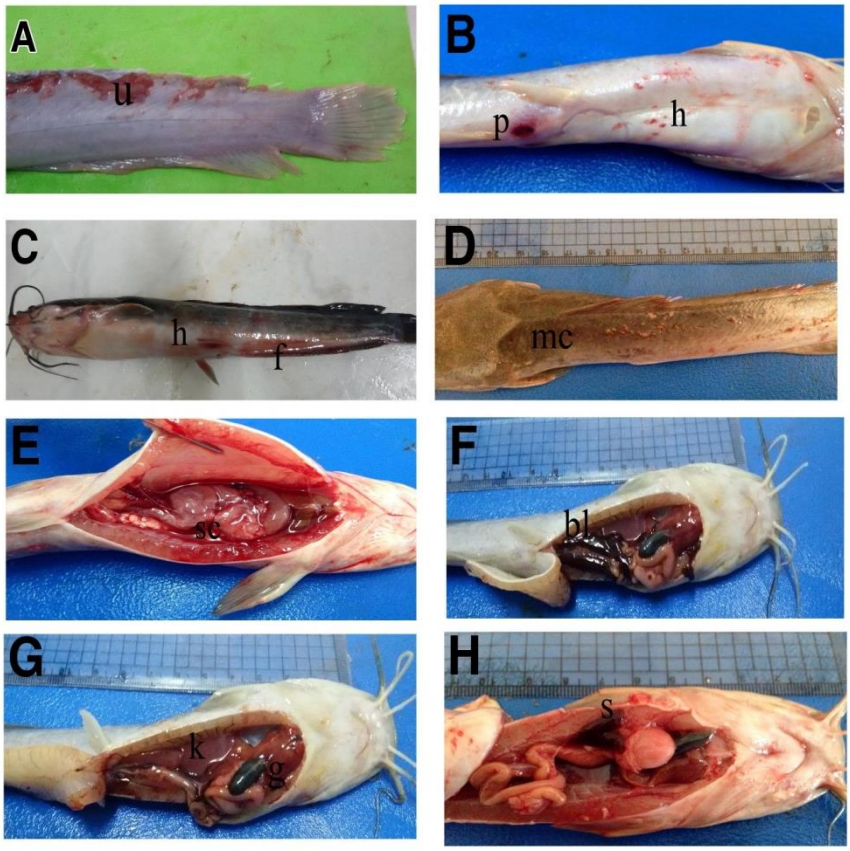

Figure 2: Sharptooth catfish, *Clarias gariepinus*, experimentally infected by intraperitoneal injection of 0.5ml of $6x10^7$cfu/m1 of *Citrobacter freundii* showing: (**A**) ulcerations with hemorrhages at the base of the dorsal fin with fin rot (u), (**B**) petechial hemorrhages at the abdominal area (h) and hyperemia of the anal opening (p), (**C**) hemorrhages scattered all over the body surface (h) and congestion of fins (f), (**D**) emaciation (mc), (**E**) accumulations of bloody ascetic fluids in the body cavity (sc), (**F**) clotted blood in the body cavity (bl), (**G**) enlargement of posterior kidney (k) and gall bladder (g), (**H**) enlargement of the spleen (s).

DISCUSSION

Extensive studies on pathogenicity and virulence and fish susceptibility to the genus *Citrobacter* are still scarce. Nevertheless, *Citrobacter* are now isolated more frequently as a possible pathogen in fish. The present study is the first report of *Citrobacter* septicemiain African sharptooth catfish in Upper Egypt and proved that *C. freundii* is most often associated with hemorrhagic septicemia. Santiago *et al.* (2014) record the first isolation of *C. freundii* from Brazilian Catfish, *Pseudoplatystoma reticulatum*. In Egypt, Elsherief *et al.* (2014) isolated *C. freundii* and *C. diversus* from, *Tilapia nilotica* and *Mugil Cephalus*.

The recent increase in the incidence of fish septicemia caused by *Citrobacter, Klebsiella, Enterobacter* and other pathogens that were not known for their pathogenicity to fish may indicate water pollution with animals and human wastes. *C. freundii* is prevalent in soil and water through contamination from the wastes (Sabrina *et al.,* 2017), and the spread to fish is possible (Allen *et al.,* 1983). This may explain the high prevalence of *Citrobacter* in the current study in catfish that inhabit the soil and mud of rivers, canals and ponds. The canal from which the catfish of the present study were collected was turbid and dark yellow in color indicating a heavy bacterial and biological pollution.

In the present study, examined catfish revealed varying signs and lesions of septicemia, as was reported by Jeremic *et al.* (2003) who isolated *C. freundii* from both cyprinids showing signs of hemorrhagic septicemia and rainbow trout suffering from gastroenteritis, and Honein *et al.* (2016) who isolated *C. freundii* from the kidneys of giant gourami, *Osphronemus goramy*, showing signs of generalized septicemia.

Definitive identification of the bacteria isolated in the current study could not be achieved based solely on the available conventional phenotypic approaches. All *Enterobacteriaceae* isolates showed the common main characteristics of the family with wide-ranging differences in the phenotypic and biochemical characters that made reaching a conclusive identification is a hard task.

The inability of the conventional phenotypic tests, used in the current study, to reach definite identification of the suspected isolates may be attributed to the comprehensive differences in the biochemical characters even within the same genus. For analysis of *Enterobacteriaceae* taxonomy, different parameters are to be included to increase the accuracy of identifying unknown strains (Navarrete *et al.,* 2010). Therefore, analysis of the 16S rDNA sequences was used as alternative diagnostic tool to resolve the identity of the suspected isolates.

Molecular identification of bacteria other than *C. freundii*, as *Klebsiella, Enterobacter* and other species of *Citrobacter*, that were originally identified as *C. freundii* by conventional approach, indicates that conventional methods are not precise enough to identify the recovered isolates to the species level, and molecular identification should be adopted.

Experimentally induced infection in catfish with the *C. freundii* isolated from a natural infected catfish showed clinical signs nearly similar to those previously reported in rainbow trout (Jeremic *et al.,* 2003), grass carp (Aijun *et al.,* 2011) and in Brazilian catfish (Santiago *et al.,* 2014). The main clinical manifestations seen in this study were related to external and internal hemorrhages with enteritis. Bacterial hemolytic toxins may cause endothelium damage leading to hemorrhage. Recent studies attributed the virulence of *C. freundii* to its ability to adhere to the gastrointestinal mucosa and cytotoxicity (Santiago *et al.,* 2014).

CONCLUSION

From the results of the present study, we can conclude that *C. freundii* can infect sharptooth catfish causing septicemia and mortalities that could lead to serious economic losses.

REFERENCES

Aijun, L.; H., X.; Lu Zheng; Aihua, Z.; Chengliang, C. and Jihong, J. (2011): Isolation and characterization of *Citrobacter* spp. from the intestine of grass carp *Ctenopharyngodon idellus*: Aquaculture 313: 156-160.

Allen, D.A.; Austin, B. and Colwell, R.R. (1983): Numerical taxonomy of bacterial isolates associated with a freshwater fishery: J Gen Microbiol 129: 2043–2062.

Austin, B. and Austin, D.A. (2007): Bacterial Fish Pathogens: Disease in Farmed and Wild Fish, 4th ed. . Godalming: Sprin-ger-Praxis.

Austin, B. and Austin, D.A. (2016): Bacterial fish pathogens, Diseases of farmed and wild fish. Springer International Publishing Switzerland, Springer.

Baeck, G.W.; KIM, J.H.; Choresca, J.R.C.; Gomez, D.K.; Shin, S.P.; Han, J.E. and Park, S.C. (2009): Mass mortality of doctor fish (*Garra rufa obtusa*) caused by *Citrobacter freundii* infection: J. Vet. Clin. 26: 150-154.

Baya, A.M.; Lupiani, B.; Hetrick, F.M. and Toranzo, A.E. (1990a): Increasing importance of *Citrobacter freundii* as a fish pathogen: FHS/ AFS Newsl. 18: 4.

Brenner, D.J. (1984): Family *Enterobacteriaceae*. U.S.A.: Williams and Baltimore.

Chuang, Y.M.; Tseng, S.P.; Teng, L.J.; Ho Yc and Hsueh, P.R. (2006): Emergence of cefotaxime

resistance in Citrobacter freundii causing necrotizing fasciitis and osteomyelitis: J. Infect. 53: 161-163.

Elkamel, A.A. and Thune, R.L. (2003): Invasion and replication of Photobacterium damselae subsp. piscicida in fish cell lines.: J Aqua. Ani. Hea. 15: 167-174.

Ellsaesser, C.F. and Clem, L.W. (1986): Hematological and immunological changes in channel catfish by handing and transport: Journal of fish biology 28: 511-521.

Elsherief, M.F.; Mousa, M.M.; Abd El-Galil, H. and El-Bahy, E.F. (2014): Enterobacteriaceae Associated with Farm Fish and Retailed Ones: Alexandria Journal of Veterinary Sciences 42: 99-104.

Honein, K.; Jagoda, S.; Arulkanthan, A.; Ushio, H. and Asakawa, S. (2016): Draft Genome Sequences of *Citrobacter freundii* Strains CF04 and A41 Isolated from Moribund, Septicemic Giant Gourami (*Osphronemus goramy*) in Sri Lanka: Genome Announcements 4(4): e00820-00816.

Jagoda, S.; Honein, K.; De Silva, D.P.N.; Arulkanthan, A.; Kinoshita, S.; Ushio, H. and Asakawa, S. (2014): Genetic characterisation of multidrug resistant *Citrobacter* spp. isolated from septicaemic fresh water ornamental fish. 9th Symposium on Diseases in Asian Aquaculture, Ho Chi Minh, Vietnam.

Jeremic, S.; Jakic-Dimic, D. and Veljovic, L. (2003): *Citrobacter freundii* as a cause of disease in fish: Acta Vet. 53: 399–410.

Martin, F.P. and Collen, M.C. (1998): Bias in template to product ratio in multitemplate PCR: Applied Environmental Microbiology 64: 3724-3730.

Navarrete, P.; Magne, F.; Mardones, P.; Riveros, M.; Opazo, R.; Suau, A.; Pochart, P. and Romero, J. (2010): Molecular analysis of intestinal microbiota of rainbow trout (Oncorhynchus mykiss): FEMS Microbiol. Ecol. 71: 148–156.

Sabrina, H.; Wimalasena, S.H.M.P. and Gang-Joon, H. (2017): Virulence Factors and Antimicrobial Resistance Pattern of *Citrobacter freundii* Isolated from Healthy Pet Turtles and their Environment: Asian Journal of Animal and Veterinary Advances 12(1): 10-16.

Santiago, B.d.P.; Debora, P.M.; Fernanda, A.S.; Fabiana, P.; Maurício, L.M. and Márcia, M.I. (2014): Isolation, characterization and pathology of *Citrobacter freundii* infection in native Brazilian catfish *Pseudoplatystoma*: Braz J Vet Pathol. 7(3): 151 - 157.

Sanz, F. (1991): Rainbow trout mortalities associated with a mixed infection with *Citrobacter freundii* and IPN virus: Bull Eur Assoc Fish Pathol 11: 222.

Sato, N.; Yamane, N. and Kawamura, T. (1982): Systemic Citrobacter freundii infection among sunfish Mola mola in Matsushima aquarium: Bull. Jpn. Soc. Sci. Fish 49: 1551–1557.

Shen, J.Y.; Gu, Z.M.; Pan, X.Y.; Zhou, B.Z.; Yin, W.L. and Cao, Z. (2005): Isolation and identification of *Citrobacter freundii* from *Cherax quadricarinatus*: J. Fish. Sci. China 12: 197-200.

التسمم الدموي بميكروب السيتروباكتر فروندي في أسماك القراميط الأفريقية: عدوي مستجدة

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يُعتبر التسمم الدموي البكتيري من أهم أسباب وجود خسائر كبيرة في مزارع الأسماك في أنحاء العالم، وهذا البحث يهدف إلى تسجيل عدوى الاينتيروبكتيريسي فى أسماك القراميط من خلال الفحص الاكلينكى لعدد مائة وعشرين سمكة من أسماك القراميط متفاوتة الأحجام والأوزان التى جُمِعت عشوائيًا من ترعة صغيرة بمركز فرشوط محافظة قنا، وقد تم إجراء الفحص الإكلينيكي والبكتريولوجي لهذه الأسماك، وقد وُجد بالفحص الاكلينيكي أعراضًا للتسمم الدموي على الجِلد والأعضاء الداخلية، وقد تم تشريح هذه الأسماك ووضعها تحت الفحص البكتريولوجي وكان من نتيجة هذا الفحص للأسماك المريضة هو عزل ٢٤٠ عِترة من عائلة الاينتيروبكتيريسي. أوضحت الطُرق البيوكيميائية التقليدية أن ١٦ عترة من المحتمل أن تكون سيتروباكتر فروندى. اعتمادًا على تحليل سلسلة الجينات للحمض النووي 16s rDNA أثبت ان ٨ من العِترات المتوقع أن تكون سيتروباكترفروندي بنسبة ٥٠% ، ولقياس ضراوة عِترات السيتروباكترفروندي المعزولة تم حقنها في مجموعة من أسماك القراميط، وقد كانت الأعراض الظاهرية لأسماك القراميط المُصابة تجريبيًا بهذه العتره هي أعراضًا للتسمم الدموي البكتيري الحاد. هذه الدراسة هي الأولى لتسجيل عدوى السيتروباكتر في أسماك القراميط بصعيد مصر.