Detection And Characterization Of Virulence Factors Among *Aeromonas* Species Isolated From Diseased Fish

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ABSTRACT

Aeromonas species are distributed world-wide and infecting different aquatic organisms. *Aeromonas* are gram -ve, oxidase +ve, rods like bacteria which metabolize glucose by both oxidative and fermentative pathways. The aim of this investigation was to detect and characterize virulence factors among *Aeromonas* species from diseased *Oreochromis niloticus*. Two hundred of diseased fish were subjected to full clinical, post-mortem and bacteriological examinations. The total prevalence of *Aeromonas* spp. infection among the examined *O. niloticus* was 65%. *A. hydrophila* was the highest incidence (40%) followed by *A. sobria* (17.5%) and *A. caviae* (7.5%). Nine *Aeromonas* isolates selected randomly (3 *A. hydrophila*, 3 *A. sobria* and 3 *A. caviae*) were tested for their virulence activity using the virulence tests as Congo red binding test, infant mice assay, vero-cells cytotoxicity assay and specific PCR tests for aerolysin and hemolysin genes detection. From the results, it was noticed that *Aeromonas* isolates had the ability to bind with Congo red and gave different degrees of red colouration. Also, from 9 tested strains, 6 *Aeromonas* isolates (66.6%) were positive for production of enterotoxin All tested isolates were able to produce cytotoxic activity with an incidence of 100 % but with different degree.

INTRODUCTION

Tilapia aquaculture is one of the most important and the fastest growing sector in Egypt and all over the world. Disease outbreaks were recognized as a significant constraint to aquaculture production and trade, affecting both the economic development and socioeconomic revenue of the sector in many countries in the world *(1)*. The causative Bacterial agents are among the most important serious fish diseases in aquaculture causing 80% of fish mortalities among the cultured fish.

Motile *Aeromonas* septicemia (MAS) is the most drastic one and distributed world-wide affecting various species of fish and shellfish, wild as well as cultured in freshwater, brackish and marine water *(2-4)*. *Aeromonas* species is opportunistic pathogen of human causing illness, dysentery like diarrhea, meningitis and septicemia particularly when fish is eaten raw or improperly cooked *(2)*. Also, the external signs of the affected fish make them unmarketable.

Aeromonas spp. produced many products that may be toxic to other cells. Some of them are released from viable cells in soluble form but others remain associated with the cell surface, and some others may be released upon cell death *(5)*. *Aeromonas* spp. produced different potential virulence factors such as haemolysins, cytotoxins, enterotoxins and proteases *(6-8)*. Presence of putative virulence genes aerolysin and hemolysin in marketed fish were provides an evidence for multifactorial activities and pathogenicity *(9)*.

Advance in molecular biology over the last 10 years have opened new avenue for microbial identification and characterization. Pathogenesis depends on detection of genes carried on DNA genome *(10)*. PCR technique was rapid, sensitive and specific for the detection of virulence factors of *Aeromonas* spp. It overcomes the handicap of time-consuming biochemical methods *(11)*.

The identification and virulence characterization of the causative agents of fish diseases are of crucial importance because it determines the choice of a potential treatment. Therefore, the aim of the current investigation was to detect and characterize different virulence factors among *Aeromonas* species

isolated from naturally infected *Oreochromis niloticus*.

MATERIAL AND METHODS

Samples

Naturally infected fish: Two hundreds of clinically and grossly diseased Nile tilapia (*O. niloticus)* at different sizes were collected from private and governmental fish farms suffered from heavy mortalities and transferred alive in plastic bag filled with 1/3 dechlorenated water or recently dead in ice container to the laboratory where they were subjected to full clinical, post-mortem and bacteriological examinations.

Clinical and postmortem examinations: The collected fish were subjected to clinical and postmortem examinations *(12).*

Bacteriological examination: Samples were collected under complete aseptic condition from the affected areas of skin, gills and internal organs then inoculated into brain heart infusion agar and incubated at 22° C. Purified isolates were identified according to standard biochemical tests *(2,4).*

Nine *Aeromonas* isolates were selected randomly (3 *A. hydrophila,* 3 *A. sobria* and 3 *A. caviae*) and tested for their virulence activity using the virulence tests, as the following:

1- **Congo red binding test:** Selected *Aeromonas* species isolates were streaked onto plate of Congo red medium and incubated at 25° C for 24 hours then left for an additional 2 or 4 days. Congo red positive (CR^+) was indicated by the development of different degree of red colonies while negative Congo red isolates (CR^-) appeared as white colonies *(13)*.

2- **Infant mice assay:** The ability to produce enterotoxins was assayed by the infant mice test *(14,15)*. Ten ml of *Aeromonas* species isolates were placed in 25 ml of media containing 2% casamino acid, 1% yeast extract and 0.4% glucose. The inoculated flasks were incubated on rotatory shaker (200 r.p.m) at room temperature (25° C) for 48 hrs, after that centrifuged at 10.000_{xg} for 30 minutes. The supernatants were filtered through Millipore membrane filter (pore size 0.22 µm) and stored at -20° C till used. One tenth ml of culture filtrate was injected through the abdominal walls into the milk-filled stomach of each of three infant mice of 3 days old. Also, negative control mouse was injected by 0.1 ml saline. After 3 hrs, the mice were sacrified and the ratio of intestine weight / remaining body weight was calculated. A ratio greater than 0.085 was considered positive for enterotoxins production.

3-**Vero-cells cytotoxic assay:** Culture supernatants of *Aeromonas* species were examined for their ability to induce cytopathic effects (CPE) in vero cells. Assays were conducted using confluent monolayers of Vero cells incubated at 37°C in 2.5% CO_2 incubator, assessed at 12, 24 and 48 hrs. *(16,17).*

4- **PCR test:** Specific PCR conditions for aerolysin and hemolysin genes among the nine isolates were performed *(18-20).*

Aero1	**5\-CTCAGTCCGTGCGACCGACT-3**	**462 bp**
Aero2	**5\-GATCTCCAGCCTCAGGCCTT-3**	
Hly1	**5\-GGCCGGTGGCCCGAAGATACGGG-3**	**597 bp**
Hly2	**5\-GGCGGCGCCGGACGAGACGGG-3**	

Specific PCR conditions, thirty cycles run under the following conditions: for aerolysin gene; Denaturation at 94°C/2 min, primer annealing at 56°C/2 min and DNA extension at 72°C/2 min. For hemolysin gene; initial denaturation at 94°C/3 min, denaturation at 92°C/30 sec, primer annealing at 65°C/30 sec, DNA extension at 72°C/1 min and final extension at 72°C/1.5 min.

RESULTS

The total prevalence of *Aeromonas* spp. infection among the examined *O. niloticus* was 65% (Table 1). *A. hydrophila* was the highest incidence (40%) followed by *A. sobria* (17.5%) and *A. caviae* (7.5%) that differentiated between them by some bacteriological investigations (Table 2).

MAS infected tilapia showed loss of appetite, loss of equilibrium, scales loss, sluggish swimming at the water surface, exophthalmia,

skin erosion and ulcer. Fin and tail rot, enlarged abdomen with ascites and vent were prolapsed. Gills might be congested or pale and anemic and covered with excessive mucus. Internally, the organs were friable and showed a generalized hyperemic appearance.

The results of Congo red binding activity were illustrated in Table 3 and Fig. 1.

For the ability of the isolates to produce the enterotoxins in infant mice, the results showed that the different types of genus *Aeromonas* had the ability to produce enterotoxins which were detected in 6 of 9 tested isolates (66.6%) (2 of 3 *A. hydrophila*, 2 of 3 *A. sobria* and 2 of 3 *A. caviae*) (Table 4).

A. hydrophila and *A. sobria* were highly cytotoxic to Vero cells (Fig. 2). While, *A. caviae* isolates gave high and low toxic activity to Vero cells as showed different degrees of cytopathic effect.

The results observed in Fig. 3 Showed that by PCR analysis with *Aeromonas* spp. (Aero) primer set, amplicons were produced only for 5 out of 9 extracted DNA (2 of 3 *A. hydrophila*, 1 of 3 *A. sobria* and 2 of 3 *A. caviae*) with a percentage of 55.5%. The results observed in Fig. 4 revealed that by PCR analysis with *Aeromonas* spp. (Hly) primer set, amplicons were produced only for 4 out of 9 extracted DNA (1of 3 *A. hydrophila*, 1 of 3 *A. sobria* and 2 of 3 *A. caviae*) with a percentage of 44.4 % of examined samples.

On the basis of specific PCR analysis, all isolates were divided into 4 different genotypes, type 1 carrying aero gene only and type 2 carrying hly gene only and type 3 carrying both aero and hly genes and type 4 for those in which no haemolytic genes were detected.

Table 1. Prevalence of *Aeromonas* spp. infection among the examined fish

Fish spp.	Total no. exam.	*Aeromonas* spp.		*A. hydrophila*		*A. sobria*		*A. caviae*	
		No.	%	No.	%	No.	%	No.	%
O. niloticus	200	130	65	80	40	35	17.5	15	7.5

Table 2. Biochemical characters of the isolated *Aeromonas* spp.

Item	*A. hydrophila*	*A. sobria*	*A. caviae*
Oxidase test	+ve	+ve	+ve
O/129 test	Resistant	Resistant	Resistant
H2S production	-ve	-ve	-ve
B- heamolysis	+ve	+ve	-ve
Catalase test	+ve	+ve	+ve
Motility test	Motile	Motile	Motile
Indole test	+ve	+ve	+ve
V. P. Test	+ve	+ve	-ve
M. R. test	+ve	+ve	-ve
Gas from glucose	+ve	+ve	-ve
Nitrate test	Varied	+ve	+ve
Urease test	Varied	-ve	-ve
Gelatine liqu. test	+ve	+ve	+ ve
O/F test	F	F	F
Citrate test	Varied	+ve	+ve
Esculine test	+ve	-ve	+ve
Arabinose test	+ve	-ve	+ve
Salicine test	+ve	-ve	+ve
Lactose	-ve	-ve	-ve

Table 3. Congo red binding activity of three isolates of *Aeromonas* spp.

Aeromonas spp.	Color of colonies
Ah1	Deep red (CR^{+++})
Ah2	Deep red (CR^{+++})
Ah3	Deep red (CR^{+++})
As1	Deep red (CR^{+++})
As2	Deep red (CR^{+++})
As3	Deep red (CR^{+++})
Ac1	Deep red (CR^{+++})
Ac2	Pale orange (CR^{+})
Ac3	Deep red (CR^{+++})

Table 4. Results of suckling mice assay of *Aeromonas* isolates.

Aeromonas spp.	Gut Weight (GW)	Body Weight (BW)	GW/BW (ratio)	Result
Ah1	0.15	1.6	0.093	+ve
Ah2	0.18	1.9	0.094	+ve
Ah3	0.12	1.8	0.066	-ve
As1	0.14	1.6	0.087	+ve
As2	0.16	1.5	0.106	+ve
As3	0.07	1.4	0.050	-ve
Ac1	0.21	1.8	0.116	+ve
Ac2	013	1.8	0.072	-ve
Ac3	0.20	1.9	0.105	+ve
Control	0.15	1.8	0.085	-ve

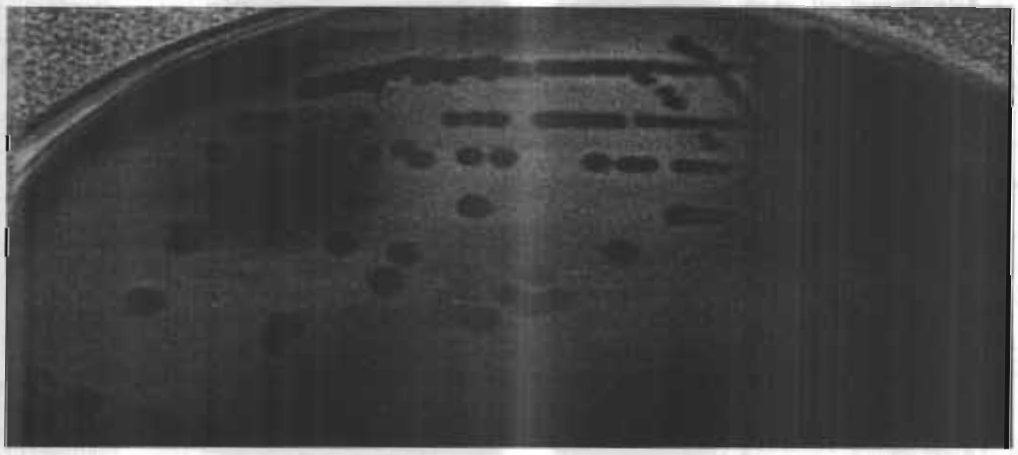

Fig. 1. Showed deep red colouration in Congo red binding activity of *Aeromonas* spp.

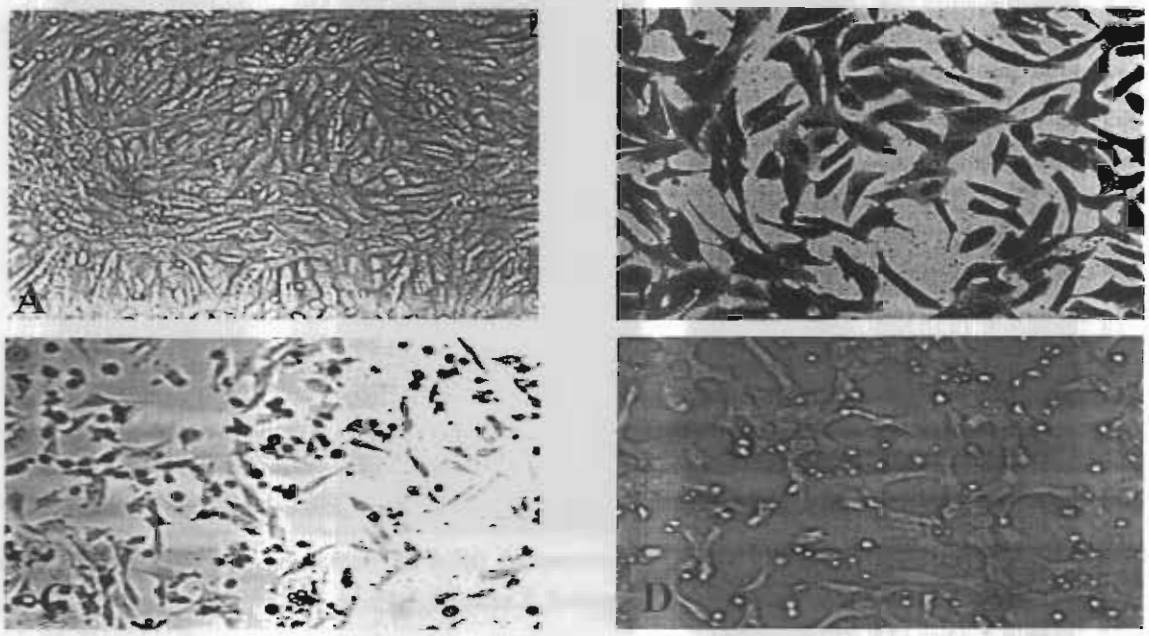

Fig. 2.Cytotoxic effect of *Aeromonas* isolates on Vero- cells after 48 hr. (A) Normal cells (B) Effect of *A. hydrophila* on Vero cells. (C) Effect of *A. sobria* on Vero cells. (D) Effect of *A. caviae* on Vero cells. Cytopathic effect showed elongation of cells and enlargement of nucleus and Stellate shaped cells, detachment, vaculation and degeneration of cell sheets.

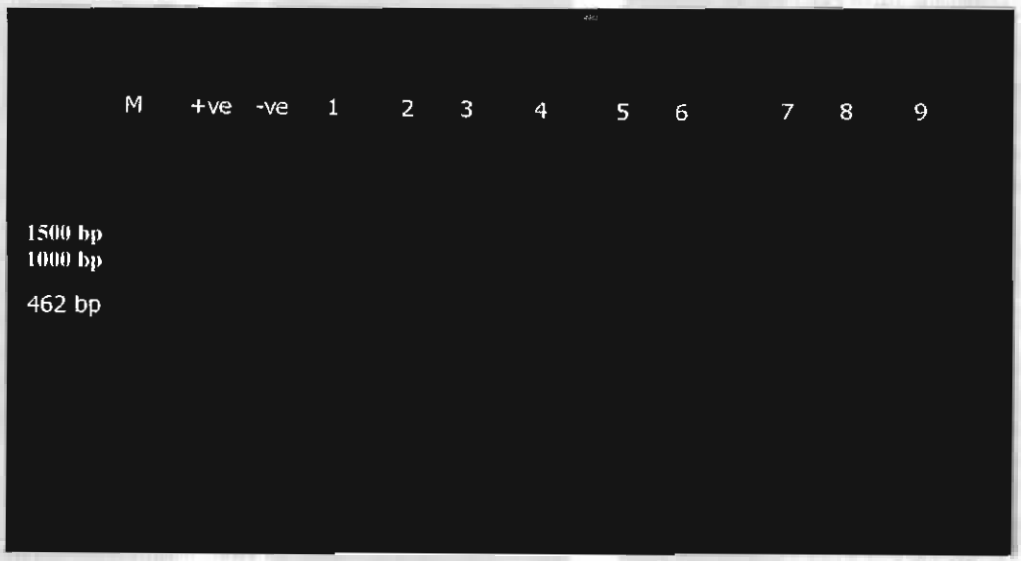

Fig. 3. Amplification of aerolysin gene from extracted DNA of *Aeromonas* spp. M: DNA marker. +ve: Positive control. -ve: Negative control. Lanes (1, 2, 3): *A. hydrophila*. Lanes (4, 5, 6): *A. sobria*. Lanes (7, 8, 9): *A. caviae*.

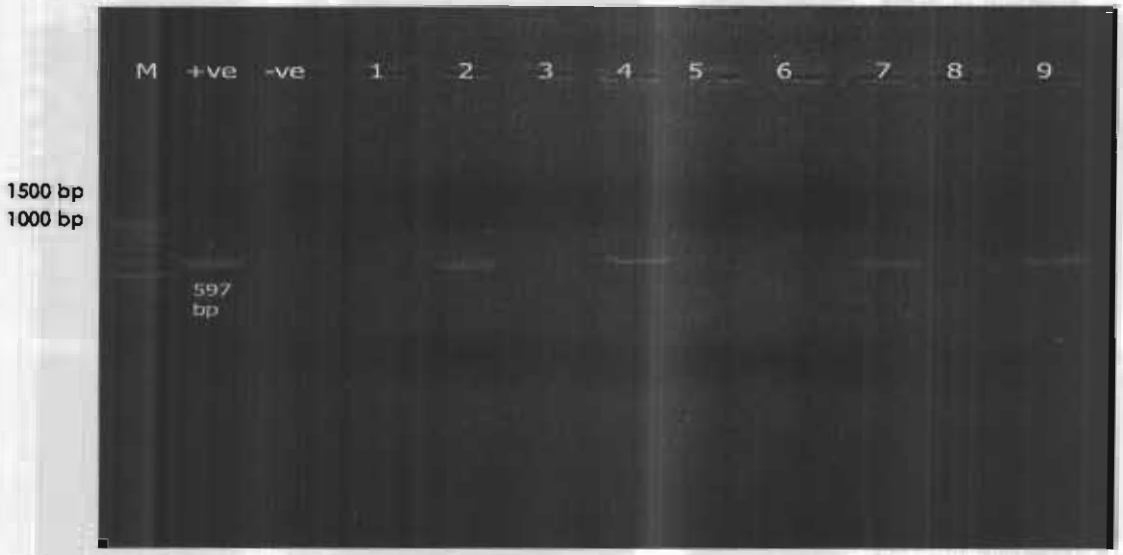

Fig. 4. Amplification of hemolysin gene from extracted DNA of *Aeromonas* spp. M: DNA marker. +ve: Positive control. -ve: Negative control. Lanes (1,2,3): *A. hydrophila*. Lanes (4,5,6): *A. sobria*. Lanes (7,8,9): *A. caviae*.

DISCUSSION

Aeromonads are serious problem for the fish farming industry in Egypt, causing heavy economic losses *(2,3)*. The results of this study showed that *A. hydrophila*, *A. sobria* and *A. caviae* were the most predominant bacterial species isolated from diseased fish but in different percentage, these results agreement with that recorded *(2,3,4,21)*. The highest prevalence of aeromonads could be attributed to its presence as a part of intestinal flora of healthy fish *(2)*. Regarding to the observed clinical signs and postmortem alterations associated with *Aeromonas* infections. Several previous studies*(2-4, 21)* described similar findings..

The morphological and biochemical investigations recorded were reported by several previous studies *(2-4,21)*. Congo red binding or lack of it, is an indicator to the virulence for several pathogenic bacteria which associated with presence of B- D glycan in the bacterial cell wall (*22*). In this study all motile *Aeromonas* isolates gave Congo red positive with different degree in red colour. These results were observed demonstrated previously (*21,23,24*).

The suckling mouse assay is a reproducible assay system for the detection of enterotoxin. The elaboration of enterotoxin may be during the colonization of bacteria which lead to damage of intestinal mucosa allowing *Aeromonas* to penetrate and create disease (*17*). From 9 tested isolates, 6 *Aeromonas* isolates (66.6 %) were positive for production of enterotoxin. Similar results were obtained by other investigation *(6,25)*. The mouse test is less expensive and not time consuming so it is better than using the ileal loop of rabbit *(26)*.

All of the tested isolates were able to produce cytolytic enterotoxin with a percentage of 100 %. The cytotoxicity in *Aeromonas* spp. was multifactorial and may involve different genes and the cytopathic effect was high in the isolates which carrying the hly and aero genes together *(27)*. On the contrary it has been proved that *A. hydrophila* and *A. sobria* strains were weak cytotoxin producers *(28)*.

PCR-based method is rapid, sensitive, and specific for the detection of virulence factors of *Aeromonas* spp. It overcomes the handicap of time-consuming biochemical methods. Aerolysin and hemolysin genes were detected by PCR, which were pathogenic factors of *Aeromonas* species. The aero gene that considered one of the major virulence factors in

gastroenteritis *(18)* was detected in 5 of 9 (55.5%) of the isolates. On the other hand, hly gene was detected in 4 of 9 (44.4%) of the isolates. *A. hydrophila* isolates were divided into three genotypes (aero$^+$hly$^-$, aero$^+$hly$^+$ and aero$^-$ hly$^-$), and *A. sobria* strains were divided into three genotypes (aero$^-$hly$^+$, aero$^+$hly$^-$ and aero$^-$ hly$^-$) while, *A. caviae* isolates were divided into two genotypes (aero$^+$hly$^+$ and aero$^-$hly$^-$). The cytolytic enterotoxin gene and hemolysin gene were detected in 46% of *Aeromonas* spp. isolated from fish. A higher percentage of aerolysin and hemolysin genes were recorded in 96% *(30)*. Both hlyA and aerA genes were detected in *A. hydrophila* by PCR, but inactivation of these genes by mutagenesis, the cytotoxic and haemolytic activity were eliminated *(19)*. In this study, the ability of the isolates to take up Congo red dye is an indication of good virulence marker for *Aeromonas* spp.

Also, it is important to mention that distribution of haemolytic genes in the isolates when compared with their toxic activities, a three detected genotypes (aero$^-$hly$^+$, aero$^+$hly$^-$ and aero$^+$hly$^+$) were enterotoxic in the suckling mice while the genotype (aero$^-$hly$^-$) was contrary to enterotoxigenic activities. The fact led us to indicate that one or two genes were responsible for enterotoxigenic activities in the suckling mice.

In this investigation, Vero cell cytotoxicity did not correlate absolutely with haemolytic genes which determined by PCR analysis. As one isolate of each *A. hydrophila, A. sobria* and *A. caviae* did not amplify any of the haemolytic genes but showed cytotoxic activity suggesting that other virulent factors are present.

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الملخص العربى

كشف وتمييز عوامل الضراوة فى عترات الأيروموناس المعزولة من الأسماك المريضة

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اجريت هذه الدراسة على عدد ٢٠٠ سمكة مريضة من البلطي النيلي والتى تم تجميعها من مزارع تعانى من نفوق الأسماك. وذلك بهدف حديد السلوك المرضى لثلاثة أنواع من بكتيريا الإيروموناس المعزوله من الأسماك و ذلك بإستخدم طرق مختلفه خاصة تقنيه الدنا. كانت نسبة الأصابة الكلية ببكتيريا الأيروموناس (٦٥٪). أشارت الدراسة ان أعلى نسبة للأصابة من الإيروموناس هيدروفيلا (٤٠٪) ثم الإيروموناس سوبريا (١٧,٥٪) و الإيروموناس كافيى (٧,٥٪). تمثلت الأعراض الأكلينيكية للأسماك المصابة فى انتشار بقع نزيفيه على أماكن مختلفة من جسم السمكة وتاكل بالزعانف والذيل وتساقط للقشور و تقرحات بالجلد مع وجود استسقاء بالبطن وعتامه فى العين مع احتقان الأعضاء الداخلية . تم إجراء اختبار الضراوة لعدد ٩ عترات عشوائية من سلالات الايروموناس (٣هيدروفيلا و٣سوبريا و٣ كافيى) بالنسبه الى خاصية الأتحاد بصبغة الكونجو الحمراء ، وجد أن الثلاث عترات للايروموناس هيدروفيلا و الإيروموناس سوبريا تتحد بها بدرجه كبيره وتعطى اللون الأحمر الغامق مما يدل على شدة الضراوة، أما الثلاث عترات للايروموناس كافيى فقد أعطت كلا اللونين الأحمر الغامق و الأحمر الخفيف . بالنسبة لقدرة المعزولات على انتاج السموم فى فئران التجارب فقد أوضحت النتائج أن الأنواع المختلفة من جنس الأيروموناس كانت لها القدرة على انتاج الأنتيروتوكسين ووجد ان ٦ عترات من ٩ عترات مختبرة بنسبة (٦٦,٦٪) كانت ايجابية لذلك. أظهرت النتائج أن العترات العشوائية التسعه بنسبة (١٠٠٪) لها القدرة على أنتاج السيتوليتك انتيروتوكسين فى خلايا الفيرو. كانت نتيجة الكشف عن وجود جين الايروليسين فى ٩ عترات عشوائية باستخدام إنزيم البلمره المتسلسل الخاص بهذا الجين, هى وجود جين الايروليسين فى ٥ عترات فقط فى حين كانت نتيجة الكشف عن جين الهيموليسين هى تواجدة فقط فى ٤ عترات.