PREVALENCE OF *AEROMONAS HYDROPHILA* INFECTION IN WILD AND CULTURED TILAPIA NILOTICA *(O.NILOTICUS)* IN EGYPT

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Abstract

Clinical signs and post mortem lesions were recorded on a large number of Nile tilapia, *O.niloticus*, specimens. Bacterial isolates were characterized to confirm the presence of *Aeromonas Hydrophila*. Our results showed that prevalence of A*. hydrophila* infection was higher in cultured fish during the summer season than in wild fish. The observed clinical signs in the examined fish suffering from Motile Aeromonas Septicemia (MAS) were varied from septicemia, ascitis, erosion, ulceration, detachment of scale and exophthalmia. The postmortem findings varied from congestion to focal lesions in the liver, spleen, and kidney. We collected 25 isolates of *A. hydrophila* from sterile extra-intestinal organs of naturally infected Nile tilapia, *O. niloticus.*

There was a pronounced variation in both the biochemical and enzymatic profiles within the motile aeromonas species. Variation was evident in the hemolytic activity. Similarly, there was variation in pathogenicity among the 25 isolates of *A. hydrophila*. The relation between pathogenicity and the biochemical activity is discussed.

Key words*: A. hydrophila*, (MAS), *Oreochromis niloticus*, clinical signs, PM, bacteriological examination, API 20 E, APIZYM system, Pathogenicity test.

INTRODUCTION

All populations of organisms, including, aquatic animals are limited partially or completely by diseases in their ecosystem (Real, 1996). Disease prevalence in the ecosystem is influenced by numerous environmental factors including infectious organisms and stressors (Nils kautsky *et. al,* 2000).

The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture, *(*Post, 1989*;* Zorrilla, *et. al.,* 2003). Fish are constantly exposed to bacteria and usually only succumb to an infection after being exposed to prolonged periods of stress. Environmental factors may act as stressors and can predispose a fish to bacterial diseases.

Aeromonas hydrophila and other aeromonads are among the most common bacteria in freshwater habitats throughout the world. Genus Aeromonas includes prominant microbiota in freshwater reservoirs where they together with other

microorganisms act as natural bio-filters and promote self purification of the water body. They are necessarily present in normal microflora and hydrobionats inhabiting fish reservoirs *(*Kompanets *et al; 1992*). However, they frequently cause problems in both feral and cultured fish (Cipriano, 2001) it is responsible for heavy economic losses caused by both high mortality and deterioration of product quality (Groff & Lapatra, 2000; *Karunasagar et al, 2003).* In the Philippines, it has reportedly caused high mortality in reared *O. mossambicus, O. niloticus* and *Tilapia zillii,* (Lio-Po *et al*., 1983). The course of the disease usually runs in an acute manner. Clinical conditions associated with systemic infection result in mortality within 24–48 hours. In more chronic types of clinical conditions, eroded fins occur as well as skin lesions and sluggish swimming (Roberts and Sommerville, 1982 and Lio-Po *et al., 1983*). The mortality was between 10% and 70% among cultured fish. Hemorrhagic septicemia has been reported in pond cultured tilapia *(O. niloticus)* in Japan, (*Miyazaki et. al., 1984*).

There are some potential risk factors associated with the main diseases of fish such as season and water temperature (Ortega *et. al;* 1995). Mortality among high thermal stressed fish was 80% due to Aeromonas. (Noga, 1996). In intensive fish culture, mortality due to *A. hydrophila* infection was highest in late spring and early summer (Faisal *et al.,* 1989).

Limited studies were carried out in Egypt regarding the seasonal Prevalence of *Aeromonas Hydrophila* Infection in wild and cultured *O. niloticus*. Therefore, this study was planned to fulfill the following objectives:

a. Studying the seasonal prevalence of *Aeromonas hydrophila* in naturally collected and cultured *O. niloticus*.
b. Registering the clinical signs and post mortem lesions of the collected fish samples.
c. Identifying the isolated *A. hydrophila* through their biochemical and enzymatic activities.
d. Investigating the pathogenicity of the isolated *A. hydrophila*.

MATERIALS AND METHODS

Studying the seasonal prevalence of *Aeromonas hydrophila* in wild and cultured *O. niloticus*.

A total number of 800 *O. niloticus* specimens were collected in different seasons of the year, according to the following categories:

Wild populations

A total of 400 *O. niloticus* specimens weighing 50-80 g and measuring 8-10 cm were collected alive from The Nile, at Giza Governorate, Egypt (Table 1). Five samples of 20

specimens each were collected in each season (winter, spring, summer, and autumn) from the same area of the Nile at the same time (9 am). Water temperature was measured at the time of collection using a digital thermometer, Fisher Scientific Company. Samples were immediately kept in containers supplied with aerated river water and transported to the wet laboratory, Dept. of Fish Disease and Management, Faculty of Veterinary Medicine, Cairo University. On arrival, the fish were subjected to clinical and post mortem examination according to Austin and Austin (1999).

Table 1. The Collected fish from River Nile, Giza Governorate, Egypt

Seasons	Number	Fish size	Water temp.(^{o}c)
Winter	100	50-80 gm & 8-10 cm	12±3
Spring	100		20±5
Summer	100		27±2
Autumn	100		23±3

Farmed populations (Semi-intensive farm)

A total of 400 specimens of average weight and length similar to the wild fish were collected from the semi-intensive fish farm, Central Laboratory for Aquaculture Research, Abbassa, Sharkia Governorate, Egypt according to the schedule shown in Table (2).

Temperature was measured upon collection of the fish and dissolved oxygen level was measured using a YSI DO meter. Fish were transported to the laboratory in aerated farm water according to Innes, (1966). Fish were maintained in 30 glass aquaria 30×40×80 cm filled with dechlorinated tap water and supplied with constant aeration**.** Fish were subjected to clinical and post mortem examination according to Austin and Austin (1999).

Table 2. The Collected fish from semi-intensive fish farm at Sharkia Governorate, Egypt

Seasons	Number	Fish size	Water temp (^{0}c)*	DO (mg/L)
Winter	100	50-80 gm & 8-10 cm	14±3	2.5-2.7
Spring	100		21±5	2.7-2.9
Summer	100		28±2	2.8-3.0
Autumn	100		23±3	2.6-2.8

Bacteriological, biochemical and enzymatic Studies of the suspected *Aeromonas hydrophila* isolates.

Individual fish were dissected and samples of the liver, spleen, and kidney were collected for bacteriological examination according to Noga (1996) and isolation of *A. hydrophila* was attempted. Swabs from the different organs of each fish were inoculated on Tryptic Soya Broth then on Tryptic Soya Agar (Gibco); Brain Heart Infusion Agar (BioTeC); and MacConky agar (BioTeC). Separate colonies were cultured into the R-S agar media prepared after Shotts and Rimler (1973). The cultivated separate colonies were subjected to biochemical identification using the cytochrome oxidase test "Biomerieux" France., Glucose fermentation, indol production, and Voges Proskauer tests in addition to API 20 E kits "Biomerieux" France, were conducted according to *Austin and Austin (1999)*; API ZYM kits "Biomerieux" France according to Sakai *et al.,* (1993), and the hemolytic activities following the method of Olivier *et al.,* (1981).

Semi-solid agar was used for testing motility. Tryptic Soya Broth +15% glycerol +2% agar was prepared after Thornton *et al.,* 1994 was used for preservation of the isolates,

Hemolytic activity was tested using T.S.A containing 5% sheep RBCs according to Olivier *et al.,* (1981)

Pathogenicity of the isolated *A. hydrophila*.

A total of 270 apparently healthy *O. niloticus* individuals were obtained from an intensive fish farm at Kalubia Governorate, Egypt. They were transferred and maintained for acclimatization for 2 weeks in glass aquaria supplied with dechlorinated tap water and aeration, in the wet laboratory, Dept. of Fish Disease and Management, Faculty of Veterinary Medicine, Cairo University. Fish were equally divided into 27 glass aquaria.

The pathogenicity test was performed following the method of Lafrentz, *et. al*., (2002) on 25 isolates of *A. hydrophila***.** For every 10 fish, 0.1 ml of 2.4×10^8 CFU/ml of the isolated *A. hydrophila* was injected intramuscularly using 21/gauge sterile needle. 10 fish were injected with PBS (PH 7.2) [control(1)] using the same procedure. Another 10 fish were held untreated [control (2)]. The observation time was 7 days**.** The pathogenicity test was considered positive when more than 50 % of the injected fish showed clinical signs and died within 96 hours. The *A. hydrophila* isolates were recovered from the dead fish under experimentation.

RESULTS

Clinical Findings

The total number of fish showing clinical abnormalities from which *Aeromonas hydrophila* was isolated and identified was 310 fish (Tables 3 - 5).

Some of the collected fish showed one or more from the following signs according to the stage of disease; darkness in the color of the skin, detachment of the scales, large irregular hemorrhages on the body surface, ulcers on the skin varied from shallow to deep necrotizing ulcers, fin erosions, inflamed vent, exophthalmia, abdominal distension with sero-hemorrhagic fluids exuded from the vent as shown in Photos (1& 2).

Post mortem findings

The Post mortem findings revealed varied signs according to the stage of the infection among the collected samples from congested to enlarged liver, spleen, kidney and gall bladder. (Photo3).

Bacteriological examination

The results of bacteriological examination revealed the presence of 25 isolates of *Aeromonas hydrophila*, based on the results obtained on R-S media and MacConky agar media. The organisms were Gram negative, motile rods that gave smooth rounded colonies, 2-3 mm in diameter, and yellow- orange in color in R-S media.

Biochemical, enzymatic identification and the hemolytic patterns of the isolated *Aeromonas hydrophila*

Results of the traditional biochemical identification match those obtained by the API 20 E system. All isolates gave positive reaction for cytochrome oxidase, gelatin hydrolysis, indol production, glucose, sucrose and mannitol fermentation, arginine dehydrolase and ß- galactosidase tests. Only 8 isolates gave positive results for Voges Proskauer, lysine decarboxylase and arabinose fermentation tests.

The enzymatic identification using the API ZYM systems revealed that all the isolates gave positive reaction for alkaline phosphatase, butyrate esterase (C4), caprylate esterase (C8), Myristate lipase (C14), Leucine arylamidase and N-acetyl- ß-glucosaminidase, Acid phosphatase and phosphomidase. Twelve isolates gave positive reaction for Trypsin. Eight isolates were positive for ß-glucosidase, ß-glactosidase, ß-glucuronidase, ∞-glucosidase.and Valine arylamidase. The negative results were for Cystine arylamidase, Chymotrypsin, ∞-Mannosidase and ∞-fucosidase. As shown in Table (6).

The hemolytic activity results showed twelve isolates were ß –hemolytic, six isolates were ∞- hemolytic and 7 isolates expressed non-hemolytic (Table 7).

Studies on the pathogenicity assay of the isolated *Aeromonas hydrophila*.

The results of the pathogenicity test are presented in Table (8). Isolate No. 1&2 were the most pathogenic as they caused 100% mortality of the injected fish within 48 hours with development of clinical symptoms. Pathogenicity was confirmed through Re-isolation of A. *hydrophila* from the internal organs. The isolates that proved to be pathogenic by the pathogenicity test were positive Voges Proskauer, lysine decarboxylase and arabinose fermentation tests.

Table 3. Occurance of *Aeromonas hydrophila* in wild *O. niloticus* after appearance of clinical signs (River Nile, Giza Governorate, Egypt)

Season	Number of fish	Water temp.(^{0}c)	Number of *Ahydrophila* isolates
Winter	100	12±3	0
Spring	100	20±5	2
Summer	100	27±2	6
Autumn	100	23±3	0
Total	400	-	8

Table 4. Occurrence of Aeromonas *hydrophila* in farmed *O. niloticus* after appearance of clinical signs.

Seasons	Number of fish	Water temp.(^{0}c)	Number of *A.hydrophila* isolate
Winter	100	14±3	0
Spring	100	21±5	4
Summer	100	28±2	10
Autumn	100	23±3	3
Total	400	-	17

Table 5. The total number of *Aeromonas hydrophila* isolates from both wild and cultured *O. niloticus*

Seasons	Total number of fish/ season	Total No. of fish with clinical abnormalities	Number of *A.hydrophila* isolates	% *A. hydrophila* isolates*
Winter	200	16	0	0
Spring	200	96	6	6.25
Summer	200	117	16	13.67
Autumn	200	81	3	3.70
Total	800	310	25	8.06

*= in relation to the total number of examined fish from both wild and cultured fish during different seasons.

Table 6. The enzymatic activities of *Aeromonas hydrophila* isolates by the APIZYM system

Number of isolates	Enzymes	Reactions	
		+	-
25	Alkaline phosphatase, butyrate esterase (C4) caprylate	25	-
	esterase (C8)	25	-
	Myristate lipase (C14)	25	-
	Leucine arylamidase	25	-
	N-acetyl-ß-glucosaminidase	25	-
	• ∞-Mannosidase	25	-
	• ∞-fucosidase	0	25
		0	25
	Acid phosphatase		
	phosphomidase.	25	-
	Trypsin	25	-
	Cystine arylamidase Chymotrypsin	12	13
		0	25
		0	
	∞-glucosidase.		
	• ß-glucosidase	8	17
	• ß-glactosidase	8	17
	• ß-glucuronidase	8	17
	Valine arylamidase.	8	17

Table 7. The hemolytic activity pattern of *Aeromonas hydrophila* isolates.

Number of Aeromonas hydrophila isolates	The hemolytic pattern
1-12 (12)	∞- hemolytic
13-18 (6)	ß -hemolytic
19-25 (7)	Non- hemolytic
25	total

Table 8. The results of pathogenicity test.

Isolate No.	Number of fish/ isolate	Fish mortality / isolate/ hr.	Mean death time/ hr.	% of pathogencity
1	10	10/48	28.8	100%
2	10	10/48	28.8	100%
3	10	8/48	30.0	80%
4	10	7/48	27.4	70%
5	10	7/72	54.8	70%
6	10	6/96	56	60%
7	10	6/96	56	60%
8	10	7/96	68.5	70%
9	10	4/96	72	40%
10	10	5/96	57.6	50%
11	10	5/96	76.8	50%
12	10	1/48	48	10%
13	10	2/72	60	20%
14	10	2/48	36	20%
15	10	1/48	48	10%
16	10	4/72	42	40%
17	10	3/72	56	30%
18	10	2/72	60	20%
19	10	5/96	62.4	50%
20	10	0%	0	0%
21	10	0%	0	0%
22	10	0%	0	0%
23	10	0%	0	0%
24	10	0%	0	0%
25	10	0%	0	0%
Control (1)	10	0%	0	0%
Control (2)	10	0%	0	0%

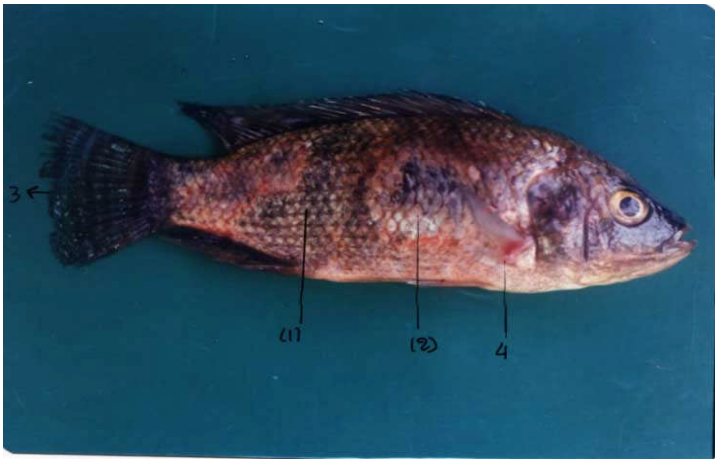

Photo 1. Nile tilapia *(O. niloticus*) showing extensive skin hemorrhage (1), ulceration (2) and tail and fin rot (3).

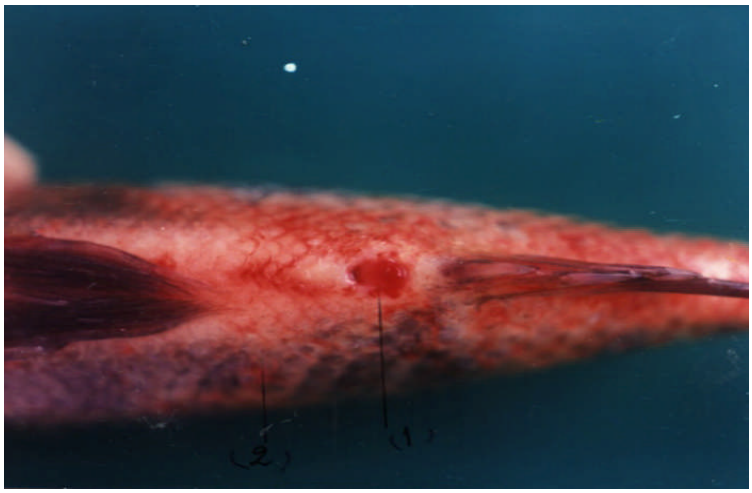

Photo 2. Nile tilapia *(O. niloticus*) showing sero-hemorrhagic fluids exuded from inflamed vent.

Photo 3. Nile tilapia *(O. niloticus*) showing distended gall bladder, the intestine is filled sero-hemorrhagic fluid and gases with congestion of the internal organs extensive skin hemorrhage, tail and fin rot and ulceration.

DISCUSSION

The presented study revealed that *A. hydrophila* infection, the cause of the motile Aeromonas septicemia (MAS), has a different seasonal distribution within the wild and the cultured *O. niloticus* ; its prevalence is higher during summer in cultured fish than wild ones. The percent of infection in the wild stock during summer was (6%) followed by (2%), (0%) and (0%) during the spring, autumn and winter, respectively (Table 3). for cultured tilapia fish, The percent of infection in the farmed stock was 10% in summer followed by (4%), (3%) and (0%) during the spring, autumn and winter, respectively(Table 4). These results are in agreement with those of Eissa *et al.*, (1994) and Company *et al.*, (1999) who reported that, the majority of

the infection occurred during the change of water temperature, spawning season, the adverse conditions during intensification. In addition to the increased environmental fluctuation, the cultured fish become more sensitive to stress than the wild population. As a result, the increase in the production of corticosteroids increases the susceptibility of the fish to *A. hydrophila* infection. Osborne *et al*., (1989) found high densities of motile aeromonads within the environment during the mid summer when sedimentary chlorophyll and water temperature were highest. Meyer (1970) stated that most epizootics among warm water fishes in southeastern United States are generally reported in late spring and early summer as the water temperature ranged from 25^{o}C-35^{o}C. However, this finding disagrees with that of Faisal *et al.*, (1989) who reported that outbreaks of MAS occurred mainly during winter in cultured fish, while in wild Nile fish mortality was observed in late spring and summer. The results also disagree with those of Topic Popovie*, et al.,* (2000) who isolated a total of 27 *A. hydrophila* from a lake in Croatia, there was a clear seasonality in occurrence of the disease and no isolation took place in summer, the same author reported that *A. hydrophila* is more likely to be an important pathogen of cultured rather than of wild fish.

The observed clinical signs in the examined fish suffering from Motile Aeromonas Septicemia (MAS) Photos (1-3), were previously reported by *Okpkowassili and Okpkowassili (1994); Viola, (1995) and Ali, (1996)* who reported that septicemia, ascitis, erosion, ulceration, detachment of scale, exophthalmia and muscular necrosis are the most predominant clinical signs of MAS in Nile tilapia. The postmortem findings (Photo 7) are supported by those of Eissa *et al.,* (1990) and Ali, (1996) who found that, the parenchymatus organs suffered from congestion with focal lesions in the liver, spleen, and kidney as well as, serosangenous fluids filling the abdominal cavity.

The successful isolation and identification of *A. hydrophila* from extra-intestinal organs of naturally infected fish is in agreement with the results of Janda., (1991) and Ali, (1996) who reported that *A. hydrophila* isolates recovered from sterile extra-intestinal organs are considered to have originated from invasive disease and the acute MAS may result in localization of colonies identified as *A. hydrophila* within the hematopoetic tissue.

The results of the biochemical characterization of the isolates were interpreted and found in agreement with those reported by Nieto *et al*., (1984) and Toranzo *et al.,* (1986); in addition variable results were obtained in voges-proskauer reaction, citrate utilization, lysine decarboxylase, arabinose and amygadalin fermentation tests. It has been demonstrated that great variation in virulence exists within the motile aeromonas species; few studies have been conducted to associate the biochemical characteristics of *A. hydrophila* species with virulence factors. Biochemical reactions such as voges -

proskauer, arabinose and amygdalin fermentation and LDC test, have been correlated with virulence. Burke *et al.,*(1982) and Santos *et al.,* (1988) reported that a significant relationship was found between virulence of *A. hydrophila* for fish and production of acid from arabinose and sucrose, LDC and V.P. test, in addition to elastase and hemolytic activities.

The virulence of some microorganisms is known to be associated with the production of particular enzymes produced by microorganisms. Our enzyme activity results were in agreement with those of Soliman, (1988) and Sakai *et al*., (1993). This indicates that the test could be used as a reliable diagnostic tool for identification of *A. hydrophila*

On studying the hemolytic activities of *A. hydrophila* isolates on T.S.A containing 5% sheep RBCs, the results indicate that 72% of *A. hydrophila* under experimentation produced 2 types of hemolytic activities. Branden and Janda (1987) and Ali, (1996) reported that *A. hydrophila* appears capable of producing extra-cellular hemolysin which induced hemolysis on blood agar. There was a strong correlation between the hemolysin and the virulence of *A. hydrophila* isolates. Thune *et al.,* (1986) found that 3 ß –hemolytic *A. hydrophila* were non virulent. The non hemolytic isolates of *A. hydrophila* in this study agree with the results of Olivier *et al*., (1981) who reported non hemolytic strains of *A. hydrophila*.

The results of the pathogenicity test showed that isolates No. 1&2 were the most pathogenic as they caused 100% mortalities of the injected fish within 48 hours with development of clinical symptoms with reisolation of the causative agent from the moribund fish. Its worth mention that this two isolates were positive in production of acid from arabinose and sucrose, LDC and V.P. test, in addition to elastase and hemolytic activities.

It could be concluded from the present investigation that *A. hydrophila* infection predominates among Tilapia nilotica during the summer season especially in semi-intensive fish farms so, it is advisable to apply a good management program and apply a preventive strategy to over come the outbreak by *A. hydrophila* during the summer seasons in the semi-intensive fish farms in Egypt.

It could be concluded also that there is strong correlation between the results of biochemical, enzymatic, hemolytic activities and pathogenicity test of *A. hydrophila* isolates with its virulence so it is recommended to include the previous tests in assessing the danger of the isolated *A. hydrophila*.

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معدلات الاصابة بميكروب الايروموناس هيدروفيلا فى اسماك البلطى النيلى المستزرعة وغير المستزرعة فى مصر

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تمت دراسة الاعراض المرضية والصفة التشريحية فى عدد كبير من اسماك البلطى النيلى وتم إجراء عزل بكتريولوجى لميكروب الايروموناس هيدروفيلا .دلت النتائج الى ان معدلات الاصابة بميكروب الايروموناس هيدروفيلا عالية فى اسماك البلطى النيلى المستزرعة خلال فصل الصيف. تم تسجيل اعراض مرضية مختلفة من الاسماك التى تم عزل الميكروب منها كاعراض الالتهاب الجلدى, القرح, تاكل الزغانف و الزيل و جحوظ العين. الصفة التشريحية تدرجت من انزفة دموية إلى إصابات في الكبد , الطحال و الكلى.

تم عزل 25 عتره من الميكروب من خارج الامعاء و الجلد. كان هناك نتائج مختلفة لنتائج الاختبارات الكيمائية و الإنزيمات و كذلك تحلل الدم , كان هناك ايضا اختلاف فى نتائج اختبار العدوى الصناعية بين ال25 عترة المعزولة.

تم مناقشة النتاج و مدى ارتباط الضراوة للميكروب بالنتائج المختلفة السايقة.