Aeromonas veronii biovar sobria a Causative Agent of Mass Mortalities in Cultured Nile Tilapia in El-Sharkia governorate, Egypt

Eissa I. A. M[1]., Maather El-lamei[1], Mona Sherif[2], E. Desuky[3], Mona Zaki[4], M. Bakry[2]

[1]Dept. of Fish Diseases and Management, Fac. of Vet. Medicine, Suez Canal Univ, Egypt.
[2]Animal Health Research Institute, Ismailia branch, Egypt.
[3]Animal Health Research Institute, Port Said branch, Egypt.
[4]Dept. of Hydrobiology, National Research Center, Egypt.
eissavet29@yahoo.com

Abstract: In this study, a total of 240*Oreochromis niloticus* species were collected randomly and monthly during an outbreak of disease mass mortalities from different fish farms in the areas of Bahr El-Baqar. Clinical signs and postmortem lesions of affected *Oreochromis niloticus* were recorded. The most isolated bacterial agent was *Aeromonas veronii biovar sobria* which identified using Vitek 2 system and traditional method as *Aeromonas sobria*. The identification was confirmed by detection of 16s rRNA gene by PCR then sequencing of this gene and by analysis of sequence was identified as *Aeromonas veronii*. The total prevalence was 86.25% in the four months. The highest prevalence was in July 95% followed by August 91.67% and June 86.67% then May 71.67%. The experimental infection was studied and the mortality was 70 and 35% of the I/P and I/M respectively.
[Eissa I. A. M., Maather El-lamei, Mona Sherif, E. Desuky, Mona S. Zaki and M. Bakry. ***Aeromonas veronii biovar sobria* a Causative Agent of Mass Mortalities in Cultured Nile Tilapia in El-Sharkia governorate Egypt.** *Life Sci Life Sci J* 2015;12(5):90-97]. (ISSN:1097-8135). http://www.lifesciencesite.com. 11

Key words: *Oreochromis niloticus*, PCR, Aeromonas sobria, *Aeromonas veronii biovar sobria*, Vitek 2 system.

1. Introduction

Without doubt, Nile tilapia (*Oreochromis niloticus*) is the most largely cultivated species in Egypt. Tilapias, under conditions of stress, are susceptible to a wide range of facultative pathogenic bacteria. These secondary invaders tend to be ubiquitous in the freshwater environment.

Aeromonas species are facultative anaerobic Gram-negative bacteria that belong to the family Aeromonadaceae ***(Aberoum and Jooyandeh 2010)***. These bacteria have a broad host spectrum, with both cold-and warm-blooded animals, including humans and are known as psychrophilic and mesophilic. Aeromonas spp. are among the most critical causes of mass mortalities in aquaculture like, *Aeromonas hydrophila* in reared *O. mossambicus, O. niloticus* and *Tilapia zillii****(Lio-Po et al., 1983)***; *Aeromonas sobria* in fingerlings of *Labeo rohita* ***(Sugumar et al., 2002)***; *A. veronii* caused in cultured *Cyprinus carpio* ***(Yu et al., 2010)***; *Aeromonas sobria* in *Garra rufa* ***(Majtán et al., 2012)***; *Aeromonas sobria* in *Carassius auratus* ***(Fard et al., 2014)***.

VITEK 2 compact system (bioMe´rieux, Marcy l'Etoile, France) is an integrated system that automatically performs rapid identification using algorithms based on fluorescence and colorimetry (***Barry et al., 2003***).

Unlike phenotypic identification, which can be modified by the variability of expression of characters, 16S rDNA sequencing provides unambiguous data even for rare isolates, which are reproducible in and between laboratories ***(Hossain, 2008)***. Moreover, confirmation of *Aeromonas veronii biovar sobria* may be obtained by sequencing the16S RNA gene (***Sreedharan et al., 2011***).

This study was concerned as a part to investigate the clinical signs and the postmortem lesions in naturally infected *Oreochromis niloticus*, isolation of the causative agent of the disease by traditional methods and confirmation using Vitek 2 and sequencing.

2. Materials and Methods

Naturally infected fish:

A total number of 240 naturally infected live and freshly dead Nile tilapia (*Oreochromis niloticus*) weighting 225±25g showing signs of septicemia (ulcers, heamorrhage allover the body surface and base of the fins with protruded anal opening) were collected during an outbreak of mass mortalities with septicemia during period from May to August 2013 from different fish farms in the areas of Bahr El-Baqar, El-Sharkia governorate and transported to the wet laboratory of Dept. of Fish Diseases and Management, Fac. of Vet. Medicine, Suez Canal University.

Water sampling:

Simultaneously to fish samplings, between 9 and 10 in the morning, precleaned polyethylene sampling bottles (1.5 liter capacity) were immersed about 50 cm below the water surface, 500 ml of water were taken at each month. Some water parameters were determined *in situ* such as: Temperature (°C) using a dry mercury

thermometer and dissolved oxygen (DO) using a digital dissolved oxygen meter (HI 9142 - Hanna instruments Inc., RI, USA).

Then the rest of water samples were acidified on spot with 10% nitric acid then transported immediately to the laboratory in an ice bath and prepared for analysis as described by APHA, 1998.

pH was measured by Orion Research Ion Analyser 399A pH meter. NH_3, NO_3 and NO_2 concentrations were determined using the colorimetric techniques according to the methods proposed by APHA (2005).

Clinical picture:

Clinical signs and postmortem alterations of the collected cultured Nile tilapia *O. niloticus* for bacterial isolation were adopted according to ***Noga (2010).***

Bacterial isolation and identification:

Under aseptic condition, skin lesions (ulcer), gills, kidneys, liver, spleen, intestine and brain inculi were streaked over BHIA plates and incubated at 25˚C for 24 hrs. The separated colonies were picked up and inoculated into TSA slant for further identification. Biochemical tests were carried out and Vitek 2 system (BioMerieux) were used for confirmatory identification.

DNA manipulations

Genomic DNA was extracted by heating at 100 C° for 4 min then centrifuged at 4000 rpm for 4 min at 4C° and take the supernatant. The specific forward primer pairs for 16S rRNAgene of 356bp were 5/-GGGAGTGCCTTCGGGAATCAGA-3/& 5/-TCACCGCAACATTCTGATTTG-3/ (***Wang et al., 2003***). The PCR mixture according to Biotek corporation company was 10µl of master mix with 1µl of primer and 7µl distilled water then add 2µl DNA template. PCR amplification was performed using PCR master cycler (Eppendorf AG Germany) under the following conditions (***Wang et al., 2003***): initial denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 0.5 min, annealing of the primers at 59°C for 0.5 min, and primer extension at 72°C for 0.5 min. with final extension at 72°C for 7 min.

Sequencing of 16S rRNA gene:

The PCR amplification product was checked on 1% (w/v) agarose gels. The PCR product was purified using the QIA quick PCR Purification Kit (QIAGEN, Hilde, Germany), following the manufacturer's protocol ***(Patil et al., 2011)***. Purified PCR products were sequenced on AB 3500 Genetic analyzer by using Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA). The resultant sequence was analyzed using the BLAST program on the NCBI and the neighbor joining blast tree (http://www.ncbi.nlm.nih.gov/blast) against the database of strain types and published valid prokaryotic nomenclature.

Experimental infection:

A total of 80 apparently healthy *O. niloticus* were collected randomly with an average body weight 100±10g and was divided into four groups each group contained 20 fish and maintained in the four fully prepared glass aquaria and kept for 1 week before injection for acclimation. Groups 1&2 were injected with 0.05ml of 0.05 ml of saline (0.85% NaCl) contained $1.2x10^8$ *A. veronii biovar sobria* CFU ***(Iqbal et al., 1999***) using insulin syringe by I/P and I/M routes respectively. While groups of 3&4 were injected with 0.05ml sterile saline (0.85% NaCl) by the same routes. They were kept in room temperature 26±2 C°

3. Results

Clinical picture:

The clinical signs of naturally infected Nile tilapia *(O. niloticus)* showed hemorrhages allover the body surface and ulcers with muscular necrosis. The postmortem findings revealed that *O. niloticus* showed septicemic lesions (congestion in gas bladder, anterior and posterior kidneys, liver, spleen, gall bladder, heart, brain and intestines) Plate (1).

Water analysis

The results of water analysis documented in table (1) revealed that the water parameters were in normal range.

Bacterial examinations:

The most isolated bacteria was identified using traditional and Vitek 2 system was *Aeromonas veronii biovar sobria* with 98% probability. The phenotypic and biochemical characteristics of A. veronii biovar sobria were illustrated in tables (2 &3).

Sequencing of 16S rRNA gene:

The neighbor joining blast tree shown in fig (3) which showed maximum relation of the isolated strain (ICI 61031) to *A. veronii* B565.

Monthly prevalence of *A. veronii biovar sobria* infection in *O.niloticus*:

The results were illustrated in Fig.(1) that showed the total prevalence in the four months was 86.25%, with highest prevalence in July 95% followed by August 91.67 % then June 86.67% while the lowest was in May 71.67%.

Experimental infection:

No fish died during acclimation period. The mortality rate was 70 and 35% of the injected fish with *A. veronii biovar sobria* for group1 and 2 respectively and there were no mortalities in groups injected with saline fig. (2). The clinical signs and postmortem findings revealed the same alterations as in the natural infection (Plate 1). *A. veronii biovar sobria* was re-isolated from the internal organs of

moribund and freshly dead fish. Phenotypical and biochemical confirmations for reisolated bacteria were performed.

Table (1) Showing water parameters during monthly examination

parameter	May	June	July	August
DO	6.20	5.60	4.20	4.50
Ammonia(mg/l)	0.53	0.57	0.59	0.57
Nitrite (mg/l)	0.19	0.21	0.24	0.20
Nitrate (mg/l)	0.090	0.095	0.100	0.100
pH	7.6	7.6	7.5	7.5
Temperature (^{o}C)	25	28	30	27

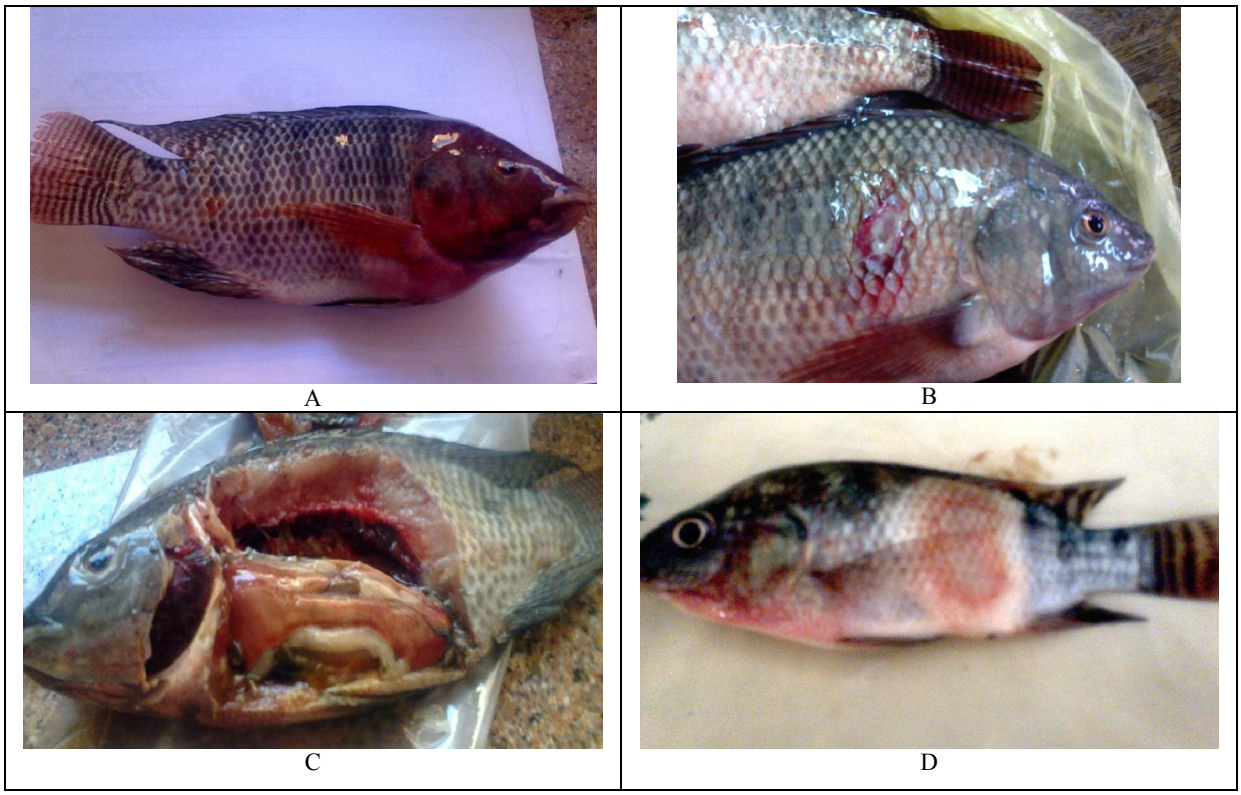

Plate (1) A: Showing naturally infected *Oreochromis niloticus* with hemorrhages allover the body surface. B:Showing naturally infected *O.niloticus* suffered from hemorrhagic ulcer. C: Showing naturally infected *O. niloticus* suffered from septicemic lesions. D: Showing experimentally infected *O. niloticus* with hemorrhagic area surrounded by area of discoloration with hemorrhages in the lower jaw and pectoral fin.

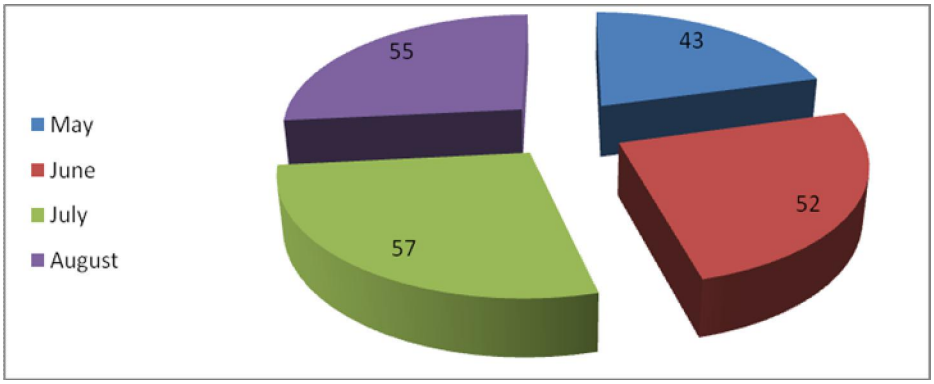

Fig (1) Monthly prevalence of *Aeromonas veroni ibiovar sobria* infection in examined *O. niloticus*.

Table (2): Showing biochemical characters of suspected *Aeromonas veronii biovar sobria* isolates from naturally infected *O.niloticus* by traditional methods

Items	1	2	3	4	5	6	7
Cytochrom oxidase	+	+	+	+	+	+	+
O/F	F	F	F	F	F	F	F
String test using KOH 3%	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
Growth at 5°C	+	+	+	+	+	+	+
Growth at 42°C	+	+	+	+	+	+	+
0% NaCl	+	+	+	+	+	+	+
2% NaCl	+	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+
6.5% NaCl	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
H2S (TSI)	+	-	+	-	-	+	+
Indole	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Esculin hydrolysis	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+
Salicin	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	-	-	+	+
Heamolysis	β	β	β	β	β	β	β
Sucrose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	-	-
Ornithene decarboxylase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	-	-	-	-	-

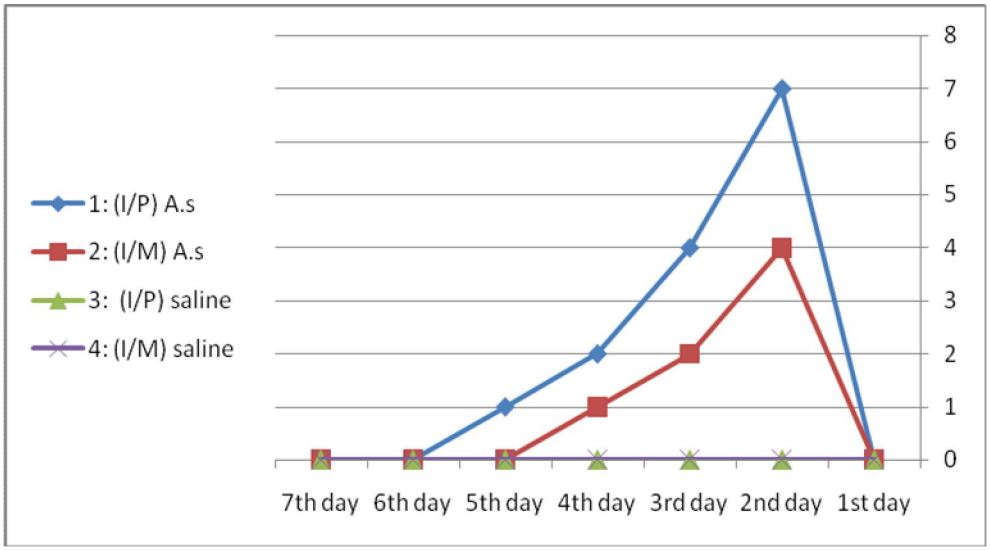

Fig. (2) Mortality curve of *O. niloticus* due to experimental infection with *A.veronii biovar sobria.*

Table (3): Showing biochemical characters of suspected *Aeromonas veronii biovar sobria* isolatefrom naturally infected *O. niloticus* using Vitek 2

WELL	TEST	RESULTS
2	APPA	+
3	ADO	-
4	PyrA	-
5	IARL	-
7	dCEL	-
9	BGAL	+
10	H2S	-
11	BNAG	+
12	AGLTp	-
13	dGLU	+
14	GGT	-
15	OFF	+
17	BGLU	-
18	dMAL	+
19	dMAN	+
20	dMNE	+
21	BXYL	-
22	BALap	-
23	ProA	+
26	LIP	-
27	PLE	-
29	TyrA	+
31	URE	-
32	dSOR	-
33	SAC	+
34	dTAG	-
35	dTRE	+
36	CIT	+
37	MNT	-
39	5KG	-
40	ILATK	-
41	AGLU	-
42	SUCT	+
43	NAGA	-
44	AGAL	-
45	PHOS	-
46	GlyA	-
47	ODC	-
48	LDC	-
53	IHISa	-
56	CMT	+
57	BGUR	-
58	O129R	+
59	GGAA	-
61	IMLTa	+
62	ELLM	+
64	ILATa	-

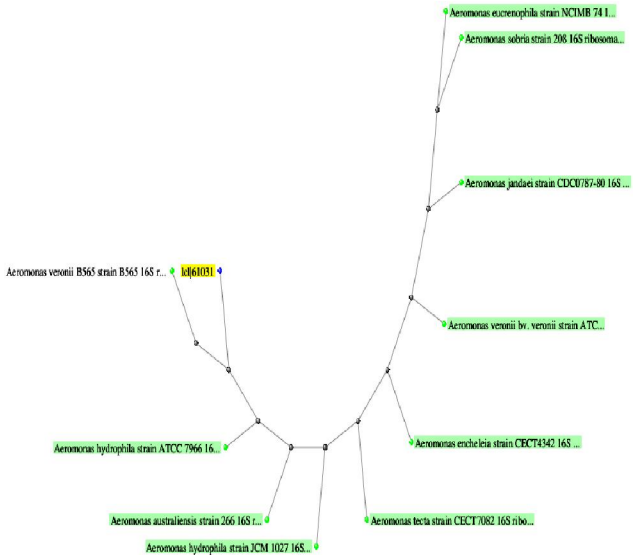

Fig. (3) Phylogenetic relationships between isolate and closely related species, based on partial lengths of 16S rDNA of ICI 61031. The evolutionary history was inferred using the Neighbor-Joining method.

4. Discussion

The aquaculture gains its important due to its high demand, market price and export value. Moreover, ***Tidwell and Allan (2001)*** reported that fish culture is being rapidly adopted in third world countries, growing approximately six times faster in developing nations than in the first world.

Tilapias, under conditions of stress, are susceptible to a wide range of facultative pathogenic bacteria. These secondary invaders tend to be ubiquitous in the freshwater environment. Outbreak of mass fish mortalities occurred in summer months of 2013 in the areas of Bahr El-Baqar, El-Sharkia governorate.

The observed clinical signs of infected *Oreochromis niloticus* in this study were hemorrhages allover the body surface and fins, ulcers with muscular necrosis. The present results were nearly similar to that recorded by ***Camus et al. (1998);Iqbal et al. (1998);Rahman et al. (2002a); Rehilka (2002); Eissa et al. (2011); Austin and Austin (2012); Majtán et al. (2012)***;***Roberts (2012); Fard et al. (2014).*** This may be due to the bacterial multiplication inside the intestine causing a hemorrhagic mucous disquamative catarrh. Toxic metabolites of Aeromonas species are absorbed from the intestine and induce toxemia. Capillary hemorrhage occurs in the dermis of fins and trunk and in the submucosa of the stomach ***(Miyazaki and Kaige, 1985)***. Moreover, ***Austin and Austin (2012)*** reported that it appears that the infection of *A. salmonicida* often starts at the site of injury to the epidermis. A haemorrhagic inflammatory process then develops between the epidermis and dermis. This red

inflammatory zone gradually extends as the infection spreads. The breakdown of tissue leads to the formation of a central ulcer, which may occur in any location on the body surface. In addition, there is evidence that proteolytic extracellular products from *A. hydrophila* have the ability to inhibit skin antimicrobial peptides of frog (***Schadich and Cole, 2009***).

The postmortem examination revealed that the affected *O. niloticus* showed septicemic lesions (congested gas bladder, anterior and posterior kidneys, liver, spleen, gall bladder, heart, brain and intestine). This come in agreement with result of ***Rehilka (2002);Eissa et al. (2011); Austin and Austin (2012); Roberts (2012); Shayo et al. (2012)*** and ***Fard et al. (2014).*** The toxic metabolites of Aeromonas species induce hepatic cells and epithelia of renal tubules to show degeneration. Glomeruli are destroyed and the tissue becomes hemorrhagic, with exudates of serum and fibrin (***Miyazaki and Kaige, 1985***).

Regarding the results of the water analysis, it was revealed that all the water parameters were in normal range and there was no role to any of water parameters in mass mortalities occurrence except the elevated water temperature.

Ling et al. (2003) mentioned that the Vitek-2 system integrates several advantages that may be of clinical interest for routine testing of Gram-negative bacilli isolated from the clinical samples: Rapid identification (three hrs), a high level of automation, a simple methodology and taxonomically updated databases. The results obtained by VITEK 2 system resembled *Aeromonas sobria–Aeromonas veronii* complex of ***Chen et al.(2013)*** except in our strain it was negative for lipase, Glu-Gly-Arg-arylamidase and also, resembled *A. veronii bv. sobria* ATCC9071 except in our strain it was negative for lipase, Glu-Gly-Arg-arylamidase and Glycine arylamidase and positive for citrate. On the other side, the examined strain differd from Ị) *A. veroniibv. veronii* CGMCC1.2205 by D-Cellobiose, α-Glucosidase, Palatinose, lipase, Ornithine decarboxylase and Glu-Gly-Arg-arylamidase which was negative in our strain. And also by Ala-Phe-Pro arylamidase and L-Malate assimilation which was positive in our strain. Ịị) *A. hydrophila* CGMCC1.2017 differed from our strain by H_2S production, lipase and L-Lactate alkalinization which were negative in our strain and also by positive result of citrate, Courmarate and L-Malate assimilation. Ịịị) *A. caviae* CGMCC1.1960 by its negative results for D-Cellobiose, α-Glucosidase, lipase, Glycine arylamidase, L-Lactate alkalinization and Glu-Gly-Arg-arylamidase and also by positive result for Ala-Phe-Pro arylamidase, D-Mannose and citrate.

In the present study, the total prevalence of *A. veronii biovar sobria* was 86.25% in the four months. Nearly similar observation was recorded by ***Fard et al. (2014)*** who found *Aeromonas sobria* in about 85 % of Goldfish (*Carassius auratus*) during the fish mass mortalities and ***Das et al.(2013)*** who identified 77.78% of isolates from catfish *(Clarias batrachus)* showing the signs of Aeromonas septicemia as *A. sobria* and ***Eissa et al. (2011)*** who found 98% of examined carp that showed clinical alteration were infected with *A. sobria* and the highest prevalence of *A. sobria* in naturally infected carp occurred in summer (70%). while, ***Sugumar et al.(2002)*** found *A. sobria*in 60.6% of fingerlings of *Labeorohita*(Hamilton) during the mass mortalities on the other side.***John and Hatha (2013)*** also, found *Aeromonas sobria* in 40.57% of examined ornamental fishes**.**

The results of this study revealed that the highest prevalence was observed in July 95% when the water temperature was the highest followed by August 91.67 % and June 86.67% while the lowest prevalence was in May 71.67%. This may be due to that outbreaks of Aeromonas septicemias and furunculosis are linked to a rise in temperature, usually occurring during spring and summer ***(Tam et al., 2011)***. Increased temperature may also lengthen the transmission season, leading to a higher prevalence of the disease and to more widespread epidemics ***(Karvonen et al., 2010)***. Furthermore, bacteria might show a greater virulence, for instance *A. hydrophila* has shown a greater virulence in largemouth bass (*Micropterus salmoides*) at warmer temperatures because of either reduced resistance of the host or to an increased expression of virulence factors ***(Marcogliese, 2008)***.

Our results of the pathogenicity test revealed that the examined isolate of *A. veronii biovar sobria* were highly pathogenic to *O. niloticus* that the use ofLD50 ($1.2x10^8$ cells/ml) caused mortalities of 70-35% of I/P and I/M groups respectively. These results were in agreement with to ***Iqbal et al. (1999***) and nearly similar to ***Eissa et al. (2011)*** who found that experimentally infected black carp and common carp with 0.2 ml of 10^8 cells\ ml of *A. sobria* by I/P were showed mortality rate 70 % and 20-100% respectively.

Regarding molecular biology, ***Lee et al. (2002)*** declared that sequence similarity between Aeromonas species is very high (ca 98–100%), but shows enough variability to discriminate among species. Confirmation of *Aeromonas veronii* biovar *sobria* may be obtained by sequencing the16S RNA gene (***Sreedharan et al., 2011***). The sequence analysis showed 99% similarity with *A. veronii* in the existing NCBI database. And by constructing a neighbor-joining tree based on the concatenated sequences the

tree revealed consistently, the strain showed maximum relation to *A. veronii*.

The result of biochemical methods (phenotypically) identified our isolates as *Aeromonas sobria* with VITEK 2 probability 98% and the result of 16S rRNA gene sequencing (genetically) identified our isolate as *Aeromonas veronii*. These results come in agreement with the last edition of Bergey`s Manual ***(Martin-Carnahan & Joseph, 2005)*** who clarified that these *A. veronii* biovar sobria (HG 8) clinical isolates were genomically *A. veronii*, but phenotypically most similar to what we had heretofore called *A. sobria*, Furthermore, the HG 7 environmental strains (*A. sobria)*and the HG 8 *(Aeromonas veronii biovar sobria*) are phenotypically very similar, they are genetically and clinically quite different. The same was reported by ***(Sharma et al., 2009*** and ***Janda and Abbott, 2010***). On the other side ***Austin and Austin (2012)*** reviewed that *A. sobria,* have been isolated from wild spawning gizzard shad (Dorosomace *pedianum)* in Maryland, USA during 1987, farmed perch *(Perca fluviatilis)* in Switzerland, tail rot in tilapia (*Oreochromis niloticus*) in China and from mass mortalities among the therapeutic fish *Garra rufa* in Slovakia while *A. veronii* biovar *sobria* was recovered from epizootic ulcerative syndrome and in infectious dropsy in cichlid oscar from India.

References

1. *Aberoum A. and H. Jooyandeh (2010)*: A Review on Occurrence and Characterization of the Aeromonas Species from Marine Fishes. World J. Fish & Marine Sci., 2 (6): 519-5230.
2. *APHA (1998)*: Standard Methods of the Examination of Water and Wastewater. in: AE Greenberg, LS Clesceri and AD Eaton (Eds.), APHA, WEF and AWWA, 20th Ed, Washington DC, USA 1193.
3. *APHA (2005):* Standard Methods for Examination of Water and Wastewater. 21stEdn. APHA, AWWA, WPCF, Washington DC, USA.
4. *Austin B. and Austin D.A.(2012)*: Bacterial fish pathogens, Diseases of farmed and wild fish Fifth Edition, © Springer Science + Business Media Dordrecht 2012.
5. Barry J., Brown A., Ensor V., Lakhani U., Petts D., Warren C. and Winstanley T.(2003): Comparative evaluation of the VITEK 2 Advanced Expert System (AES) in five UK hospitals. J. of Antimicrobial Chemotherapy. 51, 1191–1202.
6. *Camus A.C., R.M. Durborow, W.G. Hemstreet, R.L. Thune and J.P. Hawke (1998)*: Aeromonas Bacterial Infections, Motile Aeromonad Septicemia. SRAC Publication No. 478.
7. *Chen J., N. Zhu, L. Kong, Y. Bei, T. Zheng, X. Ding and Z. He (2013)*: First case of soft shell disease in Chinese soft-shelled turtle (*Trionyx sinens*) associated with *Aeromonas sobria–A. veronii* complex. Aquaculture 406–407 62–67.
8. *Das A., Rathore A., Janani C., Hemanth S. and Arvind Balakrishnan R. (2013)*: Diagnosis of motile *Aeromonas sobria* from catfish with septicemia by PCR. IOSR J. Agri. Vet. Sc. 2(6): 87- 91. doi: 10.9790/2380-0268791.
9. *Eissa I.A.M., Badran A.H., Azza Abdel-Rhman and SomayahAwad (2011)*: Studies on cultured Black Carp (*Mylopharyngodon picens*) regarding *Aeromonas sobria* septicemia for the first time in Egypt. SCVMJ, XVI (1).
10. *Fard A. N., Azadikhah D. and Abdi K. (2014)*: Occurrence reported the *Aeromonas sobria* infection in Goldfish (*Carassius auratus*). Iranian journal congress.atTehran. vol 18.
11. *Hossain* M. *(2008)*: Isolation of pathogenic bacteria from the skin ulcerous symptomatic gourami (*Colisalalia*) through 16S rDNA analysis. *University J. of Zoology, Rajshahi University* 27: 21-24.
12. *Iqbal M.M., Tajima K., Sawabe T., Nakano K. and Ezura Y. (1998a):* Phenotypic and genotypic identification of motile aeromonads isolated from fish with epizootic ulcerative syndrome in Southeast Asian countries. *Fish Pathol.* 33(4): 255-263.
13. *Iqbal M.M., Tajima K. and Ezura Y. (1999)*: Pathogenicity of Motile Aeromonas Species Isolated from Fishes with Epizootic Ulcerative Syndrome (EUS) in Southeast Asian Countries. Bull. Fac. Fish. Hokkaido Univ. 50(2).
14. *Janda J.M. and Abbott S.L.(2010):* The genus Aeromonas: taxonomy, pathogenicity and infection. *Clin. Microbiol. Rev*., 23, 35–73.
15. *John N. and Hatha A. A. M. (2013):* Distribution, Extracellular Virulence Factors and Drug Resistance of Motile Aeromonads in Fresh Water Ornamental Fishes and Associated Carriage Water. International J. of Aquaculture, Vol.3, No.17, 92-100http://ija.sophiapublisher.com.
16. *Karvonen A., Rintamkki P., Jokela J. &Valtonen E.T. (2010):* Increasing water temperature and disease risks in aquatic systems, climate change increases the risk of some, but not all, diseases. *International J. for Parasitology* 40, pp. 1483-1488.
17. *Lee* C., *Cho J.C., Lee S.H., Lee D.G. and Kim S.J. (2002):* Distribution of Aeromonas spp. as identified by 16S rDNA restriction fragment length polymorphism analysis in a trout farm. *J. Appl. Microbiol*. 93, 976-985.

18. *Ling T.K.W., Liu Z.K. and Cheng A.f.B. (2003):* Evaluation of the Vitek-2 system for rapid direct identification and susceptibility testing of gram-negative bacilli from positive blood cultures. J. of Clinical Microbiology, 41 (10): 4705-07.
19. *Lio-Po G. D., Pascual J. P. and Santos J. G. (1983):* Philippines. Fish Quarantine and fish diseases in Southeast Asia, report of a workshop held in Jakarta, Indonesia, 7–10 Dec. 1982.Ottawa Ont. IDRC,35–46.
20. *Majtán J., JaroslavČerny, AlenaOfúkaná, Peter Takáč and Milan Kozánek (2012):* Mortality of therapeutic fish *Garrarufa* caused by *Aeromonas sobria*Asian Pacific J. of Tropical Biomedicine 85-87.
21. *Marcogliese, D.J. (2008):* The impact of climate change on the parasites and infectious diseases of aquatic animals. *Revue Scientifique et Technique-Office International desEpizooties* 27, 467-484.
22. *Martin-Carnahan A. andJosephS.W. (2005):* Genus 1: Aeromonas stanier 1943, 213AL. In: Brenner, D.J.; Krieg, N.R. and Staley, J.T. (eds), Bergey,s Manual of systematic Bacteriology, 2nd edn, Vol. 2 the proteobacteria, part B, the Gammaproteobacteria. New York, Springer-Verlag, pp. 557-578.
23. *Miyazaki T. and Kaige N. (1985):* A histopathological study on motile aeromonad disease of *crucian carp*. Fish Pathology 21, 181-185.
24. *Noga E. J. (2010):* Fish Disease Diagnosis and Treatment. 2nd Edition,Blackwell Publishing 5-78.
25. *Patil C. Rajesh, Madhav. V. Upadhye, Varsha D. Kolatkar, Katchi V. I., Kulkarni G. J. and Shouche Y. S. (2011):* Isolation and identification of two new strains of Aeromonas spp. from diseased gourami fish and aquarium water. International J. of Life Sciences Volume 5 Issue 1: 25-31.
26. *RahmanM., Colque-Navaro P., Kuhn I., Huys G., Swings J. and Mollby R. (2002):* Identification and characterization of pathogenic *Aeromonas veronii* biovar sobria associated with epizootic ulcerative syndrome in fish in Bangladesh. Appl. Environ. Microbiol. 68, 650-655.
27. *RehilkaJ. (2002):* Aeromonas Causes Severe Skin Lesions in Rainbow Trout (*Oncorhynchus mykiss*): Clinical Pathology, Haematology and Bichemistry. Acta Vet. Brno 71: 351–360.
28. *Roberts* R.J. (2012): Fish Pathology Fourth Edition. by Blackwell Publishing Ltd. Page: 367-369.
29. *Schadich E, Cole AL.(2009):* Inhibition of frog antimicrobial peptides by extracellular products of the bacterial pathogen Aeromonashydrophila. LettApplMicrobiol; 49: 384-387.
30. *Sharma I., Kumar A. and Pramanik A.K. (2009):* Review of Techniques on Isolation and Identification of Aeromonads from the Food of Animal and Fish Origin Assam University. J. of Science&Technology: Biological Sci. Vol.4 Number I 73-85.
31. *Shayo S.D., Mwita C.J., Hosea K. (2012):* Ulcerative Aeromonas Infections in Tilapia (*Cichlidae: Tilapiini*) from Mtera Hydropower Dam, Tanzania. 1: 115. doi:10.4172/scientific reports.115
32. *Sreedharan K.,Philip R. and Bright Singh I.S. (2011):* Isolation and characterization of virulent *Aeromonas veronii* from ascitic fluid of Oscar *Astronotus ocellatus* showing signs of infectious dropsy. *Diseases of Aquatic Organisms* 94,29–39.
33. *Sugumar G., Karunasagar I. and Karunasagar I. (2002):* Mass mortality of fingerlings of *Labeo rohita*(Hamilton) in a nursery pond. Indian J. Fish.,49(3):305-309.
34. *Tam B., Gough W.A. & Tsuji L. (2011):* The impact of warming on the appearance of furunculosis in fish of the James Bay region, Quebec, Canada. *Regional Environmental Change* 11, pp. 123–132.
35. *Tidwe llJ. H and G. L Allan (2001)*: Fish as food: aquaculture's contribution Ecological and economic impacts and contributions of fish farming and capture fisheries Cell & Molecular Biology vol.2 issue 11.
36. *Wang G., Clark C. G., Liu C., Pucknell C., Munro C. K., Kruk T. M. A. C., Caldeira R., Woodward D. L. and Rodgers F. G. (2003):* Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR, *J. of Clinical Microbiology, 41(3)*, 1048-1054.
37. *Yu J.H., Han J.J., Kim H.J., Kang S.G. and ParkS.W. (2010):* First report of Aeromonas veronii infection in farmed Israeli carp *Cyprinuscarpio* in Korea. J.Fish Pathol., 23(2): 165-176.

4/26/2015