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PATHOGENICITY, SERUM RESISTANCE ACTIVITY AND SEROLOGICAL RELATEDNESS OF *PSEUDOMONAS FLUORESCENS* STRAINS RECOVERED FROM DISEASED *OREOCHROMIS NILOTICUS* IN EGYPT

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SUMMARY

Pseudomonas fluorescens (*Ps. fluorescens*) strains recovered from diseased Oreochromis niloticus (*O. niloticus*) were studied for their pathogenicity, serum resistance activities and serological relatedness. The results of experimental pathogenictiy in *O. niloticus* varied and generally ranged from acute septicaemic course to mild slow developing one or even none, suggesting the existence of variable virulence profiles among *Ps. fluorescens* strains. A correlation was found between serum resistance activity of *Ps. fluorescens* strains and their corresponding degrees of pathogenicity and virulence profiles as proved by the presence of a shared common resistance to the killing effect of *O. niloticus* serum among the highly pathogenic virulent strains. In addition, heterogenecity among *Ps. fluorescens* strains was biochemically and serologically proved.

INTRODUCTION

Ps. fluorescens is an opportunistic worldwide fish pathogen associated primarily with haemorrhagic septicaemia in cultured freshwater fishes and occasionally in marine and wild fishes (Roberts, 1989). However the organism may also enocountered as secondary infecting pathogen after primary viral or parasitic infection (Austin and Austin, 1989).

The pathogenesis of *Ps. fluorescens* infection in fish is multifactorial. A variety of factors belong to the host, environment and the pathogen itself work in concert to contribute to the overall course of the disease. Extracellular products appear to contribute to the establishment of *Ps. fluorescens* infections in fish (Post, 1987). However, the existence of serum resistance capability as a recorded virulence determinant for some other fish pathogens (Mittal et al., 1980; Janda et al., 1984) may also implicated in helping

Ps.fluorescens resist attack by the host's non-specific immunity such as killing.

The occurrance of variable courses of *Ps.fluorescens* infections in fish ranging from rapidly developing one in some instances to slow developing and even latent in others may agree with the recorded diversity of *Ps.fluorescens* strains and existence of environmental biotypes and miscellaneous strains (Krieg and Holt 1984 and Post 1987).

Therefore, this work was planned to characterize the pathogenicity of strains recovered from diseased *O. niloticus* in Egypt as well as to follow up serum resistance acitivities and serological relatedness.

MATERIAL AND METHODS

Fish:

A. Naturally infected fish:

Diseased *O. niloticus* showing signs of septicaemia, ascitis and fin rot were collected from some commercial fish farms and different aquaculture areas in Egypt . Fishes were subjected to clinical and postmortem examinations described by Austin and Austin (1989).

B. Fish for experimental purpose:

Healthy *O. niloticus* weighing approximately 70 ±10 g. were collected from a commercial fish farm and maintained in well-aerated water in glass aquaria at 22-25°C. All fish were fed commercial pellets at 1% body weight daily.

Recovery of Ps.fluorescens:

Isolation and identificantion of *P. Fluorescence* from diseased fish were carried out according to the methods described by Krieg and Holt (1984) and Lennette et al., (1985), using tryptic soy agar (TSA, Difco).

Pathogenicity of Ps.fluorescens strains:

Recovered Ps.fluorescens strains were tested for their experimental pathogenicity in *O. niloticus* as means of evaluating their virulence profiles. Tryptic soy broth (TSB) cultures of *Ps.fluorescens* strains were adjusted at a cell density of $1x10^7$/ml using plate count of colony forming units (CFU) according to lennette et al (1985) and separately inoculated into groups of 10 fish each at a dose of 0.2 ml/fish intraperitoneally. Experiments were monitored by continuous examination for clinical signs, lesions, mortalities and bacterial reisolation throughout 10 days experimental period.

Survival assay of Ps.fluorescens in *O. nitoticus* serum:

This assay was carried out on the bas s of Yancey et al., (1979) after modification. Briefly, serum was separated from blood of healthy *O. niloticus*. *Ps.fluorescens* strains grown in TSB at 25°C for 24 hours were collected by centrifugation and washed with PBS. The cell suspension was mixed

with serum to give a final serum concentration of 50% and the bacterial count was adjusted to $2x10^6$ CFU/ml . The mixtures were incubated at 25°C where 0.1 ml samples were removed after 2,5 and 8 hours for monitoring bacterial survival ability by viable plate counts on TSA. As a control, heat-inactivated serum at 56°C for 30 minutes (Sakai, 1981) was used . A ratio was calculated to represent the survival ability of *Ps.fluorescens* in fresh and heat-inactivated sera.

Preparation of *Ps. fluorescens* antisera and bacterial agglutination:

Selected *Ps.fluorescens* strains were grown in TSA and killed in 0.4% formalin-PBS solution. Antisera were prepared in *O. niloticus* where groups of 10 fish each were injected intraperitoneally twice at 0 and 4 weeks after the last injection. Agglutination titers were performed according to the method described by Roberson (1990).

RESULTS AND DISCUSSION

Naturally infected *O. niloticus* with *Ps.fluorescens* showed the presence of haemorrhagic skin and fin lesions in addition to occasional cases of ascitis, and fin rot externally (Fig 1). Internal visceral haemorrhages, congestion of internal organs ascitic accumulations and visceral adhesions were frequently seen. Variations in the intensity of these signs and lesions were also recorded. These results clinically reflected the presence of acute septicaemic and chronic courses of *Ps.fluorescens* infections as recorded by Bullock et al., (1971), Abdel Aziz (1988). Roberts (1989) and Inglis (1993).

Bacteriological examination of naturally infected fish resulted in the identification of twelve *Ps.fluorescens* strains closely related to three biotypes as recorded by Krieg and Holt (1984). These biotypes and their designated related strains were as follows:

Biotype 1. : included strains No. P1, P2, P4, P7 and P11.

Biotype IV. : included strains No. P5, P8.

Biotype V. : included strains No. P3, P9, P10 and P12.

The existence of different biotypes of *Ps. fluorescens* supported the findings of Lennette et al., (1985), who recorded the presence of seven biovars of Ps.fluorescens and Post (1987), who mentioned the presence of four recognized biotypes and several miscellaneous strains of *Ps. fluorescens* associated with different courses of disease conditions in fish.

Experimental induction of *Ps. fluorescens* infections in *O. niloticus* resulted in the expression of variable extents of clinical signs, lesions and mortalities or even none that could be grouped in relation to the inoculated strains as follows:

- Strains P2, P7, P11 and P12 induced 100% mortalities within 24-48 hours post inoculation with the development of sluggish movement, loss of balance and reflexes as well as abdominal dropsy (Fig. 2).Internal congestion and haemorrhages of visceral organs and variable amounts of reddish serous ascitic fluid were mostly seen (Fig. 3) . These results supported those of Bullock et al., (1971) and Roberts (1989) regarding the ascitic form of *P. fluorescens* infection. These strains could be classified as virulent on the basis of Reed and Muench (1938) and Hahnel et al., (1983).

Table (1): Serum survival ability of recovered *Ps.fluorescens* strains

Strain No.	2 hours		5 hours		8 hours	
	CFU ml-1	Serum survival ability*	CFU ml-1	Serum survival ability	CFU ml-1	Serum survival ability
P1	4.72 x10^5	0.2360	2.91 x10^5	0.1455	2.32 x10^5	0.1160
P2	2.41 x10^6	1.2050	3.62 x10^6	1.8100	4.13 x10^6	2.0650
P3	2.94 x10^5	0.1470	1.73 x10^5	0.0865	1.42 x10^5	0.0710
P4	5.36 x10^3	0.0027	3.12 x10^3	0.0016	1.94 x10^3	0.0010
P5	2.02 x10^4	0.0101	1.68 x10^4	0.0084	1.12 x10^4	0.0056
P6	2.86 x10^4	0.0143	1.93 x10^4	0.0097	1.62 x10^4	0.0081
P7	2.63 x10^6	1.3150	3.28 x10^6	1.6400	3.86 x10^6	1.9300
P8	1.22 x10^5	0.0610	7.32 x10^4	0.0366	7.10 x10^4	0.0355
P9	4.12 x10^3	0.0021	2.81 x10^3	0.0014	1.62 x10^3	0.0008
P10	1.80 x10^5	0.0900	1.27 x10^5	0.0635	1.08 x10^5	0.0540
P11	2.87 x10^6	1.4350	4.11 x10^6	2.0550	5.20 x10^6	2.6000
P12	2.19 x10^6	1.0950	3.94 x10^6	1.9700	4.72 x10^6	2.3600

* Values of serum survival ability was calculated by dividing CFU counts at the given incubation time points by the initial CFU count before incubation.

Strains P1, P3, P5, P6, P8 and P10 induced 30-70% mortalities within 72-144 hours post injection where infected fish developed darkening in colour, haemorrhagic skin and fin lesions and abdominal dropsy when necropsied, there were petechiation of the peritoneum, visceral organs and musculature as well as occasional visceral adhesions and purulent ascitic accumulations. The intensity of these signs and lesions showed considerable variations among infected fish . These results supported those of Peterinec et al., (1985) who recorded Ps. fluorescens speticaemia in naturally and experimentally infected bighead carp characterized by external and internal haemorrhages. However the recorded visceral adhesion may result from the organization of ascitic fluid with subsequent deposition of fibrin, a characteristic lesion associated with chronic type of Ps.fluorescens infections as recorded by Roberts (1989). These strains could be classified as moderately to weak virulent on the bases of Reed and Muench (1938) and Hahnel et al., (1983).

Strains P4 and P9 induced no mortalities and asymptomatic form of infection inspite of the successful recovery of the inoculated strains from internal organs of inoculated fish. Latent form of *P. fluorescens* infection was previously recorded by Ahne et al., (1982) who found that additional environmental stressor is required for the exaggeration of latent infection in tench fry. These strains could be classified as avirulent on the bases of Reed and Muench (1938) and Hahnel et al., (1983).

The experimental expression of wide range of *Ps.fluorescens* infections strongly supported the existed heterogenecity among *Ps.fluorescens* strains infecting fish in this study.

Regarding serum resistance acitivities of *Ps. fluorescens strains* Table (1) shows that all the four virulent strains (P2, P7, P11 and P12) were resistant to serum killing. These strains did not only survive in the fresh *O. niloticus* serum, but also grew in it as indicated by the increased counts of CFU. On the other hand CFU counts and their corresponding serum resistance activity values of moderately and weak virulent strains were progressively decreased. While the avirulent strains (P4 and P9) Developed serum hypersensitivity. These results may reflect the critical role played by serum resistance activities of *Ps.fluorescens* strains during the pathogenisis of their corresponding infections in fish . For all heat inactivating fresh serum (Table 2). Since the serum killing factor was heat-labile, it may be linked with complement as suggested by Yancey et al., (1979) and Saki (1981).

Table (2): Serum survival ability of recovered *Ps.fluorescens* strains in heat inactivated serum

Strain No.	2 hours		5 hours		8 hours	
	CFU ml-1	Serum survival ability*	CFU ml^{-1}	Serum survival ability	CFU ml^{-1}	Serum survival ability
P1	2.74 x10^6	1.370	4.02 x10^6	2.010	7.32 x10^6	3.660
P2	3.14 x10^6	1.570	5.21 x10^6	2.605	7.43 x10^6	3.715
P3	2.50 x10^6	1.250	3.26 x10^6	1.630	5.01 x10^6	2.505
P4	2.63 x10^6	1.315	3.32 x10^6	1.660	4.21 x10^6	3.105
P5	2.44 x10^6	1.245	3.70 x10^6	1.850	5.09 x10^6	2.545
P6	3.02 x10^6	1.510	5.10 x10^6	2.550	8.21 x10^6	4.105
P7	3.42 x10^6	1.710	5.83 x10^6	2.915	8.16 x10^6	4.080
P8	2.83 x10^6	1.415	4.25 x10^6	2.125	5.91 x10^6	2.955
P9	2.79 x10^6	1.395	3.64 x10^6	1.820	4.38 x10^6	2.190
P10	2.92 x10^6	1.460	4.66 x10^6	2.330	6.71 x10^6	3.355
P11	4.11 x10^6	2.055	6.24 x10^6	3.120	8.92 x10^6	4.460
P12	3.90 x10^6	1.950	5.73 x10^6	2.865	7.54 x10^6	3.770

Table (3): Serum agglutination titers raised against *Ps.fluorescens* strains

Antiserum / Antigen	Serum agglutintion titer*					
	P2	P6	P7	P9	P11	P12
P1	2	2	4	0	4	8
P2	4096	32	1024	8	2048	16
P3	4	4	2	2	0	4
P4	2	4	4	4	0	2
P5	128	32	32	64	2	32
P6	32	512	4	4	8	8
P7	1024	4	4096	0	512	16
P8	2	0	2	4	0	2
P9	4	2	2	32	2	4
P10	128	4	4	256	64	8
P11	2048	8	512	0	4096	16
P12	32	4	16	2	32	2048

* Reciprocal of the last dilution showing agglutination

Many authors have reviewed the characterization of virulence status of bacterial strains in-relation to the existence of some other virulence factors as as protease, haemolysins, enterotoxins, cytotoxins and others (Allan and Stevenson, 1981), however Kou (1973) and Olivier et al., (1985) recorded that the assessment of virulence status of virulent, attenuated and avirulent strains is related to the quantity of the produced toxins and not to this qualitative disposition. Meanwhile, the results of serum resistance activity recorded in this study suggested the usefulness of using such property for the assessment of the virulence profiles *P. fluorescens* strains.

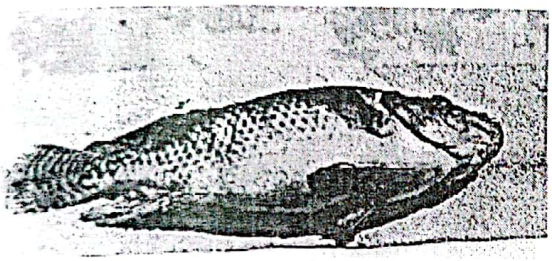

Fig. (1): Naturally infected O. niloticus showing haemorrhagic skin and fin lesions, tail rot and abdominal dropsy.

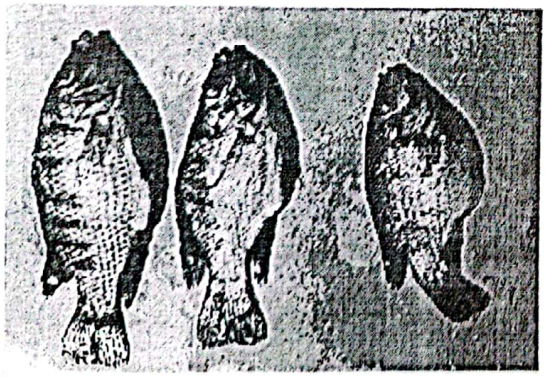

Fig. (2): Experimentally infected O. niloticus showing variable degrees of abdominal dropsy.

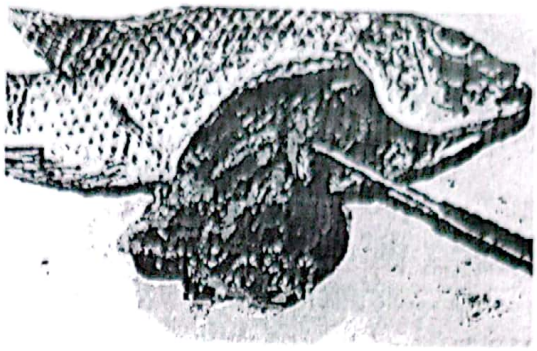

Fig. (3): Experimentally infected O. niloticus showing haemorrhagic septicaemia of visceral organs.

Serological relationship was used to group the 12 *Ps.fluorescens* strains. Antibodies were raised against the four virulent strains (P2, P7, P11 and P12), the moderately virulent strains (P6) and one avirulent strain (P9). Variations in agglutinating titers and cross reactivities as shown in table (3) suggested the presence of different serogroups and existence of heterogenicity among. *Ps. fluorescens* strains. Virulent strains could not be typed into one distinct group, however three of them (P2, P7 and P11) could be typed in a common serogroup. The antigenic relationship shared by these three virulent strains is consistent with their biotypic class, pathogenecity and serum resistance activities.

REFERENCES

Abdel-Aziz, E. S. (1988): Some studies on bacterial agents causing tail and fin rot among freshwater fishes in Egypt. M. V. Sc. thesis, Dept. Infectious diseases, Fac. Vet. Med., Cairo University.

Ahne, W.; Popp, W. and Hoffmann, R. (1982):

Pseudomonas fluorescens as a pathogen for Tench (Tinca tinca). Bull. Eur. Ass. Fish Path. 2, 36-57.

Allan B. J. and Stevenson, R. M. W. (1981): Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. Can. J. Microbiol. 27-1114-1122.

Austin, B. and Austin, D. A. (1989):Methods for the microbiological examination of fish and shell fish. Ellis Horwood Limited, England.

Bullock, G. L.; Conroy, D. A. and Snieszko, S. F. (1971): Bacterial diseases of fishes. In : Diseases of fishes, S. F. Snieszko and H. R. Axelrod (eds), T. F. H. Publications, Inc., Neptune, NJ, USA

Halmel, G. B.; Gould, R. W. and Boatman, E. S. (1983): Serological comparison of selected isolates of *Aeromonas salmonicida spp. salmonicida.* J. Fish diseases, 6, 1-11.

Inglis, V.; Roberts, R. J. and Bromage, N. (1993): Bacterial diseases of fish Blackwell Scientific Publications, Oxford, London, Edinburgh, Boston, Ch. 10: 169-174.

Janda, J. M.; Brenden, R. and Bottone, E. J. (1984): Differential susceptibility to human serum by *Aeromonas spp.* Current Microbiology. 11, 325-328.

Kou, G. H. (1973): Studies on the fish pathogen *Aeromonas liquefaciens.* II: The connections between pathogenic properties and the activites of toxic substances. J. Fish. Soc. Taiwan, 2 (1), 42-46.

Krieg, N. R. and Holt, J. G. (1984): Bergey's Manual of Systematic Bacteriology, Vol. I, Williams and Wilkins, Baltimore, M. D, USA.

Lennette, E. H.; Balows, A.; Hausler, W. J. Jr. and Shadomy, H. J. (1985): Manual of Clinical Microbiology (4th edition). American Society for Microbiology, Washington, D. C., USA.

Mittal, K. R.; Lalonde, G.; Leblance, D.; Olivier, G. and Lallier, R. (1980): *Aeromonas hydrophila* in rainbow trout : Relation between virulence and surface characteristics. Canadian J. Microbiology , 26, 1501-1503.

Olivier, G.; Evelyn, T. P. and Lallier, P. (1985): Immunogenecity of vaccines from a virulent and an avirulent strain of *Aeromonas salmonicida.* J. Fish diseases 8, 43-55.

Peterines, Z.; Naglic, T.; Matasin, Z. and Fijan, N. (1985):*Pseudomonas fluorescens* septicaemia in bighead carp (*Aristichys nobilis Rich*)), following hadnling. Veterinarski Archiv, 55 (6), 277-284.

Post, G. (1987): Textbook of Fish Health. T. F. H. Publications, inc. for revised and expanded edition.

Reed, L. J. and Meunch, H. (1938): A simple method of estimating fifty percent end points. The American J. of Hygiene, 27, 493-497.

Roberson, B. S. (1990): Bacterial agglutination. In: Techniques in fish Immunology (ed. By J. S. Stolen; T. C. Fletcher, D. P. Anderson, B. S. Roberson and W. B. Van Muswinkel), pp. 81-86. SoS Publications, Fair Haven. NJ. USA.

Roberts, R. T. (1989): Fish pathology. 2nd Ed., Bailliere Tindall, London.

Sakai, D. K. (1981)L Heat inactivation of complements and immune haemolysis reactions in rainbow trout, masu salmon, coho salmon, gold fish and tilapia. Bull. of the Japnese Society of Scientific Fisheries 47, 565-571.

Yancey, R. J.; Breeding, S. A. L. and Lankford, C. E. (1979): Enterochelin (Enterobactin): Virulence factor for *Salmonella typhimurium.* Infection and immunity. 24 (1): 174-180.

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SOME BIOCHEMICAL CHANGES IN HAEMOLYSATE OF CLARIAS LAZERA FOLLOWING EXPOSURE TO COPPER SULPHATE APPLICATION IN A RICE FARM IN SHARKIA GOVERNORATE.

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SUMMARY

Reared Nile catfish, *Clarias lazera (C.lazera)*, in a rice farm infested with rice scum and treated by copper sulphate (Cu So4) in concentration of 2.5 mg/l water showed different biochemical deviations in their red blood cells (haemolysate).

This haemolysate presented significant decreases at ($p < 0.05$, $P<0.01$ and $P<0.001$) in mineral levels of calcium (Ca), inorganic phosphorus (P) and potassium (K) at 2nd, 4th, 6th, 8th and 10th days of exposure. Sodium (Na) levels were significantly increased at ($P<0.05$ and $P< 0.01$) and levels of total protein and total lipids monitored no significant changes in haemolysate after the same periods of exposure to Cu So4.

Plasma of the same fishes showed significant elevations ($p < 0.05$, $P<0.01$ and $P<0.001$) in levels of (Ca), (P) and (Na), while (K) levels had significant declines at($P <0.01$) at the same intervals of sampling .

K/Na ratios in haemolysate and plasma of C.lazera were markedly decreased allover the period of treatment.

INTRODUCTION

In Egypt, over a million faddans are planted with rice annually and many of these faddans are used for rearing some fish spp. The Nile catfish, Clarias lazera, is one of fish species founded in rice farm and it is of commercial interest and represents an important cheep protein food source for the population and for the almost of poor people.

Rice scum is considered an important wide spread rice disease in Egypt causing several hazards for the crop and severed it during June and July . This disease is caused by Spirogyra spp. of algae, Class: Chlorophyceal (Ibrahim et al., 1974).

Cu So4 is the most effective chemical compound used as algacide for control of rice scum, where, the effect of CuSo4 on algae enclosures is through impairment of a large number of physiological reactions in these algae by copper free ion (Cu) activity (Gustavson and Wangberg, 1995).

Exposure of C.lazera to CuSo4 hazards in rice fields may constitute one of the most important factors of pollution of fish with consequent for loss of good source of animal protein. The presence of CuSo4 in rice fields linked to events as adverse effects on fish cultured in this farm as one of the most seriously limiting factor in aquaculture.

The present research was carried out to clarify the effect of CuSo4 applied in a rice field on some biochemical parameters in the red blood cells(haemolysate)of C.lazera reared in this farm.

MATERIALS AND METHODS

Collection of fish samples: C.lazera samples of this study were collected during season 1998 from a private rice farm, Bilbias, Sharkia Governorate. This Nile catfish,C.lazera, was chosen due to their capability of tolerating shallow water, high temperature (up to 35°C), low dissolved oxygen and high turbidity.

At the end of July, scum was prevalent in the rice fields, then CuSo4 as effective and successful chemical treatment was applied in rate of 1.5 kg CuSo4 / fadden to reach concentration of 2.5 mg/ l. CuSo4 was put in texture bag at water inlet during irregation for flooding to about 15 Cm of water hight.

Exposed fish groups (E) were randomly trapped off after CuSo4 introduced into the rice field with 2,4,6,8,10 days respectively. From a neighbor untreated rice field with the same conditions of scum infestion , control fish groups (C) were taken at the same periods of collection of exposed fish groups. After collection of the last groups of fish, irrigation of rice farm with CuSo4 free water was carried out to keep the rate of flooding after treatment.

Each (E) and (C)fish group was 6 fish in number which were alive , apparently healthy and sexually immature with body weight and total length were ranged between 150-200 g and 20-25 Cm respectively.

Collection of blood samples : Blood samples from catched fish were immediatly collected into heperinized tubes by tail amputation as described by (Lied et al., 1975). Blood tubes were kept on

ice till centrifuged through two hours of collection at 3000 rpm for 15 min for separation of plasma which were transferred to small vials and stored in a deep freez at - 20°C until using.

Preparation of haemolysate: 0.2 ml of the remained red cells (as sediment after deconting the plasma and buffy coat) was washed and freed of leukocytes and platelets in centrifuge tube 3times with 2ml of an ice cold isotonic aliquots of 0.65% buffer saline solution (NaCl) (wolf, 1963). Centrifugation after each washing was carried out for 10 min at 3000 rpm, where, suspension of erythrocytes was free from leukocytes and platelets. The suspended and washed erythrocytes were centrifuged in 2 ml deionized bidistaled water for 15 min at 4°C to obtain the haemolysate which was frozen and thawed twice. 0.1 ml of this haemolysate is used for assay of parameters.

Biochemical assay :

By using kits, total protein, total lipids, calcium and inorganic phosphorus in haemolysate are determined according to Doumas et al. (1981); Schmit (1964); Weissman and Pilleggi (1974) and Tietz (1970) respectively.

Na+and K+ were estimated by using flame photometer according to Varley et al. (1980).

Statistical analysis :It is done by using "t" test according to Snedecor and Cochran (1967).

RESULTS

The field oibvious clinical symptoms appeared on C.lazera were recognized as stimulation of fish activity and movement at early stages of CuSo4 application, consequently decreased later. Also, typical patho-anatomic appearance including a large amount of mucus on body surface, under gill covers and on the gills, while the skin appeared darker in colour by time.

As shown in tables (1) and (2), the levels of (Na) and (K) were significantly increased ($p < 0.05$, $P<0.01$ and $P<0.001$) and significantly decreased ($p < 0.05$, $P<0.01$ and $P<0.001$) respectively in haemolysate and plasma of C.lazera after 2,4,6,8 and 10 days of exposure to CuSo4 in the rice farm infested with rice scum. The levels of (Ca) and (P) monitored significant decreases ($p < 0.05$, $P<0.01$ and $P<0.001$) in haemolysate and significant increases ($P<0.01$ and $P<0.001$) in plasma along the whole period of exposure.

K/Na ratios in haemolysate and plasma of C.lazera of this study were markedly decreased as indicated in table (3) allover the exposure times.

Non significant changes in the levels of total lipids and total protein in haemolysate of C.lazera were detected at the same intervals of sampling as demonstrated in table (4).

Table (1): Effect of Cu So_4 exposure on haemolysate electrolytes in C.lazera.

Period exposure / Parameter	2days		4days		6 days		8 days		10 days	
	C	E	C	E	C	E	C	E	C	E
Ca++ mg/dl	16.00±1.054	11.00±1.971**	16.500±1.148	7.833±0.548***	17.166±1.065	10.833±0.863**	16.833±1.038	12.666±0.652**	16.666±0.805	13.50±0.697*
PI++ mg/dl	21.500±1.124	14.666±1.262**	21.833±0.925	11.00±1.130***	23.166±1.065	12.666±1.262***	23.333±1.262	16.833±1.140***	21.833±0.983	17.50±1.263*
Na+ mEq/l	14.500±1.467	18.666±1.217*	15.00±0.781	21.666±1.677**	15.500±1.148	22.833±1.862**	15.333±1.367	19.333±1.305**	15.333±1.018	19.333±1.990*
K+ mEq/l	18.833±1.038	13.833±0.796**	18.333±0.769	13.500±1.263**	19.333±0.962	15.666±0.962**	18.500±0.772	13.00±0.971**	18.166±0.863	13.666±0.962**

E = Each value represents the mean ± SE (Standard error of mean). C = Control group and E = exposed group. * and (** & ***) indicate a significant and a highly significant difference at P<0.05 and (P<0.01 & P<o.001) respectively.

Table (2): Effect of Cu So_4 exposure on plasma electrolytes in C.lazera.

Period exposure / Parameter	2days		4days		6 days		8 days		10 days	
	C	E	C	E	C	E	C	E	C	E
Ca++ mg/dl	5.000±1.105	6.483±0.221***	4.970±0.096	7.250±0.223***	5.050±0.107	7.900±0.270***	4.983±0.086	7.716±0.363***	5.020±0.089	7.550±0.313***
PI++ mg/dl	6.700±0.185	8.233±0.315**	6.683±0.079	8.850±0.407**	6.883±0.098	9.733±0.432***	6.883±0.114	9.550±0.344***	6.670±0.121	8.966±0.426**
Na+ mEq/l	133.667±2.950	148.833±2.291**	135.833±1.640	153.667±1.694***	134.167±1.935	157.667±2.873***	135.667±2.567	145.167±2.543*	132.00±1.810	137.500±1.350*
K+ mEq/l	6.00±0.276	5.233±0.177*	6.300±0.164	5.166±0.177**	6.420±0.172	5.150±0.189**	6.083±0.246	4.950±0.211**	6.00±0.227	4.783±134**

E = Each value represents the mean ± SE (Standard error of mean). C = Control group and E = exposed group. * and (** & ***) indicate a significant and a highly significant difference at P<0.05 and (P<0.01 & P<o.001) respectively.

Table (3): Effect of Cu So_4 exposure on K+/Na+ ratios in haemolysate and plasma of C.lazera.

Period exposure / Parameter	2days		4days		6 days		8 days		10 days	
	C	E	C	E	C	E	C	E	C	E
Haemolysate K+/Na+	1.298	0.741	1.222	0.623	1.247	0.686	1.206	1.222	1.184	0.706
Plasma K+/Na+	0.045	0.035	0.046	0.033	0.047	0.032	0.044	0.046	0.045	0.034

C = Control group.
E = Exposed group.

Table (4): Effect of Cu So_4 exposure on haemolysate total protein and total lipids levels in C.lazera.

Period exposure / Parameter	2days		4days		6 days		8 days		10 days	
	C	E	C	E	C	E	C	E	C	E
Total protein g/dl	7.500± 0.697	9.333± 0.561	8.000± 0.849	8.500± 0.874	8.166± 0.723	7.666± 0.805	8.333± 0.561	8.500± 0.874	7.833± 0.723	8.666 ±0.652
Total lipids g/L	110.000 ±9.718	129.166 ±8.450	113.333 ±8.050	118.333 ±8.871	119.166 ±8.773	100.00± 7.905	114.166 ±7.765	95.833 ±9.548	115.00 ±8.249	99.166 ±4.620

E = Each value represents the mean ± SE (Standard error of mean)
C = Control group.
E = Exposed group.

All above results were in comparing with controls which were taken at the same times of sampling and at the same conditions of the rice scum without CuSo4 exposure. Haemolysate of controls showed no significant changes in the same detected parameters in response to ambient hypoxia created by rice scum which was present in the field of controls. This may be due to adaptation and capability of C.lazera to overcome this field hypoxia as a result of presence of air breathing accessory apparatus in these species of fish.

DISCUSSION

First, increased concentration of heavy metal in freshwater can lead to disturbances in ion regulation and oxygen carrying capacity in fishes founded in this water as concluded by Giles (1984). Infestion of rice with scum makes the water in the rice field hypoxic (Ibrahim et al., 1974).

Secondly, the levels of (Cu) concentrations in gills and skin are significantly higher than in other organs as stated by Wafica and Sohair (1996) in Tilapia zilli. This may be due to the process of filtration of water against the gills and taking up of the metals through the body surface.

Thirdly, Bryan (1976) found that the ions taken up from ambient water is considered to be passive and envolved in diffusion down gradient created by adsorption or binding of the ions to the tissue and cell surfaces.

The obtained results of the present study reflected the reaction of C.lazera for recognition of the presence of high concentration of CuSo4 (2.5 mg / L) and hypoxic water due to rice scum which are leading to a heavy stress condition.

The secondary stress responses which affect water permeability of the branchial epithelium due to action of catecholamines in addition to the direct effects on the branchial ion movements leading to impaired electrolytes status as reported by Mazeaud et al. (1977). This explanation was in agreement with exposure of rainbow trout to high concentration of (Cu) to show histological alterations in the gills (Baker, 1969).

The structural damage of the gills could illustrate the increased ionic permeability of cell membranes and reduction in transport function causing ionoregulatory and (Na) fluxes disturbances as investigated in rainbow trout exposed to Cu (laurén and Mc Donald, 1985 ;Mc Donald et al., 1989; Wilson and Taylor , 1993). These disturbances were probably due to displacement of (Ca) intercellular tight junctions (Laurén, 1991). Accumulation of (Cu) in the plasma of flounder, Platichthy fleus, (Stagg and Shuttleworth, 1982b) and chinook salmon, Oncorhynchus tshawytscha, (Beckman and Zaugg, 1988) when these fishes are exposed to sublethal concentrations of (Cu) show high affinity of this (Cu) for SH-groups of transport enzymes such as Na+ / K+ -ATPase. This enzyme is an important component of current metals

and ions of active transport in teleost gills (Evans, 1980), where branchial (Na) uptake is the result of Na+ / K+ ATPase depended (Na) fluxes (Mc Donald et al., 1989).

As well accumulation of (Cu) in plasma due to the rate of (Cu) uptake is greater than that of excretion, as the red cells show decreased activity of K+ / Na+ pump, which was estimated by changes in (Na+),(K+) and (Cl-) values and resultant depletion of ATP stores (Wood, 1992). Then, Cu So4 in addition to hypoxic environment in the present study can induce sharp disturbances in ion fluxes in C.lazera leading to significant changes in electrolytes values in red blood cells as well as in plasma. This is supported by studies of Fuchs and Albers (1988) on carp, Cyprinus carpio, and Nikinmaa and Weber (1984) on lampry, Lampetra fluviatilus, which show deviations of the red cell intracellular (K+), (Na+) and (Cl-) levels on exposure to (Cu).

As well,the regulation of blood (Na) in teleosts was founded in several cell types including erythrocytes as the permeability of these cells dominating external ions (Na+) increases which support the volume regulatory process (Kregenow, 1971; Assem and Hanke, 1979).

The branchial mechanisms regulating (Na+) and (Ca++) influxes are distinctly different from one to another in hormonal control, ion specific channels or carriers in the apical membrane of the ion transporting cells (Flik et al., 1985; MC Donald et al., 1989).

A small and transient decrease in net (Ca++) uptake was founded in brown trout exposed to Cu (Sayer et al., 1991), where, the influx rates of (Ca++) are usually substainally lower than those of (Na+) as observed in rainbow trout under the effect of Cu (Reid and MC Donald, 1988).

The physiology of (Ca) and (P) are tied together in a common system (Guyton, 1991) andtherefore the levels of (P) in haemolysate and plasma of C.lazera of the present study are exactly deviated in a manner similar to that of (Ca).

Values of total protein and total lipids in haemolysate are not markedly affected. This may be due to that the molecules of these parameters are larger than that of electrolytes which show sharp deviated levels due to impaired red cell membrane permeability.

In studies of Pelgrom et al. (1995) on mature Oreochromis mossambicus and Ghazaly & Said (1995) on Oreochromis niloticus, the results were in opposite direction to those of the present study. This may be due to

(1) difference of fish spp., (2)presence of rice scum which causes water to be hypoxic, and (3) high concentration of CuSo4 (2.5 mg /L) in this study in comparing with that of the other authors,

200 ug / L and 1.14 ug /L respectively as a range of sublethal concentrations.

Therefore the results of the present study may be logical and in harmony with the results of all previous presented studies and their explanations for impairment of ionic equilibrium between red blood cells, plasma and gills of C.lazera in response to the complex reaction of high concentration of CuSo4 and rice scum of this study. This impairment of ionic equilibrium can be illustrated as investigated by Stagg and Shuttleworth (1982a) through affecting the following factors: (1) Permeability of cell membranes including those of erythrocytes and gill epithelium, (2) hormonal controling energy producing processes on which the active transport system depends, (3) transfere of electrolytes from or into one another body compartement, and (4) the water balance of the rice field may produce changes in ion composition which was interpreted as an action of (Cu) on water permeability.

REFFRENCES

Assem, H.and Hanke, W. (1979) : Volume regulation of muscle cells in the euryhaline teleost (Tilapia mossambica). Comp. Biochem. Physiol . 64 A : 17 - 24.

Baker, J.T.P.(1969): Histological and electron microscopical observations on copper poisoning in the winter flounder (Pseudopleuronectes americanus). J.Fish. Res. Bd, Can. 26, 2775 -2783.

Beckman, B.R. and Zaugg, W.S.(1988): Copper intoxication in chinook salmon (Oncorhynchus tshawytscha) induced by natural springwater: effects on gill Na+ / K+ ATPase, hematocrit and plasma glucose. Can . J. Fish. Aquat. Sci. 45 : 1430 -1435.

Bryan, G.W. (1976): Some aspects of heavy metal tolerance in aquatic organisms. In : Effects of Pollutants on Aquatic Organisms (Lockwood, A.P.M.ed.). PP. 7-34. Cambridge : Cambridge University Press.

Doumas, B.T.; Bayse, D.D.; Carter, R.J.; Peters, T.and Schaffer, R. (1981): Determination of total protein. "Clin Chem" 27 : 1642.

Evans, D.H.(1980): Kinetic studies of ion transport by fish gill epithelium. Am. J. Physiol. 238 , R224-R230.

Flik, G.;van Rijs, T.H.and Bonga, S.E.W.(1985): Evidence for high affinity Ca2+ -ATPase activity and ATPase driven Ca+2 -transport in membrane preparations of the gill epithelium of the cichlid fish (Oreochromis mossambicus). J.Exp. Biol. 119 : 335-347.

Fuchs, D.A. and Albers, C.(1988): Effect of adrenaline and blood gas conditions on red cell volume and intraerythrocytic electrolytes in the carp (Cyprinus Carpio). J.exp. Biol. 137 : 457-477.

Ghazaly, K.S. and Said, K.M. (1995): Physiological characteristics of Tilapia nilotica under acute stress of copper. J.Egypt. Ger. Soc. Zool. Vol. 16 (A), Comp. Physiol., 287-301.

Giles, M.A. (1984): Electrolyte and water balance in plasma and urine of rainbow trout (Salmo gairdneri) during chronic exposure to cadmium. Can. J. Fish. Aquat. Sci., 41: 1678-1685.

Gustavson, K. and Wangberg, S. A. (1995): Tolerance induction and succession in microalgae communities exposed to copper and atrazine. Aquatic Toxicology 32:283-302.

Guyton, A.C. (1991): "Calcium and phosphate in the extracellular fluid and plasma". In "Textbook of Medical Physiology", 8th ed., chapter 79, PP.868. W.B.Saunders Company, Philadelphia, London, Tokyo.

Ibrahim, A.E., El-Arosy, H., Mekhaeal, S. and Abd-El-Rahim, A.M. (1974): Algae and Diseases Caused by Them: In "Plant Diseases". PP. 385-387. (In arabic language). Dar El Matboat El-Gadida, Egypt.

Kregenow, F.M. (1971): The response of duck erythrocytes to nonhemolytichy potonic media : Evidence for a volume - controlling mechanism. J.Gen. Physiol. 58:372-395.

Laurén,D.J. (1991): The fish gill: a sensitive target for water borne pollutants. In : Aquatic Toxicalogy and Risk Assessment, 14 th vol. STP. 1124 (Mayes, M.A. & Barron, M.G., eds), PP. 223-244 Philadelphia: American Society for Testing and Materials.

Laurén, D.J. and Mc Donald, D.G. (1985): Effects of copper on branchial ionoregulation in the rainbow trout (Salmo gairdneri Richardson). J. Comp. Physiol. 155(B): 635-644.

Lied, E.; Gzerde, Z. and Braskhan, O.R. (1975): Simple and rapid technique for repeated blood sampling in rainbow trout. J. Fish . Res. Board of Canada, 32 (5): 699-701.

Mazeaud, M.M.; Mazeaud, F. and Donaldson, E.M. (1977): Primary and secondary effects of stress in fish : some new data with a general review. Trans. Am. Fish . Soc. 106, 201-212.

McDonald, D.G. Reader, J.P. and Dalziel, T.R.K. (1989): The combined effects of pH and trace metals on fish ionoregulation. In : Acid Toxicity and Aquatic Animals. Soc. Exp. Biol Semin. Ser. Vol. 31, edited by Morris, R.; Taylor, E.W. and Brown, J.A. Cambridge Unisesity Press. PP. 221-242.

Nikinmaa, M. and Weber, R.E. (1984):Hypoxic acclimation in the lamprey (Lampetra fluviatilis) organismic and erythrocytic responses. J. exp. Biol. 109: 109-119.

Pelgrom, S.M. G.J.; Lock, R.A.C.; Balm, P.H.M. and Bonga, S.E.W. (1995): Integrated physiological response of tilapia (Oreochromis mossambicus) to sublethal copper exposure. Aquatic Toxicology 32 : 303-320.

Reid, S.D. and McDonald, D.G. (1988): Effects of cadmuim, copper and low pH on ion fluxes in the rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci. 45: 244-253.

Sayer, M.D.J.; Reader, J.P. and Morris, R. (1991): Effects of six trace metals on calcium flux in brown trout (Salmo trutta) in soft water. J. Comp. physiol. 161 (B) : 537-542.

Schmit, J.M. (1964): Determination of total lipids. Thése, Lyon, France.

Snedecor, G.W. and Cochran, w. (1967): Statistical methods. 6th. ed., Iowa State Univ. Press. Ames., Iowa, U.S.A.

Stagg, R.M. and Shuttleworth, T. J. (1982a): The accumulation of copper in Platichthys flesus L. and its effects on plasma electrolyte concentrations. J. Fish Biol., 20: 491-500.

Stagg, R.M. and Shuttleworth, T. J. (1982b): The effects of copper on ionic regulation by the gills of the seawater - adapted flounder (Platichthys flesus). J. Comp. Physiol. 149: 83-90.

Tietz, N.W. (1970): Fundamentals of Clinical Chemistry. W.B. Saunders, Philadelphia.

Varley.H.; Gweabck, A.H. and Bell, M. (1980): "Practical cliniel chemistry, Bol. 1, General Tropical Commoner Test. 5 th Ed. London, William Heinemann Medical Books LTd.

Wafica M.A. Naga and Sohair M. Allam (1996): Heavy metal concentrations in the tissues of Tilapia zilli GERV, exposed to waste water discharge of Egyptian copper factory. J.Egypt. Ger. Soc. Zool., Vol. 19 (A), Comparative Physiology. 21-35.

Weissman, N. and Pilleggi, V.J. (1974): In clinical chemistry principles and Techinics. 2 nd R. H. Henry et al. Eds. Harper, Row, Hagerstown, M.D. PP. 646-669.

Wilson, R.W. and Taylor, E.W. (1993): The physiological responses of freshwater rainbow trout (Oncorhynchus mykiss) during acutely lethal copper exposure. J. Comp. Physiol. 163 (B): 38-47.

Wolf, K. (1963): Physiological salines for freshwater teleosts. Prog. Fish. Cult. 25: 135-140.

Wood, C.M. (1992): Flux measurements as indices of H+ and Metal effects on freshwater fish. Aquat. Toxical . 22 : 239-264.