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Original Article

Isolation and Characterization of Some Pathogenic Agents Causing Wobbling Syndrome in Cultured *Oreochromis niloticus*.

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Abstract

It is the first study all over Egypt which is dealing with fish swimming abnormalities influenced by mixed pathogens. A total of 160 *Oreochromis niloticus* were collected from some private fish farms at Sharkia governorate during the period from January to December 2008. Fish through isolation and characterization of some pathogenic agents were classified according to these groups: - A-Normal group: Free from any pathogenic agent. B-Bacterial infected group: Four bacterial isolates recovered from gas bladder affection of *O.niloticus* were *Flavobacterium columnare, Pseudomonas fluorescens, Vibrio harveyi,* and *Staph aureus*. Fish infected with bacteria showed up-right position or swim on its lateral sides. C-Mycotic infected group: Gas bladder infected with *Ichthyophonus hoferi* showed thick walled multinucleated cysts present near blood vessels confirmed by isolation in MEM containing 10% foetal bovine serum. D-Parasitic infected group: Gas bladder infected with *Goussia cichlidarum* showing white nodules variable in size contain fragile walled oocysts, have four sporocysts with two sporozoites, identified by the morphometry. E-Wobbling group: Etiological agents of the wobbling syndrome were coccidian parasite *G. cichlidarum, I. hoferi* and a strain of *F. columnare*. In-vitro sensitivity test of isolated bacterial strains to different chemotherapeutic agents was conducted to determine the effective antibiotics against isolated bacteria. No treatment is known to *I. hoferi* infected fish. Treatment of *O. niloticus* group infected with parasitic *G. cichlidarum* by Mirazid has been tried with great success. The present study has succeeded in isolation, identification and characterization of some significant pathogenic agents dealing with parasitic, bacterial, and mycotic groups which act as the causative agents of wobbling disease and shed light on ignored diagnosis of wobbling disease in Egypt cultured tilapia.

Key Words: *Oreochromis niloticus, Goussia cichlidarum, Ichthyophonus hoferi, Flavobacteerium columnare, Pseudomonas fluorescens, Vibrio harveyi, Staph aureus,* gas bladder and Mirazid.

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Introduction

Cultured tilapias expose to single or multiple pathogenic agents; parasitic, bacterial or mixed leading to diseases and mortalities (Shoemaker, et al. 2008). The gas bladder is a small epithelium-lined sac extends from just behind the head, ventral to the vertebral column of fin fish. It has a close association with blood vessels which gases can diffuse across into and out of the sac according to the needs of the fish (Perlberg, et al. 2008). Gas bladder abnormalities were important in diagnoses infectious diseases (Jennifer and Hartman, 2006). Bacterial fish diseases constitute some of the major challenges facing sustainable aquaculture production. Most bacterial pathogens of fish are aerobic gram-negative rods; the Diagnosis is by isolating the organism in pure culture from infected tissues and identifying the bacterial agent (Woo and Bruno, 1999). It is relevant to emphasis that disease is not necessarily caused by single bacterial taxa. Instead, there may well be synergistic interactions between two or more taxa. This possibility is often ignored by some scientists (Austin and Austin, 2007).

Coccidiosis in freshwater fish manifests itself as a chronic pathogen causing gradual mortality pointed out extra intestinal coccidian *Goussia cichlidarum* causing gas bladder infection in cichlid host (Paperna and Cross, 1985). The *G.cichlidarum* present within the epithelial lining, causing hypertrophy and intense desquamation of the swim bladder epithelial lining in cichlids (Landsberg and Paperna, 1985).

Ichthyophoniosis is a systemic fungal disease and once it enters the fish, there is no cure. *Icthyophonus hoferi* is worldwide distributing organism transmitted by feeding spores from dead infected fish. It is a cosmopolitan parasite that infects many organs while the primary target tissue for the parasite is the heart muscle and disseminated to other visceral organs and the somatic muscle tissue (McVicar, 1999 and Kocan, et al. 2004).

Mirazid is the oleo-resin extract from Myrrh of *comomiphora molmol* tree. It was successfully used as molluscicidal and cercaricidal drug in water (Masoud, et al. 2000). It was effective in treatment of fish ectoparasites at concentrations 10 ppm for 1 hour for two sequential days (Mai, et al. 2005). So, this study was done for isolation, identification and characterization of some significant pathogenic agents dealing with parasitic, bacterial, and mycotic groups which act as the causative agents of wobbling syndrome in cultured *O.niloticus*. Moreover, application in vitro sensitivity tests for bacterium isolation and trails for treatment of coccidian infestation by different concentration of Mirazid drug.

Materials and Methods

1- Fish

This study was conducted on (160) fish collected from some private fish farms complaining of abnormal swimming behavior at Abbasa, Sharkia Governorate, Egypt during the period from January to December 2007. Fish were transported alive in large plastic bags filled two thirds with water and provided with battery air pump to restore the oxygen needed and essential care management during transportation from fish farms to the Fish Disease Research Department in Animal Health Research Institute. Fish examined clinically in glass aquaria (100×30×50)cm immediately after reached and subjected to clinical signs, postmortem changes, and bacteriological, fungal and parasitological examinations.

2- Laboratory Examination

A- Clinical and postmortem examination of fish:

Clinical examination was done on alive fish and any abnormal swimming fish behavior was recorded. Fish examined clinically for any abnormal lesions according to Noga (1996). After euthanasia and evisceration, gas bladder

was grossly inspected for any changes or presence of cysts or nodules. Microscopical examinations were done to squash preparations from any cysts or nodules in gas bladder, spleen, liver, heart, intestine and kidney to exclude any other causes of wobbling syndrome. Samples were aseptically transferred to culture medium for bacteriological examination.

B- Bacteriological examination:

Samples from gas bladder of examined *O.niloticus* were streaked onto nutrient agar, trypticase soy agar, trypticase soy agar supplemented with 3% sodium chloride, Rimler-Shotts medium (RS) and thiosulphate citrate bile salt agar (TCBS) plates then incubated at 28ºC for 24-48hr. The growing colonies were picked up in pure form and reinoculated into trypticase soy agar for further identification. Identification of all isolates was done by cultural, morphological and biochemical characters according to Quinn et al. (2002) and through using API-20E (Biomérieux) for gram-negative fish pathogen.

C- Mycotic examination:

Samples were aseptically taken from examined *O.niloticus* gas bladder and cultured in minimum essential medium (Sigma. M 0643) supplemented with 10% bovine serum and 1% glucose (MEM.-10) pH 4, culture was incubated at 15ºC for 10-15 days. Fungal growth was identified microscopically from wet mount preparation and Lactophenol cotton blue stained slides (Spanggaard, et al. 1994).

D- Parasitological examination:

Positive impression smears from gas bladder for coccidian oocysts were air-dried, fixed in absolute methanol and stained for one hour with a pH 6.8-buffered 10% Giemsa solution for further identification by the morphometry of the isolated parasite according to Kim and Paperna (1993).

3- Treatment experiment:

A total number of 30 alive *O.niloticus* with average body weight (70±5)gm, from coccidian infected fish farm were randomly screened for the presence of *G.cichlidarum* infection in gas bladder. Fish were divided into 3 groups of 10 fish each, one of them used as a control untreated. Fish placed in aquaria measuring 40X40X80cm containing dechlorinated water and supplied with air pump at 18-20ºC. Fish starved two days before treated. The fish in the two groups were exposed to 0.2 and 0.4 ppm of the Mirazid indefinite. Fish were fed with commercial pellets 3% of body weight throughout the 2 weeks of experiment. One fish was taken for parasitological examination every 24hr throughout the treatment experiment. A treatment was considered effective when it caused a complete removal of infestation in all fish used in the assay (Tojo, et al. 1994). All fish were observed over two week's period for morbidity and mortalities.

- **Mirazid® pharco**

Chemical used in fish parasitic treatment was Mirazid. Mirazid (The oleo-resin extract from Myrrh of C.molmol tree, family: Burseraceae) is a commercial preparation made from myrrh by Pharco Pharmaceuticals (Alexandria, Egypt)

The content of soft gelatinous capsule of Mirazid is pharco (300mg).

4- In-vitro sensitivity test for isolated pathogenic bacterial agents:

It was carried out against various chemotherapeutic agents and judgment of the obtained results in comparison to interpretive standards was applied as described by Koneman (1992) and Quinn et al. (2002).

5- Histopathological studies:

Tissue specimens from gas bladder were taken from *O.niloticus* that were naturally infected. The samples were fixed in 10% formal saline, processed by conventional method, sectioned at 4µm and stained with Haematoxylin & Eosin (H&E) and periodic acid Schiff (PAS) (Bancroft and Stevens, 1996 and Roberts, 2001).

Results

Clinical and postmortem examination of naturally infected fish:

Fish showed Wobbling behavior in swimming which suspected, proved and recovered mixed infection of parasitic *G.cichlidarum*, mycotic *I.hoferi* and a strain of *F.columnare*. Postmortem examination of examined infected fish revealed hemorrhages of gas bladder, thickening, turbidity and small white nodules of its wall.

Most bacterial agents infected fish showed clinically up-right position or swim on its lateral sides and fin and tail rot, ulceration on fish body specially snout region and depigmentation of skin (Fig. 1, 3). Postmortem examination of naturally bacterial infected fish revealed paleness of gills, congestion of visceral organs, paleness of kidney, haemorrhagic enteritis and hemorrhages of gas bladder and thickening, turbidity of its wall (Fig. 4, 5). Gas bladders of infected fish with parasitic agents *G.cichlidarum* appeared with a thick wall and small white nodules and the heavily infected fish showed swim at about a 45 degree angle (Fig. 2).

Fig. 1: Bacterial infected *O.niloticus* fish showing abnormal swimming.

Fig. 2: *O.niloticus* heavily infested with *G.cichlidarum* showing 45 degree angle in swiming.

Fig. 3: *F.columnare* infected *O. niloticus* fish showing skin ulceration.

Fig. 4: *O.niloticus* showing congested gills, pale enlarged liver, haemorrhages on gas bladder.

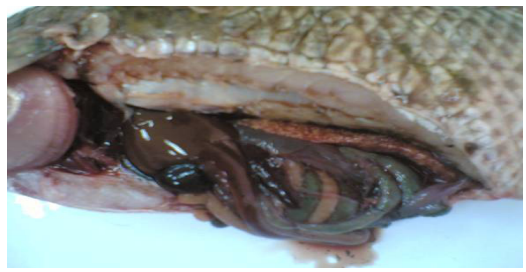

Fig. 5: *O.niloticus* showing pale gills, congested liver, bloody exudates filled the peritoneal cavity, thickening, turbidity and small white nodules of gas bladder.

Causative agents of gas bladder affection:

The study proved that fish were classified according to causative agents isolated from gas bladder affection into:

1. *Normal group:* Complete free from any infection
2. *Bacterial infected group:* gas bladder infected with *F.columnare*, *Ps.fluorescens*, *V.harvey*, and *S.aureus*.
3. *Mycotic infected group:* gas bladder infected with *I.hoferi*
4. *parasitic infected group:* gas bladder infested with *G.cichlidarum*.
5. *Wobbling syndrome group:* gas bladder infected with mixed infection of

G.cichlidarum; *I.hoferi* and a strain of *F.columnare* as shown in (Table 1).

Table 1: Incidence of the Causative agents of gas bladder affection in *O. niloticus* fish:

Causative agents Fish groups	No. of examined fish	No. of infected fish	%
Bacterial infected group		50	31.25
Parasitic infected group		70	43.75
Mycotic infected group		20	12.5
Wobbling syndrome (Mixed group)	160	24	15

Free group= 20 fish (12.5%).

I- Prevalence of infection with I. hoferi

Only 12.5 % of *O.niloticus* fish were found to be infected with *I.hoferi* in gas bladder beside heart, liver, kidneys, and spleen. Moribund fish showed grossly visible white nodules in gas bladder mainly near blood vessels. Squash preparations from the nodules revealed the presence of several thick walled multinucleated spherical bodies of variable sizes (Resting spores). Cultivating infected gas bladder on MEM-10 medium at pH (4) revealed abundant hyphal growth while staining with Lactophenol cotton blue (LPCB) showed nonseptated microhyphae with evacuated hyphal walls after germination and become rounded near the hyphal tips and separated off as large spherical thick walled spores.

II- Prevalence of infection with G. cichlidarum

43.75% *O.niloticus* fish gave positive *G.cichlidarum* parasite without external signs or showing swim at about a 45 degree angle, while internal examination of apparently healthy fish showed white nodules variable in size established in the gas bladder epithelium. Squash preparation from white nodules and cysts showed spherical oocysts with four sporocyst with two sporozoites (Fig. 6). The results of morphometry showed spherical thin and fragile walled oocyst without micropyle measuring averaged 30-35 (30.4)µ. The oocyst wall were absent while four oval sporocysts measured 12-16x8-10 (13x8.9)µ with two sporozoites and a granular residium were present.

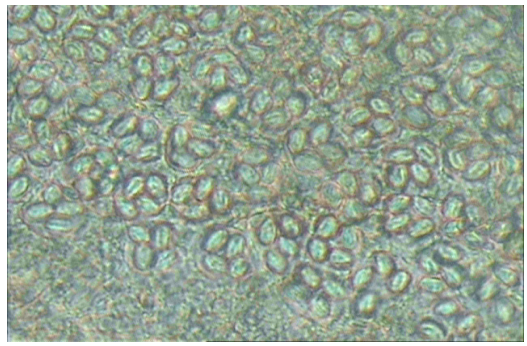

Fig. 6: Wet preparation from gas bladder of *O.niloticus* fish Showing heavily infection with *G.cichlidarum* X400.

III- Prevalence of infection with isolated bacterial agents

About 31.25% of *O. niloticus* fish were found to have bacterial infection with *F.columnare*, *Ps. fluorescence, V.harvey*i, and *S.aureus*. Among bacterial infected fish 32% were infected with *F.columnare*, 24% were infected with *Ps.fluorescens*, while *F.columnare* were found to be mixed with *Ps.fluorescens*, *V.harvey*i, and *S.aureus* at 18%, 12%, and 14%, respectively (Table 2).

Table 2: Incidence of the bacterial pathogenic causative agents of isolates from gas bladder affection in *O.niloticus* fish:

Bacterial isolates	Total No. of bacterial infected fish	No. of bacterial isolates	%
F.columnare	50	16	32
Ps.fluorescens		12	24
*F.columnare +Ps. Fluorescens**		9	18
*F.columnare +*V. harvey*		6	12
*F.columnare +*S. aureu*		7	14

*+mixed bacterial infection

In-vitro sensitivity test of pathogenic bacterial agents revealed that all tested isolates were

found to be highly susceptible to oxytetracycline while vary in susceptibility to the other chemotherapeutic agents (Table 3).

The histopathological changes in the gas bladder:

The histopathological examination of the gas bladder of natural infected fish revealed that sloughing in the epithelial layer, subepithelial edema with mononuclear cells infiltration in the submucosa (Fig. 7, 8).Congestion in the blood vessels of the submucosa with eosinophilic granular cells infiltrations (Fig. 9) were showing in gas bladder of fish infected with bacterial infection. Gas bladder showing normal structure after treatment with Mirazid (Fig. 10).

Table 3: In-vitro sensitivity tests of bacterial isolates to different chemotherapeutic agents:

Chemotherapeutic agents	Concentration per disc	Isolates			
		F. columnare	*P. fluorescens*	*V. harveyi*	*s. aureus*
Colistin sulphate	25 µg	S	S	S	R
Danofloxacin	5 µg	S	R	S	S
Gentamycin	10 µg	S	S	I	S
Nalidixic acid	30 µg	S	R	S	I
Nitrofurantoin	300 µg	S	R	S	S
Oxolonic acid	2 µg	S	R	S	S
oxytetracycline	30 µg	S	S	S	S
Sulphamethoxazole 23.7µg/ Trimethoprim 10.25 µg	25 µg	S	R	S	S

S: Sensitive I: Intermediate R: Resistant

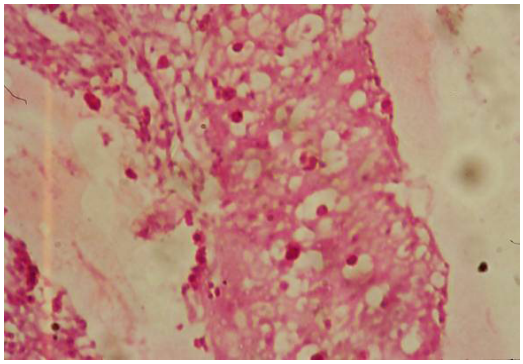

Fig. 7: Gas bladder showing epithelial desquamation and edema in the submucosa and round eosinophilc stuructures (developmental stages of coccidian) PAS stain, X400.

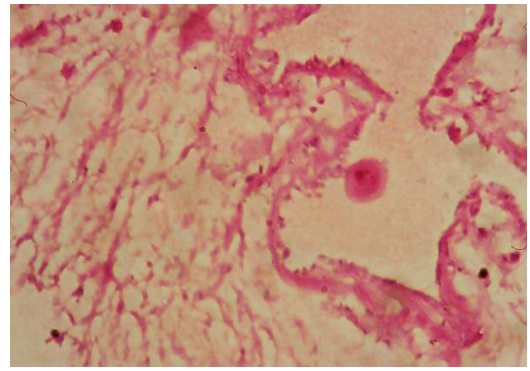

Fig. 8: Gas bladder showing epithelial desquamation and edema in the submucosa and Round eosinophilc stuructures (rest stages of I.hoferi-arow) PAS stain, X400.

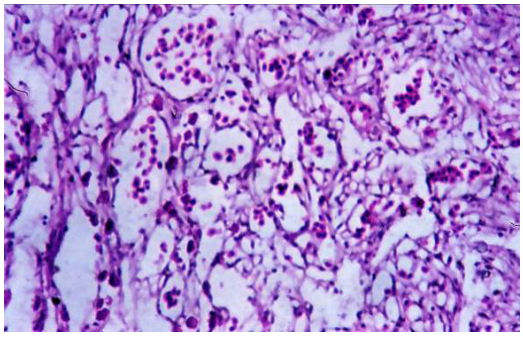

Fig. 9: Gas bladder showing congestion in the blood vessels of the submucosa with eosinophilic granular cells infiltrations H&E stain, X400.

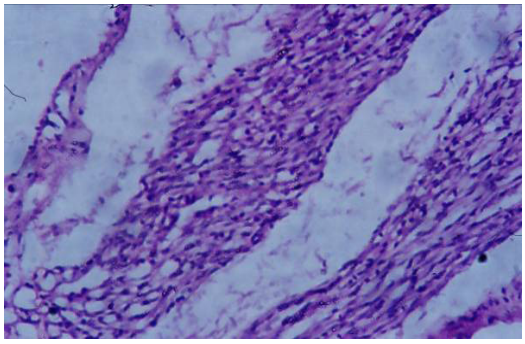

Fig. 10: Gas bladder showing normal structure after treatment with Mirazid .H&E stain, X250.

Efficacy of treatment:

The result revealed that concentration of 0.4ppm of Mirazid® pharco in water for 48 hours was sufficient to eradicate all the parasites in the infected fish.

Discussion

Gas bladder disease is a multifactorial illness, in the current work, the main clinical sign observed in infected fish with mixed bacterial, mycotic and parasitic was wobbling swimming. Numerous parasites have a predilection site for the gas bladder (Jennifer and Hartman, 2006). Fish coccidia are less host specific than mammalian coccidia and have relatively low pathogenicity. Gas bladder of infected fish with *G.cichlidarum* appeared with a thick wall and small white nodules which seems similar to findings reported by Landsberg and Paperna (1985) and El-Mansy (2008). In the present study prevalence of coccidia infection was moderate (43.75%) due to direct transmission by feeding on sporulated oocyst, which reaches their target organ via blood or predation. The obtained results agree with El-Mansy (2008) who identified *G.cichlidarum* by the morphometry of their fragile oocyst, four sporocysts having two sporozoites each with a granular residium, the site of endogenous development and their position in the host cell were similar to our findings.

A disease is the sum of the abnormal phenomena display by a group of living organisms in association with a specified common characteristic or set of characteristics by which they differ from the normal of their species in such a way as to place them at a biological disadvantage (Campbell, et al. 1979). The present work revealed that, bacterial infections had a mild prevalence (31.25%) these were *F.columnare*, *Ps.fluorescencs, V.harvey*i and *S.aureus* acting alone or in mixed infections with each other (Table 2).

Woo and Bruno (1999) results support our finding as columnaris disease is a bacterial infection of fish caused by the gram-negative bacillus *F.columnare*. It is usually of low pathogenicity and infects fish under stressful conditions. However, some strains of this bacterium are highly pathogenic and may cause disease in absence of documented stress. The initial clinical signs of columnaris disease are nonspecific and swimming near the water surface. In this concern, Wada et al. (1993) recorded the first report of an acid-fast bacterial infection in *Cheilinus undulates*, and the first observation of an imperfect fungus in the gas bladder of a tropical marine fish. Gram stained smear preparations of fluid from abdominal and gas bladder cavities demonstrated presence of protozoan spores and gram- negative rods (Kumar, et al. 1986).

Icthyophoniasis, having a wide host, is defined as one of the most economic significant affection in fish culture and wild fisheries (McVicar, 1982). In this study, majority of

infected fish with *I.hoferi* showed no external lesions except for skin darkling as those noticed by Kent et al. (2001). Prevalence of infection was 12.5%, mostly infected tissues of the gas bladder with *I.hoferi* macroscopically had white nodules mainly near blood vessels with various degrees of congestion different from neighboring transparent portions of the same specimen and this may be attributed to the relatively more affection of highly vascularised organs which identify Icthyophonus systemic nature (Spanggaard, et al. 1994 and El-Khattib and Elyas, 2003). Growth of nonseptated microhyphae and tubular club shaped macrohyphae resembled that of Kocan et al. (2004). The recorded histopathological changes in gas bladder of naturally infected *O.niloticus* were sloughing in the epithelial layer, subepithelial edema with mononuclear cells infiltration in the submucosa and congestion in the blood vessels of the submucosa with eosinophilic granular cells infiltrations. The developmental stages of coccidian parasite were detected in gas bladder epithelial cells, also rest stage of *I.hoferi* was showing positive periodic acid Schiff (Fig. 8). These results supported the results of (Landsberg and Paperna, 1985 and Kocan, et al. 2004).

In the present study, trial to treat experimentally the naturally infested fish with *G.cichlidarum* by 0.4ppm Mirazid succeeded in complete eradication of parasites after 2 days. This result is in agreement with Mai et al. (2005) who recorded that the effectiveness of Mirazid in treatment of fish parasites. In case of *I.hoferi* infection, (McVicar, 1999 and Kocan, et al. 2004) suggested that no results of chemotherapy but efforts should be focused on disease prevention. According to the present study, in-vitro sensitivity test of isolated bacterial strains to different chemotherapeutic agents revealed that all isolates were sensitive to oxytetracycline while vary in susceptibility to the other chemotherapeutic agents (Table 3). These results confirm with previous finding by El-Bouhy and Khalil (2008) who mentioned that Gentamycin was one of the effective choices for treatment of staphylococcus infection in some freshwater fish.

Conclusion

The present study succeeded in isolation, identification and characterization of some significant pathogenic agents dealing with parasitic, bacterial, mycotic groups which acting as causative agents of wobbling disease with referring to treatment with Mirazid. In addition, our study proved the in vitro sensitivity of tests to isolate pathogenic bacteria and also shed light on ignored diagnosis of wobbling disease in Egypt cultured tilapia.

Acknowledgement

This work is part of work in a project titled with epidemiological map of fish diseases in Egypt. The authors are indebted to Dr. Amany M. Kenawy, researcher, Hydrobiology Department, National Research Center, Dokki, Giza for pertinent role she played throughout the histopathology.

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