

## A CONTRIBUTION ON MYXOSOMA INFECTION IN CULTURED *OREOCHROMIS NILOTICUS* IN SHARKIA GOVERNORATE

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### Abstract

Heavy infection with myxosoma spp has been recorded among cultured *Oreochromis niloticus* from El-Abbassa fish ponds at Sharkiya governorate in Lower Egypt. Prevalence of the infection exceeded 80 % of all examined fish. Head cysts and hole in the head like lesions have been recorded among all examined fish. The causative agent "myxosoma" has been identified using classical taxonomical and histopathological techniques. The genetic non-relatedness of the salmonid *Myxosoma cerebralis* to the African cichlid myxosoma spp has been confirmed using a polymerase chain reaction (PCR) involving a specific primer of the gene encoding 18-S ribosomal RNA of *M. cerebralis*.

**Key Words:** *Oreochromis niloticus*, *Myxosoma*, Lower Egypt, Molecular Identification.

### INTRODUCTION

*Myxosoma* spp. is the best known of 1300 parasites grouped in the phylum Myxozoa and the first shown to possess a two host life cycle including fish and an aquatic oligochaete, *Tubifex tubifex* (Markiw & Wolf 1983, Wolf & Markiw 1984, Wolf, *et al.* 1986). Myxosporean research in Africa dates back to the late 19th century with Gurley (1893) being one of the earliest authors referring to the continent. The African continent boasts over a 100 myxosporean species from freshwater, brackish and marine fishes of which 84 infect primarily freshwater fishes (Fomena and Bouix 1997) and this number is continuously growing. When comparing the known African myxosporeans to more than 1,300 species described worldwide, it is evident that for a huge continent with such high fish diversity, a large gap exists in the knowledge on the occurrence and distribution of these parasites.

El-Mansy (2005) revised the taxonomy of myxosporean species, using specimens isolated from plasmodia situated in the infected cornea of *Oreochromis aureus*, *Oreochromis niloticus* or *Tilapia zillii* inhabiting the River Nile, Egypt. In addition, he described the histological effects of the parasite on the infected tissues and indicated that the spores of *M. heterosporus* had a variety of shapes expressing remarkable heteromorphism. He also, reported the presence of five main Myxobolus like spore types and tailed-spores. In the same study, El-Mansy (2005) also indicated that light and electron microscopy supported that spores of a Myxobolus-like

morphology coexisted with so-called tailed-spores in one plasmodium and some transitional stages from Myxobolus-like spore types to tailed-spores were observed. Therefore, he concluded that some tailed-spores may be simply heteromorphs of Myxobolus.

Numerous species of *myxosoma* (formerly known as *myxobolus*) have been reported among different african tilapia species within the last few decades. Among these myxosporean species , the *Myxobolus bütschli*, 1882 species from tilapia of the Okavango River and Delta, *Myxobolus africanus* (Fomena, *et al.* 1985) from the gills and fins of *Hepsetus odoe* (Bloch, 1794), *Myxobolus camerounensis* (Fomena, *et al.*, 1993) from the gill arch of *Oreochromis andersonii* (Castelnau, 1861), *Myxobolus hydrocyni* (Kostoïngue and Toguebaye, 1994) from the gills of *Hydrocynus vittatus* Castelnau, 1861, *Myxobolus nyongana* (Fomena, *et al.*, 1985) from the gills of *Barbus poechlii* (Steindachner, 1911), *Myxobolus cf. tilapiae* (Abolarin, 1974) from the buccal cavity of *Tilapia rendalli rendalli* (Boulenger, 1896), *Myxobolus etsatsaensis* sp. n. from the gills of *Barbus thamalakanensis* Fowler, 1935, *Myxobolus paludinosus* sp. n. from the gills of *Barbus paludinosus* (Peters, 1852).

There are currently 11 *Myxobolus* species parasitising cichlids in Africa (Baker 1963, Abolarin 1974, Landsberg 1985, Faisal and Shalaby 1987, Sakiti, *et al.* 1991, Fomena, *et al.* 1993). Faisal and Shalaby 1987, were first to report on the occurrence of *M. tilapiae* in Egyptian tilapia.

Despite the presence of numerous reports on the myxosporean affecting African cichlids as well as non cichlid fishes such as salmonid fishes worldwide, none of these reports described the pathological lesions reported. The current study also presents a unique variety of diagnostics used for the diagnosis of myxosoma infection in *O. niloticus*.

## MATERIALS AND METHODS

### Fish:

In the midsummer of 2006, a total of 100 *Oreochromis niloticus* (average body weight 50 gm) were collected from an earthen pond at El-Abbassa Fish Farm, Sharkia governorate in Lower Egypt and brought alive to the Fish Diseases and Management Laboratory (FDML) at Faculty of Veterinary Medicine, Cairo University. Tilapias were kept in well-aerated, temperature adjusted water aquaria until examined.

### Sampling and Sample processing:

Tilapias were euthanized with an overdose of MS 222 (tricaine methane sulfonate, Finquel- Argent Chemical Laboratories, Washington) and visually inspected for any abnormalities before examination was adopted. Lesions were photographed and documented using camera.

Skin, gill scraps were performed on all examined fish and photos for the plasmodial stage of the myxosoma spp were taken and sketched then used for taxonomical identification of the myxosoma spp. Also, eyes of the fish were examined for the presence of any myxosporean spores within both cornea and lens. Further, the fish were cut open using the standard triangular dissection technique then impression smears from different organs including liver, spleen, kidneys, gall bladder were made. Stomach and intestinal scraps were also made from the examined fish. All samples were freshly examined using regular light microscope as well as dissecting microscope. Some samples required special staining using Giemsa stain.

Sections from the head cysts, head cartilages, eyes, gills, brain, liver, spleen, kidneys, intestine and gonads were stored in 10 % neutral formalin and sent for histopathology. Histopathology was performed on 1-5  $\mu\text{m}$  sections of the above mentioned organs and stained with hematoxylin and eosin (H&E) and or special stain (Giemsa stain). Method and criteria used fro histopathological examination was adopted from Baldwin, *et al.* (2000).

Samples from the head cysts, head musculature, kidneys were collected into microfuge tubes and stored in  $-30^{\circ}\text{C}$  for PCR testing. The following method was adopted for DNA extraction: A total of 500  $\mu\text{l}$  of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each lysed myxospore preparation, and each preparation gently inverted several times to form an emulsion. The organic phase was separated from the aqueous phase by centrifugation at  $1700 \times g$  for 10 min at room temperature. The upper aqueous phase, containing DNA, was transferred to a new microcentrifuge tube and an additional 500  $\mu\text{l}$  of phenol: chloroform: isoamyl alcohol added. The solutions were mixed and centrifuged as before. The aqueous phase was again transferred to a new microcentrifuge tube, 500  $\mu\text{l}$  chloroform added, and the solutions mixed and centrifuged as before. The DNA was precipitated by adding 800  $\mu\text{l}$  of ice-cold ethanol and incubating at  $-20^{\circ}\text{C}$  for 1 h, followed by centrifugation at  $12\ 000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was discarded and the DNA pellet permitted to air dry. The pellet was re-suspended in 50  $\mu\text{l}$  of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and held overnight at room temperature. Samples were warmed to  $65^{\circ}\text{C}$  for 1 h to ensure solubilization and subsequently allowed to return to room temperature prior to use in the PCR reaction.

Primers used were adopted from that published by Andree, *et al.* (1998) as (5'-GCATTGGTTTACGCTGATGTAGCGA-3') and (5'-GGCACACTACTCCAACACTGAA TT TG-3'). The standard reaction volume was 50  $\mu\text{l}$  (45  $\mu\text{l}$  of master mix and 5  $\mu\text{l}$  of DNA template). The PCR master mix was comprised of PCR buffer (300 mM Tris, 75 mM ammonium sulfate, pH 9.0), 2.5 mM MgCl, 400  $\mu\text{M}$  dNTPs, 20 pmol of each primer,

and 2 U  $\mu\text{l}^{-1}$  taq DNA polymerase (Fisher Scientific). All reagents were stored at  $-20^{\circ}\text{C}$  and kept on ice after thawing. Taq polymerase was the last reagent added. Amplifications were performed using a thermal cycles (Barnstead/Thermolyne). A denaturation step in which samples were held at  $95^{\circ}\text{C}$  for 5 min took place before amplification cycles began. One complete cycle consisted of 1 min at  $95^{\circ}\text{C}$ , followed by 2.5 min at  $65^{\circ}\text{C}$ , followed by 1.5 min at  $72^{\circ}\text{C}$ . This cycle was repeated 35 times, after which an extended elongation step of 10 min at  $72^{\circ}\text{C}$  concluded the program. When necessary, amplified DNA samples were stored at  $4^{\circ}\text{C}$ .

## RESULTS

Clinical examination of the examined tilapia revealed the presence of number of clinical abnormalities including, frontal head cysts (Figure 1 & 2) which usually progress to hole in the head in most of cases (Figure 3), skin erosions, fin rot, and corneal opacity.

A relatively small % of the examined fish (5 %) revealed the presence of ring of cysts that surrounded the iris of the eye (Figure 4). Wet mount, Methylene blue and Giemsa stained slides examination of the affected eyes revealed the presence of high number of plasmodial stages of myxosoma spp. within each cyst. Such myxosoma spp. was sketched and further identified as *M. heterosporus*. However, there is a very close similarity between the plasmodial stage of the identified *M. heterosporus* and that of *M. tilapiae*. The polar capsules of *M. heterosporus* are, however, more pyriform, compared with the more spherical polar capsules of *M. tilapiae*. Histologically, H&E stained sections made from the corneal tissues of the eye revealed the presence of large number of the *M. heterosporus* plasmodial stages associated with localized inflammatory changes and mononuclear cell infiltration (Figure 5).

Clinical examination also revealed that over 80 % of the examined fish were associated with head cysts that usually proceed to hole in the head like lesion. Giemsa stained as well as non stained scraps made from these lesions together with impression smears made from musculature sections of the head cysts revealed the presence of the same type of myxosoma spp plasmodial spores (Figure 7) that presumptively identified as *M. tilapiae* with spore body oblong to oval with anterior and posterior ends bluntly rounded,  $14.0\text{--}15.5$  ( $15.0 \pm 0.39$ )  $\mu\text{m}$  in length. Widest region of spore observed towards centre of spore body,  $12.0\text{--}12.6$  ( $12.3 \pm 0.27$ )  $\mu\text{m}$  in width. Two almost spherical to pyriform polar capsules of equal size situated in anterior part of spore. Polar filaments have four to six coils within polar capsules.

Interestingly, another spp of myxosoma has been detected in impression smears made from kidneys and intestine of the affected fish (Figure 8). The vegetative

sporogenic stage is relatively similar to that of *M. etsatsaensis* and *Myxobolus* sp. from the kidneys and spleen of various cichlid species.

An alternative to histologic examination a polymerase chain reaction (PCR) amplification of a DNA sequence unique to *Myxobolus cerebralis* (Andree, *et al.* 1998) have been performed on the DNA extracts from the muscle sections from the head cysts, heavily infested eyes, and kidneys. Unfortunately, the characteristic 415 bp amplicon of the gene encoding 18S ribosomal RNA of *M. cerebralis* was not detected in any of the tested DNA extracts of the above mentioned tissues.

## DISCUSSION

The results came in this study, was in full accordance with that of other myxosporean studies which previously described in details the taxonomy of numerous species of myxosoma (formerly myxobolus) affecting African cichlids. However, the unique aspect of this study is tightly associated to the great linkage between rarely recorded pathological finding associated with myxosporean invasion in *O. niloticus* as well as the usage of new techniques such as molecular tools utilized to confirm the classical taxonomical methods.

The high prevalence (more than 80 %) of myxosporean infection among the examined fish highly suggests that such infection is endemic in the ponds used for rearing of these fish. This suggests that the tubificid oligochaete worm which act as an IMH for the myxosporean myxosoma spp is highly distributed in an active manner within the water- mud laden of such earthen ponds and that's why more than one spp of myxosoma have been reported in this study. The presence of myxosporean plasmodial stages within external as well as internal organs as kidneys and intestine strengthen the same conclusion.

The myxosporean *Myxosoma heterosporus* detected in the cornea of 5 % of the affected fish and its associated histological changes were similar to those of the infected cornea of *O. aureus*, *O. niloticus* and *T. zilli* described by El-Mansy (2005).

The more vigorous picture was the development of head cysts in the frontal and occipital areas in the affected tilapia. Such cysts were highly populated with large number of myxosporean *Myxosoma tilapiae* and or *M. heterosporus*. These cysts ultimately ruptured leaving a hole in the head like lesion in the frontal aspect of the head. The pathological sequence of such myxosporean invasion highly recommend the assumption that myxosporean parasites secretes certain kinds of proteolytic enzymes that liquefy the infected tissue and enables quick increase of the spores in each affected head cyst . The proteolytic enzymes might include some enzymes that digest

the cement material between the connective tissue, skin, and other tissues. Thus a hole in the head like lesion is the ultimate fate of such myxosporean invasion has developed.

The measures of the myxosoma spp. reported in the current study coincides with those of *M. tilapiae*, *M. heterosporus* reported by Reed, *et al.* (2002) and El-Mansy, *et al.* (2005). While those detected in the kidneys, intestine were in accordance with that of *M. etsatsaensis* and *Myxobolus* sp. described by Obiekezie and Okaeme (1990) from the kidneys and spleen of various cichlid species.

*Myxosoma cerebralis* specific PCR has been used to detect the gene encoding for *M. cerebralis* 18 S ribosomal RNA within the DNA extracts of affected tilapia tissues. The aim was to confirm the specificity of such test to identify the myxosporean parasite to the species level and confirm close relatedness of *myxosoma cerebralis* of salmonids to their closely related cichlid myxosporean spp as those reported in this study. Unfortunately, the specific 415bp band was not obtained with any of the above tested tissues of *O. niloticus* which suggest two main facts. First, the cichlid myxosporean are genetically different from those of salmonids. Second, in vivo expression of the cichlid myxosporean genes are mainly different from that of salmonids.

In conclusion, this study is a unique mixture between classical and recent diagnostic tools through which prevalence and severity of myxosoma infection have been clearly presented and discussed. This study can be the first bead in the chain of new diagnostic approach for the myxosporean parasites affecting African cichlids.

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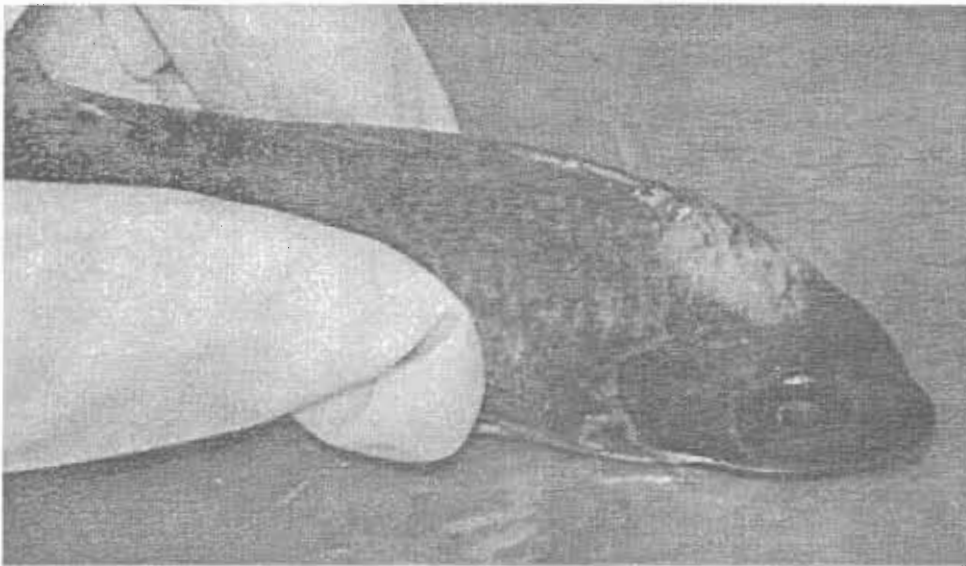


Fig. 1. *Oreochromis niloticus* with frontal head cyst (myxosporean vegetative plasmodial stages were isolated from the cyst scrap)

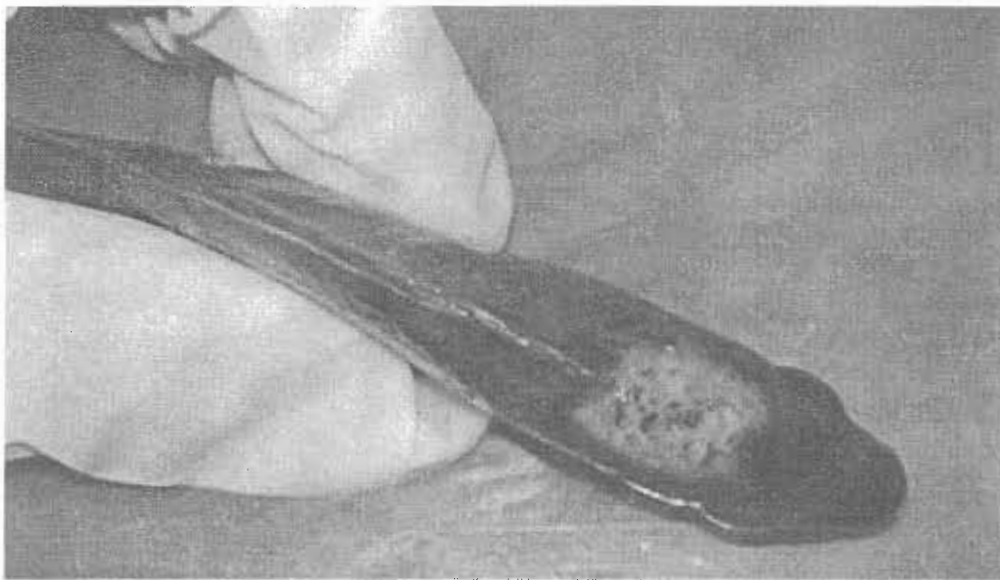


Fig. 2. *Oreochromis niloticus* with more severe frontal head cyst (notice the progression of the lesion).



Fig. 3. *Oreochromis niloticus* showing a ruptured head cyst leaving a hole in the head like lesion. *M. tilapia* and *M. heterosporus* were isolated from the infection site

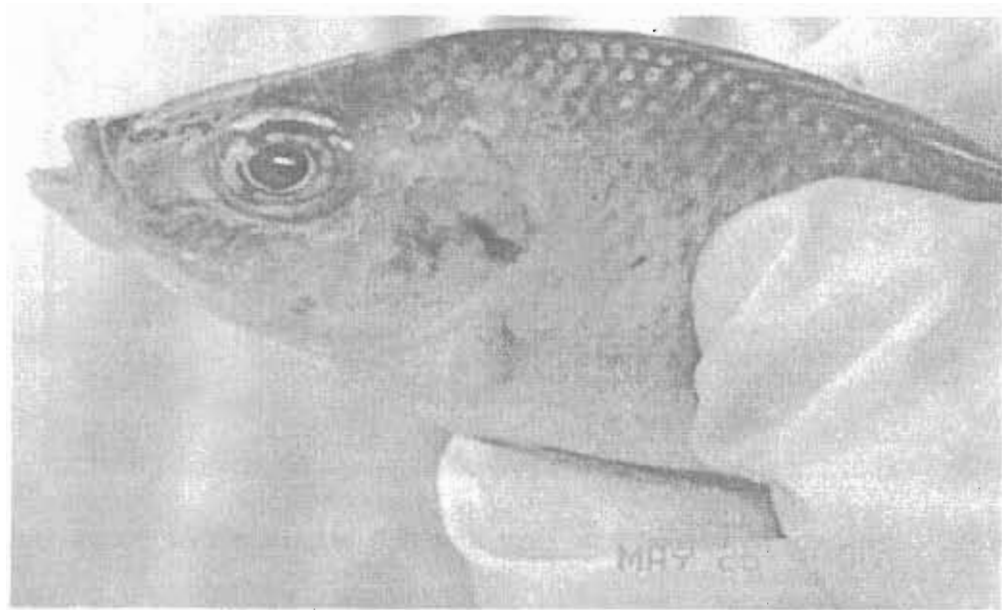


Fig. 4. *Oreochromis niloticus* with a corneal myxosporean cyst ring surrounding the iris.



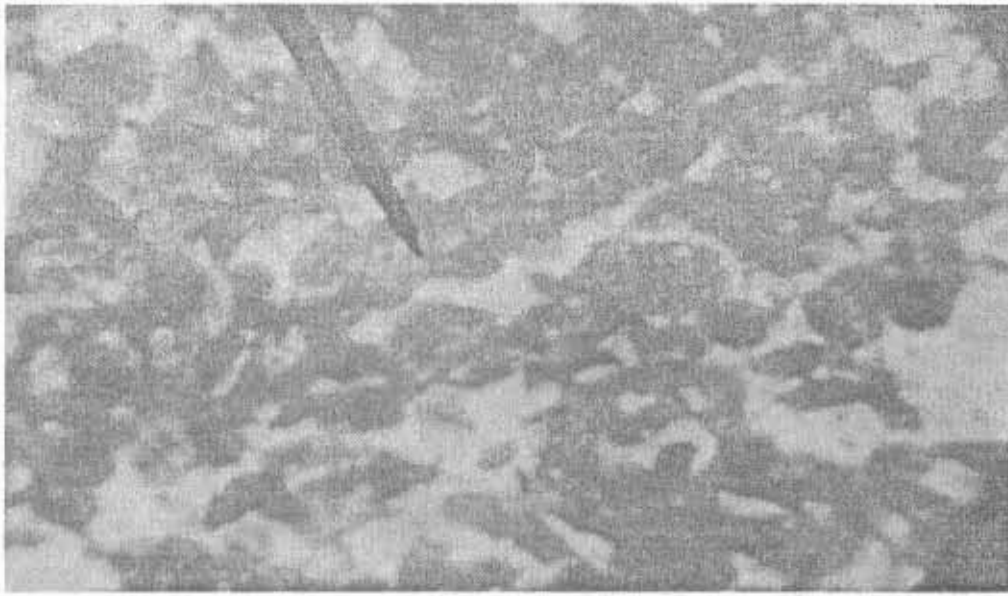


Fig. 5. A Giemsa stained histopathological section of the eye (Cornea) showing heavy infiltration of the tissue with deeply stained myxosporean vegetative plasmodial stages

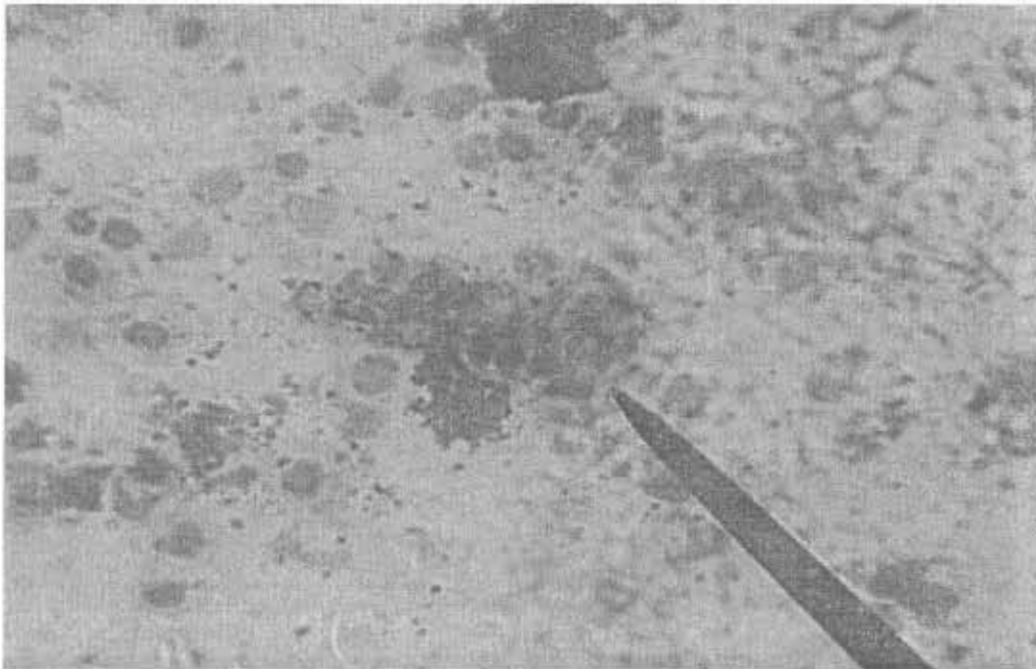


Fig. 6. A Giemsa stained wet mount from the corneal cysts showing *M. heterosporus* plasmodial vegetative spores.

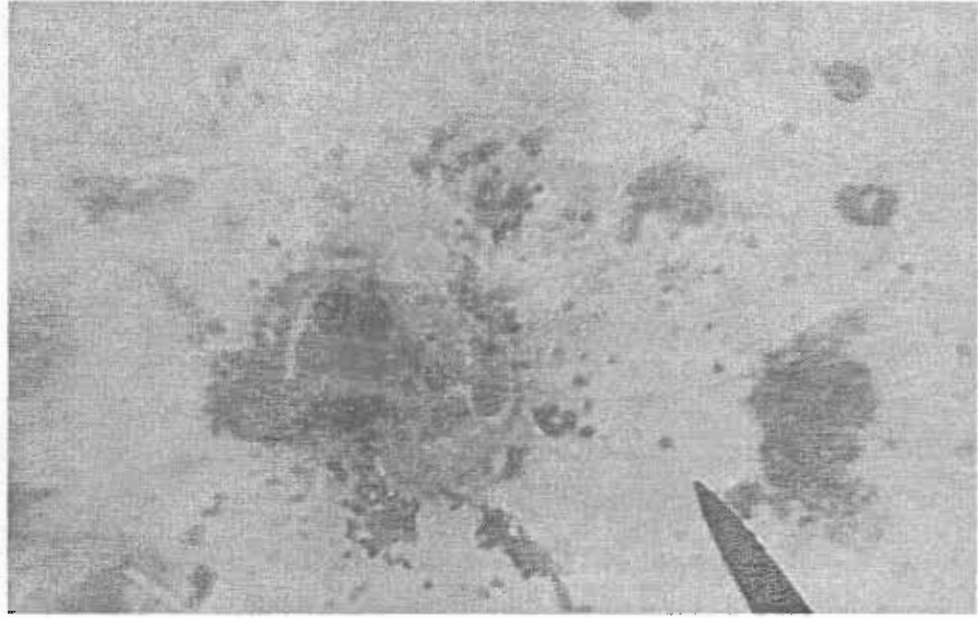


Fig. 7. Vegetative plasmodial spore of *M. tilapiae* in Giemsa stained wet mount from the head cyst.



Fig. 8. A Giemsa stained wet mount from the intestinal scrap showing deeply stained plasmodial vegetative stage of a myxosoma.

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## تسليط الضوء على عدوى الميكزوزوما فى بلطى المزارع فى الوجه البحرى بمصر

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٢. قسم الأحياء المائية - المركز القومى للبحوث- الدقى

تم تسجيل حالات إصابة شديدة بعدوى الميكزوزوما بين أسماك بلطى المزارع فى محافظة الشرقية بالوجه البحرى بمصر. معدلات العدوى تجاوزت نسبة ٨٠ ٪ من كل الأسماك التى فحصت . تكيسات و اعراض تشبه الثقب فى منطقة الرأس تم تسجيلها فى كل الأسماك التى فحصت. لقد تم التعرف على الطفيل المسبب " الميكزوزوما" باستخدام الطرق التصنيفية والهستوباثولوجية التقليدية و تم تأكيد عدم وجود علاقة وراثية بين ميكزوزوما البلطى الأفريقى والميكزوزوما المخية لأسماك السالمون الأمريكية باستخدام تقنية ال PCR بواسطة Specific Primer الخاص بالجين المشفر للحمض النووى الريبوزى من نوع 18 S-r RNA الخاص بالميكزوزوما المخية لأسماك السالمون الأمريكية .