Vet.Med.J.,Giza. Vol. 50, No.2. (2002) : 195-202.

RECENT TECHNIQUE TO DETERMINE VIABILITY OF MYXOSPOREAN SPORES

NAHLA R.H. EL-KHATIB

Fish Diseases Department, Animal Health Research Institute, Dokki, Giza, Egypt.

Received: 6-8-2001.

Accepted: 8-1-2002.

SUMMARY

A vital staining technique with fluorescein diacetate (FDA) and propidium iodide (PI) was used for determining the viability of myxosporean stage spores of Myxobolus species. Examined spores were collected in different ways from live infected tilapia fish, either naturally released from infected fish or collected from pseudocyst of infected fish. Separated spores from infected fish meal were collected and examined. In vitro survivability tests namely drying, aging, freezing and heating were done on collected spores and their viability under fluorescent microscopy was evaluated, viable spores stained green with FDA while non-viable stained red with PI. Sporicidal testing of some chemicals revealed that calcium hydroxide was the most effective among other sporicidal treatments. Formalin and sodium chloride had a moderate effect on discharged spore viability and isolated spores were relatively sensitive. Vital staining by FDA-PI can examine efficiency of manufacturing processes of infected fish meal. The results proved that pasteurization of fish meal is the only accurate preventive method.

INTRODUCTION

Fish meal is usually manufactured from fish offal consisting of fish heads, skeletons and scraps left over from fish filleting or from whole fish unfit for human consumption. Fish meal is used as animal food ingredient principally for fish and poultry because of its high protein content (Syme, 1966).

Many types of animal parasites can infest fish, either internally or externally. Mycosporean parasites are very common in teleost fish inhabiting most organs, cartilage and muscles (Lom and Dykova, 1992). Myxospora produce large number

of parasitic stages which spread the infection throughout the fish for sporogenic cycle. Myxosporean spores can infect fish directly from the water but the degree of infection depends on the maturity of the spores (Amandi et al., 1985). Freshly, isolated spores are not able to infect fish as spores must be aged in mud or water in order to be infective. Also aged spores may enter the fish with tubificid oligochates to Wolf and Markiw (1984) and El-Matbouli and Hoffman (1989).

In spite of the common occurance of myxosporeans in fish, only few reliable methods have been used to determine the parasitic cell viability, namely the methylene blue staining (Hoffman and Markiw, 1977) and a polar filament extrusion method using urea (Lom, 1964) or potassium hydroxide (Markiw and Egusa, 1974). These methods have not been used due to their inaccurate and inconsistent results. Spore viability testing in vivo is quite accurate, but due to the time (10-15) days consuming it is not recommended (El-Matbouli and Hoffmann, 1991). This problem have been overcomed by the use of vital staining of myxosporean and actinosporean spores with fluorescein diacetate (FDA) and propidium iodide (PI). FDA is deacetylated by estrase activity of viable cells to emit green fluorscein. PI permeats only through dead cells and yield a red fluorescence. By this staining method viable and non viable cells can be determined under fluorescent microscopy Jones and Senft (1985) and Yokoyama et al. (1997).

The present study was carried out to examine the viability of myxosporean spores utilizing spore survivability tests and sporicidal experiments moreover efficiency of manufacturing processes of fish meal on viability of myxosporean spores was examined by simultaneous application of FDA-PI staining method.

MATERIAL AND METHODS

1. Myxosporean spores:

A. Heavily infected tilapia fish with myxobolus sp. from Abbasa fish farm were kept in 10 L containers with chlorine free water for 1 day. Spores released from fish in water and those removed from parasitic cysts of dissected fish and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in distilled water containing penicillin (100 IU/ml) and streptomycin (100 ug/ml), to be used in vitro tests.

B. Fish meal samples collected from fish farms were examined microscopically using a drop of water. Positive samples for myxosporean spores were digested by pepsin-trypsin dextrose (PTD) (Markiw and Wolf, 1974), after which the spores of myxosporean were isolated, concentrated, resuspended in distilled water and examined for spore viability.

2. Vital staining:-

Fluorescein diacetate (FDA) No. F. 7378 and

Propidium iodide (PI) No. P. 4170, (Sigma, Mo, USA) were prepared according to Markiw (1992). Stock solution of FDA (5 mg/ml dissolved in acetone) and PI (0.02 mg/ml dissolved in distilled water) were stored at -20°C. Twenty-five microliter of freshly diluted FDA solution (20 µl of FDA stock solution diluted with 4.2 µl of distilled water) was added to 25 ml PI and 50 µl of spore suspension. This mixture left undisturbed in the dark at 5°C for 1-2 days then examined under fluorescent microscopy. Viability of spores was enumerated according to the number of spore showing bright green fluorescence sporoplasm among 100 spores.

3. Survivability tests:

Spores suspended in distilled water containing antibiotics were distributed into 8 test tubes and subjected to the following tests:

I. Aging: Three test tubes were held in the dark at 5°C for 6, 12 and 18 months and then spore viability was determined.

II. Drying: A drop of spore suspension was smeared on a glass slide and left to dry in air then stained to check the spore viability.

III. Heating: Three test tubes containing the spore suspension were incubated at 25, 40 and 60°C. Spore viability was monitored every hour for the first 24 hours and then daily over 6 months.

IV. Freezing: Two test tubes containing the spore suspension were freezed at -20°C and -4°C. Spores viability was monitored monthly over 6 months.

4. Sporicidal tests:

Calcium hydroxide, sodium chloride and formalin were added to spore suspension at different concentrations (Table, 1) that were held in the dark at 5°C for 24 hours. After centrifugation of spore suspension, the supernatant fluid was discarded, the pellet was resuspended in pure distilled water and spore viability was then determined.

RESULTS

The viability of Myxobolus spors were determined by FDA-PI stain. Viable spores showed bright green sporoplasm, polar capsules and spore valves. Non viable spores revealed red stained sporoplasm with two polar capsules. On the other hand, both red and green fluorescence were observed in the same spores. All killed control spores stained red only (Fig., 1, 2).

Myxobolus spores held in the dark at 5°C showed bright green fluorescence till end of experiments (18 months). Drying showed sporicidal effect on myxobolus spores. Spores could withstand heating at 40°C for 6 hours and only for 2 hours at 60°C. On the other hand, myxobolus spores could survive for 6 month at 25°C. Most spores preserved at -4°C, -20°C were still viable even after 6 months (Table, 1). The sporicidal effect of some

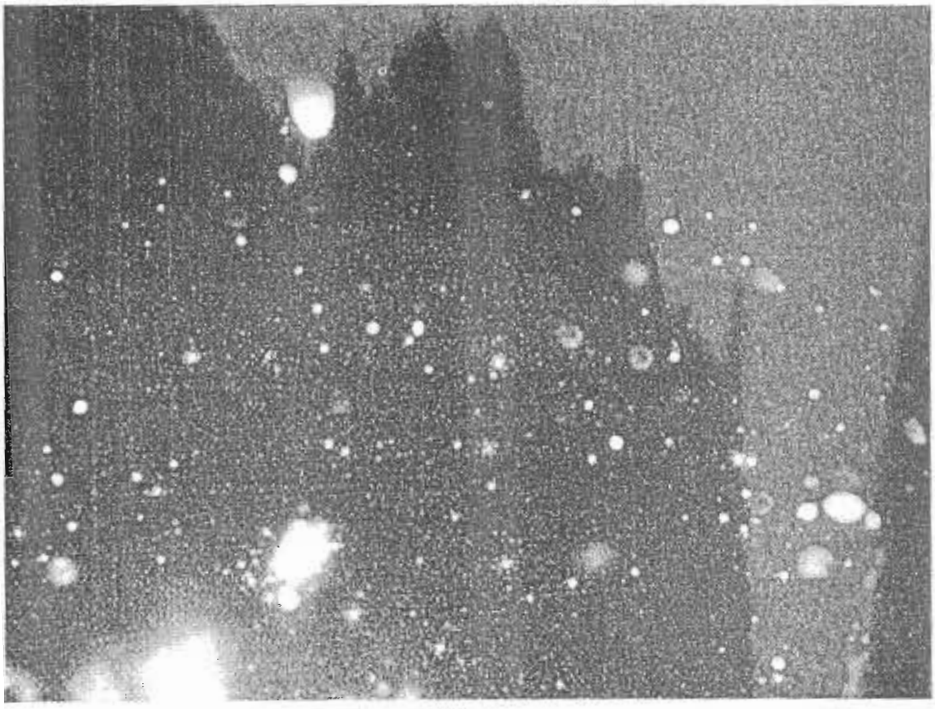

Fig. (1): Myxobolus spores released from infeced fish, FDA-PI stained, showing viable (green) and non-viable (red) spores.

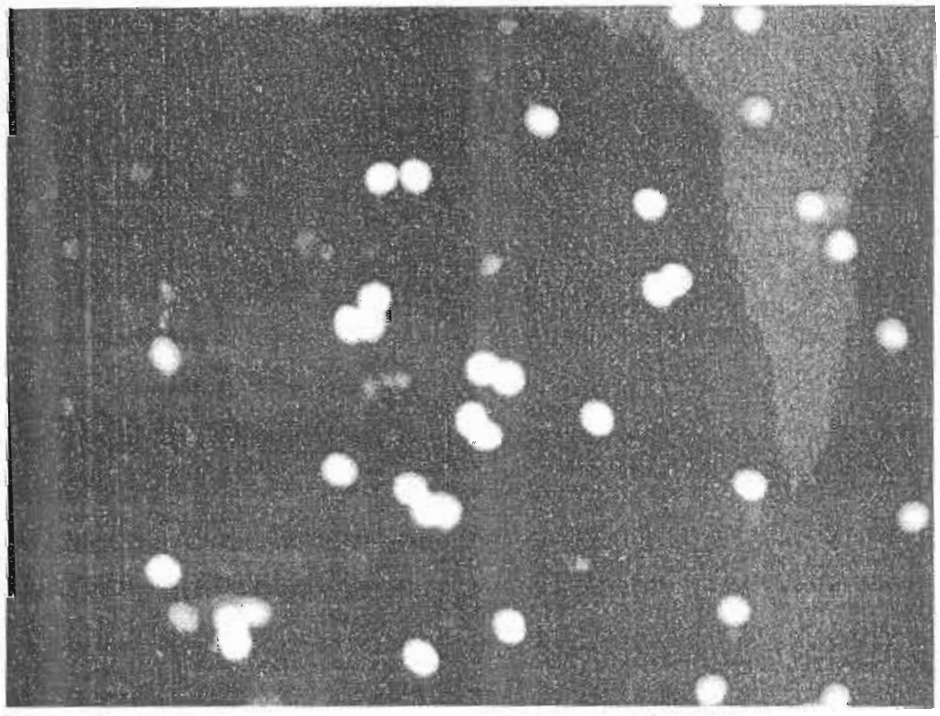

Fig. (2): ALI killed control Myxobolus spores stained only red by FDA-PI stain.

Table (1) :Effect of different survivability tests on myxobolus sp. spores viability determined by FDA-PI staining.

Survivaility	Temperature °C	Time (month)	D.spores*	C.spores**	Control***
1. Aging	5	18	100	100	0
2. Air drying	Air drying	-	0	0	0
3. Heating	25	6	98	97	0
	40	6hrs	97	97	0
	60	2hrs	94	92	0
4. Freezing	-4	6	89	87	0
	-20	6	90	85	0

* D spores = Naturally discharged spores of myxosporean.

** C. Spores = collected spores from pseudocyst of infected dissected fish.

Numbers = numbers of viable spores determined by FDA-PI staining/100 spores.

*** Control = collected spores killed by formalin

Table (2): In vitro effect of some chemicals on myxobolus sp. as spores viability as determined by FDA-PI staining.

Chemicals	Dilution (ppm)	D.spores*	C.spores**	Control***
Ca.hydroxide	2.5	55	50	0
	5	0	0	0
				0
Sod.chloride	25000	100	100	0
	50000	79	40	0
Formalin	500	96	95	0
	1000	75	46	0

* D spores = Naturally discharged spores of myxosporean.

** C. Spores = Collected spores from pseudocyst of infected fish.

Numbers = numbers of viable spores determined by FDA-PI staining/100 spores.

*** Control = collected spores killed by formalin (5000 ppm)

used chemicals on myxobolus spores was limited (Table. 2).

Positive samples of fish meal for myxosporean spores, the two red and green fluorescence were observed in the same spores beside numerous of non viable red spores and little viable bright green spores.

DISCUSSION

In this study, the viability of myxobolus spores and their ability to cause an infection were determined by using vital dyes FDA-PI, simultaneously. FDA is a non fluorescent molecule which enters spore by diffusion and is metabolized by non specific estrase releasing free fluorescein. The PI can not across intact cell membrane and only cells with disrupted or broken membrane stain with PI (Smith and Smith, 1989). The longevity of myxosporean spores varied among species and parasitic stage. Myxosporian spores after the completion of spore formation in fish are transported from site of encapsulation by phagocytosis through the host vascular system and some were discharged via the skin, intestine and gills (Yokoyama et al., 1996). Fish meal made from fish offal or whole fish may contain encapsulated spores or even free spores.

In the present study, viable spores were stained bright green, while non viable spore stain red. The presence of the red and green stains in the same spores detected in fish meal may be due to uncompleted processing of diseased fish (drying and sterilizing) which cause incomplete spores damage. FAO (1997) reported that diseases of fish can be transmitted through the use of unpasteurizied residue of the same or the relative fish species. Shuzo (1992) reported that eradication of fish, which have contracted the disease, is believed to be the only preventive method. M. spores were viable and stained bright green after 18 months of storage at 5°C. This result agree with Yokoyama et al. (1997), who found that more than 90% of the spores preserved at 5°C were still viable even after 15 months. M. spores viability depend on temperature, it could resist temperature up to 60°C for 2 hours and 6 hours at 40°C. M. spores survived for 6 months at 25°C. These results disagree with Markiw (1992) who found that M. cerebralis short-lived at 12.5°C for 3-4 days. M. spores were extremely resistant and could tolerate extended freezing at -20°C up to 6 months. These results agree with Hoffmann and Putz (1969).

Efficacy of chemical treatments on spores viability may be related to alteration of physiological activity as well as morphology at different phases of life cycle of myxosporeans. High concentration of calcium hydroxide was effective in killing all myxosporean stage spores. Formalin and sod. chloride had a moderate effect on discharged spores viability and can't completely eradicate isolated spores. These results may be due to the fact that, the isolated spores from developing

pseudocysts were relatively sensitive to chemicals but naturally released spores which contain completely formed spore surface were moderately affected by chemicals. Ogawa et al. (1992) reported that M. artus developing in common carp, after the completion of spore formation, pseudocysts were ruptured and a host reaction including phagocytic infiltration was induced. The phagocytosed spores were then transported from skeletal muscle through the host vascular system to be discharged via the skin, intestine and gills. Yokoama et al. (1996) suggested that the process of phagocystosis by host macrophages might have affected the spore surface.

Drying was also effective in killing all myxosporean spores and most spores were deformed in dry smear and stained red. These results agree with Yokoyama, et al. (1997) who suggested that drying of fish ponds might be effective to some extent in controlling Myxobolus artus infection. However, in earthen ponds, drying may not eradicate the parasite completely because oligochaete worms, possibly harboring actinosporean stages, may form solid, resistant cysts to survive extreme conditions such as drought, freezing and food deficiency.

REFERENCES

Amandi, A.; Holt, R.A. and Fryer, J.L. (1985): "Observations on Myxobolus insidiosus(Myxozoa: Myxosporea) a parasite of salmonid fishes." Fish Pathology, 20: 287-304.

El-Matbouli, M. and Hoffmann, R.W. (1989): "Experimental transmission of two Myxobolus spp. Developing bisporogeny via tubificed worms." Parsitol Res., 75: 461-464.

El-Matbouli, M. and Hoffmann, R.W. (1991): "Effects of freezing, aging and passage through the alimentary canal of predatory animals on the viability of Myxobolus cerebralis spores." J. Aquatic Animal Health, 3: 260-262.

FAO (1997): "Technical guidelines for responsible fisheries." Aquaculture Development, Rome, FAO. 5 p. 138-139.

Hoffman, G.L. and Markiw, M.E. (1977): "Control of whirling disease (Myxosoma cerebralis): use of methylene blue staining as a possible indicator of effect of heat on spores." J. Fish Biology., 10: 181-183.

Hoffman, G.L. and Putz, R.E. (1969): "Host susceptibility and the effect of aging, freezing heat and chemicals on spores of Myxosoma cerebralis." Progressive Fish Culturist, 31: 35-37.

Jones, K.H. and Senft, J.A. (1985): "An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide." J. Histochemistry and Cytochemistry, 33: 77-79.

Lom, J. (1964): "Notes on the extrusion and some other features of myxosporidian spores." Acta Protozoologica, 2: 231-237.

Lom, J. and Dykova, I. (1992): "Protozoan parasites of fishes." El-Sevier Science Publishers.

Markiw, M.E. (1992): "Experimentally induced whirling disease. II. Determination of longevity of the infective triactinomyxon stage of Myxobolus cerebralis by vital staining." J. Aquatic Animal Health, 4: 44-47.

Markiw, M.E.and Egusa, S (1974): "Sporocidal effect of some chemicals on the spore of Myxobolus Koi Kudo" Fish Pathology, 8: 161-164.

Ogawa, K.; Delegahapitiya, K.P.; Furuta, T. and Wakabayash, H. (1992): "Histological studies on the host response to Myxobolus artus Akhmerov, 1960 (Myxozoa: Myxobolidae) infection in the skeletal muscle of carp, Cyprinus carpio L." J. Fish Biology, 41: 363-371.

Shuzo, E. (1992): "Infectious diseases of fish." Brookfield, Vt. USA., p. 521-30.

Smith, A.L. and Smith, H.V. (1989): "A comparison of fluorescein diacetate and prodium iodide staining and in vito excystation for determining Giardia intestinalis cyst viability Parasitology." 99: 329-331.

Syme, J.D. (1966): "Fish and fish inspection." London, H.K. Lewis & Co. Ltd. 2nd Ed., p. 190-192.

Wolf, K. and Karkiw, M.E. (1985): "Biology contravenes taxonomy in the Myxozoa: new discoveries show alteration of invertebrate and vertebrate host." Science, 225: 1449-1452.

Yokoyama, H.; Danjo, T. Ogawa, K. and Wakabayashi, H. (1997): "A vital staining technique with fluorescein diacetate (FDA) and propidium iodide (PI) for the determination of viability of myxosporean and actinosporean spores." J. Fish Diseases, 20: 281-286.

Yokoyama, H.; Danjo, T.; Ogawa, K.; Arima, T. and Wakabayashi, H. (1996): "Hemorrhagic anemia of carp associated with spore discharge of Myxobolus artus (Myxozoa: Myxosporea)." Fish Pathology, 31: 19-23.