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Suspected Infection by *Myxobolus* Sp in Laboratory Reared *Oreochromis mossambica*

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Abstract

Oreochromis mossambica was reared at the pilot fingerling production centre at the Eastern University to supply disease free good quality fingerling for the public to venture towards stocking the reservoirs. During the brood stock rearing phase some fish showed the whirling signs and was suspected to be infected by myxosporean infection.

Different organs of the moribund fish such as kidney, head, airbladder were removed and processed for histology. Thin sections were cut and stained with haematoxylin and eosin. Spores of myxosporeans were observed in the cepahlic cartilage and in the swimmbladder. To complete the description of the *Myxobolus* sp., DNA from the spores was extracted for sequencing. PCR products were successfully obtained and the sequencing work continues for the accurate identification. The presence of *Myxobolus* sp could be a threat to the wild population. The species identification of *Myxobolus* was confirmed with the molecular diagnosis.

Key words: Brood stock, Cephalic cartilage, Fingerling, Kidney, *Myxobolus, Oreochromis mossambica,* PCR, Swimmbladder.

Introduction

Skeletal muscle has been recorded as a location for myxosporean parasites of freshwater fish since from the eighteenth century where as at present it can be found in the cephlaic cartilagenous and kidney tissues. The Phylum Myxozoa belongs to the Kingdom Protista, Sub-Kingdom Protozoa, [1]. For a long time the phylum was considered to include two classes, namely the Myxosporea, which are parasites primarily of fish hosts and the Actinosporea, which are parasites mainly of invertebrates. Nevertheless, the classification of the group has become controversial. It has been suggested that the whole phylum should be included within the Metazoa due to the fact that in some stages of the life-cycle there are situations of pluricellularity that exceed the definition of unicellular levels of organisation of the Protista [2]. Likewise, Siddal *et al*. suggested through morphological characters (e.g polar capsules) and molecular techniques (18S ribosomal gene sequences) that the phylogeny of the Myxozoa is closely related with the Cnidaria and that the whole group should be placed within the Kingdom Metazoa [3]. On the other hand, the identification of HOX genes corroborated by Anderson *et al*.and they suggested that the Myxozoa are more closely related to the Nematoda than to the Cnidaria [4]. Also, there are a number of species of Myxozoa where an actinosporean and myxosporean form part of the same life-cycle of a sole myxozoan, which has an invertebrate and a fish host [5, 6]. The pathogenic effect of muscle-dwelling myxosporeans of freshwater fish is best studied in the members of the genus *Myxobolus*, *Myxobolus pfeifferi* in particular. The same parasite causing the boil disease of the barbel, *Barbus barbus*, *M. cyprini* is the causative agent of pernicious anaemia in the common carp, *Cyprinus carpio* and *M. sandrae* producing intensive infections in the muscle of the pike perch, *Stizostedion lucioperca*, are regarded as the most pathogenic species in the muscle of freshwater fish [7, 8, 9,10]. Recently some reported heavy infections in the muscle of the common carp by *M. artus* [11]. Besides the above mentioned species, a muscle location has been described for several other *Myxobolus* spp. In most of these cases, only solitary scattered spores were observed in the muscle and similar dispersed spores were simultaneously found in other organs. *Myxobolus cyprini*, one of the most common parasites of the common carp, was considered as a

species developing in different organs in small plasmodia, because of the presence of scattered spores in these organs. However, it was proved that this species was a typical intracellular parasite of muscle cells and spores found in other organs had been carried there by the blood circulation after the maturation and disruption of the intramuscular plasmodia [12]. A similar intramuscular development was described by Baska for *M. pseudodispar*, a frequent parasite of the roach, *Rutilus rutilus*; based on the asymmetrical spores and the different sized polar capsules [13] On the other hand, *M. cyprinid* spores may show morphological variability and during maturation can assume an asymmetrical shape similar to that of *M. pseudodispar*. The taxonomic classification of Myxosporea, earlier based only on the morphology of myxospores [14] has been refined with the application of molecular biological methods [15]. Kent, Khattra, Hedrick & Devlin stated that phylogenetic trees constructed by the comparison of 18S rDNA sequences of different myxosporean species agreed with Shulman's phylogenetic hypothesis in most respects and that this sensitive method may open new possibilities for the examination of detailed relationships, such as the phylogenetic distance between closely related species [16,10]. These species were related more by their hosts and geographic origin than by spore morphology. Using 18S rDNA sequences of 10 species, it was stated that the members of the genus *Myxobolus* tend to cluster according to their tissue location [17]. Using partial 18S rDNA sequences of seven different *Myxobolus* species from cyprinid fish, it was found that these parasites segregate by spore morphology [18]. The present paper reports on morphological and molecular biological investigations of *Myxobolus* species infecting the skeletal muscle of *Oreochromis mossambica*.

A literature review has shown that there is a paucity of research in the study of fish parasites in Sri Lanka. Research effort within this area is likely, therefore, to identify the myxosporean species with maximum possible acurracy.

The aim of the study is to collect diseased fish and to identify the parasite found in the fry and fingerling production centre funded by the UNDP. Any infected specimens encountered are to be examined and the parasite found would be characterised. For identification of paraite,

a wide range of techniques was used including histological examination, molecular biology techniques and study of spore morphology.

Materials and Methods

Examination of Fish for Parasitic Infection

Organs examined for parasite infection included the gills, liver, kidney, gall bladder and swimbladder. Small pieces of tissue were removed from these organs and were examined using squash preparations. The squash preparations were prepared by placing the pieces of tissue onto a glass slide and pressing a coverslip down onto it. The squashed preparations were examined using microscopically at x10, x20, x40 and x100 magnifications. Spore measurments were made to characterise the species.

Preparation of Infected Tissue for Histological Study

Pieces of gill tissue containing the myxosporean cysts were excised using a scalpel and fixed in 10% neutral buffered formalin for 24 hours. Afterwards, the tissues were trimmed to a suitable size, placed into a cassette, labelled and auto-processed in a tissue processor. After 7 hours of tissue processing and wax infiltration, the cassettes were removed from the processor and the tissue was placed in molten paraffin wax using a histoembedder and cooled rapidly on a freezing plate. Sections from these blocks were cut at 5µm using a Rotary microtome. Tissue sections were stained using standard Haematoxylin-Eosin.

DNA Analysis of Infected Tissue

DNA was extracted from the suspected organs if they were parasitised with myxosporeans and processed as follows.

To lyse the cells and liberate DNA from the samples, whole organisms were placed in a 1.5 ml Eppendorf tubes in 400µl of TNES-Urea extraction buffer (50mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl, 1% SDS and 5M urea). The samples were then digested overnight with 100µg/ ml Proteinase K (Sigma) in a water bath at 55°C. The next day, each sample was incubated with 20µg of DNAse free RNAse (Sigma) at 37°C for one hour. The DNA was then extracted with phenol and chloroform and precipitated in ethanol. After centrifugation and

washing with 70% ethanol, the DNA content was estimated by viewing in 1.0% agarose gel.

Polymerase Chain Reaction Amplification

The polymerase chain reaction (PCR) was carried out as described by Eszterbauer *et al*. [19]. Primers (MX5 and MX3) specific for the family Myxobolidae were used for amplification of an approximately 1600 base pair (bp) fragment of the 18S rRNA gene [20]. The total volume of the PCR reactions was 50 lL, which contained 10–50 ng extracted DNA, 1· REDTaq PCR Reaction Buffer (Sigma, St Louis,MO, USA), 0.2 mmol dNTP (MBI Fermentas, Vilnius, Lithuania), 40 pmol of each primer and 2.5 U REDTaq DNA polymerase (Sigma, USA) in MilliQ purified water. A PDR 91 DNA Reproducer (BLS Ltd, Budapest, Hungary) was used for amplification. Amplification conditions were: 95°C for 30 s, 46°C for 30 s and 72°C for 60 s for 35 cycles, with a terminal extension at 72°C for 10 min. MX5 and MX3, the other primer pair used for PCR amplification, were designed in our laboratory.

Their sequences are:

MX5 (forward) 5¢-CCTGAGAAACGGCTACCACATCCA-3¢

MX3 (reverse) 5¢-GATTAGCCTGACAGATCACTCCACGA-3¢.

The contents of the PCR reactions and the equipment used for amplification were identical with those used for the MX5–MX3 primer pair. The PCR products were subjected to electrophoresis in 1.0% agarose gels (Sigma, St Louis, MO, USA) in TBE buffer. k phage DNA cut with PstI was used as the molecular weight standard.

Results

The myxosporean spores examined from the cysts of the gills of the common goby were identified as belonging to the Class *Myxosporea* Buetschli, 1881; Order *Bivalvulida* Shulman, 1959; Suborder *Platysporina* Kudo, 1919; Family *Myxobolidae* Thélohan, 1892; following the characters of the genus *Myxobolus* Bütschli,1882 [1] (Figure 1 , 2). The spore is oval in frontal view, 9.37µm (8.29-9.99) long, 9.07µm (7.74-9.95) wide and 6.59 µm (5.77-7.42) thick. The two polar capsules are approximately similar in size and pyriform 3.93µm (3.01-4.8) long and 2.78µm (2.04-

3.28) wide (Figure 3). There are 4-5 turns of the polar filament in the capsule. The presence of a mucus envelope around the spores was confirmed using Indian ink where the mucus envelope appears as a clear halo around the spore.

The results of histological staining using Haematoxylin and Eosin confirm that the preferred site of the *Myxobolus* sp. isolated from the gills are located within the gill cartilage of the gill arch (Figure 1, 2).

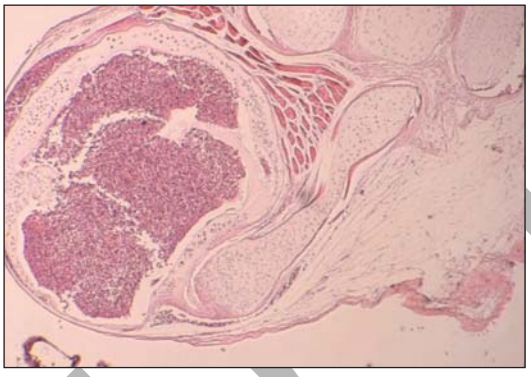

Figure 1: Stained histological tissue sections through gills of *Oreochromis mossambica* infected with a *Myxobolus* sp. (Scale bar = 10 μm)

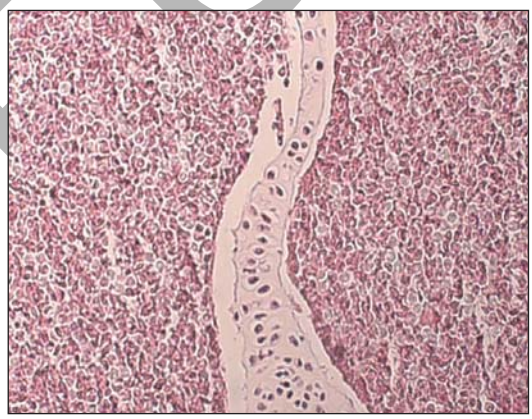

Figure 2: Stained histological tissue sections through the gills of *Oreochromis mossambica* infected with a *Myxobolus* sp. (Scale bar = 10 μm)

The parasite was contained a large polysporoblastic plasmodia causing deformation of the surrounding cartilage. The plasmodia had a thin ectoplasmic layer with spores and developmental stages admixing with the endoplasm.

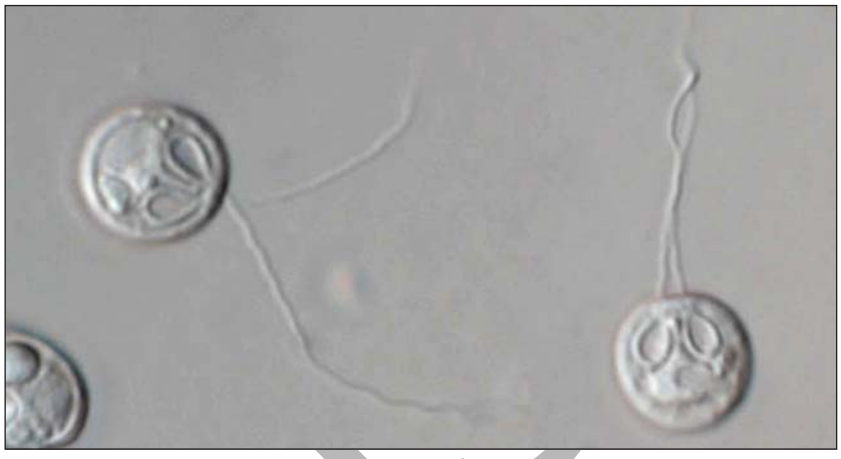

Figure 3: *Myxobolus* sp. spores from a cyst on gills of *Oreochromis mossambica* with their extended polar filaments. (Scale bar=10 μm)

PCR and phylogenetic analysis

The specific primer pairs MX5, MX3 successfully amplified approximately

1600 bp fragments, respectively, of the 18S rRNA gene from every sample of *Myxobolus* examined. The replicates of myxospores isolated from different specimens of

O.mossambica were successfully amplified with the MX5–MX3 primer pair and were sequenced with these two primers. Thus, approximately 500 bp DNA sequences from the 5¢ end and the 1600 bp from 3¢ end of DNA fragment were obtained. The DNA sequences of the PCR products of other workers have been deposited in Gene Bank and are available as Myxosporea, the parasitic protozoan responsible for whirling disease.

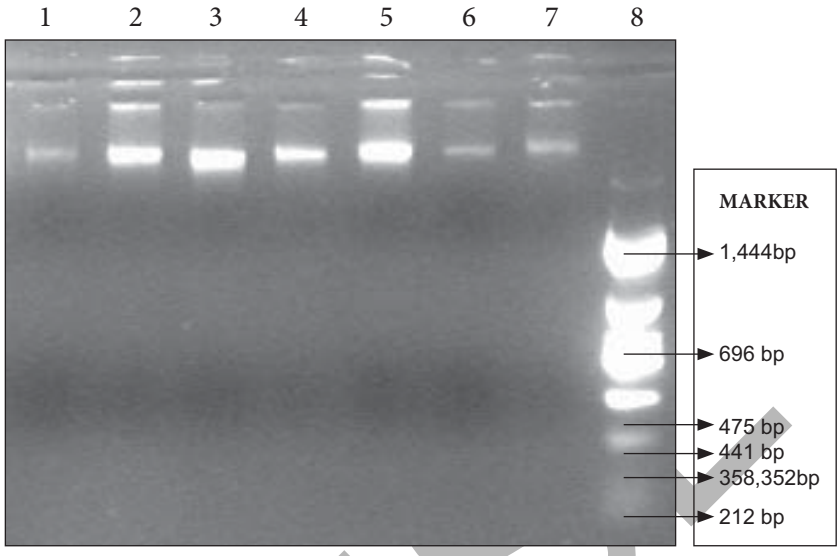

Figure 4 : DNA extraction profile of spores of *Myxobolus* sp. from gills of *O. mossambica*

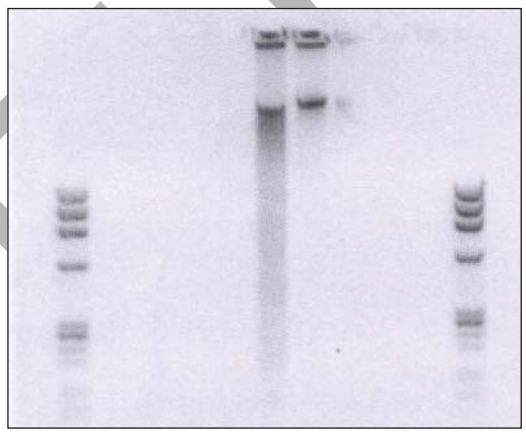

Figure 5 : DNA extraction profile of spores of *Myxobolus* sp. from head kidney of *O. mossambica*

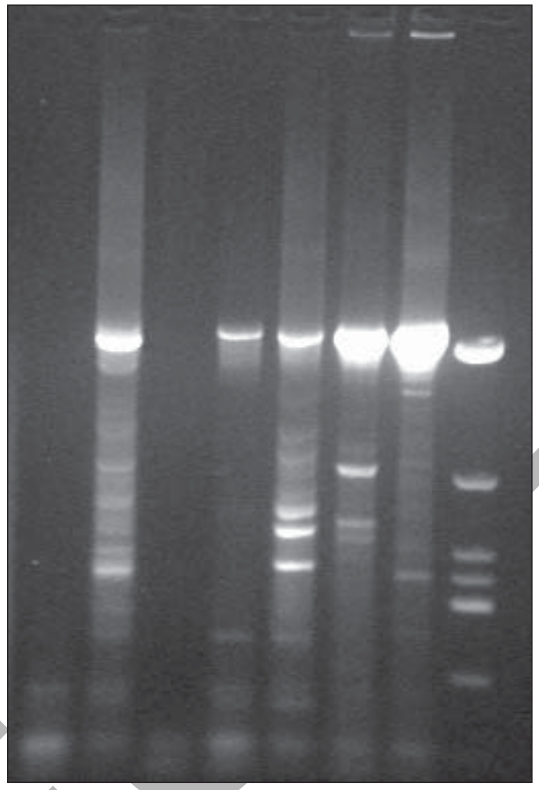

Figure 6: PCR profile of spores of *Myxobolus* sp. with universal primers (18e/18g).

The DNA extractions profile of the infected organs obtained are shown in Figures 4, 5 and 6. In most cases, the DNA extraction was successful, obtaining a clear band of high molecular weight DNA in the agarose gel. However, a few of the extractions did not have a clear band, probably representing samples where too little DNA had been extracted.

The different techniques used in the extraction of DNA from myxosporean spores obtained from infected gills of *O. mossambica* are shown in Figure 4,5 and 6. DNA was extracted from spores using the standard extraction procedure. Nevertheless, when the spores underwent thermic shock to decapsulate the spores, DNA was successfully extracted. Although PCR products of amplified *Myxobolus* DNA have been obtained, there was insufficient tissue to conduct the final sequencing reactions. There is

a likely hood that the *Myxobolus* species collected from the gills of *O. mossambica* may represent a new species.

Discussion

The spores collected from gills of infected common gobies were found to belong to the genus *Myxobolus*. In line with the description of the genus, the spores are oval in valvular view and have polar capsules that are mostly pyriform. About 453 species of *Myxobolus* have been identified in fishes, generally being histozoic in freshwater fishes and estuarine fish species [1]. According to histology evidence, the myxosporean cysts are seen to be situated within the cartilage of the gill arch.

The majority of the *Myxobolus* species recorded from cartilage have been reported to infect freshwater fish species (Table 1). Only *M. aeglefini* has been described from a range of marine fish including the haddock, *Melanogrammus aeglefinus* (Table 2). However, this is the first record of a species of *Myxobolus* been reported from *O. mossambica* in Sri Lanka.

Parasite	Fish Host	Spore shape	Spore dimensions	1	Polar capsule shape	2
M.cerebralis	Salmonids	oval to circular	7.4-9.7x7.0-10.0	+	4.2-6.0x3.0-3.5	5
M.cartilagimis	*Lepomis macrochirus*	sub-spherical	9.5-10.5-8.4-9.5	+	5.2-5.6x3.0-3.5	5
M.dentium	*Esox masquinongy*	elongate-oval	11.8-14.5x5.5-7.3	+	4.5-7.3x1.3x3.2	ND
M.divergens carassii	*Crassius auratus gibelio*	ellipsoid	11.0-12.8x7.3-7.8	+	4.2-5.2x 2.3-2.6	ND
M.eucalii	*Eucalia inconstans*	pyriform	12.0-15.6x8.4-10.8	+	9.6-12.0x3.0-4.8	9
M.filamentosus	*Puntius filamentosus*	ovoid	11.2-17.3x8.1-12.2	+	4.0-7.1x2.0-4.0	5
M.hoffmani	*Pimephales promelas*	round to oval	8.6-10.8x7.8-8.9	+	4.6-5.7-2.2-2.7	Up to 10
M.hyborhynchi	*Hyborhynchus notatus*	oval to sub-spherical	9.1-10.9x7.3-8.6	+	4.1-5.9x2.3-2.5	ND
M.indirae	*Cirrhinus mrigala*	oval to spherical	11.0-14.0x9.0-11.0	+	4.0-6.0x2.0-2.5	8
M.intrachondrealis	*Cyprinus carpio*	elongate ellipsoid	9.0-11.0x6.0-7.0	+	3.7-4.7x2.0-2.6	9

M.nuevoleonensis	*Poecilia* spp.	pyriform	ND	-	ND	10
M. petruschewskii	*Myxocephalus axilliaris*	round	11.0-12.0x11.0-12.0	+	5.5-6.5x2.7-3.6	ND
M.scleroperca	*Perca flavescens*	pyriform	10.0-19.2x7.2-9.6	-	L: 7.2-13.0x2.4-3.6 S: 7.2-12.6-2.4-3.6	ND

Table 1. *Myxobolus* species reported from cartilage of freshwater fish species. The table gives details of spore dimensions and the host they infect [21].

Column: 1 : equality in the size of the polar capsules
+ : same size of polar capsules
– : different size of polar capsules

Column: 2 : number of turns of the polar filament
ND : not determined

Of the freshwater *Myxobolus* species. That develop in the cartilage; *M. cartilaginis*, *M. divergens carassii*, *M. eucalii*, *M. intrachondrealis*, *M. nuevoleonensis*, *M. petruschewskii* and *M. scleroperca* (Guilford, 1963) are species that can be readily discriminated on their spore morphology from *Myxobolus* spp. found in gills of *O. mossambica.*

Parasite	***Fish Host***	**Spore shape**	**Spore body dimensions**	**1**	**Polar capsules size**	**2**
M. aeglefini	*Melanogrammus aeglefinus* *Gadus morhua* *Mova vulgaris* *Cyclopterus lumpus* *Micromesistius poutassou*	Elliptical or sub-spherical	11x 9.2	+	4.5 long	5
***Myxobolus* species found in this study**						
Myxobolus sp.	*O. mossambica*	oval	8.19-9.59x 7.09-7.95	+	2.91-4.5x 2.31-3.18	4-5

Table 2: *Myxobolus* species reported from cartilage of marine fishes and spore dimensions [1].

Column: 1 : equality in the size of the polar capsules
+ : same size of polar capsules
– : different size of polar capsules

Column: 2 : number of turns of the polar filament
ND : not determined

The morphological shape in valvular view, as well as the dimensions of the polar and the number of turns to polar filaments within the capsule are different to those species listed in Table 1 and 2. *M. eucalii* differs in the overall spore length and width. Of the five remaining species, *M. cerebralis* can be ruled out because of the slight differences in the size of the polar capsules and the number of polar filament coils; *M. dentium* can be discriminated from its morphological shape and longer spore body. *M. filamentosus* and *M. hybornhynchi* have longer spore bodies and polar capsules; and finally, *M. indirae* can be discriminated based on the differences in the size of the spore body and the shape of the polar capsules which are much longer. According to Longshaw *et al*. the number of turns of the polar filaments within the polar capsule is higher [22].

Of the marine *Myxobolus* species *M.aeglefini* can be ruled out based on differences in the spore shape and the length of the spore body which is slightly longer than the species recovered from *O. mossambica.*

In conclusion *Myxobolus* species found in the present study does not match any of the descriptions of existing *Myxobolus* species that have a predilection for cartilaginous tissues reported from either in freshwater or marine fishes. Further confirmation requires DNA sequencing of spores and comparison with known sequences of existing *Myxobolus* species.

A negative result in the PCR is normally interpreted as due either to the DNA being concentrated to inhibit the normal PCR reaction or uninfected or low infection rate that is too low to be detected on the gel. The optimal conditions for each PCR vary and depend on the material and region to be sequenced, the primer used and optimisation of conditions normally required. Variations in the proportion and quality of the key agents used in the PCR reaction such as $MgCl_2$, an essential cofactor binding to the DNA and primers, dNTPs and the activity of DNAse polymerase can significantly influence the running of the PCR and the products that are obtained. The annealing temperature is also an important factor in ensuring that the primers anneal to the appropriate target sites.

Further suggestions to improve the PCR reaction would involve adjusting the concentration of some agents like $MgCl_2$, primers, dNTPs and DNAse polymerase. By increasing the concentration of Mg^{2+} in the reaction it may be possible to enhance the activity of the polymerase enzyme. Adjusting other factors (annealing and elongation times,etc) involving changes in the thermocycling conditions may also be useful.

In conclusion, although PCR products have been obtained for *Myxobolus* 18S rDNA gene, further DNA sequencing and its comparison with other sequenced species will confirm whether or not this *Myxobolus* species recorded represents a new species or not.

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