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ANTIBODIES USED FOR AN IMMUNOLOGICAL CROSS-REACTIVITY BETWEEN POLAR TUBE PROTEINS OF *ENCEPHALITOZOON CUNICULI* (MICROSPORIDIA) AND PROTEINS POLAR FILAMENT OF *MYXOBOLUS EPISQUAMALIS* (MYXOZOA) PARASITE OF FLATHEAD MULLET *MUGIL CEPHALUS* (MUGILIDAE).

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ABSTRACT

*Myxobolus episquamalis (*Egusa, Maeno and Sorimachi, 1990), a myxosporean parasite, was found for the first time infecting scales with white cysts and fins of flathead mullet *(Mugil cephalus)* which is a common habitant of tropical and subtropical coastal waters and an important food fish species. Myxozoa are characterized by the presence of polar capsules each containing a coiled polar filament that serves after extrusion to anchor the parasite to the host tissue. This eversible polar filament also resembles the polar tube of Microsporidia, a large phylum of obligate intracellular eukaryotes that are fungal-related parasites. In the present study, we tested the ability of antibodies raised against microsporidian polar tube proteins (PTPs) to cross-react with antigens of *M. episquamalis*. Immunofluorescence assays (IFA) using antibodies directed against PTPs of the mammal microsporidian *Encephalitozoon cuniculi* (Ec102, anti-PTP1/PTP2 and Ec1b,anti-PTP3) showed a strong fluorescence signal associated with extruded polar filaments of *M. episquamalis* spores. In Western blot, these antibodies cross-reacted with antigens of ~50/55 kDa (Ec102) and ~150 kDa (Ec1b) in size, suggesting the existence of common epitopes between microsporidian PTPs and *M. episquamalis* polar filament antigens. Specific mouse polyclonal antibodies were then produced against *M. episquamalis* antigens corresponding to two SDS-PAGE separated protein bands recognized by the antisera Ec102. As expected, these antibodies specifically stained polar filaments in IFA and by immunogold labelling in transmission electron microscopy (TEM).

Keywords: *Myxobolus episquamalis, Mugil cephalus, polar filament, immunofluorescence assays, immunogold labelling, microsporidia, myxozoa.*

RESUME

Utilisation d'anticorps pour une étude immunologique de réaction croisée entre les protéines du tube polaire de *Encephalitozoon cuniculi* (Microsporidie) et les protéines du filament polaire de *Myxobolus episquamalis* (Myxozoa) parasite du mulet *Mugil cephalus* (Mugilidae).

Myxobolus episquamalis (Egusa, Maeno et Sorimachi, 1990), appartient au phylum des Myxozoa. Cette myxosporidie a été identifiée pour la première fois au Japon parasitant les écailles de *Mugil cephalus* qui appartient à la famille des Mugilidae.

Les Myxosporidies et les Microsporidies ont pendant longtemps, été regroupés avec les Actinomyxidies dans le sous embranchement des Cnidospora Dolfein, 1901 avant d'être séparés en deux embranchements différents grâce aux travaux de Grassé (1960). Elles sont toutes les deux caractérisées par la présence d'un système d'ancrage comprenant un tube polaire chez les Microspora et un filament polaire enroulé en spirale à l'intérieur des capsules polaires chez les Myxozoa. Bien que le filament polaire de Myxozoa ne joue pas le même rôle que le tube polaire des Microspora, une homologie structurale de leurs protéines pourrait être envisagée. C'est dans cette optique que nous avons essayé de mettre en évidence des protéines homologues entre le tube polaire des Microspora et le filament polaire des Myxozoa en utilisant des anticorps hétérologues PTP1, 2 ou 3 (Protéine du Tube Polaire) de *Encephalitozoon cuniculi*, contre des antigènes de *Myxobolus episquamalis*. Pour cela, nous avons procédé à des marquages en Immunofluorescence, en Western blot et en Microscope électronique à transmission (MET). Des anticorps polyclonaux anti-*M. episquamalis* ont été produits chez la souris et testés de la même façon. Les résultats montrent qu'en immunofluorescence, en utilisant des anticorps dirigés contre les PTP de *Encephalitozoon cuniculi* (EC102, anti-PTP1 / PTP2 et EC1b, anti-PTP3), un fort signal fluorescent associé au filament polaire de *M. episquamalis* visible sur toute sa longueur en microscopie optique au grossissement X 400 a été noté. Ceci traduit une bonne réaction croisée entre les protéines du filament polaire et ces anticorps.

Les immunomarquages réalisés en Western blot, montrent que ces anticorps utilisés contre les antigènes de *M. episquamalis,* marquent des protéines de ~ 50/55 kDa (EC102) et ~ 150 kDa (EC1b) ce qui suggère l'existence d'épitopes communs entre les protéines du tube polaire (PTP) de *E. cuniculi* et des protéines du filament polaire de *M. episquamalis*.

En microscopie électronique à transmission, la présence des particules denses au niveau des parois internes des capsules polaires ainsi qu'autour des coupes du filament polaire traduisent des réactions croisées entre les protéines de ces deux structures.

Mots clés: *Myxobolus episquamalis, Mugil cephalus, filament polaire, tube polaire, immunofluorescence, immunomarquage, microsporidie, myxozoa.*

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1. Introduction

Myxosporidia Grassé, 1970 are parasites of poikilothermic vertebrates and invertebrates, especially fishes. (Markiw and Wolf 1983; Wolf and Markiw 1984) were the first to discover to *Myxobolus cerebralis* parasite of salmonid fishes, a tubificid annelids as alternate hosts in which the myxosporean undergoes a schizogony and a gamogony, the sporogony ends with a triactinospore. This indirect two-stage life cycle was described in different marine and fish farm species (Rangel and al. 2009, Køie and al. 2013, Eszterbauer and al. 2015). Since, it has been established that, there may be an obligatory or facultative invertebrate host in most, if not all, Myxozoan life cycle. The direct-transmission fish-to-fish was demonstrated for some marine species (Diamant1997, Redondo and al. 2002, Yasuda and al. 2002). In these later cases, not spores, but the proliferative stages are responsible for the transmission of the disease.

According to Canning and al (2014) the cell organization is typically eukaryotic with nucleus, endoplasmic reticulum, mitochondria and Golgi apparatus. Centrioles are not involved in nuclear divisions.

The Myxozoans spores are made with three cell groups: valve spores, polar capsules and sporoplasm or infective stage generally with two nuclei, or two sporoplasms each is containing one nucleus. They can be coelozoic or histozoic. They are extracellular parasites even if intracellular stages were described by (Lom and Arthur 1989).

Polar capsules which are rarely present in somatic cells but omnipresent in spores are essentially characteristic of Myxozoans. Each polar capsule contains a coiled polar filament, which is everted to anchor the spore and allow the sporoplasm to emerge from the spores valves and enter into the host.

The similarities observed between the polar capsules of myxozoan and cnidocysts (nematocysts) of cnidarians led Weill, 1938 to consider myxozoan to be aberrant cnidarians. This hypothesis was supported by ultrastructural studies (Lom and de Puytorac, 1965, Lom, 1969). Phylogenetic analysis of the SSU-rDNA permitted to place Myxozoa within the Metazoa (Smothers and al. 1994, Siddall and al. 1995). Furthermore, other phylogenetic analyses provided evidence of a close relationship between Myxozoa and Cnidaria (Jimenez-Guri and al. 2007, Holland and al. 2011, Nesnidal and al. 2013, Feng and al. 2014).

Some of myxozoans can cause substantial mortalities in fishes. This is the case of *Kudoa thyrsites* that causes soft flesh syndrome in farmed atlantic *Salmo salar* and *Oncorhynchus kisutch salmon* in British Columbia (Whitaker and Kent; 1991, Kent and al, 1994); *Ceratomyxa shasta* a microscopic Myxosporean that parasite the afflicts salmonid fish of the Pacific northwest (Bartholomew and al, 1989); *Tetracapsuloides bryosalmonae* a causative agent of proliferative kidney disease in salmonids (Kumar and al. 2013).

The systematic of Myxosporidian is always made on the basis of morphoanatomical characteristics of the spore, cytological characters and rDNA gene sequence (eg. El-Matbo u li and Hoffmann 1998, Kent and al. 2011, Woo and al. 2014, Fiala and al. 2015). However, biochemical and immunol o gical techniques are increasingly used to develop both a sensitive diagnostic test (Chase and al. 2001, Kikuchi an d al. 2013, Estensoro and al. 2014).

All members of the *Myxobolus* genus possess one sporoplasm with two nuclei, two cell valves and two polar capsules each containing a coiled polar filament required for the attachment to the host and its invasion (Fall and al. 1997, Diamanka and al. 2008).

Several studies were performed on the microsporidian polar tube and to date three different PTPs (PTP1, PTP2 and PTP3) have been described in the microsporidian species infecting mammals *Encephalitozoon cuniculi* (Delbac and al. 1998, Delbac and al. 2001, Peuvel and al. 2002). Concerning the proteins of the polar filament of Myxozoa, few studies were realised.

Chase and al (2014) showed the presence of conserved epitopes of polar filaments of closely related species of *Kudoa*. F-actin was detected in the walls of the polar filament of *Myxobolus pseudodispar* (Uspenskaya and Raikova 2004). Ringuette and al (2011) showed a conservation of polar filament epitopes bands to *M. pendula* and 2 related cnidarians (the anthozoan, *Nematostella vectensis*, and the hydrozoan, *Hydra vulgaris)*.

In our study, we demonstrated the ability of antibodies directed against microsporidian polar tube proteins to cross-react with polar filaments antigens from *Myxobolus episquamalis*, a myxosporean species infecting scales and fins of flathead mulland (*Mugil cephalus*) Then, we produced specific antibodies against *M. episquamalis* polar filament proteins migrating at 50-55 kDa and confirmed the staining of polar filament by immunofluorescent assay (IFA) and by immunogold labelling in transmission electron microscopy (TEM).

2. Materials and methods

2.1 Biological material

Mugil cephalus, (Linné 1758) is a very common fish of Senegalese coasts and displays a great adaptability which enables him to live in very diverse physicochemical characteristics areas: sea, lagoons, lakes and estuaries .It can host various parasites among them *M. episquamalis* belonging to the phylum of Myxozoa. *M. episquamalis* forms compact whitish masses on the distal part of the scales (Fig.1) modifying very appreciably the external texture of fish. Each cyst mass ovoid or spherical and is formed several microcysts visible under magnifying glass.

The parastized fish were caught in the Senegalese coasts and transferred to the parasitology laboratory of animal biology department to be sacrifice. Scales from infected fishes were crushed in PBS (Phosphate Buffer Saline) with a *Potter* homogenizer to release *M. episquamalis* spores. The spore pellet was then washed three times for five minutes in PBS, centrifuged at 20 800 x g, and stored at 4°C.

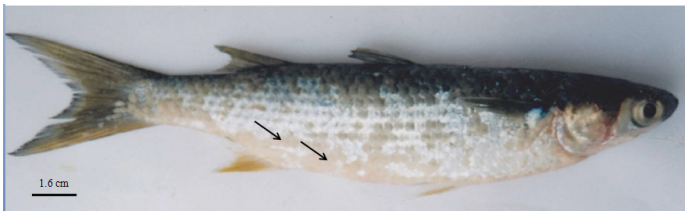

Fig. 1. Flathead mullet (*Mugil cephalus*) with numerous white cysts on its scales (arrows). These cysts contain mature spores of the myxosporean parasite *Myxobolus episquamalis*. Microcysts are 150-400 µm in length and form cystic masses of 7-9 mm x 5-6 mm.

2.2 Indirect immunofluorescence assays (IFA)

Spores of *M. episquamalis* (Fig.2) were smeared on circular cover glasses, lightly air dried and fixed with 100% methanol for 20 min at -80°C. Spores were permeabilized with PBS 0,5% Triton X-100 and saturated with PBS 5% skimmed milk. They were incubated with antibodies diluted at 1/100 in PBS 0,1% Triton X-100 for 1 hour. After washing with PBS 0,1% Triton X-100, they were incubated with a dilution of 1/1000 Alexa 488-conjugated goat anti-mouse IgG. Preparations were then examined with a DMR Leica epifluorescence microscope.

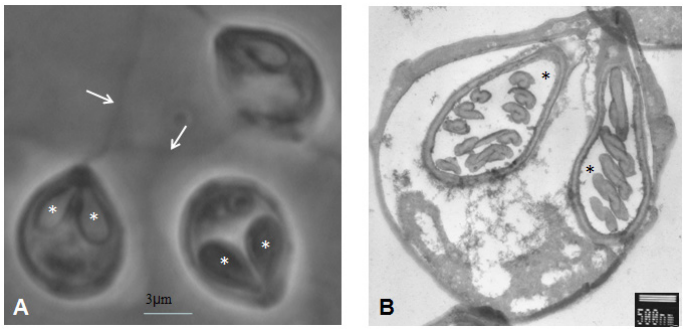

Fig. 2. Mature spores of *M. episquamalis* observed under light (A): X400 and transmission electron (B) microscope. Two polar capsules (asterisks) each containing a polar filament are present in the spores. These polar filaments can be extruded (arrows) from the capsules. Transmission electron microscopy revealed sections of the filament coiled inside each polar capsule

2.3 SDS-PAGE and Western blotting:

For SDS-PAGE and Western blotting, 6.10^7 spores of *M. episquamalis* were used to extract proteins. SDS-PAGE was performed using the method developed by (Laemli 1970). They were disrupted by 10 steps of freezing and thawing in liquid nitrogen and boiled for 15 min to solubilize proteins in a lysis buffer containing 2.5% SDS, 9 M urea and 100 mM DTT (Dithiothreitol). The proteins extract of *M. episquamalis* were separated by SDS-PAGE in denaturant conditions and transferred on polyvinyl difluoride (PVDF) membranes (Millipore). For immunological detection, membranes were saturated in PBS-milk 5% and incubated for 3 h with mouse antibodies (dilution 1:500) directed against polar tube proteins of the mammal microsporidian *Encephalitozoon cuniculi*: Ec102 a monoclonal antibody reacting with both PTP1 and PTP2 (Delbac and al. 1998, 2001), and Ec1b a polyclonal antibody specific of PTP3 (Peuvel and al. 2002). After washing, membranes were reacted for 1 h with horseradish phosphatase alcaline-conjugated goat anti-mouse IgG (1:10,000 dilutions, Promega) and revealed by NBT-BCIP (Promega).

2.4 Antibody production

Polyclonal antibody (PAb) named M1 was produced in SWISS mice from SDS-PAGE separated protein bands recognized by the *E. cuniculi* antisera Ec102. The animal house (agreement C63014.19) and the experimental staff (agreement 63-146) had been approved by the French Veterinary Services and experiments were conducted according to ethical rules. Coomassie-blue stained protein bands were excised and crushed in distilled water with a *Potter* homogenizer. Mice were injected intraperitoneally with samples homogenized with Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the next injections (days 15, 21 and 28). Sera were collected 1 week after the last injection and stored at -20°C

2.5 Immuno-labelling in electronic microscopy

For immunogold labelling in transmission electron microscopy, *M. episquamalis* spores were fixed for 1 hour with 4 % paraformaldéhyde-0.1 % glutaraldéhyde in 0,1M cacodylate buffer, pH 7, 4. After infusion for 1h at room temperature in a 25% glycerol-5% DMSO (dimethylsulfoxide) mixture, the samples were frozen in pasty nitrogen. Ultracut ultramicrotome fitted with the low-temperature sectioning system FC4, were used to do ultrathin frozen sections (90 nm), using a dry sectioning device at -110°C. Sections were mounted on collodion-coated nicked grids and stored in PBS buffer prior to immunolocalization. After saturation for 1 hour with 1% ovalbumine in PBS, ultrathin sections were incubated for 3 hours with various dilutions (1:10 to 1:100) of the primary antibody (M1), then for 1 hour with a 1/100 dilution of goat anti-mouse IgG conjugated with 10 nm colloidal gold particles (Sigma). After washing, ultrathin sections were treated with a v/v mixture of methylcellulose /4% uranyl acetate for 10 minutes, and then observed with a JEOL 1200X transmission electron microscope.

3. Results

3.1. Diseases actions of *M.episquamalis*

M. episquamalis forms compact whitish masses on the distal part of the scales changing very appreciably the external appearance of the fish (Fig.1). Each cystic mass (7-9 mm x 5-6 mm) is ovoid or spherical and is formed of several microcysts (150-400 µm in length) containing *Myxobolus* spores. As described in the figure 2, the myxosporean species *Myxobolus episquamalis* is characterized by the presence of two valves spores, two polar capsules each containing a coiled polar filament and a sporoplasm with two nuclei.

3.2. Immunological cross-reactivity in immunofluorescence assay (IFA)

For detecting a cross reaction between protein polar filament of *M. episquamalis* and protein polar tube of *Encephalitozoon cuniculi* (Microsporidian), we first used the monoclonal antibody Ec102 directed against PTP1 (55 kDa) and PTP2 (35 kDa), of *E. cuniculi* and the polyclonal antibody Ec1b who reacted with a 150 kDa protein band corresponding to PTP3 of *E. cuniculi*. Interestingly, these antibodies produced a strong green fluorescent signal associated with the extruded polar filament of *M. episquamalis* what let suppose a cross-reaction between these antibodies and certain proteins of *M. episquamalis* in IFA (Fig. 3B and 3E). Two nuclei are visualized after DAPI staining (Fig.3C and 3F).

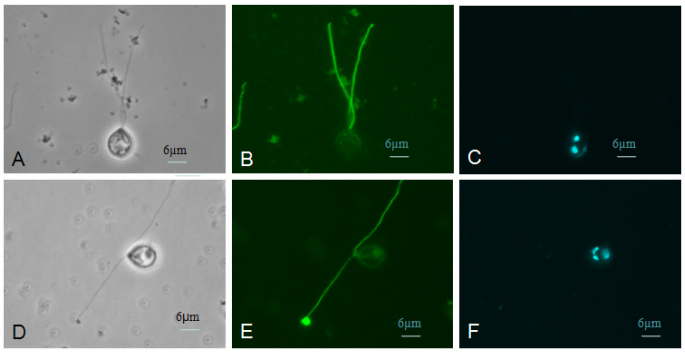

Fig. 3. Immunofluorescence (IFA) of *M. episquamalis* spores using Ec102 (A-C) and Ec1b (D-F) anti-polar tube protein antibodies (X200) . Ec102 is a monoclonal antibody directed against *E. cuniculi* PTP1 and PTP2. Ec1b are polyclonal antibodies reacting with *E. cuniculi* PTP3. Both Ec102 (B) and Ecb1 (E) cross-reacted with extruded polar filaments of *M. episquamalis*. A and D, phase contrast microscopy views; C and F, nuclei staining with DAPI.

3.3. Immunological cross-reactivity in Western blot

These antibodies were then used in Western blot against SDS-PAGE separated proteins of *M. episquamalis* (Fig.4). As shown in figure 4B, Ec1b mainly stained a protein band at ~150kDa whereas Ec102 labelled at least two protein bands migrating at 50-55 kDa in *M. episquamalis* protein lysates. We then injected the two bands around 50-55 kDa (see Fig.4A) to mice in order to produce antibody specific to *M. episquamalis* polar filament antigens. In IFA, the antiserum against the 50-55 kDa protein bands (M1 antibody) specifically reacted with the extruded polar filaments (Fig.4B), but not with polar filaments coiled within the polar capsules.

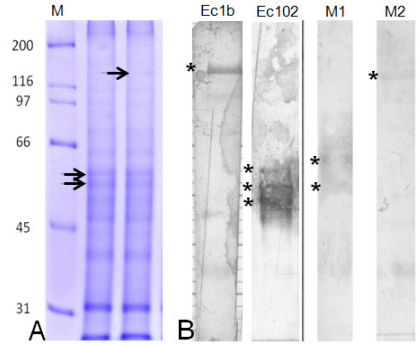

Fig. 4. SDS-PAGE (A) and immunoblotting (B) analysis of proteins from spores of *Myxobolus episquamalis* (12% polyacrylamide gel). A, In Coomassie-blue stained gel, arrows indicate the protein bands excised for mice immunization. B, In Western blot, protein bands reacting with antibodies are indicated by asterisks. The different antibodies correspond to Ec1b (anti-*E. cuniculi* PTP3), Ec102 (anti-*E. cuniculi* PTP1/PTP2), M1 (anti-50/55 kDa bands of *M. episquamalis*) and M2 (anti-150 kDa band of *M. episquamalis*). Sera were tested at 1:500 dilutions. M

3.4. Immunological cross-reactivity in microscopy electronic transmission (MET)

Immunolocalization experiments were also carried out at the ultrastructural level on thin sections *M. episquamalis* spores. As illustrated in figures 5C-D, electron-dense gold particles were distributed over cross sections of the intrasporal polar filament coiled within polar capsules and to the polar capsule walls.

4. Discussion

In the past, Myxosporidia were placed together with the Microsporidia in a new class of Protozoa called Cnidosporidia (reviewed in Lom, 1990. More recently, Microsporidia were reconsidered as fungal-related parasites (Thomarat and al. 2004) and Myxozoa as highly degenerate members of Cnidaria (Jimenez-Guri and al. 2007, Holland and al. 2011, Nesnidal and al. 2013, Feng and al. 2014).

One of the character resemblances between these phyla is the presence of a polar filament/tube coiled inside the spore and having a role in the infection machinery. After discharge, the microsporidian polar tube is used to inject the infectious spore content within the host cell whereas the myxosporean polar filament serves to anchor the parasite to the host cell tissue.

The chemical composition of the microsporidian polar tube was investigated in many species (reviewed in Weiss and al. 2014). Three main proteins referred to as PTP1, PTP2 and PTP3 were characterized in *Encephalitozoon cuniculi*, and orthologous proteins were then found in other microsporidian species. *E. cuniculi*, PTP1 (*i.e.* EcPTP1) is a proline-rich protein of 395-amino-acid in length, with a predicted molecular mass of 37 kDa and an acidic pI and characterized by the presence of 4 repeat units of 26 amino acids (Delbac and al. 1998); EcPTP2 is a 277-amino-acid basic protein, rich in lysine residues with a predicted molecular mass of 30 kDa (Delbac and al. 2001). EcPTP3 is a 1256-amino-acid protein, rich in glutamic acid, alanine, valine and lysine residues with a predicted molecular mass of 136 kDa (Peuvel and al. 2002). However, all these PTPs are subjected to post-translational modifications, including glycosylation, which increase their apparent molecular masses on electrophoresis gels as 55kDa for EcPTP1, 35kDa for EcPTP2 and 150 kDa for EcPTP3. Experiments using cross-linkers and yeast two-hybrid analysis revealed that these three PTPs can interact with each other during polar tube assembly, and this process would depend on disulfide linkages (Peuvel and al. 2002, Bouzahzah and al. 2010).PTP3 lacks cysteine residues whereas both PTP1 and PTP2 contain a high number of cysteines and have been shown to be solubilized in presence of high concentration of reducing agents. For these reasons, we used 100 mM DTT to solubilize the polar filaments of *M. episquamalis*. In particular, the myxosporean protein bands migrating at 50-55 kDa (Fig. 4) and demonstrated to correspond to polar filament antigen(s) (Fig. 5) were only solubilized in presence of DTT, suggesting that disulfide bridges would also play an essential role in stabilizing myxosporean polar filament components.

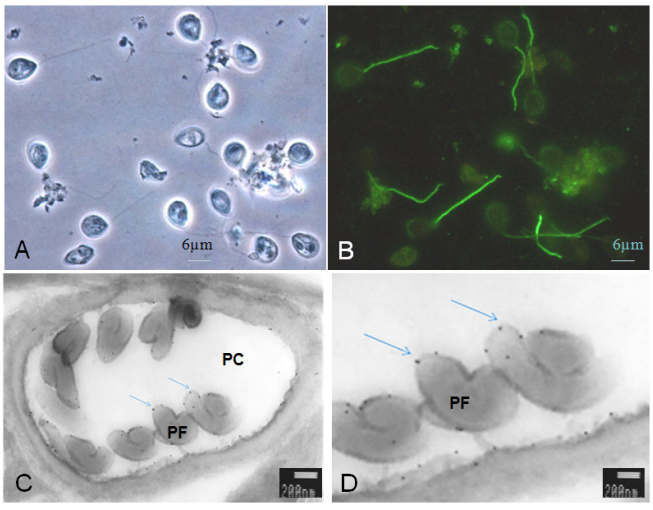

Fig. 5. Immunolabelling of *M. episquamalis* polar filaments in IFA (A-B) and transmission electron microscopy (C-D) using M1 antibodies directed against protein bands migrating at 50-55 kDa. In IFA, the extruded polar filaments are strongly labelled (B). A corresponds to phase contrast microscopy view. In electron microscope immunolabelling (C-D), gold particles are associated with the internal polar filament coiled inside a polar capsule (arrows). D corresponds to an enlargement of C. PF: Polar filament; PC: Polar capsule.

The molecular composition of the polar filament of Myxozoa is however more limited. Chase and al. (2001) generated several monoclonal antibodies (mAbs) against purified spores of *Kudoa thyrsites*, a myxozoan infecting muscle tissue of marine fishes. In immunofluorescence assays, the authors showed that two mAbs stained polar capsules and polar filaments of *K. thyrsites* and also cross-reacted with polar filaments of the closely related species *K. paniformis* and *K.crumena*. In Western blot, the mAb 3E8 specific of the polar filament reacted with protein bands of approximately 64 kDa in *K. thyrsites* and 90 to 120 kDa in *K. paniformis*, and to a doublet at 66 kDa in *K. crumena*. These cross-reactions argue for the presence of conserved epitopes between polar filament proteins of closely related species. Other components were also characterized, including F-actin that was detected in the walls of the polar filament of *Myxobolus pseudodispar* (Uspenskaya and Raikova, 2004). More recently, Ringuette and al (2001) generated a rabbit polyclonal antiserum (pAbMPPF) against the polar filament of the myxozoan *Myxobolus pendula*. In western blot, some major bands located at 35, 40, 42, 55, 70, 100, and 200 kDa were detected in *M. pendula* while a major band located at 40 kDa and 3 minor bands of 48, 70, and 130 kDa in size were detected in the closely-related species *M. bartai*. A cross-reactivity was also observed with protein bands at 15 and 165 kDa in the intestinal myxozoan parasite *Enteromyxum leei* (Estensoro and al. 2013).These results indicated once again a conservation of epitopes between myxozoan polar filament proteins. More interestingly, pAbMPPF cross-reacted in IFA with the polar filament of one type of nematocyst of the anthozoan *Nematostella vectensis* and the hydrozoan *Hydra vulgaris*, two related cnidarians. However, western blot analysis revealed an absence of immune-reactivity to nematocyst extracts, suggesting that this polyclonal antisera recognized conformational epitopes. As preliminary data, Ringuette and al (2001) indicated that the proteins recognized by the polyclonal antiserum pAbMPPF contain glycine-rich hydrophobic domains with sequence similarity to spinalin. Spinalin is a 24-kDa glycine- and histidine-rich protein found in spines of Hydra nematocysts, with regions with partial homology to loricrins and keratins. Spinalin showed a high tendency to associate into dimers both *in vitro* and *in vivo* (Koch and al. 1998, Hellster and al. 2006).

In our study, we used two different antibodies directed against polar tube proteins of the mammal microsporidian *Encephalitozoon cuniculi*: the monoclonal antibody Ec102 that reacts with three E. *cuniculi* protein bands at 55 kDa (EcPTP1), 35 kDa (EcPTP2) and 28 kDa (undetermined protein) and the polyclonal antibody Ec1b that reacts with the 150 kDa EcPTP3. Immunofluorescent assays (IFA) using these antibodies revealed a strong fluorescence signal associated with extruded polar filaments of *Myxobolus episquamalis* spores, suggesting the existence of common epitopes between microsporidian PTPs and *M. episquamalis* polar filament antigens. In Western blot, Ec102 and Ec1b cross-reacted with antigens of sizes comparable to EcPTP1 and EcPTP3: ~50/55 kDa for Ec102 and ~150 kDa, for Ec1b.. Some polar filament antigens with similar sizes (55 kDa in *M. pendula*, 120 kDa in *K. paniformis* have been also reported in other myxosporean species (Chase and al. 2001, Ringuette and al. 2011). To confirm our results, we then produced specific mouse polyclonal antibodiy against *M. episquamalis* antigens corresponding to two SDS-PAGE separated protein bands of 50-55 kDa in size recognized by the antisera Ec102. As expected, a specific labelling of the polar filament was observed by IFA and by immunogold staining in TEM (see figs. 4 and 5).

In conclusion, our study revealed immunological cross-reactivities between microsporidian PTPs and polar filament antigens from the myxosporean *Myxobolus episquamalis*, suggesting some conserved protein motifs between these antigens. However, no polar filament protein sequence with similarity to microsporidian PTPs has been identified so far. Basic Local Alignment Search Tool (BLAST) search against the fully sequenced genomes of the Myxozoa *Thelohanellus kitauei* (Yang and al. 2014) and of two free cnidarians *Nematostella vectensis* (Putnam and al. 2007) and *Hydra magnipapillata* (Chapman and al. 2010, Steele, 2012) also failed to identify protein coding genes having homology with *E. cuniculi* PTP1 and PTP2. A possible strategy to characterize some *M. episquamalis* polar filament antigens at the molecular level would be to analyze by mass spectrometry the 50-55 kDa proteins after separation by one or two-dimensional gel electrophoresis, as it has been done for the identification of the *E. cuniculi* 55 kDa PTP1 (Delbac and al. 1998).

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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CAPTIONS

Fig.1. Flathead mullet (*Mugil cephalus*) with numerous white cysts on its scales (arrows). These cysts contain mature spores of the myxosporean *Myxobolus episquamalis*. Microcysts are 150-400 µm in length and form cystic masses of 7-9 mm x 5-6 mm.

Fig.2. Mature spores of M. *episquamalis* observed under light (A) and transmission electron (B) microscope. Two polar capsules (asterisks) each containing a polar filament are present in the spores. These polar filaments can be extruded (arrows) from the capsules. Transmission electron microscopy reveals sections of the filament coiled inside each polar capsule.

Fig.3. Immunofluorescence (IFA) of *M. episquamalis* spores using Ec102 (A-C) and Ec1b (D-F) anti-polar tube protein antibodies. Ec102 is a monoclonal antibody directed against *E. cuniculi* PTP1 and PTP2. Ec1b are polyclonal antibodies reacting with *E. cuniculi* PTP3. Both Ec102 (B) and Ecb1 (E) cross-reacted with extruded polar filaments of *M. episquamalis*. Sera were tested at 1:100 dilutions. A and D, phase contrast

microscopy views; C and F, nuclei staining with DAPI.

Fig.4. SDS-PAGE (A) and immunoblotting (B) analysis of proteins from spores of *Myxobolus episquamalis* (12% polyacrylamide gel). A, in Coomassie-blue stained gel, arrows indicate the protein bands excised for mice immunization. B, in Western blot, protein bands reacting with antibodies are indicated by asterisks. The different antibodies correspond to Ec1b (anti-*E. cuniculi* PTP3) and Ec102 (anti-*E. cuniculi* PTP1/PTP2). Sera were tested at 1:500 dilutions. M: molecular weight markers in kDa.

Fig.5. Immunolabelling of *M. episquamalis* polar filaments in IFA (A-B) and transmission electron microscopy (C-D) using M1 antibodies directed against protein bands migrating at 50-55 kDa. In IFA, the extruded polar filaments are strongly labelled (B). A corresponds to phase contrast microscopy view. In electron microscope immunolabelling (C-D), gold particles are associated with the internal polar filament coiled inside a polar capsule (arrows). D corresponds to an enlargement of C. Sera were tested at 1:100 and 1:10 dilutions in IFA (B) and TEM (C and D), respectively. PF: Polar filament; PC: Polar capsule.