Vol. 106: 149–162, 2013
doi: 10.3354/dao02651

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 11

Antigenic characterization of *Enteromyxum leei* (Myxozoa: Myxosporea)

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ABSTRACT: *Enteromyxum leei*, an intestinal myxozoan parasite affecting a wide range of fish, was partially purified, and the immunogenic composition of its glycoproteins as well as the proteolytic activity were studied. Parasite extracts, mainly containing spores, were separated by SDS-PAGE, and thereafter, immunoblots were carried out with a polyclonal antiserum (Pab) raised against *E. leei*. Periodic acid/Schiff staining of gels, periodate- and Proteinase K-treated Western blots and Lectin blots were performed to analyse the terminal carbohydrate composition of the parasite's antigens. Additionally, the cross-reaction of the parasite extracts with a Pab raised against the polar filament of the myxozoan *Myxobolus pendula* was studied. Both Pabs detected proteic epitopes on antigenic proteins and glycoproteins of *E. leei*, ranging between 15 and 280 kDa. In particular, 2 glycoproteic bands (15 and 165 kDa), immunoreactive to both Pabs and with glucose and mannose moieties, could correspond to common antigens shared among myxozoans. The 165 kDa band also presented galactose, N-acetyl-galactosamine and N-acetyl-glucosamine, pointing to its possible origin on chitin-built spore valves and to its possible involvement in host–parasite interactions. The molecular weight of the 15 kDa glycoproteic antigen matches that of minicollagen, a cnidarian-specific protein of nematocysts with a myxozoan homologue. Several proteases with apparent molecular weights ranging between 43 and 245 kDa were found in zymographies of *E. leei* extracts, and these may have a potential role in the parasite's pathogenesis. This is a useful approach for further studies to detect targets for antiparasitic therapy.

KEY WORDS: Western blot · Polyclonal antibody · Glycoprotein · Lectin · Protease · Parasite

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INTRODUCTION

Intensively cultured fish are susceptible to parasitic infections, a primary concern for the aquaculture sector (Mennerat et al. 2010). Among fish parasites, the members of the myxozoan genus *Enteromyxum* infect intestines, mainly of marine fish. They cause severe enteritis that results in weight loss, poor feed conversion, delayed growth and even death (Rigos & Katharios 2010, Sitjà-Bobadilla & Palenzuela 2012). *E. leei* affects a wide range of hosts (Sitjà-Bobadilla & Palenzuela 2012), including gilthead sea bream (GSB) *Sparus aurata*, the most important marine finfish in Mediterranean aquaculture (APROMAR 2012). In this sparid, *E. leei* provokes a chronic intestinal infection with epithelial destruction and intense inflammatory reaction leading to emaciation, anemia and mortalities (Fleurance et al. 2008, Sitjà-Bobadilla et al. 2008, Estensoro et al. 2011).

Little is known about the antigen composition of this parasite, and this is a key aspect for the understanding of parasite infectivity and survival in the host. No efficacious treatments are available for enteromyxoses (Bermúdez et al. 2006, Golomazou et al. 2006, Yokoyama & Shirakashi 2007), and thus it is important to identify potential targets for the development of chemotherapeutants and vaccines. Previous lectin histochemistry studies have shown that the

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different *Enteromyxum leei* stages have different surface-associated carbohydrate moieties (Redondo & Álvarez-Pellitero 2009). Such terminal sugar residues are believed to play a paramount role in host–parasite interactions and may form a protective sheath for the parasites that helps them to evade host recognition (Hicks et al. 2000, Theodoropoulos et al. 2001). Therefore, some parasites, such as *Trypanosoma cruzi* (Buscaglia et al. 2006), employ an antigen variation strategy through modification of N- and O-glycosides of glycoepitopes in their surface glycoproteins (Gagneux & Varki 1999, Knaus & El-Matbouli 2005). In fish, as in all vertebrates, the lectin pathway of the complement system is an ancient first line defense mechanism of the innate immune system that relies on recognition of pathogen-associated glycan epitopes (Sunyer & Lambris 1998, Nakao et al. 2006, Kania et al. 2010). In previous studies, the glycoproteins of some piscine parasites have been studied by lectin blotting (Feng & Woo 1998b, Kim et al. 1999, Muñoz et al. 2000a, Knaus & El-Matbouli 2005).

Another approach to studying the antigenicity of myxozoans is the use of polyclonal antibodies (Pabs) (Bartholomew et al. 1989, Saulnier & deKinkelin 1996, Muñoz et al. 1999b, 2000a, Lu et al. 2002, Knaus & El-Matbouli 2005, Zhang et al. 2010). In the present study, the antigenic glycoproteins of *Enteromyxum leei* were analysed using 2 Pabs; one raised against *E. leei* and another raised against the polar filament of *Myxobolus pendula* (Ringuette et al. 2011). The latter Pab was chosen to check its possible cross-reaction with *E. leei* antigens and for its proven capacity to detect common myxosporean and other cnidarian antigens.

Parasitic invasion mechanisms imply penetration and colonization of host tissues. Proteolytic enzymes generated by parasites are often involved in such processes, as studied for the myxozoans *Kudoa* sp. (Martone et al. 1999, Funk et al. 2008), *Myxobolus cerebralis* (Kelley et al. 2004, Dörfler & El-Matbouli 2007) and *Sphaerospora dicentrarchi* (Muñoz et al. 2000a), as well as for other piscine parasites (Zuo & Woo 1998, Paramá et al. 2004, Piazzón et al. 2011). There is no current knowledge about the mechanisms that *Enteromyxum leei* employs to disrupt cell junctions between enterocytes, to penetrate and invade the intestinal epithelium and to migrate along the digestive tract. Parasite-derived proteinases may play an important role in pathogenesis as well as in lesion formation and evasion of the host immune response in enteromyxosis due to *E. leei*, and thus they may represent targets for antiparasitic chemotherapy (McKerrow et al. 1993).

The antigenic characterization of *Enteromyxum leei* is an issue of great importance with little available information. Here we probed the reactivity of *E. leei* antigens with a set of lectins to determine its terminal carbohydrate composition, and with 2 different antibodies. Additionally, the proteolytic activity of *E. leei* antigen preparations was determined. Such information may help to develop further studies aiming to select parasite antigens with immunopreventive potential against enteromyxosis.

MATERIALS AND METHODS

Fish and parasite extracts

Parasite-free and clinically healthy GSB from a commercial fish farm were kept in 5 µm filtered and UV irradiated sea water at temperatures always above 18°C. Some fish were used as control (C) and others as recipient fish (R) for *Enteromyxum leei* experimental infections (see Sitjà-Bobadilla et al. 2007, Estensoro et al. 2010). All efforts were made to minimize suffering of the fish used for the experiments in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and current European Union legislation on handling experimental animals.

C and R fish were starved for 2 d, euthanized by overexposure to MS-222 (Sigma) and bled by caudal puncture to avoid sample contamination by blood cells. Infective status of R intestines was checked by light microscopy observation of fresh smears. Intestines were opened lengthwise under sterile conditions and the mucosa lightly scraped with a scalpel. The intestinal scrapings of 7 C and 7 intensively infected R fish were pooled separately, homogenized in sterile HBSS (Gibco-Life Technologies) with an antibacterial and antimycotic mixture (10 000 units ml^{-1} of penicillin, 10 000 µg ml^{-1} of streptomycin and 25 µg ml^{-1} of Fungizone, ® Gibco-Life Technologies) using a syringe, and the remaining cell aggregations and debris were retained by a 40 µm cell strainer (BD). The parasite/cell suspensions were then centrifuged at 2200 × *g* (10 min at 4°C) and the pellets resuspended in lysis buffer (Tris-HCl 0.1 M, $MgCl_2$ 0.05 M, 1% Triton X-100, sucrose 0.3 M). Suspensions were then centrifuged at 1100 × *g* (5 min at 4°C) and the pellet resuspended in ether:HBSS (1:2) before a subsequent centrifugation at 2500 × *g* (5 min at 4°C). Antigen pellets from R pools contained mainly spores and unlysed disporoblasts (about 66% of the parasite extract) together with some small-sized cel-

lular debris, and those from C pools contained intestinal epithelium cellular debris. They were collected separately and washed twice in cold HBSS (2500 × *g*, 5 min at 4°C).

Parasites contained in antigen preparations were counted with a haemocytometer. The protease inhibitor cocktail cOmplete ULTRA Tablets (® Roche Diagnostics) was added to the protein extracts. After determining their total protein content via Bradford staining (Bio-Rad), the extracts were aliquoted and stored at −20°C until used. The parasite extracts obtained from R fish also contained host cells because complete parasite purification was not achieved. Therefore, all subsequent analyses aimed to detect parasite bands present in R intestinal extracts that were absent in C intestinal extracts.

SDS-PAGE

C and R antigen preparations were boiled for 5 min and then an equal volume of reducing SDS-PAGE sample buffer (0.62 Tris-HCl, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 0.1 M DTT) was added. They were boiled again for 5 min, centrifuged (13 000 × *g*, 30 min at 4°C), and the supernatants containing the soluble protein fraction were recovered and stored at −20°C until used. For the preparation of native antigens, extracts were not denatured by boiling, and non-reducing sample buffer lacking DTT was used. Thereafter, antigen preparations were separated by SDS-PAGE either on 12% or 5% polyacrylamide gels at 180 V for approximately 50 min. Gels were used either to visualize proteic antigen bands (Coomassie staining), to visualize glycoproteic antigen bands (Periodic acid/Schiff staining, PAS) or used for further blotting. In all gels, prestained broad range molecular weight (MW) standards (Bio-Rad; 7–209 kDa) were loaded.

For protein staining, 0.25% Coomassie brilliant blue R-250 (IBF Pharmindustrie Rèactifs) in 40% methanol, 10% acetic acid was employed, followed by washing with 40% methanol, 10% acetic acid. For PAS staining, gels were fixed in 25% isopropyl alcohol, 10% acetic acid overnight; then in 10% isopropyl alcohol, 10% acetic acid for 2 h; 0.5% periodic acid for 2 h; 0.5% sodium arsenate, 5% acetic acid for 40 min; 0.1% sodium arsenate, 5% acetic acid for 20 min, twice; and acetic acid for 20 min. Gels were then stained with Schiff's reagent overnight and washed with 0.1% sodium metabisulphite in 0.01 N HCl several times until the rinse solution was no longer coloured pink.

Polyclonal antibodies

The production and characterization of the Pab raised against *Enteromyxum leei* (PabEleei) is described in a previous work (Estensoro et al. 2013). This antiserum was adsorbed with normal GSB intestinal scrapings to avoid background noise due to host cell detection. The adsorbed PabEleei (aPabEleei) was used in Western blots for the current antigenic study.

Before blotting, the Pab against the polar filament of *Myxobolus pendula* (PabMPPF) was checked for cross-reactivity with *Enteromyxum leei* on paraffin histological sections of infected GSB intestines. Routine immunohistochemical procedures were applied as described by Estensoro et al. (2012) and sections eventually counterstained with Gill's haematoxylin, dehydrated and mounted in di-N-butyl-phthalate in xylene (DPX). PabMPPF was obtained and kindly provided by Ringuette et al. (2011) and is directed against an evolutionary conserved glycoepitope of polar filaments detected in 3 *Myxobolus* species as well as in some cnidarian nematocysts.

Western blots

The antigen proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (0.45 µm pore size; Bio-Rad) at 250 mA for 1 h. Membranes were blocked with 5% skimmed milk (Merck) in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2) overnight at 4°C and washed 10 min with TTBS (0.05% Tween 20 in TBS). All washing procedures consisted of successive 10 min immersions in TTBS, and all washing and incubation steps were performed at room temperature (RT) with gentle shaking. After washing, membranes were incubated for 2 h either with Pabs or with biotinylated lectins and washed again 4 times. Detailed information about the used Pabs and lectins can be found in Table 1. Secondary incubations for Western blots were performed with goat anti-rabbit horseradish peroxidase-conjugated antibody (1:2000; Sigma) and for lectin blots with avidin-biotin-peroxidase complex (VECTOR Laboratories) for a further 2 h. Blots were washed 4 times and finally rinsed in TBS before bound peroxidase was visualized by addition of 3,3′-diaminobenzidine tetrahydrochloride chromogen (DAB; Sigma) for 2 min. The reaction was stopped with deionised water and blots were allowed to dry. Reactive antigen bands in gels and blots were visualized with a Multi-Image light cabinet (Alpha Innotech Corporation) and digitally analysed with Quantity-One Quantitation Software (Bio-

Table 1. Detector molecules used in Western blots. Ab: antibody; GalNac: N-acetylgalactosamine; Gal: galactose; Glc: D-glucose; GlcNAc: N-acetylglucosamine; Man: mannose; NeuNAc: N-acetylneuraminic acid; Fuc: Fucose; IATS: Instituto de Acuicultura Torre de la Sal

Detector molecule (antibody or lectin)	Abbreviation	Binding specificity	Dilution used	Source
Rabbit polyclonal Ab anti-*Enteromyxum leei*	aPabEleei[a]	Unknown	1:500	IATS
Rabbit polyclonal Ab anti-polar filament	PabMPPF[b]	Non-collagenous glycoprotein of *Myxobolus pendula*	1:30	University of Toronto
Canavalia ensiformis-Agglutinin	ConA	Manα-1 > Glcα-1 > GlcAcα-1	2 µg ml^{-1}	Sigma
Bandeiraea simplicifolia-Lectin	BSL I	D-Gal > D-GalNac	5 µg ml^{-1}	Sigma
Glycine max-Agglutinin	SBA	Terminal α,βGalNac > α,βGal	5 µg ml^{-1}	Vector Lab.
Triticum vulgaris-Agglutinin	WGA	GlcNAc (β1,4GlcNAc)$_{1-2}$ > β1,4GlcNAc > NeuNAc	10 µg ml^{-1}	Vector Lab.
Sambucus nigra-Agglutinin	SNA	NeuAc-α2,6Gal = NeuAcα2,6GalNAc	20 µg ml^{-1}	Vector Lab.
Ulex europaeus-Agglutinin	UEA	L-Fucα1,2Galβ1,4	20 µg ml^{-1}	Sigma

[a]This antibody was obtained and titred by the Fish Pathology group, IATS-CSIC, Spain (Estensoro et al. 2013); [b]This antibody was produced by the Department of Cell and Systems Biology, University of Toronto, Canada (Ringuette et al. 2011)

Rad). Band sizes were inferred from the linear range of the migration of MW standards.

Periodate oxidation

Periodate oxidation was carried out as described by Woodward et al. (1985) to demonstrate the carbohydrate nature of the epitopes recognized by the Pabs. Following the blotting, membranes were rinsed with 0.05 M sodium acetate buffer (pH 4.5) and incubated for 1 h in the dark at RT with graded periodate dilutions (0.1 mM; 1 mM; 10 mM) in sodium acetate buffer. Blots were then rinsed with sodium acetate buffer, incubated with 0.05 M sodium borohydride in phosphate-buffered saline for 30 min at RT and washed 5 min with TTBS and 5 min with TBS. Thereafter, membranes were blocked, exposed to the primary and secondary antibodies, exposed to DAB and finally dried and visualized as described in the previous section.

Proteinase K digestion

Blots were digested with Proteinase K to confirm the polypeptide nature of the antigen epitopes detected by the Pabs, as described by Feng & Woo (1998a). Briefly, blotted membrane strips were incubated for 1 h at 37°C with Proteinase K (0.2 µg ml^{-1}, 2 µg ml^{-1}, 20 µg ml^{-1}) in Tris-HCl (50 mM, pH 8) and then washed in TTBS and 12% trichloracetic acid to inactivate the proteinase. Thereafter, membranes were blocked, incubated with the primary and secondary antibodies, exposed to DAB and finally dried and visualized as described in the previous section.

Zymography

The proteolytic activity of the antigen preparations was tested by separating them through SDS-PAGE in 10% polyacrylamide gels co-polymerized with 0.2% gelatine. Cell extracts employed therefore were native and not reduced, thus, not boiled and did not contain DTT in the SDS-PAGE sample buffer. After electrophoresis, gels were washed in 2.5% Triton X-100 (Sigma) for 1 h to remove SDS and restore proteolytic activity, rinsed with 0.1 M phosphate buffer (pH 5.5) and then incubated with this same buffer for 12 h at 37°C to allow proteolysis. Finally, gels were stained with 0.25% Coomassie brilliant blue R-250 and visualized with a Multi-Image light cabinet (Alpha Innotech Corporation) as previously described. Proteases appeared as clear bands on the blue background where digestion of co-polymerized gelatine occurred.

RESULTS

SDS-PAGE: Coomassie and PAS

The parasite content of R extracts ranged between 6.1×10^6 and 4.1×10^7 parasite stages ml^{-1} and their total protein content ranged between 297 and 904 mg ml^{-1}. In Coomassie brilliant blue R-250

stained gels (Fig. 1, Lanes A–D), denatured and reduced parasite extracts showed R proteic bands of MWs ranging between 10 and 49 kDa. The detected R antigenic bands were 3 intense bands of low MW (10, 15 and 17 kDa) and 3 weakly stained bands of 30, 31 and 42 kDa, respectively. Except the 42 kDa band, all of these R bands were absent in C extracts. Native R extracts presented a similar band pattern with a slightly lower relative mobility (native proteic bands: 11, 16, 18.5, 31, 33 and 49 kDa).

In PAS-stained gels (Fig. 1, Lanes E,F), C extracts did not show PAS-positive bands, whereas in R parasite extracts, a single high-MW glycoprotein band of 193 kDa was weakly stained. The MW of this glycoproteic band was confirmed in 5% polyacrylamide gels.

Western blots

The immunoblots with aPabEleei showed several immunoreactive bands in R antigen preparations (Fig. 2, Lane B). In reduced and denatured R extracts, aPabEleei detected reactive bands with MWs of 15, 23, 42, 67 and 165 kDa together with an intense smear ranging from 24 to 37 kDa. In native R extracts, aPabEleei detected only 30 and 43 kDa reactive bands as well as a weakly stained 33 kDa reactive band. In C antigens, this antibody detected only 29 and 42 kDa reactive bands.

The immunoblots with PabMPPF showed reactive antigens between 15 and >209 kDa in R reduced and denatured extracts (Fig. 2, Lanes E,F). Reactive bands had MWs of 15, 29, 42, 154 and 166 kDa, and a reactive smear of >209 kDa (mean MW 280 kDa) was also detected. Only a 42 kDa band was detected in C extracts. Using immunohistochemistry, this PabMPPF clearly stained primary and secondary cells of proliferative parasite stages and polar filaments, polar capsules, spore valves and accompanying cells of sporogonic stages of *Enteromyxum leei* (Fig. 3). No label was detected either in negative control slides omitting primary antibody, secondary antibody and avidin-biotin-peroxidase complex, respectively, or in non-parasitized tissues. For comparative purposes, an image of the staining obtained with aPabEleei is also provided (Fig. 3).

Terminal sugar moieties of glycoproteic bands were determined with 6 biotinilated plant lectins, which detected a broad array of reactive bands in lectin blots, some of them exclusive of R extracts. The lectin *Canavalia ensiformis*-Agglutinin (ConA) detected mannose/glucose (Man/Glc) residues in glycoproteic bands of 15, 27, 31, 33, 34, 36, 39, 46, 49, 165 and 311 kDa, which were all absent in C extracts (Fig. 4, Lanes B,C). *Bandeiraea simplicifolia*-Lectin (BSL I) detected galactose/N-acetyl-galactosamine (Gal/GalNac) moieties in 2 exclusive bands of R extracts with MWs of 148 and 165 kDa (Fig. 4, Lanes D,E). *Glycine max*-

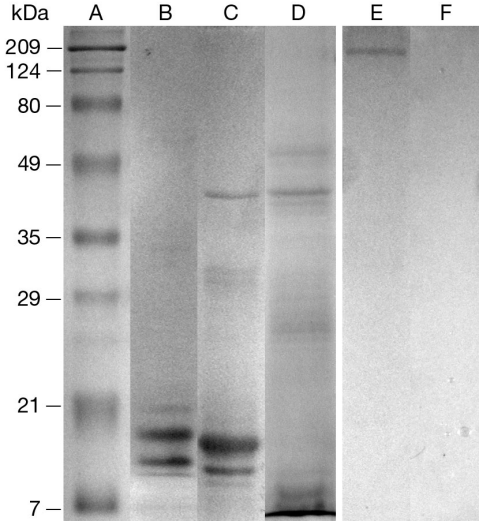

Fig. 1. SDS-PAGE soluble protein profile of parasite extracts of *Enteromyxum leei* (Lanes B, C, E) and host tissue extracts (Lanes D, F) stained with R-250 Coomassie brilliant blue (Lanes A-D) and PAS (Lanes E-F). Lane A, molecular weight standards; Lane B, native *E. leei* extract; Lanes C and E, denatured and reduced *E. leei* extract; Lanes D and F, denatured and reduced host tissue extracts

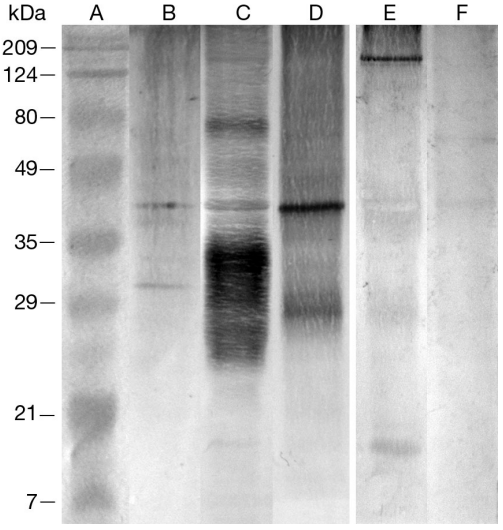

Fig. 2. Western blot detection of *Enteromyxum leei* (Lanes B, C, E) and host tissue (Lanes D, F) extracts with the polyclonal antibody raised against *E. leei* (Lanes B–D) and by the polyclonal antibody against polar filament epitopes of *Myxobolus pendula* (Lanes E, F). Lane A is the molecular weight standard

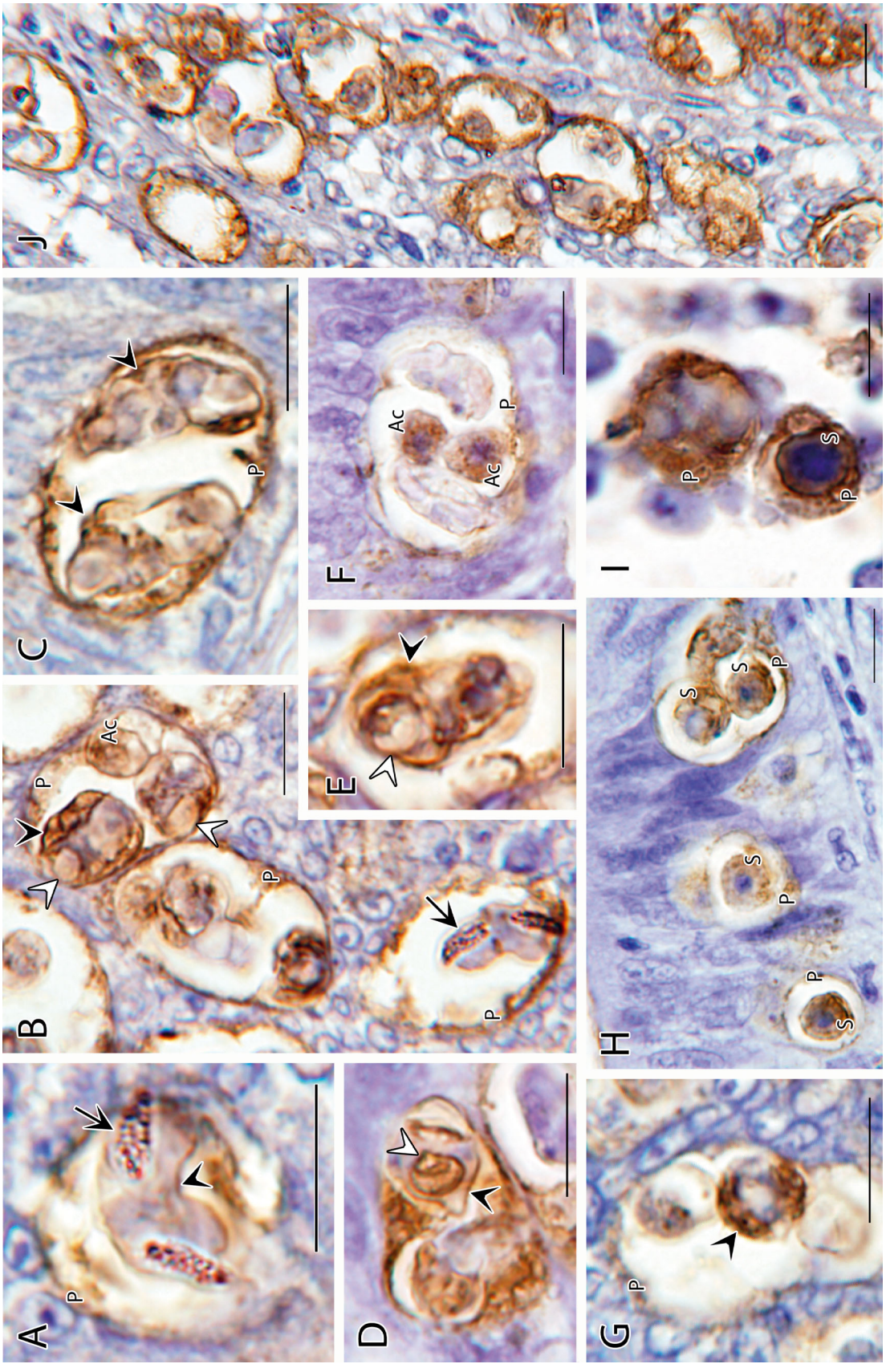

Fig. 3. Immunohistochemical labelling of *Enteromyxum leei* with the polyclonal antibody (Pab) against polar filament epitopes of *Myxobolus pendula* (A–I) and with the Pab against *E. leei* (J) in infected intestinal gilthead sea bream paraffin sections counterstained with haematoxylin. Immunoreactive parasite components are indicated by arrows (polar filament), black arrowheads (valves and valvogenic cells), white arrowheads (polar capsules). P: primary cells; S: secondary cells; Ac: accompanying cells. (A) Front view of a spore in a disporoblast (DEB). (B) Differently orientated DEB with visible valves, polar filaments, polar capsules, P and Ac. (C) DEB with lateral view of the 2 spores with immunoreactive valves and P. (D) Polar capsule and valve of a spore in a DEB. Note that the typical protrusion of the spore valve of this species is visible. (E) Cross section of a polar capsule in a spore within a DEB. (F) Two Ac in a DEB. (G) Intensively stained valvogenic cell/s of an immature spore within a DEB. (H) Intraepithelial proliferative stages with labelled P and S. (I) P and S cells of free proliferative stages in the intestinal lumen after epithelial desquamation. (J) Parasite labelling with the Pab against *E. leei*. Scale bars = 10 µm

Agglutinin (SBA) reacted with N-acetyl-galactosamine/galactose (GalNac/Gal) residues in 2 high-MW bands only present in R extracts, 164 and 271 kDa (Fig. 4, Lanes F,G). With *Triticum vulgaris*-Agglutinin (WGA), 3 glycoprotein bands of 158, 165 and 239 kDa containing N-acetyl-glucosamine/neuraminic acid (GlcNac/NeuNac) were visualized exclusively in R extracts (Fig. 4, Lanes H,I). The 108 kDa reactive band detected with *Sambucus nigra*-Agglutinin (SNA) (specific for NeuAc) was found in both R and C extracts (Fig. 4, Lanes J,K), and no clear results could be obtained with the *Ulex europaeus*-Agglutinin (UEA) lectin (specific for fucose) due to the high background noise.

The optic density profiles obtained for reduced and denatured R extracts blotted with aPabEleei, PabMPPF and ConA are represented in Fig. A1 in Appendix 1.

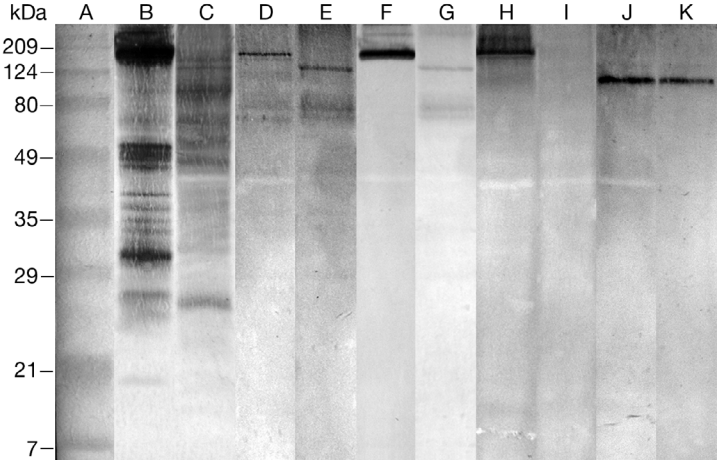

Fig. 4. Lectin blot detection of *Enteromyxum leei* (Lanes B,D,F,H,J) and host tissue (Lanes C,E,G,I,K) extracts with the biotinylated plant lectins ConA (Lanes B,C), BSL I (Lanes D,E), SBA (Lanes F,G), WGA (Lanes H,I) and SNA (Lanes J,K). Lane A is the molecular weight standard

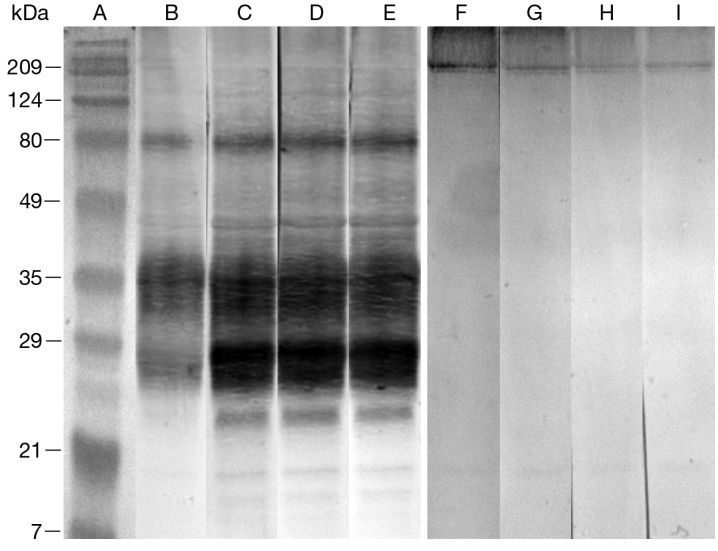

Fig. 5. Western blot reactivity of *Enteromyxum leei* parasite extract with the polyclonal antibody against *E. leei* (Lanes B–E) and with the polyclonal antibody against *Myxobolus pendula* polar filament (Lanes F–I) after sodium periodate oxidation (Lanes B and F, 0 mM; Lanes C and G, 0.1 mM; Lanes D and H, 1 mM; Lanes E and I, 10 mM). Lane A is the molecular weight standard

Periodate oxidation

Following the treatment with sodium periodate, no reduction in the number of reactive bands recognized by aPabEleei was observed (Fig. 5, Lanes B–E). Similarly, the immunoreactive bands detected by the PabMPPF did not decrease after the sodium periodate treatment (Fig. 5, Lanes F–I). Band detection with both Pabs was maintained at the different periodate concentrations used.

Proteinase K digestion

The reactivity of R proteins with aPabEleei in Western blots was reduced after Proteinase K digestion (Fig. 6, Lanes B–E). A progressive reduction of the antibody's label was observed at increasing Proteinase K concentrations, and the label almost disappeared after incubation with Proteinase K at 20 µg ml^{-1}. Likewise, the intensity of the PabMPPF immunoreactive bands was progressively reduced after incubation with increasing concentrations of Proteinase K, eventually disappearing at 20 µg ml^{-1}.

Zymography

The zymography detected gelatinolytic proteases with a broad MW range, from 27 up to >209 kDa, for C and R extracts (Fig. 7). Accurate assessment of proteinase molecular mass through zymography was not possible since migration is affected by gelatine inclusion in the gel, and moreover, we used native extracts that had neither been boiled nor reduced (Hummel et al. 1996). In any case, proteolytic bands only present in R extracts had apparent MWs of 43, 49, 113 and 245 kDa, together with a smear of a mean MW of 174 kDa and 2 weak bands of 58 and 66 kDa (Fig. 7, Lane B).

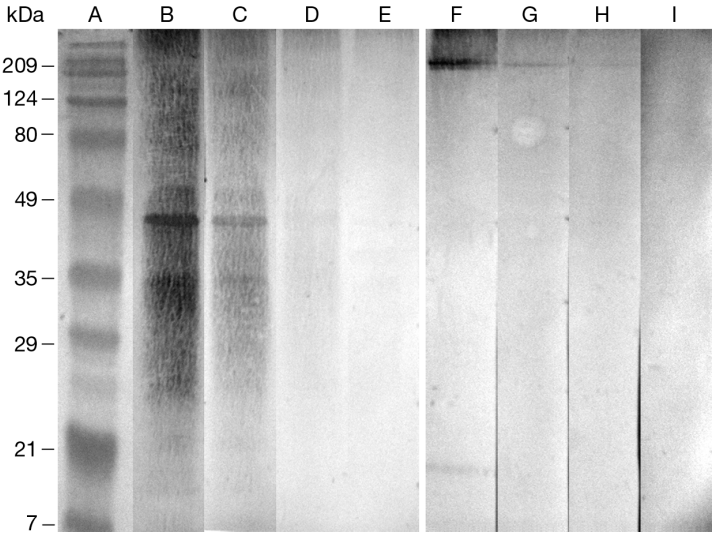

Fig. 6. Western blot reactivity of *Enteromyxum leei* parasite extract with the polyclonal antibody against *E. leei* (Lanes B–E) and with the polyclonal antibody against *Myxobolus pendula* polar filament (Lanes F–I) after Proteinase K digestion (Lanes B and F, 0 mM; Lanes C and G, 0.2 mM; Lanes D and H, 2 mM; Lanes E and I, 20 mM). Lane A is the molecular weight standard

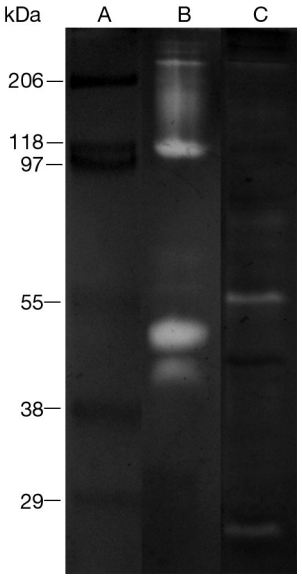

Fig. 7. Protease zymography of *Enteromyxum leei* (Lane B) and host tissue (Lane C) extracts using gelatine non-reducing SDS-PAGE. Lane A is the molecular weight standard

DISCUSSION

The antigenic characterization of *Enteromyxum leei*, a parasite with economic impact on the aquaculture industry, has been a postponed issue due to its complex purification. In the current study, *E. leei* antigens were obtained and analysed by several immunological and biochemical techniques leading to their identification and partial characterization.

Large amounts of spores together with some disporous sporoblasts were contained in the R parasite extracts yielding concentrations of over 6×10^6 parasite stages ml^{-1}. Spores constitute a large proportion of the parasite stages found in well established *Enteromyxum leei* infections in GSB, either free or as part of sporoblasts. Nevertheless, the specific role of *E. leei* spores in the life cycle of the parasite and its infectivity for the fish host is uncertain (Diamant et al. 2006). However, as described in other myxozoans, the role of myxospores is most likely the transmission to an annelid intermediate host (Yokoyama et al. 2012). Indeed, an actinosporean (Unicapsulactinomyxon type) with an SSU rDNA sequence that is 80 to 84% related to *Enteromyxum* spp. was found in Portugal (Rangel et al. 2011). *E. leei* spores were demonstrated to be immunogenic for rabbits, as the aPabEleei specifically detected surface epitopes on spore valves by immunohistochemistry (Estensoro et al. 2013). The immunogenicity of myxospores in organisms different than the corresponding hosts (mice and rabbits) has already been observed in other studies (Clouthier et al. 1997, Chase et al. 2001, 2003). Furthermore, the role of myxospores in resistance development in fish has been suggested. In the case of *Myxobolus cerebralis*, common antigens for actinospores and myxospores were discovered, and the latter generated an antibody response in fish (Morris et al. 2004). In any case, the presence of sporoblasts in the parasite extracts provided antigens from primary and secondary accompanying cells, both of importance for the parasite's pathogenicity because they are infective for fish (Cuadrado et al. 2008). Obtaining a pure *E. leei* extract was not possible due to its localization inside the intestinal epithelium and its adherence to host enterocytes, but most host-derived cells were eliminated and highly enriched parasite extracts were achieved. In all assays, host background in parasite extracts was identified by comparison with parasite-free C intestinal extracts. Indeed, a 42 kDa band apparently of piscine origin was detected in all gels and Western blots of R extracts. However, it cannot be discounted that parasite antigens could mimic host antigens to evade immune attack (Bartholomew et al. 1989, Lu et al. 2002, Villavedra et al. 2007).

SDS-PAGE, lectin and Western blotting have proved to be useful techniques for the visualization and char-

acterization of antigen proteins and glycoconjugates of *Enteromyxum leei*. Protein profiles in Coomassie brilliant blue stained gels revealed 6 low-MW antigenic bands (10 to 49 kDa) of parasite origin which differed slightly in their relative migration when separated under native or under reducing denaturalized conditions. Such variation in the speed of migration suggests a non-linear polypeptide structure of the detected antigen proteins, which contain disulphide bonds (Feng & Woo 1998a). Thus, after the reducing and heating treatment, the unfolded polypeptide presented a higher relative migration.

Further proteic/glycoproteic bands, which were not visualized with Coomassie brilliant blue staining, were detected by Western blotting. Numerous examples exist in the literature in which antibodies or lectins detect additional proteic or glycoproteic bands in blotted membranes that were previously undetected in the Coomassie brilliant blue stained gels (Schumacher & Krause 1995, Heimann et al. 1997, Newlands et al. 1999, Muñoz et al. 2000a, Tanaka et al. 2007). The reason is the limited sensitivity of the Coomassie staining to detect trace proteins in antigen preparations, in contrast with the high sensitivity of antibodies and lectins. aPabEleei detected 5 different *Enteromyxum leei* antigens in reduced and denatured cell extracts. All of them had proteic antigen determinants because they were not affected by the periodate oxidation, but by Proteinase K digestion. The detection of fewer bands in native extracts suggests that aPabEleei does not detect conformational epitopes, but rather unfolded polypeptide epitopes, some of them only exposed after reducing and denaturalizing. Although aPabEleei showed specificity for proteic epitopes, some of these bands corresponded to glycosylated proteins also detected by lectins (Table 2; Appendix 1). This was the case of the 34 kDa band positive for Man/Glc sugar moieties (ConA), which coincided with the reactive smear to aPabEleei. Intense smears in Western blotting are characteristic of heavily glycosylated molecules (Chase et al. 2001, Villavedra et al. 2007), in accordance with the observed aPabEleei smear coinciding with a ConA reactive band. The 15 kDa protein was detected by both Pabs and was also positive for Man/Glc. Moreover, the 165 kDa (±1 kDa) aPabEleei-positive proteic band immunoreacted with ConA, BSL I, SBA and WGA, indicating the presence of Man/Glc, Gal, GalNac and GlcNac terminals. The affinity of Pabs for proteic epitopes of parasites has been reported, while Mabs more frequently recognize carbohydrate epitopes (Bartholomew et al. 1989, Clouthier et al. 1997, Chase et al. 2001, Villavedra et al. 2010), which are the predominant surface molecules. Accordingly, the present aPabEleei exclusively recognized peptidic antigens, which are thought to be generally more potent immunogens than glycans and are probably the common epitopes shared between parasites (Villavedra et al. 2007).

Parasite surface glycoconjugates contribute to protecting the parasite from harsh environments and enable host–parasite interactions. Important events among such interactions are recognition and adhesion leading to penetration of the parasite, but also trapping of the parasite by host mucins leading to its removal. Although not all glycopeptids present in our extracts are surface molecules, some could have a role in such interactions. The detected glycoproteic antigens of 15, 34 and 165 kDa of *Enteromyxum leei* contained Man/Glc moieties, which were the most abundant monosaccharides among the parasite's antigens, compared to the other monosaccharides tested. This result agrees with the ConA staining of *E. leei* proliferative and sporogonic stages observed by light and electron microscopy (Redondo & Álvarez-Pellitero 2009) and is a common trait among myxozoans (Muñoz et al. 1999a, 2000b, Morris & Adams 2004, Kaltner et al. 2007, Redondo et al. 2008). In fact, Man/Glc moieties were present at the host–parasite interface in enteromyxoses (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). The presence of surface Man moieties on offending microorganisms can trigger an innate immune response via complement system by the lectin pathway or via phagocytosis by Man-binding receptors on macrophages (Kaltner et al. 2007). In fact, it is known that complement through the alternative pathway and macrophages through the respiratory burst activity are involved in

Table 2. *Enteromyxum leei* antigens immunoreactive to the adsorbed polyclonal antibody (Pab) against *E. leei* (aPabEleei), which were detected by other probes and techniques ±1 kDa difference. Coomassie: Coomassie brilliant blue stain; PabMPPF: Pab against *Myxobolus pendula* polar filament epitopes; ConA: *Canavalia ensiformis*-Agglutinin; BSL I: *Bandeiraea simplicifolia*-Lectin; SBA: *Glycine max*-Agglutinin; WGA: *Triticum vulgaris*-Agglutinin

	E. leei antigens (molecular weight in kDa)		
	15	34	165
Coomassie	+	–	–
PabMPPF	+	–	+
ConA	+	+	+
BSL I	–	–	+
SBA	–	–	+
WGA	–	–	+
Gelatinolytic activity	–	–	+

the innate immune response against *Enteromyxum* spp. (Cuesta et al. 2006, Sitjà-Bobadilla et al. 2006, Estensoro et al. 2011). Furthermore, down-regulation of GSB mannose binding lectin 2 during enteromyxosis was considered as parasite-induced immunodepression (Davey et al. 2011).

In addition to the Man/Glc, the 165 kDa glycoproteic antigen also presented reactivity for WGA, which binds to GlcNac with high affinity. The presence of chitin, a polymer of D-GlcNac, in polar capsules positive for WGA has been described for some myxozoans (Lukes et al. 1993, Muñoz et al. 1999a, 2000b, Kaltner et al. 2007). Myxospore valves also contain substantial amounts of chitin (Lukes et al. 1993, Muñoz et al. 1999a). Thus, bacterial chitinases degrading spore valves of the myxozoan *Thelohanellus kitauei* have been suggested as alternative biological agents to control myxozoan diseases (Liu et al. 2011). Interestingly, polar capsules of *Enteromyxum leei* were negative for WGA while other spore structures were positive and putatively contained chitin, as was also the case with the spores of the congener *E. scophthalmi* (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). Since these authors detected no GlcNac in proliferative stages of *E. leei*, we can hypothesize that the high-MW glycoprotein of 165 kDa is located in spores, and could have a structural, protective function as described for chitin in polar capsule walls. In accordance with this, the 165 kDa glycoproteic band of *E. leei* was also labelled by BSL I and SBA, both of them only binding to spores of *E. leei* in light microscopy preparations (Redondo & Álvarez-Pellitero 2009). These lectins recognize Gal and GalNac carbohydrate residues, which are frequently involved in host–parasite interactions and have also been detected in spores of several myxozoans (Muñoz et al. 1999a, 2000b, Kaltner et al. 2007, Redondo et al. 2008) and in antigenic bands of *Myxobolus cerebralis* (Knaus & El-Matbouli 2005). The 165 kDa carbohydrate-rich antigen was not detected by PAS staining, but primary cells, secondary cells and polar capsules of *E. leei* were PAS positive in histological sections (Álvarez-Pellitero et al. 2008). However, the lack of expected PAS-positive bands has been reported before, due to the higher sensitivity of lectins in Western blotting over PAS staining in SDS-PAGE gels (Wilkinson & Hames 1983, Schumacher & Krause 1995).

SNA, specifically binding NeuAc, did not recognize any antigenic band exclusive of *Enteromyxum leei*. The intense 108 kDa band observed in R and in C blots apparently belongs to the host in accordance with the absence of NeuAc residues previously reported for *E. leei* and *E. scophthalmi* by immunohistochemistry (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009) as well as for *Myxobolus cerebralis* by lectin blot (Kaltner et al. 2007). However, surface exposed sialic acids may play a role in self/non-self recognition, and therefore some parasites would acquire sialic acid moieties from the host by trans-sialidases as a biological mask to evade immune detection by phagocytes or complement (Feng & Woo 1998b, Kaltner et al. 2007). Therefore, this band might be alternatively interpreted as a sialylated glycoconjugate of the parasite. Nevertheless, even without sialic acids, *E. leei* seems to evade the initial immune response of GSB, suggesting the involvement of other glycans in the parasite's masking.

PabMPPF cross-reacted with *Enteromyxum leei* epitopes on polar filaments, polar capsules, spore valves, and primary and secondary cells. This antibody is directed against a spinalin-like non-collagenous glycoprotein present in *Myxobolus pendula* cyst extracts which is resistant to cyanogen bromide digestion. Spinalin is a nematocyst-specific, resistant protein tolerating high mechanical stress and with partial homology to avian keratins. Spinalin presented an MW of 24 kDa in Western blot and was localized in stylets, spines and opercula of cnidarian nematocysts (Koch et al. 1998). Immunoreactive spore structures of *E. leei* to PabMPPF putatively present the spinalin-like peptides which confer a high mechanical strength, but whose function in the primary, secondary and accompanying cells of *E. leei* is yet to be revealed. The current results show that PabMPPF cross-reacts with several peptidic epitopes of *E. leei*, 2 of which are glycoproteins also detected by aPabEleei (Table 2). In addition, *M. pendula* blotted with PabMPPF presented a 35 kDa antigenic band (Ringuette et al. 2011) almost coinciding with the 34 kDa glycoproteic band detected in the current study by aPabEleei and ConA. Common antigenic determinants shared between *E. leei* and *Sphaerospora* spp. have been detected with the aPabEleei, but *Myxobolus* spp. have not been tested for cross-reactivity (Estensoro et al. 2013). The glycoproteic antigens found in the present study (15, 34 and 165 kDa) (Appendix 1) may represent further common antigens shared between *E. leei* and other myxosporeans. Such common myxozoan epitopes have been found previously for other species (Muñoz et al. 1998, 1999a,b). In support of the phylogenetic affinity between Cnidaria and Myxozoa, Ringuette et al. (2011) also found cross-reactivity of the PabMPPF in polar filament epitopes of *M. pseudokoi*, *M. bartai*, the anthozoan *Nematostella vectensis* and the hydrozoan *Hy-*

dra vulgaris and, in the current study, we demonstrated cross-reactivity with *E. leei* and *E. scophthalmi* (not shown). Recently, phylogenomic analyses of new genomic sequences of *M. cerebralis* firmly placed Myxozoa as a sister group to Medusozoa within Cnidaria (Nesnidal et al. 2013). Interestingly, minicollagens are phylum-specific genes encoding cnidarian nematocyst proteins, and a minicollagen homologue was discovered in the malacosporean *Tetracapsuloides bryosalmonae* involving a further link between Myxozoa and Cnidaria (Holland et al. 2011). The inner wall of *Hydra magnipapillata* nematocyst capsules consists of fibril bundles formed by polymers of small 12 to 15 kDa minicollagens (Koch et al. 1998), coinciding in MW with the 15 kDa glycoproteic band of *E. leei* recognized by PabMPPF, aPabEleei and ConA. However, at the moment we can only speculate about the existence of a 15 kDa minicollagen monomer in *Enteromyxum*, and further investigation is required to confirm the nature of this band.

Enteromyxum leei proteases are probably the key to the pathogenesis of enteromyxosis. Different proteases at different stages of the parasite's life cycle are released to degrade host proteins, contributing to invasion, parasite proliferation and tissue damage (McKerrow et al. 1993). Our results showed several functional parasite proteases in *E. leei* parasite extracts (not detected in C intestinal extracts). Their *in vivo* functions may be involved in intra- and extracellular digestion of nutrients and/or destruction of immune relevant host molecules. The release of *E. leei* stages directly to the gut and hence to the environment might depend on epithelial disruption by proteolysis. This release, though causing intestinal tissue damage, might be considered less harmful than the damage caused by other myxozoan species that need to induce host death to be discharged from the host. In the myxozoan *Kudoa rosenbushi*-infected muscle of *Merlucius hubbsi*, a protease allows spores to enter muscle fibres and to use the breakdown products as a major nutrient source for the parasite (Martone et al. 1999), and in *Oncorhynchus mykiss* exposed to *Myxobolus cerebralis*, the gene expression of *Myx*SP1 protease in gills increases significantly after exposure (Kelley et al. 2004). Moreover, resistance of an *O. mykiss* strain to *M. cerebralis* infection is suggested to derive from a difference in susceptibility to parasite proteases (Dörfler & El-Matbouli 2007). In any case, further characterization of the detected proteases by their inhibitors and their peptide substrates is still needed to decipher the underlying mechanisms and functions of parasite-induced proteolysis during enteromyxosis.

In this first approach to the antigenic characterization of *Enteromyxum leei*, several glycoproteic antigens were detected, one of them possibly derived from myxospores. Nevertheless, further improvement of the parasite purification technique is needed to avoid all possible interference of host cells with binding and detection and to definitively discard host-derived antigens. The function played by each glycoconjugate in the host–parasite interaction as well as the characterization of the individual glycoproteic antigens deserve further investigation. The role played by parasite proteases during intestinal invasion and their importance for virulence and pathogenesis are still to be investigated in depth.

Acknowledgements. The PabMPPF was kindly provided by M. J. Ringuette and S. Desser from the Department of Cell and Systems Biology of the University of Toronto (Canada). This work was funded by MICINN through project AGL2009-13282-C02-01, and by the 'Generalitat Valenciana' (projects PROMETEO 2010/006 and ISIC 2012/003). I.E. received a PhD FPI fellowship.

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Appendix

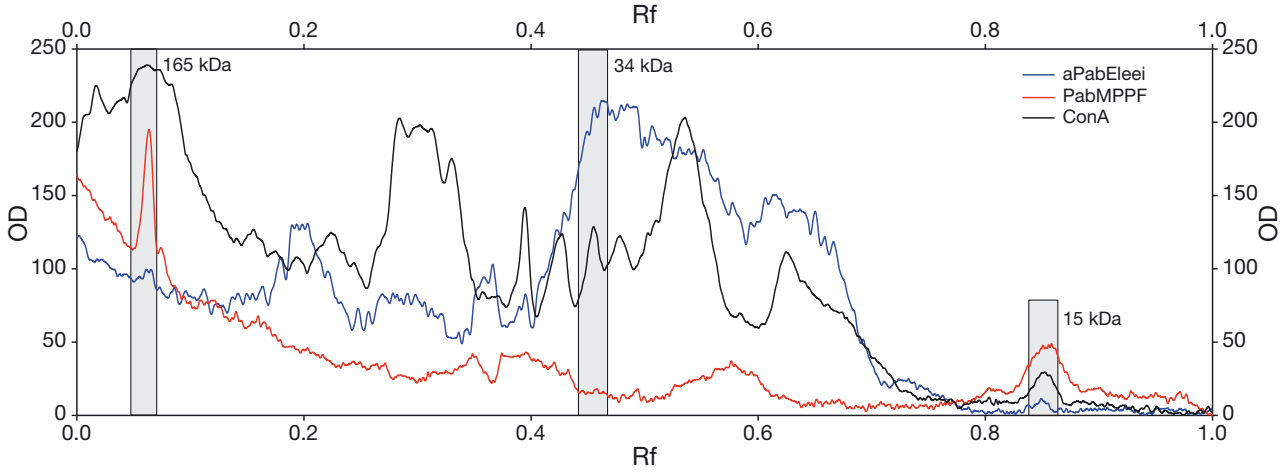

Fig. A1. Optic density (OD) profile along the relative migration distance (Rf) of reduced and denatured *Enteromyxum leei* extracts in Western blots. Peaks corresponding to the bands of the same molecular weight detected by the polyclonal antibodies against *E. leei* (aPabEleei) and against *Myxobolus pendula* polar filament (PabMPPF) and detected by *Canavalia ensiformis*-Agglutinin (ConA) are highlighted by grey shading

Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: May 6, 2013; Accepted: July 12, 2013
Proofs received from author(s): September 20, 2013