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Comparative studies on carbohydrates of several myxosporean parasites of fish using lectin histochemical methods

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Abstract. A histochemical study using lectin methods was performed on myxosporean parasites from vastly different fish hosts from marine and fresh waters. Six biotinylated lectins were used (WGA, SBA, BS-I, Con-A, UEA-I and SNA). The binding pattern of Con-A and WGA revealed the presence of mannose and/or glucose, and N-acetyl-D-glucosamine respectively, in polar capsules and valves of most of the myxosporea assayed. Thus, chitin may be present in polar capsules and/or valves of myxosporean spores. The BS-I binding pattern showed the presence of α-D-galactose and/or N-acetyl-D-galactosamine residues in polar capsules of *Kudoa* sp., *Zschokkella mugilis* Sitjà-Bobadilla et Alvarez-Pellitero, 1993 and *Leptotheca* sp., and in the valves of the latter. Scarce amounts of N-acetyl-D-galactosamine and/or α-D-galactose were demonstrated by SBA binding in *Sphaerospora dicentrarchi* Sitjà-Bobadilla et Alvarez-Pellitero 1992, *Leptotheca* sp. and *Kudoa* sp. valves, and in *Leptotheca* sp. polar capsules. The UEA-I staining indicated the absence of α-L-fucose in all the myxosporea assayed except in *Leptotheca* sp. N-acetylneuraminic acid was detected with SNA in the polar capsules and sporoplasms of *Polysporoplasma sparis* Sitjà-Bobadilla et Alvarez-Pellitero, 1995 and in the polar capsules and valves of *Kudoa* sp. These results indicate that, although Myxosporea may have conserved carbohydrate structures, some of them can show significantly different binding patterns, which may be useful in diagnostic and functional studies.

Lectins are defined as proteins or glycoproteins, derived from both animal and plant material, that recognise and reversibly bind to specific sugar moieties or certain glycosidic linkages of polysaccharides, glycoproteins and glycolipids (Sharon and Lis 1989). These proteins have been invaluable tools for the structural and functional analysis of animal cell glycoconjugates because of their ability to discriminate among the myriad of complex carbohydrate structures that are found on the surface cells, in extracellular matrices, and attached to soluble glycoproteins. All parasites have carbohydrates on their surfaces, as part of their cytoskeletons or in their internal structures, and these molecules have been found responsible for many of the biological functions of cells (Reuter et al. 1988). Therefore, lectins have been used in parasitology to probe the carbohydrate topography (Mutharia and Pearson 1987), to demonstrate differences between parasite growth and stationary phases (Jacobson and Schnur 1990), to distinguish between the different stages of the life cycle (Andrade et al. 1991) or to differentiate between parasite species (Maraghi et al. 1989).

Although Myxosporea have been studied for a long time, many questions about their biology, physiology and life cycle remain unanswered for the vast majority of species, and the study of carbohydrate residues of myxosporean parasites is very limited (Hedrick et al. 1992, Lukeš et al. 1993, Marín de Mateo et al. 1996, 1997). The increasing economic importance of the fish culture industry, the pathogenic impact of some Myxosporea and the lack of reliable measures for their prevention and treatment, have turned these organisms into threats for fish production. From a practical point of view, the advance in the studies with lectins might help in defining the developmental stages of parasites and the characterisation, localisation and structural composition of myxosporean glycoconjugates. Therefore, the aim of this work was to study lectin specificities for myxosporean glycoconjugates, which could be shared by Myxosporea from vastly different hosts.

MATERIALS AND METHODS

Tissue samples. Periodic surveys of European sea bass (*Dicentrarchus labrax* L.), gilthead sea bream *(Sparus aurata* L.), grey mullet (*Mugil cephalus* L.), and common dentex (*Dentex dentex* L.) were conducted for myxosporean parasite examination. They included fish of different stocks and ages from the Mediterranean area. Fish were killed by overdose with the anaesthetic MS-222 (Sigma Chemical Co., St. Louis, MO, USA), necropsied and all their organs excised for parasite diagnosis. Sea bass tissues infected by *Sphaerospora dicentrarchi* Sitjà-Bobadilla et Alvarez-Pellitero, 1992, *Sphaerospora testicularis* Sitjà-Bobadilla et Alvarez-Pellitero, 1990 or *Ceratomyxa diplodae* Lubat, Radujkovic, Marques et Bouix, 1989; gilthead sea bream tissues harbouring *Polysporoplasma sparis* Sitjà-Bobadilla et Alvarez-Pellitero, 1995 or *Ceratomyxa sparusaurati* Sitjà-Bobadilla, Palenzuela et Alvarez-Pellitero, 1995; grey mullet tissues infected by *Zschokkella mugilis* Sitjà-Bobadilla et Alvarez-Pellitero, 1993, and common dentex tissues with *Ceratomyxa* sp. or *Leptotheca* sp. were fixed in 10% v/v buffered formalin, dehydrated through a graded ethanol series, and embedded in Technovit 7100 acrylic resin

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(Kulzer, Heraeus, Germany). Other fish tissue samples parasitised by different myxosporean species, fixed and embedded as above, were obtained from other sources. They included rainbow trout (*Oncorhynchus mykiss* Walbaum) intestines infected by *Ceratomyxa shasta* Noble, 1950, poutassou (*Micromesistius poutassou* Risso) muscle harbouring *Kudoa* sp., rainbow trout trunk kidney infected by PKX (the causative agent of proliferative kidney disease) and barbel (*Barbus barbus* L.) trunk kidney with *Myxobolus* sp.

Lectin histochemistry. The concentrations of the lectins used in this study, their acronyms and major sugar specificities are listed in Table 1. They were obtained from Sigma except SNA lectin, which was purchased from Vector (Vector Lab., Burlingame, CA, USA). Sections of 1.5 µm thickness were adhered to standard microscopy glass slides previously treated with Poly-L-Lysine solution (Sigma). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 7 min at room temperature. After rinsing in Tris buffered saline (TBS, 20 mM Tris, 0.5 M NaCl, pH 7.4), the sections were incubated with biotinylated lectin solutions, in TBS containing 0.05 % (v/v) Tween 20 (TTBS) for 1 h at 20ºC. After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC) (Vector) for 30 min at 20ºC and bound peroxidase was finally revealed by adding the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) for 10 min. The reaction was stopped with deionised water and the sections counterstained using Gill's hematoxylin, and finally mounted in DPX. Incubation of ABC with the tissue sections alone served as control to discard the presence of endogenous biotin-binding proteins. An equal amount of each lectin and its corresponding blocking sugar (0.2 M) were incubated for 1 h at 20ºC, before application of the solutions to the sections as a control for binding specificity. The blocking sugars used were: lactose for BS-I, α-N-acetyl-D-galactosamine for SBA, α-N-acetyl-D-glucosamine for WGA, α-L-fucose for UEA-I, methyl-α-D-mannopyranoside or methyl-α-D-glucopyranoside for Con-A, and N-acetylneuraminic acid for SNA.

RESULTS

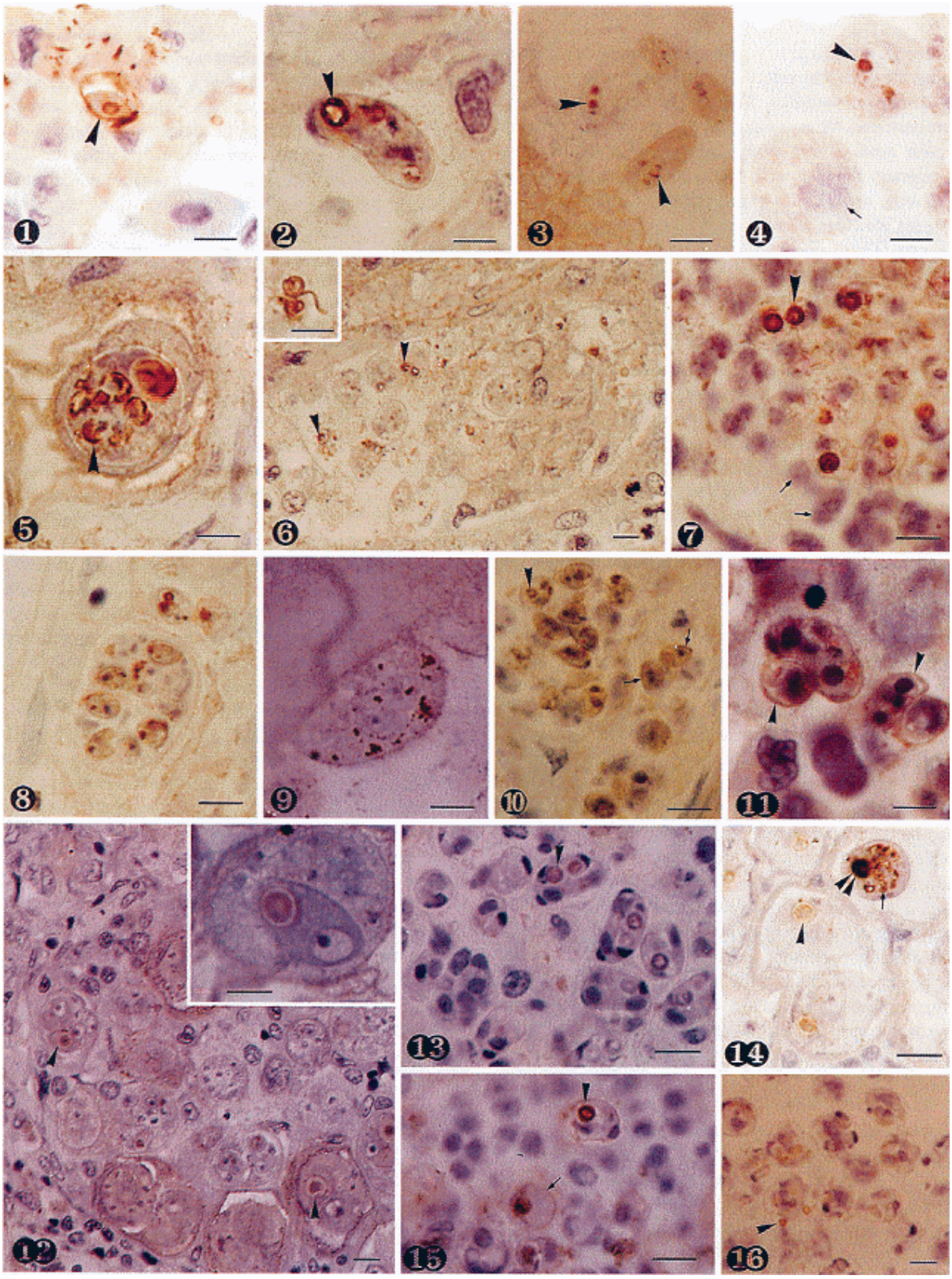
The different lectin-binding patterns observed for the myxosporeans assayed are summarised in Table 2. Histochemistry with *Canavalia ensiformis* lectin (Con-A) resulted in moderate to strong staining of the polar capsules of all the myxosporean parasites assayed, and weak to strong of the valves of *Sphaerospora dicentrarchi, S. testicularis, Zschokkella mugilis, Ceratomyxa shasta, Ceratomyxa* sp., *Kudoa* sp., and *Myxobolus* sp. (Figs. 1-8).

Triticum vulgaris lectin (WGA) bound weakly to moderately to the polar capsules of all the parasites assayed except those of *Ceratomyxa* sp. from common dentex, and weakly to strongly to the valves of *S. dicentrarchi*, *C. diplodae, C. shasta, P. sparis, Leptotheca* sp., *Z. mugilis*, *Kudoa* sp., and *Myxobolus* sp. (Figs. 9-12)

Glycine max lectin (SBA) stained weakly the valves of *S. dicentrarchi, Kudoa* sp. and *Leptotheca* sp., and the polar capsules of the latter (Fig. 13). *Griffonia simplicifolia* lectin (BS-I) bound strongly to the polar capsules of *Leptotheca* sp. (Fig. 14), and weakly to its valves and to *Kudoa* sp. and *Z. mugilis* polar capsules. *Ulex europaeus* lectin (UEA-I) only stained weakly the polar capsules of *Leptotheca* sp. and material surrounding *P. sparis*. *Sambucus nigra* lectin (SNA) labelled strongly the polar capsules and sporoplasms of *P. sparis* mature spores, and weakly the polar capsules and valves of *Kudoa* sp. (Figs. 15, 16).

Histochemistry with all the lectins used in this study stained mostly mature stages, as only developmental stages of *Leptotheca* sp. were weakly labelled with WGA. Binding to host tissues was only detected in the brush border of the proximal tubule of trunk kidney, in goblet cells and in the epithelia of intestine and gall bladder. Lectin staining was inhibited in all cases after blocking with each specific sugar.

Figs. 1-15. Lectin histochemical staining of myxosporean parasites in different tissue sections. **Figs. 1-8.** Sections stained with Con-A. **Fig. 1.** Barbel trunk kidney infected by *Myxobolus* sp. Note the strong labelling in polar capsules and valves (arrowhead). **Fig. 2.** Rainbow trout intestine infected by *Ceratomyxa shasta.* Strong staining in polar capsules (arrowhead). **Fig. 3.** Common dentex gall bladder infected by *Ceratomyxa* sp. Moderate staining in polar capsules (arrowheads). **Fig. 4.** Gilthead sea bream gall bladder infected by *Ceratomyxa sparusaurati.* Note the staining in polar capsules (arrowhead) and the lack of labelling in valves and in secondary cells (arrow). **Fig. 5.** Gilthead sea bream trunk kidney infected by *Polysporoplasma sparis*. Note the strong labelling in polar capsules and sporoplasms (arrowhead). **Fig. 6.** Sea bass testis infected by *Sphaerospora testicularis*. Note the weak labelling in valves and the strong staining in polar capsules (arrowheads). Stained polar filament (Inset). **Fig. 7.** Common dentex trunk kidney infected by *Leptotheca* sp. Strong labelling in polar capsules (arrowhead) and lack of staining in developmental stages (arrows). **Fig. 8.** Sea bass intestine infected by *Sphaerospora dicentrarchi*. **Figs. 9-12.** Sections stained with WGA. **Fig. 9.** Grey mullet gall bladder infected by *Zschokkella mugilis*. Note the scattered labelling in primary and secondary cells. **Fig. 10.** Sea bass intestine infected by *Sphaerospora dicentrarchi*. Note the labelling in polar capsules (arrowhead) and valves (arrows). **Fig. 11.** Rainbow trout intestine infected by *Ceratomyxa shasta*. Moderate staining in valves (arrowheads). **Fig. 12.** Gilthead sea bream trunk kidney infected by *Polysporoplasma sparis*. Note the moderate staining in polar capsules (arrowheads). **Fig. 13.** Section of common dentex trunk kidney infected by *Leptotheca* sp. stained with SBA. Note the weak labelling in polar capsules (arrowhead) and valves. **Fig. 14.** Section of gilthead sea bream trunk kidney infected by *Polysporoplasma sparis* stained with SNA. Note the strong labelling in mature polar capsules (double arrowhead) and sporoplasms (arrow), and weak in immature polar capsules (arrowhead). **Figs. 15, 16.** Sections stained with BS-I. **Fig. 15.** Strong staining in *Leptotheca* sp. polar capsules (arrowhead) and weak in valves (arrow). **Fig. 16.** Section of poutassou muscle harbouring *Kudoa* sp. Note the weak labelling in polar capsules (arrowhead). Scale bars = 5mm.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DISCUSSION

Lectin histochemistry provided detailed information about the chemical nature of glycoconjugates present in myxosporean parasites. Variable binding patterns were demonstrated for myxosporean spores with the different lectins used in this study. Since Con-A binding was completely blocked with methyl-α-D-mannopyranoside and with methyl-α-D-glucopyranoside, all the myxosporean parasites assayed must contain mannose and/or glucose. These observations indicate the existence of conserved carbohydrate structures in polar capsules of Myxosporea, even belonging to different orders, from different hosts from marine and fresh waters. Mannose has been found in trematodes such as *Trichobilharzia szidati* (Horák 1995) or *Schistosoma mansoni* (Pearce et al. 1996), flagellates such us *Cryptobia* spp. (Feng and Woo 1998), or nematodes such as *Onchocerca volvulus* (Taylor et al. 1986). The presence of mannose residues may be important in the interaction of these parasites with the host cells, especially those of the immune system. Thus, the mannose receptors of some cells, such as macrophages, may bind carbohydrates found on the surfaces of pathogenic microorganisms and are believed to play a role in the innate immune response (Drickamer and Taylor 1993).

Triticum vulgaris lectin (WGA) labelled the polar capsules of all the parasites assayed except those of *Ceratomyxa* sp. from common dentex, and the valves of all of them except those of *Sphaerospora testicularis*, *Ceratomyxa* sp. and *C. sparusaurati*. This lectin has been used to detect chitin, a polymer of N-acetyl-D-glucosamine, in several organisms (Jacobson and Doyle 1996, Muzzarelli and Peter 1997). Nevertheless, WGA also binds to sialic acid. The absence of SNA labelling (which binds to sialic acid more specifically) in all the parasites assayed except in *Polysporoplasma sparis* and *Kudoa* sp., indicates that WGA must have detected N-acetyl-D-glucosamine or its polymers in the SNA-negative parasites. Other authors (Lukeš et al. 1993) have reported the presence of chitin in the polar capsule walls of some Myxosporea. Chitin could have several functions in myxosporean spores such as protection against mechanical or chemical stress from the environment, or shape maintenance.

Biotinylated SBA lectin, specific for N-acetyl-D-galactosamine and α-D-galactose, stained only *Sphaerospora dicentrarchi, Leptotheca* sp. and *Kudoa* sp. valves, and *Leptotheca* sp. polar capsules. Marín de Mateo et al. (1997) observed staining in spores and developmental stages of several *Sphaerospora* spp. with SBA, so they considered this lectin as a useful probe for the detection of *Sphaerospora*. Nevertheless, in our study *S. testicularis* was not labelled with this lectin and *S. dicentrarchi* only weakly. In general, the labelling pattern demonstrated a scarce amount of the terminals recognised by this lectin, but some masking of them could occur. Sialylation is a frequent cause of such masking (Kelm and Schauer 1997), and could explain the absence of SBA labelling in *Polysporoplasma sparis*, which showed a strong staining with SNA (specific for sialic acid). Nevertheless, the remaining SBA negative myxosporean spores are also negative for SNA. Thus, they must have no α-D-galactose or N-acetyl-D-galactosamine detectable with this method.

Griffonia simplicifolia lectin (BS-I) was considered by Marín de Mateo et al. (1996) to be specific for sporogonic and extrasporogonic stages of PKX. In our experiments, we could not stain PKX with this lectin, even when the tissue was embedded in JB4, a highly recommended resin for histochemistry (Horák 1995). However, we have obtained positive staining of the parasites using paraffin-embedded, PKX-infected tissues (data not shown). The differences between the paraffin and the resin techniques employed in both studies are probably responsible for these different patterns. Nevertheless, variable results have also been reported with BS-I in *Sphaerospora* spp. samples. Neither *S. dicentrarchi* nor *S. testicularis* were labelled in the present study, and *Sphaerospora* sp. from Italian brown trout was not labelled with a rapid fluorescence method (Hedrick et al. 1992). *S. oncorhynchi* sporogonic stages were not stained with BS-I in Marín de Mateo et al. report (1996), but their spores and trophozoites were considered positive by Kent et al. (1993), using the same procedure. On the other hand, galactosyl residues were labelled by this lectin in *Kudoa* sp., *Zschokkella mugilis* and *Leptotheca* sp. spores in our study. Although a useful tool for the differential staining of PKX organisms in paraffin-embedded salmonid tissues, in our opinion BS-I should not be considered a specific diagnostic probe for PKX, as other myxosporean parasites, even with a similar renal location, can be stained with the lectin.

Histochemistry using UEA-I lectin revealed the absence of α-L-fucose in the myxosporean parasites assayed, except a scarce amount in *Leptotheca* sp. and in the material surrounding *Polysporoplasma sparis*. This carbohydrate is known to have an important role in the morphogenetic process of plasmatic membranes submitted to rapid turnover (Bennet et al. 1974), and an immunological relevance in both mammalian and parasitic systems (Wisnewski et al. 1993). Fucose has been previously reported in other parasites such as *Trichinella spiralis* (Wisnewski et al. 1993).

Histochemistry using SNA lectin revealed N-acetylneuraminic acid (sialic acid) only in the polar capsules and sporoplasms of *P. sparis* and in the polar capsules and valves of *Kudoa* sp. Several functions have been described for this carbohydrate, such as to regulate cellular and molecular interactions, and to contribute both to the structural properties of glycoproteins and

Table 1. Lectins used in this study: their acronyms, the concentration used and their sugar-binding specificities.

Lectin source	Acronym	Concentration (mg/ml)	Specificity*
Glycine max	SBA	20	GalNAcα (or ß)-1,3Gal >D-Gal
Griffonia simplicifolia	BS-I	50	D-Gal > D-GalNAc
Triticum vulgaris	WGA	50	(GlcNAc ß-1,4) $_n$ >NeuNAc
Canavalia ensiformis	Con-A	10	Man α-1 >Glcα-1>GlcNAcα-1
Ulex europaeus	UEA-I	20	L-Fucα-1,2Gal
Sambucus nigra	SNA	20	Neu5Acα-2,6Gal>GalNAc

* Abbreviations: GalNAc – N-acetylgalactosamine; Gal – galactose; GlcNAc – N-acetylglucosamine; NeuNAc – neuraminic acid; Man – mannose; Glc – D-glucose; Fuc – fucose.

Table 2. Lectin binding patterns found in myxosporean spores and extrasporogonic stages of PKX embedded in acrylic resin. Intensity of staining: +++ = intense; ++ = moderate; + = weak; – = none.

Parasite	Host	Location	Spore structure	WGA	SBA	BS-I	Con-A	UEA-I	SNA
Ceratomyxa diplodae	Sea bass	Gall bladder	pc vv	+ +	– –	– –	++ +	– –	– –
Ceratomyxa shasta	Trout	Intestine	pc vv	+ ++	– –	– –	+++ +	– –	– –
Ceratomyxa sparusaurati	Sea bream	Gall bladder	pc vv	+ –	– –	– –	++ –	– –	– –
Ceratomyxa sp.	Common dentex	Gall bladder	pc vv	– –	– –	– –	++ +	– –	– –
Kudoa sp.	Poutassou	Muscle	pc vv	+ +	– +	+ –	+++ +++	– –	+ +
Leptotheca sp.	Common dentex	Trunk kidney	pc vv	+++ +++	+ +	+++ +	+++ –	+ –	– –
Myxobolus sp.	Barbel	Trunk kidney	pc vv	+ ++	– –	– –	+++ +++	– –	– –
PKX	Trout	Trunk kidney		–	–	–	–	– –	–
Polysporoplasma sparis	Sea bream	Trunk kidney	pc vv	++ +	– –	– –	+++ –	– –	+++ –
Sphaerospora dicentrarchi	Sea bass	Intestine and gall bladder	pc vv	+++ ++	- +	– –	+++ ++	– –	– –
Sphaerospora testicularis	Sea bass	Testes	pc vv	+ –	– –	– –	++ +	– –	– –
Zschokkella mugilis	Grey mullet	Gall bladder	pc vv	+ +	– –	+ –	++ +	– –	– –

Spore structure: pc = polar capsules; vv = valves

glycolipids, and to negative charge of cells surfaces and glycoproteins (Kelm and Schauer 1997). Nevertheless, the possible role of sialic acid in the metabolism of *P. sparis* and *Kudoa* sp. is unknown.

The parasite surface coat is involved in many host-parasite interactions and it is made up of oligosaccharide side-chains of glycoproteins and glycolipids (Peters 1988). Therefore, the carbohydrate composition of valves is of great importance in understanding myxosporean biology. In the case of the parasites assayed, valves mainly presented N-acetylglucosamine terminals and also α-D-mannose. They can be present as oligomers or polymers, or making part of N-linked glycoproteins, as both N-acetylglucosamine and α-D-mannose residues are normally found in N-linked oligosaccharide chains (Osawa and Tsuji 1987).

The myxosporean staining patterns observed using lectins were similar to the patterns detected using polyclonal antisera raised against *Sphaerospora dicentrarchi* (Muñoz et al. 1998), *S. testicularis* and *Ceratomyxa labracis* (Muñoz et al. 1999). Thus, carbohydrate components may be involved in antigenic recognition, as previously reported by Roth et al. (1997).

The lectins assayed stained mostly mature stages, as only *Leptotheca* sp. immature stages were stained with WGA. It could be possible that developmental stages had

no carbohydrates on their surfaces, as previously reported for larval stages from skin microfilaria (Ham et al. 1988). Other explanations could be that myxosporean immature stages have other carbohydrates not recognised by the lectins used in this study or amounts too small to be detected or they may be masked. The change in carbohydrate composition during the life cycle could be a specific defence mechanism developed by myxosporean parasites to avoid host immune response, as it has been previously demonstrated for other parasites, such as *Leishmania major* (Jacobson and Schnur 1990), or *Trichobilharzia szidati* (Horák 1995), and also suggested for some myxosporean parasites (Pauley 1974, Bartholomew et al. 1989).

Scarce binding was detected at the epithelial surfaces of some fish hosts. Similar binding patterns were observed in rainbow trout by Castagnaro et al. (1991) using several lectins. The presence of carbohydrates in epithelia could explain these results. Scocco et al. (1996) have described the existence of α-N-acetylgalactosamine and α-galactose in the stomach epithelium of *Tilapia* spp., which could contribute to the protective functions of the mucose. Our results indicate that some structures of Myxosporea, mainly spore polar capsules, and host epithelial surfaces share some carbohydrate structures. This phenomenon has been used by other authors as an argument for antigenic mimicry (McArthur and Sengupta 1982) in Myxosporea.

In the present study, the results obtained indicated the existence of differences in the staining pattern of some lectins among several myxosporean parasites. Nevertheless, they share carbohydrate structures, mainly in their polar capsules and valves. Further studies are needed to elucidate the biological function of these carbohydrates, and their possible role in the interaction with the immune system.

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