

Analysis of *Kudoa thyrsites* (Myxozoa: Myxosporea) spore antigens using monoclonal antibodies

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ABSTRACT: A method employing Percoll™ gradient centrifugation was developed to purify *Kudoa thyrsites* spores from somatic muscle tissue of Atlantic salmon *Salmo salar*. Highly purified spores were then used to immunize inbred BALB/c mice for derivation of hybridomas secreting *Kudoa*-specific monoclonal antibodies (mAbs). Analysis of mAbs by immunofluorescence microscopy and flow cytometry showed that several were specific for antigens on the surface of *K. thyrsites* spores whereas other mAbs reacted with polar capsules or with polar filaments of spores of *K. thyrsites*, *K. paniformis* and *K. crumena*. Immunoblots on spore lysates using the surface-binding mAbs showed a broad band of 46 to >220 kDa, whereas mAbs specific for antigens of polar capsules and polar filaments detected sharper bands of various molecular masses, depending on the *Kudoa* species. The dominant epitope of the *K. thyrsites* spore surface antigen was shown to be carbohydrate as determined by its sensitivity to treatment with anhydrous trifluoromethane sulfonic acid and by its resistance to treatment with Proteinase K. Immunofluorescence microscopy using the *K. thyrsites*-specific mAbs on isolated, intact, permeabilized plasmodia and on thin sections of somatic muscle tissue containing plasmodia revealed intense labeling of spores both within the spore-producing plasmodia and in the flesh of infected Atlantic salmon. As few as 100 spores were detected by immunoblotting, indicating that these mAbs have potential for use in developing a field-based diagnostic test.

KEY WORDS: Atlantic salmon · *Salmo salar* · *Kudoa thyrsites* · Multivalvulida · Marine myxosporean · Soft flesh syndrome · Plasmodia · Spores · Monoclonal antibodies · Immunodetection

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INTRODUCTION

Kudoa thyrsites is a myxosporean parasite that causes soft flesh syndrome (i.e. post-mortem myoliquefaction) in farmed Atlantic *Salmo salar* and coho *Oncorhynchus kisutch* salmon in British Columbia (Whitaker & Kent 1991, Kent et al. 1994). It is of concern to both the aquaculture and commercial fisheries of

British Columbia (B.C.) and elsewhere due to its worldwide distribution and wide host range (Moran et al. 1999a). It is important to develop both a sensitive diagnostic test and an effective vaccine for this parasite because of its effect on a wide variety of marine fishes and the resulting economic impact. We have therefore identified molecules of *K. thyrsites* parasites that have potential for use in detecting infections in susceptible fish species and for eventual development of immunological control strategies.

Kudoa thyrsites is an intracellular myxosporean parasite that is not restricted to salmonids but rather

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infects the myocytes of many species of marine fish (Gilchrist 1924, Moran et al. 1999a). The infective stage of the parasite is unknown, although based on studies of freshwater myxozoans (El-Matbouli et al. 1995) it has been hypothesized that an actinospore stage is released by annelids (Moran et al. 1999b) and subsequently gains entry into a susceptible fish host. Approximately 4 mo after natural seawater exposure, plasmodia can be detected in the somatic musculature using light microscopical analysis of histological sections. These presporogonic plasmodia are as small as 7 μm in diameter and may contain only a few internal cells or nuclei. As sporogenesis progresses, the plasmodia grow along the length of the muscle fibers and increase to >10 mm in length and 100 μm in diameter. These polysporic plasmodia contain hundreds or even thousands of mature and developing myxospores (for photomicrographs see Moran et al. 1999b). Late in the infection, the plasmodia and the infected muscle fibers rupture, releasing the myxospores and other cytoplasmic material. At this stage, chronic inflammation is evident and is characterized by granuloma formation and macrophage ingestion of spores. This is followed by complete resolution of the infection and the associated lesions. However, if a fish is infected with sufficient numbers of parasites and is harvested, soft flesh syndrome occurs. Depending on handling conditions, this usually does not occur until several days after death, at which point the fish may have already reached the market place. It is therefore important to determine the prevalence and intensity of *K. thyrssites* infections in populations of wild-caught and farmed salmon prior to or at harvest so that susceptible, infected fish can be removed from commercial sales. As heavily infected fish appear normal immediately post-harvest and during processing, a rapid and inexpensive technique for identifying fish prone to developing soft flesh syndrome is necessary. The research presented here is the first step in the molecular characterization of *Kudoa* with the ultimate goal of developing immunodiagnostic procedures and vaccination strategies.

MATERIALS AND METHODS

Fish infection and maintenance. Spores and plasmodia of *Kudoa thyrssites* used in this study were obtained from a naturally infected stock of Atlantic salmon (75 to 100 g) which were maintained at the Pacific Biological Station in Nanaimo, B.C. The fish acquired their infections naturally during exposure to seawater enzootic for *K. thyrssites*. All fish were maintained in one tank and were fed a commercial artificial diet. Fish were periodically screened to determine the

prevalence and intensity of infection within the tank population. Screening was performed using microscopic examination of wet mount preparations of minced somatic muscle tissue in saline under phase contrast illumination at 160 to 200 times magnification. Heavy infections were defined as >50 spores field⁻¹. Fish found to be heavily infected were filleted for spore purification and plasmodia collection.

Kudoa spore purification. To purify spores, heavily infected Atlantic salmon muscle tissue (~7 to 10 g) was minced and sequentially sieved through 220 μm and 100 μm nylon mesh screens using a stainless steel Cell Dissociation Sieve/Tissue Grinder Kit (Sigma, St. Louis, MO, USA). During the sieving process, 100 ml of Hank's balanced salt solution (HBSS; Life Technologies, Burlington, ON, Canada) containing 1.0% lyophilized antibiotic-antimycotic mixture, were gradually added. The resultant slurry was collected into two 50 ml polypropylene centrifuge tubes and was centrifuged at 1500 $\times g$ for 15 min at 20°C. The pellets were combined, resuspended in 20 ml of HBSS and 1.0 ml amounts of the suspension were added to 15 ml polypropylene centrifuge tubes containing discontinuous gradients of Percoll™ (Sigma, St. Louis, MO) (2.5 ml layers of 15% and 30% Percoll™ in phosphate buffered saline, PBS pH 7.4). The tubes were centrifuged at 1500 $\times g$ for 30 min at 10°C and the resulting pellets of *Kudoa* spores were washed free of Percoll™ by resuspending in 50 ml of HBSS followed by centrifugation at 1500 $\times g$ for 30 min at 10°C. Finally, spores were collected in a 1.5 ml Eppendorf microcentrifuge tube, a sample was taken, counted using a Neubauer haemocytometer and the number of spores in the lot was determined. The sample was then centrifuged at 1500 $\times g$ for 5 min, excess HBSS was removed and the pellet was snap frozen at -70°C. Spores of *K. paniformis* from Pacific hake *Merluccius productus* and *K. crumena* from yellowfin tuna *Thunnus albacares* were purified using this same protocol.

Isolation of plasmodia and slide preparation. Approximately 5.0 g of heavily infected somatic muscle from a freshly sacrificed Atlantic salmon were placed in a glass Petri dish with sufficient HBSS to cover the tissue. The Petri dish was stored in a refrigerator at 5°C for 3 to 5 d to allow myoliquefaction. Under a dissecting microscope, the HBSS was examined and any free floating intact plasmodia were removed using a 3.0 ml syringe fitted with a 1.0 mm bore needle. Free plasmodia were transferred to a slide containing 4 \times 2 mm diameter wells (Marienfeld Superior, Marienfeld Laboratory Glassware, Germany). Excess HBSS was removed and the slides were allowed to air dry. Slides were then gently rinsed with distilled water (to remove excess salts) and were air dried and fixed in 70% ethanol for 1 min. In addition, histological sec-

tions of plasmodia in infected muscle tissue were made and sections were mounted as described previously (Moran et al. 1999b). All slides were stored in the presence of a desiccant at room temperature prior to use in immunofluorescence microscopy.

Derivation of monoclonal antibodies. Purified *Kudoa thyrsites* spores used for immunization were adjusted to $5 \times 10^7 \text{ ml}^{-1}$ in PBS, pH 7.4, and were frozen in $5 \times 0.2 \text{ ml}$ aliquots. At intervals of 3 d, 2 female BALB/c mice were given a total of five subcutaneous bilateral injections of $50 \mu\text{l}$ freshly thawed spore suspension per leg in the diffuse adipose tissue between the calcaneous tendon and the posterior aspect of the tibia. Three days after the last injection, the mice were sacrificed by cervical dislocation, the heart severed and the blood collected using a sterile Pasteur pipette. The skin of the tibia and femur of each injected leg was removed and the draining inguinal and femoral lymph nodes were excised and placed in a sterile 1.5 ml Eppendorf microcentrifuge tube containing complete serum-free Dulbecco's Minimal Essential Medium (DMEM). The lymph nodes were minced with fine scissors, the cell fragments left to settle and the supernatant collected in a sterile 15 ml conical-bottom centrifuge tube. One ml of sterile, serum-free DMEM was added to the remaining fragments which were again minced and the supernatant collected into the 15 ml tube. This was repeated an additional 2 times and the volume was topped to 10 ml with serum-free DMEM that had been warmed to ambient temperature. The collected lymph node cells were then fused with X63-Ag8.6.5.3 parental myeloma cells and single step selection and cloning of hybridomas was performed using the ClonaCell-HY™ system (StemCell Technologies Inc., Vancouver, BC) following the instructions of the manufacturer. Essentially, the cell fusion mixture was diluted and plated in a semi-solid methylcellulose containing HAT selective medium and B-cell growth factors, allowing a single-step selection and cloning of the hybridomas. After isolation and growth of individual clones, hybridoma tissue culture supernatants were screened by indirect enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assays. Monoclonal antibodies in hybridoma supernatants were screened for surface binding by ELISA on glutaraldehyde-fixed intact spores using a method first developed for African trypanosomes (Richardson et al. 1986). Purified *Kudoa thyrsites* spores ($50 \mu\text{l}$ per well of $1 \times 10^6 \text{ ml}^{-1}$) were coated onto round bottom, 96-well microplates (Falcon 3911 Microtest III™ Flexible Assay Plates, Becton-Dickinson, Oxnard, CA, USA) and were fixed with glutaraldehyde prior to addition of primary and secondary antibodies and substrate. Hybridoma supernatants were also screened by indirect ELISA (Tolson

et al. 1989) using, as antigen, spores that had been disrupted by sonication in distilled water. Spores were sonicated using a narrow probe and a Sonifier Cell Disruptor W 185E (Heat Systems-Ultrasonics, Inc, Plainview, NY, USA, Setting #3, $3 \times 30 \text{ s}$, on ice). Lysates were dried onto flat-bottom ELISA plates (Falcon 3915 PRO-BIND™ Assay Plates, Becton-Dickinson, Oxnard, CA) ($100 \mu\text{l}$ per well of 1.7×10^5 spore equivalents per ml in distilled water). Isotyping of mAbs was performed using hybridoma tissue culture supernatants and an antigen-capture ELISA kit (American Qualex, La Mirada, CA) according to instructions supplied by the manufacturer. Essentially the method involves an antibody-mediated capture of murine mAbs from hybridoma tissue culture supernatants and determination of the isotypes of the bound mAbs by isotype-specific secondary antibodies that are directly enzyme-labelled.

Immunofluorescence microscopy and flow cytometry. Frozen *Kudoa* spores (1×10^7 in a 1.5 ml Eppendorf microcentrifuge tube) were thawed and resuspended in $500 \mu\text{l}$ PBS. For each immunoreaction, $50 \mu\text{l}$ of spore suspension were transferred to fresh 1.5 ml Eppendorf microcentrifuge tubes and centrifuged at $2900 \times g$ to pellet the spores. Primary antibodies (undiluted hybridoma tissue culture supernatants) were added in $50 \mu\text{l}$ volumes to resuspend the pelleted spores and the tubes were incubated for 30 min on ice. The spores were washed twice with 1.0 ml PBS by microcentrifugation for 2 min at $2900 \times g$ at 4°C and then incubated for 30 min on ice with $50 \mu\text{l}$ of a 1:50 dilution of goat-anti-mouse IgG/IgM (H + L) FITC conjugate (Caltag Laboratories, Burlingame, CA). The spores were then washed twice with 1.0 ml ice-cold PBS, resuspended in $20 \mu\text{l}$ PBS and $10 \mu\text{l}$ volumes were placed on a slide prior to microscopic examination. The slides were examined and photographs taken using a Zeiss UV microscope fitted with a digital camera or with a Zeiss Confocal microscope using a $\times 63$ oil immersion objective. For quantification of surface binding of mAbs, spores were resuspended in 1.0 ml PBS and flow cytometry was performed using a FACS-Calibur™ flow cytometer (Becton-Dickinson, San Jose, CA). For every sample 1×10^4 spores were analyzed.

Immunofluorescence analysis of *Kudoa thyrsites* plasmodia was performed on either intact, isolated plasmodia or on thin sections of infected Atlantic salmon muscle tissue. Isolated plasmodia and infected tissue on slides were fixed and permeabilized in ice-cold acetone for 10 min at -20°C , air-dried and then incubated with $10 \mu\text{l}$ of primary antibody (undiluted hybridoma tissue culture supernatants) at 37°C for 20 min in a humidified chamber. The slides were then washed in PBS twice for 10 min and once for 30 min and excess PBS was removed by blotting. FITC-labeled goat-anti-mouse IgG/IgM (H + L) secondary antibody (Caltag

Laboratories, Burlingame, CA) was diluted 1:50 in hybridoma culture medium containing 10% foetal bovine serum and applied in 10 μ l volumes to the sections. After 30 min incubation at 37°C the slides were washed 3 \times 5 min in PBS. Slowfade™ equilibration buffer and fluorescence fading inhibitor (Molecular Probes, Eugene, OR, USA) were then added, followed by a coverslip prior to microscopic examination and photography.

Chemical deglycosylation of spores. Purified *Kudoa thyrsites* spores (7.5×10^6) were lyophilized in each of 2 Reacti-Vials™ (1.0 ml, Pierce Chemical Co. Rockford, IL, USA). To 1 vial, 600 μ l anhydrous trifluoromethane sulfonic acid (TFMSA; Sigma Chemical Co., St. Louis, MO) were added and the vial was flushed with dry nitrogen to maintain an anhydrous environment. The vial was then incubated on ice for 2 h with constant stirring. The control vial was incubated on ice for 2 h without adding any reagent. After the incubation, 5.4 ml of 2 M Tris base was added to the first vial to neutralize the TFMSA and a mixture of TFMSA /Tris base was added to the second, sham-treated (control) vial. The mixtures were immediately desalted by chromatography (3.0 ml volumes on 10 ml Sephadex G-10 columns). Fractions absorbing at 280 nm were collected, pooled, dialyzed against several changes of distilled H₂O/0.2% SDS for 7 h and lyophilized. Finally, the lyophilized materials were each dissolved in 25 μ l Laemmli sample buffer (Laemmli 1970) and analyzed by SDS-PAGE and immunoblotting with mAb 4H2, specific for surface antigens of *K. thyrsites* spores.

Proteinase K-treatment of spores. Purified *Kudoa thyrsites* spores (3×10^6) were suspended in 100 μ l of PBS/5 mM EDTA, pH 7.4 containing 25 units of Proteinase K and incubated for 2.5 h at 45°C. An identical spore suspension lacking Proteinase K served as a control. After incubation the spores were pelleted by centrifugation, resuspended in 25 μ l Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with mAb 4H2.

Gel electrophoresis and immunoblotting. *Kudoa* spores of 3 different species were resuspended in Laemmli sample buffer at 3.3×10^7 spores ml⁻¹ and 15 μ l volumes of each were run on lanes of 1-dimensional SDS-PAGE minigels. Separation of proteins using 10% gels and immunoblotting using Immun-Blot™ polyvinylidene (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) were performed as previously described (Beecroft et al. 1993) except that Supersignal Dura chemiluminescence substrate (Pierce Chemical Company, Rockford, IL) was used with a 1:5 dilution of primary antibodies (hybridoma tissue culture supernatants) and 1:50 000 dilution of secondary antibody (goat anti-mouse IgG/IgM-horseradish peroxidase conjugate; Caltag Laboratories, South San

Francisco, CA). Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) was used to detect chemiluminescence.

Dot-blot arrays and immunoassay. Purified *Kudoa thyrsites* spores were suspended in 1.0 ml distilled water at 2×10^7 ml⁻¹ and divided into 2 portions, 1 of which was sonicated as described in the ELISA section of the methods. Dilutions of both intact and sonicated spores were made in water and 5 μ l volume (ranging from 10^5 to 10^1 spores or spore equivalents) were pipetted onto Immun-blot™ PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and dried using a heat lamp. Pooled tissue culture supernatant containing mAbs 2A2, 2F4, 4H2 (spore surface-specific) and 3E8 (polar capsule-specific) (i.e., a 1/4 dilution of each mAb) was used as primary antibody. Immunodetection with horseradish peroxidase conjugated secondary antibody and prolonged duration, enhanced chemiluminescence substrate was performed as described above.

RESULTS

Spore purification

Following centrifugation through Percoll™ gradients, *Kudoa thyrsites* spores were present only at the bottom of the gradient and were free of contaminating muscle cells or debris. A Nomarski differential interference contrast photomicrograph of purified spores from a representative run is shown in Fig. 1. It can be seen that the spores are intact, with only a few free polar capsules in the preparation. Immunofluorescence analysis using 1 of the surface-binding mAbs (4H2) showed that all of the purified material was immunoreactive, confirming that it consisted entirely of spores. The material at the interface of the gradient was free of spores (not shown) indicating high efficiency in the separation of spores from both the plasmodia and muscle tissue. Yields ranged from 1.0×10^6 to 5.0×10^6 spores per gram of heavily infected muscle tissue. In more than 20 different isolations, the purified spores appeared to be intact (as shown in Fig. 1). Similar results were obtained with spores of *K. paniformis* and *K. crumena*, indicating that this purification method may be applicable to other *Kudoa* species.

Monoclonal antibody screening and selection

More than 800 hybridoma clones were visible in the ClonaCell™ medium 10 d post-fusion. Of these, 480 clones were isolated by pipetting and were grown in

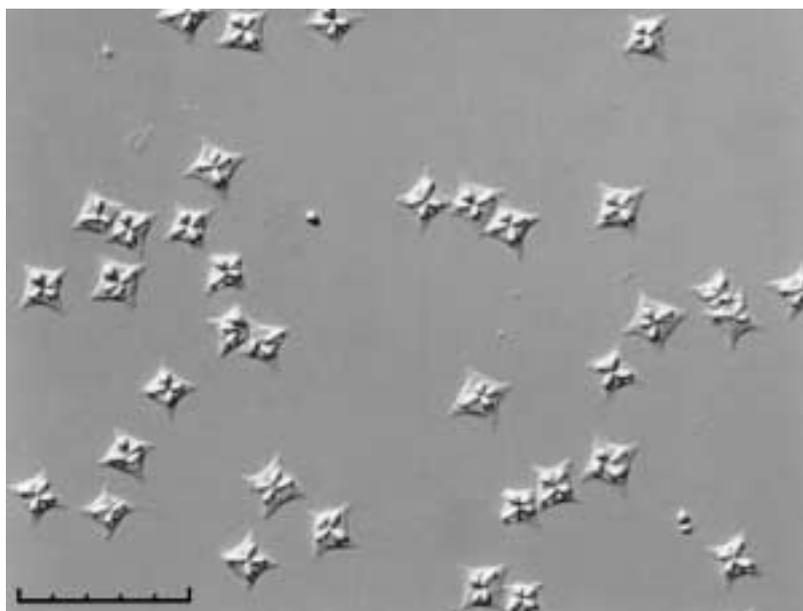


Fig. 1. Nomarski differential interference contrast photomicrograph of Percoll™-purified *Kudoa thyrsites* spores. Scale bar: 50 μm in 10 μm divisions

96-well microplates for 4 d prior to screening by ELISA using both glutaraldehyde-fixed (intact) and lysed *Kudoa thyrsites* spores as antigen. Forty-eight positive hybridomas were selected after screening for non-specific 'sticky' mAbs using human transferrin as control antigen. Of these, 10 were selected for isotyping, for cryopreservation and for further research. The clone designation and isotypes of the selected mAbs are shown in Table 1.

Detection of spore antigens by immunofluorescence and flow cytometry

The 10 selected mAbs were tested in immunofluorescence assays on Percoll™-purified spores of *Kudoa thyrsites*, *K. paniformis* and *K. crumena* (Table 1). Five of the mAbs (1B2, 2A2, 2F4, 3B5 and 4H2) showed bright fluorescence that covered the entire surface of *K. thyrsites* spores but were completely negative on spores of *K. paniformis* and *K. crumena* (i.e., these mAbs were species-specific). An example of this staining pattern is shown with mAb 4H2 in Fig. 2A. One mAb (3E8) localized to the internal polar capsules (Fig. 2B) and 2 mAbs (1H2 and 2D10) localized to polar capsules and polar filaments of *K. thyrsites* or to polar filaments of *K. paniformis* or *K. crumena* (mAb 2D10 binding to polar filaments is shown in Fig. 2C). The 2 remaining mAbs (1E5 and 4F2) did not

show any immunofluorescence staining. The surface-binding mAbs were used in flow cytometry experiments on intact *K. thyrsites* spores. A large increase in fluorescence intensity was seen with the surface-binding mAbs when compared with a control mAb. An example (mAb 4H2) of the shift in fluorescence intensity is shown in Fig. 3.

A light micrograph of a toluidine blue-stained thin section of an intact plasmodium of *Kudoa thyrsites* is

Table 1. Clone designation, isotype and specificity of anti-*Kudoa* monoclonal antibodies determined by immunofluorescence microscopy

Mab	Isotype ^a	Immunofluorescence ^b			
		<i>K. thyrsites</i> spores	<i>K. paniformis</i> spores	<i>K. crumena</i> spores	<i>K. thyrsites</i> plasmodia ^c
1B2	IgG1	Surface	-	-	4+
1E5	IgG1	-	-	-	-
1H2	IgM	Polar capsules+filaments	Polar filaments	-	2+
2A2	IgM	Surface	-	-	3+
2D10	IgM	Polar capsules+filaments	Polar filaments	Polar filaments	-
2F4	IgM	Surface	-	-	4+
3B5	IgM	Surface	-	-	3+
3E8	IgM	Polar capsules	Polar capsules	Polar capsules	-
4F2	IgM	-	-	-	2+
4H2	IgM	Surface	-	-	4+

^aDetermined by antigen-capture ELISA

^bIndirect immunofluorescence on intact, purified spores

^cIndirect immunofluorescence on acetone-permeabilized plasmodia and thin sections of plasmodia in infected Atlantic salmon somatic muscle. Fluorescence intensity graded from 4+ (very strong) to 1+ (weak)

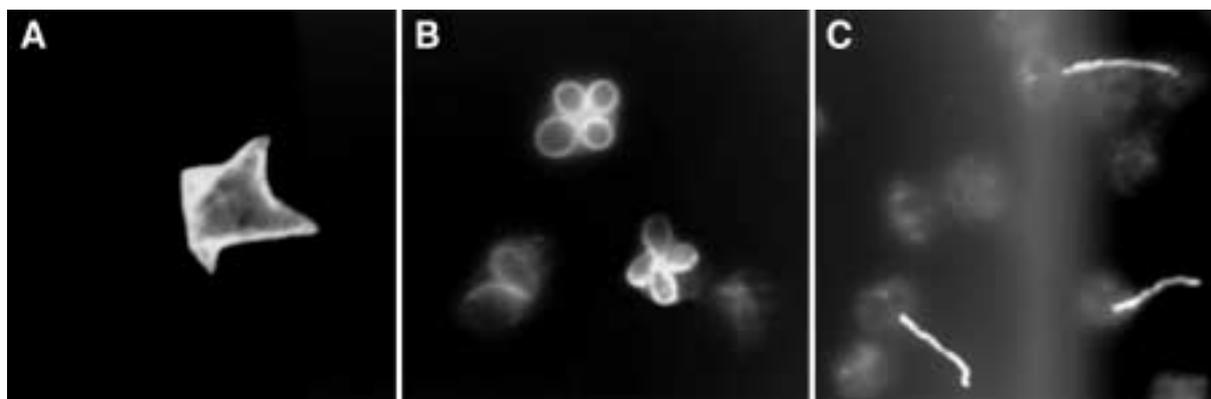


Fig. 2. Immunofluorescence photomicrographs of purified *Kudoa* spores labeled with spore-specific mAbs. Indirect immunofluorescence performed on slides using undiluted hybridoma tissue culture supernatants and a 1:50 dilution of fluorescein-conjugated goat anti-mouse IgG/IgM secondary antibody. (A) Surface-specific mAb 4H2, *K. thyrssites* spores. (B) Polar capsule-specific mAb 3E8, *K. thyrssites* spores. (C) Polar filament-specific mAb 2D10, *K. paniformis* spores

shown in Fig. 4A. The crystal-like interior shows that the plasmodium contains many spores, although the resolution is not definitive at this low magnification. Immunofluorescence microscopy was therefore used to show more clearly the spores within a plasmodium. Two mAbs (1B2 and 4H2), that bound to *K. thyrssites* spore surfaces in fluorescence microscopy and flow cytometry experiments, were tested on isolated, acetone-permeabilized *K. thyrssites* plasmodia. Both showed strong fluorescence. An example using mAb 4H2 is shown in Fig. 4B. The fluorescence patterns were complex, presumably due to large amounts of spores distributed throughout the intact plasmodium. Using the same mAb on thin sections of *K. thyrssites*-infected

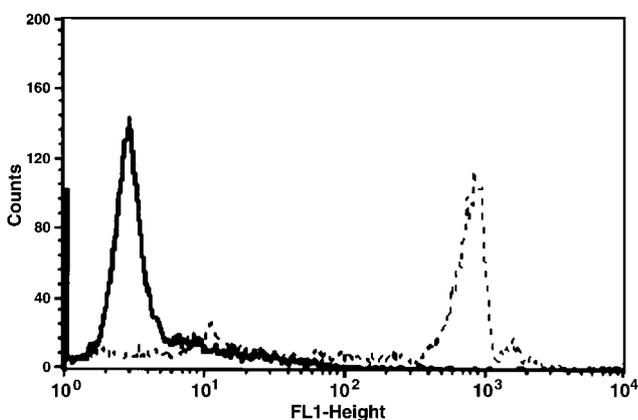


Fig. 3. Flow cytometry analysis of mAb binding to surface of purified *Kudoa thyrssites* spores. Undiluted hybridoma tissue culture supernatants were used with a 1:50 dilution of fluorescein-conjugated goat anti-mouse IgG/IgM secondary antibody. (----), anti-*K. thyrssites* mAb 4H2; (—), anti-African trypanosome (*Trypanosoma brucei brucei*) procyclin mAb 247 (negative control). 1×10^4 spores were counted in each case

muscle tissue, fluorescent spores could clearly be seen in a highly organized array inside a cross section of a plasmodium (Fig. 4C). In some cases, spores could be seen free, outside of plasmodia, in fish tissue (Fig. 4D).

Spore antigen identification by immunoblotting

Immunoblotting with the 10 selected mAbs was performed on SDS-PAGE-separated molecules of *Kudoa thyrssites* spores. The results with positive mAbs are shown in Fig. 5. Six of the mAbs recognized molecules of a range of varying apparent molecular masses which appeared as relatively defined bands whereas the remaining 3 (2A2, 2F4 and 4H2) detected molecules that migrated on SDS-PAGE as very intense broad bands of 45 to >220 kDa. When tested on immunoblots of spore molecules from different *Kudoa* species, these latter 3 mAbs bound only to molecules of *K. thyrssites* and not to molecules of *K. paniformis* or *K. crumena*, confirming that they were species-specific. The immunoblot pattern of 1 of these mAbs (4H2) on spores of different *Kudoa* species is shown in Fig. 6A. This mAb, like its surface-binding counterparts 2A2 and 2F4 (not shown) bound strongly to molecules of *K. thyrssites* spores but not to molecules of *K. paniformis* or *K. crumena*. Some of the other mAbs cross-reacted with molecules of other *Kudoa* species. For example, mAb 2D10 that bound to polar capsules in immunofluorescence experiments, detected molecules of approximately 70 kDa in *K. thyrssites* and 220 kDa in *K. paniformis* (Fig. 6B). Similarly, mAb 3E8 that bound to polar filaments in immunofluorescence reactions, bound to molecules of approximately 64 kDa and 90 to 120 kDa in *K. thyrssites* and *K. paniformis* respectively, and to a doublet of approximately 66 kDa in *K. crumena* (Fig. 6C).

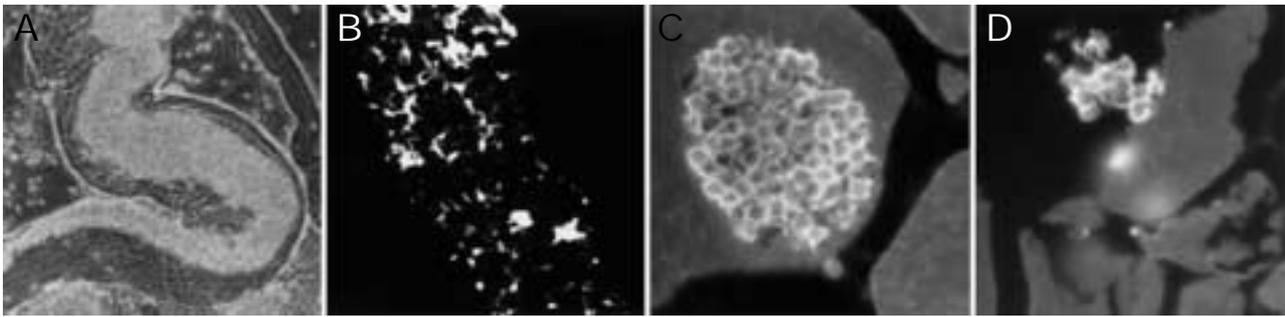


Fig. 4. Light microscopic and immunofluorescence photomicrographs of *Kudoa thyrsites* plasmodia in muscle tissue of infected Atlantic salmon. (A) Toluidine blue-stained thin section of intact plasmodium inside muscle tissue of infected Atlantic salmon. (B) Intact, acetone-permeabilized plasmodium isolated from liquified *Salmo salar* muscle tissue (immunofluorescence). (C) Thin section through single plasmodium inside muscle cell (immunofluorescence). (D) Thin section through infected *S. salar* muscle tissue showing extra-plasmodial *K. thyrsites* spores (immunofluorescence). Indirect immunofluorescence was performed on slides using undiluted hybridoma supernatant containing spore surface-specific mAb 4H2 and a 1:50 dilution of fluorescein-conjugated goat anti-mouse IgG/IgM secondary antibody

Chemical deglycosylation and Proteinase K treatment of *Kudoa thyrsites* spores

Immunoblotting was performed to detect *Kudoa thyrsites* spore surface epitopes in both untreated spores and spores chemically deglycosylated by treatment with anhydrous TFMSA. The results are shown in Fig. 7. Treatment with TFMSA completely abrogated mAb 4H2 binding (lane 1) whereas a characteristic immunoblot profile with mAb 4H2 was seen with untreated spores (lane 2). Treatment of spores with Proteinase K did not affect the binding of the same mAb in immunoblotting experiments (not shown).

Detection of spore antigens by dot-blotting

Kudoa thyrsites-specific mAbs were used in dot-blotting experiments to test their ability to detect low numbers of spores. Lysates from sonicated spores were dried onto PVDF membrane and antigens were detected by indirect immunoassay using extended duration enhanced chemiluminescence substrate. The surface-binding mAb 4H2 detected molecules from as few as 500 spores (Fig. 8A). A mixture of mAbs (1H2, 2D10, 3E8 and 4H2), selected on the basis of their specificity for different *K. thyrsites* antigens (i.e., polar capsules, polar filaments and spore surface antigens), was able to detect antigen from as few as 100 parasites (Fig. 8B). When intact, non-sonicated spores were used as antigen, at least 10 times as many spores were required for detection (data not shown).

DISCUSSION

Spores constitute a large proportion of mature plasmodia in *Kudoa*-infected fish and are thus a rational target for the development of immunodiagnostic tests and for immunization. By using a simple Percoll™ gradient technique we were able to isolate, with high efficiency and purity, large quantities of spores from infected muscle tissue, as determined by Nomarski differential interference contrast and immunofluorescence microscopy with spore-specific mAbs. The technique was used with muscle tissue from 3 different fish species (Atlantic salmon, Pacific hake and yellowfin tuna), each infected with a different species of *Kudoa* and yielded highly purified spores in all cases, indicating that the Percoll™ gradient technique may be of general utility. Yields varied but we could consistently purify more than 1×10^6 spores per gram of infected fish tissue, enough for immunological and biochemical characterization.

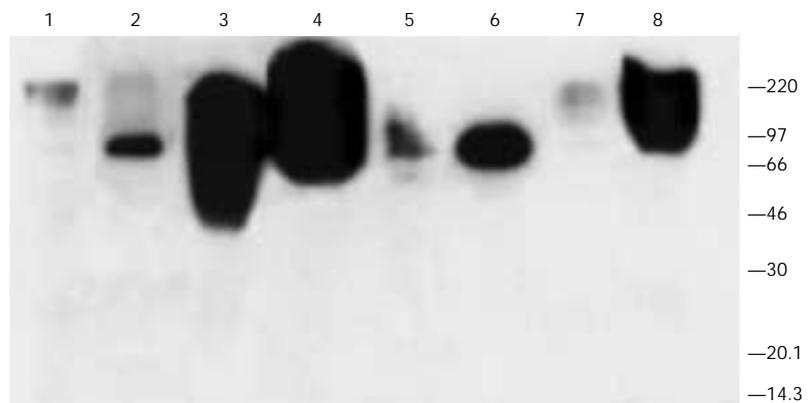


Fig. 5. Immunoblot analysis of anti-*Kudoa* spore-specific mAbs. Lane 1: mAb 1B2; Lane 2: mAb 1H2; Lane 3: mAb 2A2; Lane 4: mAb 2F4; Lane 5: mAb 3B5; Lane 6: mAb 3E8; Lane 7: mAb 4F2; Lane 8: mAb 4H2. Migration positions of molecular mass markers (in kDa) on right

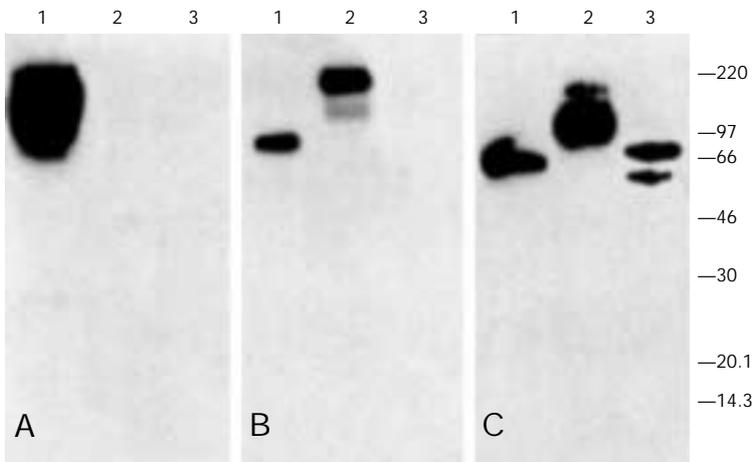


Fig. 6. Immunoblot analysis of anti-*Kudoa* spore-specific mAbs on spores of various *Kudoa* species. (A) mAb 4H2; (B) mAb 2D10; (C) mAb 3E8. In each panel, Lane 1: *K. thyrsites*; Lane 2: *K. paniformis*; Lane 3: *K. crumena*. Migration positions of molecular mass markers (in kDa) on right

Purified *Kudoa thyrsites* spores were used, without adjuvant, to immunize mice for mAb production and proved to be highly immunogenic. More than 800 hybridomas were obtained from a single fusion, which was performed after only a 15 d immunization period. Only 10 mAbs were selected for further characterization and even though this number was less than 25% of the total number of positive hybridomas identified, they were specific for a variety of antigens as revealed by immunofluorescence patterns and by immunoblotting on 3 different species of *Kudoa* spores. MAbs that recognized antigens of polar capsules, polar filaments and of the spore surface were identified. Some antigens were shared between all 3 species of *Kudoa* spores that were tested and it is interesting that the molecules detected in each species were of different apparent molecular masses. What is most intriguing is that all 4 of the mAbs that bound to the spore surface epitopes were specific for *K. thyrsites* and did not bind at all to anti-

gens of the other *Kudoa* species tested, *K. paniformis* or *K. crumena*, (i.e., they were species-specific). Perhaps this is an indication that the spore surface molecules play a role in infection of salmon. Alternatively they may play a role in infection of the as yet unidentified alternate host as this would be a natural molecular interface for host-parasite interactions. The immunoblot patterns seen with the surface-binding mAbs were unusual as they were extremely intense smears that extended through a large molecular mass range, from 45 to >220 kDa. This pattern is often characteristic of heavily glycosylated molecules or those with high ionic charge, both of which influence the amount of SDS bound to the molecules. This affects the charge to mass ratio, thus impeding electrophoretic migration in polyacrylamide gels. It is also possible that different

genes encoding different isoforms of proteins are simultaneously expressed and that the different protein gene products migrate uniquely in gels. This has been observed with the major surface proteins, the procyclins, of the African trypanosome *Trypanosoma brucei brucei* (Roditi et al. 1998, Pearson et al. 2000). In this case, the protein isoforms differ in the number of proline-glutamic acid repeats they contain and in whether or not they have an N-linked glycosylation site, both features which clearly influence migration in gels.

All 5 mAbs that were specific for spore surface antigens showed intense immunofluorescence on purified spores. In addition, flow cytometry experiments using purified spores with the surface-binding mAbs showed that the fluorescence intensity shifted more than 1000-fold when compared to a control, non-binding mAb. Along with the extremely strong immunoblot

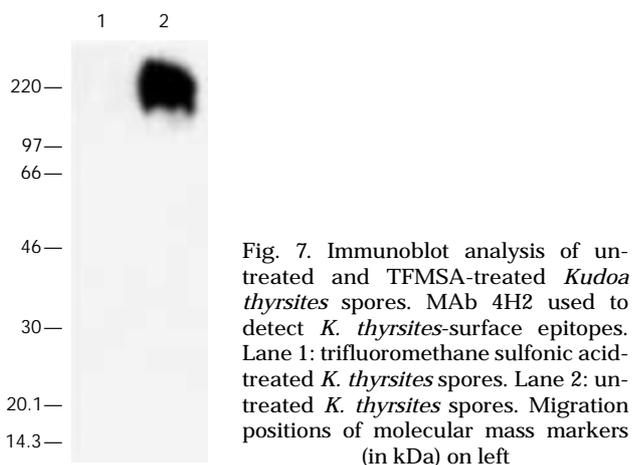


Fig. 7. Immunoblot analysis of untreated and TFMSA-treated *Kudoa thyrsites* spores. MAb 4H2 used to detect *K. thyrsites*-surface epitopes. Lane 1: trifluoromethane sulfonic acid-treated *K. thyrsites* spores. Lane 2: untreated *K. thyrsites* spores. Migration positions of molecular mass markers (in kDa) on left

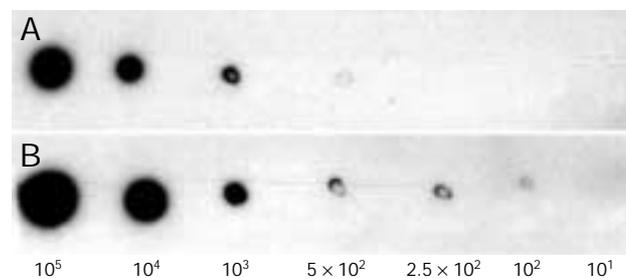


Fig. 8. Immunodetection of *Kudoa thyrsites* spores by dot-blot titration analysis using spore-specific mAbs. (A) mAb 4H2 (1:4 dilution of hybridoma culture supernatant); (B) mAbs 1H2, 4H2, 2D10 and 3E8 (undiluted mixture of hybridoma tissue culture supernatants). Numbers of purified, sonicated spores dotted in 5 μ l volumes onto the PVDF membrane indicated at bottom of figure

patterns, the fluorescence microscopy and flow cytometry data indicate that the surface epitopes are abundant, a characteristic that is desirable for antigen detection in an immunodiagnostic test. The epitopes were shown to be carbohydrate, as they were unaffected by digestion with Proteinase K, yet were completely destroyed by treatment with anhydrous TFMSA, a reagent that breaks glycosidic bonds while leaving peptide bonds intact.

Immunofluorescence microscopy, using the spore-specific mAbs on isolated plasmodia and on thin sections of plasmodia in infected Atlantic salmon muscle tissue, showed that the plasmodia contained large numbers of spores that could easily be detected with individual mAbs. In some cases the spores could be seen outside of the plasmodia and free of muscle tissue, implying that the spores had been released from the plasmodia into extracellular spaces. Whether the free spores in the particular samples we observed were an artifact of the tissue preparation technique or truly represent the release of spores from mature plasmodia during the natural life cycle of *Kudoa* is unknown. It is clear however, that macrophages containing ingested spores can often be seen in infected muscle of Atlantic salmon, implying that they have been released from plasmodia.

The ease of detection of the *Kudoa thyrsites* spores in salmon tissue suggested to us that the mAbs could be used to develop a diagnostic test for *Kudoa* infections. The sensitivity of detection with selected mAbs was therefore tested with known numbers of purified spores in a dot-blot array using a sensitive chemiluminescence based immunoassay. It was clear that a mixture of mAbs specific for several different internal and surface disposed spore epitopes was more sensitive than individual mAbs and that sonication of the spores was also important for antigen exposure. As few as 100 spores could be detected. It has been estimated that a threshold of infection of 20 000 spores per gram of infected muscle tissue is required before post-mortem myoliquefaction occurs (St-Hilaire et al. 1997). In our lab-based assay we used 5 µl of spore suspension to spot onto the detection membrane. Hypothetically, if 5 µl of an infected tissue suspension (1 g ml⁻¹ at a threshold level of 20 000 spores per gram) were spotted onto the membrane they would contain 100 spores, a number which is clearly detectable in our assay. It is likely however, that the cells and debris in crude salmon tissue extracts would interfere with the binding and detection of so few spores in a complex mixture. By using an antigen-capture ELISA or by enriching for spores, perhaps using differential centrifugation techniques, we should be able to markedly isolate spores from infected fish tissue, enabling the development of a sensitive diagnostic test. Such a test would be useful for screening subpopulations of fish to forewarn fish farmers of a potential problem in a population des-

tinued for harvest. In addition, fish could be tested on the processing line to allow removal of fish at risk of developing soft flesh syndrome.

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