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NOTE

# Epitopes associated with mature spores not recognized on *Kudoa thyrsites* from recently infected Atlantic salmon smolts

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ABSTRACT: Atlantic salmon *Salmo salar* skeletal muscle was examined for *Kudoa thyrsites* by polymerase chain reaction (PCR) and positive fish were further examined by *in situ* hybridization (ISH) and immunohistochemistry (IHC). The infection was detected in 42% of salmon by PCR following a 60 d exposure to infective seawater at a temperature of 10°C (= 600 degree-days, °D). The parasite was detected by ISH in skeletal and cardiac muscle but not in gill, kidney, spleen, liver, stomach, intestine, pyloric caeca and skin. None of 4 monoclonal antibodies (2F4, 4H2, 1H2, 3E8) raised against mature *K. thyrsites* spores reacted with the stages identified by ISH following a 600 °D exposure, but they did react with ISH-identified stages following a 1600 °D exposure. In contrast, a polyclonal antibody reacted with *K. thyrsites* stages in salmon with both 600 and 1600 °D exposures, suggesting that the parasite observed in 600 °D infections represents an antigenically distinct developmental stage of *K. thyrsites*.

KEY WORDS: *Kudoa thyrsites* · Myxosporea · *Salmo salar* · *In situ* hybridization · Immunohistochemistry



## INTRODUCTION

*Kudoa thyrsites* (Myxozoa: Myxosporea) has a global distribution and wide host range, including commercially important Atlantic *Salmo salar*, chinook *Oncorhynchus tshawytscha*, and coho *Oncorhynchus kisutch* salmon (Kabata & Whitaker 1989, Moran et al. 1999b). The life cycle of *K. thyrsites* is unknown but, as with other Myxosporea (Kent et al. 2001), it is thought to include developmental stages within both the fish and an alternate invertebrate host (Moran et al. 1999c). Infection in salmon occurs upon seawater entry by exposure to a presumptive water-borne infective stage. Members of some myxosporean genera, such as *Myxobolus* and *Sphaerospora* have one or more developmental stages within their fish hosts in tissues remote from the ultimate site of sporulation (Csaba 1976, El-Matbouli et al. 1995). Moran et al. (1999c) showed that *K. thyrsites* infection could be transmitted to naïve Atlantic salmon by injecting blood from an infected fish, suggesting that an extra-sporogonic stage (or stages) may occur in the blood of salmonids.

Mature spores and plasmodia of *Kudoa thyrsites* are detected by microscopic examination of fresh or histological preparations of skeletal muscle (Whitaker & Kent 1991), by PCR (Hervio et al. 1997) or by *in situ* hybridization (ISH) (Jones et al. 2003). Earlier developmental stages of the parasite are neither commonly reported nor well characterized. For example, PCR was used to detect infection in several Atlantic salmon tissues as early as 4 wk post seawater-exposure (p.e.) (Moran et al. 1999a). The earliest reported detection by histology occurred at about 2 mo p.e., with what were apparently pre-sporogonic plasmodia observed in the

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skeletal muscle, whereas developmental stages were first reported 17 wk p.e. in cardiac muscle (Moran et al. 1999a). The immunohistochemical examination of *K. thyrsites* stages in recently (60 d) exposed Atlantic salmon formed the basis of the present study.

## MATERIALS AND METHODS

Approximately 100 Atlantic salmon smolts (average length: 173 mm, average weight: 61.2 g) were held in tanks and exposed to flowing seawater from Departure Bay, Nanaimo, British Columbia, in which the parasite is enzootic (Moran et al. 1999c). The fish were fed a commercial diet for the duration of the study. After 60 d exposure at a constant seawater temperature of 10°C (= 600 degree-days, °D), fish were killed by immersion in MS-222 and samples of blood, heart, spleen, liver, anterior kidney, posterior kidney, eye, gill, stomach, pyloric caeca, lower intestine and skeletal muscle (including skin), were collected. Tissues were preserved in phosphate-buffered 10% formalin (pH 7.0) for 24 h, followed by two 24 h washes with 95% ethanol. Additional skeletal muscle samples were harvested aseptically and frozen at –20°C for examination by polymerase chain reaction (PCR). Fixed tissues were dehydrated through an increasing alcohol gradient and embedded in paraffin. We mounted 5 µm sections onto aminoalkylsilane-treated glass slides (Sigma Diagnostics) for ISH and immunohistochemistry. Similar tissues from exclusively freshwater-reared salmon (negative controls) and from 1600 °D seawater-exposed salmon with previously confirmed infections (positive controls) were collected and processed as described above.

DNA was extracted from ~25 mg skeletal muscle from each fish, using the Qiagen Dneasy tissue kit according to manufacturer's instructions. Aliquots of 1.5 µl of extracted DNA (<50 µg $ml^{-1}$) were then used as template in PCR reactions to amplify a 531 base-pair fragment of the *Kudoa thyrsites* 18S rRNA gene (Jones et al. 2003).

Tissues from PCR-positive fish were assayed by ISH using the protocol of Jones et al. (2003). Briefly, tissue sections were de-paraffinized in an increasing alcohol gradient and permeabilized for 10 min in cold (–20°C) acetone. Sections were rinsed twice in phosphate-buffered saline (PBS), followed by an incubation in acetic anhydride and 0.1 M triethanolamine (pH 8.0), and a subsequent wash in PBS and 2 washes in 4× SSC (0.6 M NaCl, 0.06 M sodium citrate). Sections were incubated for 1 h in hybridization buffer (51% deionized formamide, 20% 20× SSC, 1% dextran sulfate, 0.5 mg $ml^{-1}$ heat-denatured herring sperm DNA, 1× Denhardt's [0.02% acetylated bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400], and 0.025% sodium dodecyl sulfate). The DIG_KUDISH5r probe (5′-ATTTGGCACTTGCGTACGC-3′) was applied at 3 ng $µl^{-1}$ and the sections were coverslipped and heated to 95°C for 5 min. The covered sections were incubated overnight at 37°C. After hybridization, sections were washed twice in 4× SSC. The hybridized probe was detected by following the DIG Nucleic Acid Detection Kit protocol (Roche Diagnostics). Sections were counterstained in Bismarck Brown Y (2 min), dehydrated in alcohol and xylene, and coverslipped with Permount prior to microscopic observation and photography.

Cardiac and skeletal muscle sections from three 600 °D-exposed fish that were identified by ISH as heavily infected were also examined by immunohistochemistry (IHC). IHC with each primary poly- and monoclonal antibody was also performed on skeletal muscle sections from fish with 1600 °D exposure. Serial sections (5 µm) were alternately assayed with either ISH or IHC, allowing direct comparison between locations on sequential slides. We tested 4 monoclonal antibodies (mAb: 2F4, 4H2, 1H2 and 3E8; Chase et al. 2001) and 1 polyclonal antibody (pAb) raised in rabbits against mature *Kudoa thyrsites* spores as described in Chase et al. (2003). Tissue sections were deparaffinized and rehydrated in phosphate-buffered saline (PBS) (pH 7.4), plus 0.15% Tween-20 (PBS/T). Tissues were permeabilized in ice-cold acetone at –20°C for 10 min, and rinsed in PBS/T. Slides were incubated with 200 µl of primary antibody (mAb undiluted, pAb dilution 1:10,000, in PBS/T), at room temperature for 1 h in a humid chamber. Monoclonal antibodies were tested individually or combined in pools of 2 (IH2 and 3E8, 2F4 and 4H2) or 4. Slides were first rinsed (5 × 1 ml), then washed 3 times in PBS/T and incubated with 200 µl of secondary antibody (1:50 dilution in PBS/T) (mAb: alkaline phosphatase [AP] conjugated goat anti-mouse immunoglobin, IgG and IgM [H+L]; pAb: AP-conjugated goat anti-rabbit IgG [H+L]; Jackson Immunoresearch Laboratories), for 45 min in a humid chamber. Slides were washed as above. Sections were incubated for 2 h with 200 µl of nitro blue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP) substrate (1/50 dilution), and washed in deionized, distilled $H_2O$ to stop the reaction. Sections were counterstained in Bismarck Brown Y, dehydrated in alcohol and xylene, and coverslipped with Permount prior to microscopic observation and photography. Controls included incubation of primary and secondary antibodies with tissue sections from non-exposed fish, as well as incubation of infected muscle with a non-specific primary antibody, followed by secondary antibody. ISH and IHC preparations were viewed with a Leitz Dialux 22 series microscope and micrographs were taken using a Nikon Coolpix 995 camera.

## RESULTS

### Polymerase chain reaction and *in situ* hybridization

*Kudoa thyrsites* was detected by PCR in the skeletal muscle of 42% (43/102) of salmon sampled after 600 °D of seawater exposure. Positive ISH signals were detected in the skeletal muscle of 51% (22/43) of PCR-positive fish. Of these, 16 fish also showed strong ISH signals in cardiac muscle. Variability in the intensity of the ISH signal among samples was noted, with some sections showing multiple strong signals, while others displayed much smaller and weaker signals. Staining was observed in both white and red skeletal muscle, as well as in muscle fibers associated with the eye and gill. No staining was observed in any other tissue. Strong signals were observed in all positive control sections, whereas sections from naïve fish were consistently negative.

### Immunohistochemistry

None of the 4 mAbs, either alone or as pools, reacted with *Kudoa thyrsites* in 600 °D-exposed fish (Fig. 1B). The presence of the parasite was confirmed by ISH staining in flanking serial sections (Fig. 1A,C). In contrast, strong IHC reactivity was observed in serial sections from 600 °D-exposed fish assayed with the pAb (Fig. 1D). Strong staining was observed for each of the 4 mAbs and the pAb in muscle sections from fish with 1600 °D exposure (Fig. 2). Negative control assays consistently showed no staining following incubation with either the mAb or pAb.

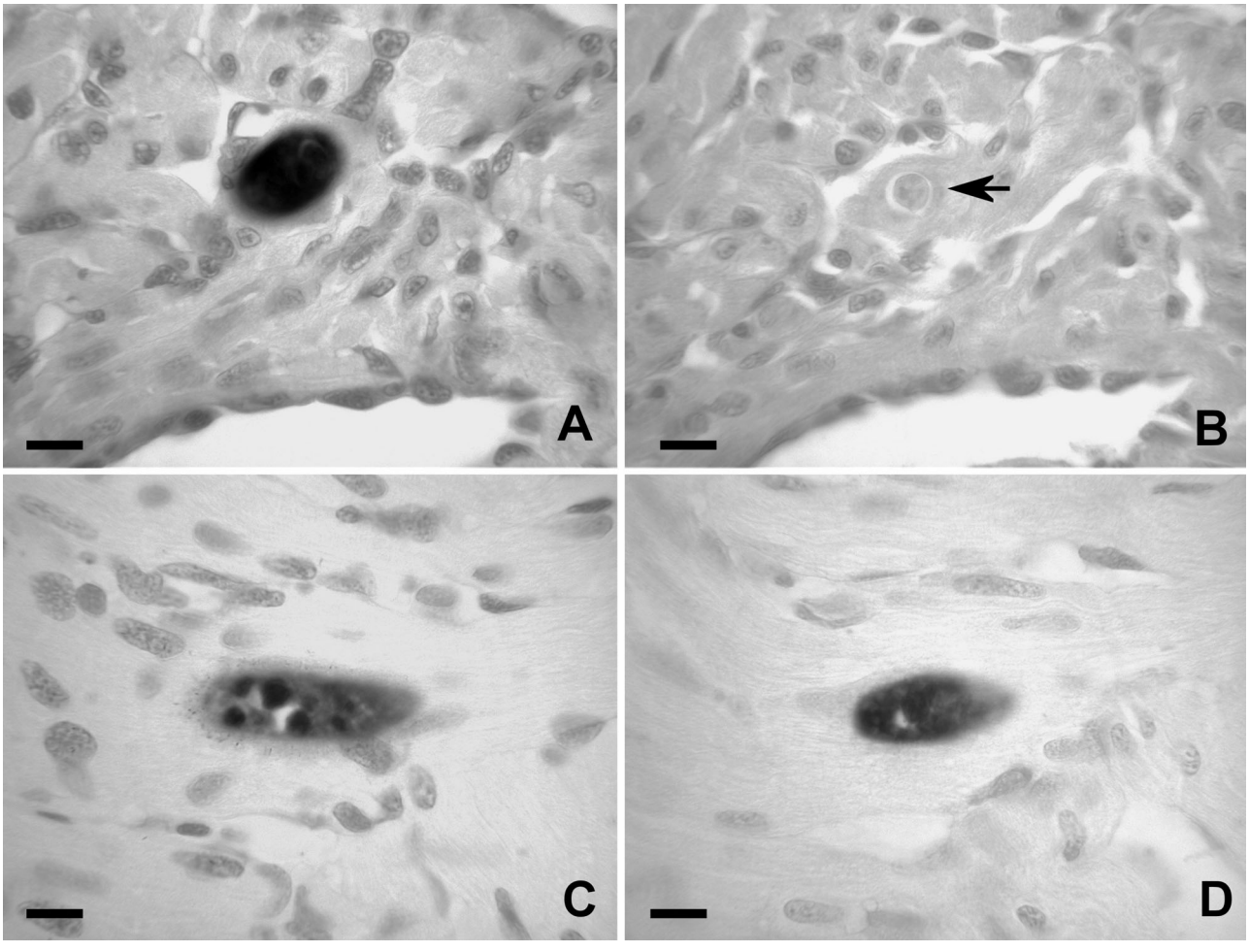


Fig. 1. Detection of *Kudoa thyrsites* in cardiac muscle from Atlantic salmon *Salmo salar* with 600 degree-day seawater exposure by *in situ* hybridization (ISH) and immunohistochemistry (IHC). Serial sections show staining by (A) ISH and (B) monoclonal antibody 1H2 . Arrow marks location of parasite in (B). Results for 1H2 are representative of those for 3 additional monoclonal antibodies tested (2F4, 4H2, 3E8), each of which failed to react with *K. thyrsites* stages in both cardiac and skeletal muscle, whereas *K. thyrsites* in flanking sections was intensely stained with ISH. In contrast, *K. thyrsites* was detected in serial sections of cardiac muscle stained with (C) ISH and (D) polyclonal antibody. Similar results were observed for skeletal muscle. Scale bars = 10 µm

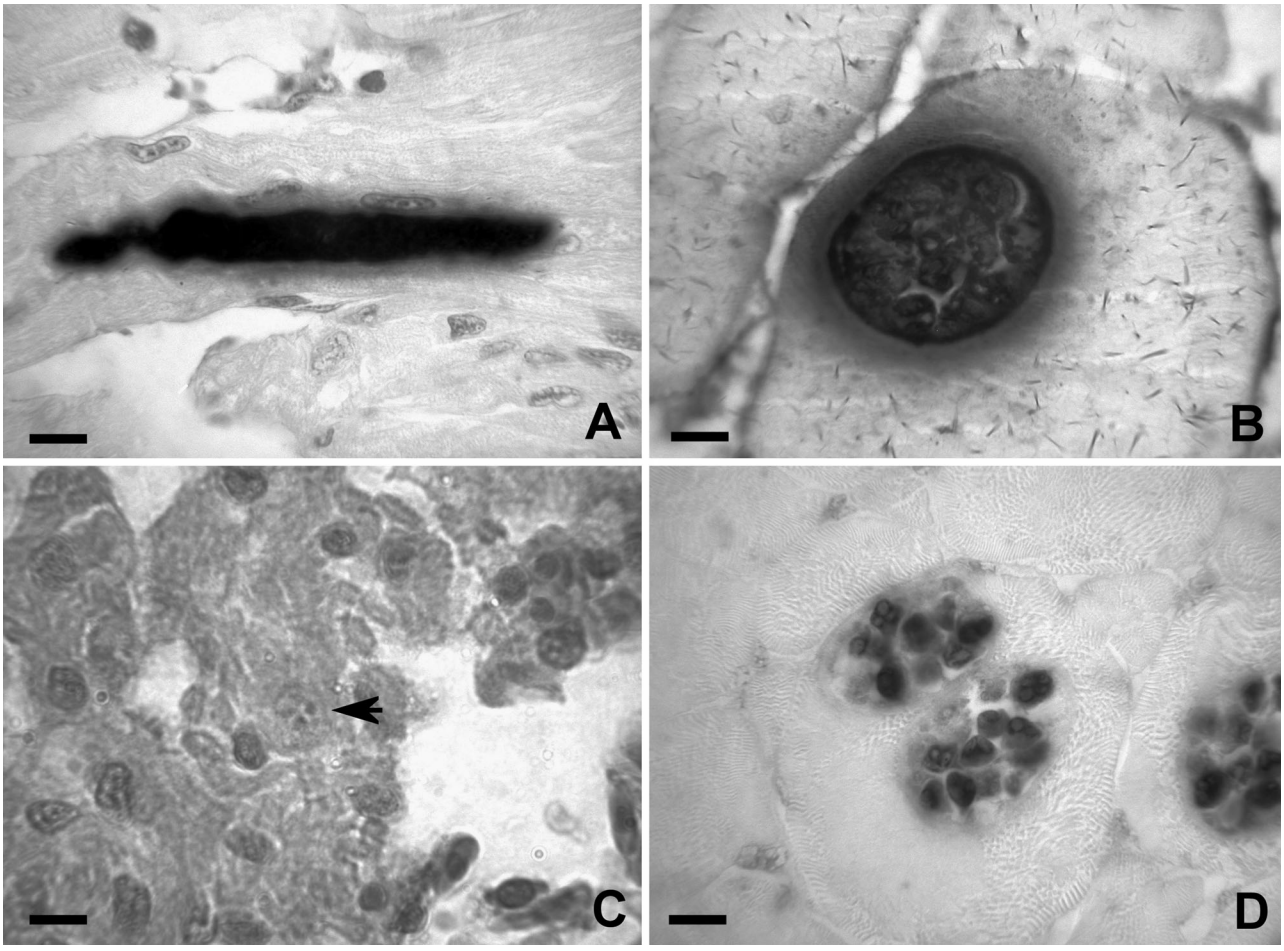


Fig. 2. Detection of *Kudoa thyrsites* in Atlantic salmon *Salmo salar* by immunohistochemistry. Polyclonal antibody detects *K. thyrsites* in muscle tissue with (A) 600 and (B) 1600 degree-days (°D) seawater exposure. In contrast, monoclonal antibodies (mAbs) do not react with stages of *K. thyrsites* from 600 °D-exposed salmon (arrow, C), but do react strongly with mature spores (D) from 1600 °D-exposed salmon. Results shown are for mAb 3E8, and are representative of those for each mAb tested (2F4, 4H2, 1H2) individually and pooled. Scale bars = 10 µm

## DISCUSSION

The presence of *Kudoa thyrsites* in Atlantic salmon smolts following a relatively brief (600 °D) exposure was demonstrated using polymerase chain reaction and confirmed by ISH. The apparent difference in infection rates suggested by PCR and ISH was probably due to differences in the amounts of tissue assayed or to differences in the sensitivities of these assays. Well-differentiated spore-like structures were clearly visualized within the 600 °D plasmodia and, at this time, stained strongly using ISH. In contrast, ISH of *K. thyrsites* from the 1600 °D infections resulted in strong staining around the periphery of the plasmodia but the mature spores remained largely unstained probably due to poor permeability of the probe, similar to observations of Jones et al. (2003). Further examination of histological preparations using reagents that better resolve structural detail, such as Gram or Giemsa stains, or transmission electron microscopy are required to accurately identify these *K. thyrsites* developmental stages. Their relatively intense ISH staining, however, suggested that they were pre-sporogonic.

Immunohistochemical assays showed all 4 monoclonal antibodies to be reactive with *Kudoa thyrsites* in formalin-fixed, paraffin-embedded sections from fish exposed for 1600 °D. However, evidence of a previously unrecognized developmental complexity for *K. thyrsites* was provided by the failure of the 600 °D stages to react with any of the 4 monoclonal antibodies despite the ready detection of these stages using a polyclonal antibody. The strong ISH signals observed in flanking sections confirmed the specific location of the stages that failed to react with the monoclonal antibodies. This suggested that certain epitopes present in mature spores are sequentially expressed during

parasite development, and that those recognized by the monoclonal antibodies were not yet present in the stages examined after the 600 °D exposure. It is unlikely that the differences in antibody reactivity were the result of the loss or masking of the epitopes, since samples from both 600 and 1600 °D infections were fixed and assayed in an identical manner. The monoclonal antibodies recognized epitopes associated with the polar capsule and filament (1H2, 3E8) and with the spore surface (2F4, 4H2) (Chase et al. 2001). Furthermore, the surface epitope recognized by mAb 4H2 is associated with an abundant carbohydrate (Chase et al. 2001). Reactivity with the high-titer polyclonal antibody further suggested that in addition to those that are sequentially expressed, other epitopes are consistently expressed throughout development. Together, results from the ISH and IHC assays suggested that pre-sprogonic stages of *K. thyrsites* present after 600 °D of exposure do not express epitopes that are associated with the polar capsule, polar filament and valve surface of mature spores. We are presently attempting to better characterize these early stages by purification and immunoblotting and to establish the expression kinetics of serologically reactive parasite antigens.

Detection and characterization of the early development of *Kudoa thyrsites* within muscle had previously relied on PCR and light microscopy (Moran et al. 1999a). PCR is a sensitive assay and allows early detection but provides little information on parasite locality in specific tissues. Similarly, localization of recognizable mature infections is possible with histological staining, but does not allow characterization of obscure developmental stages. This study has shown the effectiveness of combining ISH and immunohistochemistry to characterize previously undescribed developmental stages of *K. thyrsites*. ISH was confirmed to be an effective tool in localizing *K. thyrsites* to specific tissues and in visualizing cryptic stages. Confirming the presence of these cryptic stages provided the opportunity to examine their reactivity by IHC. While polyclonal antisera may be useful in the early detection of *K. thyrsites* infection, the monoclonal antibodies, by recognizing epitopes only expressed on mature spores, will be poorly suited to this application.

Several questions relating to the transmission and early pathogenesis of *Kudoa thyrsites* remain unresolved. Exposure to the infective stage of *K. thyrsites* is measured from seawater entry, and infection is assumed to occur as of that time, but whether infection occurs by continuous or intermittent exposure to a putative infective stage is not clear. The relatively low prevalence (42%) of infection following 600 °D exposure suggested that infection occurs gradually, perhaps by trickle-exposure to a water-borne infective stage. The nature of this exposure pattern could be explored by direct monitoring of seawater for the infective stage and through an improved understanding of parasite development within the fish.

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