

Detection of the oyster parasite *Bonamia ostreae* by fluorescent *in situ* hybridization

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ABSTRACT: *Bonamia ostreae* is an economically significant protistan parasite of the flat oyster *Ostrea edulis* in Europe and North America. Management of this parasite depends partly upon its reliable identification in wild and aquacultured oyster populations, but *B. ostreae* is small and difficult to detect by traditional microscopic methods. We designed a fluorescent *in situ* hybridization (FISH) assay to sensitively detect *B. ostreae* in standard histopathological sections of *B. ostreae*-infected oysters using fluorescently labeled DNA oligonucleotide probes. Hybridization using a cocktail of 3 presumptively *B. ostreae*-specific, fluorescein iso(thio)cyanate (FITC)-labeled oligonucleotides produced an unambiguous staining pattern of small green rings inside infected oyster hemocytes that was easily distinguished from host tissue background. This pattern is diagnostic for *B. ostreae*. A negative control cocktail of oligonucleotides containing 2 mismatches relative to target sequences, on the other hand, failed to hybridize at all. *B. ostreae*-specific probes did not cross-react with a related protist, *Haplosporidium nelsoni*.

KEY WORDS: *Bonamia ostreae* · *Ostrea edulis* · Fluorescent *in situ* hybridization

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INTRODUCTION

Bonamia ostreae (Pichot et al. 1980) is a protistan parasite of *Ostrea edulis* Linné, a commercially important flat oyster that occurs widely in Europe and locally on both coasts of North America. It proliferates within oyster hemocytes (Balouet et al. 1983), often resulting in massive inflammation and death of its host (Bucke & Feist 1985). A haplosporidian protist (Pichot et al. 1980, Perkins 1990, Carnegie et al. 2000), *B. ostreae* was described after causing catastrophic oyster mortality in 1979 in Brittany, France (Comps et al. 1980, Pichot et al. 1980), but was first observed in California, USA a decade earlier (Katkansky et al. 1969, Elston et al. 1986). It now occurs in Europe from Ireland to Spain and in California, Washington, and Maine, USA (Comps et al. 1980, Pichot et al. 1980, Bucke & Feist 1985, van Banning 1985, Elston et al. 1986, Friedman et al. 1989, McArdle et al. 1991, Montes et al. 1991,

Barber & Davis 1994, Friedman & Perkins 1994, Zabaleta & Barber 1996). The disease *B. ostreae* causes, bonamiosis, is notifiable to the Office International des Epizooties (OIE), and is a serious threat to flat oyster aquaculture.

Bonamia ostreae transmission is direct (Elston et al. 1987). Bonamiosis outbreaks in Europe began when *B. ostreae* was transmitted to naïve French *O. edulis* populations from infected juvenile *O. edulis* imported commercially from California (Elston et al. 1986). This may not have occurred if the oysters had been screened before transfer, *B. ostreae* had been recognized as an infectious agent, and the transfer had been denied. *Bonamia ostreae* is not easily detected, however. Because it is small (2 to 3 µm in diameter; Pichot et al. 1980), it may be overlooked in microscopic examinations of lightly infected oysters (Bucke & Feist 1985). Such oysters may seem healthy. Early *B. ostreae* infections are typically focal (Bucke & Feist 1985,

Bucke 1988); foci may be missed during sampling. Recently developed PCR assays for *B. ostreae* (Carnegie et al. 2000, Cochenne et al. 2000), on the other hand, may detect the parasite *too* sensitively. Degraded or non-viable pathogen DNA may generate false positive PCR results. False negative results, as with histopathology, may arise because of sampling error (Burreson 2000).

In situ hybridization (ISH) is a powerful complement to traditional and PCR-based detection methods. It combines advantages of both. Like traditional histopathology (i.e. the microscopic examination of fixed and stained tissue sections), ISH reveals the tissue location of an infection and the host response. Like PCR, ISH is specific and very sensitive. Successful hybridization provides an unambiguous phylogenetic confirmation that a specific pathogen is associated with a specific host tissue. The usefulness of ISH goes beyond routine diagnostics. An ISH for *Haplosporidium nelsoni* (Stokes & Burreson 1995), for example, has been used with PCR in attempts to resolve this oyster parasite's life cycle (Stokes et al. 1997), and was used to discover its geographic source (Burreson et al. 2000). An ISH for *Bonamia ostreae*, similarly, could confirm routine PCR results, illuminate *B. ostreae*'s life cycle and transmission dynamics, and resolve its translocation history. The development of a fluorescent ISH (FISH) for *B. ostreae* was the objective of this project.

MATERIALS AND METHODS

Sample collection and histological processing. Seventy *Ostrea edulis* were collected at Gun Point Creek, Maine, USA (43° 46' 28" N, 69° 56' 50" W) on 4 June 2000. Hemolymph was non-destructively drawn from the adductor muscle of each, diluted in artificial seawater (~1:10; 30‰), and placed on a glass slide. Hemocytes adhering after 10 min were fixed and stained using Hemostat solutions (Fisher Scientific; see Culloty & Mulcahy 1996, Zabaleta & Barber 1996). Slides were screened for *Bonamia ostreae* using a compound microscope at 400× magnification until 5 oysters with abundant *B. ostreae* were identified. These individuals were sacrificed and fixed whole in Davidson's Solution (Shaw & Battle 1957) for 24 h. Transverse sections (~4 mm) posterior to the palps were dehydrated, cleared, and embedded in paraffin. Serial sections (5 µm) were cut from these blocks and dried at 50 to 55°C on 3-aminopropyltriethoxysilane (APTS)-treated slides (Schwarzacher & Heslop-Harrison 2000). The first slide in each series was stained with hematoxylin and eosin (H&E) for standard histopathological evaluation. Others were used for FISH. Thin sections (5 µm) of paraffin-embedded *Crassostrea virginica* infected

with *Haplosporidium nelsoni* were included to test for cross-reactivity of the *B. ostreae*-specific probes.

Design and preparation of probes. An alignment of haplosporidian and oyster SSU rDNA gene sequences was constructed for DNA oligonucleotide probe selection. *Bonamia ostreae* (GenBank accession number AF-262995), *Haplosporidium nelsoni* (U19538), *H. costale* (U20858), *H. louisiana* (U47851), *Minchinia teredinis* (U20319), *Urosporidium crescens* (U47852), and *Ostrea edulis* (U88709) sequences were aligned using the program Se-Al (Oxford University Evolutionary Biology Group). Seven probes were designed. UME-BO-1 (5'-CGAGGCAGGGTTTGT-3'), UME-BO-2 (5'-GGG-TCAAACCTCGTTGAAC-3'), and UME-BO-3 (5'-CGC-TCTTATCCACCTAAT-3') in theory were specific for *B. ostreae*. They composed a multi-oligoprobe cocktail, an effective way to increase sensitivity without sacrificing specificity (Trembleau & Bloom 1995). Potential cross-reactivity of these probes with other SSU rDNA sequences was discounted after a GenBank BLAST search for the probes and their targets revealed no close matches. Negative control probes UME-BO-1M (5'-CGAGCCAGGCTTTGT-3'), UME-BO-2M (5'-GGC-TCAAACCTCCTTGAAC-3'), and UME-BO-3M (5'-CCC-TCTTATCCTCCTAAT-3') each differed from corresponding *B. ostreae*-specific probes by 2 substitutions. Thus, they were slightly mismatched to theoretical target sequences. UME-OE-385 (5'-TCATGCTCCCT-CTCCGG-3') was a positive control oligonucleotide designed to bind to both *O. edulis* and *B. ostreae*.

The probes were synthesized by Operon Technologies). Each carried a 5' fluorescein iso(thio)cyanate label (FITC; absorbance λ = 490 to 496 nm; emission λ = 514 to 521 nm). All probes were purified using thin-layer chromatography (TLC; plate: 20 cm × 20 cm × 250 µm Whatman PE SIL G/UV, polyester-backed and silica-coated; running buffer: 55 ml n-propanol, 35 ml ammonium hydroxide, 10 ml H₂O) and resuspended in Tris-EDTA (pH = 7.4).

FISH. Our FISH methodology was derived from published ISH protocols (Dubilier et al. 1995, Stokes & Burreson 1995, Stokes et al. 1995). Four consecutive *Ostrea edulis* sections and 2 *Crassostrea virginica* sections were deparaffinized in xylene (3 × 10 min), rehydrated through a descending ethanol series (100, 95, 80 and 70% for 10 min each), and equilibrated in phosphate-buffered saline (PBS; once for 10 min, once for 5 min). The sections were then digested with Proteinase K (100 µg ml⁻¹ in PBS for 15 min at 37°C, followed by a wash in PBS plus 0.2% glycine for 5 min); acetylated using acetic anhydride (5% [v/v] in 0.1 M triethanolamine-HCl [pH 8.0] for 10 min at room temperature, followed by a wash in PBS for 10 min; see Schwarzacher & Heslop-Harrison 2000); and equilibrated in 5 × SET (750 mM NaCl, 6.4 mM

EDTA, 100 mM Tris base) (10 min at room temperature). Excess SET was drained off, and 200 μ l of prehybridization buffer (5 \times SET, 0.02% bovine serum albumin, 0.025% sodium dodecyl sulphate [SDS]) was added to each section. After incubation for 30 min at 45°C, the prehybridization buffer was drained off and replaced with 10 to 12 μ l of prehybridization buffer containing 2 to 10 ng μ l⁻¹ of the appropriate oligonucleotide(s). One *O. edulis* section received theoretically *Bonamia ostreae*-specific probes UME-BO-1, UME-BO-2, and UME-BO-3; a second received mismatched probes UME-BO-1M, UME-BO-2M, and UME-BO-3M; a third received no probe; and a fourth received the control probe UME-OE-385. The *C. virginica* sections (*Haplosporidium nelsoni* controls) received probes UME-BO-1, UME-BO-2, and UME-BO-3. The sections were coverslipped with parafilm and incubated overnight in humid chambers at 45°C. They were washed the next day in 0.2 \times SET (3 \times 5 min at 42°C), air dried, and then covered using Vectashield Mounting Medium (Vector Laboratories) and glass coverslips.

Slides were examined (600 to 1000 \times) using a Nikon Labophot-2 epifluorescent microscope with a dual FITC-Texas Red filter. Specific fluorescence patterns were expected to result from the 5 experimental conditions. Binding of the *Bonamia ostreae*-specific probes to *B. ostreae* rRNA was expected to result in the appearance of green rings of 2 to 4 μ m outside diameter within oyster hemocytes. The mismatched probe condition would display a near or complete absence of green fluorescence. The no probe condition and the *Haplosporidium nelsoni* control would show no green fluorescent signal (orange background alone). Finally, in the positive control, all tissues would exhibit green fluorescence.

Fluorescent images were captured on 35 mm slide film and scanned. The images were prepared for publication using Jasc Paint Shop Pro Version 7.00 (Jasc Software). Diameters of stained structures were estimated by calculation from pixel measurements using this software.

RESULTS

Probes UME-BO-1, UME-BO-2, and UME-BO-3 hybridized to *Bonamia ostreae* rRNA (Fig. 1A). Small FITC-stained objects occurred in many hemocytes in all tissues, and were sometimes free in hemolymph sinuses. Each resembled a small green ring, as green fluorescence surrounded an eccentric dark region. They resembled rings because the labeled probes were concentrated at the ribosomes within the cytoplasm, surrounding the eccentric *B. ostreae* cell nucleus. The distribution of these small green rings

precisely matched the distribution of *B. ostreae* cells observed in the H&E-stained section. The outside diameter of these rings (2.4 to 5.6 μ m) also conformed to that expected for *B. ostreae*.

Negative control treatments showed only an orange background autofluorescent signal. Fig. 1B illustrates the level of background autofluorescence in the absence of probes. The mismatched-probe control treatment (Fig. 1C) and the *Haplosporidium nelsoni* control treatment (Fig. 1D) resembled the no probe condition in Fig. 1B. They displayed none of the green fluorescence characteristic of probe binding. The failure of mismatched probes UME-BO-1M, UME-BO-2M, and UME-BO-3M to hybridize (Fig. 1C) indicated that the theoretically *Bonamia ostreae*-specific probes UME-BO-1, UME-BO-2, and UME-BO-3 hybridized to specific target sequences. The failure of UME-BO-1, UME-BO-2, and UME-BO-3 to hybridize to *H. nelsoni* (Fig. 1D) confirmed that these target sequences were, if not unique to *B. ostreae*, restricted to certain haplosporidians. Importantly, the specific probes failed to hybridize to one of *B. ostreae*'s closest phylogenetic relatives.

DISCUSSION

This FISH assay for *Bonamia ostreae* provided significant advantages over traditional histopathological techniques. The assay generated unambiguous signs when *B. ostreae* was present. *B. ostreae*-specific fluorescence possessed a characteristic color, shape, and size, and occurred in a specific tissue. It was green, ring-shaped, and small (2.4 to 5.6 μ m), and was primarily observed inside hemocytes. The color reflected specific binding of the FITC-conjugated probes; the shape reflected the cytoplasmic location of the ribosomes to which the probes hybridized; and the size reflected the actual diameter of *B. ostreae* cells. The tissue location of the fluorescence reflected the tissue specificity documented for *B. ostreae* itself (Balouet et al. 1983). Unambiguous *B. ostreae*-specific staining possessed all 4 traits.

Traditional histopathological detection of *Bonamia ostreae* depends on 3 possible observations: small size, a 'fried egg' appearance to the presumptive *B. ostreae* cells, and association of these cells with hemocytes (Bower et al. 1994). *B. ostreae* cells are easily observed when they are numerous; for example, in oysters with systemic infections. In oysters with light or prepatent infections, however, small *B. ostreae* cells may be mistaken for routine intracytoplasmic inclusions (Bucke & Feist 1985). Traditional histopathological detection of *B. ostreae* is insensitive partly for this reason. With FISH, even a small number of *B. ostreae* cells stand out from the host tissue background because, in a section, they alone are stained. Thus, FISH is more sensitive.

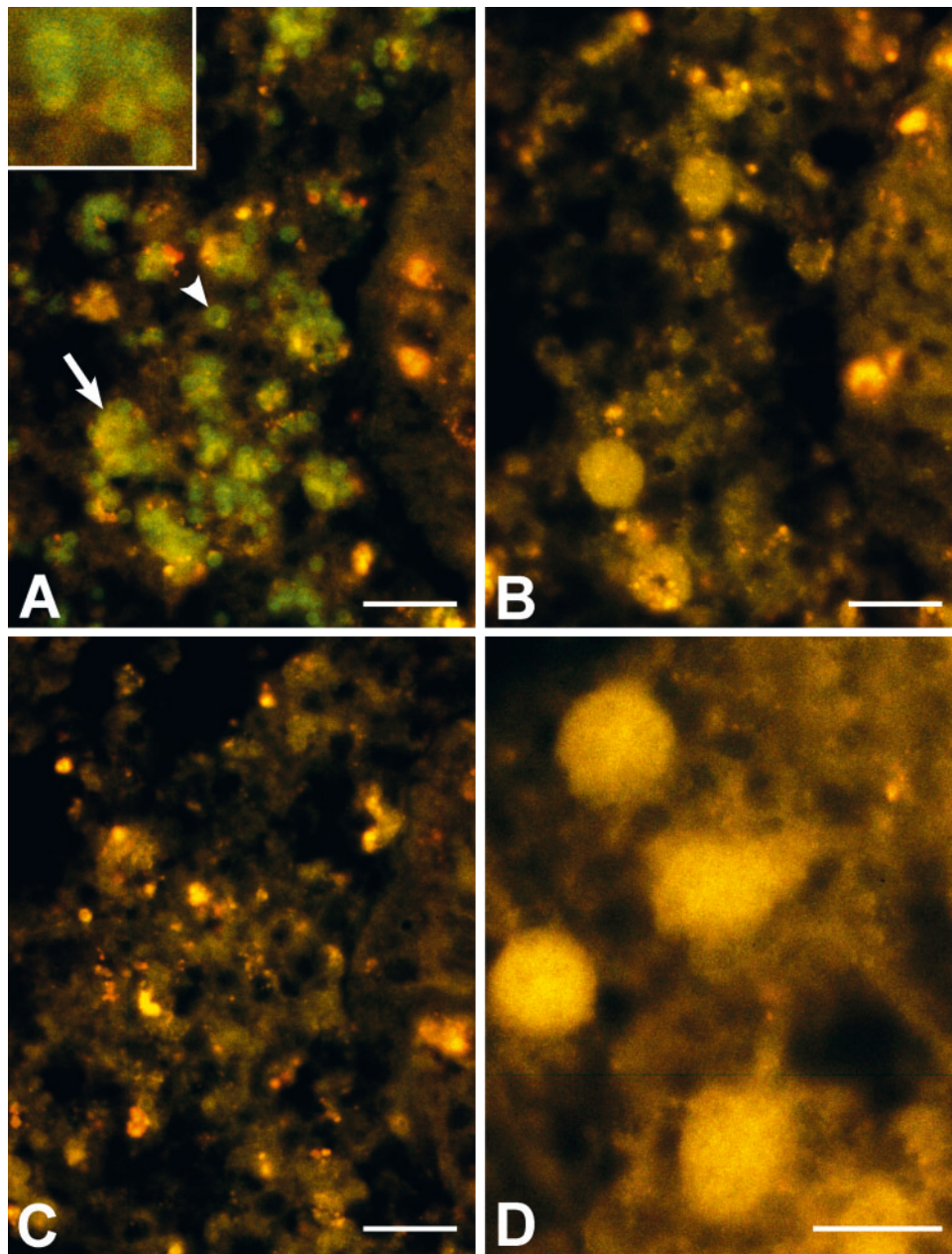


Fig. 1. Photomicrographs of the fluorescent *in situ* hybridization (FISH) trials (scale bars = 20 μ m). (A) to (C) are serial images of hemocytes aggregated at the gut wall of a *Bonamia ostreae*-infected flat oyster (A) *B. ostreae*-specific probe treatment. Green rings indicate specific binding of the *B. ostreae*-specific probes to parasite cells. Arrowhead indicates 1 individual *B. ostreae* cell; arrow indicates a cluster of cells. Inset: Detail of *B. ostreae* cells. Note the dark eccentric areas representing *B. ostreae* cell nuclei. (B) No probe treatment, indicating the level of background autofluorescence in the absence of probes. (C) Mismatched probe treatment. A cocktail of probes mismatched to target 2 positions failed completely to bind to *B. ostreae* target. (D) *Haplosporidium nelsoni* (MSX) control. *B. ostreae*-specific probes failed to bind to MSX plasmodia, the 4 areas of intense yellow autofluorescence surrounded by *Crassostrea virginica* hemocytes

FISH provided a phylogenetic confirmation of the pathogen's identity that histopathology could not. The mismatched probe control indicated that the experimental (presumptively *Bonamia ostreae*-specific) probes hybridized to very specific target sequences.

The absence of hybridization in the *Haplosporidium nelsoni* control indicated that these target sequences might be unique to *B. ostreae*. (In a partial SSU rDNA sequence for *B. exitiosus* that appeared on GenBank [AF337563] after this work was done, one probe bind-

ing site was identical to *B. ostreae*, while another matched *B. ostreae* at only 11/15 positions. The sequence at the third binding site was unknown, but weak cross-hybridization to *B. exitiosus* should occur because of the single perfectly matched probe.) Thus, in the northern hemisphere where no other *Bonamia* spp. are known to occur, this FISH assay should not generate false positive results by cross-hybridization. This is significant for 2 reasons. First, as *H. nelsoni* advances northward (Ford & Tripp 1996, Barber et al. 1997), it may eventually co-occur with *B. ostreae* in the northeastern USA. Second, the Pacific oyster *Crassostrea gigas* parasite *Mikrocytos mackini*, while not a haplosporidian (Hine et al. 2001), is indistinguishable from *B. ostreae* at the light microscope level (Farley et al. 1988), and infects *Ostrea edulis* in British Columbia, Canada where *C. gigas* and *O. edulis* are cultured together (Bower et al. 1997).

The specificity of this FISH assay, therefore, should compare favorably to *Bonamia ostreae*-specific PCRs (Carnegie et al. 2000, Cochenne et al. 2000). This assay is more specific than the *B. ostreae*-specific ISH described by Cochenne et al. (2000), which cross-reacted with both *Bonamia* sp. (now *Bonamia exitiosus*) from New Zealand and *Haplosporidium nelsoni*. The sensitivity of this FISH relative to PCR awaits validation. While FISH may be less sensitive than PCR, it should also generate fewer false positive results, because a positive signal can be validated in terms of tissue location and cell morphology. The degree of host response can also be determined with FISH, but not with PCR. In contrast, low cost and high throughput are the PCR's strongest advantages.

Bonamia ostreae is directly transmissible between oysters in a population (Elston et al. 1987). Infections probably begin when oyster hemocytes phagocytose *B. ostreae* cells that have penetrated the gill epithelium or digestive tract. The parasite proliferates in these hemocytes and disperses throughout the oyster. With eventual necrosis and death of the host, *B. ostreae* passes via the water column to nearby oysters, and the cycle begins anew (Bucke 1988, Montes et al. 1994). This hypothesized life cycle may be tested using FISH. Prepatent infections may be detected without the visual cues (e.g. host response) that permit location of *B. ostreae* in H&E-stained sections. Cryptic forms may be identified as well. *B. ostreae*-specific probe hybridization to objects of unexpected size or shape or in an unusual tissue location may reveal the presence of cryptic or non-canonical *B. ostreae* cell types. Here *fluorescent* ISH is particularly powerful. Probe signal is very precisely localized in FISH because the fluorescent probes may be directly observed (Schwarzacher & Heslop-Harrison 2000). Thus, morphological charac-

teristics of a stained object may be described more completely than if a chromogenic ISH were used.

This FISH assay is a powerful tool for detecting *Bonamia ostreae*. It sensitively identifies *B. ostreae* cells *in situ*. It provides a degree of morphological resolution that will expedite the search for cryptic parasite forms and resolution of the parasite's life cycle. Its primary weakness is low throughput. However, it is faster than chromogenic ISH assays for *Haplosporidium nelsoni* (Fong et al. 1993, Stokes & Burreson 1995), *Minchinia teredinis* (Stokes et al. 1995), and *Marteilia refringens* (Le Roux et al. 1999) because it requires fewer post-hybridization steps. It has great potential to advance our understanding of *B. ostreae* and improve our management of the parasite in wild and cultured flat-oyster populations.

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