Short communication

Molecular evidence of release of *Tetracapsula bryosalmonae*, the causative organism of proliferative kidney disease from infected salmonids into the environment

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*Tetracapsula bryosalmonae* is the malacosporean parasite that causes the commercially important proliferative kidney disease (PKD) in cultured salmonids (Canning, Curry, Feist, Longshaw & Okamura 1999). Kent & Hedrick (1986) first identified sporoblasts in the kidney tubule lumens of PKD affected fish and identified them as specific stages in the parasite’s development. Subsequent molecular, lectin and immunochemical studies confirmed the identity of the stages as *T. bryosalmonae* (Marín de Mateo, Adams, Richards, Castagnaro & Hedrick 1993; Kent, Khattria, Hervio & Devlin 1998; Morris, Adams & Richards 1999). However, because of the lack of hard spore valves and the inflammatory nature of the disease it was widely considered that the observed spores were not viable (Kent & Hedrick 1986). The discovery that species of freshwater bryozoans could be infected with *T. bryosalmonae* demonstrated that within these hosts the parasite formed spores that did not possess hard spore valves (Canning et al. 1999). *Tetracapsula bryosalmonae* stages obtained from bryozoans were subsequently proven to be able to infect rainbow trout, *Oncorhynchus mykiss* (Walbaum), resulting in clinical PKD. This suggested that the spore stages observed in the bryozoan hosts were viable and capable of transmitting the parasite (Feist, Longshaw, Canning & Okamura 2001).

It is well documented that the sporogonic stages of *T. bryosalmonae* can persist in the tubule lumens of salmonids many months after the fish have recovered from clinical PKD (Kent & Hedrick 1986; Kent et al. 1998; Morris, Adams, Feist, McGeorge & Richards 2000). This has led to suggestions that the *T. bryosalmonae* spore stages observed in salmonids are viable and capable of infecting bryozoan hosts (Kent, Khattria, Hedrick & Devlin 2000). Limited work has been performed examining the release of spore stages from infected salmonid species. Kent & Hedrick (1986) examining 10 rainbow trout collected from a hatchery in North America enzootic for PKD reported the presence of a small number of sporoblasts in the urine of one of the fish which were presumed to be *T. bryosalmonae*. However, so far *T. bryosalmonae* spores or sporogonic stages have yet to be identified in collecting ducts, ureters or the urinary bladder of any salmonid fish.

This short communication describes both molecular evidence confirming that *T. bryosalmonae* is released from salmonids into the freshwater environment and the results of a preliminary experiment to transmit *T. bryosalmonae* from brown trout, *Salmo trutta* L., to a freshwater bryozoan.
Forty fingerling brown trout were collected from a PKD enzootic farm in Scotland in August 2000. They were maintained in a circular 100 L plastic tank that was supplied by de-chlorinated, aerated mains water. The tank was thoroughly disinfected before use. After 2 months the kidneys of 10 fish were removed and examined using immunohistochemistry for the presence of parasite stages using MAbs C5 and B4 (Morris, Adams & Richards 1997). After the fish had been in the tank for 10 months, a 10-μm mesh was placed over the outlet and a third of the tank drained through the mesh. The filtrate collected on the mesh was then subjected to a DNA extraction protocol using a Nucleon Soft Tissue DNA Extraction Kit (Tepnel Life Sciences PLC, Manchester, UK), chloroform extraction and ethanol precipitation, following the manufacturer’s instructions. Filtrate collected from a tank containing rainbow trout obtained from a source with no reported PKD was used as a negative control.

The extracted DNA from the tank was amplified using PCR 25 μL reaction tubes with Ready to Go™ PCR beads (Amersham Pharmacia Biotech Ltd, Chalfont, Bucks, UK) providing 1.5 U Taq polymerase, 1.5 mM MgCl2, 200 mM of each dNTP, 10 mM Tris–HCl (pH 9.0) and 50 mM KCl. Sample DNA (2.5 μL), 10 pmol (1 μL) of each primer and 20.5 μL of nanopure water were added. Primers used were 3F and 4R developed for the detection of *T. bryosalmonae* by Kent et al. (1998). Cycling was 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and a 7-min extension at 72 °C, using a Perkin-Elmer GeneAmp™ 9700 Thermocycler (Perkin-Elmer Corp., Foster City, CA, USA). Samples and a 7-min extension at 72 °C, using a Perkin-Elmer GeneAmp™ 9700 Thermocycler (Perkin-Elmer Corp., Foster City, CA, USA). Samples and positive and negative controls were resolved in 1.5% agarose gels with 6 μL containing forward or reverse primer, Big Dye™ terminator ready reaction mix (Perkin-Elmer), and the products were then sequenced. After 2 months the kidneys of 10 fish in the same tank were removed and examined using immunohistochemistry to confirm PKD. The resulting product matched identically the sequence for *T. bryosalmonae* as reported by Kent & Hedrick (1986). The 10 brown trout obtained for immunohistochemistry were all infected with *T. bryosalmonae* sporogonic stages as determined by staining with MAb B4. These kidneys all appeared to have recovered from the clinical disease with an absence of pathological changes and no detectable extrasporogonic stages.

This data demonstrates that *T. bryosalmonae* DNA is released from fish. We consider it unlikely that any DNA introduced into the tank along with the fish would remain in the tank over such a long period of time, especially because of the flow-through nature of the tank and absence of invertebrates in the system. Therefore the most likely source of the DNA was from the brown trout. The molecular data reported here supports the report of Kent & Hedrick (1986) of possible *T. bryosalmonae* sporoblasts being present in the urine of salmonids. Lesions attributed to *T. bryosalmonae* that can appear late in the clinical disease may also be a route of exit for the parasite (Fernandez de Luca, Peribanex, Garcia & Castillo 1997). However, such lesions were not noted on the fish in this study, suggesting that the parasite is released into the environment via the urine of the fish. However, whether the stages released are viable to infect an alternate host or undergo further development outside the host remains unclear.

A preliminary series of experiments were conducted to ascertain whether the sporogonic stages observed in the kidney tubules could infect bryozoans. Transmission experiments from fish to...
bryozoans were conducted within the PKD season. Naïve colonies of the bryozoan *Plumatella repens* L. were collected from a PKD-free site. A portion of each of the colonies was tested by PCR using the primers 3F and 4R to confirm that they were *T. bryosalmonae*-negative. The colonies were washed under running tap water and macro-invertebrates removed by eye, before they were introduced into the experimental aquarium tanks. The bryozoans were kept in 100 L tanks in June/July 2000 (15–18 °C), with six colonies per tank. One tank included two 2-year-old brown trout obtained from a PKD enzootic trout farm while the other tank did not contain any fish. The bryozoan colonies were removed from the tanks daily and immersed into buckets for 2 h containing water obtained from a small eutrophic pond that is known to be *T. bryosalmonae* free to maintain the colonies. At the end of the experiment, *T. bryosalmonae* infection was confirmed in the experimental fish by PCR and immunohistochemistry.

Naïve bryozoan colonies were also maintained in two aerated, 50 L, static, aquarium tanks (15–18 °C), six colonies a tank. These colonies were exposed to an uninfected (control) or infected 2-year-old brown trout kidney, or uninfected (control) or clinically diseased *T. bryosalmonae* infected rainbow trout kidney. The clinically diseased kidney was grade 3 using the scale of Clifton-Hadley, Bucke & Richards (1987) and infections were confirmed using immunohistochemistry. All kidney tissue was disrupted by force through 50 μm mesh before adding to the tanks. Infected kidneys were from a PKD enzootic site from a batch of fish previously tested positive for the parasite. Control brown trout and rainbow trout were from a PKD-free site and a batch of fish tested negative for the parasite. These colonies were maintained in the tank by completely replacing the tank water weekly with water obtained from the eutrophic pond.

After 4 and 8 weeks of exposure, DNA was extracted from samples of all of the bryozoan colonies and specifically amplified by PCR using the primers 3F and 4R. Products obtained from the PCR were sequenced as previously described.

All bryozoan colonies kept with fish were negative as were the colonies exposed to the disrupted control kidneys and the rainbow trout kidney with clinical PKD. However, two colonies exposed to disrupted infected brown trout kidney tested positive by PCR. On sequencing the product, a 100% match was obtained with the expected sequence.

Although the results suggest that successful transmission occurred from brown trout kidney homogenate to a bryozoan, the results from this preliminary transmission study are inconclusive in that only DNA is being detected. As such it is difficult to assess if the transmission to the bryozoans occurred, whether it was residual DNA that was detected adhering to the colonies or an unidentified, infected, invertebrate host was present in the sample. Further transmission experiments are currently in progress to elucidate the role (if any) of the spore stages observed in the salmonid host in the life-cycle of *T. bryosalmonae*.

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**References**


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