International Journal for Parasitology 42 (2012) 511–515

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

In vitro cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates

P.B.B. Crosbie, A.R. Bridle, K. Cadoret, B.F. Nowak *

National Centre for Marine Conservation and Resource Sustainability, Australian Maritime College, University of Tasmania, Launceston, Tasmania 7250, Australia

ARTICLE INFO

Article history:
Received 22 February 2012
Received in revised form 9 April 2012
Accepted 10 April 2012
Available online 24 April 2012

Keywords:
Amoeba
Salmon
Gill
Disease causation
Aquaculture

ABSTRACT

Amoebic gill disease (AGD) in marine farmed Atlantic salmon is of growing concern worldwide and remains a significant health issue for salmon growers in Australia. Until now the aetiological agent, *Neoparamoeba perurans*, has not been amenable to in vitro culture and therefore Koch's postulates could not be fulfilled. The inability to culture the amoeba has been a limiting factor in the progression of research into AGD and required the maintenance of an on-going laboratory-based infection to supply infective material. Culture methods using malt yeast agar with sea water overlaid and subculturing every 3–4 days have resulted in the establishment of a clonal culture of *N. perurans*, designated clone 4. Identity of the amoeba was confirmed by PCR. After 70 days in culture clone 4 infected Atlantic salmon, causing AGD, and was re-isolated from the infected fish. Diagnosis was confirmed by histology and the infectious agent identified by PCR and in situ hybridisation using oligonucleotide primers and probes previously developed and specific to *N. perurans*. This study has fulfilled Koch's postulates for *N. perurans* as a causative agent of AGD and illustrates its free-living and parasitic nature.

© 2012 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Amoebic gill disease (AGD) is a widespread condition affecting salmonids farmed in the marine environment and other cultured fish. While it has affected salmonids farmed in Tasmania, Australia and Washington State, USA for many years (Kent et al., 1988; Munday et al., 2001; Nowak et al., 2002), it is now becoming a significant problem in major salmon producing countries (Steinum et al., 2008; Young et al., 2008; Bustos et al., 2011). Increased severity of infection has been reported during the last 12 months in Scotland (Armstrong, personal communication) and Ireland (Mitchell, personal communication).

While AGD was described more than 21 years ago (Kent et al., 1988), the aetiological agent was identified and named as *Neoparamoeba perurans* only recently (Young et al., 2007). Phylogenetic analyses of the 18S and 28S rRNA gene sequences from the virulent, non-cultured, freshly isolated *Neoparamoeba* showed it to be distinct from *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila*, and it was consequently described as a new species, *N. perurans*, by Young et al. (2007). The application of oligonucleotide probes, that discriminate between the three *Neoparamoeba* spp. showed that *N. perurans* was the only detectable amoeba associated with gill pathology in all cases (Young et al., 2008). Species-specific molecular tools developed for *N. perurans* have been used on presumptive AGD samples from Japan (Crosbie et al., 2010a) and Chile (Bustos et al., 2011) which showed that *N. perurans* was the only detectable amoeba. In the first cases of AGD reported from Norway, Steinum et al. (2008) analysed 18S cDNA sequences produced by reverse transcriptase PCR (RT-PCR) on RNA derived from AGD lesions and found high similarity to *N. perurans*. In all investigated cases of AGD from fish in the marine environment, *N. perurans* was associated with AGD lesions (Young et al., 2008; Crosbie et al., 2010a; Bustos et al., 2011). *Neoparamoeba perurans* was discovered and described using a molecular approach (Young et al., 2007). On the basis of molecular guidelines for establishing microbial disease causation (Fredricks and Relman, 1996; Desnuses et al., 2010) it was shown that *N. perurans* causes AGD (Young et al., 2007), however Koch's postulates have not been fulfilled due to the inability to culture the amoeba.

Previously the causative agent of AGD in Australia and elsewhere was thought to be *N. pemaquidensis* based on morphology and on the presence of a parasome (an endosymbiont) in the proliferating amoebae associated with gill pathology, but then isolation and characterisation of a new *Neoparamoeba* spp., *N. branchiphila*, by Dyková et al. (2005) from salmon displaying AGD, questioned the aetiology. Both species are routinely cultured in vitro but despite numerous attempts in the past it has not been possible to reproduce AGD in tank-housed salmon infected with either *N. pemaquidensis* (see Kent et al., 1988; Morrison et al., 2005) or *N. branchiphila* (see Vincent et al., 2007). The only way

* Corresponding author. Address: National Centre for Marine Conservation and Resource Sustainability, Australian Maritime College, University of Tasmania, Locked Bag 1370, Launceston, Tasmania 7250, Australia. Tel.: +61 3 63243814; fax: +61 3 63243804.

E-mail address: B.Nowak@utas.edu.au (B.F. Nowak).

0020-7519/$36.00 © 2012 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijpara.2012.04.002

to produce AGD in the laboratory has been by using amoebae freshly isolated post-mortem from AGD-affected fish or by co-habitation of infected fish with naive fish (Morrison et al., 2004). This situation has led to the maintenance of an experimental tank (University of Tasmania, Launceston, Tasmania, Australia) where amoebae are passaged through naive fish to ensure a supply of infective material. Experimental challenges with freshly isolated amoebae have in all cases resulted in the induction of AGD (Bridle et al., 2005; Embar-Gopinath et al., 2005; Morrison et al., 2005; Attard et al., 2006; Crosbie et al., 2007; Florent et al., 2007).

Until now in vitro culture of *N. perurans* has not been successful. The only *Neoparamoeba* spp. isolated by culture on yeast agar plates from AGD-affected salmon have been either *N. pemaquidensis* or *N. branchiphila* (see Dyková et al., 2005). *Neoparamoeba perurans* could only be maintained for a short time after isolation from AGD-affected salmon before the culture was overgrown by ciliates, flagellates and bacteria.

Neoparamoeba pemaquidensis which had been freshly isolated from AGD-affected salmon was cultured in sterile sea water with a cocktail of antibiotics and heat-killed *Escherichia coli*, but after 34 and 98 days in culture the amoebae failed to reproduce AGD when exposed to Atlantic salmon (Morrison et al., 2005). At the same time these authors did show that amoebae freshly isolated from AGD-affected salmon could elicit AGD after 72 h in culture (Morrison et al., 2005). As all evidence currently shows that *N. perurans* is the exclusive agent of AGD, these observations suggest that traditional culture methods select for non-virulent species of *Neoparamoeba* even when the virulent species is present in the inoculating material.

We believe that this paper describes the first known successful culture of *N. perurans* and evidence of continued virulence after 125 days. Koch's postulates have been fulfilled for *N. perurans* and thus it has been shown that it is the causative agent of AGD in Atlantic salmon.

2. Materials and methods

2.1. Amoebae culture

Amoebae were isolated from AGD-affected Atlantic salmon according to the method of Morrison et al. (2004) and then approximately 40 cells were inoculated onto malt yeast agar (MYA; 0.01% malt, 0.01% yeast, 2% Bacto Agar, sea water at 35‰ salinity) plates overlaid with 15 mL of 0.2 μm-filtered sea water and incubated at 18 °C. Amoebae were subcultured weekly. Free-floating cells were removed and inoculated to fresh MYA plates with an additional overlay of filtered sea water. Clonal strains were established by seeding single cells to individual wells containing MYA in 12 well cell culture plates, then overlaid with 1 mL of 0.2 μm-filtered sea water.

2.2. Infection trial with a clonal culture of N. perurans and experimental animals

A clonal cultured strain of *N. perurans* (clone 4) was maintained as described in Section 2.1 and trophozoites were harvested from several agar plates by flushing with 0.2 μm-filtered sea water. The amoebae suspension was centrifuged at 450*g* for 5 min, then cells were counted. A control group of *N. perurans* directly isolated from AGD-affected fish according to Morrison et al. (2004) was used as a positive control. Viability of each isolate was assessed by a neutral red inclusion assay; briefly 50 μL of the amoebae suspensions were added to 50 μL of a neutral red solution (50 μg mL^{-1} in PBS) in a microcentrifuge tube and incubated at room temperature for 25 min. Excess dye was removed when tubes were filled with filtered sea water and centrifuged at 14,000*g* for 10 s. Most of the supernatant was removed apart from 20 to 30 μL which contained the amoebae. The percentage of viable cells, which had taken up the dye, was then determined for all groups of amoebae on microscopic examination using a haemocytometer and 10 μL of the cell suspensions. A small sample of the cell suspensions was kept for DNA extraction and identity confirmation by PCR. All amoebae were then resuspended in 400 mL of filtered sea water and used for infection experiments within 1 h of collection. At the time of the challenge the non-clonal *N. perurans* population, from which clone 4 was isolated, had been in culture for 125 days and clone 4 for 70 days.

All fish used in this trial were approved for experimentation by the University of Tasmania, Australia (Animal Ethics Committee Permit No. A0011594). Atlantic salmon (approximately 130 g) were held in three × 250 L recirculating sea water tanks each with a 250 L sump and external biofilter, and stocked at 10 fish per tank. After a 2 week system acclimation period, fish in one tank were challenged with cultured *N. perurans*, in another with freshly isolated *N. perurans* and the fish in the third tank were negative controls with no amoebae added. For each challenge the water level was reduced to 150 L and amoebae were inoculated to tanks using a watering can to ensure uniform distribution at 5,000 cells L^{-1}. Four hours after addition of amoebae, all of the tanks were re-filled to 250 L. For the duration of the challenge fish were fed 1.5% of body weight per day, water quality was monitored every 2–3 days and the temperature maintained at 16 °C. All of the sea water was nominally 0.2 μm filtered and 50% water exchanges occurred weekly. At 20 days post-challenge, one fish from each tank was randomly selected and euthanased with a lethal dose of anaesthetic (Aqui-S, Aqui-S New Zealand Ltd., New Zealand) at 40 mg L^{-1} and gills removed and fixed in sea water/Davidson's fixative and assessed for gross signs of AGD. After 24 h the fixative was replaced with 70% ethanol and gills were processed for routine histology, paraffin embedded gills were sectioned at 5 μm and stained with H&E. As fish became moribund they were removed, euthanased and gills sampled as above. The trial was terminated after 38 days and all surviving fish euthanased and gills removed. Two hemibranchs were placed into 50 mL tubes with 0.2 μm-filtered sea water for amoebae isolation and the remaining hemibranchs were fixed for routine histology as described above and for in situ hybridisation (ISH) experiments.

2.3. Amoebae re-isolation and culture post-challenge

Amoebae were re-isolated using the method of Morrison et al. (2004). Hemibranchs in filtered sea water were gently inverted several times and then decanted into individual sterile Petri dishes and amoebae allowed to adhere to the surface for approximately 40 min. Unattached debris was then washed from each plate, remaining sea water removed and the amoebae detached with 1 mL of trypsin/EDTA solution in PBS (0.05% trypsin, 0.53 mM NA_4EDTA; Gibco, Burlington, Canada). Cells were immediately collected and added to MYA plates with a sea water overlay and incubated at 18 °C. After 1–4 days some cells were harvested and DNA extracted for identification by PCR.

2.4. PCR and ISH

All PCRs were performed on DNA extracted from amoebae directly isolated from gill material or from amoebae directly isolated and cultured on MYA plates. All amoebae were subjected to lysis and DNA extraction using a MasterPure Complete DNA and RNA extraction kit (Epicentre, Wisconsin, USA) following the manufacturer's instructions. Total nucleic acids were resuspended in 35 μL of Tris–EDTA buffer following ethanol washing and drying. Aliquots

Download English Version:

https://daneshyari.com/en/article/2436150

Download Persian Version:

https://daneshyari.com/article/2436150

Daneshyari.com