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Changes in antigenic profile during culture of *Neoparamoeba* sp., causative agent of amoebic gill disease in Atlantic salmon

Margarita Villavedra[a,c,*], Kristy McCarthy[a,c], Joyce To[a,c], Richard Morrison[b,c], Philip Crosbie[b,c], Kevin Broady[a,c], Robert L. Raison[a,c]

[a]*Institute for the Biotechnology of Infectious Diseases, University of Technology, Westbourne Street, Gore Hill, Sydney, NSW 2065, Australia*
[b]*School of Aquaculture, Tasmanian Aquaculture & Fisheries Institute (TAFI), University of Tasmania, Tasmania, Australia*
[c]*Aquafin CRC, Henley Beach, SA 5022, Australia*

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Abstract

Amoebic gill disease (AGD), the most serious infectious disease affecting farmed salmon in Tasmania, is caused by free-living marine amoeba *Neoparamoeba* sp. The parasites on the gills induce proliferation of epithelial cells initiating a hyperplastic response and reducing the surface area available for gaseous exchange. AGD can be induced in salmon by exposure to freshly isolated *Neoparamoeba* from AGD infected fish, however cultured *Neoparamoeba* are non-infective. We describe here antigenic differences between freshly isolated and in vitro cultured parasites, and within individual isolates of the parasite cultured under different conditions. Immunoblot analysis using polyclonal antisera, revealed differences in the antigen profiles of two cultured isolates of *Neoparamoeba* sp. when they were grown on agar versus in liquid medium. However, the antigen profiles of the two isolates were very similar when they were grown under the same culture conditions. Comparison of these antigen profiles with a preparation from parasites freshly isolated from infected gills revealed a very limited number of shared antigens. In addition monoclonal antibodies (mAbs) raised against surface antigens of cultured parasites were used in an indirect immunofluorescence assay to assess the expression of specific surface antigens of *Neoparamoeba* sp. after various periods in culture. Significant changes in antigen expression of freshly isolated parasites were observed after 15 days of in vitro culture. The use of mAb demonstrated progressive exposure/expression of individual antigens on the surface of the freshly isolated parasites during the period in culture.

Keywords: Antigen; Neoparamoeba; Amoebic gill disease; Atlantic salmon; Culture

1. Introduction

Neoparamoeba sp., the etiological agent of amoebic gill disease (AGD), is a free-living amoeba that under appropriate conditions can parasitise the gills of farmed Atlantic salmon (*Salmo salar* L.) in Tasmania. (Munday et al., 2001). *Neoparamoeba pemaquidensis* was the first species identified as an agent of AGD. While a second species (*Neoparamoeba branchiphila*) has been isolated from the gills of diseased fish, a definitive role in the in the aetiology of AGD has not been established (Dyková et al., 2005).

The parasites on the gill surface of the fish induce hyperplasia of the lamellar epithelium (of the gills) resulting in the fusion of the secondary lamellae and formation of macroscopic mucoid gill patches with the consequent reduction of gill surface available for gaseous exchange (Adams and Nowak, 2001; Clark and Nowak, 1999; Dykova et al., 1998; Powell et al., 2000).

Neoparamoeba sp. isolated from the gills of AGD infected salmon can be grown on lawns of bacteria on malt–yeast–sea water agar or maintained in sea water liquid cultures (Dykova et al., 1998; Kent et al., 1988; Munday et al., 1990). However, naïve salmon do not acquire AGD

* Corresponding author. Address: Institute for the Biotechnology of Infectious Diseases, University of Technology, Westbourne Street, Gore Hill, Sydney, NSW 2065, Australia. Tel.: +61 2 9514 4129; fax: +61 2 9514 4026.
E-mail address: margarita.villavedra@uts.edu.au (M. Villavedra).

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when exposed to cultured amoebae (Kent et al., 1988; Morrison et al., in press). Experimental infections of AGD can only be established by cohabitation with infected fish, or by exposure to freshly isolated parasites (Zilberg et al., 2001).

Salmon naturally infected with *Neoparamoeba* sp. do not develop an effective protective immune response against the parasite and are therefore susceptible to re-infection. Furthermore, immunization with crude preparations of *Neoparamoeba* has also been unsuccessful in protecting fish from AGD despite the appearance of *Neoparamoeba* specific antibodies in the serum of immunized fish (Akhlaghi et al., 1996; Douglas-Helders et al., 2003b; Zilberg and Munday, 2001). There are at least two possible reasons why attempts to induce a protective immune response may have been unsuccessful. Firstly, the route of immunization used in previous immunization trials may not have been appropriate for the development of a mucosal immune response at the gill epithelium, the site of attachment of the parasite. Secondly, as it is clear that in vitro cultured *Neoparamoeba* are non-infective it is likely that the antigenic profile of these parasites will be different from that of infective parasites. To address this issue, and to establish the degree of variation of antigen expression of parasites cultured in vitro under different conditions, we compared the antigenic profiles of infective and non-infective parasites grown in liquid or on agar culture medium using polyclonal antisera. To evaluate the changes in antigen expression on the surface of the parasite we used monoclonal antibodies (mAbs) to follow the changes in the antigenic profile of freshly isolated parasites established in liquid culture over a period of 1 month.

2. Materials and methods

2.1. Parasites

2.1.1. Cultured parasites

PA027 is a non-infectious clone of *N. pemaquidensis,* originally isolated from AGD affected salmon and established in continuous culture since 1994. These parasites were grown on malt yeast seawater (MYS) agar plates (0.1 g/L malt, 0.1 g/L yeast, 75% filtered seawater, 25% reverse osmosis water, 6.25 mg/L pimaricin (Sigma-Aldrich, USA), which had been seeded with heat inactivated *Escherichia coli* (ATCC 25922) as a food source. The plates were kept at 20 °C in atmospheric conditions. *Neoparamoeba pemaquidensis* were harvested from the plates using sterile seawater and a transfer pipette. The amoebae suspensions were washed three times with sterile seawater at 400 g for 5 min to reduce bacterial load. After washing, the pellet was resuspended in 45 mL of sterile seawater and amoebae cells counted using a haemocytometer.

NP251002 is a non-infectious clone of *N. pemaquidensis,* (Morrison et al., in press) originally isolated from AGD affected salmon in October 2002 and kept in continuous liquid culture. The isolate was maintained at 18 °C in 0.2 μm filtered, autoclaved seawater supplemented with heat killed *E. coli* (ATCC 25922) (approximately 1×10^9 cells/mL), streptomycin sulphate (0.7 μM; Sigma), benzyl penicillin (2.8 μM.; CSL Ltd, Australia), carbenicillin (2.4 μM; Sigma), ampicillin (7.1 μM; Sigma), erythromycin (1.4 μM; Sigma), sulphadiazine (2.5 μM; Sigma) and trimethoprim (0.5 μM; Sigma)

2.1.2. Freshly isolated parasites

Three fresh isolates of *Neoparamoeba* sp., E1, E2 and E3, were obtained directly from the gills of individual salmon from the same infection tank maintained at the aquatic centre of the University of Tasmania. The gills were excised from dead fish and amoebae isolated according to the method of Morrison et al. (2004).

2.2. Parasite cultures

The cultured *N. pemaquidensis* strains (PAO27 and NP251002) and the three batches of freshly isolated parasites (E1, E2 and E3) were established and maintained in identical liquid cultures as follows. The gill-derived parasite preparations were vortexed for 15 s to release adherent amoebae, centrifuged at $500\times g$ for 15 min at 10 °C and the parasite pellet washed twice, counted and adjusted to 2×10^5 amoebae per tube. The parasites were then centrifuged (500 g for 15 min at 10 °C) and resuspended in 10 mL of sterile filtered seawater supplemented with streptomycin sulphate (0.7 μM; Sigma), benzyl penicillin (2.8 μM.; CSL Ltd., Australia), carbenicillin (2.4 μM; Sigma), ampicillin (7.1 μM; Sigma), erythromycin (1.4 μM; Sigma), sulphadiazine (2.5 μM; Sigma) and trimethoprim (0.5 μM; Sigma) and 1×10^7 heat killed *E. coli* (ATCC 25922) cells per culture, and maintained at 10 °C in normal atmospheric conditions. Viable cell counts were determined by trypan blue exclusion. All the cultures were sampled at day 1, 5, 10, 15, 20, 25 and 30 from initiation of culture. At each time point, 2×10^5 amoebae were removed for antigen extraction and sterile filtered sea water added to maintain a constant total volume of the culture. After day 30 of culture, the presence of *N. pemaquidensis* was confirmed in all three cultures (E1, E2 and E3) by PCR using specific primers (Wong et al., 2004). All cultures (PAO27, NP251002 and a representative of the freshly isolated amoebae) were also confirmed as *N. pemaquidensis* by morphological characterisation (Dyková et al., 2005).

2.3. Antigens

2.3.1. Neoparamoeba antigens

Parasites were centrifuged at $400\times g$ for 15 min at 10 °C, resuspended in SDS-PAGE sample buffer (Laemmli, 1970),