

Chemotactic responses of the fish-parasitic scuticociliate *Philasterides dicentrarchi* to blood and blood components of the turbot *Scophthalmus maximus*, evaluated using a new microplate multiassay

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Abstract

This study describes a new capillary-type microplate multiassay for characterization of protozoal chemotactic responses, allowing up to 32 assays to be run simultaneously. We used the new multiassay to evaluate the chemoattractant activity of turbot blood components and turbot cells for the facultative parasite *Philasterides dicentrarchi*, which is responsible for significant losses in turbot farming. Preliminary tests indicated that the assay requires 3–4 h for detection of chemoattractant activity, that it can be performed effectively using the ciliate axenic culture medium, and that it distinguishes clearly between different concentrations of chemoattractant. Application of the assay indicated that whole blood and serum from normal turbot, and especially infected turbot, have strong chemoattractant activity for *P. dicentrarchi* trophozoites, whereas neither turbot blood cells nor other turbot cells nor bacteria were significant chemoattractants. These results raise the possibility that turbot serum components are involved in host detection and host invasion by *P. dicentrarchi*, in line with previous findings indicating that turbot with skin lesions show increased susceptibility to *P. dicentrarchi* infection.

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1. Introduction

Histiophagous ciliates causes severe damage in farmed turbot (Dyková and Figueras, 1994; Sterud

et al., 2000; Iglesias et al., 2001). The species responsible for ciliate outbreaks in turbot farming in Spain has been identified as *Philasterides dicentrarchi* (Iglesias et al., 2001), a species that can also infect farmed sea bass (*Dicentrarchus labrax*) (Dragesco et al., 1995). The natural route of infection is probably through lesions in the gills and/or skin, and then via the bloodstream to other parts of the body in which the ciliate divides rapidly and feeds

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on cells and tissue components in various vital organs and tissues (Iglesias et al., 2003; Paramá et al., 2003).

Chemotaxis is a class of response by which the direction of locomotion of cells or organisms is determined by changes in the concentration of substances in their environment. Ciliates exhibit known chemokinetic responses to gradients of various chemical substances, and these responses have been extensively studied in recent years (Fenchel and Blackburn, 1999; Leick et al., 1994). It is also known that several ciliates are capable of transforming their ingestive apparatus in response to chemical stimuli released by prey (Gómez-Saladin and Small, 1993) and chemotactic motility is essential for invasion and virulence in fish (O'Tool et al., 1999). To date, the stimuli that induce the free-living predatory species *P. dicentrarchi* to become a fish parasite are unknown.

The present study describes a versatile assay based on capillary tubes for investigation of ciliate chemotactic responses. We use this assay to characterize the chemotactic responses of *P. dicentrarchi* trophozoites to turbot tissue components, and we explore possible relationships between chemotactic responses and the facultative adoption of parasitic lifestyle by this species.

2. Materials and methods

2.1. Ciliate culture

Axenic culture of the ciliate *P. dicentrarchi* was done as previously described (Iglesias et al., 2003).

2.2. Turbot and experimental infections

Turbot were obtained from a fish farm (Insua-mar) in Galicia (northwest Spain). Body weight ranged from 20 to 40 g. The fish were maintained in 50-l closed-circuit aerated tanks at 17–18 °C and 30‰ salinity. Experimental infections were done by intraperitoneal injection of 200 µl of sterile physiological saline containing 10^4 ciliates. Before bleeding (see below), infected fish were maintained in their tanks for a week postinoculation.

2.3. Chemoattractants

To obtain turbot blood, normal or infected fish were bled by caudal vein puncture. After addition of heparin (4 mg/ml, 1 vol of heparin to 10 vols of blood), the blood was centrifuged at $1000 \times g$ for 5 min, then resuspended in L-15 medium at 2.5×10^6 cells/ml. Turbot serum (from normal or infected fish; NTS or ITS, respectively) was separated by centrifugation of the coagulated blood at $2000 \times g$ for 10 min. Spleen and kidney cells were obtained as previously described (Leiro et al., 1999): briefly, the organs were disaggregated by pushing through a steel mesh into L-15 medium and, after mixing by drawing into and out of a pipette, the cell suspension was filtered (80 µm pore size) to remove nondisaggregated tissue and adjusted in L-15 medium to 2.5×10^6 cells per ml.

The bacterium *Vibrio anguillarum* ATCC 19264 was cultured on trypticase soy agar (DIFCO) plates containing 1% NaCl. Bacteria were harvested by washing with L-15 medium, and bacterium concentration was adjusted to 2.5×10^6 cells per ml.

2.4. Chemotaxis assay

The microplate capillary assay used for investigation of the chemotactic responses of *P. dicentrarchi* is shown schematically in Fig. 1. The assay was performed in sterile 96-well flat-bottomed plates (Sterilin, England), using a capillary tube with inverted U shape (total length 34 mm internal section 1 mm, thus total internal volume 27 µl) to connect two wells containing A) 200 µl of a trophozoite suspension at 6×10^4 trophozoites per ml and B) 200 µl of a solution or suspension of the candidate chemoattractant (see Fig. 1). First, the capillary tubes were positioned in the microtitration plate; then the B wells were filled (200 µl), so that the tubes filled by capillary action; finally the A wells were filled (200 µl). Note that the capillary tube lies across an empty intervening well that has no functional importance in the assay. After incubation at 20 °C under 5% CO₂, viability was evaluated on the basis of ciliate motility using an inverted microscope with phase-contrast illumination. Viable trophozoites present inside the tubes and in the B wells were fixed with 1% glutaraldehyde, and the number of cells was determined using a Neubauer haemocytometer.

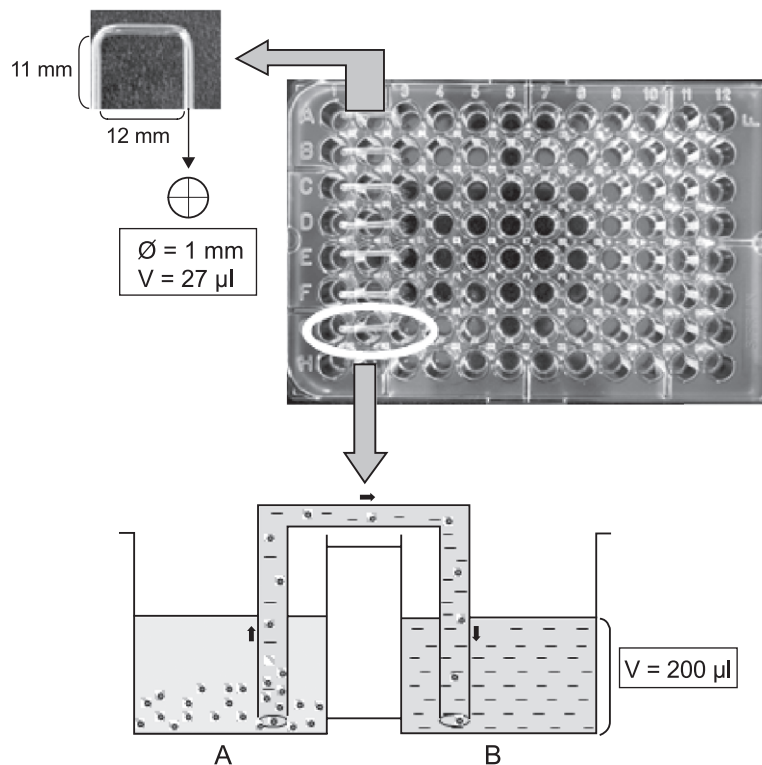


Fig. 1. Schematic representation of the microplate capillary assay used to study chemotaxis of *P. dicentrarchi* trophozoites. Each individual assay used three wells: well A containing 200 µl of ciliate suspension, well B containing 200 µl of the chemoattractant solution or suspension, and a third well left empty, between A and B. Wells A and B were linked by a U-shaped capillary tube allowing migration of the ciliates.

2.5. Statistics

Data are expressed as means \pm S.E.M. Means were compared ($p=0.05$) by one-way ANOVA followed by Tukey–Kramer tests for multiple comparisons.

3. Results

In a preliminary experiment, we evaluated optimal assay time. For this experiment, we used as chemoattractant the FBS (50% in L-5 medium) that had been used to maintain the axenic cultures of *P. dicentrarchi*. As shown in Fig. 2, the trophozoites migrated to the wells containing FBS; the minimum time required for a clear chemotactic response was about 2 h, and the maximum response was observed after 3–4 h. After this incubation period ciliate viability was 100%, and we therefore used an assay

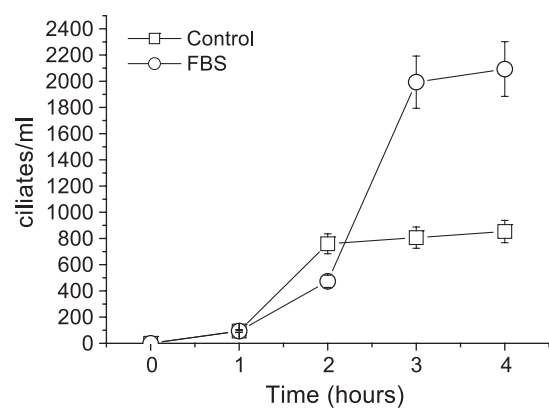


Fig. 2. Time course (0–4 h) of chemotaxis of *P. dicentrarchi* trophozoites with 50% foetal bovine serum (FBS) as chemoattractant. The assay was performed at 20 °C. Symbols show means \pm standard errors ($n=5$) of number of ciliates per ml inside the capillary tube and well B.

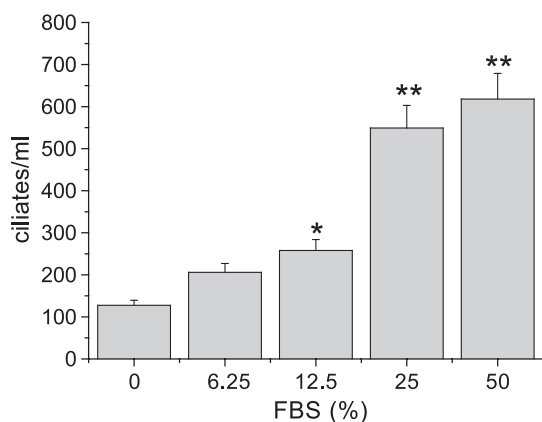


Fig. 3. Chemoattractant activities of different concentrations of FBS for *P. dicentrarchi* trophozoites. Each bar shows mean \pm standard error ($n=5$) of number of ciliates per ml inside the capillary tube and well B at the end of the 4-h assay. * $p<0.05$ and ** $p<0.01$ with respect to ciliates incubated without FBS.

time of 4 h in the subsequent assays. This preliminary assay also discriminated among different concentrations of chemoattractant, with a clearly dose-dependent response over the FBS concentration range 0–50%, and statistically significant chemoattraction observed at concentrations of 12.5% or higher (Fig. 3).

Since *P. dicentrarchi* trophozoites must be cultured in L-15 medium for maximal viability, we next performed assays to assess whether L-15 itself shows chemoattractant activity. As can be seen from Fig. 4, ciliates maintained in PBS migrated towards L-15, indicating that this medium contains chemoattractants. In addition, migration towards normal turbot serum (NTS) in PBS was much more marked than migration towards NTS in L-15.

Fig. 5 summarizes the results of the assays of the chemoattractant activity of different turbot tissue components (considering those tissues invaded by *P. dicentrarchi*), and of bacteria (the natural prey of *P. dicentrarchi* in the free-living state). Neither bacteria nor turbot blood cells nor cells from other turbot tissues had significant chemoattractant effects. However, whole turbot blood or turbot serum showed strong chemoattractant activity (Fig. 5). Finally, the chemoattractant activities of both whole blood and serum from infected turbot were significantly increased with respect to whole blood and serum from non-infected turbot (Fig. 5).

4. Discussion

Currently, capillary tube assays (Leick and Helle, 1983) are the most widely used assays of chemotactic responses, in view of their precision. In the present study, we have developed a capillary tube assay using 96-well microplates, permitting up to 32 assays to be run in each microplate (since each assay uses three wells, with an empty well between each A and B well): if we assume three replicates per assay, this means that up to 10 candidate chemoattractants can be tested simultaneously in a single plate. A similar assay, but with only two chambers, was used to investigate the effects of various hormones on ciliate chemotaxis (Köhida et al., 1994; Csaba et al., 2000). Reported optimal incubation time in assays of this type has ranged from 15 to 20 min (Köhida et al., 1994; Köhida et al., 1997; Köhida and Csaba, 1998; Köhida et al., 2000) to 2–3 h (Leick and Helle, 1983; Leick and Hellung-Larsen, 1985); in the present study we found the optimal incubation time to be 3–4 h.

In assays with mammalian cells, it has been demonstrated that FBS at concentrations of 10% has a significant chemoattractant effect (Murata et

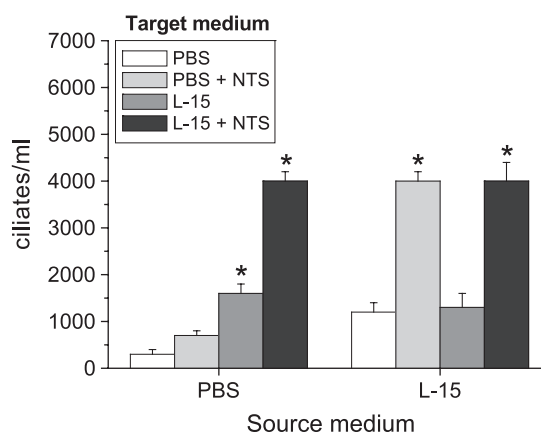


Fig. 4. Chemoattractant activities of L-15 medium for *P. dicentrarchi* trophozoites. At the start of each assay, well A contained trophozoites in PBS or L-15 medium ("source medium"); well B contained PBS only, L-15 only, PBS plus normal turbot serum (NTS), or L-15 plus NTS ("target medium"). In wells containing NTS, the final NTS concentration was 50%. Each bar shows mean \pm standard error ($n=5$) of number of ciliates per ml inside the capillary tube and well at the end of the 4-h assay. * $p<0.01$ with respect to the corresponding control (target medium PBS only).

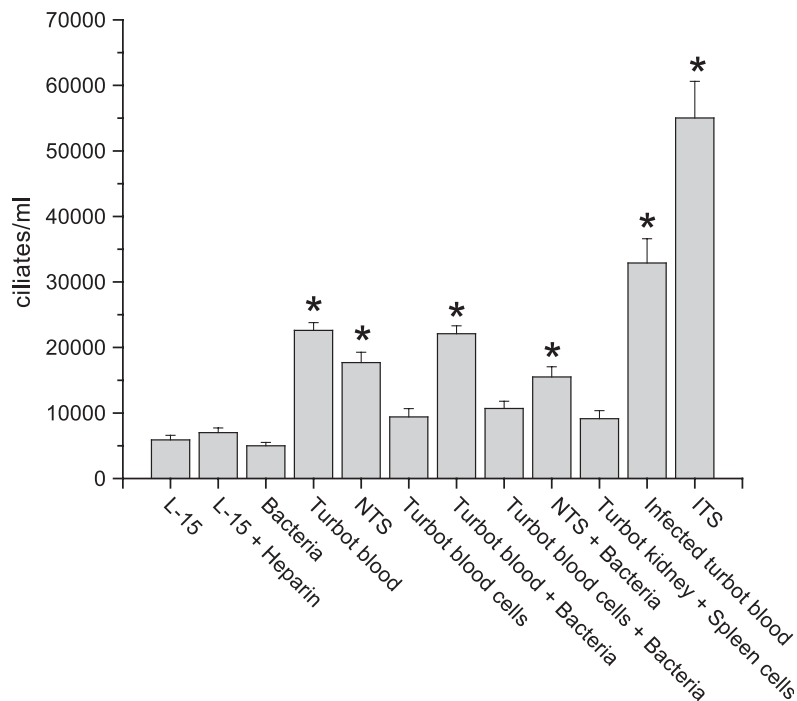


Fig. 5. Chemoattractant activities of turbot blood, turbot cells and bacteria for *P. dicentrarchi* trophozoites. Each bar shows mean \pm standard error ($n=5$) of number of ciliates per ml inside the capillary tube and well B at the end of the 4-h assay. * $p < 0.01$ with respect to the control (target medium L-15 medium only). NTS = normal turbot serum; ITS = infected turbot serum.

al., 1999). In the present study, we likewise found that FBS at concentrations of 10% or higher had a strong chemoattractant effect on *P. dicentrarchi*. Various previous studies have clearly shown that serum from a number of teleosts induces unidirectional chemotaxis by infective-stage ciliates (Buchmann and Nielsen, 1999), and that host serum factors affect penetration behaviour (Lom and Cerkosovova, 1974). Likewise, ciliates respond positively to those chemical stimuli that signal the presence of bacteria or other kinds of food in the external environment (Leick and Hellung-Larsen, 1985). The results of the present experiments indicate that turbot blood and turbot serum are strong chemoattractants for *P. dicentrarchi* trophozoites; however, we did not detect any significant chemoattractant effects of other cells (including blood cells), or of bacteria, at least at the concentrations tested. The chemoattractant activity of non-coagulated blood (containing heparin as anticoagulant) appears to reside in the serum fraction, since blood cells alone do not attract *P. dicentrarchi*

trophozoites. The difference in chemoattractant activity between blood and serum may be due to inactivation of complement factors during the coagulation process used for serum obtention; it has previously been demonstrated that complement factors have chemoattractant activity for infective-stage ciliate pathogens (Buchmann and Nielsen, 1999). This hypothesis is also supported by significantly higher chemoattractant activity of whole blood and serum from infected turbot than of whole blood and serum (respectively) from non-infected turbot. A possible influence of heparin itself can be ruled out, since heparin alone showed no chemoattractant activity; indeed, studies with some mammalian cells (eosinophils) have indicated that heparin may inhibit chemotactic responses (Culley et al., 2003). The chemoattractant activity of blood for *P. dicentrarchi* trophozoites may explain the greater susceptibility of fish with dermal lesions (such as microhaemorrhages) to infections by this ciliate (Paramá et al., 2003).

In conclusion, the present results suggest that turbot serum components possess chemoattractant properties for trophozoites of *P. dicentrarchi*, and that chemotactic responses to these components may play a role in host detection and host tissue invasion.

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