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A serum cytotoxicity assay for measuring the resistance of turbot to the ciliate *Philasterides dicentrarchi*

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ABSTRACT

In previous studies we have found that the complement system plays an essential role in the resistance of fish to infection by the ciliate parasite *Philasterides dicentrarchi*. The present study shows that it is possible to predict resistance to *P. dicentrarchi* in the turbot *Scophthalmus maximus* by using a complement-dependent serum cytotoxicity assay. Fish were immunised against *P. dicentrarchi*, and fish mortality following experimental challenge, specific serum antibody levels and serum cytotoxicity were compared. We found a correlation between the results obtained regarding the killing activity of serum and the protection generated in turbot by the vaccine after experimental challenge. The study findings suggest that the serum cytotoxicity assay can be used to assess the protection generated by the vaccine against *P. dicentrarchi* in turbot, thereby reducing both the number of fish required for the experiments and the pain and distress associated with experimental infection

Introduction

Philasterides dicentrarchi is a marine, free-living ciliate known to be a pathogen of many fish species, particularly in aquaculture and in aquarium settings. Cultured flatfish are particularly strongly affected, and high rates of mortality have been associated with the parasite [1]. Fortunately, the administration of vaccines can help to increase protection and reduce the mortality caused by the parasite [2,3]. The complement system and the coagulation system have been reported to be key components of defence against the scuticociliate *P. dicentrarchi* in fish [4–7]. Vaccine administration increases serum levels of specific antibodies and resistance to the ciliate via activation of the complement system, which is particularly efficient for killing parasites when it is activated through the classical pathway [5,6]. The presence of specific antibodies in serum may be a good indicator of resistance to the parasite in turbot. However, turbot are known to be affected by several strains of *P. dicentrarchi*, not all of which induce cross-protection when included in vaccines [8,9]. After conducting several trials with turbot to test vaccines containing several types of antigens, Palenzuela et al. [10] obtained contradictory results, with a general lack of correlation between serum antibody levels and protection. Thus, although different *P. dicentrarchi* strains share many antigens, the protective antigens may differ between strains, and their proportion relative to the total antigenic repertoire is very low. We have carried out numerous assays to identify these antigens, performing experimental challenges that involve large numbers of fish. The 3R principle in bioethics, Replacement, Reduction and Refinement, is aimed at minimizing the use of animals in scientific research. Although the use of fish cannot be avoided when testing the vaccines, the number of fish can be reduced and pain/stress thus minimized by avoiding conducting experimental infections. Based on our knowledge of the interactions between fish complement and *P. dicentrarchi* and after several experiments, we found that the killing activity of turbot serum obtained from vaccinated fish was correlated with the resistance of fish to *P. dicentrarchi*. We suggest that by using this assay it is possible to decrease the number of fish required in testing and thus reduce the pain and distress associated with experimental

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infections.

Materials and methods

Animals and ethical statement

All experimental protocols carried out in the present study followed the European legislation (Directive 2010/63/EU) and the Spanish legislative requirements relating to the use of animals for experimentation (RD 53/2013). The protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain) (authorization number: 15,012/2022/008). Specimens of turbot, *Scophthalmus maximus* (L.), each weighing about 20 g, were obtained from local fish farms. The fish were maintained in the aquarium in 250 l tanks with recirculating aerated seawater (16 °C) and were fed daily with commercial pellets (Skretting, Burgos, Spain). The fish were acclimatized to the aquarium conditions for 2 weeks before the start of the experiments. The fish were anaesthetized by immersion in a solution of MS-222 (tricaine methane sulfonate; Merck, Spain) in seawater, before vaccination (60 mg MS-222 /L), and before being euthanized (100 mg MS-222 /L seawater) by pithing.

Parasites

Ciliates (*Philasterides dicentrarchi*, isolate S6) were obtained from ascites of naturally infected turbot suffering an outbreak of scuticociliatosis that occurred in a turbot farm in Galicia (NW Spain). The ciliates were cultured and maintained in the lab at 18 °C in supplemented L-15 medium, as previously described [11]. To maintain their virulence, parasites were injected intraperitoneally (i.p.) into healthy turbot every 6 months (200 μL of sterile physiological saline containing 5×10^5 trophozoites). The ciliates were also passaged through fish prior to their use in vaccines or for inducing experimental infections [2]. After three days, the ciliates were recovered from the peritoneal cavity of injected fish and cultured as indicated above. Before being used in vaccination experiments, the parasites were concentrated by centrifugation (650 x g for 5 min) to 1×10^7 cells ml^{-1} of PBS [9].

Fish vaccination and challenge

The vaccine was prepared as previously described [2]. Groups of 60 fish each were injected i.p. on day 0 (water temperature, 16 °C) and day 30 with 0.1 ml of PBS, an emulsion of adjuvant (50 %) (Montanide ISA 763A VG (M763); Seppic, Paris, France) and PBS, or with an emulsion of adjuvant and ciliates (10^6 ciliates ml^{-1}) in PBS containing formalin (0.1 %) [8]. In some experiments, turbot were vaccinated with ciliates cultured in the laboratory for at least 6 months (OldC) or with ciliates obtained after passage through fish (NewC) ((10^6 ciliates ml^{-1} in PBS) and M763 (50:50, v:v). Additionally, other groups of fish were injected with lyophilized yeast (*Saccharomyces cerevisiae*, 1 mg/fish), with yeast and ciliates (10^6 ciliates ml^{-1}), or with M763 and different number of ciliates (5×10^5, 1×10^6 5×10^6 ciliates ml^{-1} in PBS containing formalin (0.1 %). Thirty days after administration of the booster, 10 fish per group were bled by caudal vein puncture. The blood was allowed to clot overnight at 4 °C before being centrifuged, and the serum was then collected and used to determine the antibody levels by ELISA or to evaluate its cytotoxic activity [5]. For the cytotoxic assays, the serum was used on the day after blood sampling. For the enzyme-linked immunosorbent assay (ELISA), the serum was diluted 1:1 (v/v) in glycerol and stored at − 20 °C until use.

The challenge (50 fish per group) was carried out one month after administration of the booster (water temperature, 18 °C). Before the challenge, each fish group was marked using subcutaneous injections of coloured elastomer tags for identification purposes. After the challenge, fish were mixed in the tanks (25 fish per tank). Fish were injected i.p. with 0.1 ml of inoculum containing 10^6 ciliates ml^{-1} in PBS, and mortality was recorded daily for 20 days and expressed as cumulative mortality. The cause of death was verified by microscopic analysis of the parasite isolated from internal organs or ascites. The relative percentage survival (RPS) was also determined as follows:

RPS= (1- mortality in vaccinated group/mortality in control group) X 100

Determination of serum antibody levels

Serum antibody levels were determined in ten fish per group, in triplicate, using an indirect enzyme-linked immunosorbent assay (ELISA), as previously described [9]. One hundred μl of carbonate–bicarbonate buffer (pH 9.6) containing 1 μg of *P. dicentrarchi* total soluble antigen was added to each well of a 96-well ELISA plate (high binding, Greiner Bio-One, Germany) and incubated overnight at 4 °C. The plates were incubated, washed three times with TBS (50 mM Tris, 0.15 M NaCl, pH 7.4) and blocked for 2 h with at room temperature with TBS containing 0.2 % Tween 20 (TBS-T) and 5 % non-fat dry milk. The plates were then incubated with fish serum, diluted 1:200 in 0.2 % TBS-T for 12 h at 4 °C, and finally washed five times with 0.05 % TBS-T. For detection of turbot IgM, 100 μl of monoclonal antibody (Mab) anti turbot IgM (UR3 [12], diluted 1:500 in 0.2 % TBS-T was added to each well of the plates, which were then incubated for 30 min at room temperature in a microplate shaker (750 rpm). The plates were washed 5 times with 0.05 % TBS-T, and the bound mouse antibodies were detected with HRP-conjugated polyclonal rabbit anti-mouse Ig (Dako, Denmark), diluted 1:1000 in 0.2 % TBST-T, for 30 min at room temperature and orbital shaking at 750 rpm. The plates were washed five times with 0.2 % PBS-T, and a colour reaction was mediated by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB, Thermo Scientific). The reaction was stopped after 20 min, by the addition of 0.18 M H_2SO_4, and the optical density (OD) at 450 nm was measured in an ELISA reader (Titertek Multiscan, Flow Laboratories). Controls with pre-immune turbot serum, without turbot serum, or without anti-turbot IgM were also included.

Cytotoxicity assay

The cytotoxicity assay was carried out using turbot serum and heat-inactivated turbot serum (heated at 56 °C for 30 min, to inactivate the complement) [5]. As previously described [5], heat-inactivated serum showed no cytotoxic activity against *P. dicentrarchi*. Serum samples were obtained from ten fish per group (1–1/128) in 96-well flat bottom microtitration plates (Iwaki, Japan). Ciliates were washed three times by centrifugation in L-15 medium and then added to the plates, at a concentration of 3×10^2 ciliates/well, and mixed with the serum. After incubation of the plates at 18 °C for 2 h, the lowest serum dilution that killed all the ciliates in each well was determined in an inverted microscope (Nikon Eclipse TE300, Nikon, Japan). Cytotoxicity data are presented as the $\log_2$ of the serum dilution that results in complete ciliate killing. Dead ciliates were easily identified by the immobile cilia and changes in the shape, as dead ciliates are spherical while live ciliates are elongated (Figs. 1A, B).

Data and protocols

A file containing the results obtained from each experiment and the corresponding statistical analysis is included. All raw data and detailed protocols are available upon request.

Statistical analysis

Differences in survival rates were determined by comparing the changes in mortality among groups over time using Kaplan–Meier and Log-Rank tests (SPSS for MS Windows, version 11.5.1). The Spearman's rank correlation coefficient (ρ) and p-value were used to determine any

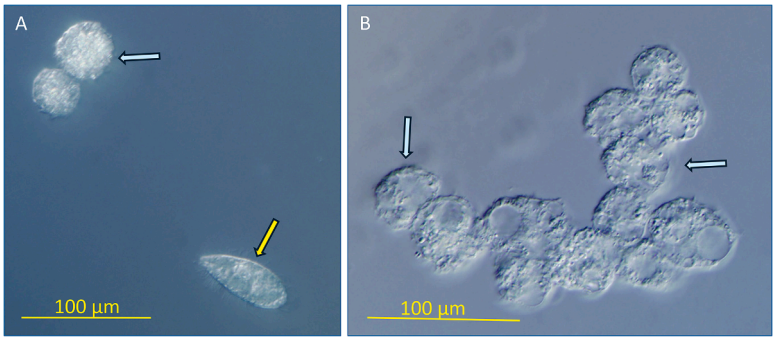

Fig. 1. A and B. Differential interference contrast microscopy photomicrographs of ciliates incubated with serum from immunised turbot, showing dead (light blue arrows) and live ciliates (yellow arrow). Dead ciliates are spherical, and live ciliates are elongated in shape.

correlation between the two variables, and it was calculated by using SPSS for Windows, version 11.5.1. In the three experiments, we have compared antibody titres and serum cytotoxicity with the cumulative mortality observed following an experimental challenge with the pathogen. The correlation coefficient was calculated within groups of each individual experiment, without comparisons across different experiments. For the other assays, a one-way ANOVA was performed, and if significant differences were found, Tukey's HSD (Honest Significant Difference) post hoc test was applied for multiple comparisons between experimental groups (see supplementary material). Differences were considered significant at $p < 0.05$.

Results

In previous studies, we have shown that the complement and coagulation systems play a central role in the resistance of fish to infections caused by the ciliate parasite *P. dicentrarchi* [4–6]. In tests including serum, the cytotoxic activity was related to complement activation, and the activity disappeared after complement was heat inactivated. The purpose of the present study was to determine whether measurement of the cytotoxic activity in fish serum raised against this ciliate could be used to evaluate the resistance of fish to the pathogen, without the need to conduct an experimental challenge. This would have the benefit of reducing the number of fish used in vaccination experiments.

We compared the responses (serum antibody levels, serum cytotoxicity to the parasite and fish resistance to infection) in several turbot vaccination assays. In one of these assays, we used two groups of ciliates: ciliates cultured in the laboratory for at least 6 months and ciliates obtained after passage through fish. These were designated as OldC (long-term cultured ciliates) and NewC (freshly passaged ciliates), respectively. Both groups of ciliates were used in vaccination and in cytotoxic experiments. Specific serum antibody levels and serum cytotoxicity were higher in fish vaccinated with ciliates (whether OldC or NewC were used) than in fish injected with PBS (Table 1). After the challenge, and as expected, mortality was lower in immunised fish than in PBS-injected fish, with the latter showing about 70 % mortality in both ciliate groups. In both cases, where the challenge was carried out with OldC or NewC, fish mortality was lower when fish were immunised with NewC (Figs. 2A, B). We assessed the correlation between serum cytotoxicity and ELISA results with turbot resistance to *Philasterides dicentrarchi* following an experimental infection. A negative correlation was observed between serum cytotoxic activity (against both OldC and NewC) and fish resistance to *P. dicentrarchi* (correlation coefficient (ρ) = −0.75); however, this association was not statistically significant (p-value = 0.084). In contrast, the correlation between the results obtained by ELISA and resistance to infection was weaker ($\rho = -0.40$; $p = 0.42$). Comparison of sera from fish immunized with OldC and NewC revealed that turbot vaccinated with NewC showed higher cytotoxic activity than those immunized with OldC. However, no significant differences in antibody titres were observed between the two groups

In the second experiment, we compared the serum antibody levels, serum cytotoxicity to the parasite and fish resistance to infection in turbot injected with PBS, M763, yeast, M763 + ciliates and yeast + ciliates. The inclusion of adjuvants (M763 or yeast) did not induce any increase in serum cytotoxicity, as also observed in the group injected with PBS (Table 2). The cumulative mortality in the groups injected with M763 or yeast was high and comparable to that found in the PBS-injected group (Fig. 3). Serum cytotoxicity was higher in fish injected with ciliates together with an adjuvant (M763 or yeast, particularly the former) than in fish injected with ciliates only. As expected, the groups with the highest serum cytotoxicity showed the lowest cumulative mortality after experimental infection. In this experiment, serum cytotoxicity and resistance to infection were correlated (ρ = - 0.894; p: 0.041). Regarding the ELISA assay, sera obtained from fish injected with yeast or with yeast and ciliates showed similar absorbance (Table 2), but an increase in protection was only observed in the group immunized with yeast and ciliates. In this assay, a correlation was found between the results obtained by ELISA and the resistance to the *P. dicentrarchi* ($\rho = -0.894$; $p = 0.041$).

In the third experiment, fish were immunized with vaccines containing different concentrations of antigens. An increase in antigen concentration was correlated with an increase in the serum cytotoxicity activity, although no statistically significant differences between groups were observed, mainly because not all the fish responded to vaccination (Table 3). After the challenge, the cumulative mortality was lower in the groups immunized with higher concentrations of antigen, although the

Table 1
Cytotoxicity assay in cultured ciliates exposed to turbot serum obtained from fish injected either with PBS or with a vaccine containing previously passaged ciliates (OldC) or freshly passaged ciliates (NewC) and adjuvant (M763). Each group consisted of 10 fish. Ciliates cultured in the laboratory during at least 6 months were designated "old" and ciliates obtained after one passage through fish, "new". Data are presented as the $\log_2$ of the inverse serum dilution that completely killed the ciliates. The levels of specific antibodies in serum, measured by ELISA (expressed as absorbance), are also included. Values indicated by different letters (a–d) differ significantly ($p < 0.05$).

Serum	PBS		Vaccine (OldC)		Vaccine (NewC)	
Cultured ciliates	OldC	NewC	OldC	NewC	OldC	NewC
Cytotoxicity, mean±SD	1.9 ± 0.32^{a}	3.1 ± 0.32^{b}	3.8 ± 0.42^{c}	3.5 ± 0.52^{c}	5.2 ± 0.42^{d}	5.0 ± 0.47^{d}
ELISA (absorbance), mean±SD	0.120 ± 0.045^{a}	0.175 ± 0.041^{a}	0.696 ± 0.025^{b}	0.583 ± 0.225^{b}	0.597 ± 0.030^{b}	0.476 ± 0.049^{b}

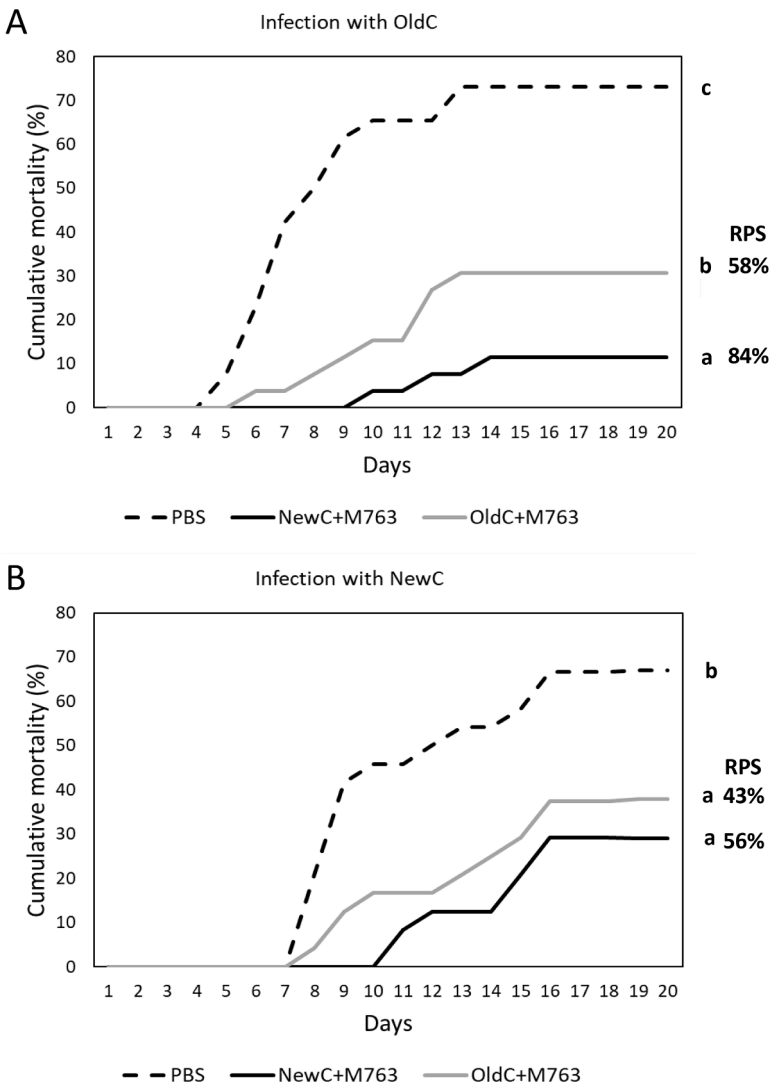

Fig. 2. A and B. Turbot were injected with PBS, adjuvant (M763) and ciliates cultured for at least 6 months (OldC+M763) or M763 and freshly passaged ciliates (NewC+M763) and then challenged with OldC (A) or NewC ciliates (B). The percentage cumulative mortality observed 20 days after challenge is shown. Values indicated by different letters (a–c) differ significantly ($p < 0.05$). The RPS values are also included.

Table 2
Cytotoxicity assay in cultured ciliates exposed to turbot serum obtained from fish injected with PBS, M763, yeast (lyophilized *S. cerevisiae*, 1 mg per fish), ciliates and M763 or ciliates and yeast. Each group consisted of 10 fish. Data are presented as the $\log_2$ of the inverse serum dilution that completely killed the ciliates. The levels of specific antibodies in serum, measured by ELISA (expressed as absorbance), are also shown. Values indicated by different letters (a–c) differ significantly ($p < 0.05$).

Serum	PBS	M763	Yeast	Ciliates + M763	Ciliates + yeast
Cytotoxicity, mean±SD	1.9 ± 0.57[a]	1.7 ± 0.48[a]	1.6 ± 0.52[a]	4.2 ± 0.63[b]	3.4 ± 0.52[c]
ELISA (absorbance), mean±SD	0.208 ± 0.072[a]	0.248 ±0.061[a]	0.452 ±0.012[b]	1.001± 0.266[c]	0.465 ±0.066[b]

differences were not always statistically significant (Fig. 4). There were no differences in the total number of specific antibodies between groups (Table 3). A strong negative correlation was found between the serum cytotoxicity and the resistance to the parasite ($\rho = -1.000$; $p = 0.000$). In contrast, the correlation between ELISA-detected antibody levels and parasite resistance was high but not statistically significant ($\rho = -0.8$; $p = 0.2$).

Discussion

In the present study, we demonstrated that the cytotoxicity of serum to *P. dicentrarchi* measured in an *in vitro* cytotoxic assay is correlated with the protection generated by a vaccine against this parasite. All sera from control fish were able to kill the parasites at low dilutions; however, the killing activity was much higher in the presence of specific antibodies, as expected from the findings of previous studies [5,6]. Complement-dependent cytotoxicity assays can be used to evaluate the ability of antibodies or other molecules to mediate cell death via the complement system [13]. This assay can be adapted to other pathogens, such as bacteria and parasites, and to cells infected with virus or neoplastic cells [14,15]. It seems to work particularly well for evaluating the cytotoxicity of turbot serum to *P. dicentrarchi* because of complement-dependent cytolysis of the parasites, which increased in the presence of specific antibodies [5,6]. Although this assay can be carried out in different ways [13,16–18], the method used in the present study is very simple, effective and easy to carry out.

After administration of almost all vaccines, prevention of infection was correlated with the induction of specific antibodies [19]. An ELISA

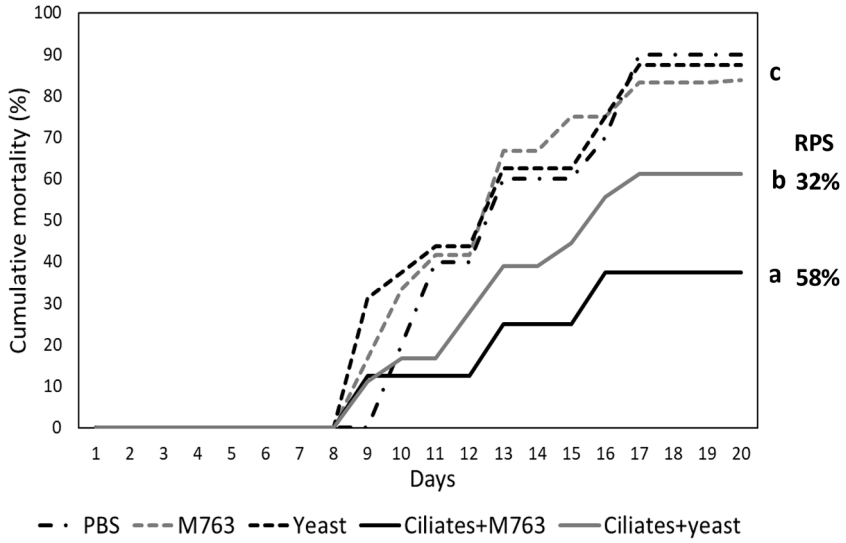

Fig. 3. Turbot were injected with PBS, adjuvant (M763), yeast (lyophilized *S. cerevisiae*, 1 mg per fish), M763 and ciliates or yeast and ciliates. The percentage cumulative mortality observed 20 days after challenge is shown. Values indicated by different letters (a–c) differ significantly ($p < 0.05$). The RPS values are also included.

Table 3
Cytotoxicity assay in cultured ciliates exposed to turbot serum obtained from fish injected with PBS, or with M763 and 5×10^5, 1×10^6 or 5×10^6 ciliates per mL. Each group consisted of 10 fish. Data are presented as the $\log_2$ of the inverse serum dilution that completely killed the ciliates. The table also shows the levels of specific antibodies in serum, measured by ELISA (expressed as absorbance). Values indicated by different letters (a–b) differ significantly ($p < 0.05$).

Serum	PBS	5.00E+06	1.00E+06	5.00E+05
Cytotoxicity, mean ±SD	2.2 ± 0.42^{a}	4.2 ± 0.79^{b}	4.0 ± 0.67^{b}	3.8 ± 0.42^{b}
ELISA (absorbance), mean±SD	0.200 ± 0.120^{a}	0.920 ± 0.143^{b}	0.781 ± 0.150^{b}	0.833 ± 0.142^{b}

can be used to evaluate the host response, in terms of antibody production, to vaccination or infection as to demonstrate that vaccination was successful. However, when the vaccine includes several different antigens, such as whole pathogen, the ELISA results may not reflect the protection generated by the vaccine, as only the antibodies generated against a few antigens will be protective. Among the three assays carried out in the present study, only one showed a statistically significant correlation between ELISA results and protective efficacy ($p < 0.05$). The complement-dependent cytotoxicity assay is a better method to evaluate the protective effects of fish vaccination against *P. dicentrarchi.* A statistically significant correlation between cytotoxic activity and protection was observed in two of the three experiments. Although a similar trend was noted in the third assay, the correlation did not reach statistical significance ($p = 0.085$).

We were not able to include very large number of groups of fish in the experimental challenges, due to space limitations, which would be desirable to improve the robustness of the statistical analysis. However, we compared the results of three different experiments to show that the complement cytotoxicity assay could be used to measure the resistance of turbot to *P. dicentrarchi* in the presence of protective specific antibodies generated after vaccination. Based on the results presented here and those of other unpublished studies, we found a good correlation between the results obtained in the complement-dependent cytotoxicity

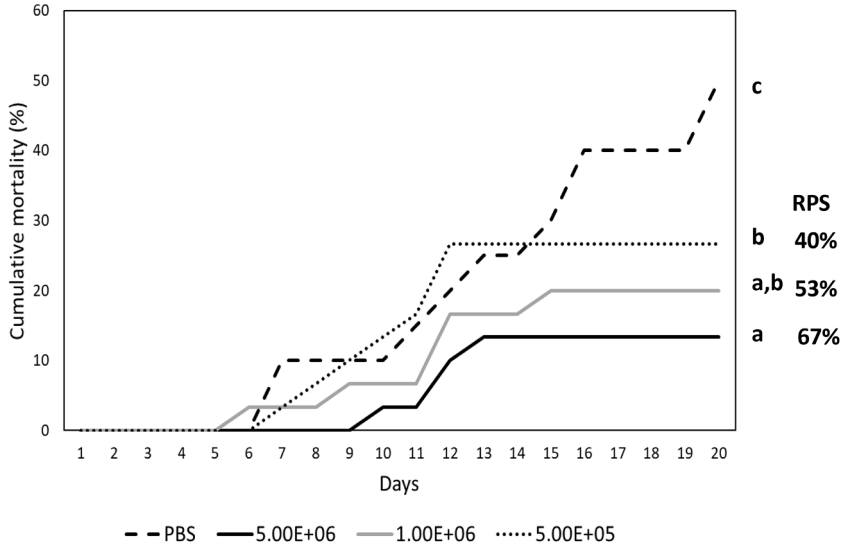

Fig. 4. Turbot injected with PBS, or with the adjuvant (M763) and different amounts of antigen (5×10^5, 1×10^6, 5×10^6 ciliates/ml). The percentage cumulative mortality observed 20 days after challenge is shown. Values indicated by different letters (a–c) differ significantly ($p < 0.05$). The RPS values are also included.

assays applied to *P. dicentrarchi*, using serum from immunised or control fish, and the protection generated by the vaccine after an experimental challenge. These results support previous findings showing that, in the presence of complement, the cytotoxic activity of turbot serum to *P. dicentrarchi* can be increased by increasing the levels of specific antibodies [5]. In vaccination experiments, the serum antibody titres were usually correlated with the resistance of turbot to infection by *P. dicentrarchi* after experimental challenge, when the homologous strain is used in the challenge. Several studies in both fish and mammals have reported a high correlation between antibody titres and the level of post challenge protection against a particular pathogen [19,20], although this association was not always found [19]. In pathogens with several serogroups, ELISA can detect humoral responses, but only bactericidal assays have been shown to correlate with protection [21]. In cases of parasite antigenic variation, ELISA may not reflect the protective immunity due to the parasite's ability to switch surface antigens [22]. We have identified several strains of *P. dicentrarchi*, which when used in vaccines generate large amounts of specific antibodies in turbot serum; however, cross-protection was not always found between heterologous strains [8,9]. In this case, it was not possible to predict the protection generated by the vaccine by measuring only the serum antibody titres, mainly because the vaccine contained whole ciliates that share many antigens. However, as usually occurs in the challenge, serum from turbot immunized with the heterologous strains, which did not induce cross-protection, did not kill the heterologous strain. In this respect, the complement-dependent cytotoxicity assay performed better than ELISA for predicting the resistance to infection in different vaccinated groups. When the two methods are compared, ELISA has the advantage that the sera can be maintained frozen at −20 °C for a long time and live ciliates are not required. We have found that the levels of serum complement decline considerably after serum is frozen and that the cytotoxic assays should be carried out with live ciliates and fresh serum for optimum results. However, this method is not applicable unless these conditions are met. Despite this limitation, the cytotoxicity assay is technically simpler than ELISA, as it does not require secondary antibodies or additional reagents. This cytotoxicity assay enables the rapid assessment of whether the serum from vaccinated fish can lyse *P. dicentrarchi* trophozoites under field conditions, without the need for sophisticated equipment. Moreover, when fresh serum and live ciliates are available, by using the complement-dependent cytotoxicity assay, it is possible to greatly reduce the number of fish required for *P. dicentrarchi* vaccination experiments, which are needed to identify the protective antigens for future vaccine development.

CRediT authorship contribution statement

Paola Guliás: Investigation, Methodology. **Rosa Ana Sueiro:** Formal analysis, Investigation, Methodology. **Oscar González:** Funding acquisition, Resources. **Cristina Brea:** Resources. **Joaquín Macías:** Resources. **José Manuel Leiro:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Writing – review & editing. **Jesús Lamas:** Conceptualization, Funding acquisition, Supervision, Writing – original draft.

Declaration of competing interest

The authors of the manuscript entitled "A serum cytotoxicity assay for measuring the resistance of turbot to the ciliate *Philasterides dicentrarchi*" by Giuliás et al. declare that they do not have competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cirep.2025.200259.

Data availability

Data will be made available on request.

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