Susceptibility of turbot to *Aeromonas salmonicida* subsp. *salmonicida* during a mixed experimental infection with *Philasterides dicentrarchi*

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Abstract

This study assesses the effect of the simultaneous infection of two common pathogens in turbot cultures, the bacterium *Aeromonas salmonicida* subsp. *salmonicida* (ASS) and the parasite *Philasterides dicentrarchi* (PD). Sublethal doses of both pathogens were used in an intraperitoneal infection. The total number of samples positive for ASS recovery from internal organs was significantly higher among fish with mixed treatments (ASS+PD) than among fish with single (ASS) ones ($p = 0.004$). Furthermore, a lower survival rate among fish exposed to co-infection with bacteria and parasite (71%), than among fish exposed to the same doses of monocultures of parasite or bacteria (100% and 96% respectively) ($p=0.0129$) was found. Slight differences were also found in the mean survival time (from 7.6 to 8 d). Although the effects were quite subtle, together they suggest that the ciliate increased the virulence of the bacteria, promoting the bacterial colonization of internal tissues and resulting in death of the fish.

Introduction

Aquaculture is an advantage for multiple host-pathogen interactions due to the high stocking density used in culture systems and prevalence of pathogens. Fish may then be co-infected by different pathogens, and as a consequence the infective dose of one of the pathogens could be affected. In fact, some authors have analyzed the effect of co-infection of bacteria and parasites in different species of fish, showing that the parasite infection could be a stress factor for fish which enhances bacterial invasion (Bandilla et al., 2006; Xu et al., 2012a, 2012b; Zhang et al., 2015).

Aeromonas salmonicida subsp. *salmonicida* (ASS) is the etiological agent of furunculosis, a systemic disease characterized by an ulcerative dermatitis in fish, which produces lethargy and multiple hemorrhages, both externally and internally and finally death of fish. It is typically associated with salmonids (Toranzo et al., 2005); however, ASS has also frequently been isolated from diseased turbot (*Scophthalmus maximus*) (Farto et al., 2011). To our knowledge, there is currently no information available on the effect of parasitism on infection of the bacterium ASS, and if its virulence could be enhanced. In particular,

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Philasterides dicentrarchi (PD) is a highly virulent ciliate endoparasite of turbot. It can invade the host and migrate through the bloodstream and connective tissues to reach various organs and tissues. Ultimately PD can result in the death of fish (Dyková and Figueras, 1994; Iglesias et al., 2001). PD induces apoptosis of leukocytes in turbot, mediated by cysteine proteases (Paramá et al., 2007), and causes immunosuppression in fish. Studies using a co-infection with PD and ASS will give a broader understanding of how virulence and colonization of ASS could be increased by multiple pathogen interactions on the host and they will also provide information in order to design new therapeutic strategies to protect fish from this pathogen.

In this study the effect caused by co-infection with ASS and PD on the susceptibility of turbot to experimental infection was analyzed in order to confirm the effect of parasitism interaction on virulence of ASS. Doses that were sublethal when bacteria and parasite were inoculated as monocultures were used in co-infection experiments.

Material and methods

Bacteria. A. salmonicida

An ASS strain (RIM 33.1) isolated from a diseased farmed turbot in Galicia during 2004 was used in this experiment; the positive identification was confirmed (Lago et al., 2012) using the specific primers and the PCR assay previously developed to identify ASS strains (Byers et al., 2002).

The bacterial isolate was cryopreserved in trypticase soy broth (TSB; Cultimed), supplemented with 15 % glycerol (v/v; Panreac) at -80 °C. Prior to use, the ASS isolate was resuscitated and grown on trypticase soy agar (TSA; Cultimed) and incubated at 18 °C for 72 h.

Ciliate. P. dicentrarchi

The ciliates were isolated by abdominocentesis under aseptic conditions and after peritoneal washing with physiological saline solution (PSS) of the abdominal cavity of infected turbot. Ciliates were kept in axenic culture, with L-15 medium supplemented with fetal bovine serum (100 mL/L of FBS, Sigma), previously inactivated at 56 °C for 30 min, and supplemented with 100 units/mL penicillin G, 0.1 mg/mL of streptomycin sulfate and 0.25 mg/mL amphotericin B (Antibiotic Antimycotic Solution, Sigma). PD was periodically subcultured at 20ºC.

Fish

Unvaccinated turbot (*Scophthalmus maximus*, L.) were provided by an aquaculture facility from Galicia (NW Spain). They were moved to the Marine Science Station of Toralla (ECIMAT), belonging to the University of Vigo, where they were kept. No history of fish disease had previously been recorded in either of the facilities. The average weight of the fish used was 94.46 ± 7.33 g.

Before starting each experiment, 12 fish per tank were acclimatised for 72 h, in tanks containing 40 L of filtered (0.22 μm) sea water in flow through systems (three exchanges per day). Temperature was 14°C and salinity was maintained at 33°/$_{oo}$ with a 12L:12D photoperiod. The number of fish used was the minimal to obtain accurate statistical analyses.

Effluents were collected and treated for 24 h with sodium hypochlorite to eliminate pathogens. The physicochemical parameters were

recorded daily and maintained less than 0.08 mg/L ammonia (measured using a Nutrafin ammonium Test Kit), between 7.5 and 7.9 for pH levels and greater than 80% oxygen saturation. The fish were monitored daily for 8 d and fed a maintenance ration (0.1% of weight/day). When disease symptoms appeared the fish were anesthetized and then euthanized by an overdose of tricaine methanesulfonate (MS-222; Sigma) and were analysed for bacteria and parasite presence.

Determination of bacterial sublethal dose

A preliminary test to determine the bacterial sublethal dose for fish, allowing 8 days of survival after injection, was performed.

To prepare the bacterial suspension, 5 ASS colonies were inoculated and grown in TSB shake cultures (20 mL, 18 °C, 24 h) (Farto et al., 2011). The cells were harvested and resuspended in PSS to an optical density of 0.5 ($OD_{600nm,}$ 10^9 cfu/mL). Serial dilutions were prepared in PSS and abundance (cfu/mL) was determined on TSA plates.

The bacterial suspension (0.1 mL) was inoculated in turbot by injection into the abdominal cavity, with doses of 2.7×10^3 cfu/mL, 2.7×10^4 cfu/mL and 2.7×10^6 cfu/mL. Fish intraperitoneally injected (0.1 mL) with PSS were used as negative controls. After the experimental period, the surviving fish were euthanized as above. Liver and kidney samples from euthanized and dead turbot were sampled for bacteria by inoculation onto TSA plates. After incubation at 22 °C for 48 h, identification by determination of presumptive ASS colonies was carried out as described by Lago et al. (2012).

Determination of ciliates sublethal dose

A preliminary test to determine the ciliates sublethal dose for fish was not performed. According to Paramá et al. (2003), 5×10^5 ciliates/fish were used.

For the following experiments the total amount of ciliates present in each culture flask was calculated by examining an aliquot of the culture to which was added a final concentration of 0.25 % glutaraldehyde. The sample was centrifuged at 600 G for 5 min and the ciliates were counted in a Neubauer chamber. The original suspension was then washed and adjusted with PSS to achieve the required concentration of ciliates.

Experimental infections with monocultures of ASS and PD

Based on the previous results of sublethal doses for bacteria and ciliates, fish were exposed (in duplicate experiments) to monocultures (0.1 mL) of ASS and PD, at a final dose of 1.91×10^2 cfu and 5×10^5 ciliates per fish respectively.

Experimental infections with mixed cultures of ASS and PD

At the same time of experimental exposures to monocultures of the bacterium and parasite, turbot were exposed (in duplicated experiments) to mixed cultures of ASS (0.1 mL) and PD (0.1 mL), at a final dose of 1.91×10^2 cfu and 5×10^5 ciliates per fish. Control tanks in which the fish were injected with 0.1 mL of PSS were included. After 8 d, the surviving fish were euthanized. All fish were examined for ASS as described previously. Ciliate presence was determined by direct microscopic examination of aliquots of blood of euthanized and dead fish.

Ethical approval

All experimental protocols were approved and supervised by the National Committee on Animal Research and Ethics in accordance with European regulation on animal protection (Directive 2010/63/UE).

Statistical analysis

Significant differences in responses were explored using a one-way ANOVA. Kaplan–Meier survival curves were generated from data from two replicates, and they were compared by means of the Log-rank (Mantel–Cox) test. Analyses were calculated using SPSS version 15.0.

Results

Determination of bacterial sublethal dose

The sublethal dose of bacteria per fish, to be used in the mixed experimental infection, was determined to be 10^2 cfu/fish (Table 1). This dose did not generate mortality within 8 d post challenge and bacteria were not recovered from internal organs of surviving fish.

Experimental infections with monocultures of ASS and PD

The PD dose used did not result in turbot mortalities within 8 d post challenge; with PD presence confirmed in the blood of 2 surviving turbot (in both replicates). When a pure culture of bacteria was used for treatment, 1 dead fish from 12 infected was recorded (in one replicate). ASS was isolated from internal organs of dead and surviving turbot (in both replicates) (Table 2).

Experimental infections with mixed cultures of ASS and PD

When experimental treatments mixing ASS and PD were applied, 3 and 4 dead fish were recorded in each replicate tank respectively. The ciliate was detected in the blood of only one of the dead turbot and in one surviving fish from each replicate. However, the bacteria were always recovered from the internal organs of all dead fish and from 7 of the 9 surviving fish in one replicate or 4 of the 8 in the another

Table 1. Fish mortalities and detection of bacterial recovery from internal organs in three bacterial experimental infections.

Mean weight (g)	Group	Doses[1]	Mortality[2]	Recovery ASS D[3]	Recovery ASS S[4]
90.66 ± 10.3	*A. salmonicida*	2.7×10^2	0/12	0/0	0/12
92.32 ± 7.15	*A. salmonicida*	2.7×10^3	4/12	4/4	2/8
94.10 ± 11.48	*A. salmonicida*	2.7×10^5	11/12	11/11	0/1
91.95 ± 9.58	Control	0	0/12	0/0	0/12

[1] Injected dose, cfu/fish.
[2] Mortality: number of dead fish/total tested fish.
[3] Number of positive fish for ASS recovery/total dead fish.
[4] Number of positive fish for ASS recovery/total surviving fish.

Table 2. Fish mortalities and detection of ASS and PD from dead and surviving fish after experimental infections mixing ASS and PD.

Infection	Pathogen	Mortality[1]	Detection D[2]	Detection S[3]
	A. salmonicida	0/12	0/0	1/12
Single	*A. salmonicida*	1/12	1/1	1/11
	P. dicentrarchi	0/12	0/0	2/12
	P. dicentrarchi	0/12	0/0	2/12
Mixed	*A. salmonicida*	3/12	3/3	7/9
	P. dicentrarchi		0/3	1/9
Mixed	*A. salmonicida*	4/12	4/4	4/8
	P. dicentrarchi		1/4	1/8
	Control	0/12	0/0	0/12

[1] Mortality: number of dead fish/total tested fish.
[2] Number of positive fish for ASS or PD detection/total dead fish.
[3] Number of positive fish for ASS or PD detection/total surviving fish.
Injected dose/fish: 1.91 x 10^2 cfu of bacteria and 5 x 10^5 of ciliates.

replicate. No mortality was recorded in control fish (Table 2).

ANOVA was used to test for statistical differences in the number of positive fish for ASS or PD detection after experimental infections using a mix of ASS and PD and using each of the pathogens separately as inoculum. The results indicated significant differences ($p = 0.004$) in the total number (dead and surviving fish, D+S) of positive fish for ASS recovery between fish inoculated with only bacteria and those treated with a mixed preparation. No significant differences ($p = 0.934$) were found for the ciliate detection (Figure 1).

Kaplan-Meier survival curves for the fish populations in the three experimental infections are shown in Figure 2, showing different profiles. The analysis showed significantly smaller final survival rates among fish co-infected with 10^2 cfu/fish of bacteria and 10^5 of ciliates/fish (71%), compared with fish exposed to the same doses of monocultures of parasite or bacteria (100% and 96% respectively) ($p=0.0129$), slight differences were also found in mean survival time (from 7.625 to 8 d) resulting in death of fish.

Discussion

An interaction between PD and ASS was demonstrated in this study, as the susceptibility of turbot to ASS was increased as a result of coinfection. The assays were performed using approximately 100g turbot under normal culture conditions and two relevant pathogens for this host, ASS and PS.

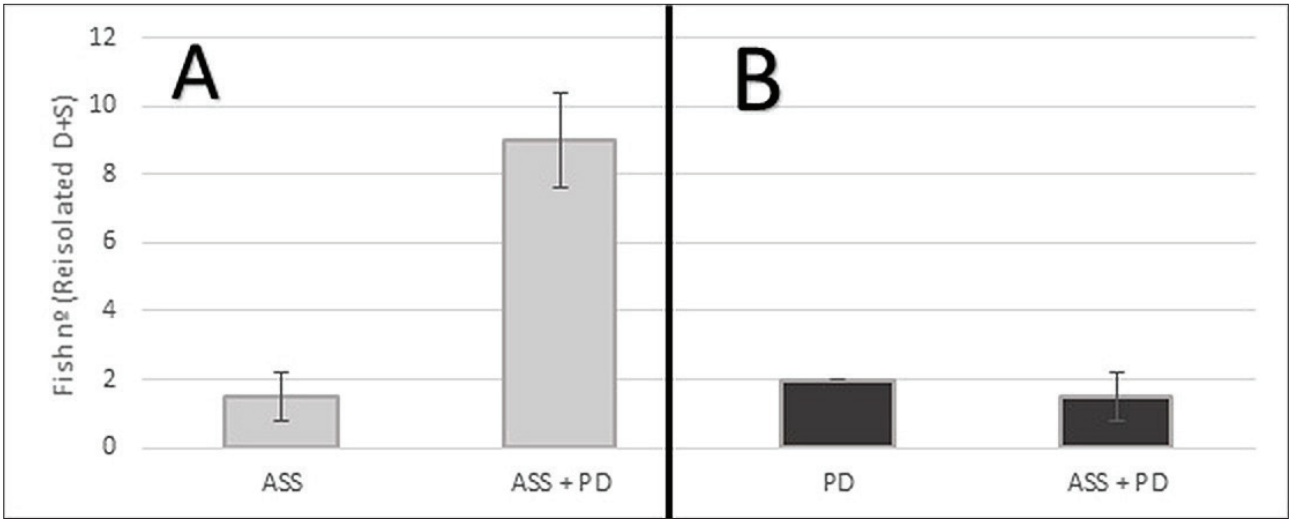

Figure 1. Number of positive fish (dead, D and surviving, S) for ASS recovery and PD detection in blood, after experimental infections mixing ASS and PD or using both pathogens separately as inoculum. Bars represent the average obtained for replicates, with standard deviations. There are significant differences (p = 0.004) in the total number (D+S) of positive fish for ASS recovery (A) when single (ASS) and mixed treatments (ASS+PD) were compared. No significant differences (p = 0.934) were found for the ciliate detection (B).

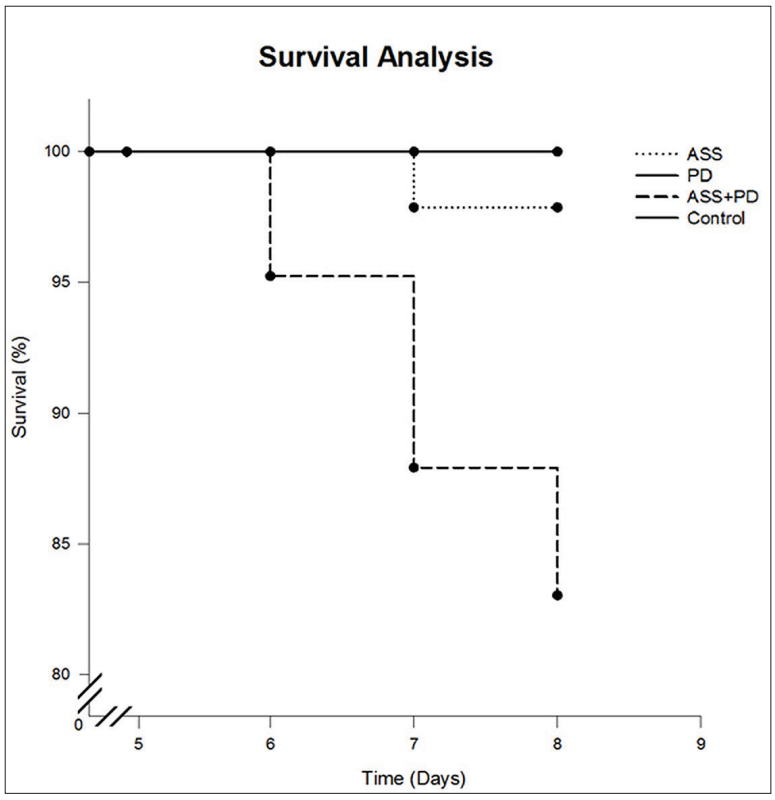

Figure 2. Kaplan-Meier survival curves of turbot after experimental infection with ASS and PD. Each curve was painted from data of two replicates. Each replicate used 12 fish. As inoculum a mix of bacteria and ciliate (ASS+PD) was used or each pathogen separately. According to the long-rank analysis, significant differences were found between % survival of fish after treatment using mix of ASS+PD or using each one separately (p = 0.0129).

In order to show the interaction between both pathogens, it was necessary to select doses that were sublethal when bacteria and parasite were inoculated as monocultures. In our assays with ASS, it was demonstrated that a dose of 10^2 cfu/fish was ideal for the purposes of the experiment, since the bacteria were not re-isolated in culture from surviving fish. This dose had the advantage of producing no mortality before 8 days, whereas the other tested doses resulted in much more rapid mortality. The higher doses used allowed confirmation of the virulence of the strain previously described. In fact, the strain generated 33% or 92% of mortality at doses of 2.7 x 10^3 cfu/fish or 2.7 x 10^5 cfu/fish, respectively, whereas in previous studies 100% of mortality was observed in a few days (before 5 d post inoculation) at doses of 2.2 x 10^4 cfu/fish using 30g fish (Lago et al., 2012).

The monoculture treatments showed that both ciliates and bacteria would affect the fish at interval stages of the experiment, but the immune system of fish was able to control infection throughout the monitoring period. The detection of PD in blood after mixed treatment suggests that *Philasterides dicentrarchi* weakens *S. maximus* immune response, as suggested from the results obtained previously (Piazzon et al., 2014). Furthermore, this parasite promoted the colonization of internal organs of fish by ASS and increased the number of positive fish for ASS recovery after a mixed treatment.

Statistically significant differences in the response of fish were noted in the survival of fish exposed to a monoculture bacterial infection with those after having suffered a mixed infection of bacteria and ciliates. Lower final survival rates among fish co-infected than among fish exposed to the same doses of monocultures of parasite or bacteria were found. Differences were also found in the mean survival time, resulting in a slight average delay of 10 h to death of fish.

In summary these results are suggestive that coinfection with a ciliate parasite slightly increases the susceptibility of turbot to infection with ASS. In general, there is very limited information on the effects of coinfections with more than one pathogen on fish mortality and morbidity. Although the apparent effects in this study were subtle, it is important that they are documented and made available for the benefit of other fish disease coinfection workers.

An important implication could be that the time available to treat fish with antibiotics and other treatments will likely be reduced if there are concurrent infections with PD and other pathogens.

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