

# Differential susceptibility to furunculosis of turbot and rainbow trout and release of the furunculosis agent from furunculosis-affected fish

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**ABSTRACT:** The different susceptibilities to furunculosis of turbot held in sea water and of rainbow trout in fresh water were compared using intraperitoneal, bath, and intragastric exposures. The intraperitoneal LD<sub>50</sub> of *Aeromonas salmonicida* was  $3 \times 10^5$  cfu (colony-forming units) fish<sup>-1</sup> for 25 g rainbow trout *Oncorhynchus mykiss* and  $2 \times 10^4$  cfu fish<sup>-1</sup> for 30 g turbot *Scophthalmus maximus*. The minimal lethal dose by bath for rainbow trout was  $10^8$  cfu ml<sup>-1</sup> in fish exposed over a challenge period of 12 h. With turbot, the same mortality percentage was obtained with  $10^5$  cfu ml<sup>-1</sup> after an exposure for 12 h. By both challenge methods turbot therefore proved to be more susceptible than rainbow trout to *A. salmonicida*. Both species proved refractory to challenge by the intragastric method. The release of bacteria from exposed fish was studied and the recovery of culturable *A. salmonicida* was only possible from dead or moribund rainbow trout. It is also interesting that only the more resistant rainbow trout appeared to become carriers of *A. salmonicida* following exposure to the pathogen. The implications for farming are discussed.

**KEY WORDS:** *Aeromonas salmonicida* · Turbot · Rainbow trout · Infectivity routes

## INTRODUCTION

Furunculosis is a major disease in fresh- and seawater aquaculture and is caused by *Aeromonas salmonicida* subsp. *salmonicida*. It has been widely described as causing losses in salmonid fish (Austin & Austin 1983, Toranzo & Barja 1992, Munro & Hastings 1993), and its virulence factors have been reviewed by Ellis (1991).

To our knowledge, only a limited number of cases of *Aeromonas salmonicida* subsp. *salmonicida* in seawater fish such as turbot *Scophthalmus maximus* have been reported (Nougayrede et al. 1990, Toranzo et al. 1991). The primary purpose of this study was to compare the infectivity of *A. salmonicida* for rainbow trout

*Oncorhynchus mykiss* in fresh water and turbot in sea water by intraperitoneal, intragastric, and bath challenge, using the same conditions for all the experiments. In addition, we examined the shedding of *A. salmonicida* from fish surviving exposure to the pathogen and from fish dead of furunculosis.

## MATERIAL AND METHODS

**Bacteria.** The challenge strain of *Aeromonas salmonicida*, AI<sub>130B</sub>, had been isolated from the spleen of a brown trout *Salmo trutta* during an outbreak of furunculosis. It was grown on trypticase soy agar (TSA, Oxoid), was identified as *A. salmonicida* subsp. *salmonicida*, showed the A<sup>+</sup>LPS<sup>+</sup> phenotype (Fernández 1993), and was agglutinated with an antiserum raised against whole cells of reference strain ATCC

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33658 (Toranzo et al. 1991). The strain was kept frozen at  $-70^{\circ}\text{C}$  in tryptic soy broth (TSB, Adsa-micro) containing 15% (v/v) glycerol.

**Fish.** Fish free of *Aeromonas salmonicida* were used for the experiment. Absence of *A. salmonicida* in these fish was confirmed by the carrier test (McCarthy 1977). Rainbow trout with an average weight of 25 g and turbot averaging 30 g were used for intraperitoneal and bath challenge experiments; fish of both species with an average weight of 150 g were used for the intragastric challenge. Rainbow trout were maintained in 25 l aerated freshwater tanks at  $15^{\circ}\text{C}$  and water was changed every 12 h. Turbot were maintained in the same conditions in sea water. Experiments were performed on groups of 8 fish. After inoculation, fish were monitored at 4 h intervals.

**Challenge experiments.** The strain of *Aeromonas salmonicida* used for the challenge was grown in TSB at  $22^{\circ}\text{C}$  for 24 h. Cells were harvested by centrifugation and were resuspended in phosphate buffered saline (PBS) to  $10^9$  colony-forming units (cfu)  $\text{ml}^{-1}$  (optical density at 540 nm = 1). Serial dilutions in PBS were prepared to obtain the challenge dose. The cfu  $\text{ml}^{-1}$  were determined on TSA plates.

(1) Intraperitoneal injection: The intraperitoneal (i.p.) challenge was carried out according to Nieto et al. (1985). Fish were injected with 0.1 ml of the bacterial suspensions which contained from  $10^3$  to  $10^7$  cfu  $\text{ml}^{-1}$ . Mortalities were recorded for 7 d. The lethal dose 50 ( $\text{LD}_{50}$ ) values were determined by the Reed & Muench (1938) method.

(2) Bath challenge: Rainbow trout and turbot were exposed to  $10^3$  to  $10^8$  cfu  $\text{ml}^{-1}$  bacteria for 12 h. Throughout the challenge period the water was aerated. After each exposure period fish were monitored for up to 19 d post-challenge.

(3) Intragastric challenge: For these experiments, fish were anaesthetized with benzocaine (20 to 50 ppm solubilized in ethanol; Sigma). A soft silicone plastic tube, diameter 3 mm, attached to a syringe, was pushed 3 to 5 cm into the oesophagus and 0.1 ml of different challenge suspensions ( $10^4$  to  $10^7$  cfu  $\text{ml}^{-1}$ ) was inoculated. Mortalities were recorded for 14 d.

In each challenge experiment, kidney samples from all recently dead fish (less than 4 h) were inoculated on TSA to confirm the presence of *Aeromonas salmonicida*.

**Carrier test.** Surviving as well as pre-challenged fish were tested according to McCarthy (1977) to determine whether they were carrying *Aeromonas salmonicida*. Briefly, the fish were anaesthetized with benzocaine (Sigma) and injected intramuscularly (i.m.) with dexamethazone (Fluka Chemicals) (0.5 mg per 10 g of fish) suspended in PBS. Water temperature was maintained at  $15^{\circ}\text{C}$  and fish were monitored for 10 d. After

this, kidney samples were taken from all fish to test for the presence of *A. salmonicida*.

**Shedding experiments.** Following challenge and monitoring, release of *Aeromonas salmonicida* from recently dead and surviving fish was investigated (shedding from survivors was measured after the carrier test). Individual fish were placed in 1 l of filtered fresh (rainbow trout) or sea (turbot) water for 1 h, after which the water was well mixed and triplicate samples of 10  $\mu\text{l}$  and 100  $\mu\text{l}$  were spread onto TSA plates supplemented with  $10 \mu\text{g ml}^{-1}$  streptomycin (Fluka Chemicals). The antibiotic was added as a solution in PBS to TSA at  $40^{\circ}\text{C}$ . This strain was resistant to streptomycin (Toranzo et al. 1990).

**Statistical analysis.** Differences in susceptibility of rainbow trout and turbot to *Aeromonas salmonicida* were tested for using the chi-square test of independence and homogeneity. The tests were part of the statistical program SPSS/PC+ V 2.0.

## RESULTS AND DISCUSSION

### Infectivity experiments

(1) Intraperitoneal injection: The AI<sub>130B</sub> strain of *Aeromonas salmonicida* subsp. *salmonicida* injected i.p. showed a  $\text{LD}_{50}$  of  $3 \times 10^5$  cfu fish<sup>-1</sup> for 25 g rainbow trout maintained in fresh water and  $2 \times 10^4$  cfu fish<sup>-1</sup> for 30 g turbot held in sea water (Tables 1 & 2). It is known that the susceptibility of different fish species to *A. salmonicida* is affected by, among other factors, the size and age of fish (Jofre 1988). For this reason, we used immature fish of equivalent size in all our tests and we employed standardized challenge test conditions. Even though these  $\text{LD}_{50}$  were not significantly different on the basis of the chi-square test, they appeared to suggest that turbot was more susceptible than rainbow trout.

(2) Bath challenge: The mortalities of fish exposed by bath to *Aeromonas salmonicida* are summarized in Tables 1 & 2.

The  $\text{LD}_{50}$  was  $3.16 \times 10^7$  cfu fish<sup>-1</sup> for rainbow trout and  $3.16 \times 10^4$  cfu fish<sup>-1</sup> for turbot. As with the findings suggested by the i.p. challenge, the results from the bath challenge showed turbot to be more susceptible to *Aeromonas salmonicida* than rainbow trout. By bath exposure, the dose of *A. salmonicida* required to kill 100% of the turbot was a thousand times less than that required to kill 100% of the rainbow trout. Using the chi-square test it was found that the lethal dose for turbot was significantly smaller ( $p < 0.001$ ) than that for rainbow trout, the turbot showing a susceptibility similar to that of *Salmo salar* held in sea water (Rose et al. 1989).

Table 1 Results obtained with rainbow trout *Oncorhynchus mykiss* challenged with *Aeromonas salmonicida* (A.s.) by various routes: intraperitoneal injection (i.p.), bath exposure, and intragastric intubation (i.g.)

Route	Dose (cfu ml <sup>-1</sup> )	Mortality <sup>a</sup>	Recovery of A.s. from mortalities <sup>b</sup>	Average no. of A.s. cells shed from dead fish	No. of A.s.-positive survivors <sup>c</sup>	Average no. of A.s. cells shed from survivor fish <sup>d</sup>
i.p.	10 <sup>3</sup>	0/8			0/8	0
	10 <sup>4</sup>	3/8	3/3	0	0/5	0
	10 <sup>5</sup>	4/8	4/4	0	1/4	0
	10 <sup>6</sup>	5/8	5/5	0	2/3	0
	10 <sup>7</sup>	8/8	8/8	0		
Bath	10 <sup>4</sup>	0/8			0/8	0
	10 <sup>5</sup>	0/8			0/8	0
	10 <sup>6</sup>	0/8			4/8	3.5 × 10 <sup>4</sup> (1.8 × 10 <sup>4</sup> ) <sup>e</sup>
	10 <sup>7</sup>	0/8			8/8	10 <sup>5</sup> (8 × 10 <sup>4</sup> ) <sup>e</sup>
	10 <sup>8</sup>	8/8	8/8	10 <sup>5</sup> (2.3 × 10 <sup>4</sup> ) <sup>e</sup>		
i.g.	10 <sup>4</sup>	0/8			0/8	0
	10 <sup>5</sup>	0/8			0/8	0
	10 <sup>6</sup>	0/8			0/8	0
	10 <sup>7</sup>	0/8			0/8	0

<sup>a</sup>No. of fish dead/no. tested. <sup>b</sup>No. of A.s.-positive/no. tested. <sup>c</sup>No. of A.s.-positive survivors/no. tested  
<sup>d</sup>Shedding determinations were made at 17 d (i.p. method), 29 d (bath method), and 24 d (i.g. method) post-challenge  
<sup>e</sup>SD in parentheses

(3) Intragastric challenge: No mortalities were recorded from any group of fish challenged with 10<sup>4</sup> to 10<sup>7</sup> cfu fish<sup>-1</sup>. This result agrees with an earlier study (Rose et al. 1989), which found intragastric exposure to be less effective than bath exposure for infecting *Salmo salar*.

*Aeromonas salmonicida* was reisolated from the kidneys of all of the dead trout and from the kidneys of all of the dead i.p.-challenged turbot. It was also reisolated from 67% of the dead bath-challenged turbot.

These data suggest that *A. salmonicida* was responsible for the deaths of all or most of the challenged fish. Failure to recover *A. salmonicida* from all of the bath-challenged turbot may have been due to overgrowth of the isolation plates with fast-growing opportunistic bacteria (*Pseudomonas* and *Moraxella* spp.) which appeared to be present in the kidneys of some turbot. These bacteria have been shown to be avirulent in fish (Fernández et al. 1993) are generally viewed as part of the normal water microbiota (Allen et al. 1983, Austin

Table 2. Results obtained with turbot *Scophthalmus maximus* challenged with *Aeromonas salmonicida* (A.s.) by various routes: intraperitoneal injection (i.p.), bath exposure, and intragastric intubation (i.g.)

Route	Dose (cfu ml <sup>-1</sup> )	Mortality <sup>a</sup>	Recovery of A.s. from mortalities <sup>b</sup>	Average no. of A.s. cells shed from dead fish	No. of A.s.-positive survivors <sup>c</sup>	Average no. of A.s. cells shed from survivor fish <sup>d</sup>
i.p.	10 <sup>3</sup>	1/8	1/1	0	0/7	0
	10 <sup>4</sup>	3/8	3/3	0	0/5	0
	10 <sup>5</sup>	5/8	5/5	0	0/3	0
	10 <sup>6</sup>	8/8	8/8	0		
	10 <sup>7</sup>	8/8	8/8	0		
Bath	10 <sup>4</sup>	0/8			0/8	0
	10 <sup>5</sup>	8/8	4/8	0		
	10 <sup>6</sup>	8/8	nt	nt		
	10 <sup>7</sup>	8/8	6/8	0		
	10 <sup>8</sup>	8/8	6/8	0		
i.g.	10 <sup>4</sup>	0/8			0/8	0
	10 <sup>5</sup>	0/8			0/8	0
	10 <sup>6</sup>	0/8			1/8	0
	10 <sup>7</sup>	0/8			0/8	0

<sup>a</sup>No. of fish dead/no. tested. <sup>b</sup>No. of A.s.-positive/no. tested. <sup>c</sup>No. of A.s.-positive survivors/no. tested  
<sup>d</sup>Shedding determinations were made at 17 d (i.p. method), 29 d (bath method), and 24 d (i.g. method) post-challenge

1983, Nieto et al. 1984). The capacity of these bacteria to inhibit growth of *A. salmonicida* is well known (Cornick et al. 1969, Dubois-Darnaudpeys 1977).

### Carrier test

Details of the carrier test results are given in Table 1 for rainbow trout and Table 2 for turbot.

In rainbow trout challenged by i.p. injection, the percentage of *Aeromonas salmonicida*-positive survivors (carriers) increased with the dose of cells used. In the bath-challenged rainbow trout, 50% of the fish exposed to  $10^6$  cfu ml<sup>-1</sup> and all of the fish exposed to the smaller doses proved to be non-carriers. The other rainbow trout survivors were positive in the carrier test.

With turbot, the carrier test was negative for all fish surviving the i.p. and bath challenges and for all but one of the fish receiving the gastric challenge. The single positive intragastrically challenged fish had been dosed with  $10^6$  cfu fish<sup>-1</sup> and it is possible that injury to the oesophagus received during the challenge contributed to the infection.

The foregoing results with rainbow trout are consistent with the fact that salmonids, following exposure to *Aeromonas salmonicida*, can become carriers of the pathogen. The results with turbot are, however, more surprising because, despite their relative susceptibility to the pathogen, they appeared to free themselves of it if they survived challenge with the organism.

### Shedding of bacteria from infected fish

Details on the shedding of *Aeromonas salmonicida* by infected rainbow trout and turbot are given in Tables 1 & 2, respectively.

Dead bath-challenged trout shed on average  $1 \times 10^5$  cfu h<sup>-1</sup> fish<sup>-1</sup>. This value is similar to those of  $1.7 \times 10^5$  to  $1 \times 10^7$  cfu h<sup>-1</sup> fish<sup>-1</sup> released from dead *Salmo salar* in sea water (Rose et al. 1989) after bath challenge and is slightly smaller than that ( $10^8$  cfu ml<sup>-1</sup>) reported from dead rainbow trout by McCarthy (1980). Rainbow trout survivors of bath infection showed, after the carrier test, a smaller or similar shedding rate to dead fish, but *Aeromonas salmonicida* could still be recovered at 29 d post-challenge from fresh water in which the fish had been held for shedding experiments.

*Aeromonas salmonicida* was not detected in the water where trout or turbot surviving intragastric and i.p. challenge were immersed for shedding experiments. The test used to recover culturable *A. salmonicida* cells has been demonstrated to be unsuitable for detecting very small numbers of culturable cells (Pérez et al. 1995).

In summary, from these results we conclude that turbot are more susceptible than rainbow trout to *Aeromonas salmonicida* infection. However, the differences in the shedding rate between trout and turbot together with the fact that turbot tend not to become carriers of the pathogen lead us to conclude that *A. salmonicida* would be less easily spread in turbot farms than in salmonid farms.

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