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Protective Responses of Rainbow Trout Following Intraperitoneal Injection with a Live Virulent or Avirulent Isolate of *Renibacterium salmoninarum*

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Abstract.- Understanding the immune response of fish to *Renibacterium salmoninarum* (*Rs*), the causative agent of bacterial kidney disease (BKD), would help in the development of a vaccine against the disease. The aim of the present study was to examine the protection and antibody response of rainbow trout following intraperitoneal (i.p.) injection of either a live virulent (NCIMB 1113) or an avirulent (NCIMB 1111) isolate of *Rs* and subsequently, also after a following challenge with the virulent strain. Following subsequent challenge with *Rs* 1113, fish initially injected with *Rs* 1113 demonstrated a higher rate of survival and higher antibody levels with a longer duration compared to fish initially immunized with *Rs* 1111. In addition, higher numbers of mortalities were observed in fish initially immunized with the avirulent isolate *Rs* 1111 than fish injected with Rs 1113 and phosphate buffered saline (PBS) injected group. These results show that there appears to be an increased susceptibility to BKD from initially challenging the fish with avirulent *Rs* 1111 and this increased susceptibility might be due to antibodies produced which may be counterproductive to protection.

Key words: *Renibacterium salmoninarum*, antibody, Virulent, Avirulent, protective response.

INTRODUCTION

Bacterial kidney disease (BKD) is a chronic bacterial infection which is slow to develop in infected fish, and has a serious impact on both wild and farmed salmonid stocks (Evenden *et al*., 1993). Control of BKD is difficult to achieve, and chemotherapy provides only temporary relief. A greater understanding of the immune response of fish to *R. salmoninarum* (*Rs*) is required to control the disease.

Specific antibody responses against *Rs* have been detected in the serum of naturally infected, vaccinated or challenged salmonids such as chinook salmon (*Oncorhynchus tshawytscha)*, Atlantic salmon (*Salmo salar* L.) and rainbow trout *(Oncorhynchus mykiss)*, while the duration of the humoral response in fish following injection with the bacterium has been reported by other researchers (Olivier *et al*., 1992; Daly *et al*., 2001).

Piganelli *et al*. (1999) demonstrated some protection in coho salmon (*Oncorhynchus kisutch*) treated with an oral vaccine consisting of formalin-killed *Rs* with the 57 kDa antigen removed by heat treatment. Griffiths *et al.* (1998), also showed that vaccination with live presumptive *Arthrobacter* species and a nutritional mutant strain of *Rs* decreased the level of detection of the pathogen in fish kidney. Furthermore, Daly *et al.* (2001) demonstrated some protection with live, attenuated *Rs*. (Rhodes *et al*., 2004; Salonius *et al*., 2005) also showed that Atlantic salmon injected with live *Arthrobacter davidanielli* was resistant to *Rs*.

Recently, interest in the use of live attenuated vaccines against bacterial pathogens in fish has increased (Temprano *et al*., 2005; Itano *et al*., 2006; Lan *et al*., 2007). Live attenuated vaccines generally provide better protection than killed vaccines, and seem to elicit a stronger cell-mediated response than bacterin vaccines (Marsden *et al*., 1996; Igarashi and Iida, 2002). Despite many studies examining the immune response to BKD, there is still need to understand the immune response of fish injected with live virulent and avirulent bacterin.

The aim of this study was to examine if survivors from an experimental infection with BKD

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developed protection against the bacterium following infection with live virulent or avirulent *Rs*. In addition we measured the duration of the antibody response in infected rainbow trout and examined whether there was any relationship between this response and the level of protection in fish to subsequent challenge with the virulent isolate of *Rs*.

MATERIALS AND METHODS

Preparation of bacterial strains

Two isolates of *Rs*, NCIMB 1111 and NCIMB 1113 determined as avirulent and virulent (Turgut, 2002) were cultured on selective kidney disease medium (SKDM) for three weeks at 15°C. The bacteria were then washed off the plates with sterile phosphate buffered saline (PBS, pH 7.2) and the cell concentration of the suspension was preliminary determined from its absorbance at 610 nm and also colony forming unit (CFU) was determined (Farias, 1995). The concentration of the suspension was adjusted to 1 $x10^8$ cells mL^{-1} with PBS.

Experimental challenge with live Rs

Rainbow trout (30-50 g), were maintained in 200 L tanks with flow-through freshwater at a temperature of 12±2°C. The fish were acclimatised to these conditions for two weeks prior to the challenge. Kidney samples of four fish were examined by PCR prior to the experiment in order to establish that the stock was *Rs* free. Throughout the experiment fish were fed a commercial pelleted diet (Ewos, Westfield, UK) twice daily at 2 % body weight day^{-1}. Fish were distributed in replicate thanks with 90 fish in each and challenged by i.p injections of 0.1ml of virulent *Rs* 1113 (n= 360); avirulent *Rs* 1111 (n= 180) a concentration of 1x 10^8 cfu mL^{-1}, and as control, with PBS (n= 180). Fish were anesthetised before injection with benzocaine (ethyl p-aminobenzoate, Sigma). Ten weeks post-challenge, 40 fish from each group were re-challenged by i.p injection of 0.1ml of virulent *Rs* 1113 at a concentration of 1 $x10^8$ cfu mL^{-1}. Two fish from each tank were sampled at 2 weekly intervals. The blood were collected from the caudal vein, which was allowed to clot for 1 to 2 h at 22°C before collecting the serum by centrifuging at 10 000 g for 10 min. Sera were stored at -70°C until tested**.**

Culture, ELISA and Gram-staining were used to confirm the presence of *Rs* in dead and sampled fish (Turgut *et al*., 2008). Statistical analysis of the time-mortality relationship was performed with Kaplan-Meier survival analysis. Differences in mortality due to isolate differences were also assessed. Test values of $P<0.05$ were accepted as significant.

Determination of antibody levels by ELISA

The relative anti-*Rs* antibody concentrations (RACs) were quantified by ELISA with sera pooled from two fish in each case (Harlow and Lane, 1988). The ELISA plate was coated with a 1:1 mixture of *Rs* 1113 and *Rs* 1111 at 1 $x10^8$ cell ml^{-1} and antibody titres determined using a two-fold dilution of the pooled serum, diluted in PBS. Samples were added to duplicate wells at 100 µl $well^{-1}$ and incubated overnight at 4°C. Negative controls consisted of replacing sera with PBS. Supernatant containing MAb 4C10 (anti-trout IgM) (Thuvander *et al*., 1990) was added to each well for 1 h at 22°C. The plates were incubated with anti-mouse immunoglobulin-G labelled with Horseradish peroxidase (IgG-HRP) (Diagnostics Scotland), diluted 1/1000 in PBS, for 1 h at 22°C and then chromogen/substrate solution (100 mL $well^{-1}$) was added to each well. The reaction was stopped with the addition of 2M H_2SO_4. The absorbance of each well was assessed in a multiscan spectrophotometer ELISA reader (MR 5000, Dynatech) at a wavelength of 450 nm. The negative-positive threshold value (0.34) of samples collected from challenged fish was based on the mean OD value at 450 nm plus 3 standard deviations of the non-challenged fish (n=10) included in the experiment as negative controls (Harlow and Lane, 1988). For the purposes of comparison, a mean negative control (PBS) was calculated and the readings for each individual were corrected for differences in background absorbance, by subtracting the difference of the mean negative control for that pooled sera from the overall mean negative control (Harlow and Lane, 1988). The RAC of fish from the first challenge, collected at 2 weekly intervals, were

compared between the three groups challenged with *Rs* 1111, *Rs* 1113 and PBS. Challenge isolates were designed as fixed factors and time of sampling was used as a covariate, and they were compared using a 2-way analysis of covariance (ANCOVA). The ANCOVA was followed by Turkey multiple comparisons. The RACs of re-challenged survivors were then compared by a 2-way ANCOVA with interaction, using the first challenge isolates, and presence or absence of a re-challenge, as fixed factors, and time of sampling as a covariate. The ANCOVA was followed by Tukey multiple comparisons. Test values of $P<0.05$ were accepted as significant in all tests.

RESULTS

The level of mortality in challenged fish

In the first challenge, 53.6 % of fish died over the course of the trial following i.p. injection with the virulent isolate *Rs* 1113 and all mortalities were *Rs* positive by culture on SKDM and ELISA. Only 0.5% of fish died following challenge with *Rs* 1111 but no *Rs* was isolated by culture from these fish and no mortalities occurred in fish injected with PBS (Fig. 1A). There were no significant differences between the replicate groups challenged with *Rs* 1113 ($P>0.05$), however, there were significant differences between the mortalities in fish injected with *Rs* 1113 compared with *Rs* 1111 and PBS ($P<0.05$).

After 10 weeks post-challenge, 40 survivors (no mortalities occurred after 6 weeks post-challenge) from each group were re-challenged with *Rs* 1113. Fish previously changed with *Rs* 1113, *Rs* 1111 and PBS showed 15 %, 40 % and 20% mortality after re-challenging with *Rs* 1113, respectively. Fish previously challenged with *Rs* 1111 showed significantly higher levels of mortality than fish previously challenged with *Rs* 1113 ($P<0.05$), although the level of mortality among fish previously challenged with *Rs* 1113 was not significantly different to that of fish injected with PBS (Fig. 1B)

The antibody response in challenged fish

In the initial challenge, maximal antibody responses were reached at 6 and 8 weeks post-injection with *Rs* 1111 and *Rs* 1113 respectively (Fig. 2B,C). RACs of the control group was significantly lower compared to the RAC of fish challenged with *Rs* 1113 and *R* 1111 ($P<0.05$) (Fig 2 a,b,c) and the RACs of fish challenged with *Rs* 1113 were significantly ($P<0.05$) higher than those challenged with *Rs* 1111 at all sample time points (Fig. 2B,C). When sera, collected 2, 4, 6, 8 and 10 weeks post-injection from fish re-challenged with *Rs* 1113, were analysed by ELISA, it was found that the peak antibody response was reached four weeks post-challenge in groups previously challenged with *Rs* 1111 and *Rs* 1113, and 6 weeks post-challenge for the group previously injected with PBS (Fig 2).

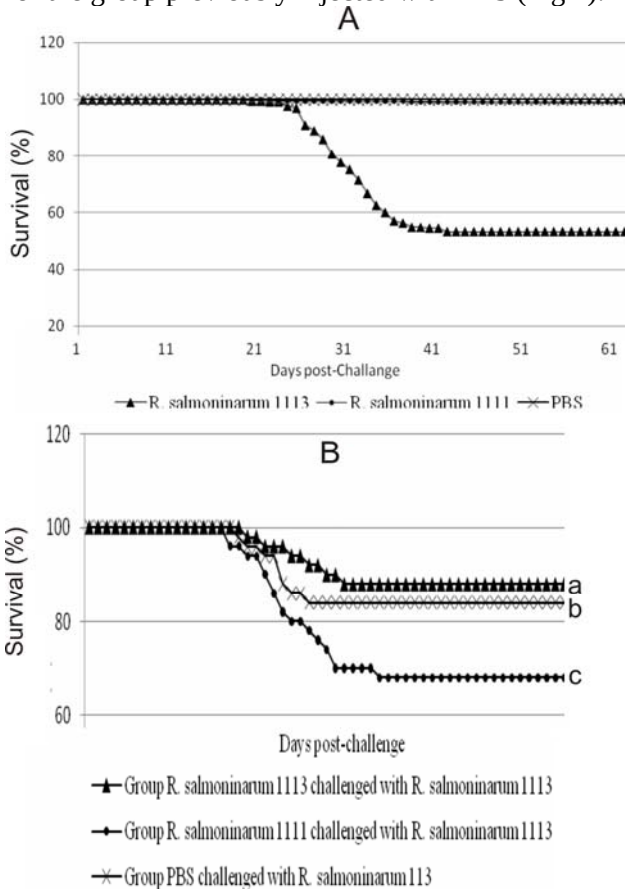

Fig. 1. Survival percentage (%) of rainbow trout following (A) an intraperitoneal challenge with virulent *R. salmoninarum* 1113 (n=360) and avirulent *R. salmoninarum* 1111 (n=180); (B) intraperitoneal challenge of survivors in (a) with *R. salmoninarum* 1113 (n=40); different letter indicate significant differences in cumulative survival

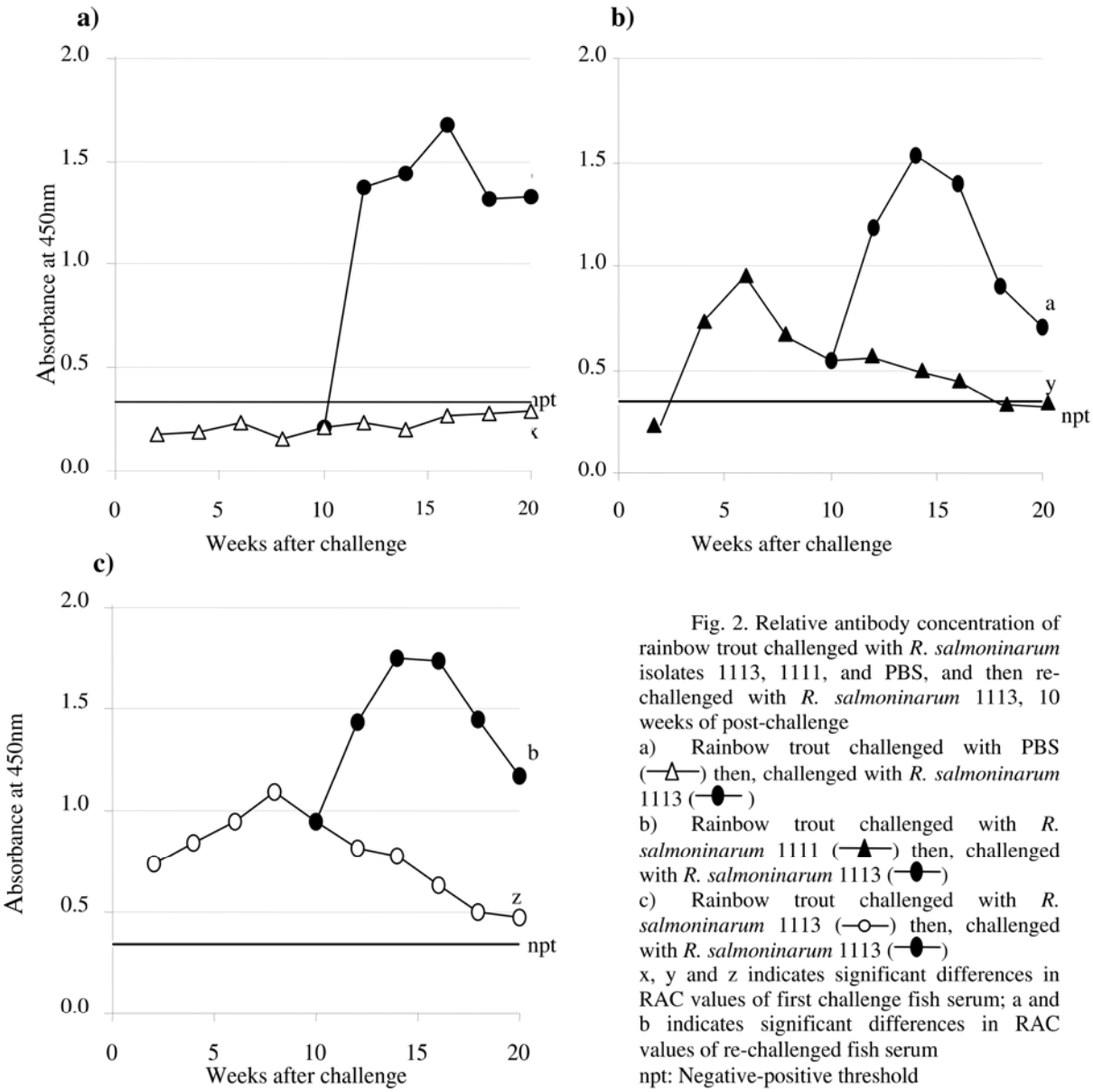

Fig. 2. Relative antibody concentration of rainbow trout challenged with *R. salmoninarum* isolates 1113, 1111, and PBS, and then re-challenged with *R. salmoninarum* 1113, 10 weeks of post-challenge
a) Rainbow trout challenged with PBS (—△—) then, challenged with *R. salmoninarum* 1113 (—●—)
b) Rainbow trout challenged with *R. salmoninarum* 1111 (—▲—) then, challenged with *R. salmoninarum* 1113 (—●—)
c) Rainbow trout challenged with *R. salmoninarum* 1113 (—○—) then, challenged with *R. salmoninarum* 1113 (—●—)
x, y and z indicates significant differences in RAC values of first challenge fish serum; a and b indicates significant differences in RAC values of re-challenged fish serum
npt: Negative-positive threshold

There were significant differences between the RAC values of re-challenged fish, with the RAC of fish previously challenged with *Rs* 1113 being higher than those challenged with *Rs* 1111 at all sample points ($P<0.05$). However, there was no significant difference in RAC of re-challenged fish between those previously challenged with *Rs* 1111 and those injected with PBS ($P>0.05$). Moreover, there was no significant difference between the RAC of fish previously challenged with *Rs* 1113 compared to the PBS group ($P>0.05$).

DISCUSSION

Fish challenged with live *Rs* 1111 showed higher levels of mortality after re-challenge with *Rs* 1113 compared to other groups, and lowest mortality levels were among fish challenged with *Rs*

1113 when they were re-challenged with *Rs* 1113, although these were not significantly different between the PBS injected group and those infected with *Rs* 1113. In addition, antibody levels in the *Rs* 1113-challenged group were significantly higher than in the *Rs* 1111-challenged group after re-challenging with *Rs* 1113, whereas there was no significant difference in antibody levels between the PBS-injected group, compared to those infected with live *Rs* 1111 or *Rs* 1113 after re-challenging with *Rs* 1113.

The antibody response of the group challenged with *Rs* 1113 was higher and lasted longer than the response of the group challenged with *Rs* 1111. Evelyn (1971) was the first to demonstrate production of antibodies to *Rs* in sockeye salmon lasting 16 months after a primary injection with heat-killed *Rs*. A second injection given 13 months after the first injection elicited a secondary immune response. Development of an antibody response to *Rs* after *i.p.* challenge has also been reported in rainbow trout by other researchers (Olivier *et al*., 1992; Jansson and Ljungberg, 1998). Levels of sera-positive fish were found to be greatest 8 weeks after immersion challenge (Jansson and Ljungberg, 1998). Also, Olivier *et al*. (1992) detected antibodies to soluble antigens up to 20 weeks post-challenge although mortality ceased after 13 weeks. The humoral antibody response to a heat-stable *Rs*-antigen was found to increase up to 10 weeks after immersion challenge, but after 27 weeks was reduced to a level similar to that of the non-challenged fish. In the present study, no mortalities were recorded after six weeks post-challenge and a detectable level of antibodies remained for 20 weeks in fish challenged with *Rs* 1113. By contrast, the group challenged with *Rs* 1111 gave no BKD-related mortalities and no detectable antibodies after 16 weeks post-challenge.

Turgut *et al*. (2008) showed as part of this study that fish tested positive for the bacterium up to 10 weeks and 12 weeks post-challenge by ELISA and culture on SKDM, respectively, with kidney samples from fish infected with *Rs* 1113, while the kidneys sampled from fish infected with *Rs* 1111 were all negative by ELISA from week 2 onwards. Furthermore, no growth was observed on SKDM with samples taken from fish infected with the avirulent isolate. Since the virulent isolate established an infection, it would have been multiplying within the fish. Isolate *Rs* 1111 did not establish a persistent infection in the fish and was therefore most likely not replicating within the fish. This might help to explain the higher antibody response observed against *Rs* 1113 compared to the response in fish initially infected with *Rs* 1111. The more effective clearance of *Rs* 1111 from the fishes system than *Rs* 1113 may explain why antibodies did not persist as long as in fish infected with *Rs* 1111.

The survivors of the challenges did not show any significant protection compared with the PBS control group even though they had antibodies at the time of the second challenge. The evidence would suggest that the antibodies produced during the initial challenge did not protect the fish against subsequent challenge. Thus, neither the virulent or the avirulent isolate appeared to stimulate a protective response in surviving fish. Furthermore, there appear to be increased sensitivity to BKD from initially challenging the fish with virulent *Rs* 1111, although *Rs* 1111 did not caused any BKD related mortalities during the initial challenge and resulted insignificantly higher mortality compared to groups the initially injected with *Rs* 1113 and PBS. This increased susceptibility might be due to antibodies produced against *Rs* 1111, and *Rs* 1111 might have different antigenic properties compared to *Rs* 1113, which might result susceptibility to BKD.

The experimental challenges with *Rs*, presented here, confirm *Rs* 1113 to be virulent and *Rs* 1111 to be avirulent, causing no BKD-related mortalities in challenged fish. There are several possible reasons for the difference in virulence between *Rs* isolates. Virulence has been reported to be associated with auto-agglutination of the pathogen (Bruno, 1988). Bruno (1988, 1990) reported that isolates became less hydrophobic and non-autoagglutinating after repeated sub-culture with the tendency to lose the 57 kDa surface protein, with the result that the bacterium becomes less virulent than auto-agglutinating strains with the 57 kDa protein (Bruno, 1988, 1990; Senson and Stevenson, 1999). The 57 kDa protein is considered to be a major virulence factor of *Rs* (Bruno, 1988, 1990; Senson and Stevenson, 1999). However, both

Rs isolates used in this study retained the surface antigen 57 kDa protein and showed no antigenic differences between virulent and avirulent isolates as determined by SDS-PAGE and Western blots analysis. However, *Rs* 1113 showed strong auto-agglutination while *Rs* 1111 was non-autoagglutinating (Turgut, 2002).

Kaattari and Piganelli (1997) showed that antibody responses against *Rs* appeared to be counterproductive in protection. Furthermore, it was reported that antibodies to the p57 protein were capable of opsonising the bacteria, thus enhancing phagocytosis (Bandin *et al*., 1995). This may explain the increased susceptibility to BKD observed in fish after induction of antibodies to *Rs* (Kaattari and Piganelli, 1997).

Alcorn *et al*. (2005) have compared efficacies of several vaccines using cohabitation challange and none of the bacterins evaluated induced protective immunity against Rs, while Griffiths *et al*. (1998) and Daly *et al*. (2001) reported some protection against Rs using a live Rs TSAI.

Specifically-induced antibodies are not always related to protection. Temprano et al. (2005) showed that circulating antibodies against *Yersinia ruckeri* were not protective; suggesting that protection against Enteric Red Mouth may be because of cell-mediated immunity rather than antibodies. Also, a live (delta aroA) *Aeromonas salmonicida* vaccine for furunculosis was found to preferentially stimulate T cell responses relative to B cell responses in rainbow trout (Marsden *et al*., 1996). Itano *et al*. (2006) showed protection in yellowtail (*Seriola quinqueradiata*) using several live *Nocardia* species (*i.e. Nocardia seriolae, Nocardia soli, Nocardia fluminea* and *Nocardia uniformis)*. Immunisation with live bacteria may produce bacterial proteins that function as virulence factors and are induced within macrophages (Marsden *et al*., 1998; Gudding *et al*., 1999; Griffiths *et al*., 1998) and which can strongly stimulate T cell responses (Marsden *et al*., 1996). Therefore, further research is required in order to understand and identify the protective antigens in virulent and avirulent isolates of *Rs* and their role in the immune response against the bacterium, especially for the cellular response.

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REFERENCES

BANDIN, I., RIVAS, C., SANTOS, Y., SECOMBES, C.F., BARJA, J.L. AND ELLIS, A. E., 1995. Effect of serum factors on the survival of *Renibacterium salmoninarum* within rainbow trout macrophage. *Dis. Aquat Organ.*, **23**: 221-227.

BRUNO, D.W., 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiol. Lett.*, **51**: 135-140.

BRUNO, D.W., 1990. Presence of a saline extractable protein associated with virulent strains of the fish pathogen, *Renibacterium salmoninarum*. *Bull. Eur. Assoc. Fish Pathol.*, **10**: 8-10.

DALY, J.G., GRIFFITHS, S.G., KEW, A.K., MOORE, A.R. AND OLIVIER, G., 2001. Characterization of attenuated *Renibacterium salmoninarum* strains and their use as live vaccines. *Dis. Aquat. Anim. Hlth.*, **44**: 121-126.

EVELYN, T.P.T., 1971. The agglutinin response in sockeye salmon vaccinated intraperitoneally with a heat-killed preparation of the bacterium responsible for salmonid kidney disease. *J. Wildl. Dis.*, **7**: 328-335.

EVENDEN, A.J., GRAYSON, T.H., GILPIN, M.L. AND MUNN, C.B., 1993. *Renibacterium salmoninarum* and bacterial kidney disease–the unfinished jigsaw. *Annu. Rev. Fish Dis.*, **3**: 87-104.

FARIAS, C.R.S., 1995. *Immmunopathological studies on* Renibacterium salmoninarum. Ph.D. thesis, University of Stirling, Scotland, UK.

GRIFFITHS, S.G., MELVILLE, K.J. AND SALONIUS, K., 1998. Reduction of *Renibacterium salmoninarum* culture activity in Atlantic salmon following vaccination with avirulent strains. *Fish Shellfish Immunol.*, **8**: 607-619.

GUDDING, R., LILLEHAUG, A. AND EVENSEN, Q., 1999. Recent development in fish vaccinology. *Vet. Immunol. Immunopathol.*, **72**: 203-212.

HARLOW, E. AND LANE, D., 1988. *Antibodies, a laboratory manual.* Cold Spring Harbour Laboratory, New York, USA.

IGARASHI, A. AND IIDA, T., 2002. A vaccination trial using live cells of *Edwardsiella tarda* in tilapia. *Fish Path.*, **37**: 145-148.

ITANO, T., KAWAKAM, H., KONO T. AND SAKAI, M., 2006. Live vaccine trials against nocardiosis in yellowtail *Seriola quinqueradiata*. *Aquaculture*, **261**:

1175-1180.

JANSSON, E. AND LJUNGBERG, O., 1998. Detection of humoral antibodies to *Renibacterium salmoninarum* in rainbow trout, *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar* challenged by immersion and in naturally infected population. *Dis. Aquat. Organ.*, **33**: 93-99.

KAATTARI, S.L. AND PIGANELLI, J.D., 1997. Immunization with bacterial antigens: Bacterial kidney disease. In*: Fish vaccinology* (eds. R. Gudding, A. Lillehaug, P.J. Midtlyng and F. Brown). Dev Biol Stand, Karger, Basel, pp. 145-152.

MARSDEN, M.J., VAUGHAN, L.M., FITZPATRICK, R.M., FOSTER, T.J., AND SECOMBES, C.J., 1998. Potency testing of a live genetically attenuated vaccine for salmonids. *Vaccine*, **16**: 1084-1094

MARSDEN, M.J., VAUGHAN, L.M., FOSTER, T.J. AND SECOMBES, C.J., 1996. A live (Delta aroA) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). *Infect. Immu.*, **64**: 3863-3869.

OLIVIER, G., GRIFFITHS, S.G., FILDES, J. AND LYNCH, W.H., 1992. The use of western blot and electroimmunotransfer blot assays to monitor bacterial kidney disease in experimentally challenged Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, **15**: 229-241.

PIGANELLI, J.D., WIENS, G.D., ZHANG, J.A., CHRISTENSEN, J.M. AND KAATTARI, S. L., 1999. Evaluation of a whole cell, p57- vaccine against *Renibacterium salmoninarum*. *Dis. Aquat. Organ.*, **36**: 37-44.

RHODES, L.D., RATHBONE, C.K., CORBETT, S.C., HARRELL, L.W. AND STORM, M.S., 2004. Efficacy of cellular vaccines and genetic adjuvants against bacterial kidney disease in Chinook salmon (*Oncorhynchus tshawytscha*). *Fish Shellfish Immunol.*, **16**: 461-474.

SALONIUS, K., SIDERAKIS, C., MACKINNON, A.M. AND GRIFFITHS, S.G., 2005. Use of *Arthrobacter davidanieli* as a live vaccine against *Renibacterium salmoninarum* and *Piscirickettsia salmonis* in salmonids. *Dev. Biol. Stand.*, **121**: 189-197.

SENSON, P.R. AND STEVENSON, R.M.W., 1999. Production of the 57kDa major surface antigen by a non-agglutinating strain of the fish pathogen *Renibacterium salmoninarum*. *Dis. Aquat. Organ.*, **38**: 23-31.

TEMPRANO, A., RIANO, J., YUGUEROS, J., GONZALEZ, P., CASTRO, L., VILLENA, A., LUENGO, J.M. AND NAHARRO, G., 2005. Potential use of a *Yersinia ruckeri* 01 auxotrophic aroA mutant as a live attenuated vaccine. *J. Fish Dis.*, **28**: 419-427.

THUVANDER, A., FASSUM, C. AND LORENZEN, N., 1990. Monoclonal antibodies to salmonid immunoglobulin, characterization and applicability in immunoassays. *Dev. Comp. Immunol.*, **14**: 415–423.

TURGUT, E., 2002. *Characterisation and detection of* Renibacterium salmoninarum *cultured* in-vivo *and* in-vitro. Ph.D. thesis. University of Stirling, Scotland, UK.

TURGUT, E., THOMPSON, K.D., ELLIS, A.E. AND ADAMS, A., 2008. Persistence of *Renibacterium salmoninarum* in experimentally infected rainbow trout (*Oncorhynchus mykiss*). *Bull. Eur. Assoc. Fish Pathol.*, **28**: 65-75.

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