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Summary — The effect of Baypamun, a non-specific immune system stimulant, on the treatment and control of furunculosis was studied in experimentally infected rainbow trout. A statistically significant association between the Baypamun treatment and a reduction in cumulative incidence of the clinical symptoms of Furunculosis and the infection level, despite the presence of the bacteria in blood and organs was observed. A reduction in the mortality rate in Baypamun-treated fish was also observed but this was not statistically significant. The results suggest that the treatment of fish diseases with non-specific immune stimulants could be, in the future, a good defense strategy leading to a reduction in disease symptoms and a greater control of the asymptomatic carriers after an outbreak.

immunization / Furunculosis / Baypamun / rainbow trout / treatment

Résumé — Contrôle de la furunculose grâce à l'utilisation d'un immunostimulant (Baypamun) chez la truite arc-en-ciel. *Dans ce travail, nous avons étudié l'effet du Baypamun, un stimulant de l'immunite non spécifique dans le contrôle de la furonculose de truites arc-en-ciel expérimentalement infectées par* Aeromonas salmonicida. *Nous avons observé une liaison statistique entre le traitement par le Baypamun, la réduction de l'incidence de la furunculose, et le niveau d'infection dans le sang et les organes. On a aussi observé une réduction du taux de mortalité des animaux traités, mais sans avoir d'association statistiquement significative dans ce dernier cas. Les résultats obtenus suggèrent que le traitement des maladies des poissons par des stimulants de l'immunité non spécifique pourrait être, dans le futur, un bon moyen pour réduire l'effet de la maladie et contrôler les porteurs asymptomatiques après la maladie.*

immunisation / furunculose / Baypamun / rainbow trout / traitement

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INTRODUCTION

Aeromonas salmonicida is the aetiological agent of Furunculosis, the disease that has the most significant economic effects in the salmonid fish farms of northeastern Spain (Ortega *et al*, 1993; Sanz *et al*, 1993). The disease was named Furunculosis because the subacute or chronic form of the disease can be recognized by the presence of lesions resembling boils in the skeletal muscle tissue (Austin and Austin, 1987). This form of the disease is more common in adult fish, and is accompanied by exophthalmus, lethargy, blood-shot fins and multiple haemorrahages at the skeletal muscle and other tissues (De Kinkelin et al, 1985; Austin and Austin, 1987). The acute form, which is the most common in growing fish, shows a general septicaemia accompanied by a darkening of the skin colour and haemorrhages at the base of the fins (Austin and Austin, 1987). Fish surviving the disease can become carriers of *A salmonicida* for a long time following the original outbreak (Jensen and Larsen, 1985).

At present, Furunculosis has been detected in salmonid and non-salmonid fish species, in both freshwater and saltwater, and causes high mortality rates (Cornik et al, 1984; Nougayrède et al, 1990; Wiklund, 1990). One of the reasons for which Furunculosis continues to be an acute problem on fish farms, is the persistent presence of asymptomatic carriers in the affected populations, both in salmonid and non-salmonid fish species (Jensen and Larsen, 1985; Markwardt and Klontz, 1989a,b).

Furunculosis has been shown to be associated with certain fish or environmental 'risk factors'. The most important associated factors are the age of the fish, as the disease causes higher mortality rates in younger fish (Barja and Estévez, 1988; Ortega et al, 1995), and the water temperature, with 15–18 °C being optimal for the disease's development (Michel and Dubois-Darnaudpeys, 1980; Ohtsuka et al, 1984; Jensen and Larsen, 1985; Ortega et al, 1995).

Currently, the disease is controlled through the use of chemotherapy, but, unfortunately, this treatment results in the persistence of asymptomatic carriers and/or strains that are resistant to antibiotics (Paterson, 1983; Inglis et al, 1992). At present, the antibiotics amoxicilin, enrofloxacin, oxolinic acid, or oxytetracyclin are used, but the results of the treatments are variable (Barja and Estévez, 1988; Barnes et al, 1991; Inglis and Richards, 1991a,b; Hoie et al, 1992; Inglis et al, 1992).

Together with these antibiotic treatments, Furunculosis is controlled using vaccines in fish combined with strict disinfection of water, pools and working materials (Austin and Austin, 1987; Sako et al, 1988).

Observations made over the past 10 years in the hatcheries of northeastern Spain have demonstrated that treatment of disease outbreaks with antibiotics reduced the number of diseased animals but did not control the infection. The agent persisted in the hatchery in a latent status capable of producing new outbreaks resulting in significant losses, as soon as a potential risk factor, environmental or management, changes (Ortega et al, 1993; Sanz et al, 1993).

Because of the low efficacy of the current treatments, it was decided to investigate the possible use of a non-specific immune response stimulant, such as Baypamun, as a 'treatment' to control Furunculosis outbreaks and to potentially reduce the number of asymptomatic carriers.

Baypamun (Bayer) is a natural non-specific immune response stimulant. It is formulated from a parapoxvirus that is isolated and cultured in bovine kidney cells (*Parapoxvirus ovis* D-1701) and then inactivated with β-propiolactone (Mayr, 1986).

The objective of this study was to evaluate the efficacy of Baypamun as a 'treatment' during the first days of Furunculosis outbreaks in experimentally infected rainbow trout. The study was carried out during the first 30 days post-infection. This time period was selected based on the knowledge that paraimmunization with Baypamun begins to increase immune activity 2–4 h after induction and lasts for 8–12 days post-treatment (studies undertaken in other species especially in swine and cattle, with very good results in the treatment of infectious bovine rhinotracheitis (IBR) and Aujezsky disease virus infections or *Pasteurella multocida*, *Escherichia coli* and *Pseudomonas aeruginosa* infections (Büttner, 1986; Mayr, 1986, 1992; Strübe et al, 1989; Egbering, 1991), but not in fish).

Epidemiological concepts such as mortality rate, disease symptoms, cumulative incidence, infection status or epidemiological study were used in this report (Toma et al, 1991).

MATERIALS AND METHODS

Design

The research was designed following the model of Rothman (1987) and Thrusfield (1990) and used three different groups of fish: *Group a*: *A salmonicida*-infected fish treated with Baypamun (treatment procedure is explained below); *Group b: A salmonicida*-infected fish treated with sterile saline solution (SSS) at pH 7–7.2; and *Group c:* SSS inoculated (inoculation equivalent to *Group a*) fish treated with SSS. Analysis was made between *Groups a* and *b*, with *Group c* being the negative control. In the study, the unit of analysis was the fish.

Sample selection

In every group 8-month-old rainbow trout were used, obtained from a fish farm in Zaragoza (Spain) where symptoms of Furunculosis had never been detected and no *A salmonicida* had been isolated in the past ten years. This ensured that the fish had not previously been immunized against this aetiological agent.

The sample size of the groups was estimated using the computer program Episcope (Frankena et al, 1990), which was used to determine the number of fish in every group. In the sample size determinations, a confidence level (∂) of 90% and a power (β) of 80% were used, as well as an expected recovery in the Baypamun-treated group of at least 50% and in SSS-treated group 20% (natural recovery rate from the disease observed in affected fish farms in northeastern Spain) (Sanz et al, 1993).

The programme determined that 22 fish should be selected for every group for a one-tailed test (proportion of expected recovery in *Group a* was higher when compared to *Group b*).

Infection and treatment procedure

Three 200 L tanks were used. The tanks had a closed and cooled water circuit with oxygen injection. The temperature was maintained at 15 ± 1 °C (this temperature was selected as being optimal for *A salmonicida* development (Barja and Estévez, 1988; Sanz et al, 1993). Each tank contained 22 rainbow trout that were adapted to the new environment for 7 days prior to being experimentally infected.

The treatment procedure is described in table I. The experimental bacterial infection was induced through intraperitoneal inoculation in the infected fish groups (*a* and *b*). The dose was 0.5 mL bacterial suspension with 3 x 10^8 cfu/mL (Markwardt and Klontz, 1989a,b). There were two bacterial inoculations at 0 and 48 h, respectively. The aetiological agent was a strain of *A salmonicida* Salmonicida isolated on a fish farm in Huesca (Spain) in 1990 (Sanz et al, 1993).

Baypamun treatment was made by the intramuscular route in animals of *Group a* at a dose of 0.5 mL suspension (a dose of 2 mL of Baypamun has 10^7–10^8 $TCID_{50}$ of Paramixovirus). There were two inoculations at 72 and 120 h as recommended by the manufacturer (Bayer). Sterile saline solution pH 7–7.2 was used as a placebo control at a dose of 0.5 mL by intraperitoneal route (equivalent to infection) in *Group c* at 0 and 24 h and via intramuscular route (equivalent to treatment) in *Groups b* and *c* at 72 and 120 h.

Table I. Treatment procedure.

Inoculation time (h)	Group a	Group b	Group c
0	*A salmonicida*	*A salmonicida*	SSS
48	*A salmonicida*	*A salmonicida*	SSS
72	Baypamun	SSS	SSS
120	Baypamun	SSS	SSS

SSS: sterile saline solution inoculation

Disease study and infection status

Daily observations were taken for the presence or absence of dead fish and for clinical symptoms of Furunculosis in the fish of the three groups. Clinical signs of Furunculosis were considered to be the presence of boils in the musculature and skin, exophthalmus, lethargy, blood-shot fins and haemorrhages at the base of the fins (Austin and Austin, 1987). Blood samples were taken daily from two fish in every group. These samples were cultured on *A salmonicida* isolation media (see below) (Austin and Austin, 1987; Barja and Estévez, 1988).

An autopsy was carried out on the dead fish and tissue samples were cultured on isolation culture media (see below). Samples were taken from the liver, spleen, kidney, gonads, affected musculature, skin and gills (Austin and Austin, 1987; Barja and Estévez, 1988).

Fish were sacrificed daily from each group if necessary to maintain the same number of fish in each group. Tissue samples from the dead or sacrificed fish were used to compare the infection status between groups. When there were no deaths, the tissues samples were taken from the gills and affected skin or musculature. All the tissue samples were cultured on bacterial isolation media (see below).

On the 30th day postinfection all the remaining living fish were sacrificed, using a high dose of the anaesthetic 2-phenoxyethanol. Tissue samples were again taken for bacterial isolation on culture media (see below).

Isolation of the bacteria

A sa.monicida was isolated on microbiological culture media tryptic soy agar (TSA) and brain-heart infusion agar (BHIA). The media were cultured at 22 °C for 48–72 h. Isolated bacteria were identified using microscopy, biochemical and staining procedures (Austin and Austin, 1987; Barja and Estévez, 1988).

Statistical and epidemiological analysis

The statistical analysis was carried out using the computer programs EPI-INFO (WHO) and Episcope (Frankena et al, 1990). We used the likelihood ratio test on which the difference in deviance between groups was compared against a chi-square distribution. The level of the difference between groups was determined by relative risk (RR) and confidence interval (CI) of the RR (Martin et al, 1987; Thrusfield, 1990), using the computer program Episcope (Frankena et al, 1990). When RR = 1, the treatment had no effect on disease control, when RR < 1 the treatment reduced the disease effect and when RR > 1 the treatment increased the disease effect.

A treatment effect was only accepted when CI did not include the value 1. The *P* value for chi-square distribution means the probability that the observed effect was a consequence of the treatment (Martin et al, 1987; Thrusfield, 1990).

RESULTS

In the control group *(Group c)* no disease symptoms were observed and no death occurred that could be attributed to Furunculosis.

The results of the mortality rate, cumulative incidence of Furunculosis, disease symptoms and the isolation of *A salmonicida* from the blood and organs in the *Groups a* and *b* are shown in table II.

The CI for the RR showed a statistical association between the treatment with Baypamun, and reduction in Furunculosis cumulative incidence, reduction in *A salmonicida* isolation from organs and blood. When the value 1 was included in the CI (table II) no statistical association could be shown between the Baypamun treatment and mortality rate. The study of the mortality rates during the first 30 days postinfection revealed differences between both groups (18%), but the difference was not statistically significant.

Baypamun treatment was statistically associated with reduction (31%) of Furunculosis cumulative incidence. The observed RR, 0.61, indicates that for every case of Furunculosis in the SSS-treated group, 0.61 cases of furunculosis could be found in the Baypamun-treated group.

A statistically significant relationship was also observed between the Baypamun treatment and the bacterial isolation from the fish organs and blood. In both cases, a significant reduction in the number of bacteria isolations was observed in the Baypamun-treated group of 32% cases in organs and 26% cases in blood. The detected RR, 0.22 for bacterial isolation from organs and 0.19 for bacterial isolation from blood, indicate that per case of *A salmonicida* isolation in the SSS-treated group (organs or blood), *A salmonicida* could be isolated from organs in 0.22 cases and from blood in 0.19 cases for the Baypamun-treated group.

DISCUSSION

The absence of death and disease symptoms in the control group confirmed that the disease occurrence in infected groups was due to *A salmonicida* activity. Both results together with the reduction of the disease and bacterial isolation in Baypamun-treated group confirmed that this reduction was due to Baypamun activity.

Table II. Relative risk (RR), confidence interval of the relative risk (CI), and probability of a real association *(P)* for several variables following injection of either Baypamun (a non-specific immuno-stimulant; *Group a*) or saline *(Group b)* in trout experimentally infected with *A salmonicida*.

Parameter	*Number of positive cases (%)*		*RR*	*CI*	P
	Baypamun (n=22)	*SSS (n=22)*			
Mortality	9 (41)	13 (59)	0.69	0.38; 1.28 [a]	NS
Furunculosis incidence	11 (50)	18 (81)	0.61	0.39; 0.97	< 0.05
Bacterial isolation in organs	2 (9)	9 (41)	0.22	0.05; 0.91	< 0.05
Bacterial isolation in blood	1 (6)	7 (32)	0.19	0.07; 0.52	< 0.001

SSS: sterile saline solution-treated; [a] The CI includes the value 1 which means that no statistical association exists.

A high dose of bacteria was used for the infection (not 'challenge' because it occurred before the treatment) as well as a non-natural route, because it was important for each fish to develop infection and for the bacteria to reach the organs of all the fish. This high dose permitted us to focus our attention on the bacterial isolation from the organs and blood rather than merely considering incidence of the disease and mortality rates, which only are asociated with the peracute and acute forms.

The difference in mortality rate between the treated and non-treated groups was not statistically significant because the number of dead fish in both groups was low and also when death was observed in one group, the same number of fish was killed in the other group. Both situations hindered the detection of the real association between the mortality rate and Baypamun treatment effect. The statistically significant reduction in the occurrence of disease symptoms might indicate that Baypamun could also be associated with a significant mortality rate reduction.

No comparison can be made between the results presented here and other authors' results, because such studies have not previously been performed in fish. Nevertheless, studies in other species such as swine and bovines have demonstrated that Baypamun is an effective treatment for animals during disease outbreaks (bacterial and viral diseases). An incidence of symptom reduction has been observed in Baypamun-treated animals 20–60% in cattle infected with IBR virus and 60–80% in swine infected with Aujezsky disease virus depending on the route and dose of Baypamun). The efficacy of Baypamun has also been observed in swine and bovines infected with *Pasteurella multocida*, *Escherichia coli* and *Pseudomonas aeruginosa* (Büttner, 1986; Mayr, 1986, 1992; Strübe et al, 1989; Egbering, 1991).

It seemed from these previous studies, therefore, that the Baypamun treatment could induce an interesting 'protection level' in Furunculosis outbreaks and may result in the control of asymptomatic carriers.

In this study, the Baypamun treatment produced a decrease in the mortality rate, in the cumulative incidence of clinical symptoms of Furunculosis, and in the presence of *A salmonicida* in the organs and blood (negative isolation of the bacteria in culture media, which suggested that the aetiological agent of the disesase was probably not in the organs or blood). Studies undertaken in other species, also showed a reduction of 3–5 days in the elimination of different aetiological agents in swine (Strübe et al, 1989; Egbering, 1991). This result suggested that Baypamun treatment could play an important role in reducing the infection level. This in turn implies a reduction in the number of asymptomatic carriers of the disease which is the most serious problem in the control of Furunculosis in infected fish farms.

Various studies using synthetic non-specific immune stimulants, mainly with peptide Fk-565 or β-1,3-glucan, have been developed in rainbow trout and chinook salmon *(Oncorhynchus tshawytscha)*, both as a prophylactic method, or as a mechanism for increasing the vaccine potency in both cases against *A salmonicida* and also as a treatment procedure during disease outbreaks (Nikl et al, 1993). In the first case, a 60% reduction in the mortality rate was observed. This was associated with an increase in the phagocytic cell response (Kitao and Yoshida, 1986). In the second case, similar mortality rates were observed in the studied groups, vaccinated and vaccinated plus association with a non-specific immune stimulant. Similar to our results, the bacteria isolation in organs and blood of the latter group (vaccinated and associated with a non-specific immune stimulant) happened with a lower frequency than that of first group (vaccinated alone) (Nikl et al, 1993). In the case of the use of non-specific immune stimulants associated with vaccination, it is necessary to consider that they have a non-specific cellular response but also a specific response to *A salmonicida* (antibody production).

Our results suggested that the treatment of fish diseases with non-specific immune response stimulants, 'paraimmunization', could be, in the future, an interesting method for the control, not only at a preventive level but also as a treatment of fish diseases such as Furunculosis. The objective of their use would be to restrict the latent status of the agent after the outbreak of the disease.

Non-specific immune stimulants play important roles in disease control at different levels. They may be used as a defence against disease outbreaks during stressful situations (changes in management or environment), or they may be associated with vaccine immunization systems (because the activity begins 2 or 3 weeks post-induction of the vaccine, while paraimmunization begins to be active 3 or 4 h postinduction of the product and persists for 8–12 days). Finally, non-specific immune stimulants could play an important role in disease treatment, at the same level as antibiotics but without the problem of resistance to the antibiotics (the most important problem with this type of treatment). They reduce the incidence of clinical symptoms and also the presence of the disease agent in the organs and blood (this implies a potential reduction in the latent status of the pathogen after disease outbreak).

This study gathered preliminary information about the potential role of paraimmunization in the treatment of fish diseases and in reducing the number of asymptomatic carriers after a disease outbreak. The results suggest that more studies are needed to obtain more information regarding the efficacy of this type of treatment over longer time periods and using different activity mecanisms.

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