Vol. 23: 51–57, 1995 | DISEASES OF AQUATIC ORGANISMS Dis aquat Org | Published September 14

In vitro killing of *Pasteurella piscicida* by fish macrophages

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ABSTRACT: The capacity of *Pasteurella piscicida* strains to survive contact with macrophages obtained from rainbow trout *Oncorhynchus mykiss*, sea bass *Dicentrarchus labrax* and gilthead sea bream *Sparus aurata* was evaluated using an *in vitro* assay. Both virulent and avirulent isolates were killed by all the macrophages tested after 3 and 5 h incubation. The increased production of superoxide anion (O_2^-) by rainbow trout macrophages infected with *P. piscicida* coinciding with the highest bactericidal activity (5 h incubation) suggests that the O_2^- could be involved in the killing of *P. piscicida*. Bactericidal activity of cell-free generated O_2^- confirms this hypothesis, although this indicates that some other microbicidal factors must also contribute to the killing of *P. piscicida* by fish macrophages.

KEY WORDS: *Pasteurella piscicida* Rainbow trout Sea bass Gilthead sea bream Macrophages Killing

INTRODUCTION

Pasteurella piscicida, an halophilic fish pathogenic bacteria, is the causative agent of pasteurellosis. The disease causes important economical losses in many different species of wild and farmed fish in Japan, USA and Europe (Hawke et al. 1987, Toranzo et al. 1991, Kusuda & Salati 1993).

Gross external pathological signs of pasteurellosis are usually inconspicuous without surface lesions being present; only some affected fish may exhibit a darkening of body colour and/or slight haemorrhagic areas in head and gills (Tung et al. 1985, Toranzo et al. 1991). Internally, white tubercles of about 0.5 to 3.5 mm diameter have been observed in spleen, kidney, and to a lesser extent in the liver (Kubota et al. 1970, Tung et al. 1985, Hawke et al. 1987, Toranzo et al. 1991). Histologically these internal organs exhibit multifocal necrosis and bacterial accumulations, free and within phagocytes, in the capillaries and the interstitial spaces (Kubota et al. 1970, Nelson et al. 1981, Tung et al. 1985, Hawke et al. 1987, Toranzo et al. 1991, Noya et al. 1995).

The pathogenesis of *Pasteurella piscicida* infections is at present poorly understood. Recently, it has been reported that the extracellular products (ECP) could be involved in the ability of this microorganism to invade and proliferate in the host (Magariños et al. 1992). On the other hand, contradictory reports exist on the intracellular location of *P. piscicida* and its role in the disease process. Early histopathological studies of adult yellowtail *Seriola quinqueradiata* affected by pasteurellosis suggest that *P. piscicida* can survive within fish macrophages (Kubota et al. 1970, Nelson et al. 1981). In contrast, Noya et al. (1995) using gilthead sea bream *Sparus aurata* of different sizes (0.5 g and 20 to 30 g body weight) experimentally infected with a highly virulent *P. piscicida* strain concluded that, in the largest fish, both macrophage and granulocytes may be involved in phagocytosis and killing of *P. piscicida*.

In the present study, the capacity of 2 *Pasteurella piscicida* strains with different degrees of virulence to survive contact with kidney macrophages of rainbow trout *Oncorhynchus mykiss*, sea bass *Dicentrarchus labrax* and gilthead sea bream has been evaluated *in vitro*. The experiments were performed using a colorimetric assay based upon the reduction of a tetrazolium dye, MTT [3(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide], that is reduced in direct

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proportion to the number of viable bacteria present. This indirect method is fast and reproducible and has been proved to give numbers of surviving bacteria similar to those yielded by the conventional colony counting technique (Graham et al. 1988).

MATERIALS AND METHODS

Macrophage monolayers. Monolayers of fish macrophages were prepared as described by Secombes (1990) with slight modifications. Briefly, the head kidney of rainbow trout, sea bass and gilthead sea bream (300 to 500 g body weight) was removed and pushed through a nylon mesh with L-15 medium (Gibco) containing 2% foetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (P/S), and 0.2% heparin (10 U ml^{-1}) (Sigma). The cell suspension was layered onto a 34–51% Percoll density gradient and, following centrifugation at 400 × *g* for 30 min, the band of cells lying above the 34–51% interface was collected and washed with L-15, containing 0.1% FBS. The cell suspension was adjusted to 2×10^7 cells ml^{-1} with L-15 medium plus 0.1% FBS and 1% P/S and 100 µl was added per well to 96-well microtitre plates. After 3 to 5 h at 15°C, non-adherent cells were washed off and the remaining monolayers were fed with L-15 containing 5% FBS and 1% P/S and maintained at 15°C for 1 to 3 d before use.

Bacterial strains. Two strains of *Pasteurella piscicida* with different geographic origin, source of isolation and degrees of virulence were used in this study. Strain DI-21 was isolated from gilthead sea bream in Spain and strain EPOY-8803-II from red grouper *Epinephelus akaara* in Japan. Both isolates had previously been tested for pathogenicity in trout and gilthead sea bream; DI-21 was established as pathogenic (LD_{50} values ranging between 10^3 and 10^5) and EPOY-8803-II as non-pathogenic ($LD_{50} > 2 \times 10^7$) for both fish species (Toranzo et al. 1991, Magariños et al. 1992).

Both strains were cultured on brain heart infusion agar or broth (BHI, Difco) with 2% (w/v) NaCl added (BHI-2) during 48 h at 20°C. A starting concentration of bacteria was adjusted to 5×10^8 bacteria ml^{-1} and then serially diluted in phosphate buffered saline (PBS, pH 7.4) for use in bactericidal assays.

Bactericidal assay. The assay used was basically that described by Graham et al. (1988). Macrophage monolayers were washed twice with L-15 medium to remove all traces of medium containing antibiotics and then supplemented with 100 µl of L-15 medium containing 5% FBS. Wells were inoculated with 20 µl of each of six 4-fold bacterial dilutions (5×10^8 ml^{-1} to 9.8×10^3 ml^{-1}) in PBS. The plate was then centrifuged for 5 min at 150 × *g* to bring the bacteria into contact with the macrophages and incubated at 20°C for 0, 3 and 5 h. At the end of each incubation period, the supernatants were removed and the killing stopped by lysing the macrophages with 50 µl of cold sterile distilled water. The water was removed and replaced with 100 µl of BHI-2 added to support an overnight growth of the surviving bacteria. After 18 h at 20°C, 10 µl of MTT (5 mg ml^{-1} distilled water) was added per well, shaking the plate and reading the optical density at 600 nm 15 min later on a multiscan spectrophotometer (Flow). Mean of the values of quadruplicate wells were calculated. The difference in the optical density readings at any one bacterial concentration between times T_0 and T_x incubation represented the degree of killing. The data were adjusted to give killing percentage by:

$$\frac{T_0\text{MTT reduction} - T_x\text{MTT reduction}}{T_0\text{MTT reduction}} \times 100$$

where the T_0 value represents 100% bacterial growth. Results are means of 3 separate experiments.

The accuracy of MTT assay to detect killing activity of rainbow trout macrophages was compared with that of conventional colony counting on suitable media. After killing was stopped, the contents of quadruplicate wells were diluted in BHI broth and spread over agar plates. The enumeration of the colony-forming units (CFU) was performed after 72 h incubation at 20°C.

For each experiment 3 extra wells of monolayers were prepared for total cell counts. The medium was removed and 100 µl of lysis buffer (0.1 M citric acid, 1% Tween 20 and 0.05% crystal violet) was added. After 2 min, the nuclei were counted in a hematocytometer.

Superoxide anion (O_2^-) detection assay. The generation of extracellular and intracellular superoxide anion by rainbow trout macrophages was determined by the reduction of cytochrome C (Sigma) and nitroblue tetrazolium (NBT), respectively. The assays were performed essentially as described by Secombes (1990). Macrophage monolayers were infected with *Pasteurella piscicida* for 5 or 3 h and non-infected macrophages were used as control. Both assays were repeated at least 3 times.

Extracellular O_2^-. Cell monolayers were covered with 100 µl of 160 µM cytochrome C dissolved in phenol red-free Hank's balanced salt solution (HBSS) with phorbol myristate acetate (PMA, Sigma) at 1 µg ml^{-1}. Superoxide dismutase (SOD, Sigma) (300 U ml^{-1}) was also added to some of the wells to confirm O_2^- was reducing the cytochrome C. The macrophages were incubated for 30, 60 and 90 min at 18 to 20°C and optical density was read at 550 nm in a multiscan spectrophotometer (Flow). Optical densities were converted to nM O_2^-, using the conversion factor described by Pick (1986), and then adjusted to 10^5 cells.

Intracellular O_2^-. Macrophages monolayers were covered with 100 µl of 1 mg ml^{-1} NBT in L-15 with PMA at 1 µg ml^{-1} for 30 min at 18 to 20°C. During this reaction, NBT is reduced by O_2^- into an insoluble blue formazan. The reduced formazan within macrophages was solubilised in KOH/DMSO after fixing and washing the cells in 100 and 70% methanol, respectively. Macrophage monolayers incubated without bacteria were used as control. The optical density was read at 620 nm.

Generation of superoxide anion. The superoxide anion was generated in a cell-free system basically following the procedure described by Karczewski et al. (1991), except for riboflavin concentrations of 0.05 to 0.4 mM and methionine (25 mM).

Bactericidal activity of superoxide anion. A 25 mM methionine solution was prepared in HBSS. A 0.4 mM riboflavin solution was prepared in the methionine solution and serially diluted from 0.4 to 0.05 mM.

Pasteurella piscicida cells were serially diluted in HBSS in 4-fold steps from 5×10^8 ml^{-1} to 4.9×10^5 ml^{-1} riboflavin , and 50 µl of the bacterial suspensions were mixed with 50 µl of the riboflavin-methinonine solutions in a 96-well microtiter plate. The plates were exposed to a blue light source (Kodak No. 47 blue filter) for 5 min to initiate the superoxide anion production and subsequently incubated for 5 h. After incubation the supernatant was carefully removed and fresh media added to allow bacterial growth. The number of viable bacteria was determined as for the macrophage bactericidal assay.

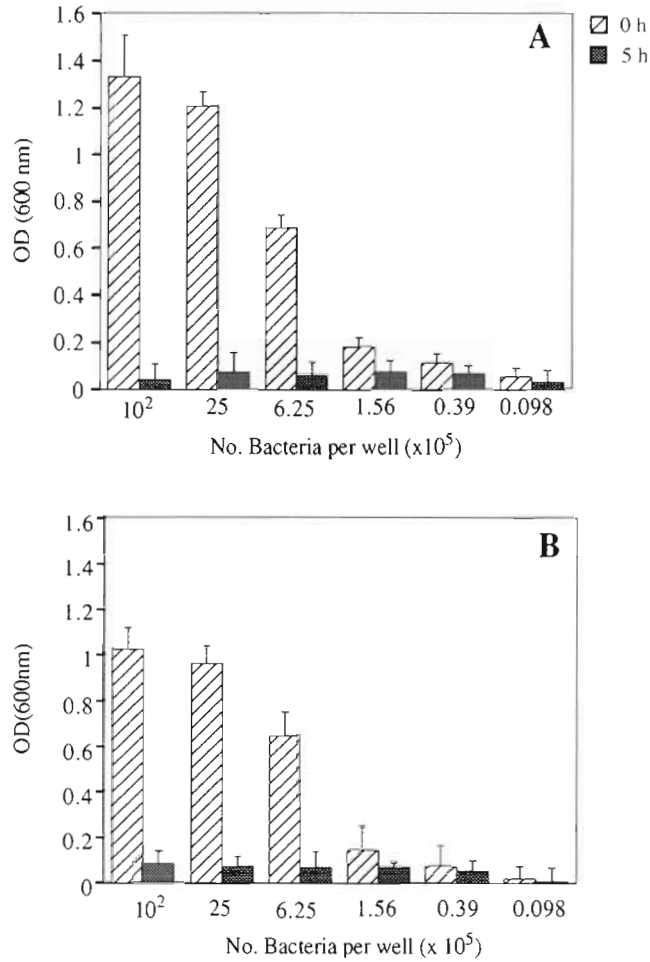

Fig. 1 Bactericidal activity (+ SD) of rainbow trout macrophages against *Pasteurella piscicida* strain DI-21 (A) and strain EPOY-8803-II (B) after 0 or 5 h incubation with macrophages. Results are mean of 3 separate experiments

RESULTS

Bactericidal assay

Prior to bactericidal assays the optimal length of time to allow the bacteria to 'grow-up' before addition of MTT was determined. Bacterial incubation was thus standardized to 18 h at 20°C.

The incubation of bacteria with the macrophages for 3 and 5 h (T_x) showed clearly that both strains were killed by all the macrophages tested in comparison with the control (T_0), although the highest rate of bactericidal activity was always observed after 5 h incubation. Figs. 1 & 2 show the killing activity of trout and sea bass macrophages.

Although the bactericidal assays performed with gilthead sea bream and sea bass macrophages resulted in a high degree of killing, the highest killing percentage was obtained using rainbow trout macrophages (Table 1).

Apparent differences were observed in the killing ability of fish macrophages against the 2 strains tested, suggesting that, in general, the virulent DI-21 strain was killed to a greater extent than the non-virulent EPOY-8803-II isolate (Table 1). However, these differences were only statistically significant ($p < 0.05$) when sea bass macrophages were used.

Considering the killing percentages (Table 1), it is clear that progressively more killing occurred with increasing concentrations of bacteria per well, regardless of the origin of the macrophages employed. The decrease in this index with lower bacterial concentrations could be due to the low MTT readings at both T_0 and T_x. (Figs. 1 & 2). This hypothesis is supported by the results obtained using the conventional colony counting method, since the killing percentage obtained with low bacterial concentrations (from 3.9×10^4 to 9.8×10^3 bacteria per well) was significantly higher ($p < 0.05$) than that obtained using MTT values (Table 2).

Table 1. Killing percentages (%) obtained after 5 h incubation of 2 strains of *Pasteurella piscicida* with fish macrophages, at different bacterial concentrations. Bactericidal assay carried out in 96-well microtiter plates

Strain	Fish species	Bacteria well^{-1}					
		1.0×10^7	2.5×10^6	6.25×10^5	1.56×10^5	3.9×10^4	9.8×10^3
DI-21	Sea bass	90.6	87.7	73.8	42.2	40.8	21.6
	Sea bream	89.2	88.9	80.9	90.6	72.7	25.0
	Trout	86.9	95.0	92.2	78.1	65.2	38.6
EPOY-8803-II	Sea bass	89.0	76.8	65.2	27.9	20.0	10.0
	Trout	92.1	92.2	89.2	51.4	26.7	25.0

Respiratory burst assay

The production of superoxide anion (O_2^-) by macrophages infected with *Pasteurella piscicida* for different periods of time (3 and 5 h) was measured by the reduction of Nitroblue tetrazolium (NBT) and cytochrome C. The macrophages were stimulated with PMA. Both bacterial strains stimulated the respiratory burst pathway. Fig. 3 shows the results obtained with the virulent strain DI-21, which indicate an increase in intracellular O_2^- production (reduction of NBT) after 3 h, compared with the non-infected macrophages, which became

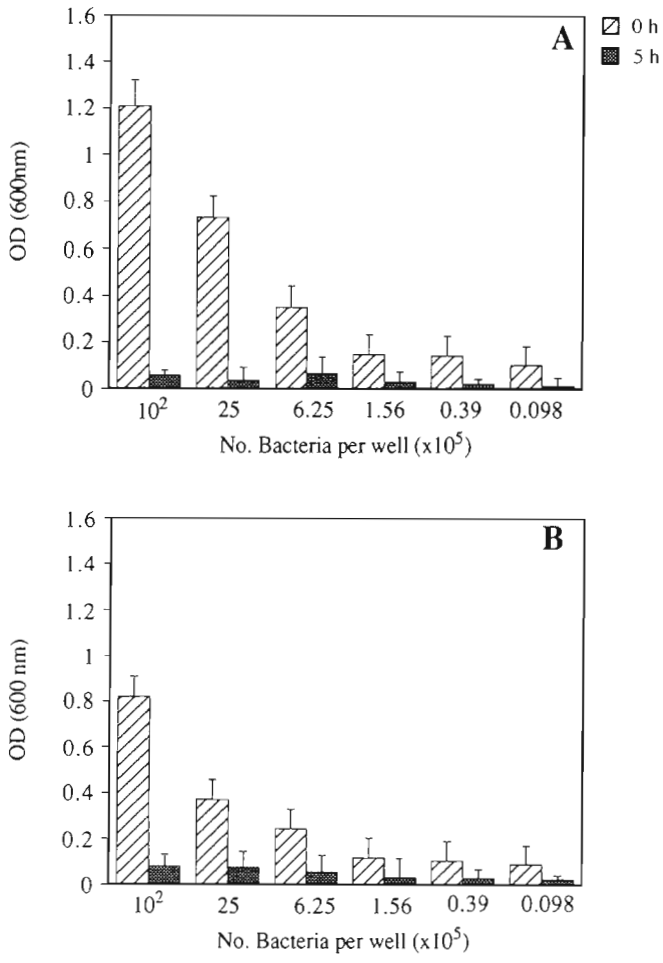

Fig. 2. Bactericidal activity (+SD) of sea bass macrophages against *Pasteurella piscicida* strain DI-21 (A) and strain EPOY-8803-II (B) after 3 or 5 h incubation with macrophages. Results are mean of 3 separate experiments

Table 2. Killing percentages obtained after incubation of *Pasteurella piscicida* DI-21 with rainbow trout macrophages for 5 h. Comparison of MTT and colony counting to detect surviving bacteria. Bactericidal assay carried out in 96-well microtiter plates. Colorimetric assay based upon the reduction of a tetrazolium dye, MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide], in direct proportion to the number of viable bacteria

Bacteria well^{-1}	MTT	Colony count
1.0×10^7	86.9	96.7
2.5×10^6	95.0	96.0
6.25×10^5	92.2	94.5
1.56×10^5	78.1	81.2
3.9×10^4	65.2	80.5
9.8×10^3	38.6	53.5

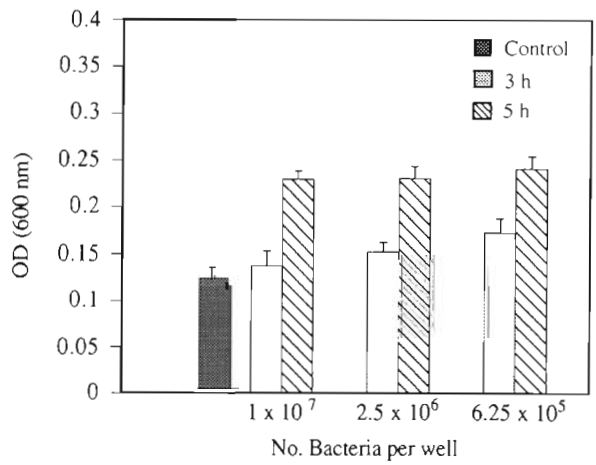

Fig. 3. Reduction (+SD) of NBT in the presence of PMA by rainbow trout macrophages infected with *Pasteurella piscicida* DI-21 for 3 or 5 h. Control: non-infected macrophages

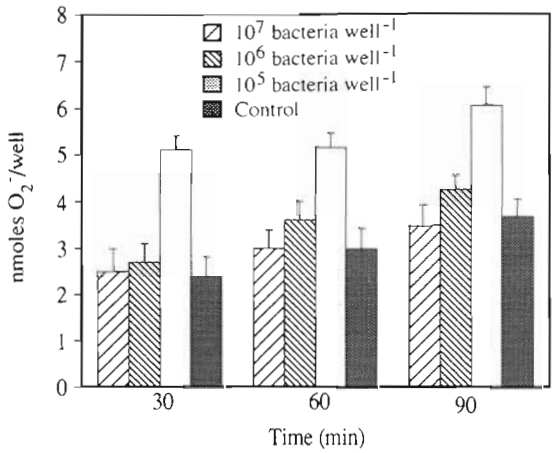

Fig. 4. Reduction (+SD) of cytochrome C in the presence of PMA by rainbow trout macrophages infected with *Pasteurella piscicida* DI-21 for 3 or 5 h. Control: non-infected macrophages

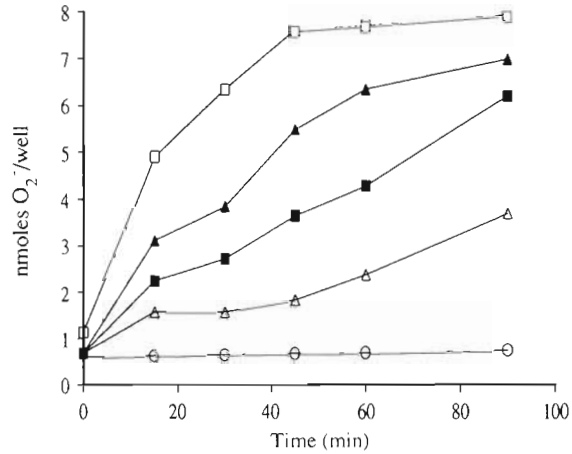

Fig. 5. Generation of superoxide anion by the photoreduction of 0.4 mM (□), 0.2 mM (▲), 0.1 mM (■) or 0.05 mM (△) riboflavin in the presence of 25 mM methionine. The effect of coincubation with superoxide dismutase is also shown for 0.05 mM riboflavin (○)

more evident at bacterial concentrations of 10^5 bacteria well^{-1}. After 5 h incubation this production clearly increased, coinciding with the highest rate of bactericidal activity, although at this incubation there were no big differences among the bacterial concentrations (Fig. 3).

It was also observed that incubation of *Pasteurella piscicida* strains with macrophages stimulated the extracellular O_2^- production, as shown by the reduction of cytochrome C compared with non-infected macrophages (Fig. 4). A progressive increase in the O_2^- production was observed at longer exposures to cytochrome C, and it was also clear that the increase was higher with bacterial concentrations of 10^5 bacteria well^{-1} (Fig. 4). The results of the assay to detect extracellular O_2^- after incubation of macrophages with *P. piscicida* strains for 3 and 5 h followed basically the same pattern and no differences were observed between virulent and avirulent strains. Because of these only the results obtained with strain DI 21 after 5 h incubation with macrophages are shown in Fig. 4 and the remaining data are omitted to avoid redundancy.

Bactericidal activity of cell-free generated superoxide anion

The generation of superoxide anion by the reduction of different concentrations of riboflavin in the presence of methionine is shown in Fig. 5. The effect of co-incubation with SOD is also shown for 0.05 mM riboflavin. Depending on the concentration of riboflavin used, between 3 and 8 mM of O_2^- were produced. The presence of SOD inhibited the reduction of cytochrome C.

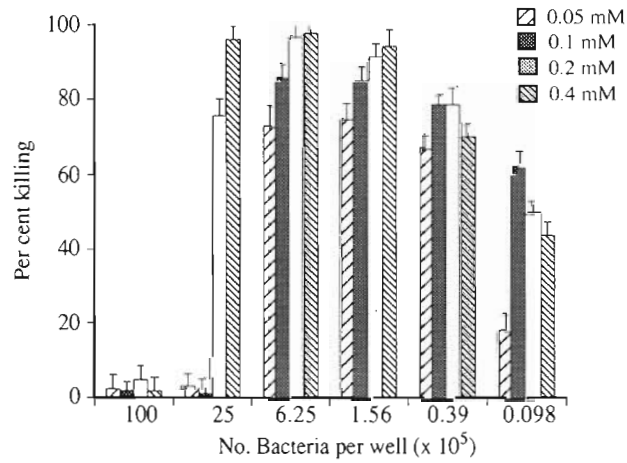

Fig. 6. Percent killing of *Pasteurella piscicida* DI-21 after exposure to superoxide anion generated with different riboflavin concentrations

A high degree of killing of the DI 21 strain was observed in the bactericidal assay (Fig. 6). However, in the presence of large numbers of bacteria (10^7 bacteria well^{-1}) the bactericidal activity was substantially reduced regardless of the riboflavin concentrations. Similar results were obtained with the avirulent strain EPOY-8803-II (data not shown).

DISCUSSION

Little is known about the mechanisms of establishment of a *Pasteurella piscicida* infection. The histopathological studies performed by Kubota et al. (1970) and Nelson et al. (1981) indicated the presence of

macrophages with engulfed intact bacteria, which led to these authors to postulate that *P. piscicida* would be able to survive within yellowtail macrophages. More recently, Noya et al. (1995) also reported the existence of unaffected *P. piscicida* within macrophages of small sea bream (0.5 g body weight), supporting the hypothesis of intracellular survival. However, the same authors indicated that macrophages from larger sea bream (20 to 30 g body weight) did kill the pathogen. It seems clear that further studies are necessary to clarify the role of the interaction between fish macrophages and *P. piscicida* in the pathogenesis of pasteurellosis.

In the present study the ability of *Pasteurella piscicida* strains to survive *in vitro* contact with macrophages obtained from rainbow trout, seabass and gilthead sea bream (300 to 500 g body weight) has been evaluated using a colorimetric assay, MTT. Our results indicated that the phagocytic cells of the 3 fish species tested were able to kill *P. piscicida* strains and maximum killing always occurred at high bacterial concentrations (from 10^5 to 10^7 bacteria well^{-1})

Comparison of the MTT assay with colony counting (Table 2) indicated that both methods gave similar killing percentages when high bacterial concentrations (10^5 to 10^7 bacteria well^{-1}) were used, but the MTT method was faster and more reproducible. From these results it can be concluded that MTT is a useful method of evaluating killing activity of fish macrophages against *Pasteurella piscicida*. However, it must be taken into account that the MTT assay is not sufficiently sensitive to detect the real extent of killing when low bacterial numbers (10^3 to 10^4) are employed.

Although generally more virulent fish pathogens are not killed as well (or not killed at all) compared to less virulent pathogens (Olivier et al. 1986, Graham et al. 1988), in our study, the virulent strain DI-21 was killed to a higher extent than the non-pathogenic isolate EPOY-8803-II by sea bass macrophages. The differences in killing of bacterial strains can be attributed to many factors, such as differential stimulation of the respiratory burst, phagocytosis and resistance to oxygen free radicals (Stave et al. 1985, Olivier et al. 1986, Graham et al. 1988).

The production of reactive oxygen species (ROS) is considered to be one of the most important bactericidal pathways in mammalian phagocytes (Babior 1988). A number of ROS have been shown to be released from fish phagocytes (Nash et al. 1987, Secombes et al. 1988, Plyzycz et al. 1989, Sharp & Secombes 1992) and have been implicated in bactericidal activity (Karcewski et al. 1991, Sharp & Secombes 1992). The increased production of O_2^- by rainbow trout macrophages infected with *Pasteurella piscicida* demonstrates that the phagocytic cells became activated by the bacterium, and suggests that the products of the respiratory burst pathway may be involved in the killing of *P. piscicida* by macrophages, since the highest level of O_2^- production (after 5 h incubation) coincided with the highest bactericidal activity.

In addition, bactericidal assays performed with cell-free generated O_2^- indicate that this agent has a potent bactericidal activity against *Pasteurella piscicida*, although large numbers of bacteria seem to be able to avoid its effect. The low killing percentages obtained with high bacterial concentrations in this assay are in contrast with the high bactericidal activity of macrophages against the same bacterial numbers. These findings suggest that other antimicrobial factors must also be implicated in the killing of *P. piscicida* by fish macrophages.

Internalisation is a necessary condition for intracellular killing of bacteria (Sharp & Secombes 1992) and the observation of engulfed intact *Pasteurella piscicida* in yellowtail and sea bream macrophages (Kubota et al. 1970, Nelson et al. 1981, Noya et al. 1995) does not necessarily demonstrate that they are alive. In fact, the results of the histological studies can be also interpreted as a preliminary step to killing.

These results demonstrate the *in vitro* killing of *Pasteurella piscicida* by macrophages of adult fish from 3 different species and the implication of superoxide anion in the killing process.

Acknowledgements. This investigation was supported by grants MAR91-1113-C02-01 and AGF94-1360-C03-01 from the Comisión Interministerial de Ciencia y Tecnología, Spain. A.M.S. thanks the Instituto de Cooperación Iberoamericana (Spain) for a research fellowship.

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Responsible Subject Editor: O. Kinne, Oldendorf/Luhe, Germany

Manuscript first received: March 20, 1995
Revised version accepted: June 1, 1995