Dynamic Biochemistry, Process Biotechnology and Molecular Biology ©2011 Global Science Books

An Attenuated Strain of *Edwardsiella ictaluri* is Killed by Channel Catfish (*Ictalurus punctatus*) Macrophages and Confers Protection in Few Days

Riccardo Russo[1,2]

[1] Aquatic Animal Health Research Unit, United States Department of Agriculture, Agriculture Research Service, Auburn, AL 36832
[2] Present address: Public Health Research Institute-University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 225 Warren Street, Newark, New Jersey 07103, USA

Corresponding author: * **ri.russo@hotmail.com**

ABSTRACT

In channel catfish protective immunity against *Edwardsiella ictaluri* is mediated by cellular immune response, but *E. ictaluri* is able to survive inside macrophages. The aim of this study was to determine if catfish macrophages were able to kill an avirulent strain of E. *ictaluri* and to observe if there was a difference in the dispersion in the catfish body among virulent and avirulent *E. ictaluri.* Channel catfish macrophages were exposed to a virulent or to an attenuated strain of *E. ictaluri* transformed with plasmids coding a green or a red fluorescent protein. Macrophage killing efficiency, reactive oxygen species and nitric oxide production were determined. Catfish were intraperitoneally (IP) injected with fluorescent virulent or attenuated *E. ictaluri* to determine the location of the bacteria within internal organs of the fish. Catfish were also IP injected or bath exposed to the attenuated strain of *E. ictaluri* 3, 2, or 1 day before, or at the same time of the challenge with the virulent strain of *E. ictaluri*. The virulent strain of *E. ictaluri* survived and replicated inside macrophages, while the attenuated strain was killed. Fluorescent virulent bacteria were observed in all organs of the fish and attenuated bacteria were observed only in lymphoid organs. Injection of the attenuated strain of *E. ictaluri* at least one day prior to challenge conferred protection (survival from 83 to 100%) in fish and upregulated the expression of the toll-like receptor 5. The results of this study suggest that the channel catfish immune system can kill and control the dispersion of the attenuated *E. ictaluri* strain within the fish. The protection conferred by the *E. ictaluri* attenuated strain highlights the possibility to use this strain as a "therapeutic" during disease outbreak.

Keywords: *Edwardsiella ictaluri*, macrophages, catfish, vaccine, immunostimulation, live vaccine

INTRODUCTION

Edwardsiella ictaluri, an Enterobacterium closely related to *Salmonella* spp., is one of the most important pathogens of channel catfish (*Ictalurus punctatus*) that causes considerable economic losses to the aquaculture industry. *E. ictaluri* is able to survive and replicate inside macrophages (Booth *et al*. 2006; Russo *et al*. 2009b), and similarly to *Salmonella* spp. (Wigley *et al.* 2001; Tierrez and García-del Portillo 2005; Prost *et al.* 2007). Macrophages have been implicated in the dispersion of *E. ictaluri* within the fish and may represent a reservoir in the body (Klesius and Sealey 1995; Thune *et al*. 1997; Russo *et al*. 2009b). In channel catfish, protective immunity against *E. ictaluri* is mediated mainly by a cellular immune response and passive immunization is not very effective (Shoemaker and Klesius 1997; Shoemaker *et al.* 1997; Thune *et al*. 1997; Elibol-Flemming *et al.* 2009; Russo *et al.* 2009b). Several studies have shown that an attenuated strain of *E. ictaluri* (RE-33) used for prophylactic immunization is efficacious in protecting catfish fingerlings, fry, and eyed-eggs following immersion vaccination (Wise *et al*. 2000; Shoemaker *et al*. 2002, 2007).

Several studies have determined the temporal expression of genes involved in the innate immune response of catfish during acute infections of *E. ictaluri* (Bilodeau and Waldbieser 2005; Bilodeau *et al.* 2006, 2008; Elibol-Flemming *et al.* 2009; Pridgeon *et al*. 2009, 2010) with the purpose of identifying and gaining a better understanding of the different components of the innate immune system that are involved in fighting the infection. Several genes have been identified, and especially the toll-like receptors 3 and 5 (TLR5) have been shown to be upregulated during the early stage of infection (Bilodeau and Waldbieser 2005; Bilodeau *et al*. 2006, 2008; Pridgeon *et al*. 2009, 2010).

The aim of this study was five-fold: 1) compare the ability of catfish macrophages to kill the attenuated strain and the virulent strain of *E. ictaluri*; 2) determine the distribution of the attenuated and virulent strain within catfish organs after intraperitoneal injection (IP) with the attenuated and virulent strains transformed with fluorescent protein; 4) demonstrate that immunization with the attenuated strain up regulated the expression of the TLR5 in catfish and 5) determine when protective immunity was stimulated following immunization of catfish with the attenuated vaccine.

MATERIALS AND METHODS

Bacterial strains culture and transformation

The characteristics of the virulent (AL-93-58) and of the attenuated strain (RE-33) of *E. ictaluri* used in this study were previously reported (Panangala *et al.* 2005). The AL-93-58 virulent strain was isolated from a channel catfish; the attenuated strain RE-33, used as a live attenuated vaccine, was derived from the EILO parent strain (Klesius and Shoemaker 1999). Both strains of *E. ictaluri* were transformed by electroporation (Russo *et al.* 2009a) with the pZsGreen or pDsRed2 vectors (Clontech, Mountain View, CA) carrying a green or red fluorescent reporter gene and an ampicillin resistance marker. Untransformed bacteria were cultured in brain heart infusion (BHI) broth or agar at 27°C; transformed bacteria were grown in BHI media containing 50 µg/ml

Received: 18 January, 2010. Accepted: 16 October, 2010.

Original Research Paper

ampicillin. Bacterial cultures were verified for purity using API 20E test strips (bioMerieux, Hazelwood, MO) and by PCR using specific primers for *E. ictaluri* (Russo *et al.* 2009b).

Macrophage isolation and killing assay

Macrophages were isolated from five catfish by peritoneal lavage (Jenkins and Klesius 1998). Briefly, catfish were injected intraperitoneally with 150 µl of squalene (Sigma, St. Louis, MO, USA) and macrophages were harvested 4-5 days after treatment by lavage with 20 ml of sterile ice-cold phosphate buffered saline solution (PBS). Harvested macrophages were centrifuged at 500 × *g* for 15 min, washed once more by centrifugation in 12 ml of ice-cold PBS, and resuspended in 5 ml of channel catfish macrophage medium (CCMM; Booth *et al.* 2006). Samples of harvested macrophages were stained with Hema-3 (Biochemical Sciences Inc., Swedesboro, NJ, USA) for total macrophage counts. Viability was assessed with trypan blue exclusion (Sigma) and observed microscopically using a hemacytometer. Macrophages were then aliquoted into 16-well fibronectin-coated, glass chamber slides at 1×10^5 cells per well and incubated for 2 h at 28°C with 5% CO_2 in a humidified chamber.

Following incubation, the macrophages were exposed to green or red fluorescent *E. ictaluri* (1) untreated or (2) opsonized with serum from vaccinated catfish, with a macrophage: bacteria ratio of 1: 20. Green fluorescent bacteria were used when macrophages were exposed only to one strain of *E. ictaluri.* Green and red fluorescent bacteria were used when macrophages were exposed to both the virulent and the attenuated bacterial strains; these experiments were repeated using red fluorescent virulent + green fluorescent attenuated bacteria and green fluorescent virulent + red fluorescent attenuated bacteria. The slides were incubated in a humidified chamber for 1 h. Each treatment was conducted in triplicate. The medium was discarded and the wells gently washed with warm PBS and replenished with fresh CCMM containing gentamicin 100 µg/ml, streptomycin 100 µg/ml and penicillin 100 U/ml. The slides were monitored at 4 h and thereafter at intervals of 24 h for a total of 96 h with an inverted fluorescent microscope (Olympus 1X 70, Center Valley, PA) equipped with a digital camera (Olympus DP11). A sample of macrophages was aspirated from each well with a sterile pipette at 4 h and thereafter at 24 h intervals up to 96 h. Aspirated macrophages were briefly treated with 2.5 g/L trypsin (Invitrogen Corp., Carlsbad, CA) and 0.2 g/L versene (Invitrogen) to break cell aggregates, placed in a hemacytometer chamber and observed with a fluorescent microscope (Olympus BX 41) equipped with a digital camera (Olympus DP 70) at a 600X magnification. The number of macrophages with or without engulfed bacteria was counted. Data were recorded at each time point based on a total count of 200 macrophages for each treatment. The experiment was repeated three times.

Reactive oxygen species production

A chemioluminescensce assay was conducted according to the procedure described previously (Leung *et al*. 1995) with modifications. A subsample of macrophages collected for the killing assay was used for these experiments. Briefly, macrophages were washed twice in ice cold PBS, resuspended in Hank's balanced salt solution without phenol red, calcium chloride and magnesium sulphate (HBSS) (Gibco, Invitrogen Corp., Carlsbad, CA, US) and seeded at 1×10^5 cells per well in a 96-well opaque Luminometer plate (Promega, Madison, WI, USA). After 30 min, (1) untreated *E. ictaluri* (2) and *E. ictaluri* opsonized with serum from vaccinated fish were added to each respective well to contain a macrophage: bacteria ratio of 1: 20 per well. Luminol [5-amino-2-3-dihydro-1, 4-phthalazinedione (Sigma)] in 0.2 M sodium borate pH 9.0 was added in a volume of 20 µl/well to each well. Controls included wells with macrophages stimulated with zymosan, HBSS alone, macrophages with luminol, and macrophages free of luminol. Plates were read with the Glomax 96 microplate luminometer (Promega). All tests were conducted in triplicate. The experiment was repeated three times.

Nitrogen oxide production

Nitric oxide production by macrophages was measured by the Griess reaction with a commercial kit (Promega) according to the protocol provided (Schmidt and Kelm 1996). A subsample of macrophages harvested for the killing assay experiment were washed and resuspended in cold HBSS. Aliquots of 100 µl of the macrophage suspension adjusted to 1×10^5 cells was transferred into 3 microcentrifuge tubes and 100 µl of *E. ictaluri* were added to each tube to provide a macrophage: bacteria ratio of 1: 20. At intervals of 0.5, 1, 2, 2.5 and 3 h following bacterial challenge, the reaction was stopped by placing the tube on ice and 50 µl of the macrophage/bacteria suspension transferred to a well of a 96-well flat-bottom plate. Each test was performed in triplicate and the complete experiment was repeated twice. Sulfanilamide in a volume of 50 µl was added to each test well and to wells containing the nitrite reference standard provided in the test kit and run in parallel. The test plates were incubated at room temperature for 10 min in the dark. The Griess reagent [N-1-napthylethylenediamine dihydrochloride (NED)] equilibrated to room temperature was added in a volume of 50 µl to each well and the plates incubated for 10 min in the dark. The colorimetric-reactions were measured with a BioRad (BioRad, Hercules, CA, USA) microplate reader at an absorbance of 540 nm. The average absorbance per test sample for each time interval was determined and compared with the nitrite standard reference curve generated for each assay.

Localization of *E. ictaluri* in fish

Two groups of nine catfish were injected intraperitoneally (IP) with ~ 5×10^8 CFU of either green-fluorescent *E. ictaluri* AL-93-58 or RE-33. Three fish from each treatment were picked after 12, 24 and 48 h and killed by immersion in 300 mg/L of tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA, USA). Samples were taken from gills, skin, fins, muscle, head and trunk kidney, spleen, liver, stomach, intestine, heart and brain. Thin sections (approximately 1-2 mm) of fresh tissues were mounted on a microscope slide and examined by fluorescence microscopy. Bacteria were isolated from spleen, head and trunk kidney and grown on BHI agar with ampicillin and identified by API 20E test strips or by PCR using specific primers for *E. ictaluri* (Russo *et al*. 2009b). The experiment was repeated twice.

Expression of the Toll like receptor 5

Three catfish were injected IP with ~ 5×10^8 CFU of the virulent (AL-93-58) or of the attenuated strain (RE-33) of *E. ictaluri*, or with PBS. After 6 hrs, fish were killed by immersion in 300 mg/L of MS-222, head kidney extracted within few minutes and stored at -80°C. Total RNA and cDNA synthesis were performed as previously described (Pridgeon *et al*. 2009, 2010). The cDNA was used for a quantitative PCR (qPCR) using the SYBR®Green qPCR System kit (Invitrogen) using specific forward and reverse primers as previously described (Pridgeon *et al*. 2009, 2010). Channel catfish 18S ribosomal RNA were used for each head kidney sample as an internal control and for the normalization of the cDNA amount of primers (Jørgensen *et al*. 2008; Pridgeon *et al*. 2009, 2010).

Fish and challenge protocol

Catfish were reared in 38 L flow-through tanks with constant aeration and a water temperature of 26 ± 2°C. Tanks were stocked with 15 fish. The average weight and total length of the fish were 72.8 ± 17 g and 17.6 ± 0.5 cm, respectively. Fish were acclimated for two weeks, then injected IP with ~ 5×10^7 CFU of the attenuated strain (RE-33) of *E. ictaluri* or immersed in water with ~ 1×10^7 CFU/ml for 30 minutes 3, 2, or 1 day before, or at the same time of the IP challenge (~ 5×10^8 CFU) with the virulent strain (AL-93-58) of *E. ictaluri*. A negative control included fish not exposed to the attenuated strain of *E. ictaluri* and challenged with the virulent strain. Fish mortality was recorded for 14 days and bacteria were isolated from all dead fish examined (approximately 70% of dead fish). Three tanks were used for each treatment and the experiment was repeated three times.

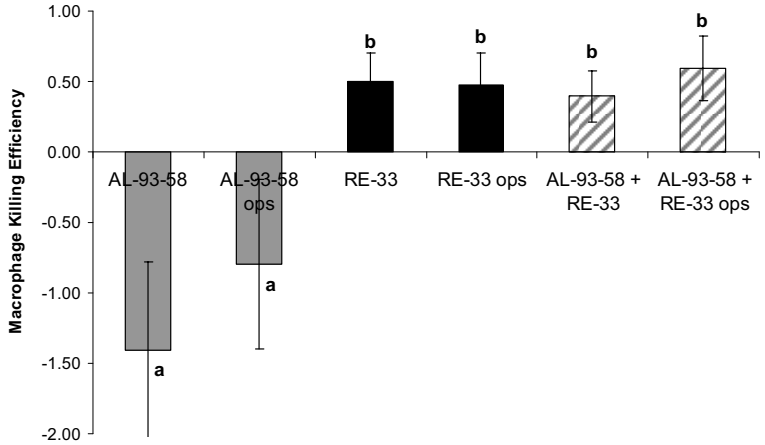

Fig. 1 Efficiency (mean ± Standard Deviation, S.D.) of macrophages in killing the virulent (AL-93-58) or attenuated (RE-33) strains of *E. ictaluri*. Macrophages were exposed to one or both bacterial strains. Untreated or bacteria coated with serum from vaccinated fish (ops), were used in different treatments. The macrophage killing efficiency, expressed as Relative Percent Killing (RPK), was calculated comparing the number of macrophages with engulfed bacteria at 1 and 48 hours after exposure to *E. ictaluri.* Superscripts above the bars indicate significant difference at $P < 0.05$ between treatments based on Tukey's post-*hoc* test.

Statistical analysis

A one-way ANOVA and Tukey's post-*hoc* test, run with the statistical program SPSS-12.0 (SPSS Inc., Chicago, IL) were used for analysis of data obtained from the phagocytosis assays and for the ROS and nitric oxide production. An arcsin square root transformation was performed on data expressed as percentages or as ratios. $P \leq 0.05$ was considered statistically significant. Because the percentage of macrophages with bacteria was different among treatments at the beginning of the experiments, the relative percent killing (RPK) was calculated to compare the efficiency of bacterial killing by macrophages among treatments at the end of each phagocytic assay using the following formula:

$$RPK = \frac{MbBe\ (\%) - MbEe\ (\%)}{MbBe\ (\%)}$$

where MbBe = the percent of macrophages with bacteria at beginning of experiment, MbEe = the percent of macrophages with bacteria at end of experiment. An ANOVA and Tukey's test was run on the calculated RPK for replicate treatments and a negative RPK was assigned a value of 0.0001 for the purpose of statistical analysis.

The following formula was used to determine the relative transcriptional level of the TLR5 (Pridgeon *et al*. 2008):

$$\Delta C_t = C_t\ (sample) - C_t\ (calibrator)$$

where C_t is the cycle threshold of the relative transcriptional level of the TLR 5, or of the 18S rRNA (calibrator). The following formula was used to determine the relative expression level of the TLR5 in infected fish compared to the negative control (fish injected with PBS):

$2^{\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (*E. ictaluri* injected fish) - ΔC_t (PBS injected fish)

A one-way ANOVA was run with the statistical program SPSS-12.0 to analyze the relative expression level data.

RESULTS

Macrophage isolation and killing assay

Macrophages were not able to kill internalized virulent *E. ictaluri* (AL-93-58) (**Fig. 1**). After 36 h from the beginning of the experiment, free *E. ictaluri* were observed in proximity of lysed macrophages, and after 48 h the percentage of macrophages with internalized bacteria was higher than at 1 h. Macrophages were able to kill the attenuated strain of *E. ictaluri* (RE-33) or the virulent and attenuated strains when macrophages were exposed simultaneously to both strains. Opsonization of bacteria with immune serum did not significantly ($P > 0.05$) influence macrophage killing efficiency.

Reactive oxygen species and nitrogen oxide production

Opsonization of *E. ictaluri* with serum from vaccinated fish significantly stimulated higher production of ROS and of nitric oxide compared to untreated bacteria (**Figs. 2, 3**). There was no significant difference in ROS production

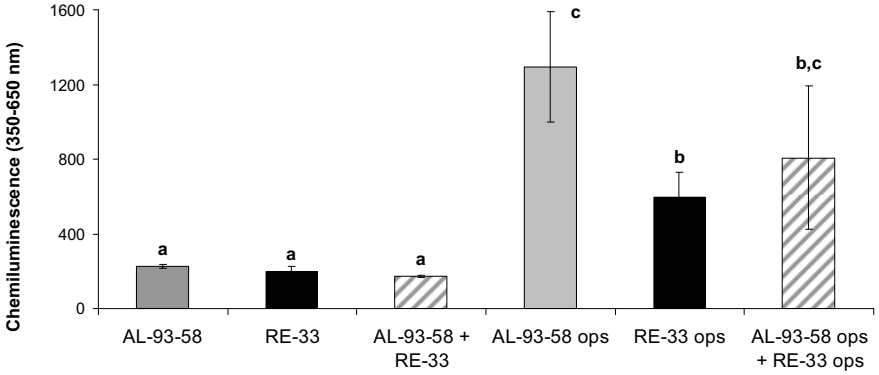

Fig. 2 Mean maximum chemioluminescensce (absorbance at 350-650 nm) (± S.D.) measured in macrophages following exposure to the virulent (AL-93-58) or attenuated (RE-33) strains of *E. ictaluri* or to both strains. The chemioluminescensce assay was used to quantify the ROS production. Macrophages were exposed to untreated bacteria (first three bars) or bacteria opsonized (ops) with serum from vaccinated fish (fourth, fifth and sixth bars). Superscripts above the bars indicate significant difference at $P < 0.05$ between treatments based on Tukey's post-*hoc* test.

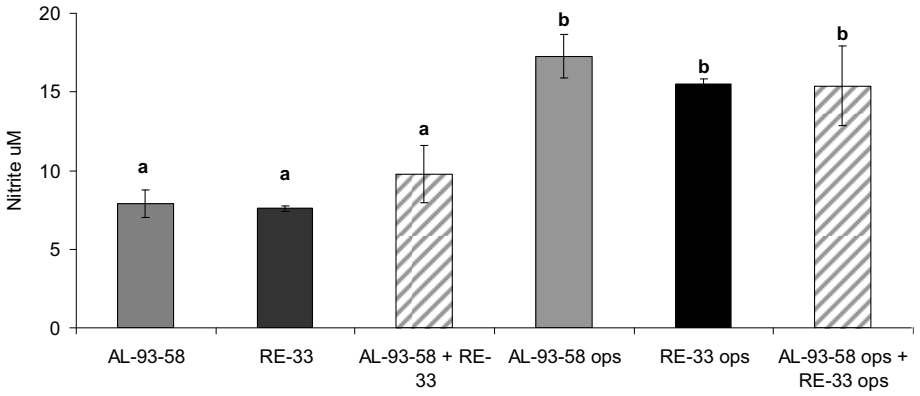

Fig. 3 Mean maximum nitrite (µM) (± S.D.) measured in macrophages following exposure to the virulent (AL-93-58) or attenuated (RE-33) strains of *E. ictaluri* or to both strains. Macrophages were exposed to untreated bacteria (first three bars) or bacteria opsonized (ops) with serum from vaccinated fish (fourth, fifth and sixth bars). Superscripts above the bars indicate significant difference at $P < 0.05$ between treatments based on Tukey's post-*hoc* test.

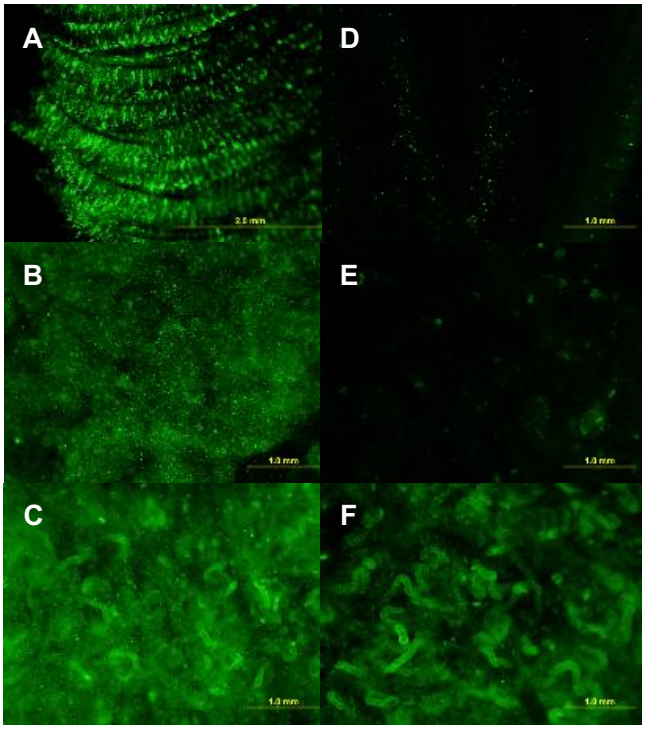

Fig. 4 Views of organs containing green fluorescent *E. ictaluri*. Representative views of gills (*a* and *d*), head kidney (*b* and *e*), trunk kidney (*c* and *f*) at 40x magnification 48 hours post intraperitoneal injection of approximately 5×10^8 CFU of the green fluorescent virulent strain (AL-93-58) (pictures from *a* to *c*) or of the green fluorescent attenuated strain (RE-33) (pictures from *d* to *f*) of *E. ictaluri* into catfish.

among macrophages exposed to the virulent (AL-93-58) or/ and the attenuated (RE-33) untreated strains of *E. ictaluri* (**Fig. 2**, first, second and third bars). Instead, there was a difference in the amount of ROS production among macrophages exposed to the opsonized attenuated and virulent *E. ictaluri*: the highest ROS production was seen in macrophages exposed to the virulent strain (**Fig. 2**, fourth bar), a lower ROS level was seen in cells exposed to both strains (**Fig. 2**, sixth bar), and the lowest ROS production was seen in macrophages exposed to the attenuated *E. ictaluri* (**Fig. 2**, fifth bar). There was no significant difference in nitric oxide production among macrophages exposed to the virulent and/or the attenuated untreated or opsonized *E. ictaluri* (**Fig. 3**).

Localization of *E. ictaluri* in fish

In fish injected with the virulent AL-93-58 strain of *E. ictaluri*, green fluorescent bacteria were observed in all organs, with the exception of the brain (**Fig. 4A-C**). Bacteria isolated from these organs were positively identified as *E. ictaluri* by culture, API 20E test strips and confirmed by PCR. The greatest amount of bacteria (highest fluorescence intensity) was observed in the gills (a), head (b) and trunk kidneys (c) and in the spleen. All other organs, such as liver, intestine and muscle proximal to the peritoneal cavity (not shown) presented fewer bacteria (lower fluorescence intensity). The gills (**Fig. 4D**), head (e) and trunk kidneys (f) and the spleen of fish injected with the attenuated strain RE-33 of *E. ictaluri* presented a lower fluorescent intensity compared to the previous group of fish. Almost no fluorescence was detected in all other organs.

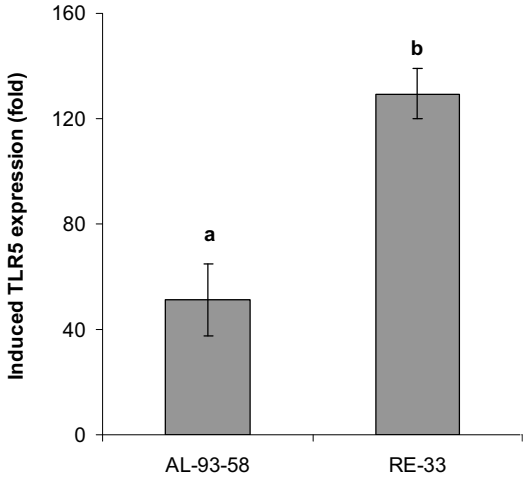

Fig. 5 Relative transcriptional level (± S.D.) of the TLR5 in fish injected with the AL-93-58 virulent, or the RE-33 attenuated strain of *E. ictaluri* compared to the transcriptional level of the TLR5 of fish injected with PBS (negative control). Superscripts above the bars indicate significant difference at $P < 0.05$ between treatments based on Tukey's post-*hoc* test.

Expression of the Toll-like receptor 5

The injection of either the virulent (AL-93-58) or the attenuated strain (RE-33) of *E. ictaluri* increased the level of the TLR5 expression (**Fig. 5**) 6 h after the bacterial challenge, compared to fish injected with PBS (negative control). Furthermore, the expression of the TLR5 was significantly higher ($P < 0.05$) in fish injected with the attenuated bacterial strain compared to fish injected with the virulent *E. ictaluri*.

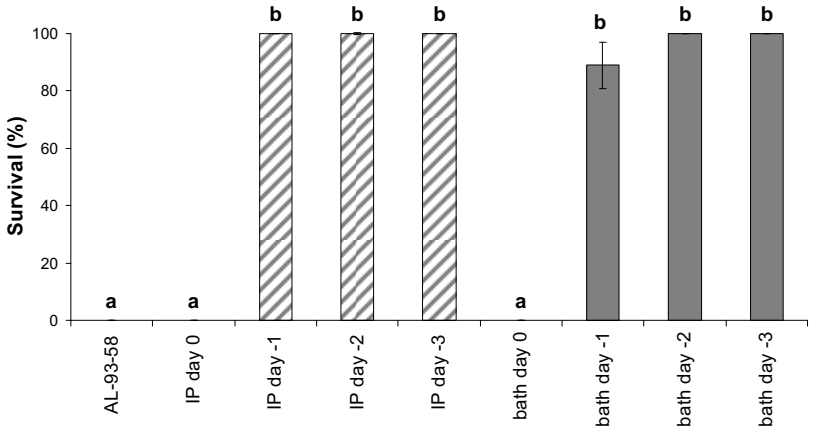

Fig. 6 Percent survival (mean ± S.D.) of catfish 14 days after the intraperitoneal injection (IP) of the virulent strain of *E. ictaluri.* Fish in some treatments were IP injected (striped bars) or bath (solid bars) exposed to the attenuated strain of *E. ictaluri* 1, 2, 3 days before, or at the same time of the challenge with the virulent bacterial strain. Fish in the AL-93-58 treatment (first bar) were not exposed to the attenuated strain of *E. ictaluri*. Superscripts above the bars indicate significant differences at $P < 0.05$ between treatments based on Tukey's post-*hoc* test.

Fish challenge

Fish exposed to the attenuated strain of *E. ictaluri* by injection or bath immersion at least 1 day prior to challenge were protected against challenge with the virulent strain of *E. ictaluri* (**Fig. 6**). In these treatments, survival varied between 83 to 100% and there was no significant difference in survival between fish exposed to the attenuated strain by bath or by intraperitoneal injection. There was no improvement in survival rate (0%) in fish exposed simultaneously to the virulent and attenuated strains of *E. ictaluri* compared to the control fish (fish not exposed to the attenuated bacterial strain).

DISCUSSION

It is well known that neutrophils and macrophages play an important role in the defense against microbial pathogens in fish (Secombes and Fletcher 1992; Shoemaker *et al.* 1997; Neumann *et al.* 2001; Secombes *et al*. 2001; Magnadottir 2006; Elibol-Flemming *et al*. 2009). In this study we were able to demonstrate that the difference in infection efficiency between a virulent and an attenuated strain of *E. ictaluri* is related to the ability of macrophages to kill the bacterium and to a more efficient stimulation of the immune system through upregulation of TLR5. Virulent strain survived and replicated in macrophages, as also seen in previous studies (Shoemaker *et al.* 1997; Russo *et al.* 2009b), even in the presence of ROS and nitric oxide, while attenuated strain were killed. A higher ROS and nitric oxide production was observed in macrophages exposed to bacteria opsonized with specific antibodies, as also observed previously (Shoemaker *et al.* 1997; Russo *et al.* 2009b). Similar results have been documented for *Aeromonas salmonicida* and *Salmonella* spp. and several studies have established a crucial role for phagocytic cells in host resistance to these bacteria (Graham *et al.* 1988; Garduno and Kay 1992; Daly *et al.* 1996; Wigley *et al*. 2002; Burr *et al.* 2003; Ebanks *et al.* 2005; Tierrez and García-del Portillo 2005; Prost *et al.* 2007). Macrophages were able to kill both the virulent and the attenuated strain when simultaneously exposed to both strains. The main difference between the virulent and the attenuated strains is that the attenuated strain lacks the high molecular weight bands of the lipopolysaccharide (HMW-LPS) (Arias *et al.* 2003). It might be possible that the absence of the HMW-LPS might make the attenuated strain more susceptible to the macrophage killing activity. Subsequently, the bacteria killing process may enhance the macrophage killing activity by a positive feedback loop. This positive feedback process might explain why virulent bacteria are killed when macrophage engulf both virulent and attenuated cells. It has been demonstrated by several studies, that *Salmonella* spp. cells internalized by macrophages are able to modify their lipopolysaccharide layer and their membrane protein composition. These changes help the bacteria to resist the action of antimicrobial peptides and reactive oxygen species present inside the macrophage phagosome (O'Brien *et al.* 1980; Ernst *et al.* 2001; Gibbons *et al*. 2005). These modifications alter the membrane permeability making the bacterial surface more hydrophobic. Studies on other fish pathogens have also shown that more virulent strain of bacteria are more resistant to ROS than less virulent bacteria (Nematolahi *et al.* 2005). It might be possible that the absence of the HMW-LPS in the attenuated strain of *E. ictaluri* prevents these modifications. Changes in the polysaccharide layer might also make bacteria more susceptible to complement-mediated killing. For example, Lawrence *et al*. (2001, 2003) and Lawrence and Bane (2005) demonstrated that a gene deletion mutant of *E. ictaluri* lacking the O polysaccharide, a major component of the polysaccharide layer, was more susceptible to killing by the fish serum. It has been demonstrated in other animals that the O polysaccharide is involved in resistance to killing by complement (Merino *et al.* 1996, 2000) and by macrophages (Price *et al.* 1990).

Edwardsiella ictaluri might be able to evade the fish immune response sequestered within macrophages and at the same time, macrophages can also be involved in the dispersion of *E. ictaluri* in the body and might represent a possible reservoir (Thune *et al*. 1997; Russo *et al.* 2009b), as demonstrated for *Salmonella* spp. (Vazquez-Torres *et al.* 1999; Wigley *et al.* 2001; Prost *et al.* 2007). Fluorescent virulent *E. ictaluri* were observed in all organs, and the high amount of bacteria observed in the gills indicated the presence of free and dividing bacteria in the blood. Instead, fluorescent attenuated bacteria were observed mainly in the lymphoid organs (spleen, head and trunk kidney) suggesting that the fish immune system was able to control the dispersion of the attenuated bacteria. Similar results were observed in studies conducted on different families of catfish: families more resistant to *E. ictaluri* presented a significant lower load of bacteria compared to susceptible families (Withler and Evelyn 1990; Camp *et al*. 2000; Bilodeau *et al.* 2005), and an increased amount of macrophage aggregates were observed in the kidney and in the spleen of resistant families. Macrophages and neutrophils are among the first cells to migrate on the site of the infection and they release several cytokines and chemokines that activate several other cells and components of the immune system. Macrophages recognize bacteria and other pathogens through different toll-like receptors. Many studies have demonstrated that in channel catfish exposed to virulent *E. ictaluri* there is a rapid increase in the expression of the TLR3 and TLR5 (Bilodeau and Waldbieser 2005; Bilodeau *et al*. 2006; Peat-

man *et al.* 2007; Bilodeau *et al.* 2008; Elibol-Flemming *et al.* 2009; Pridgeon *et al.* 2009, 2010). In this study we confirmed previous observed increased in the expression of the TLR5 in fish injected with *E. ictaluri*. Interestingly, the attenuated strain of *E. ictaluri* stimulated a significant higher expression of the TLR5 compared to the virulent strain. The higher expression of the TLR5 in combination to the ability of macrophages in killing the attenuated strain of *E. ictaluri* might explain the protective state observed in fish just after one day from the exposure to the RE-33 strain. In this study we only analyzed the expression of the TLR5 in head kidney six hours after the bacterial challenge based on the results of previous studies (Pridgeon *et al.* 2009, 2010) that showed that the anterior kidney and this time frame were the ideal for the detection of increased levels of TLR5 expression. The same studies (Pridgeon *et al*. 2009, 2010) also demonstrated that in channel catfish exposed to *E. ictaluri*, the mRNA expression of the TLR5 was a better indicator of regulation of the immune system compared to the expression of other toll like receptors.

The results of this study are supported by previous observations that the attenuated strain of *E. ictaluri* is able to stimulate a protective immune response in larval fish and eyed fish eggs, when cells and molecules of the innate immune response are present in the fish, but the adaptive arm of the immune system is still not fully developed (Klesius and Shoemaker 1999; Shoemaker *et al.* 1999; Petrie-Hanson and Ainsworth 2001; Shoemaker *et al.* 2002, 2007; Bebak *et al*. 2009). Live attenuated vaccines work in immunologically incompetent animals due to persistence of the attenuated bacteria and/or stimulation of the proper immune response (Mast and Goddeeris 1999). The protective state might be the result of activation of macrophages and other antigen processing cells followed by the release of cytokines and chemokines that stimulate the other components of the immune system. A rapid stimulation of the immune system has been also documented for other vaccines against intracellular pathogens where the innate immunity plays an important role (Mast and Goddeeris 1999). For example a vaccine against foot-and-mouth disease virus conferred complete clinical protection in swine and cattle in a few days; indicating stimulation of innate immunity was responsible for early protection (Chinsangaram *et al*. 2003; Golde *et al*. 2005; Pacheco *et al*. 2005). The authors of these studies suggested the possibility to use such vaccines during disease outbreaks. Similar results were observed in chicken eggs vaccinated against avian pneumovirus or *Salmonella typhimurium* STM-1 (Coloe *et al.* 1994; Worthington *et al*. 2003). The rapid protection conferred by the attenuated strain of *E. ictaluri* highlights the possibility to use this strain as a "therapeutic" during disease outbreak to stimulate a protection status in animals not yet infected or that have been infected with a low number of bacteria. Therapeutic use of vaccines might also be beneficial for treating asymptomatic or carrier animals, as several studies have suggested (Hunter *et al*. 1980; Inglis *et al*. 1996; Evans *et al.* 2006).

ACKNOWLEDGEMENTS

This work was supported by the US Department of Agriculture, Agricultural Research Service, Current Research Information Systems Project No. 6420-32000-022-00D. The authors gratefully acknowledge the technical assistance of Stacey LaFrentz, Elizabeth Peterman and, Mrs. Paige Mumma. This research was conducted in compliance with all relevant federal guidelines and institutional policies.

REFERENCES

Arias CR, Shoemaker CA, Evans JJ, Klesius PH (2003) A comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin-mutant (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biolog, API 20E and genomic analyses. *Journal of Fish Diseases* **26**, 415-421

Bebak J, Matthews M, Shoemaker C (2009) Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*. *Vaccine* **27**, 4297-4301

Bilodeau AL, Waldbieser GC (2005) Activation of TLR3 and TLR5 in channel catfish exposed to virulent *Edwardsiella ictaluri*. *Developmental and Comparative Immunology* **29**, 713-721

Bilodeau AL, Small BC, Wise DJ, Wolters WR (2005) Pathogen levels, lysozyme, and cortisol response in channel catfish with susceptibility differences to *Edwardsiella ictaluri*. *Journal of Aquatic Animal Health* **17**, 138-146

Bilodeau AL, Peterson BC, Bosworth BG (2006) Response of toll-like receptors, lysozyme, and IGF-I in back-cross hybrid (F1 male (blue x channel) x female channel) catfish challenged with virulent *Edwardsiella ictaluri*. *Fish and Shellfish Immunology* **20**, 29-39

Bilodeau-Bourgeois L, Bosworth BG, Peterson BC (2008) Differences in mortality, growth, lysozyme, and toll-like receptor gene expression among genetic groups of catfish exposed to virulent *Edwardsiella ictaluri*. *Fish and Shellfish Immunology* **24**, 82-89

Booth NJ, Elkamel A, Thune RL (2006) Intracellular replication of *Edwardsiella ictaluri* in channel catfish macrophages. *Journal of Aquatic Animal Health* **18**, 101-108

Burr SE, Wahli T, Segner H, Pugovkin D, Frey J (2003) Association of Type III secretion genes with virulence of *Aeromonas salmonicida* subsp. *Salmonicida. Diseases of Aquatic Organisms* **57**, 167-171

Camp KL, Wolters WR, Rice CD (2000) Survivability and immune responses after challenge with *Edwardsiella ictaluri* in susceptible and resistant families of channel catfish, *Ictalurus punctatus*. *Fish and Shellfish Immunology* **10**, 475-487

Chinsangaram J, Moraes MP, Koster M, Grubman MJ (2003) Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. *Journal of Virology* **77**, 1621-1625

Coloe PJ, Gerraty NL, Christopher W, Alderton MR, Smith SC (1994) Vaccination of poultry with *Salmonella typhimurium* STM-1. Laboratory and field evaluations. In: Jensen M (Ed) *Proceedings of the Western Poultry Disease Conference*, Sacramento, CA, pp 37-39

Daly JG, Kew AK, Moore AR, Olivier G (1996) The cell surface of *Aeromonas salmonicida* determines *in vitro* survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. *Microbial Pathogenesis* **21**, 447-461

Ebanks RO, Goguen M, McKinnon S, Pinto DM, Ross NW (2005) Identification of the major outer membrane proteins of *Aeromonas salmonicida. Diseases of Aquatic Organisms* **68**, 29-38

Elibol-Flemming B, Waldbieser GC, Wolters WR, Boyle CR, Hanson LA (2009) Expression analysis of selected immune-relevant genes in channel catfish during *Edwardsiella ictaluri* infection. *Journal of Aquatic Animal Health* **21**, 23-35

Ernst RK, Guina T, Miller SI (2001) *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes and Infection* **3**, 1327-1334

Evans JJ, Klesius PH, Shoemaker CA (2006) Therapeutic and prophylactic immunization against *Streptococcus iniae* infection in hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). *Aquaculture Research* **37**, 742-750

Garduno RA, Kay WW (1992) Interaction of the fish pathogen *Aeromonas salmonicida* with rainbow trout macrophages. *Infection and Immunity* **60**, 4612-4620

Gibbons HS, Kalb SR, Cotter RJ, Raetz CR (2005) Role of Mg2þ and pH in the modification of *Salmonella* lipid A after endocytosis by macrophage tumour cells. *Molecular Microbiology* **55**, 425-440

Golde WT, Pacheco JM, Duque H, Doel T, Penfold B, Ferman GS, Gregg DR, Rodríguez LL (2005) Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: Use in emergency outbreak response. *Vaccine* **23**, 5775-5782

Graham S, Jeffries AH, Secombe CJ (1988) A novel assay to detect macrophage bactericidal activity in fish: Factors influencing the killing of *Aeromonas salmonicida. Journal of Fish Diseases* **11**, 389-396

Hunter VA, Knittel MD, Fryer JL (1980) Stress-induced transmission of *Yersinia ruckeri* infection from carriers to recipient steelhead trout *Salmo gairdneri* Richardson. *Journal of Fish Diseases* **3**, 467-472

Inglis V, Robertson D, Miller K, Thompson KD, Richards RH (1996) Antibiotic protection against recrudescence of latent *Aeromonas salmonicida* during furunculosis vaccination. *Journal of Fish Diseases* **19**, 341-348

Jenkins JA, Klesius PH (1998) Elicitation of macrophages from the peritoneal cavity of channel catfish. *Journal of Aquatic Animal Health* **10**, 69-74

Jørgensen SM, Afanasyev S, Krasnov A (2008) Gene expression analyses in Atlantic salmon challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality. *BMC Genomics* **9**, 179

Klesius PH, Sealey WM (1995) Characteristics of serum antibody in enteric septicemia of catfish. *Journal of Aquatic Animal Health* **7**, 205-210

Klesius PH, Shoemaker CA (1999) Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. In: Schultz R (Ed) *Advances in Veterinary Medicine* (Vol 14), Academic Press, San Diego, CA, USA, pp 523-537

Lawrence ML, Banes MM, Williams ML (2001) Phenotype and virulence of a

transposon-derived lipopolysaccharide O side-chain mutant strain of *Edwardsiella ictaluri*. *Journal of Aquatic Animal Health* **13**, 291-299

Lawrence ML, Banes MM, Azadi P, Reeks B (2003) The *Edwardsiella ictaluri* O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. *Microbiology* **149**, 1409-1421

Lawrence ML, Banes MM (2005) Tissue persistence and vaccine efficacy of an O polysaccharide mutant strain of *Edwardsiella ictaluri*. *Journal of Aquatic Animal Health* **17**, 228-232

Leung KY, Low KW, Lam TJ, Sin YM (1995) Interaction of the fish pathogen *Aeromonas hydrophila* with tilapia, *Oreochromis aureus* (Steindachner), phagocytes. *Journal of Fish Diseases* **18**, 435-447

Magnadóttir B (2006) Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20**, 137-151

Mast J, Goddeeris BM (1999) Development of immunocompetence of broiler chickens. *Veterinary Immunology and Immunopathology* **70**, 245-256

Merino S, Rubires X, Aguilar A, Alberti S, Hernandez-Alles S, Benedi VJ, Tomas JM (1996) Mesophilic *Aeromonas* sp. serogroup O: 11 resistance to complement-mediated killing. *Infection and Immunity* **64**, 5302-5309

Merino S, Altarriba M, Izquierdo L, Nogueras MM, Regue M, Tomas JM (2000) Cloning and sequencing of the *Klebsiella pneumoniae* O5 *wb* gene cluster and its role in pathogenesis. *Infection and Immunity* **68**, 2435-2440

Nematollahi A, Pasmans F, Haesebrouck F, Decostere A (2005) Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **64**, 23-28

Neumann NF, Stafford JL, Barreda D, Ainsworth AJ, Belosevic M (2001) Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental and Comparative Immunology* **25**, 807-825

O'Brien AD, Rosenstreich DL, Scher I, Campbell GH, MacDermott RP, Formal SB (1980) Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *Journal of Immunology* **124**, 20-24

Pacheco JM, Brum MC, Moraes MP, Golde WT, Grubman MJ (2005) Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus-vectored FMDV subunit vaccine. *Virology* **337**, 205-209

Panangala VS, van Santen VL, Shoemaker CA, Klesius PH (2005) Analysis of 16S-23S intergenic spacer region of the rRNA operons in *Edwardsiella ictaluri* and *Edwardsiella tarda* isolates from fish. *Journal of Applied Microbiology* **99**, 657-669

Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, Li P, Wang S, Somridhivej B, Dunham R, Liu Z (2007) Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. *Developmental and Comparative Immunology* **31**, 1183-1196

Petrie-Hanson L, Ainsworth AJ (2001) Ontogeny of channel catfish lymphoid organs. *Veterinary Immunology and Immunopathology* **81**, 113-127

Price RE, Templeton JW, Adams LG (1990) Survival of smooth, rough and transposon mutant strains of *Brucella abortus* in bovine mammary macrophages. *Veterinary Immunology and Immunopathology* **26**, 353-365

Pridgeon JW, Becnel JJ, Clark GG, Linthicum KJ (2008) Developmental and environmental regulation of the *AaeIAP1* transcript in *Aedes aegypti*. *Journal of Medical Entomology* **45**, 1071-1079

Pridgeon JW, Russo R, Shoemaker CA, Klesius PH (2010) Expression profiles of toll-like receptors in anterior kidney of channel catfish acutely infected by *Edwardsiella ictaluri*. *Journal of Fish Diseases* **33**, 497-505

Pridgeon JW, Shoemaker CA, Klesius PH (2009) Identification and expression profile of multiple genes in the anterior kidney of channel catfish induced by *Edwardsiella ictaluri* vaccination. *Veterinary Immunology and Immunopathology* **134**, 184-198

Prost LR, Sanowar S, Miller SI (2007) *Salmonella* sensing of anti-microbial mechanisms to promote survival within macrophages. *Immunological Reviews* **219**, 55-65

Russo R, Panangala V, Wood R, Klesius P (2009a) Transformation efficiency of *Edwardsiella ictaluri* with three different plasmids and using three different transformation protocols. *FEMS Microbiologial Letters* **298**, 105-10

Russo R, Shoemaker C, Panangala V, Klesius P (2009b) *In vitro* and *in vivo* interaction of macrophages from modified live vaccinated and non-vaccinated channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri*. *Fish and Shellfish Immunology* **26**, 543-552

Schmidt H, Kelm M (1996) Determination of nitrite and nitrate by the Griess reaction. In: Feelisch M, Stamler JS (Eds) *Methods in Nitric Oxide Research*, John Wiley and Sons Inc., New York, pp 491-497

Secombes CJ, Fletcher TC (1992) The role of phagocytosis in the protective mechanisms of fish. *Annual Reviews in Fish Diseases* **1992**, 53-71

Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, Cunningham C, Zou J (2001) Cytokines and innate immunity of fish. *Developmental and Comparative Immunology* **25**, 713-723

Shoemaker CA, Klesius PH (1997) Protective immunity against enteric septicaemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following controlled exposure to *Edwardsiella ictaluri*. *Journal of Fish Diseases* **20**, 361-368

Shoemaker CA, Klesius PH, Plumb JA (1997) Killing of *Edwardsiella ictaluri* by macrophages from channel catfish immune and susceptible to enteric septicemia of catfish. *Veterinary Immunology and Immunopathology* **58**, 181-190

Shoemaker CA, Klesius PH, Bricker JM (1999) Efficacy of a modified live *Edwardsiella ictaluri* vaccine in channel catfish as young as seven days post hatch. *Aquaculture* **176**, 189-193

Shoemaker CA, Klesius PH, Evans JJ (2002) *In ovo* method for utilizing the modified live *Edwardsiella ictaluri* vaccine against enteric septicemia in channel catfish. *Aquaculture* **203**, 221-227

Shoemaker CA, Klesius PH, Evans JJ (2007) Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. *Vaccine* **25**, 1126-1131

Thune RL, Collins LA, Pena MP (1997) A comparison of immersion, immersion-oral combination, and injection methods for the vaccination of channel catfish *Ictalurus punctatus* against *Edwardsiella ictaluri*. *Journal of World Aquaculture Society* **28**, 193-201

Tierrez A, García-del Portillo F (2005) New concepts in *Salmonella* virulence: The importance of reducing the intracellular growth rate in the host. *Cell Microbiology* **7**, 901-909

Vazquez-Torres A, Jones-Carson J, Bäumler AJ, Falkow S, Valdivia R, Brown W, Le M, Berggren R, Parks WT, Fang FC (1999) Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature* **401**, 804-808

Wigley P, Berchieri A Jr., Page KL, Smith AL, Barrow PA (2001) *Salmonella enterica* serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. *Infection and Immunology* **69**, 7873-7879

Wigley P, Hulme SD, Bumstead N, Barrow PA (2002) *In vivo* and *in vitro* studies of genetic resistance to systemic salmonellosis in the chicken encoded by the SAL1 locus. *Microbes and Infection* **4**, 1111-1120

Wise DJ, Klesius PH, Shoemaker CA, Wolters WR (2000) Vaccination of mixed and full-sib families of channel catfish *Ictalurus punctatus* against enteric septicemia of catfish with a live attenuated *Edwardsiella ictaluri* isolate (RE-33). *Journal of World Aquaculture Society* **31**, 206-212

Withler RE, Evelyn TPT (1990) Genetic variation in resistance to bacterial kidney disease within and between two strains of coho salmon from British Columbia. *Transactions of the American Fish Society* **119**, 1003-1009

Worthington KJ, Sargent BA, Davelaar FG, Jones RC (2003) Immunity to avian pneumovirus infection in turkeys following *in ovo* vaccination with an attenuated vaccine. *Vaccine* **21**, 1355-1362