Fish & Shellfish Immunology 27 (2009) 773–776

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Combined immersion and oral vaccination of Vietnamese catfish (*Pangasianodon hypophthalmus*) confers protection against mortality caused by *Edwardsiella ictaluri*

N.H. Thinh [a,1], T.Y. Kuo [b,c,1], L.T. Hung [a], T.H. Loc [a], S.C. Chen [e], Ø. Evensen [d], H.J. Schuurman [f,*]

[a] *Nong Lam University, Linh Trung Ward, Thu Duc District, Hochiminh City, Vietnam*
[b] *Department of Animal Science, National Ilan University, Ilan, Taiwan*
[c] *Institute of Biotechnology, National Ilan University, Ilan, Taiwan*
[d] *Norwegian School of Veterinary Science, PO Box 8146 Dep., N-0033 Oslo, Norway*
[e] *National Pingtung University of Science and Technology, Department of Veterinary Medicine, Animal Vaccine and Adjuvant Research Center, Taiwan*
[f] *Schweizer Biotech Company, 4F., No. 501, Sec. 2, Tiding Blvd., Neihu Technology Park, Taipei 11493, Taiwan*

ARTICLE INFO

Article history:
Received 20 June 2009
Received in revised form
13 August 2009
Accepted 30 August 2009
Available online 10 September 2009

Keywords:
Edwarsiella ictaluri
Tra catfish
Vaccination
Immersion
Oral boost

ABSTRACT

Edwardsiella ictaluri septicemia occurs worldwide and causes high mortality and considerable economic damage to the catfish industry especially in Vietnam and the USA. To control Edwardsiella septicemia farmers extensively use antibiotics and various vaccination methods. Vaccination with inactivated vaccines has come with variable efficacy.

In this trial the results of an approach of controlling Edwardsiella septicemia of Tra catfish (*Pangasianodon hypophthalmus*) in Vietnam through vaccination via mucosal surfaces are presented. The results show that a combination of primary vaccination by immersion with inactivated *E. ictaluri* followed by an oral boost with a formulated antigen preparation induces a statistically significant level of protection against mortality caused by experimental infection 4 weeks post-boost. Fish immunized by immersion only show significantly lower level of protection but significantly higher than the controls. Repeated boosts result in improved duration of immunity with a relative percent survival (RPS) of 47% at 90% control mortality. The immunization procedure provides an alternative for disease control through vaccination.

Infections with *Edwardsiella ictaluri* play a major role in catfish (*Pangasianodon hypophthalmus*) farming in Vietnam [2,5] and were first observed in the Mekong River Delta in 1999 [3]. *E. ictaluri* infections are seen in USA in channel catfish (*Ictalurus punctatus*) and also in walking catfish in Thailand [7].

Inactivated vaccines as a preventive measure to control infections with *E. ictaluri* have been tried in various catfish species, especially in the USA [9]. The use of inactivated vaccines (whole cell) has not given a long-lasting immunity [15] while a live attenuated vaccine based on an *E. ictaluri* strain RE-33 has elicited a protection of short duration following one inoculation of the vaccine [9]. Different ways of protecting the catfish in Vietnam against *E. ictaluri* infection using inactivated vaccines have been tried but none have so far been successful.

Oral delivery of vaccine antigens to fish is the preferred method for several reasons however the limitations have been the lack of efficacy [4,6,15]. In this study we describe an oral delivery method based on an oil-based formulation used for feed top-dressing to control *E. ictaluri* infection. We report the findings from combined immunization regimes including primary vaccination by immersion or injection combined with oral boosting and show that repeated oral boosts confer a moderate level of protection in immunized fish under high challenge pressure with 90% control mortality.

1. Materials and methods

Two batches of healthy non-infected catfish (*P. hypophthalmus*) weighing 5–6 g and 8–10 g were used in this study. The fish were fed twice daily to satiation. During the period of experimental infection, the fish were not fed. The water in fish tanks was regularly checked for dissolved oxygen, NH_3, pH and temperature, and daily volume exchange was 20–30%. The water temperature was controlled to be within the range of 25–28 °C.

* Corresponding author.
E-mail address: shield_001@yahoo.com (H.J. Schuurman).
[1] These authors contributed equally to the work.

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.08.012

Healthy, non-infected, catfish were vaccinated using injection, immersion or oral vaccination or combinations of these delivery methods (Fig. 1). The various vaccinations and vaccination combinations and challenges were performed in 3 different experiments. A total of about 1500 fish were used in the challenge studies. Experiment 1 was carried out to decide the optimal challenge route. Experiment 2 describes the combinations of primary vaccination by immersion followed by oral boosts while Experiment 3 outlines the injection/oral vaccination combinations.

In Experiment 2, the fish were given a primary immersion vaccination (Group A) at Day 1 of the experiment (immersion-prime) or a primary vaccination by oral delivery only (Group E, oral-prime) through days 8–21 of the experiment. Group C was given a combination of immersion followed by an oral boost (Fig. 1) and Group F was given a second oral boost through experimental days 101–107 (Group F), 80 days after the 1st boost (Fig. 1). The first challenge was carried out by experimental day 48 (4 weeks post oral boost was completed) while the second challenge was carried out 3 weeks after the second boost was completed (experimental day 121; Fig. 1).

In Experiment 3 fish were immunized first with injection vaccination and given an oral boost through days 8–21 of the experiment (Fig. 1). Group B was given an injection vaccination only, while a Group D was boosted once orally (Fig. 1). Challenges were initiated at experimental days 48 and 121 as for Experiment 2.

Two types of commercial tra catfish pellet feed (diameter of 2.0–2.2 mm) produced by Woosungvina Co. were fed to the fish based on fish size.

An *E. ictaluri* strain VL33 isolated from diseased Tra catfish from the Vinh Long Province in June, 2007 was used for vaccination and challenge. The bacterial strains were characterized as described [2]. Bacterial culture grown in Brain Heart Infusion (BHI) broth for 18 h at 25–30 °C were used for experimental challenge and identification. Bacterial counts were done by spread plate on BHI agar. Inactivation of bacteria was done by adding formalin at a final concentration of 0.5% (w/v) and incubation for a minimum of 24 h at room temperature. Inactivated bacterial suspensions were stored at +4 °C. Documentation of inactivation was performed by spreading on BHI agar followed by incubation as described above.

1.1. Bath challenge

Fifty fish per tank in three parallel tanks per dose (4 doses total) were challenged by pouring bacteria grown in BHI broth into water to give a final concentration of 5.5×10^3–5.5×10^6 colony forming units of *E. ictaluri* per ml of water. Exposure to the challenge dose lasted for 30 min. Strong aeration was supplied to the water in the tanks during challenge. The control groups were immersed in clean aerated water. Mortalities were monitored for 14 days.

1.2. Injection challenge

Fish were injected intraperitoneally with 0.1 ml of a bacterial dilution at 4 different bacterial concentrations ranging from 5.5×10^3 to 5.5×10^6 colony forming units (10-fold steps) of *E. ictaluri* per ml or sterile saline water for control. Mortalities were monitored for 14 days. From both experiments moribund and freshly dead fish were submitted for bacterial isolation from the liver, spleen and kidney. Surviving fish at the end of monitoring period were checked for bacterial infection.

Combined immersion/oral immunization studies (Experiment 2) are summarized in Table 1. The groups included Group A with immunization by immersion only (day 1) and challenge at 48 and 121 days after primary vaccination, Group C (imm-oral boost-1) with combined immersion (day 1) and oral boost by days 8–21 and Group F (imm-oral boost-2) boosted a second time through experimental days 101–107. Group E (oral-prime) was given primary immunization at days 8–21 by the oral route and challenged at the two time points (Fig. 1). Non-vaccinated control groups were included at both challenge times.

Combined injection/oral immunization studies (Experiment 3) included primary vaccination intraperitoneally with an inactivated, water-based vaccine preparation at day 1 (inj-prime) while Group D (inj-oral boost-1) was given an oral boost by days 8–21 post-prime (Fig. 1). Challenge by immersion was carried out at experimental days 48 and 121 (as in Exp. 2, Fig. 1).

The immersion vaccine consisted of a sterile water-based, killed bacterial suspension of 5.0×10^9 bacteria per ml. The injection vaccine consisted of a sterile water-based, killed bacterial suspension of 3.85×10^9 bacteria per ml. The oral vaccine was made by formulating the killed bacteria at a concentration of 3.85×10^8 bacteria per ml in an oil-emulsion followed by top-dressing on feed pellet (see below).

Immersion vaccination was performed by immersing 1200 fish in 2 L of vaccine diluted in 18 L (5.56×10^8 bacteria per ml final concentration) of clean water for 1 min with strong aeration (dip vaccination). The injection vaccine (water-based) was delivered to each fish intraperitoneally, in the midline right between the pectoral fins and the anus, with 0.1 ml of vaccine (3.85×10^8 bacteria per fish). Before injecting, fish were anaesthetized with

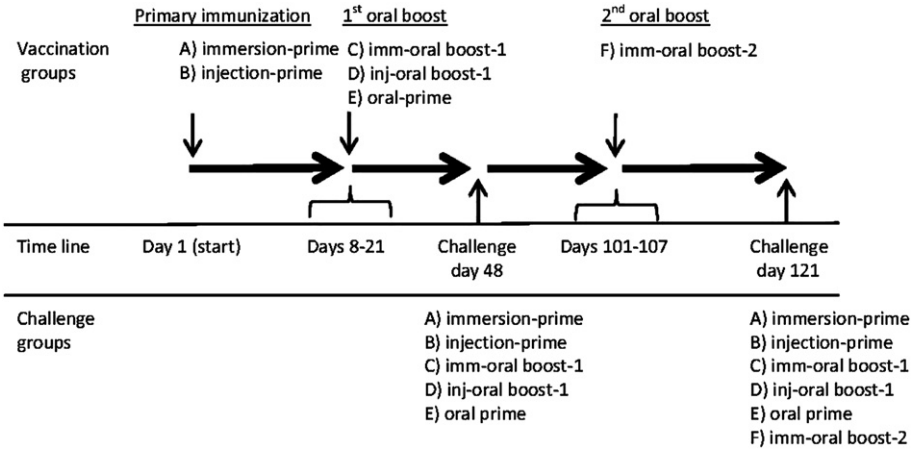

Fig. 1. The figure shows the layout and the time line of the experiments and the identification of different vaccination groups included in the study.

Table 1
Trial set up for studies of efficacy following single and combined immersion and oral vaccination. Group F was made up from the remaining fish of the 48 day challenge and given a 2nd boost by days 101–107. The first boost included a feeding period for 2 weeks while the second oral boost period lasted for 1 week.

Group	Number of replicates	Prime immunization	1st boost	2nd boost	Challenge at day 48 (50 fish per parallel)	Challenge at day 121 (40 fish per parallel)
A	3	Immersion-prime	–	–	Yes	–
	1	Immersion-prime	–	–	–	Yes
C	3	Immersion-prime	Oral (day 8–21)	–	Yes	–
	1	Immersion-prime	Oral (day 8–21)	–	–	Yes
F	1	Immersion-prime	Oral (day 8–21)	Oral (day 101–107)	–	Yes
E	3	Oral-prime (day 8–21)	–	–	Yes	–
	1	Oral-prime (day 8–21)	–	–	–	Yes
Ctrl	3	Non-vaccinated controls			Yes	–
	1	Non-vaccinated controls			–	Yes

0.2% MS222 in the water. The oral vaccine was made by spray-coating the emulsified antigen on the outside of the pelleted feed at 2% (volume/weight). The coated pellet was then sprayed with squid oil at 0.1% (v/w). The coated feed pellets were prepared daily and used within 1–2 days after preparation and fed to satiation.

The immersion challenge was performed in 96 L tanks containing 70 L of clean water (Fig. 1). At the first challenge (day 48), 40 or 50 fish (number of fish depending on the experiment, Table 1) from each tank were transferred to a bucket containing 10 L of clean water and then bacteria grown in BHI broth was poured into water to given concentrations of 7.6×10^6 or 4.3×10^6 bacteria/ml of water for Experiments 2 and 3, respectively (Table 1). Exposure to the challenge dose lasted for 1 h. At the second challenge (121 days), the same immersion method was applied with a concentration of 8.1×10^6 bacteria/ml of water for both Experiments 2 and 3. The non-challenged controls were immersed in clean aerated water. After challenge the fish were observed for 14 days.

Fisher's exact test was used to analyze differences between groups at end-point. A *P*-value below 0.05 was considered to represent significant differences between groups/treatments.

2. Results

In this experiment challenge by immersion was compared with challenge by injection. For immersion challenge the end-point mortality varied from 1.3% (±1.15 SD) at 5.5×10^3 CFU/ml to 66% (±8.5 SD) at 5.5×10^6 CFU/ml. For the injection challenge it ranged from 93 (±1.5 SD) to 99.3% (±0.6 SD) end-point mortality over the dose range tested. Based on these results immersion challenge was used for assessing vaccination efficacy and it was considered that the immersion dose of $>10^6$ gave sufficient mortality, i.e. more than 60% control mortality.

Immersion/oral immunization studies (Experiment 2) showed a cumulative mortality in the non-vaccinated controls of 87% by day 48 (Fig. 2). In Group A (immersion-prime) the average cumulative mortality was 65% (±3.1 S.D.) ($p < 0.02$), while in Group E (oral-prime) average, cumulative mortality was 74% ± 3.5 ($p > 0.1$), and in Group C (immersion-prime/oral boost-1) average cumulative mortality was 42% ± 4.0, ($p < 0.001$), giving RPS values of 25, 15, and 52, respectively.

At experimental day 121 the cumulative mortality in the controls was 90% (Fig. 3). In Group A (immersion-prime) cumulative mortality was 80% (RPS = 11, $p = 0.26$), while Group E (oral-prime) showed a mortality of 82% (RPS = 9, $p = 0.388$). In Group C (immersion-prime/oral boost-1) mortality was 64% (RPS = 29, $p = 0.0037$) while in Group F (immersion-prime/oral boost-2) cumulative mortality was 48% (RPS = 47, $p = 0.0001$).

Vaccination by injection combined with oral boost (Experiment 3) showed a cumulative mortality in the non-vaccinated controls of 91.7% ± 2.9 while Group B (injection-prime) had average cumulative mortality of 76.7% ± 8.0 ($p = 0.115$). In Group D (injection-prime/oral boost) average cumulative mortality was 80% ± 2.5 ($p > 0.2$). At experimental day 121 the cumulative mortality in the controls was 96%. In Group B (injection-prime) cumulative mortality was 92.5% (RPS = 3.6, $p > 0.3$), while Group D (injection-prime/oral boost) showed 85% mortality (RPS = 11.5, $p > 0.2$).

3. Discussion

The main conclusion that can be drawn from the present study is that a combination of primary immunization by immersion and an oral boost vaccination gives a moderate level of protection against lethal challenge with *E. ictaluri* in Tra catfish.

Immersion vaccination is considered a practical method for vaccination of large number of small fish however the main obstacle for its use has been lack of efficacy [10]. Previous studies have shown that live *E. ictaluri* gain access to circulation through the gills while killed bacteria were found not to be taken up over external surfaces at all [11]. Thus the level of protection obtained in the immersion group (Group A) at 7 weeks post-primary vaccination is higher than would be expected while the duration of protection attained from a single immersion is limited and no protection was seen by 4 months post-vaccination.

In general there are few reports in the literature of successful oral vaccination of fish and oral vaccines, in contrast to immersion vaccines, are used to a very limited extent in aquaculture in any part of the world [6]. Thune and coworkers [15] studied vaccination of channel catfish using immersion and or oral vaccination for

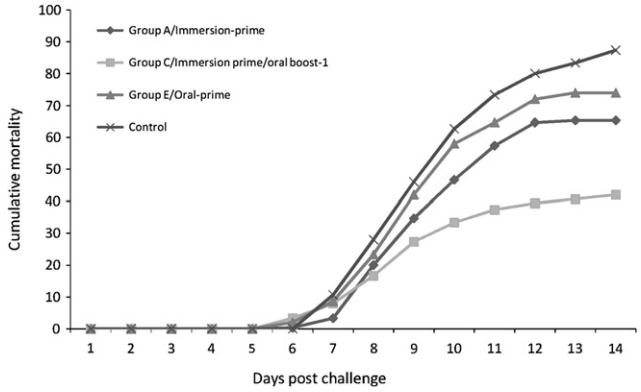

Fig. 2. Challenge performed at 48 days post-vaccination using an immersion challenge in Groups A, C and E. The end-point in the non-vaccinated controls was 87%. In Group A (immersion-prime) end-point mortality was 65% ($p < 0.02$), in Group E (oral-prime) 74% ($p > 0.1$), and in Group C (immersion-oral boost-1) 42% ($p < 0.001$). Corresponding RPS values are 25, 15, and 52, respectively.

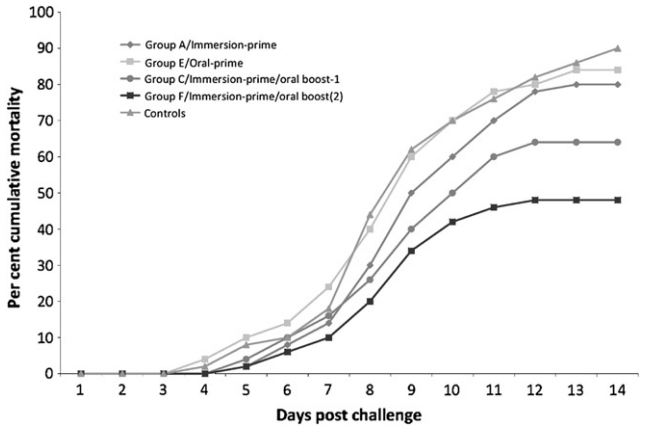

Fig. 3. Immersion challenge at 121 days post-primary immunization for Groups A, C, E and F. The end-point mortality in the controls was 90%. In Group A (immersion-prime) end-point mortality was 80% (RPS = 11, $p = 0.26$), for Group E (oral-prime) 82% (RPS = 9, $p = 0.388$), in Group C (immersion-oral boost-1) 64% (RPS = 29, $p = 0.0037$) while in Group F (immersion-oral boost-2) mortality was 48% (RPS = 47, $p = 0.0001$).

E. ictaluri and their findings were ambiguous. In one field study a very good protection was obtained in groups of fish vaccinated with a combination of immersion and oral delivery while in parallel groups, no difference was found between vaccinees and controls [15]. The studies of Thune and coworkers [15] are partly in line or contrast with what others have found for immersion and/or oral vaccine against *E. ictaluri* infection in channel catfish. More specifically, one study [12] reported that immersion and immersion plus oral boost vaccination of channel catfish with *E. ictaluri* bacterin gave strong immunity. Others [14] found that a combination of immersion and oral vaccination with killed *E. ictaluri* in channel catfish was found non-efficacious in the field. Our findings are based entirely on laboratory experiments and it will be necessary to document similar findings from field studies to substantiate our laboratory findings.

The number of fish available for challenge at the 121 day challenge did not allow us to include parallel tanks, which obviously is preferable based on a well-known tank-effect, although it has been found less important than previously thought [8]. The variation between parallels tanks in Experiment 1 in the immersion and injection challenge was in general found low for both challenge methods. Further, in Experiment 2 at the 48 day the variation between tanks was relatively low which would substantiate that the results obtained at the 121 day challenge are representative of true difference between immunization methods.

The combination of primary immunization by immersion followed by oral boosting (Group C) seems to be of importance since a single oral immunization (Group E) is not conferring a significant protection. The level of protection attained by the combination of immersion and one boost – in Group C – is reduced by experimental day 121 (approximately 3 months post-boost) but still the protection is twice as high as in the single immunized fish and still significantly different from controls ($p = 0.0037$). Interestingly, when the fish are given a second oral boost increased protection is obtained and almost half the fish survive at very high challenge pressure by day 121 (2 weeks after boost); ($p = 0.0001$).

Injection vaccination alone induces a non-significant low level of protection by 7 weeks post-immunization (equal to the oral group at this time point). Further, an interesting observation is the lack of combination effects between injection-prime and an oral boost, in conformity with previous studies of turbot using primary vaccination by injection and an oral boost against furunculosis [13]. It is tempting to speculate that priming via parenteral routes will not elicit mucosa-associated immunity and thus boosting via this route will be of no value possibly pointing towards compartmentalization of the immune system also of fish as seen in higher vertebrates [1].

In summary, combined immersion-oral prime-boost vaccination confers relatively good protective immunity against lethal challenge with *E. ictaluri*. Repeated oral boosting might be an attractive alternative to maintain level of immunity in Tra catfish against lethal exposure to the pathogen.

References

[1] Brandtzaeg P, Johansen FE. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. Immunol Rev 2005;206:32–63.
[2] Dung TT, Haesebruck F, Tuan AG, Sorgeloos P, Baele M, Decostere A. Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam. Microb Drug Res 2008;14:311–6.
[3] Ferguson HW, Turnbull JF, Shinn A, Thompson K, Dung TT, Crumlish M. Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage) from the Mekong Delta, Vietnam. J Fish Dis 2001;24:509–13.
[4] Gudding R, Lillehaug A, Evensen Ø. Recent developments in fish vaccinology. Vet Immunol Immunopathol 1999;72:203–12.
[5] Ho TT, Areechon N. Identification and antibiotic sensitivity test of the bacteria isolated from striped catfish (*Pangasianodon hypophthalmus* [Sauvage, 1878]). In: Proceeding of Fisheries. Bangkok, Thailand: Kasetsart University; 2008. p. 23–5.
[6] Håstein T, Gudding R, Evensen Ø. Bacterial vaccines for fish – an update of the current situation world wide. Dev Biol Stand 2005;121:55–74.
[7] Kasornchandra J, Rogers WA, Plumb JA. *Edwardsiella ictaluri* from walking catfish, *Clarias batrachus* L., in Thailand. J Fish Dis 1987;10:137–8.
[8] Kjøglum S, Grimholt U, Larsen S. Non-MHC genetic and tank effects influence disease challenge tests in Atlantic salmon (*Salmo salar*). Aquaculture 2005; 250:102–9.
[9] Klesius PH, Shoemaker CA. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. Adv Vet Med 1999; 41:523–37.
[10] Nakanishi T, Ototake M. Antigen uptake and immune responses after immersion vaccination. Dev Biol Stand 1997;90:59–68.
[11] Nusbaum KE, Morrison EE. Entry of 35S-labelled *Edwardsiella ictaluri* into channel catfish. J Aquatic Anim Health 1996;7:146–9.
[12] Plumb JA. Vaccination of channel catfish, *Ictalurus punctatus* (Rafinesque), by immersion and oral booster against *Edwardsiella ictaluri*. J Fish Dis 1993; 16:65–71.
[13] Santos Y, García-Marquez S, Pereira PG, Pazos F, Riaza A, Silva R, et al. Efficacy of furunculosis vaccines in turbot, *Scophthalmus maximus* (L.): evaluation of immersion, oral and injection delivery. J Fish Dis 2005;28(3):165–72.
[14] Shoemaker C, Klesius P. Protective immunity against enteric septicaemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following controlled exposure to *Edwardsiella ictaluri*. J Fish Dis 1987;20(5):361–8.
[15] Thune RL, Hawke JP, Johnson MC. Studies on vaccination of channel catfish, *Ictalurus punctatus*, against *Edwardsiella ictaluri*. J Appl Aquacult 1994; 3(1–2):11–24.