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Cross-protection elicited in channel catfish (*Ictalurus punctatus* Rafinesque) immunized with a low dose of virulent *Edwardsiella ictaluri* strains

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Abstract

Cross-protection of channel catfish (*Ictalurus punctatus* Rafinesque) immunized with a low dose of virulent *Edwardsiella ictaluri* and challenged with six *E. ictaluri* strains was examined in four trials. The relative per cent survival among low-dose immunized and then challenged fish ranged from 27.7% to 100%. Significant protection ($P < 0.05$), with the exception of strain ATCC-33202, was conferred by immunization with a given *E. ictaluri* strain challenged either with a homologous or a heterologous strain. Antibody titres of pooled serum collected on day 22 from surviving fish examined by enzyme-linked immunosorbent assay (ELISA) ranged from 1:40 to 1:320, but no differences were apparent among different vaccinated groups. The protein profiles of six *E. ictaluri* strains examined by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a relatively homogeneous pattern. Immuno-blots probed with pooled serum from immunized and challenged fish showed a pattern similar to LPS-reaction patterns observed with *E. ictaluri* in other studies. Since the present studies further corroborate that *E. ictaluri* is a clonal bacterial species with no apparent antigenic differences, it is possible that immunization with a single *E. ictaluri* field strain should confer protection against any other strain.

Keywords: *Edwardsiella ictaluri*, cross-protection, catfish, immunity

Introduction

Enteric septicaemia of channel catfish (ESC) caused by *Edwardsiella ictaluri* is recognized as one of the most significant causes of morbidity and mortality in the commercial catfish farming industry in the southeastern United States (Klesius 1992; Plumb 1999; Wagner, Wise, Khoo & Terhune 2002). *Edwardsiella ictaluri* is considered to be an obligate pathogen with a disease-causing potential predominantly in fish belonging to the ictalurid species, although infrequently reported from ornamental fish species such as Siamese fighting fish, *Betta splendens* (Regan), and rosy barbs, *Puntius conchonius* (Hamilton) (Humphrey, Lancaster, Gudkovs & McDonald 1986); green knife fish, *Eigenmannia virescens* (Valenciennes) (Kent & Lyons 1982); danio, *Danio devario* (Hamilton) (Waltman, Shotts & Blazer 1985); and freshwater catfish, *Pangasius hypophthalmus* (Sauvage) (Crumlish, Dung, Turnbull, Ngoc & Ferguson 2002). In experimental infection attempts with *E. ictaluri*, it has been demonstrated that an intraperitoneal (i.p.) injection with 1.5×10^8 organisms could produce mortality in tilapia, *Oreochromis aureus* (Linnaeus), but not in golden shiners, *Notemigonus crysoleucas* (Mitchill), largemouth bass, *Micropterus salmoides* (Lacepede), and bighead carp, *Hypophthalmichthys nobilis* (Richardson) (Plumb & Sanchez 1983). Susceptibility to *E. ictaluri* infection in two *salmonid* species, Chinook salmon, *Oncorhynchus tahawstscha* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), by immersion exposure was reported by Baxa, Groff, Wishkovsky and Hedrick (1990). Klesius, Lovy, Washuta and Arias (2003) isolated *E. ictaluri* from ulcerative cranial lesions from three tadpole madtom, *Noturus gyrinus* (Mitchill), during an aquatic fish-health assessment survey in the Cohansey River in New Jersey. Thus, with few exceptions, *E. ictaluri* can be considered to be a

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pathogen with a propensity for natural infection within a limited fish-host range.

While some studies to antigenically characterize *E. ictaluri* isolates obtained from outbreaks of infection occurring in different geographic locations have led to the concept that *E. ictaluri* are comprised of a single antigenic group (Rogers 1981; Bertolini, Cipriano, Pyle & McLaughlin 1990), other investigations have yielded conflicting results. Early studies based on the analysis of *E. ictaluri* outer membrane protein profiles by gel electrophoresis revealed an apparent antigenic homogeneity among several isolates (Plumb & Klesius 1988; Newton, Blevins, Wilt & Wolfe 1990; Klesius & Horst 1991). Contrasting results revealing antigenic differences among some isolates of *E. ictaluri* were reported in later studies (Lobb, Ghaffari, Hayman & Thompson 1993; Klesius & Shoemaker 1997). From a different perspective, antigenic variations between *E. ictaluri* isolates were also found on the basis of red blood cell agglutination inhibition by the monosaccharide D-mannose (Wong, Miller & Janda 1989; Ainsworth 1993). More recent studies based on analysis of the 16S-23S intergenic spacer regions (ISR) of the rRNA operons of 19 isolates of *E. ictaluri* showed a lack of length polymorphisms in the ISR regions and only a sparse heterogeneity in the individual nucleotide positions in the gene sequences (Panangala, van Santen, Shoemaker & Klesius 2005). A companion study of the same 19 *E. ictaluri* isolates evaluated on the basis of three phenotypic parameters (electrophoretic protein profiles, antigenic epitopes and cellular fatty acid composition) led to the conclusion that *E. ictaluri* represents a rather cohesive, clonal bacterial population (Panangala, Shoemaker, McNulty, Arias & Klesius 2006). In this study, we examined the cross-protection conferred by select strains of *E. ictaluri* to determine whether an appreciable difference in the virulence potential exists and to further explore the possibility of identifying a suitable candidate strain and/or strains that possess a distinctive ability to confer the optimal level of protection to challenge infection in channel catfish.

Materials and methods

Bacterial strains

The *E. ictaluri* strains were cultured from stocks maintained in the culture repository at the Aquatic Animal Health Research Unit at Auburn, AL. Two of the strains AL-93-58 and AL-93-75 were originally isolated from diseased catfish in Alabama, and strain S-94-1051 was from an outbreak of ESC in Mississippi. The type strain ATCC-33202 originally isolated from a diseased catfish in Georgia was obtained from the American Type Culture Collection and NJ #3 from an infected catfish in New Jersey. Strain EILO was originally cultured from a walking catfish, *Clarias batrachus* (Linnaeus), (Kasornchandra, Rogers & Plumb 1987) and re-isolated from a channel catfish in our laboratory following an experimental infection trial (Klesius & Shoemaker 1997). Each *E. ictaluri* isolate was passaged at least once in fish by an i.p. injection of disease-free channel catfish ($n = 3$–4), and the bacteria were isolated in a pure culture from the head kidney of aseptically dissected fish.

Fish and culture conditions

Channel catfish (National Warm-water Aquaculture Center strain 103) weighing 12–15 g, obtained from a disease-free stock [as determined by bacteriological techniques (Panangala *et al.* 2006) of random samples] and reared at the Aquatic Animal Health Research Unit, were used in the experiments. The fish were initially maintained in 60 L glass aquaria with flow-through dechlorinated tap water, constant aeration, a water temperature of 26 ± 2 °C and a rhythmic 12:12-h light:dark photoperiod. Fish were fed daily to satiation with a commercial feed (Aquamax Grower 400 Brentwood, MO, USA). Experiments were conducted in four trials as indicated in Table 1, A–D, in groups ranging from 5 to 7 with 75 fish per group, except trial C, which comprised 51 fish per group.

Immunization and challenge

Approximately 250 fish per treatment were immunized by immersion in 15 L of water, to which 15 mL of a broth culture of the respective *E. ictaluri* isolate (optical density reading of 1.0–1.2 at OD_{600}) was added to yield a concentration of $\sim 1 \times 10^6$ bacteria mL^{-1}, and allowed to remain in contact for 15 min. Following immersion exposure, the fish were removed using a net and transferred into their respective 60 L holding tanks. Immunized fish were monitored daily for a period of 28-days and dead or moribund fish were removed. On day 28, fish were exposed to the challenge strain of *E. ictaluri* by immersion in batches of 25 fish per 4 litres of water containing 40 mL of the respective *E. ictaluri* broth culture containing 3–4 × 10^7 *E. ictaluri* per mL for

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Table 1 Relative per cent survival (RPS) of *Edwardsiella ictaluri* immunized and challenged channel catfish

Trial	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
A							
I	AL-93-58	S-94-1051	S-94-1051	AL-93-58	BHI	BHI	BHI
C	AL-93-58	S-94-1051	AL-93-58	S-94-1051	AL-93-58	S-94-1051	BHI
%Mortality	0	0	1	4	100	100	0
RPS*	100	100	99	96	–	–	
B							
I	ATCC-33202	ATCC-33202	EILO	EILO	BHI	BHI	BHI
C	AL-93-58	EILO	AL-93-58	ATCC-33202	AL-93-58	EILO	ATCC-33202
%Mortality	47	52	1	0	65	72	0
RPS	27.7	27.7	98.6	100	–	–	
C							
I	NJ #3	NJ #3	AL-93-75	AL-93-75	BHI	BHI	BHI
C	S-94-1051	AL-93-75	S-94-1051	NJ #3	NJ #3	S-94-1051	AL-93-75
%Mortality	0	0	0	6	40	51	32
RPS	100	100	100	81	–	–	–
D							
I	AL-93-75	EILO	NJ #3	BHI	BHI	BHI	
C	AL-93-75	EILO	NJ #3	AL-93-75	EILO	NJ #3	
%Mortality	5	9	7	64	56	58	
RPS	92.5	83.9	87.9	–	–	–	

I,Immunizing strain; C,Challenge strain; BHI,Brain Heart Infusion medium.
*RPS (Relative Per cent Survival) according to Amend [27].
For a given trial, RPS values were obtained by comparing mortality in fish exposed to the same challenge strain, with the control group immunized with BHI.

30 min and distributed into individual 40 L tanks, with 25 fish per tank (three tanks per treatment). Following challenge, fish were monitored daily up to a period of 22 days for signs of disease and the dead fish were promptly removed and the head kidneys were aseptically dissected for bacteriological examination. On day 22, all surviving fish were anaesthetized in batches by immersion in tricaine methanesulphonate (Argent Chemical, Redmond,WA, USA) 100 μg L^{-1} and a blood sample (~ 200 μL $fish^{-1}$) was collected by vein puncture of the caudal vein with a hypodermic syringe and needle. Sera from 5 to 8 fish from the same group were pooled into individual tubes and stored at − 20 °C until used. The fish were then euthanized using 300 mg L^{-1} of tricaine methanesulphonate.

Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was performed according to the indirect procedure described by Crowther (1995) with essential modifications. Nunc, MaxiSorp 96-well microtitre plates (Nalge Nunc International, Rochester, NY, USA) were coated (100 μL $well^{-1}$) with sonicated *E. ictaluri* antigen (20 μg protein mL^{-1}) in 0.05 M L^{-1} carbonate/bicarbonate buffer, pH 9.6. The plates were incubated with antigen overnight at 4 °C and washed thrice with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). Blocking buffer (PBS containing 1% w/v bovine serum albumin and 0.5% w/v nonfat dry milk) was added in a volume of 100 μL $well^{-1}$ and incubated at room temperature for 30 min. After washing thrice with PBST, the pooled channel catfish sera obtained from each experimental group of *E. ictaluri* immunized/challenged fish were two-fold serially diluted with PBS in the microtitre plate wells. Sera were tested in duplicate wells in microtitre plates coated with the *E. ictaluri* antigens of the immunizing strain (homologous) and separately in plates coated with the challenge strain, for groups that were challenged with a heterologous strain of *E. ictaluri*. A minimum of two pooled serum samples representing each group was tested and the mean ELISA antibody titre for the respective group was estimated. Known positive (catfish anti-*E. ictaluri*) serum from pooled catfish hyperimmunized by an i.p. injection and negative serum from naïve catfish were serially diluted in each plate as controls. Following incubation for 1 h at room temperature, the plates were washed with PBST and goat anti-catfish IgM (prepared by us) diluted 1:200 in PBS

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was added (100 μL well^{-1}) to each well and incubated for 45 min at room temperature. The plates were washed with PBST and horseradish peroxidase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:2000 in PBS (100 μL well^{-1}) was added, and the plates were incubated for 1 h at room temperature. Plates were washed once more and the chromogenic substrate, 3,3′,5,5′-tetramethylbenzidine (TMB)-Ultra (Pierce, Rockford, IL, USA) was added to visualize the reactions. The reactions were stopped by adding 1 M sulphuric acid (50 μL well^{-1}), and the absorbency was read at 450 nm using a microplate reader (BioRad, Hercules, CA, USA). The endpoint serum titre was determined as the highest dilution that gave an absorbance reading above the cutoff (negative controls).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described previously (Panangala *et al.* 2006) using the Laemmli (1970) buffer systems and Criterion (BioRad) 12.5% precast slab gels. Briefly, 30 μg of each *E. ictaluri* protein sample (~ 3 μg μL^{-1}) in Laemmli (1970) sample buffer (2% w/v SDS, 1 M Tris HCl, pH 6.8, 10% v/v glycerol and 0.005% w/v bromophenol blue) was loaded onto the gel-wells and electrophoresed at 75 V (constant voltage) at room temperature for ~3.5 h. *Edwardsiella ictaluri* protein profiles were visualized by staining the gels with 0.25% Coomassie brilliant blue R-250 in 50% v/v methanol and 10% v/v acetic acid in distilled water. Unstained gels containing *E. ictaluri* proteins were electroblotted onto nitrocellulose membranes according to the method of Towbin, Staehelin and Gordon (1979), using a Criterion (BioRad) electroblotting unit with a setting of 100 V for 30 min. The nitrocellulose membranes were equilibrated in Starting-Block (Pierce, Rockford, IL, USA) blocking buffer for 30 min. The blots were rinsed and immersed for 1.5 h in 1:40 or 1:50 dilution of pooled catfish anti-*E. ictaluri* antiserum obtained from the different immunized/challenged groups of fish separately. A goat anti-catfish second antibody (prepared by us) at a dilution of 1:400 was added and incubated for 1 h at room temperature and the blots were rinsed as before. Finally, the blots were incubated for 1 h in horseradish peroxidase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:4000, and specific antibody-bound epitopes were detected with the chromogenic substrate 4-chloro-1-naphthol (BioRad) used according to the instructions provided.

Statistical analysis

The relative per cent survival (RPS) was calculated according to the formula derived by Amend (1981) as follows:

$$\mathrm{RPS} = \left(1 - \frac{\%\text{vaccinate mortality}}{\%\text{ control mortality}}\right) \times 100$$

Comparative survival rates between groups were statistically analysed using normal proportions or Fisher's exact test (Snedecor & Cochran 1989), and a *P*-value of ≤ 0.05 was considered to be significant.

Results

Low-dose immunization and challenge

Mortality among fish in each of the four trials (A–D) following immunization ranged between 10% and 20% within the initial observation period of 9–14 days. Trial C had the highest mortality, ~20%. Thereafter, the surviving fish were healthy and robust until the challenge exposure on day 28. The comparative percentage survivals of fish following challenge in each respective trial are presented in Fig. 1a–d. The highest mortality following challenge occurred within days 3–7 in all the trials. In trial A, all the placebo BHI-treated and challenged fish (groups 5 and 6) were dead within the first seven days following challenge (Fig. 1a).

In trial A (Table 1), fish in group 1, immunized with *E. ictaluri* strain AL-93-58 and challenged with the homologous strain, the RPS was 100%. The RPS for the heterologous challenge with *E. ictaluri* strain S-94-1051 (group 4) was 96%. Likewise, in group 2, immunization and challenge with the homologous strain S-94-1051 resulted in an RPS of 100% compared with an RPS of 99% with the heterologous (strain AL-93-58) challenge (group 3). The protection conferred by either homologous or heterologous immunization was not significantly different ($P \geq 0.05$) between the two strains AL-93-58 (groups 1 and 4) or S-94-1051 (groups 2 and 3). However, when compared with the BHI-treated controls (groups 5 and 6) challenged with *E. ictaluri* strains AL-93-58 or

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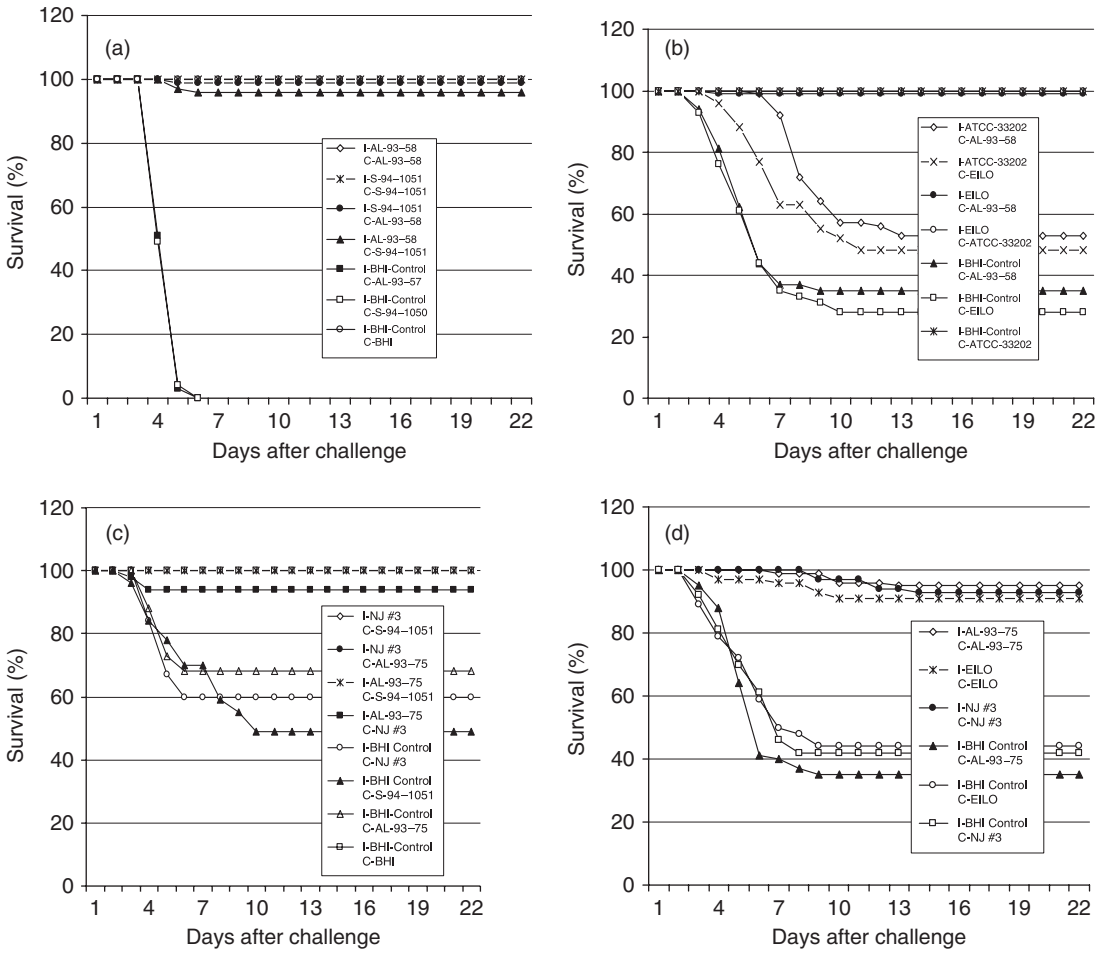

Figure 1 (a–d) Cumulative per cent survival of channel catfish in each group following immunization and challenge with different *Edwardsiella ictaluri* strains. The survival rates are schematically presented for each of the trials a, b, c and d from the day of challenge (day 1) until the experiment was terminated on day 22.

S-94-1051, the protection conferred by either strain was significantly ($P \leq 0.05$) high.

In the second trial (Table 1, Trial B), immunization with *E. ictaluri* strain ATCC-33202, followed by challenge with either strain AL-93-58 (group 1) or strain EILO (group 2), resulted in an RPS of 27.7%. Immunization with strain EILO and challenge with strain AL-93-58 resulted in an RPS of 98.6% (group 3) or with ATCC-33202 an RPS of 100% (group 4). A significant difference ($P \leq 0.05$) was observed in the RPS between group1 (immunized with strain ATCC-33202) and group 3 (immunized with strain EILO) when the challenge strain was AL-93-58. Further, a significant difference ($P \leq 0.05$) in the RPS was observed between group 2 (immunized with strain ATCC-33202) compared with group 4 (immunized with EILO). An equivalent challenge dose of either strains EILO (group 2) or AL-93-58 (group 3), when compared with the placebo BHI-treated control groups 5 and 6, demonstrated a moderately significant ($P = 0.019$) and a highly significant ($P = 0.001$) difference, respectively, in the RPS. No mortality was observed in the BHI-treated fish in control group 7 that were challenged with strain ATCC-33202.

In trial C (Table 1), immunization with *E. ictaluri* strain NJ #3 conferred the same degree of protection against challenge with either strain S-94-1051 (group 1) or strain AL-93-75 (group 2) (RPS = 100%). When the immunogen was strain AL-93-75 and the challenge strain was S-94-1051 (group 3), the RPS was 100% and when the challenge strain was NJ #3 (group 4), the RPS was 81%. However, when control groups 5, 6 and 7 were exposed to strains NJ #3 or S-94-1051 or AL-93-75, respectively, mortality ranged from 32% to 51%. When compared with the placebo-treated controls (groups 5, 6 and 7),

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protection in the vaccinates (groups 1, 2, 3 and 4) was highly significant ($P \leq 0.001$).

In trial D (Table 1), immunization with the *E. ictaluri* strain AL-93-75, followed by challenge with the homologous strain, resulted in an RPS of 92.5% (group 1) as compared with immunization and challenge with the homologous strain EILO, with a RPS of 83.9% (group 2) and NJ #3 with an RPS of 87.9% (group 3) respectively. A moderately significant difference ($P = 0.013$) was observed between groups 1 and 2 in the RPS. However, there was no significant ($P = 0.731$) difference between group 1 (immunized and challenged with strain AL-93-75) and group 3 (immunized and challenged with NJ #3). Similarly, no significant difference was observed between group 2 (immunized and challenged with EILO) and group 3 ($P = 0.547$). However, an equivalent challenge dose of the same three *E. ictaluri* strains resulted in mortality that ranged from 56% to 64% in the control groups 4, 5 and 6. With the exception of strain ATCC-33202, overall, immunization with a low dose of any virulent *E. ictaluri* field strains protected equally well compared with the control groups irrespective of the challenge being performed with either a homologous or a heterologous strain.

ELISA antibody titres post challenge

The antibody titres of the immunized and challenged groups were between 1:80 and 1:320 (Table 2). The titre was 1:320 when the pooled serum was tested against the heterologous antigen (AL 93-58) in trial A group 3, and against the homologous antigen (ATCC-33202) in trial B, group 2. The titres in the remaining groups were between 1:80 and 1:160 among the different groups when antibody titres were measured against the homologous or heterologous antigens (Table 2). Overall, the ELISA antibody titers remained considerably low in all groups and showed no appreciable difference in titre irrespective of the antigen (homologous or heterologous strain of *E. ictaluri*) used for testing. In the BHI-treated and challenged groups, the antibody titers ranged between 1:20 and 1:80 with an antibody titre of 1:40 in the majority of samples. In most of the tests ($\sim$ 90%), the titre was 1:5120 for positive control serum from hyperimmunized fish.

Protein and antigen profile of *E. ictaluri* strains

A relatively homogeneous banding pattern was observed among all strains in SDS-PAGE-resolved solubilized whole bacterial suspensions (Fig. 2). In Western blots of SDS-PAGE-resolved profiles of the bacteria probed with pooled serum from immunized and challenged catfish, a typical reaction pattern with high-molecular-mass epitopes that were relatively uniform among the six *E. ictaluri* strains [Fig. 3a and b (only two of 15 blots are shown)] was revealed. The high-molecular-mass reaction patterns were typical of LPS antigens. A faint band at a low-molecular-mass position ($\sim$ 35 kDa) was observed in all blots.

Discussion

Efforts to reduce fish production losses due to ESC of channel catfish, have spurred a quest for prophylactic vaccines and vaccination methods (Saeed & Plumb 1986; Klesius & Shoemaker 1997; Shoemaker & Klesius 1997; Vinitnantharat, Gravningen & Greger 1999). The fact that vaccines composed of attenuated bacteria are far superior to killed bacteria (bacterins) or bacterial products (antigenic components) as immunogens are well founded (Klesius & Shoemaker 1999; Shoemaker, Klesius & Bricker 1999; Seder & Hill 2000; Janeway, Travers, Walport & Shlomchik 2001). An ideal vaccine would be one that would be arrayed with the widest spectrum of antigenic determinants (epitopes) that would confer a protective immune response in the broadest population within a species (Janeway *et al.* 2001). An objective of this research was to determine the degrees of protection conferred by different *E. ictaluri* strains and to identify any apparent diversity or homogeneity in the putative antigenic determinants widely shared among strains. Our approach took advantage of previous studies that showed that exposure to low doses of live *E. ictaluri* results in strong acquired immunity (Klesius & Shoemaker 1997; Shoemaker & Klesius 1997). In this study, initial immunization with low doses of the organism resulted in 10–20% mortality. This result was expected, because at the inception of the experiments, the fish originated from a naïve population with no previous exposure to *E. ictaluri* as confirmed by bacteriological testing of random samples. With one exception (i.e., immunizing strain ATCC-33202), in the challenge experiments of catfish we observed significant protection following immersion exposure to low doses of virulent *E. ictaluri* irrespective of whether the challenge strain of *E. ictaluri* was homologous or heterologous. The relative per cent survival was significantly higher compared with

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Table 2 ELISA antibody titres of immunized and challenged channel catfish pooled-sera sampled at termination of experiment

Trial	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Positive control	Negative Control
A									
I	AL-93-58	S-94-1051	S-94-1051	AL-93-58	BHI	BHI	BHI		
C	AL-93-58	S-94-1051	AL-93-58	S-94-1051	AL-93-58	S-94-1051	BHI		
Ab titre (Ho)	1:80	1:160	1:160	1:80	–	–	1:40	1:5120	1:20
Ab titre (Het)	–	–	1:320	1:160	–	–	–	1:5120	1:20
B									
I	ATCC-33202	ATCC-33202	EILO	EILO	BHI	BHI	BHI		
C	AL-93-58	EILO	AL-93-58	ATCC-33202	AL-93-58	EILO	ATCC-33202		
Ab titre (Ho)	1:160	1:320	1:80	1:160	1:40	1:80	1:40	1:5120	1:20
Ab titre (Het)	1:80	1:160	1:80	1:80	–	–	–	1:5120	1:20
C									
I	NJ #3	NJ #3	AL-93-75	AL-93-75	BHI	BHI	BHI		
C	S-94-1051	AL-93-75	S-94-1051	NJ #3	NJ #3	S-94-1051	AL-93-75		
Ab titre (Ho)	1:80	1:160	1:80	1:80	1:80	1:40	1:40	1:10 240	1:20
Ab titre (Het)	1:160	1:160	1:80	1:80	–	–	–	1:10 240	1:20
D									
I	AL-93-75	EILO	NJ #3	BHI	BHI	BHI	-		
C	AL-93-75	EILO	NJ #3	AL-93-75	EILO	NJ #3			
Ab titre (Ho)	1:160	1:160	1:80	1:20	1:40	1:40	–	1:5120	1:20

I, = Immunizing strain; BHI, = Brain Heart Infusion medium. Values expressed as the mean antibody (Ab) titer of simultaneously tested paired-samples of pooled serum within each respective group against antigens of homologous (Ho) and/or heterologous (Het) strains of *Edwardsiella ictaluri*; C, = Challenge strain; Ho, = Homologous; Het, = Heterologous.

the BHI-treated control groups that were challenged with the same dose of homologous or heterologous *E. ictaluri* strain, except in trial B, where the BHI-treated group 7 showed no mortality following challenge with strain ATCC-33202. It has been acknowledged previously that *E. ictaluri* strain ATCC-33202 representing the type strain of this genospecies undergoes numerous *in vitro* passages in culture (Panangala *et al.* 2005). Therefore, it is likely that this strain has undergone some change that has rendered it less immunogenic and less virulent than the other field strains. Klesius and Shoemaker (1997) found that differences exist among *E. ictaluri* strains in their ability to confer protective immunity against a heterologous challenge. Our results, however, have demonstrated otherwise, i.e., there appear to be no differences among strains in their ability to confer protection against either a homologous or a heterologous *E. ictaluri* challenge. Nonetheless, it should be pointed out that Klesius and Shoemaker (1997) used the Marion strain of channel catfish at 10–12 months of age and weighing 25–30 grams in their studies. Although five of the strains used by Klesius and Shoemaker (1997) (AL-93-75, AL-93-58, S-94-1051, ATCC-33202 and EILO) were the same as those used in the present study, the challenge dosage of the bacteria was higher in that (Klesius & Shoemaker 1997) study. Thus, apparent dissimilarities in the two studies may not yield an accurate comparison.

Many studies have examined the humoral antibody response of channel catfish to a variety of bacterial and viral antigens (Heartwell III 1975; Thune, Fernandez & Battista 1999; Moore & Thune 2002; Nasbaum, Smith, DeInnocentes & Bird 2002; Harbottle, Plant & Thune 2005). Their results indicate that despite the occurrence of protection in channel catfish to challenge, the antibody responses to the cognate bacterial and/or viral antigens are not substantially high. In general, our findings agree with this observation. Admittedly, there was a measurable serum antibody titre on day 22 post challenge among all *E. ictaluri*-immunized and challenged groups. But these titres were very low compared with the findings of others (Baldwin, Collins & Newton 1997). Immunization and challenge studies with other species of fish have also revealed measurable but low antibody levels notwithstanding appreciable degrees of protection. To cite a few examples, antibody responses in primed and immersion-immunized European sea bass (*Dicentrarchus labrax* L.) (Angelidis, Karagiannis & Crump 2006) and gadoid haddock (*Melanogrammus aeglefinus* L.) immunized against the bacterial pathogen *Vibrio anguillarum* (Corripio-Miyar, Mazorra de Quero, Treasurer, Ford, Smith & Secombes 2007). Catfish, like

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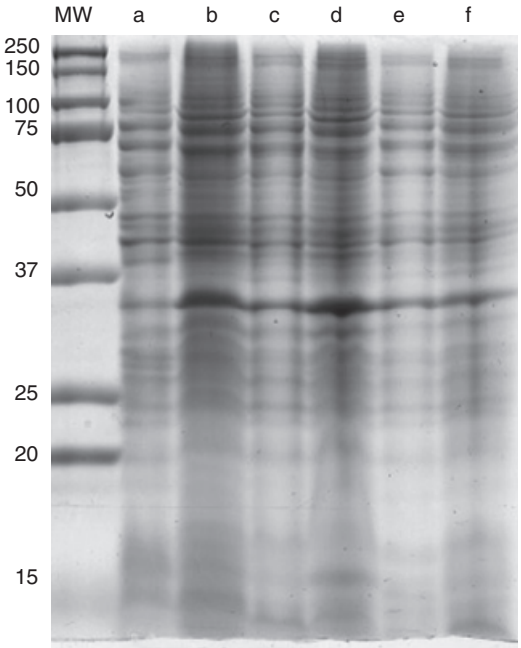

Figure 2 Coomassie blue-stained comparative Sodium dodecyl sulphate-polyacrylamide gel electrophoresis-resolved whole-cell profiles of the six *Edwardsiella ictaluri* strains used for immunization and challenge arranged in lanes: (a) AL-93-58; (b) ATCC-33202; (c) NJ#3; (d) EILO; (e) AL-93-75; and (f) S-94-1051. No apparent differences were seen in the protein profiles of the six *E. ictaluri* strains. Recombinant Precision Plus (BioRad) prestained molecular weight markers are shown in the lane marked MW (kDa).

other teleosts, possess a tetrameric IgM as the major serum immunoglobulin in addition to a homologue of the mammalian IgD (Bengten, Clem, Miller, Warr & Wilson 2006). However, the IgD-like gene is chimeric and contains the Cμ1 exon, which is critical for incorporation of an internal disulphide (Wilson, Bengten, Miller, Clem, Du Pasquier & Warr 1997). There is no evidence yet for true Ig-class switching (VHDJH rearrangement) in catfish or any other teleost (Cannon, Haire, Rast & Litman 2004; Bengten *et al.* 2006). Perhaps due to this reason affinity maturation of the antibody response in teleost remains a moot point.

SDS-PAGE profiles of all six *E. ictaluri* strains revealed a uniform banding pattern, analogous to the relatively homogeneous banding patterns observed in previous studies (Plumb & Klesius 1988; Panangala *et al.* 2006). The immunoblots probed with pooled immune serum from immunized and challenged catfish showed a broad band within the high-molecular-mass region, with a very faint band at an apparent molecular mass position of ~35 kDa. The broad banding pattern is reminiscent of the lipopolysaccharide (LPS) moieties present in *E. ictaluri* and found to be reactive with immune serum from channel catfish (Baldwin *et al.* 1997; Arias, Shoemaker, Evans & Klesius 2003). Considerable evidence from other studies with Gram-negative organisms and the immune response in fish (Espelid, Rødseth & Jørgensen 1991; Lund, Børdal, Kjellsen, Mikkelsen & Schrøder 2006; Nikoskelainen, Verho, Järvinen, Madetoja, Wiklund & Lilius 2007), as well as other vertebrates (Lucas 2004; Munford & Varley 2006) supports the conclusion that bacterial polysaccharide antigens are capable of stimulating an efficient innate and adaptive immune response in the hosts. A growing body of evidence regarding the different components that contribute to the catfish immune system argues in favour of remarkably well-developed innate and adaptive immune mechanisms that are capable of defensively responding to invading pathogens (Plouffe, Hanington, Walsh, Wilson & Belosevic 2005; Bengten *et al.* 2006).

Attempts to explain immune-protection in channel catfish exposed to *E. ictaluri* have suggested various mechanisms implicating different components of the immune system. In the wake of more recent knowledge, we postulate a scenario in the catfish immune response in the event of *E. ictaluri* infection that hinges on the coordinate interaction of many components. Upon initial encounter with *E. ictaluri*, the innate immune mechanisms comprised of antimicrobial peptides, lysozyme, Toll-like receptors (TLRs 2, 3 and 5) (Bilodeau, Peterson & Bosworth 2006; Baoprasertkul, Peatman, Abernathy & Liu 2007), complement (Nonaka & Smith 2000), acute-phase proteins and cytokines such as TNF-α, (Zou, Secombes, Long, Miller, Clem & Chinchar 2003) and NK cells (Shen, Stuge, Bengten, Wilson, Chinchar, Naftel, Bernanke, Clem & Miller 2004) attempt to stem the infection. The TLRs (perhaps TLR2 or a homologue of TLR4) react with LPS and signals the presence *E. ictaluri*. A receptor on macrophages (a putative homologue of CD 14, a receptor for bacterial LPS) then reacts with *E. ictaluri*, enabling the macrophages to phagocytose the bacterium (Shoemaker, Klesius & Plumb 1997; Booth, Elkamel & Thune 2006). Once this line of defence is overwhelmed, the macrophages and co-stimulatory signal transduction molecules express in the adaptive immune response, which includes *E. ictaluri* antigen processing by the macrophages and presentation of the antigens in the context of the relevant class I (Antao,

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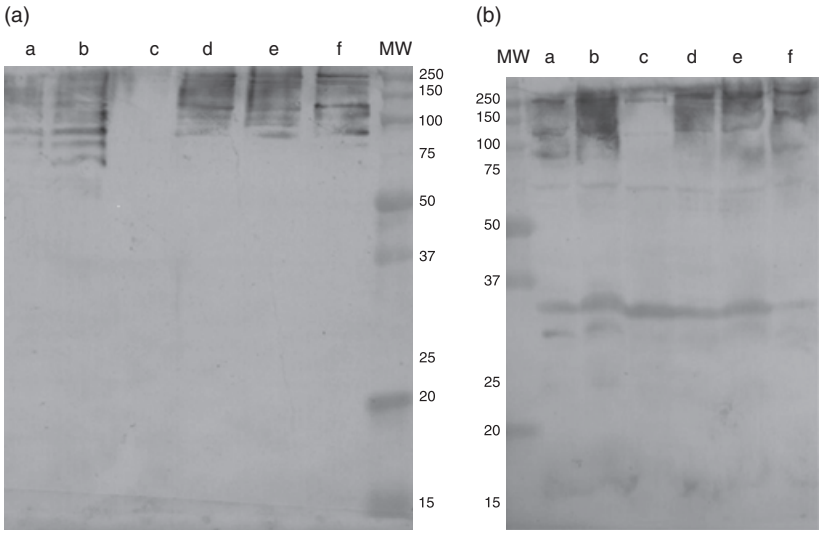

Figure 3 (a) Immunoblots of *Edwardsiella ictaluri* strains arranged in lanes: (a) AL-93-58; (b) ATCC-33202; (c) NJ #3; (d) EILO; (e) AL-93-75; and (f) S-94-1051 probed with pooled serum taken on day 22 from channel catfish immunized with *E. ictaluri* strain S-94-1051 and challenged with strain AL-93-58 (trial A, group 3); (b) Immunoblot of *E. ictaluri* strains arranged in lanes as in (a) and probed with pooled serum from catfish immunized with *E. ictaluri* strain NJ #3 and challenged with strain NJ #3 (trial D, group 3). Recombinant Precision Plus (BioRad) prestained molecular weight markers are shown in the lane marked MW (kDa). A broad band characteristic of LPS was observed within the high-molecular-mass region of all lanes in all blots (only two of 15 shown). A faint band at an apparent molecular mass position of 35 kDa was present in all blots.

Wilson, Wang, Bengten, Miller, Clem & Chinchar 2001; Peatman, Terhune, Baoprasertkul, Xu, Nandi, Wang, Somridhivej, Kucuktas, Li, Dunham & Liu 2008) or class II (Goodwin, Flores, Quiniou, Wilson, Miller, Clem & McConnell 2000) major histocompatibility complex (MHC) molecules for recognition and response by the appropriate sub-populations of T and B cells. This inference was reached through analysis of previous studies reviewed herein and is worthy of further empirical investigation. The present findings indicate that with the exception of strain ATCC-33202 (type strain), there is no difference among *E. ictaluri* field strains in their ability to confer immune protection against each other. Our previous studies suggested that *E. ictaluri* is comprised of a single clonal population based on 16S–23S ISR of the rRNA operon (Panangala *et al.* 2005) and phenotypic characterization (Panangala *et al.* 2006). Therefore, logically, immunization with a single *E. ictaluri* strain should provide protection against most *E. ictaluri* strains that are present in an aquatic environment.

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