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Comparison of the efficacy of selected bacterins against *Edwardsiella tarda* in immunized Japanese eel (*Anguilla japonica*)

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Abstract

An effective vaccine against *Edwardsiella tarda* has not been reported in substitution for high concentration of formalin for the prevention of edwardsiellosis disease. In this study, the efficacy of inactivated *E. tarda* was evaluated and compared by intraperitoneal (IP) injection-immunization or challenge against Japanese eel (*Anguilla japonica*). Formalin, formalin with heat, citric acid, pressure and electric current were used for inactivation of the bacteria, and the relative percent survival (RPS) values of pressure (600 psi for 5 min) killed cells was determined. PKC-inactivated vaccine showed-89-93 protection that was higher than others. PKC-inactivated vaccine at a concentration of 10^6 cells/fish was sufficient to induce high protection (RPS>89). Protection of the different-inactivated vaccines was evaluated at different time post immunization, and the peak of protection was observed at 9 days post-challenge. Fish immunized with PKC showed significantly ($P<0.05$) higher serum and mucus antibody titers elicit both systemic and mucosal adaptive immune responses, and induce specific humoral immune responses in eel. Coincident with higher protection, sera of fish immunized with the PKC vaccine had higher agglutination titers than FKC, FHKC, CAKC and ECKC. All these data strongly suggested that PKC vaccine is an effective strategy to protect eel against edwardsiellosis.

Keywords: *Edwardsiella tarda*, Protective immunity, Antibody titer, *Anguilla japonica*, Vaccine, Pressure

Introduction

Edwardsiella tarda is an enteric Gram-negative bacterium of the Enterobacteriaceae (Ewing *et al*., 1965), first isolated from pond-cultured eel by Hoshina in 1962. It is the causative agent of the systemic disease edwardsiellosis, which leads to extensive losses in many fresh water and marine water fish worldwide, including many commercially important fish, such as eel (Wakabayashi and Egusa, 1973), channel catfish (Meyer and Bullock, 1973), mullet (Kusuda *et al*., 1976), tilapia (Kubota *et al*., 1981), chinook salmon (Amandi *et al*., 1982), olive flounder (Nakatsugawa, 1983) and carp (Sae-Oui *et al*., 1984). *E. tarda* is widely distributed in nature, having been isolated from reptiles, birds, mammals (Van Damme and Vandepitte, 1984) including humans (Wilson and Waterer, 1989) and environmental water (Pitlik *et al*., 1987) and has been found in 14 countries and 39 states of the USA (Ewing *et al*., 1976). For the treatment and prevention of this infection, the use of biological control methods such as vaccination should be developed.

The use of synthetic chemicals and antibiotics (DePaola *et al*., 1995) for the control of fish disease may result with the emergence of antibiotic-resistant microbes, drug residues and environmental impacts. *E. tarda*, antibiotic resistance has been reported widely in the world (Aoki *et al*., 1989). To limit the use of chemicals and antibiotics, vaccination is highly recommended (Chinabut and Puttinaowarat, 2005).

Over the last decade vaccination has become increasingly important for the prevention of infectious diseases in farmed marine and freshwater fish (Gudding *et al*. 1999). To induce protection against edwardsiellosis, including formalin-killed *E. tarda* bacterin (Gutierrez and Miyazaki, 1994), cellular lipid (Salati and Kusuda, 1986) and lipopolysaccharides, LPS (Salati *et al*., 1987) several studies have been reported.

Several types of experiments were performed on the inactivation of bacteria by heat treatment to induce protection against fish pathogenic bacterium (Kotrola *et al*., 1997). Heat inactivation is not suitable for large-scale treatment and culturing of fish (Mann *et al*., 2001). Numerous high-pressure carbon dioxide or hydrostatic pressure inactivation of bacterial cells are potentially available (Erkmen, 2001), but seems to be difficult to apply to food industry, have little inactivation effects on the bacterial cells (Sonoike, 1997).

Use of pulsed electric fields or low amperage electric current for the inactivation of bacteria has been reported (Jong-Chul *et al*., 2003), the efficacies of those treatments were all evaluated but generated a toxic substrates H_2O_2 (Liu *et al*., 1997), furthermore, represents a major investment for manufacturers. Therefore, in the present study, low concentration of formalin with heat, citric acids, pressure and low amperage electric current inactivation on *E. tarda* would be the most promising way to prevent edwardsiellosis diseases of fish.

The aim of this study was to induce protection of Japanese eel (*Anguilla japonica*) against edwardsiellosis disease by intraperitoneal (IP)-immunization of inactivated-*E. tarda* vaccine. The efficacies of the vaccines prepared by different methods were compared and the optimal conditions for intraperitoneal immunization were established.

Materials and Methods

Bacterial strain and growth conditions: *E. tarda* strain V-1 was originally isolated from kidney of diseased eel (*Anguilla japonica*) in Japan. Sixty-one different serotypes of *E. tarda* have been differentiated according to the O-antigen (Tamura *et al*., 1998). *E. tarda* strain V-1 was temporally differentiated into serotypes by a cross absorption test of the O-antigen (Tamura *et al*., 1998). Different serotype strains of *E. tarda* V-1 strain was used as the strain for antigen preparation and infection to test the vaccine efficacy against edwardsiellosis (Liu *et al*., 2005). The bacterial strain *E. tarda* V-1 used in this study to prepare a vaccine, was pre-cultured for 24 h at 30ºC in brain heart infusion (BHI, Difco) broth and was inoculated into 1000 ml BHI broth, cultured with shaking at 30ºC for 18 h. The cells were harvested by centrifugation at 4000×g for 15 min at 4ºC and were stored at −80ºC freezer until used.

Preparation of inactivated bacterin: Either formalin, formalin combined with heat, citric acid, pressure or low amperage electric current were used to inactivate *E. tarda* separately. Formalin or citric acid was added into bacteria supernatant to a final concentration of 0.1% to 0.9% separately. For the second means of inactivation, formalin was mixed with bacteria supernatant to a final concentration of 0.05% or 0.1% and heated with 60ºC or 70ºC for 10 min. The time needed to achieve the treatment with pressure (French pressure cell press, or French press; No 5501, OHTAKE Mfg, 941, Tokyo, Japan) was approximately 200-1600 ksc. The decompression time was approximately 1-10 min. For the electric current inactivation, the current in the range of 1-150 mA at 12v DC was applied for 1-30 sec (Jong-Chul *et al*. 2003). The electrolysis vessel with two electrodes was used to inactivate bacteria supernatant that was connected with electrophoresis machine. After inactivation, the bacterial culture was incubated separately at 15ºC for one day to perform inactivation (Table 1).

The vaccines were confirmed to be completely inactivated by viable counts, showing moderate antigenicity, expressing major protective antigen by sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE (Fig. 4) and Western blot analysis (Fig. 4 and Table 1), were used for the following immunization tests.

Safety test of vaccine: Two experimental groups: vaccinated pressure (600 psi for 5 min)-killed cells, PKC; formalin (0.4%)-killed cells, FKC; formalin (0.1%) with heat (70^{o}C for 10 min)-killed cells, FHKC; citric acid (0.9%)-killed cells, CAKC; electric current (100mA for 5sec at 12v DC)-killed cells, ECKC and PBS (phosphate buffer saline) control, each with 20 eels, were used to evaluate the safety of the inactivated antigen preparation. Fish were implanted with visible alphanumeric tags in two different colors (Nacalai Tesque.Inc, Japan) for group identification. Fish were then distributed into 100 l tanks. Fish were starved 24 h before injection, anesthetized by immersion for 2 min in fresh water containing with 0.03 ml/l of 2-phenoxyethanol (ethylene glycol monophenyl ehter $C_6H_5OCH_2CH_2OH$, Nacalai Tesque.Inc, Japan). The vaccine group was injected with the dose as opposed to 10× the designed dosage of 5×10^6 cells/fish of (1 mg per fish) antigen, and the control group was injected with PBS only. Fish were observed for mortality, abnormal swimming behavior, and appetite for 60 days post vaccination (PV). They were then killed for pathological and histopathological examination. This procedure was repeated for each test of vaccine.

Table 1. Summary for effective inactivator of *E. tarda* after different treatments

Inactivation		Killing activity*	Agglutination titer (log_2)[1] (Average±S.D)	Western blot (kDa) Total antigens	Confirmed vaccine (Symbols)
Formalin	0.2	L	15.78 ± 0.41	-	
	0.4	M	14.53±2.72	74, 37+	FKC
	0.9	R	ND		
Formalin (%) combined with heat (for 10 min)	0.05, 60ºC	L	15	-	
	0.05, 70ºC	L	14		
	0.1, 60ºC	L	14	-	
	0.1, 70ºC	M	13.43±1.98	84, 74, 43, 37	FHKC
Citric acid (%)	0.2	L	16	-	
	0.6	L	14	-	
	0.9	M	13.03±1.27	97, 78, 45, 37	CAKC
PKC (ksc for 5 min)	200	L	15.10 ± 1.41	-	
	400	L	14.83 ± 0.76	-	
	600	M	12.90 ± 0.14	37	PKC
	800	R	12.85 ± 0.38	-	
	1,000	R	12.22 ± 0,35	-	
	1,200	R	ND	-	
	1,400	R	ND	-	
	1,600	R	ND	-	
Electric current (mA at 12v DC for 5 sec)	1	L	17.10 ± 0.32	-	
	25	L	15.67 ± 0.71	-	
	50	L	15.23 ± 0.30	-	
	75	L	14.98 ± 1.14	-	
	100	M	14.07 ± 0.54	76, 73, 69	ECKC
	125	R	ND	-	
	150	R	ND	-	

* L, low killing where bacteria still remain alive after 24h; M, moderate killing in 24h, where almost all bacteria were killed within 24h; R, rapid killing in 6h or 12h, where all bacteria were killed by this time.
[1]Figures indicate geometric mean reciprocal log_2 value of the highest dilution of the serum that showed positive agglutination. ND, agglutination value was not determined by autoagglutination.
(-), indicates, no clear bands (remaining high antigenicity) were observed in the bacterial cells inactivated with above treatment. + 37 kDa is the major protective antigen of this bacterium, kDa, kilo Dalton.

Fish and vaccination

As described in Table 1, five independent vaccine groups or one control group were performed. Test fish, Japanese eels *Anguilla japonica* of an average weight 102.8±6.6g (mean±SD, N = 210) were obtained from an eel farm in Yoshikawa at Kochi Prefecture, Japan. The stock had 35 fish and was used for each group. The fish prepared for this study had no previous occurrence of infection with *E. tarda* in this farm. Before immunization, each group of the fish were allowed to adapt for 1 week in a 150 l-tank supplied with well-aerated flowing water at 25°C and fed with 0.5mm commercial dried pellets (Nissui) corresponding to 3% of the fish body weight per day for the entire experiment.

The vaccines used in this study were all prepared by inactivating the culture supernatant of bacteria-infected cells instead of purified bacterial particles. The bacterial supernatant was titrated before inactivation, and the titer of the bacteria ($TCID_{50}$/ml) was used herein to represent the concentration of the inactivated vaccine. To compare the efficacies, the fish were intraperitoneal injection -immunized with same doses (10^6 $TCID_{50}$/ml) of five independent inactivated *E. tarda* vaccines.

After vaccination, fish were maintained at 25°C for 8 weeks. Ten (10) fish in each group were randomly sampled for blood and skin mucus collection at 4 weeks after immunization. The other fish (25 fish/each group) were used in challenge tests. For surface mucus collection, fish were anaesthetization with 0.03 ml/l of 2-phenoxyethanol (ethylene glycol monophenyl ehter $C_6H_5OCH_2CH_2OH$, Nacalai Tesque.Inc, Japan), placed in empty sterile vinyl bags for 2 min, and the secreted mucus was filtered through 0.45 µm pore-size membranes (Millipore), then blood was drawn from the caudal vein. The serum was collected by centrifugation at 1000xg for 20 min and stored at – 20°C until further use.

Serum agglutinating antibody titers

Fish were bled from the caudal vessels at 4 weeks after immunization and the sera was heat treated (44°C, 20 min) to inactivate complement activity (Sakai, 1981). Serum agglutination titers against *E. tarda* were determined by the microtitre method according to Hirst and Ellis (1994). A two-fold dilution series of 25µl of each serum sample was made in PBS in wells of a 96 well microtitre plate. Then 25µl of FKC, FHKC, CAKC, PKC and ECKC suspension containing 10^8 bacteria.ml was added to each well. After 2 h at 25ºC and overnight at 4ºC incubation, titers were scored as the highest serum dilution showing agglutination.

Table 2. Serum agglutinating antibody titer of Japanese eel using eel antiserum by ELISA

Method of inactivation	*Symbol*	Number of fish (tested serum) in each group	Agglutination antibody titer (log2) (Average*±S.D)
Control	CON	10	8.83±0.41
Formalin 0.4%	FKC	10	12.53±2.72
Formalin 0.1% + 70ºC for 10 min	FHKC	10	11.43±1.98
Citric acid 0.9%	CAKC	10	10.03±1.27
Pressure 600 ksc for 5 min	PKC	10	13.93±3.46 *
Electric current 100mA/12v DC for 5 sec	ECKC	10	10.70±1.62
		F-Value	*P*-Value
One-way ANOVA	Serum agglutination antibody titer	4.28	0.004

Asterisk: geometric mean reciprocal log2 value of the highest dilution of the serum that showed positive agglutination. The averages of agglutinating antibody titers of the PKC were significantly different as determined by Tukey's test (P<0·05).

Enzyme linked immunosorbant assay (ELISA) for detecting antibody titers of serum and mucus

Flat-bottomed 96-well plates (Corning) were coated with 50 µl of five independent vaccine groups (4 mg/ml) in PBS for 2 h at 60°C. The plates were then washed thoroughly with PBST (PBS containing 0.1%Tween 20) and blocked with 200 µl of 2% BSA in PBS for 1 h at 37°C. Subsequently, the plates were washed thoroughly with PBST and incubated with 75 µl of eel serum or mucus at 27°C for 30 min. The plates were washed with PBST and incubated with 75 µl of rabbit anti-eel antiserum (1:1000) for 30 min, washed 3 times with PBST, and further incubated with 75 µl of goat anti-rabbit IgG conjugated with alkaline phosphatase (1:1000, KPL, Gaithersburg, MD 20878, USA) for 1 h at room temperature. The plates were washed with PBST and developed with the substrate p-nitrophenyl phosphate in substrate buffer at dark. After 30 min incubation, the optical density was measured at 415 nm using an automated ELISA reader (Bio-Rad).

SDS-PAGE and immunoblotting analysis

Protein electrophoresis was performed by using the method of Laemmli (Laemmli, 1970). The inactivated *E. tarda* V-1 strain was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) which containing 4% stacking gel and 14% separating gel. After staining with Coomassie brilliant blue (CBB) R-250, the total gel of each strain was subjected to Western blotting. The proteins were electrophoretically transferred to nitrocellulose paper (0.45µm pore size, Bio-Rad) by using a semi-dry apparatus (Bio- Rad) as described by (Towbin *et al*., 1979) after blocking with 1% skim milk at 4°C for overnight, the membrane was reacted with rabbit anti-V-1 serum (diluted 1:1000 in PBS containing 0.05% Tween 20 and 5% skim milk) as the first antibody, and then goat antirabbit IgG (diluted 1:2000 in PBS containing 0.05% Tween 20 and 5% skim milk) was used as the secondary antibody. Then the blots were immunostained to procedure a color reaction by using Konica immunostaining HRP-1000 (Konica).

Challenge test

Injection (i.p.) challenge tests were done 6 weeks post immunization and the control fish were challenged accompanied with vaccine groups at the respective time points. The *E. tarda* dose for i.p. challenge test was 5×10^6 $TCID_{50}$/ml and 5×10^3 $TCID_{50}$/ml. Control fish that had been injected with PBS were challenged as well. The cumulated mortality was recorded 12 days post challenge (Table 3). *E. tarda* was confirmed by analysis of kidney isolates cultured on Salmonella Shigella agar (SS agar, Nissui) and the infection of *E. tarda* was confirmed by observation of black pigments. Slide agglutination titer was also performed to confirmed *E. tarda* using eel antiserum mixed with bacterin on PBS; visible granular clumps (agglutination) were observed under the microscope. The severity was characterized with common morphological anomaly features including reddish spot, external blood hemorrhage, abnormal swimming behavior, and loss of appetite at post vaccination.

Table 3. Efficacy of vaccines against *Edwardseilla tarda* in Japanese eel (*Anguilla japonica*)

Vaccine	Challenge dose cells/fish	Total fish	Dead fish	Number of abnormal fish	Mortality (%)	Abnormality (%)	RPS (%)
CON	(5×10^6)	25	18	5	72	20	-
FKC	,,	25	7	1	28*	4	61
FHKC	,,	25	12	3	48	12	33
CAKC	,,	25	17	3	68	12	6
PKC	,,	25	2	0	8*	0	89
ECKC	,,	25	16	2	64	8	11
	,						
CON	(5×10^3)	25	14	4	56	16	-
FKC	,,	25	4	0	16*	0	71
FHKC	,,	25	7	2	28	8	50
CAKC	,,	25	12	2	48	8	14
PKC	,,	25	1	0	4*	0	93
ECKC	,,	25	10	1	40	4	29

Asterisk: significantly (P<0·05) lower than control

Relative percentage survival, RPS (Croy and Amend, 1977) was calculated from the end cumulative mortalities as:

$$\mathrm{RPS}(\%) = \left(1 - \frac{\text{Mortality of vaccinated group}}{\text{Mortality of unvaccinated control group}}\right) \times 100$$

Acute toxicity of PKC (600 ksc for 5 min pressure-killed E. tarda)

To determine the acute toxicity of the PKC preparation, PKC groups of 10 Japanese eel were intraperitoneally injected with 0, 10, 20, or 40µg PKC respectively (negative controls were injected with PBS). Injected fish were maintained at 25°C and mortality was recorded daily for 2 weeks.

Statistical analysis: Differences in mortality were tested for statistical significance by the Chi-square contingency table test with the Yates' correction (Pagano and Gauvereau, 2000). Data from each treatment were subject to one-way ANOVA or t-test where appropriate. When overall differences were significant (P<0·05), Tukey's test was used to compare the mean values between individual treatments (Zar, 1984). Statistical analysis was performed using the StatPlus 2007 Professional.

Results and Discussion

Safety of the vaccine: The safety of the vaccine was evaluated by injection of a dosage tenfold that used in the treatment study (1 mg per fish). All fish survived with no abnormality in swimming behavior and no observed morphological or pathological changes during the 60 day observation. Fish in the vaccine group consumed about 15% less food in week 1, as also reported by Midtlyng (1994); but then resumed normal intake. Fish in vaccine group developed adhesions in the abdominal cavity at the injection site (data not shown). Fish injected with PBS were not affected, indicating that the adhesion and loss of appetite were possibly related to the injection of vaccine.

Specific antibody titers of serum and mucus: Fish immunized with PKC showed significantly higher both serum and mucus antibody titers than both fish immunized with FKC, FHKC, CAKC, ECKC or control fish (Fig. 1). The agglutinating antibody titers by ELISA of vaccinated and control groups of eel are shown in Table 2. The highest antibody titers were in sera from the PKC vaccinated fish, followed by sera from the FKC, FHKC, ECKC and CAKC vaccinated fish. Differences between the PKC vaccinated fish and the FKC, FHKC, ECKC, CAKC vaccinated fish, PKC was significant ($P<0.05$).

Efficacy of vaccines: To compare the efficacy of differently-inactivated vaccines, the final concentrations of the vaccines during injection (i.p.) immunization were adjusted to 10^6 $TCID_{50}$/ml. There were no abnormalities in any of the vaccinated or control fish before challenge. The fish in each group were then injection (i.p.)-challenged 6 weeks post immunization. The cumulative mortality of the challenged fish was 56-72% for the control group, 16-28% for FKC group, 28-48% for FHKC group, 48-68% for CAKC group, 4-8% for PKC group and 40-64% ECKC group (Fig. 2 and 3). The RPS value of the fish immunized by PKC was 89-93, i.e. much higher than that of the fish immunized by FKC (RPS = 61-71) or other vaccinated group (Table 3). In addition, all efficacy trials the dead fish showed clinical signs typical of Edwardsiellosis disease. No pathogen other than *E. tarda* was isolated from dead fish.

SDS-PAGE and Western blotting analysis of inactivated vaccine: In the SDS-PAGE of the total protein extracted from all inactivated conditions of *E. tarda* V-1 strain, many proteins were detected in each inactivation conditions; however, in the western blotting assay, only the 37, 45, 73, 75, 76 and 97 kDa locations of *E. tarda* V-1 strain was reacted with rabbit antiserum against the FKC, FHKC, CAKC, PKC and ECKC of *E. tarda.* A major protective antigen at 37 kDa was strongly detected by western blotting analysis with PKC of *E. tarda* (Fig. 4).

Acute toxicity of PKC: Intraperitoneal injection of Japanese eel with 10μg or 20 μg (adjusted to a density of 5×10^5 cells/fish) PKC did not result in any mortality. However, 25% mortality occurred in fish injected with 40μg PKC.

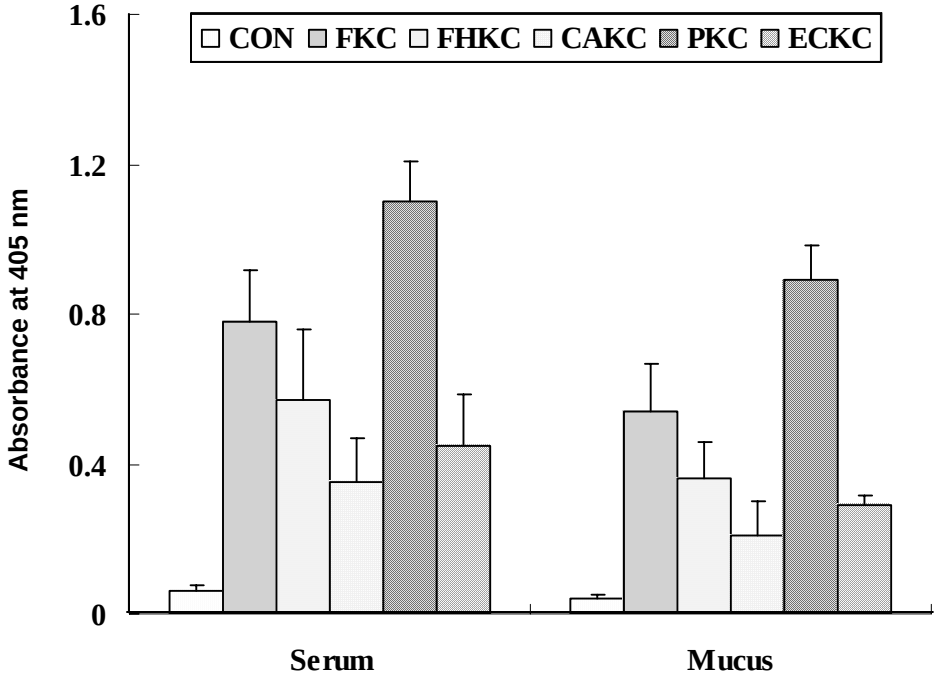

Fig. 1. Serum (dilution ratio 1:50) and cutaneous mucus (dilution ratio 1:20) antibody responses in Japanese eel (*Anguilla japonica*) following i.p. immunization with formalin-killed cell (0.4% formalin, FKC), formalin and heat-killed cells (0.1% formalin and 70°C for 10 min, FHKC), citric acid-killed cell (0.9% citric acid, CAKC), pressure-killed cell (600 ksc/5min, PKC), low amperage electric current-killed cell (100mA/5sec/12v DC, ECKC) and sterile distilled water alone (CON) as determined by ELISA. Asterisks are significantly different as determined by Tukey's test (P<0.05). One-way ANOVA: Serum antibody response: *F*-value (13.38) and *P*-value (0.003).

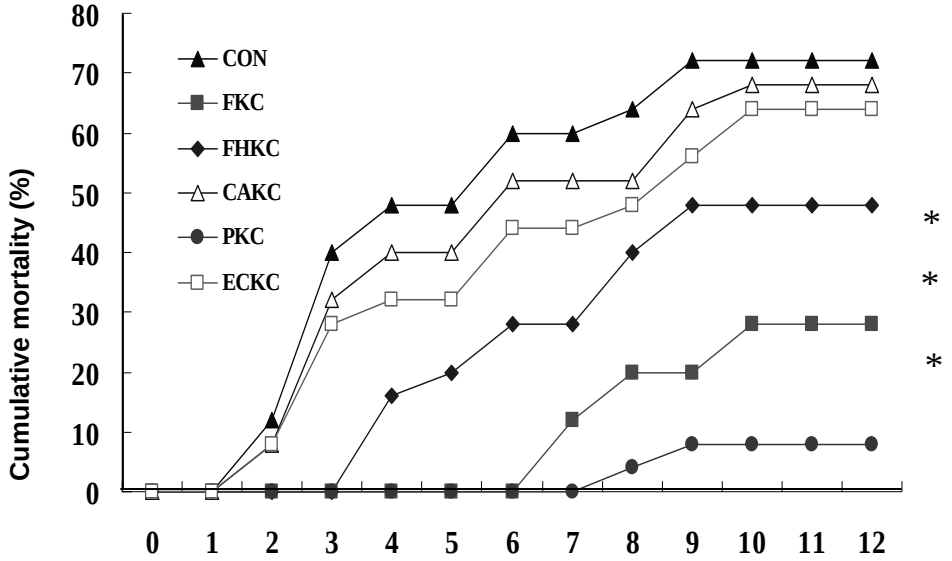

Fig. 2. Cumulative mortality of Japanese eel (*Anguilla japonica*) immunized through intraperitoneal injection with formalin-killed cell(0.4% formalin, FKC), formalin and heat-killed cells (0.1% formalin and 70°C for 10 min, FHKC), citric acid-killed cell (0.9% citric acid, CAKC), pressure-killed cell (600 ksc/5min, PKC), low amperage electric current-killed cell (100mA/5sec/12v DC, ECKC) and sterile distilled water alone (CON) after challenge with *E. tarda* by intraperitoneal injection with $5x10^6$ cells/fish. The mortalities reached 72% by day 12 post-challenge for the groups injected with a high dose. *p< 0.001 (Chi-square)

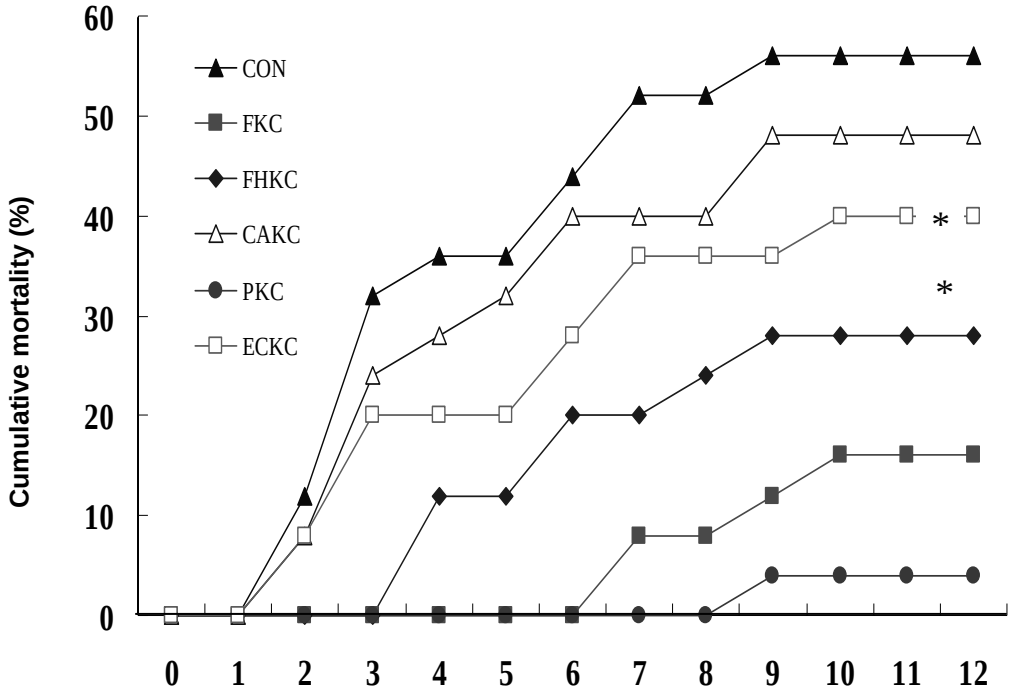

Fig. 3. Cumulative mortality of Japanese eel (*Anguilla japonica*) immunized through intraperitoneal injection with formalin-killed cell(0.4% formalin, FKC), formalin and heat-killed cells (0.1% formalin and 70°C for 10 min, FHKC), citric acid-killed cell (0.9% citric acid, CAKC), pressure-killed cell (600 ksc/5min, PKC), low amperage electric current-killed cell (100mA/5sec/12v DC, ECKC) and sterile distilled water alone (CON) after challenge with *E. tarda* by intraperitoneal injection with $5x10^3$ cells/fish. The mortalities reached 56% by day 12 post-challenge for the groups injected with a high dose. *p< 0.001 (Chi-square)

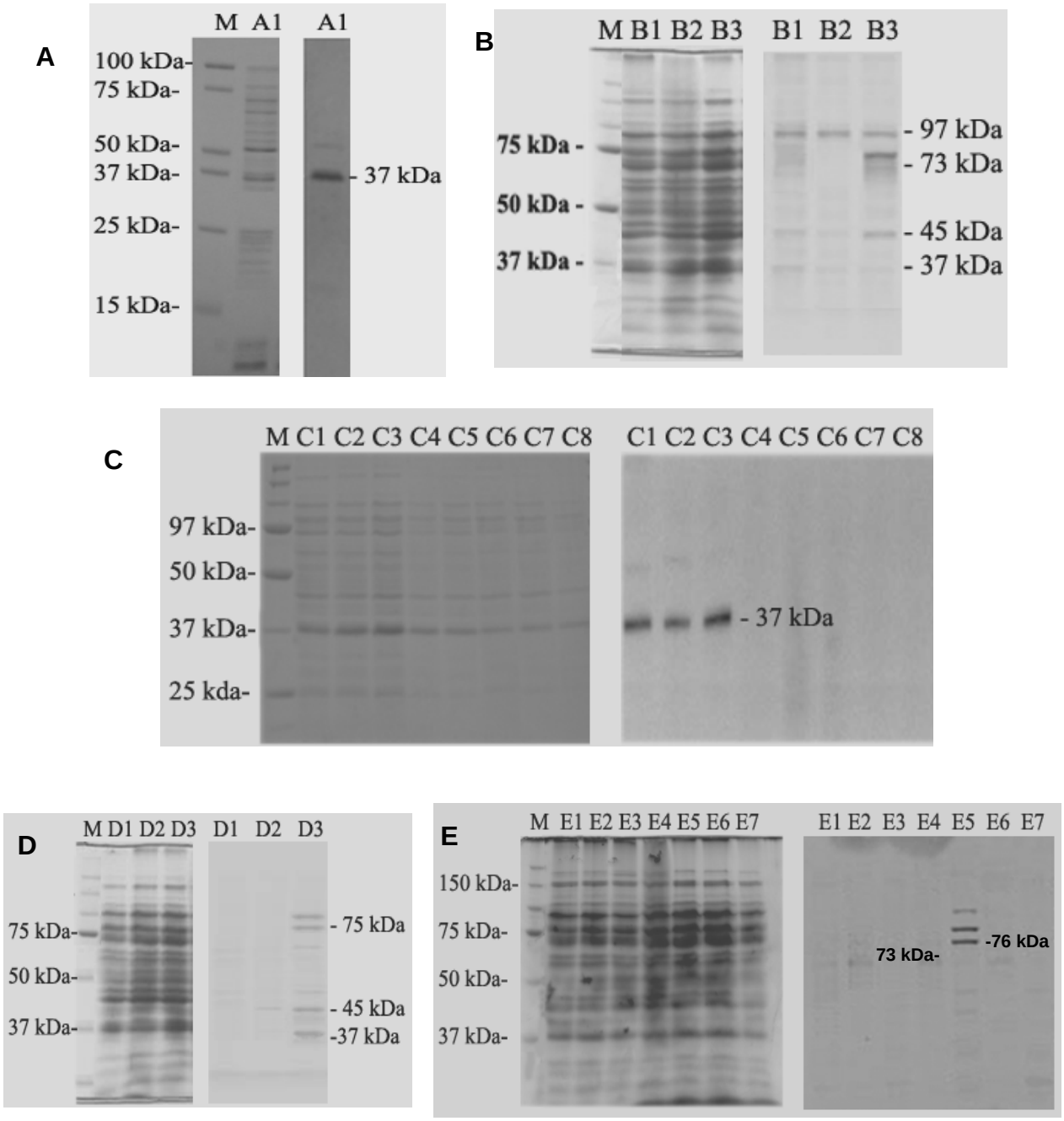

Fig. 4. SDS-PAGE and Western blotting profiles of formalin, citric acid, pressure, formalin combined with heat and electric current inactivated *Edwardsiella tarda* V-1 strain. Left, SDS-PAGE; right, Western blot. M, molecular weight marker; A, formalin (%) [A1 0.4]; B, citric acid (%) [B1 0.3, B2 0.6 and B3 0.9]; C, pressure (ksc/5 min) [C1 200, C2 400, C3 600, C4 800, C5 1000, C6 1200, C7 1400 and C8 1600]; D, formalin with heat (D1 0.05% at 70ºC for 10 min, D2 0.05% at 60ºC for 10 min and D3 0.1% at 70ºC for 10 min); E, electric current (mA/5 sec/12v DC) [E1 1, E2 25, E3 50, E4 75, E5 100, E6 125 and E7 150)

The problems of vaccination with formalin-killed vaccine against bacterial infection have been reviewed by a number of authors (Shin *et al*., 2007). Until recently, they related to a limited understanding of the immune response - in particular the role of the systemic and mucosal adaptive immune responses - and the relationship of both the humoral and cell-mediated systems to the vaccination with low concentration of formalin with heat, citric acid, pressure and electric current-killed vaccine against *E. tarda* infection. Studies have not revealed to successful vaccination in these areas.

More recent studies of protective efficacy in fish with inactivated *E. tarda* against edwardsiellosis has been reported (Swain *et al*., 2007) but until recently there has been a paucity of inactivation on *E. tarda* with low concentration of formalin with heat, citric acid, pressure and electric current in constitutions of high concentration of formalin.

This study has reported that five independent vaccine preparations from *E. tarda* cells were immunogenic, with the development of antibody reactivity against *E. tarda* infection, including the major protective protein 37 kDa (Kawai *et al*., 2004), although their identity remains to be confirmed by monoclonal antibodies. Evidence of protective efficacy in eel by IP vaccination with PKC indicated the acquisition of passive immunity. Additionally, antigenicity superiority was also found in PKC-inactivated *E. tarda* vaccines (RPS = 89-93), whereas the RPS values of formalin-inactivated *E. tarda* vaccines were 61-71, which happily avoids morbidity of eels encourages further investigation of this vaccine in several species.

All vaccines had a wide representation of bacterial antigens, including the major protective protein. It was encouraging to be positively identified serum and mucus antibody titers, this showed significantly higher in PKC than FKC, FHKC, CAKC or ECKC, it elicited both systemic and mucosal adaptive immune responses, and more favorable to induce specific humoral immune responses. The vaccines of many bacteria prepared with differently inactivated were reported to be antigenicically superior and induce specific antibodies or systemic humoral immune responses in several fish species (Romalde *et al*., 2004).

It was encouraging that PKC gave a better protection than other vaccines in the in vivo experiments - which may be due to the preservation of antigenicity by pressure. However, another possibility is that quantity of the protective antigens 37 kDa (Kawai *et al*., 2004) in this preparation is higher, destroyed less of the protective epitopes, particle structure between the vaccines, mechanisms of inactivation (Taisuke *at al*., 2003), and antigen form (Schirmbeck *et al*. 1995). Method of the vaccine preparation may vary to allow increased protein content and PKC could also raise bacteria-specific antibody titres above the rather low levels observed in other vaccine preparation by ELISA. Antibody titer measured by ELISA was useful in evaluating the efficacy of vaccines in several fish species (Gudmundsdottir *et al*., 2003). Elicited antibody titers or immunogenicity of the inactivated vaccine have been reported against Gram-negative bacteria in fish as well as in mammals (Rahman and Kawai 2000, Swain *et al*. 2002, Lan *et al*., 2007). In addition ELISA data in this study also indicated that the immune response in vaccinated eel correlated well with the protection efficacy.

The observed increased in the survival rate of PKC of any cause and the strong protection against severe edwardiselossis due to *E. tarda* indicate the potential to induce protective adaptive immunity of the PKC vaccine than other vaccine. Although the present survival rates were positively correlated to serum and mucus antibody titers, it cannot be excluded that the cell-mediated immunity may be involved in the protection. In fish, a number of studies have associated increased in the survival rate with high antibody titers (Ashida *et al*., 1999, Castro *et al*., 2008, Carrias *et al*., 2008).

In summary, PKC bacterins to vaccinate eel demonstrated that a single dose of the vaccine was sufficient to induce an immune response and prevent disease in eel. Vaccination of eel contributes to the understanding of vaccination in the health management of fish farming. Further investigations are required in order to verify the ability of PKC bacterin to induce specific cell-mediate immunity in fish or improve antigenicity, and to develop a commercial product, the optimal antigen formulation and vaccination dose.

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