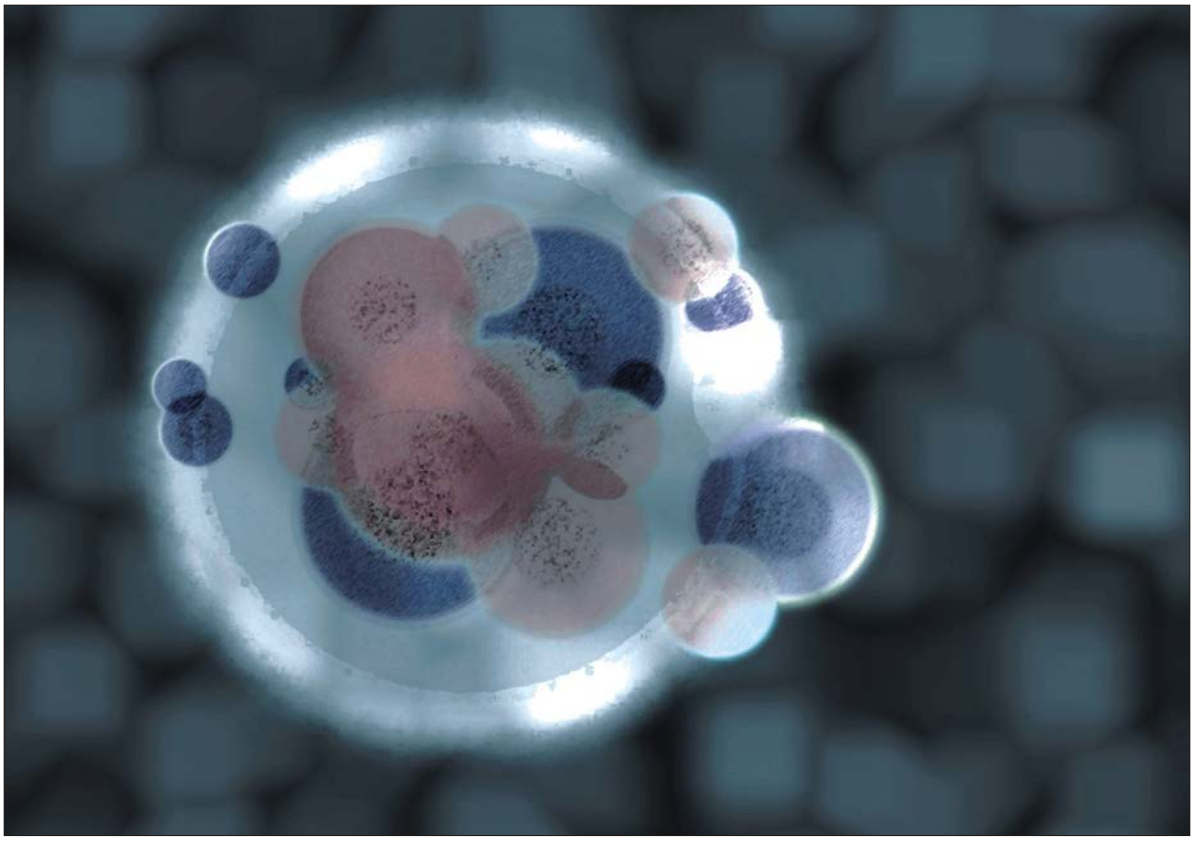

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Effective Inactivation of *Edwardsiella tarda* for the Development of Vaccine for Fish

Md. Mer Mosharraf Hossain, Kenji Kawai and Syunichirou Oshima
Department of Aquaculture, Faculty of Agriculture, Kochi University,
Nankoku Shi, Kochi 783-8502, Japan

Abstract: To find out adequate inactivator in substitution for the high concentration of formalin, different organic and inorganic chemicals, heat and chemicals combined with heat were tested for the inactivation of a fish-pathogenic bacterium *Edwardsiella tarda* strain. The survivality, sustainability of the cell surface antigenicity, bacterial protein and the total cell antigens of *E. tarda* were determined after treated with those inactivators. A major antigen at 37 kDa was detected by Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Formalin (0.1%) with heat (70°C×10 min) and 0.9% citric acid killed *E. tarda* resulted moderate killing activity with enough antigen-sustainability. This study suggests that formalin (0.1%) combined with heat (70°C×10 min) and citric acid (0.9%) killed *E. tarda* as a new vaccine candidate.

Key words: Survivality, antigenicity, chemicals, heat, fatty acids, vaccine

INTRODUCTION

Edwardsiella tarda is an enteric Gram-negative bacterium of the Enterobacteriaceae, first isolated from pond-cultured eel by Hoshina (1962). It is the causative agent of the systemic disease, edwardsiellosis, in many freshwater and marine fishes worldwide (Thune *et al*., 1993; Ling *et al*., 2000; Castro *et al*., 2008), Such as eel, *Anguilla japonica* (Wakabayashi and Egusa, 1973), chinook salmon, *Oncorhynchus tshawytscha* (Amandi *et al*., 1982), Japanese flounder, *Paralichthys olivaceus* (Nakatsugawa, 1983), tilapia, *Tilapia niloticus* (Kubota *et al*., 1981), carp, *Cyprinus carpio* (Sae-Oui *et al*., 1984), channel catfish, *Ictalurus punctatus* (Meyer and Bullock, 1973) striped bass, *Morone saxatilis* (Thune *et al*., 1993). *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).

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 (Aoki and Takahashi, 1987; 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 (Kwon *et al*., 2007; Carrias *et al*., 2008; Castro *et al*., 2008). Relatively very few research has been performed on the inactivation of *E. tarda* concerning to vaccine preparation. Most of the available vaccines for fish are inactivated with formalin. Several attempts made to induce protection by immunizing fish against edwardsiellosis with formalin-killed whole cells, cellular lipid and lipopolysaccharides (Bachmann *et al*., 1993; Inaba *et al*., 1973; Mussgay and Weiland, 1973; Salati *et al*., 1987; Huang *et al*., 2008; Dumrongphol *et al*., 2008), produced variable results.

Inactivation by heat in the preparation of inactivated vaccine (Ahmed *et al*., 1995; Farber and Brown, 1990) is not suitable for large-scale treatment as well as in fish culture (Mann *et al*., 2000) resulted heat resistance of pathogenic bacteria including harmless derivatives (Blatchley *et al*., 2001; Smith *et al*., 2000).

To produce microbicidal effects on Gram-negative or Gram-positive bacteria (Kabara *et al*., 1972; Kabara, 1978; Shibasaki ad Kato, 1978; Isaacs *et al*., 1995; Bergsson *et al*., 1998) with variety of lipids have been extensively studied in recent years.

The effective inactivator for the development of *E. tarda* vaccine for fish other than formalin has been

Corresponding Author: Md. Mer Mosharraf Hossain, Fish Disease Laboratory, Department of Aquaculture, Faculty of Agriculture, Kochi University, 200 Monobe otsu, Nankoku shi, Kochi 783-8502, Japan
Tel: (81) 088-863-4755

receiving increasing attention. In this study, the effect of inactivation with chemicals, fatty acids, heat and chemicals combined with heat treatment were compared.

MATERIALS AND METHODS

Bacterial strains and growth conditions: This study was conducted at Fish Disease Laboratory, Department of Aquaculture, Faculty of Agriculture, Kochi University, Japan. From October 2006 to October 2008, *E. tarda* strain V-1, originally isolated from kidney of diseased eel (*Anguilla japonica*) in Japan, was pre-cultured for 24 h at 30°C in Brain Heart Infusion (BHI, Difco) broth and was inoculated into 1000 mL BHI broth, cultured with shaking at 30°C for 18 h followed by centrifugation at 4000 x g for 15 min at 4°C, harvested and stored at -80°C until used.

Inactivation by chemicals: Broth culture of the bacterium was centrifuged at 5,000 xg for 15 min, cells washed three times with PBS, resulting pellet suspended in Phosphate Buffered Saline (PBS) containing below 1% (from 0.1 to 0.9%) of formalin, ethanol (0.3 and 0.6%), Na_2SO_3 (0.3 and 0.6%), $(NH_4)_2SO_4$ (0.4 and 0.7%), KCl (0.4, 0.6 and 0.9%) and amphotericin B (0.3 and 0.6%), incubated overnight at 15°C for 24 h. Excess chemicals was removed by three washes with PBS and stored at 4°C.

Inactivation by fatty acids: To achieve the treatment of fatty acids (0.1 to 0.9%), lauric acid (0.3, 0.6 and 0.9%), myristic acid (0.3, 0.6 and 0.9%), tannic acid (0.3, 0.6 and 0.9%) and citric acid (0.3, 0.6 and 0.9%) were used as inactivating fatty acids. Treated suspensions were incubated overnight at 15°C for 24 h.

Heat inactivation: The bacterial solution was heat at 60, 70, 80, 90 and 100°C for 10 min (Heating at 70°C for 10 min was also repeated). Treated solution was incubated overnight at 15°C for 24 h.

Inactivation by chemical combined with heat: Inactivation was performed with formalin with 0.05, 0.1% at 60 and 70°C for 10 min (0.05, 0.1% Phenol at 60 and 70°C for 10 min was repeated) incubated overnight at 15°C for 24 h. Excess chemicals was removed by three washes with PBS and stored at 4°C.

Viable counts (confirmation of bacterial inactivation): The number of cells surviving chemicals, fatty acids, heat and chemicals combined with heat treatments was estimated from colony counts (Table 1). The drop plate counting method was employed (Reed and Reed, 1948). The viable count was also calculated from at least two different dilutions, with weighting for dilution (Farmiloe *et al*., 1954).

Table 1: Agglutination titers of *Edwardsiella tarda* V-1 strain inactivated with chemicals and fatty acids

Inactivators	Concentration (%)	Killing activity*	Agglutination titer (log_2)¶[2]
Chemicals			
Formalin	0.2	L	13
	0.4	M	14
	0.9	R	ND
Ethanol	0.3	L	15
	0.6	L	14
Na_2SO_4	0.3	L	15
	0.6	L	14
$(NH_4)_2SO_3$	0.4	L	15
	0.7	L	14
KCl	0.4	L	17
	0.6	L	16
	0.9	L	16
Amphotericin B	0.3	L	16
	0.6	L	15
Fatty acids			
Citric acid	0.3	L	16
	0.6	L	14
	0.9	M	13
Lauric acid	0.3	L	16
	0.6	L	15
	0.9	L	14
Tannic acid	0.3	L	17
	0.6	L	15
	0.9	L	14
Myristic acid	0.3	L	15
	0.6	L	14
	0.9	L	14

*L: Low killing where bacteria still remain alive after 24 h, M: Moderate killing in 24 h, where almost all bacteria were killed within 24 h, R: Rapid killing in 6 or 12 h. ¶Values indicate the highest dilution times of the serum with positive agglutination. [2]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

Agglutination: The inactivated *E. tarda* suspension was adjusted to an OD of 1.0 at 550 nm and 30 μL of the suspension (McFarland No. 2) was added to serum dilutions. Rabbit anti-*E. tarda* whole-cell antiserum served as a positive control and PBS served as a negative control. Plates were incubated 2 h at 25°C and overnight at 4°C and the agglutination was scored (Table 1).

SDS-PAGE and Western blots: Protein electrophoresis was performed by using the method of Laemmli (1970). The proteins were electrophoretically transferred to nitrocellulose paper (0.45 μm 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 overnight, the membrane was reacted with rabbit anti-*E. tarda* V-1 serum.

Statistical analysis: Data were analyzed by the student's t-test/ANOVA. Tukey's test was used to compare the mean values (Zar, 1984) using the StatPlus 2007 Professional.

RESULTS

Effect of chemical inactivation: Compared to different treatment of *E. tarda* with formalin, only the treatment formalin (0.4%) that showed moderate killing activity in Fig. 1 and Table 1. Inactivation profile of *E. tarda* with different chemicals (Fig. 2, 3, Table 1), ethanol (0.3, 0.6%), Na_2SO_3 (0.3, 0.6%), $(NH_4)_2SO_4$ (0.4, 0.7%), KCl (0.4, 0.6, 0.9%) and amphotericin B (0.3, 0.6%) showed low killing activity, where the treated bacteria still remained alive after 24 h.

Effect of fatty acids inactivation: Compared to fatty acids killed *E. tarda* vaccine (Fig. 4), citric acid 0.9% only the treatment that showed moderate killing activity. The bacteria still remained alive after 24 h with expressing low killing activity by the treatment of lauric acid (0.3, 0.6, 0.9%), myristic acid (0.3, 0.6, 0.9%), tannic acid (0.3, 0.6%, 0.9%) and citric acid (0.3, 0.6%).

Effect of heat and chemicals combined with heat inactivation: The bacterium was completely inactivated by 6 to 12 h with heating at 80, 90 and 100°C for 15 min. Bacterium still remains alive after 24 h heating at 60 and 70°C for 15 min, 0.05% formalin with heating at 60 and 70°C for 10 min, 0.1% formalin with heating at 60°C for 10 min and 0.05 and 0.1% phenol with heating at 60 and 70°C for 10 min. Compared to different bactrins of *E. tarda* only the treatment 0.1% formalin with heating at 70°C for 10 min showed moderate killing activity in 24 h (Fig. 5).

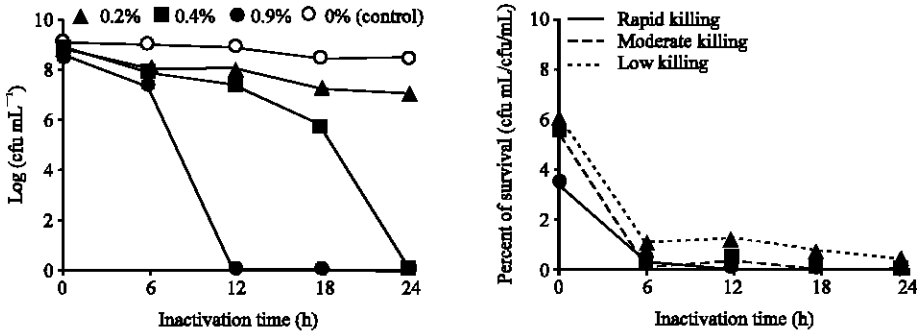

Fig. 1: Inactivation and survival of *Edwarsiella tarda* V-1 strain after incubation with different concentration of formalin

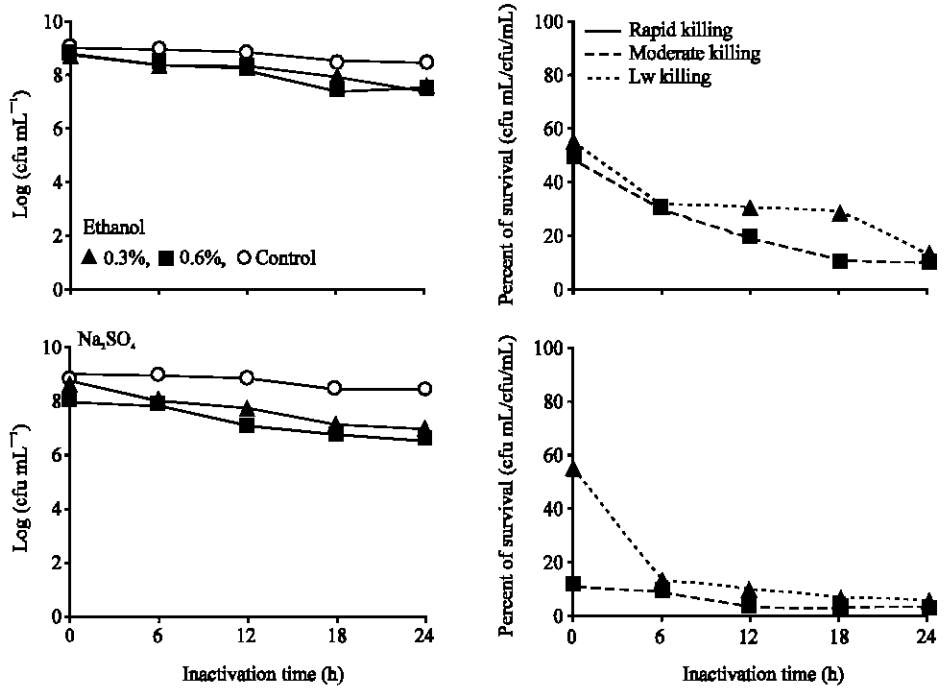

Fig. 2: Inactivation profile and survival of *Edwarsiella tarda* V-1 strain after incubation with different concentration of chemicals

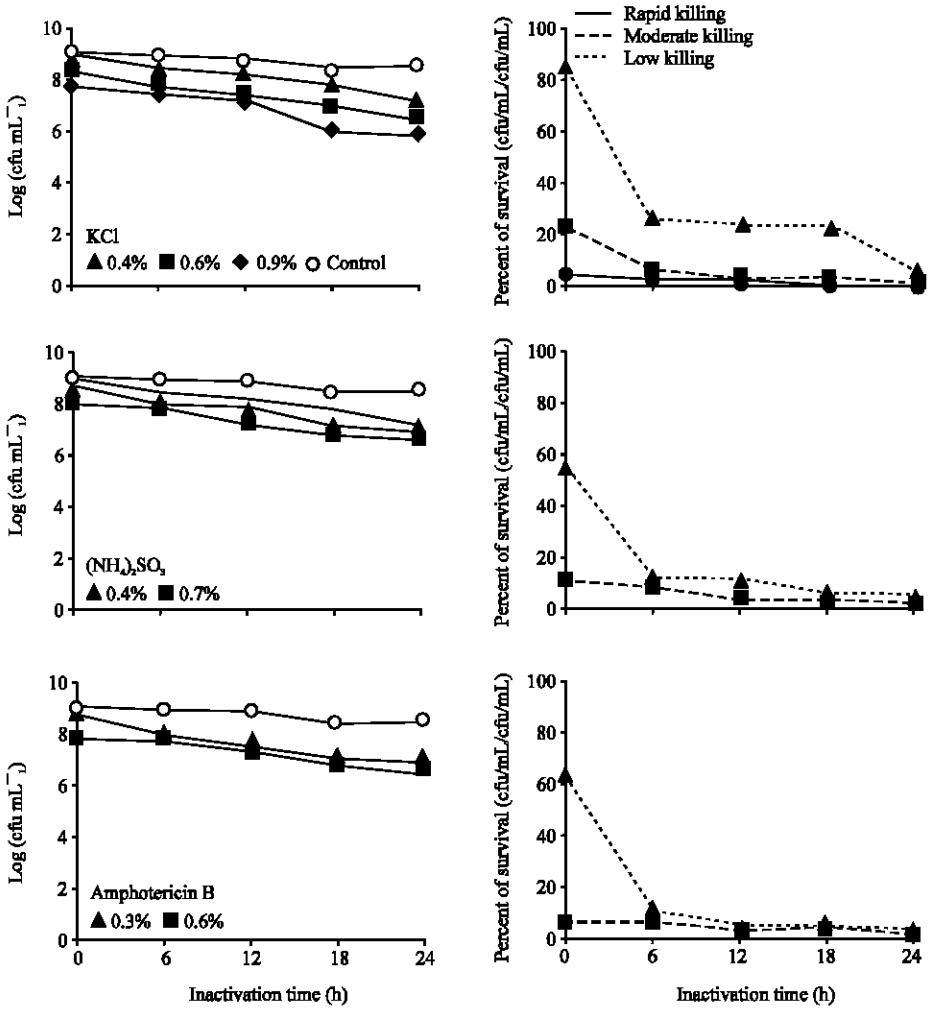

Fig. 3: Inactivation profile and survival of *Edwarsiella tarda* V-1 strain after incubation with different concentration of chemicals

Antigenicity of inactivated *E. tarda* measured by agglutination reaction

Inactivation by chemicals: The agglutination titers of different bactrins of *E. tarda* against rabbit antiserum were shown in Table 1. Higher percentage of formalin (0.9%) rose autoagglutination which hid the agglutination titer. Moderately high agglutination titer was observed with formalin (0.4%).

Inactivation by fatty acid: The agglutination titers with *E. tarda* cells inactivated by lauric acid, myristic acid, tannic acid and citric acid against rabbit antiserum are shown in Table 1. The 0.9% citric acid brought moderately high agglutination titer.

Inactivation by heating and chemicals combined with heating: Compared to different bactrins of *E. tarda*, only the treatment 0.1% formalin with 70°C for 10 min that showed moderately high agglutination titer in Table 2. The agglutination titer was hid by autoagglutination with treatment at 80, 90 and 100°C for 15 min, where the treatment with 60 and 70°C for 15 min, 0.05% formalin with 60 and 70°C and 0.1% formalin with 60°C for 10 min; 0.05 and 0.1% phenol with 60 and 70°C for 10 min brought higher agglutination titer in Table 2.

Antigenicity of inactivated *E. tarda* measured by Western blot: The profiles of the total proteins (SDS-PAGE) and major protective antigen of this bacterium at 37 kDa location of *E. tarda* V-1 strain was strongly reacted with rabbit antiserum against citric acid (0.9%) and formalin (0.1%) with heat (70°C for 10 min) killed cells in Fig. 6. At 97, 75, 74, 45, 43 and 37 kDa locations were reacted with rabbit antiserum against

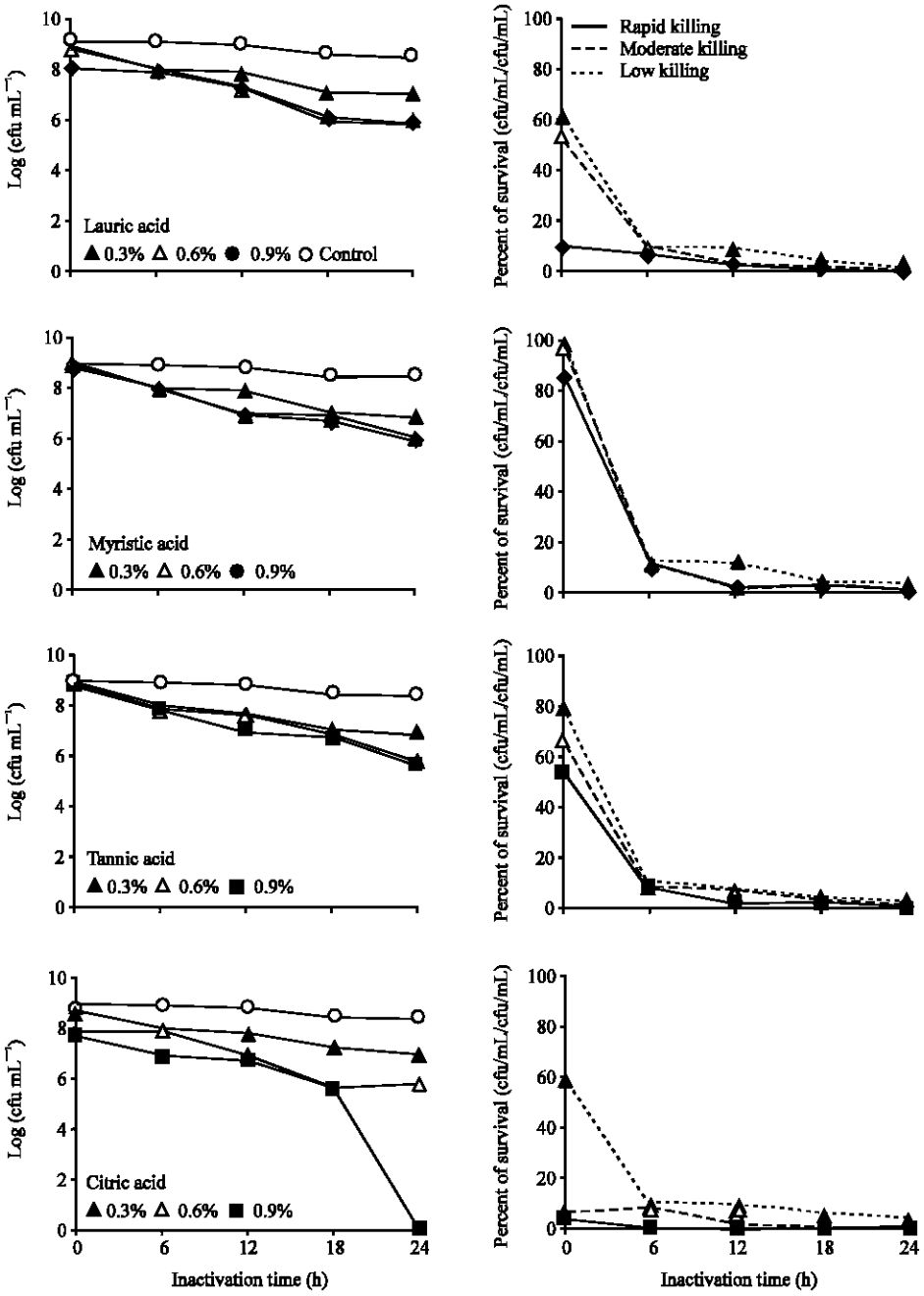

Fig. 4: Inactivation profile and survival of *Edwardsiella tarda* V-1 strain after incubation with different concentration of fatty acids

formalin (0.4, 0.9%), myristic acid (0.3, 06, 0.9%), citric acid (0.3, 0.6%) and formalin (0.05%) with heat (60°C for 10 min) killed *E. tarda* in Fig. 6.

DISCUSSION

E. tarda is a pathogen of aquaculture industry which can lead to serious diseases. Present new approach to produce an inactivated *E. tarda* vaccine considering awareness of residual effect to human is based on the chemicals fatty acids, heat, low concentration of formalin combined with heat other than formalin inactivation of cells with high concentration which has been extensively investigated in this study.

In the present study, the high levels of inactivating activity (Bergsson *et al*., 1998; Lampe *et al*., 1997) showed

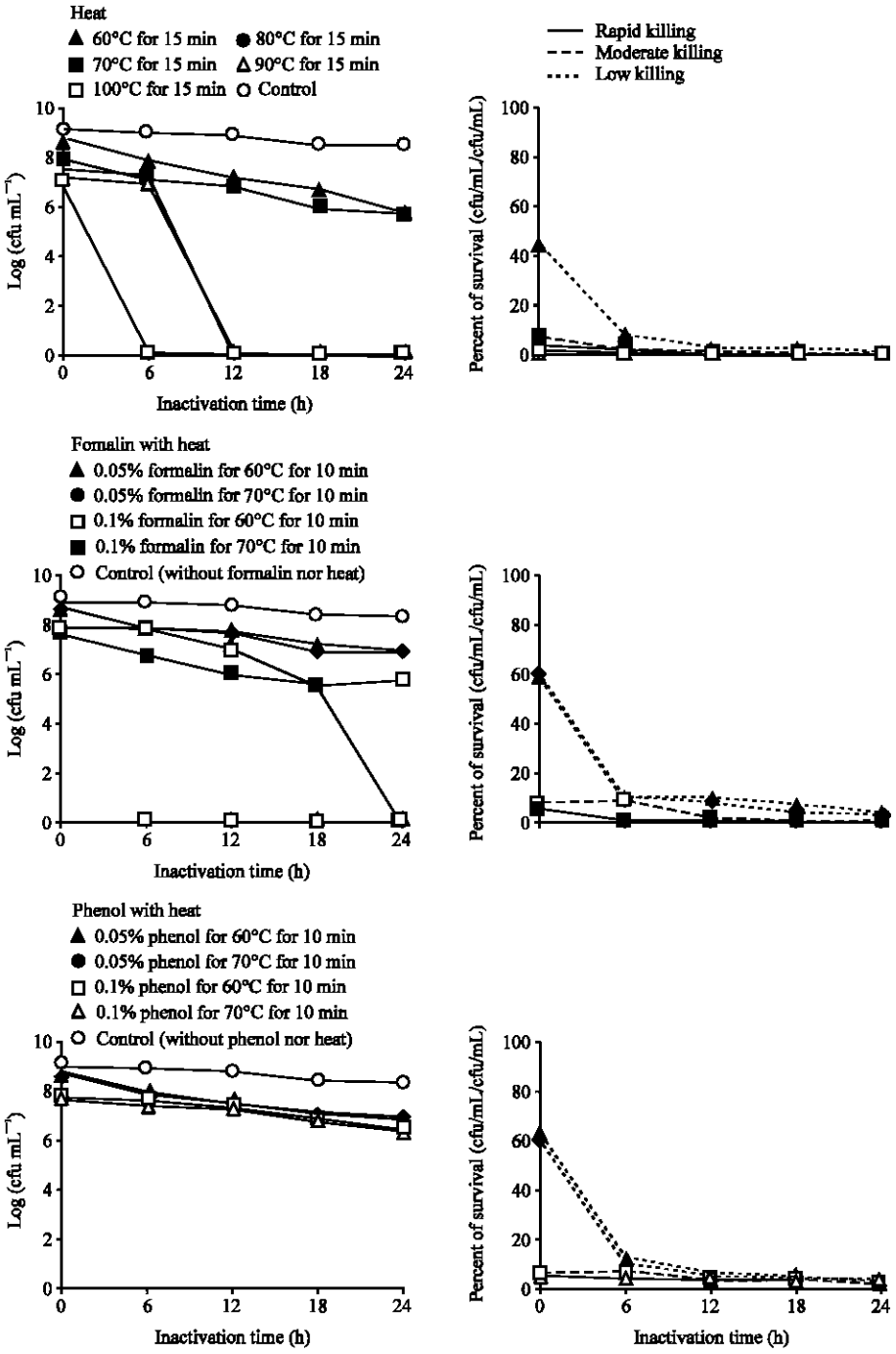

Fig. 5: Inactivation profile and survival of *Edwarsiella tarda* V-1 strain after incubation with heat and chemicals combined with heat

high concentrations of citric acid that had not been seen with fatty acids. We therefore hypothesized that citric acid kills the bacterium by affecting outer membrane by citric acid, leading to disruption the elementary bodies of the cell membrane and remaining the bacterial cell membrane intact (Bergsson *et al*., 1998; Lampe *et al*., 1997).

Significantly elevated agglutination titer and resistance against *E. tarda* with formalin killed cells were found by Swain *et al*. (2007), which was similar to the treatment with low concentration of formalin combined with heat. *E. tarda*, strains positively responded to heating at 60°C for 30 min and it was rapidly destroyed by heating at 100°C for 15 min (Bockemühl *et al*., 1983).

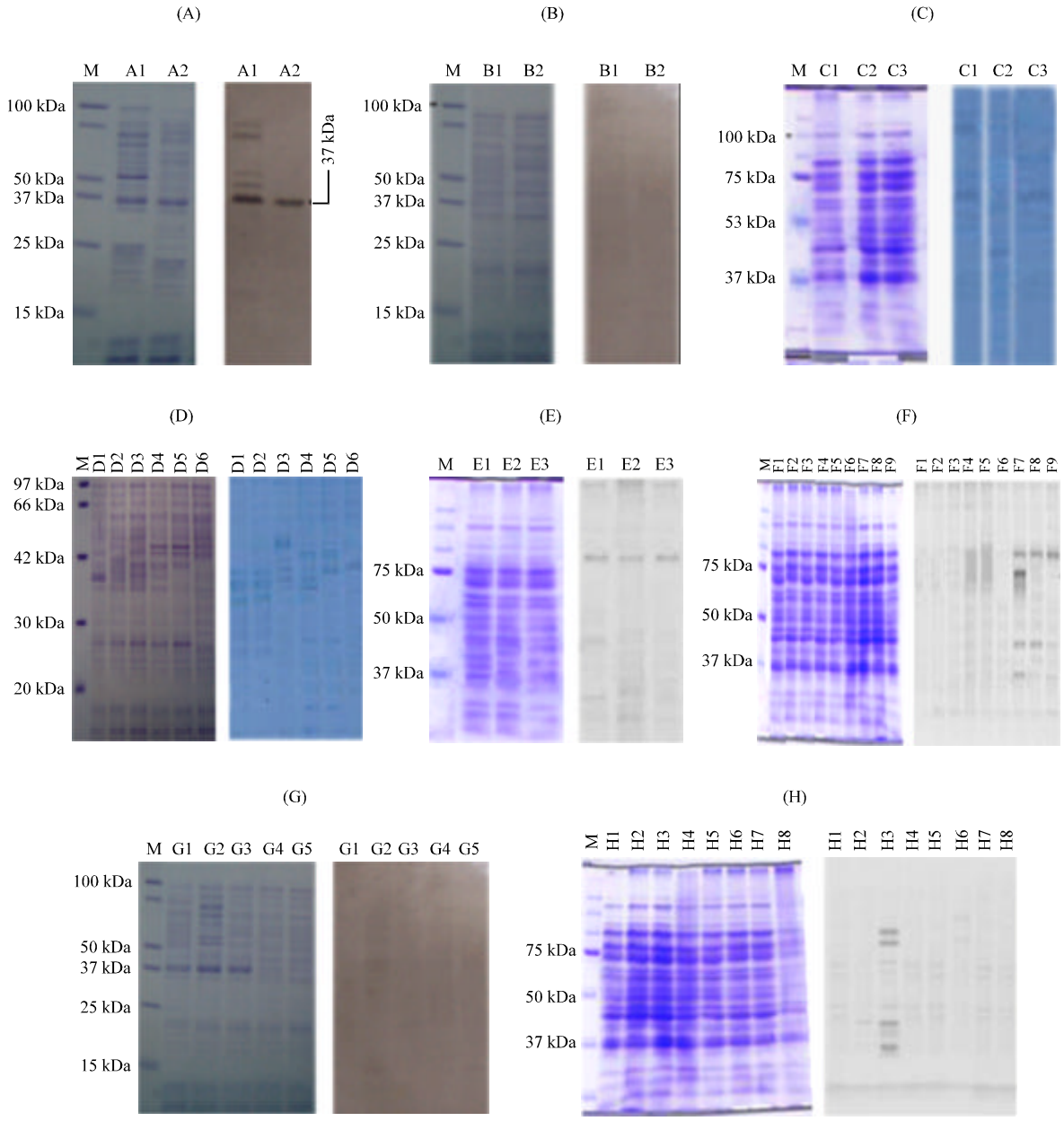

Fig. 6: SDS-PAGE and Western blotting profiles of chemicals, fatty acid, heat and chemicals combined with heat inactivated *Edwardsiella tarda* V-1 strain. Left, SDS-PAGE; Right, Western blot. M: Molecular weight marker; A1, 0.4% formalin; A2, 0.9% formalin; B1, 0.3% ethanol; B2, 0.6% ethanol; C1, 0.4% KCl; C2, 0.6% KCl; C3, 0.9% KCl; D1, 0.3% $NaSO_4$; D2, 0.6% $NaSO_4$; D3, 0.4% $(NH_4)_2SO_4$; D4, 0.7% $(NH_4)_2SO_4$; D5, 0.3%amphotericin B; D6, 0.6% amphotericin B; E1, 0.3% myristic acid; E2, 0.6% myristic acid; E3, 0.9% myristic acid; F1, 0.9% tannic acid; F2, 0.6% tannic acid; F3, 0.3% tannic acid; F4, 0.9% lauric acid; F5, 0.6% lauric acid; F6, 0.3% lauric acid; F7, 0.9% citric acid; F8, 0.6% citric acid; F9, 0.3% citric acid; G1, heat at 60°C for 15 min; G2, heat at 70°C for 15 min; G3, heat at 80°C for 15 min; G4, heat at 90°C for 15 min; G5, heat at 100°C for 15 min; H1, 0.05% formalin with heat at 70°C for 10 min; H2, 0.05% formalin with heat at 60°C for 10 min; H3, 0.1% formalin with heat at 70°C for 10 min; H4, 0.1% formalin with heat at 60°C for 10 min; H5, 0.05% phenol with heat at 70°C for 10 min; H6, 0.05% phenol with heat at 60°C for 10 min; H7, 0.1% phenol with heat at 70°C for 10 min and H8, 0.1% phenol with heat at 60°C for 10 min

Table 2: Agglutination titers of *Edwardsiella tarda* V-1 strain inactivated by heat and chemicals combined with heat

Inactivation	Method of inactivation	Killing activity*	Agglutination titer (log_2)¶[2]
Heat	60°C, 15 min	L	14
	70°C, 15 min	L	14
	80°C, 15 min	R	11
	90°C, 15 min	R	ND
	100°C, 15 min	R	ND
Chemicals combined with heat			
Formalin	0.05%, 60°C, 10 min	L	15
	0.05%, 70°C, 10 min	L	14
	0.1%, 60°C, 10 min	L	14
	0.1%, 70°C, 10 min	M	13
Phenol	0.05%, 60°C, 10 min	L	16
	0.05%, 70°C, 10 min	L	15
	0.1%, 60°C, 10 min	L	15
	0.1%, 70°C, 10 min	L	14

*L: Low killing where bacteria still remain alive after 24 h, M: Moderate killing in 24 h, where almost all bacteria were killed within 24 h, R: Rapid killing in 6 or 12 h.¶Values indicate the highest dilution times of the serum with positive agglutination. [2]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.

Compared to different bactrins of *E. tarda* (Salati and Kusuda, 1985; Salati *et al*., 1987; Kwon *et al*., 2007; Swain *et al*., 2007), only the treatment citric acid (0.9%) and formalin (0.1%) with heat (70°C for 10 min) killed *E. tarda* that showed enough-sustainability and expressing moderate killing activity where the major protective antigen of this bacterium at 37 kDa location (Tu and Kawai, 1999) was strongly reacted with rabbit antiserum against *E. tarda*.

Over the last decade effective inactivation and antigenic potency of the bacterin after inactivation was not properly evaluated with *E. tarda*. Compared to heat or formalin killed bacterial vaccines (Gutierrez and Miyazaki, 1994; Crosbie and Nowak, 2004; Castro *et al*., 2008), in present study, citric acid and low concentration of formalin combined with heat could be the potential tool for *E. tarda* inactivation. Also, suggested as an alternate approach for the inactivation of bacteria (Nakayama *et al*., 1996; Sonoike, 1997) which have frequently caused the reduction of antigenicity, residual effect (Smith *et al*., 1956).

Exactly why the low concentration of formalin with heat at 70°C for 10 min and 0.9% citric acids induced effective inactivation is remained unclear. The quantity of the protective antigens 37 kDa in this preparation is higher, but another possibility is that the method of production destroyed less of the protective epitopes than bacterins of whole cells. A further influencing factor might be differences in particle structure between the vaccines (Taisuke *et al*., 2003). We can only speculate about the structure of the PKC vaccine; however another study showed that the antigen form can influence immunogenicity (Schirmbeck *et al*., 1995).

In conclusion, as this promising type of an inactivation bacterin preparation seems to be favorable over conventional vaccines, we suggested formalin (0.1%) with heat (70°C for 10 min) and citric acid (0.9%) killed *E. tarda* as a new vaccine candidate.

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