EFFECT OF SODIUM CHLORIDE ON PRODUCTION AND TOXICITY OF EXTRACELLULAR PRODUCTS OF *EDWARDSIELLA TARDA*

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ABSTRACT

The effect of NaCl concentration in the culture medium on the production and toxicity of *Edwardsiella tarda* extracellular products (ECP) was studied. *Edwardsiella tarda* (FK1051) was cultured in a peptone-yeast extract broth supplemented with 3% NaCl (3%-NaCl culture) and without NaCl (0%-NaCl culture). The ECP of both cultures were prepared by the cellophane plate method. The wet bacterial weights and the protein concentrations were detected at different time intervals. The bacterial weights decreased after 2-3 days incubation and thereafter the ECP protein levels increased. The protein concentration in ECP of 3%-NaCl culture was higher than that of 0%-NaCl culture. SDS-PAGE revealed the appearance of new bands (35 and 70 kDa) and intensification of other bands in the ECP of 3%-NaCl culture. The intramuscular injection of ECP in goldfish revealed higher toxicity in the 3%-NaCl culture. These results suggest that the NaCl-induced ECP toxicity of *E. tarda* may play a vital role in its virulence.

Key words: *Sodium chloride, Extracellular products, Toxicity, Edwardsiella tarda*

INTRODUCTION

Edwarsiella tarda as a member of family enterobacteriaceae has a wide host range including not only fish but also reptiles, amphibians and mammals (Evance *et al*., 2011). *E. tarda* infection (edwardsiellosis) leads to extensive losses in many commercially important freshwater and marine fish such as Nile tilapia *Oreochromis niloticus* (Badran, 1993; Galal *et al*., 2005 and Ibrahem *et al*., 2011), channel catfish *Ictalurus punctatus*, Japanese eel *Anguilla japonica*, carp *Cyprinus carpio*, goldfish *Carassius auratus*, mullet *Mugil cephalus*, Chinook salmon *Oncorhynchus tshawytscha*, Japanese flounder *Paralichthys olivaceus*, and striped bass *Morone saxatilis* and others (Evance *et al*., 2011).

Though the exact pathogenic mechanism of this bacterium is still not clearly explained, some virulence factors have been proposed including Type III and Type IV secretion systems, invasion of epithelial cells, resistance to serum and production of toxins (Ullah and Arai, 1983; Suprapto *et al*., 1995; Ling *et al*., 2000; Sirinivasa Rao *et al*., 2001 and Leung *et al*., 2012).

Extracellular products (ECP) of many fish pathogens include substances such as enzymes and proteins that exhibit toxicity to fish. For example, *Aeromonas hydrophila* produces hemolytic and proteolytic exotoxins lethal to Nile tilapia *Oreochromis niloticus* (Khalil and Mansour, 1997). *Vibrio splendidus-Vibrio lentus* related group also produces proteolytic enzyme as one of its ECP (Farto *et al*., 2006). Besides, *Vibrio anguillarum* ECP elicited lethal toxicity against goldfish, Japanese eel, ayu *Plecoglossus altivelis* and mouse and this toxic effect was attributed to the possession of proteolytic enzyme (Inamura *et al*., 1984). The most potent lethal neurotoxin (acetylcholinestrase) was determined in ECP of 42 strains of the family vibrionaceae including fish-pathogenic *V. anguillarum* (Pérez *et al*., 1998). Cytotoxic effect for fish cell lines was also reported to be induced by the ECP of *V. damselae* (Wang *et al*., 1998). Balb/c mice, injected with ECP of *A. salmonicida* subsp. *achromogenes*, displayed symptoms similar to toxic shock syndrome (Gudmundsdóttir and Gudmundsdóttir, 2001).

In *Edwardsiella tarda*, the ECP has been considered a virulence factor. Ullah and Arai (1983) demonstrated the dermatotoxic effect of *E. tarda* when injected into rabbits. ECP toxicity to Japanese eel and Japanese flounder was verified (Suprapto *et al*., 1995). Subsequently, the lethal toxin of *E. tarda* was purified and proved to be a protein with 37 kDa molecular weight (Suprapto *et al*., 1996).

Previously, both hemagglutinating activity (HA) and adhesion of *E. tarda* were found to be induced by the

high NaCl in the culture medium (Mahmoud *et al*., 2006; Yasunobu *et al*., 2006). The objective of the present study is to investigate the effect of NaCl concentration on the production and toxicity of the ECP.

MATERIALS and METHODS

Bacterial strain and culture conditions:
E. tarda (FK1051), isolated from diseased Japanese flounder was used. The bacteria were pre-cultured on Trypto-Soya Agar (TSA, Nissui) at 25ºC for 24 h.

Following the method described before (Yasunobu *et al*., 2006), *E. tarda* was inoculated into 10 mL of a liquid medium consisted of 1% peptone-0.5% yeast extract (pH 6.5). The bacterial suspension was diluted 100-fold in the same medium, and then 100 μL of the dilution was inoculated into 10 mL of the liquid medium supplemented with 3% NaCl or without NaCl. The bacterial culture was incubated at 25ºC overnight with shaking (100 rpm) and the bacterial cells were harvested by centrifugation (5,000 ×*g*, 10 min). The bacterial cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and the cell concentration was adjusted to A_{530} = 1.0 (ca. 10^9 CFU/mL).

Preparation of ECP:
The ECP was prepared by the cellophane plate method (Liu, 1957). Cellophane was cut to fit the bottom of petri dish and then was sterilized by autoclaving (121ºC, 15 min) and overlaid on peptone-yeast agar medium (1% peptone-0.5% yeast extract and 1.5% agar) supplemented with 3% NaCl or without (0%) NaCl. Each cellophane-overlaid agar plate was inoculated with 200 μL (approximately 1 mg/plate; 10^9 CFU/mL) of the pre-cultured bacteria either in the medium containing 3%- or 0%-NaCl. The cultures were incubated at 25ºC for 13 days. This culture technique allows the bacteria to draw nutrients freely from the medium beneath the cellophane sheet, and at the same time prevent mixing of the ECP proteins released by the bacteria with the proteins present in the underlying culture medium.

Bacterial cells cultured on both 0%- and 3%-NaCl cellophane-overlaid agar were harvested with 2 mL of PBS (pH 7.4) per each plate. The cell suspensions were centrifuged at 12,000 ×g at 4ºC for 20 min. The resultant supernatants containing ECP were sterilized by 0.45-μm membrane syringe filters. The protein concentration of the supernatants was measured following the method of Bradford (1976) with bovine serum albumin as the standard.

SDS-PAGE of the ECP:
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a 10% acrylamide slab gel in presence of 2-mercaptoethanol. The gel was stained with Coomassie brilliant blue.

Fish:
Goldfish *Carassius auratus* (6 ± 1 g) were used for exploring the toxicity of ECP. The fish were acclimated for at least 2 weeks prior to the experiment. The aquaria were supplied with tap dechlorinated water and maintained at 25 ± 1ºC. The feeding was discontinued during the toxicity test.

Toxicity test:
The ECP were collected and pooled from the 0%- and 3%-NaCl cultures at 9-13 days post-incubation. Two groups of fish (10 fish/group) were injected intramuscularly with the ECP from 0%-NaCl culture or 3%-NaCl culture. Fish received 50 μL (19 μg protein/fish) of each ECP. Another group of 10 fish was injected with 50 μL sterile PBS and served as a control group. The survival rate was calculated for 2 weeks.

RESULTS

Preparation of ECP:
The bacterial growth, represented by the wet bacterial weight, of the 0%-NaCl culture was higher than that of the 3%- NaCl culture. The bacterial growth in both cultures decreased gradually after 2-3 days incubation. On the contrary, the concentration of protein in ECP of 0%-NaCl culture was lower than that of 3%- NaCl culture. The protein concentration of the ECP of 0%-NaCl culture could be detected only 9 days after incubation at 25ºC. Whereas, for ECP of 3%- NaCl culture, the protein level was detectable from the third day after incubation. The protein concentration of ECP in the 3%- NaCl culture reached its peak at 9 days post-incubation while that in 0%- NaCl culture continuously increased until the end of experiment (Table 1, Fig. 1).

SDS-PAGE of the ECP:
SDS-PAGE analysis of the ECP showed differences in the composition and concentration of the ECP of 0%- and 3%-NaCl cultures. At least 2 bands (about 35 and 70 kDa) were identified as being unique to the ECP of 3%-NaCl culture. There were also differences in the staining intensity of several shared bands (Fig. 2).

Toxicity test:
The intramuscular injection of the pooled ECP of 3%-NaCl culture exhibited higher mortality (90%) than that (40%) displayed by the ECP of 0%-NaCl culture (Table 2, Fig. 3). No mortalities were recorded in the fish injected with sterile PBS (control group).

Table 1: Effect of NaCl concentration on bacterial growth and protein concentration of extracellular products (ECP) of *E. tarda* at different incubation periods.

Incubation period (days)	0%-NaCl culture		3%-NaCl culture	
	Wet bacterial weight (g)	Protein conc. (mg/mL)	Wet bacterial weight (g)	Protein conc. (mg/mL)
1	0.0497	UN*	0.0216	UN
2	0.0540	UN	0.0258	UN
3	0.0448	UN	0.0269	0.03
5	0.0426	UN	0.0218	0.06
7	0.0417	UN	0.0187	0.30
9	0.0396	0.27	0.0160	0.48
11	0.0386	0.34	0.0135	0.45
13	0.0339	0.55	0.0096	0.34

*Undetectable

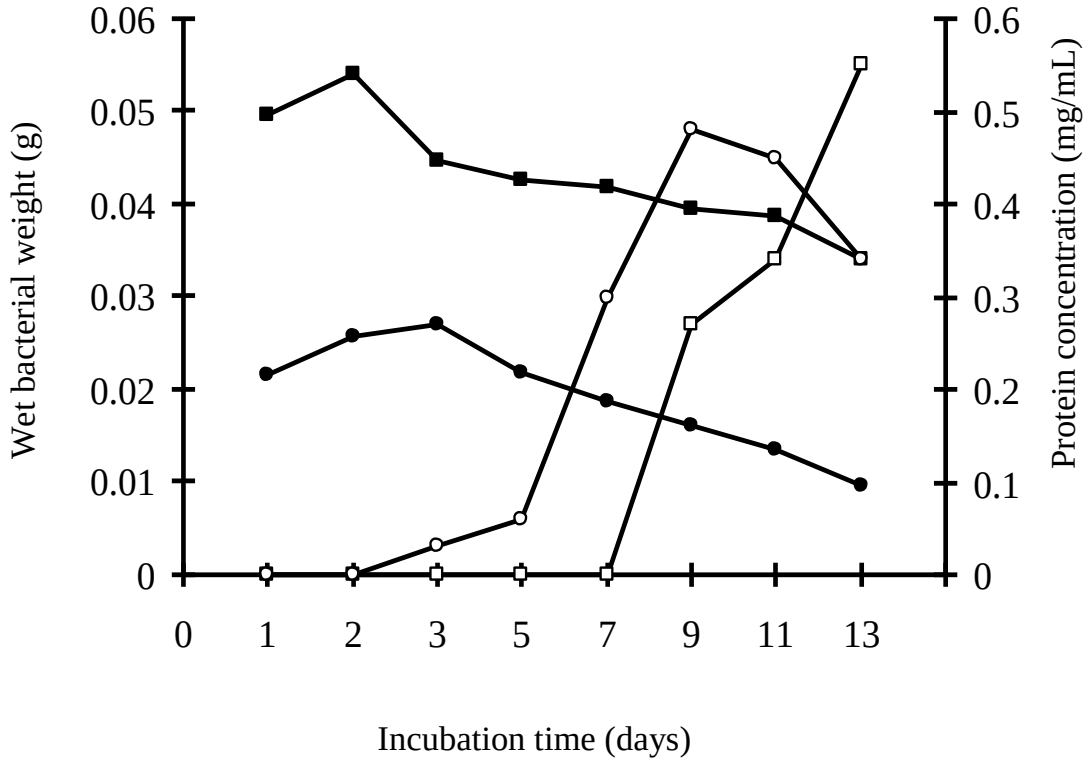

Fig. 1: Effect of NaCl concentration on bacterial growth and protein concentration of extracellular products (ECP) of *E. tarda* at different incubation periods. Wet bacterial weight: 0%-NaCl culture (■), 3%-NaCl culture (●). ECP protein concentration: 0%-NaCl culture (□), 3%-NaCl culture (○).

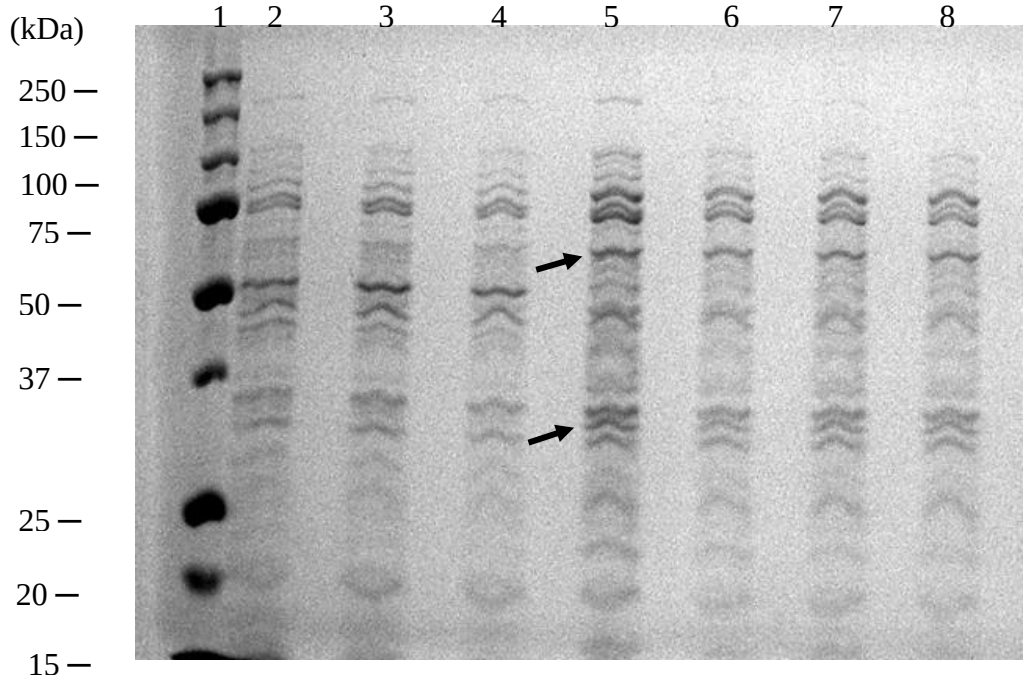

Fig. 2: SDS-PAGE of ECP of 0%- and 3%-NaCl cultures. Lane 1, molecular weight marker; lanes 2-4, ECP of 0%- NaCl cultures at 9, 11 and 13 days incubation, respectively; lanes 5-8, ECP of 3%- NaCl cultures at 7, 9, 11 and 13 days incubation, respectively. Arrows refer to bands specific to ECP of the 3%-NaCl culture. The gel was stained with Coomassie brilliant blue.

Table 2: Toxicity of the *E. tarda* extracellular products (ECP) to goldfish. A dose of 50 µL (19 µg protein/fish) of ECP/ fish was injected intramuscularly and mortalities were recorded for 2 weeks

NaCl concentration	Mortality % (dead/tested)	Mean days to death
0%-NaCl culture	40 (4/10)	2
3%-NaCl culture	90 (9/10)	2
Control	0 (0/10)	-

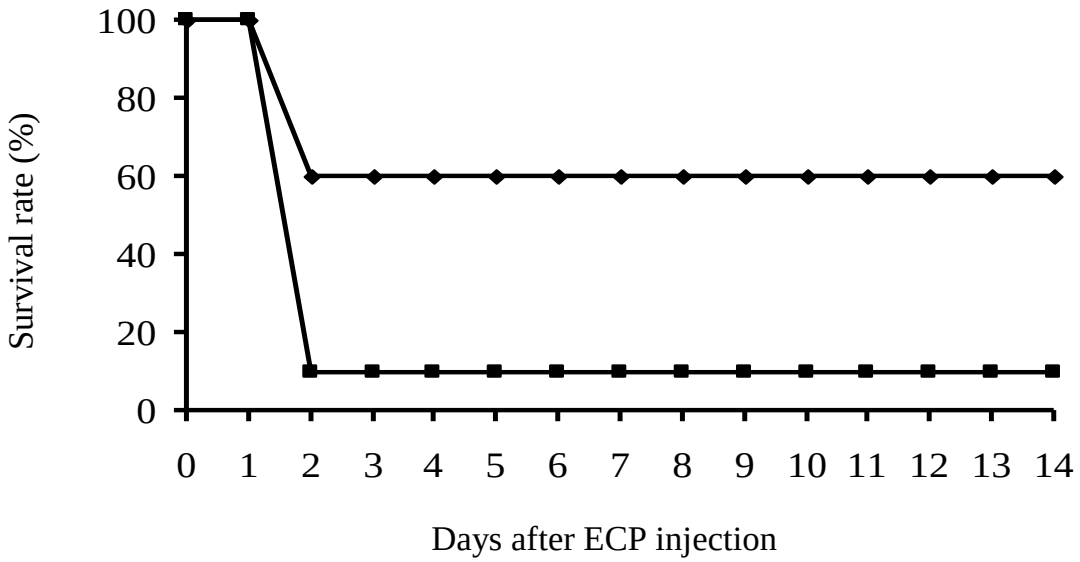

Fig. 3: Survival rate of goldfish injected intramuscularly with pooled *E. tarda* ECP. A dose of 50 µL (19 µg protein/fish) of ECP/fish was applied. ECP of 0%- NaCl culture (♦) and of 3%-NaCl culture (■).

DISCUSSION

ECP mostly consisted of enzymes that facilitate the propagation of infectious bacteria by causing extensive host tissue damage, thereby degrading host tissues to provide readily-available nutrients for bacterial growth. Furthermore, ECP counteract the host defense system by degrading immunoglobulins and components of the complement system. The involvement of ECP in the pathogenicity of *E. tarda* is currently not fully recognized. Some studies were previously conducted regarding the production of lethal toxins by *E. tarda* and their assumed role in the virulence of this pathogen (Ullah and Arai, 1983; Suprapto *et al*., 1995 and Han *et al*., 2006).

The present study investigated the influence of the increase in the NaCl concentration in the culture medium on the ability of *E. tarda* to produce ECP and its toxicity to fish. The culture at high NaCl (3%-NaCl culture) resulted in lowered bacterial growth indicating that the high salt condition is not optimal for its growth though *E. tarda* can grow in a medium supplemented with as much as 4% NaCl. However, the production of ECP was higher in 3%-NaCl culture than in 0%-NaCl culture. The NaCl-promoted ECP production, in addition to the induced adhesion by high NaCl (Mahmoud *et al*., 2006), may help the *E. tarda* to escape the unfavorable high-salt marine environment by adhering to (using adhesins) and penetrating (by the enzymes of ECP) the host tissue.

The SDS-PAGE revealed the appearance of two protein bands (approximately 35 and 70 kDa) specific to the ECP of the 3%-NaCl culture. Suprapto *et al.* (1996) purified a lethal toxin from the ECP of *E. tarda* with a molecular weight of 37 kDa. These bands seam to indicate proteins responsible for the toxicity of ECP.

The toxicity of the ECP of *V. anguillarum* to goldfish was established (Inamura *et al*., 1984). In the current study, injection of the same dose (19 μg protein/fish) of pooled ECP to goldfish demonstrated an obvious increase in the mortality rate in case of the 3%-NaCl culture.

In conclusion, the stimulation of toxicity of *E. tarda* ECP by the high NaCl may be one of the pathogenic mechanisms utilized by the bacterium to establish the infection especially in the saltwater environment. Further investigations are still needed to elucidate the components of *E. tarda* ECP and their exact role in its pathogenicity.

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تأثير كلوريد الصوديوم على إنتاج وسمية المنتجات الخارجية لميكروب الإدواردسيللا تاردا

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تناولت هذه الدراسة تأثير تركيز كلوريد الصوديوم المستخدم فى بيئة زرع البكتريا على إنتاج وسمية المنتجات خارج خلايا ميكروب الإدواردسيللا تاردا. حيث تم زرع البكتيريا على بيئة تحتوى على ٣% كلوريد الصوديوم و أخرى لا تحتوى على كلوريد الصوديوم. تم الحصول على المنتجات المفروزة بواسطة البكتيريا خارج الخلايا باستخدام طريقة أطباق السيلوفان. وقد تم تحديد أوزان البكتيريا وتركيزات البروتين المفروز خارجها على فترات زمنية مختلفة. أظهرت النتائج أن أوزان البكتيريا انخفضت بعد ٢-٣ أيام من الحضانة عند ٢٥ درجة مئوية، فى حين ارتفعت معدلات إنتاج البروتين المفروز خارج الخلايا البكتيرية. وكان تركيز البروتين المفروز خارج الخلايا البكتيرية النامية باستخدام ٣% كلوريد الصوديوم أعلى منه فى تلك التى نمت فى عدم وجود كلوريد الصوديوم. وباستخدام طريقة التفريد الكهربى (SDS-PAGE) اتضح ظهور بروتينات جديدة عند أوزان جزيئية ٣٥ و ٧٠ كيلو دالتون فى البكتريا النامية فى وجود ٣% كلوريد الصوديوم وكذلك زيادة معدل إفراز بروتينات أخرى عنه فى البكتيريا النامية فى غياب كلوريد الصوديوم. وعند الحقن العضلى لهذه السموم المفروزة خارج خلايا البكتيريا فى الأسماك الذهبية، أظهرت النتائج زيادة سمية هذه المواد المنتجة من البكتيريا المستزرعة فى وجود ٣% كلوريد الصوديوم عن مثيلتها فى المستزرعة فى عدم وجود كلوريد الصوديوم. ومن هذه النتائج يتضح تأثير تركيز كلوريد الصوديوم على زيادة إفراز السموم خارج الخلايا البكتيرية وكذلك زيادة سميتها مم يشير إلى الارتفاع المحتمل فى ضراوة هذا الميكروب بزيادة تركيز كلوريد الصوديوم فى الوسط المحيط به.