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Temperature effects on pathogenicity of selected *Edwardsiella tarda* strain to Japanese eel, *Anguilla japonica*

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Abstract
Temperature effect on the pathogenicity of selected *Edwardsiella tarda* V-1 strain to Japanese eel, *Anguilla japonica* was investigated. To evaluate the effects of both pathogen incubation temperature and fish cultivation temperature on pathogen pathogenicity a two-factor design was conducted. *E. tarda* was incubated at 15, 20, 25, 30 and 37±1°C, and the fish (mean weight: 100g) were reared at 15, 20, 25 and 28±1°C respectively. The fish reared at different temperatures were infected with the *E. tarda* incubated at different temperatures. The results of a 4-day LD50 test showed that temperature significantly affected the pathogenicity of *E. tarda* ($p<0.01$) and the interaction between the two factors was also significant ($p<0.01$). For fish reared at 20°C the pathogenicity of *E. tarda* was the highest at 30°C of pathogen incubation. When the fish rearing temperature was raised to 25 and 28°C, the pathogenicity of *E. tarda* incubated at all temperatures increased. Isolation testing demonstrated results similar to those of LD50. The selected isolate was virulent to eel, but pathogenicity varied with temperature.

Keywords: *Edwardsiella tarda*, Pathogenicity, Japanese eel (Anguilla Japonica)

Introduction

Edwardsiella tarda, the causative agent of edwardsiellosis in fish, is responsible for extensive losses in both freshwater and marine aquaculture. *E. tarda* infection of many commercially important cultured and wild fish has been reported, namely, channel catfish, eels, mullet, Chinook salmon, flounder, carp, tilapia, and striped bass (Thune *et al.* 1993). It has a wide host range, thus causing infection in higher vertebrates such as birds, reptiles (White *et al.* 1973), and mammals (Van Damme and Vandepitte 1984), including humans (Plumb 1993). In fish, it causes septicemia with extensive skin lesions, affecting internal organs such as the liver, kidney and spleen and muscle. These bacteria systemically avoid host defense mechanisms, thereby rapidly proliferating within the host and causing death. Pathogenesis of *E. tarda* is multifactorial, and many potential virulent factors have been suggested, namely dermatotoxins (Ullah and Arai 1983),

antiphagocyte killing (Ainsworth and Chen 1990), hemolysins (Hirono *et al.* 1997), serum resistance, and the ability to invade epithelial cells (Janda *et al.* 1991 and Ling *et al.* 2000). Although both virulent and avirulent strains were able to invade cultured cells in vitro, only the virulent strain could enter fish in large numbers via mucus, gills, and the gastrointestinal tract (Ling *et al.* 2001) and multiply inside various internal organs, causing death. It was also found that pathogenicity of *E. tarda* did not correlate with plasmid content, chemotactic motility, serum resistance, and expression of selected enzyme activities (Janda *et al.* 1991).

Serological studies have been carried out on strains isolated from Japanese eel, Japanese flounder, sea bream, and so on. The strains were classified into several serotypes. Pathogenicity of strains belonging to serotype A was the highest (Park *et al.* 1983 and Mamnur *et al.* 1994b). Some substances that may relate to the pathogenicity have been reported, such as siderophore (Kokubo *et al.* 1990 and Mathewl *et al.* 2001), hemolysin (Kusuda and Kitadai 1993), superoxide dismutase (SOD) (Yamada and Wakabayashi 1998), extracellular products (ECPs) and intracellular components (Icc) (Suprato *et al.* 1995). The invasion of *E. tarda* was also studied (Ling *et al.* 2000), however, very little is known about the pathogenesis of *E. tarda* in disease occurrence.

The present study was carried out to investigate the interaction of *E. tarda* with fish and environmental conditions that affect the occurrence of fish diseases, specially the effects of temperature on the pathogenicity of *E. tarda* to Japanese eel.

Materials and methods

Bacterial strain and growth conditions

E. tarda strain V-1 which is 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 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 was used in this study to prepare a vaccine, 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. The cells were harvested by centrifugation at 4000×g for 15 min at 4°C and were stored at −80°C freezer until used. In the temperature experiment, *E. tarda* was incubated on plates covered with cellophane at 15±1°C for 15 days, 20±1°C for 10 days, 25±1°C for 5 days, 30±1°C for 3 days and 37±1°C for 1 day respectively. The medium contained yeast extract 1500, beef extract 1500, tryptone 5000, glucose 1000, NaCl 3500, K2HPO4 4800, KH2PO4 1320 and agar 15000 mg/L.

Experimental fish

Test fish, Japanese eels *Anguilla japonica* of an average weight 102.8±6.6g

(mean±SD, N = 25) were obtained from an eel farm in Yoshikawa at Kochi Prefecture, Japan and the fish had no previous occurrence of infection with *E. tarda* in this farm. Approximately 10 fish in each group and also for control were reared in 100 l tanks with well-aerated flowing water at 25°C. Fishes were fed with 0.5mm commercial dry pellets (Nissui) corresponding to 3% of the fish body weight per day for the entire experiment.

Infection and LD50 test

In order to evaluate the effects of temperature in both pathogen incubation and fish rearing, a two-factor experiment was designed. The temperature for the incubation of *E. tarda* was designed with five levels at 15, 20, 25, 30 and 37±1°C and the water temperature for the eel rearing at four levels, namely, 15, 20, 25 and 28±1°C. *E. tarda* was washed down from the cellophane with sterile saline solution. The bacteria of each temperature or salinity treatment were fivefold diluted into four dilutions. In the temperature experiment, the pathogen concentration was 106–109 CFUml/L when the fish rearing temperature was at 15±1°C, and 106–108CFU ml/L at 20, 25 and 28±1°C. Each dilution of 0.1ml was intraperitoneally injected into each fish in the three replicate tanks to ensure that each fish was infected with exactly same dose of the bacteria. The fish were fasted after injection, but other daily management practices were kept the same as before. After injection, the 4-day LD50 results were calculated. The results were expressed as Log CFU ml/L. A relatively short period (4 days) was used for LD50 calculation to more precisely evaluate the effect of pathogen incubation conditions (temperature) on its pathogenicity. A longer evaluation period probably abates the effects of the environmental factor treatments for pathogen incubation, because the pathogen and host will live in the same environment after challenge and then its pathogenicity probably changes with its environment in a longer duration.

Infection and isolation test

Based on the results of LD50 test, the concentration of *E. tarda* was varied in the different treatments of the isolation test. In temperature experiment, the pathogen concentration of the dilutions was 3×108 CFU ml/L when water temperature was at 15±1°C, and 3×107 CFU ml/L at 20±1, 25±1 and 28±1°C. Each dilution was injected intramuscularly into 20 fish with 0.1 ml per fish. Two fish in each group were randomly sampled to remove liver collection at 24, 48 and 72 h after injection. Fish were anaesthetization with 0.03 ml/L of 2-phenoxyethanol (ethylene glycol monophenyl ehter C6H5OCH2CH2OH, Nacalai Tesque. Inc, Japan). Liver was weighed and homogenized in a glass homogenizer containing 10 ml of sterile saline. The suspensions were diluted 10-fold to a series of dilutions. Each dilution of 0.1ml was incubated on plates of SS-agar at 25°C. For the 24 h sampling, the first to third dilutions of the suspensions were used for plate incubation, the second to fourth and third to fifth dilutions were used for the incubations of 48 and 72 h samplings respectively. After 48 h incubation the black clones of *E. tarda* were counted. The results were expressed as Log CFU g/L liver.

Statistical analysis

Data from each treatment were subject to one-way ANOVA, two-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

LD50 test

In the temperature experiment, the LD50 test results are shown in Table 1. The results showed that both the pathogen incubation temperature and challenge temperature significantly affected the pathogenicity of *E. tarda* ($p<0.01$) and the interaction between them was also significant ($p<0.01$). At 20°C fish rearing temperature, the highest pathogenicity of *E. tarda* was observed at 30°C of pathogen incubation, followed by 15, 20, 25 and 37°C. When the fish rearing temperature was raised to 25 and 28°C, the pathogenicity of *E. tarda* incubated at all the temperatures increased. The mean value of LD50 decreased from 1010.3 to 107.9 CFU ml/L, and to 107.5 CFU ml/L respectively. The pathogen incubated at 37°C had a more obvious increase in its pathogenicity to Japanese eel. Its pathogenicity, however, was still lower than those incubated at 20 and 25°C. Incubation at 15°C always resulted in relatively lower pathogenicity.

Table 1. LD50 of *Edwardsiella tarda* incubated at different temperatures to *Anguilla japonica* reared at 15, 20, 25 and 28°C, respectively (mean Log CFU ml/L±SD)*

Fish rearing temperature	Pathogen incubation temperature				
	15°C	20°C	25°C	30°C	37°C
15°C	7.98±0.97ay	7.79±0.43by	7.65±0.21by	7.32±1.11by	8.89±0.29ay
20°C	9.91±1.38abx	9.92±0.23abx	8.63±0.21bx	11.40±1.84bx	11.92±0.56abx
25°C	8.27±0.11ay	7.27±0.30cz	7.18±0.14cz	7.18±0.11by	8.19±0.10ay
28°C	8.12±0.14ay	7.17±0.30cz	7.01±0.10cz	7.38±0.11by	8.57±0.12ay

Two-way ANOVA	F-value	P-value
Pathogen incubation temperature	112.83	9.27E−14
Fish rearing temperature	17.11	7E−05
Interaction	3.66	0.00311

* Means in the same row or column sharing a common superscript letter (a, b, c for rows, and x, y and z for columns) are not significantly different as determined by Tukey's test ($p>0.05$).

Isolation test

Table 2 showed that the pathogen incubation temperature significantly affected the proliferation rate of *E. tarda* in the liver of Japanese eel at 20°C of fish rearing temperature ($p<0.05$). The slopes of linear regression of *E. tarda* proliferation rates indicated that the bacteria incubated at 20°C had the highest proliferation rate during the 72 h infection test, followed by 25, 30 and 37°C. The slopes at 37 and 15°C were significantly lower than the slopes at 20, 30 and 25°C. At 20°C of fish rearing, the number of isolated *E. tarda* incubated at 15°C maintained at the lowest level, and the numbers of isolated *E. tarda* incubated at 20, 25, 30 and 37°C were not significantly different from each other ($p>0.05$) after 72 h infection. All the remaining fish died by the fifth day.

Table 2. Proliferation of *Edwardsiella tarda* incubated at different temperature in the liver of Japanese eel reared at 20°C (mean Log CFU g/L liver±SD)*

Incubation temperature	Time			Linear regression slope
	24 h	48 h	72 h	
15°C	4.33±0.58	4.54±0.12	4.87±0.11	0.0112b
20°C	4.25±0.35	5.54±0.36	6.22±0.61	0.0398a
25°C	5.43±0.18	6.43±0.43	6.76±0.39	0.0431a
30°C	5.49±0.32	5.65±0.43	6.61±0.63	0.0487ab
37°C	5.23±0.51	5.77±0.23	6.43±0.35	0.0190b
Analysis of covariance		F-value	P-value	
		6.321	$0.001<p<0.0025$	

* Slopes sharing a common superscript letter are not significantly different as determined by Tukey's test ($p>0.05$).

When water temperature of fish rearing was at 25°C (Table 3), the results showed that *E. tarda* incubated at 20°C could not be isolated during the 3 days, whereas the numbers of other treatments came up to about 106 CFU g/L liver. At the third day after infection the number of *E. tarda* incubated at 20°C was the highest, followed by 25, 30 and 37°C, but no significant difference between them was found ($p>0.05$). The remaining fish that were injected with *E. tarda* incubated at 20°C remained alive for 10 days till the experiment termination, but the others died by the fifth day. Again, the bacteria incubated at 20°C had the highest proliferation rate; the slopes of 20, 25, 30 and 37°C were 0.0743, 0.0554, 0.0734 and 0.0787 respectively. However, analysis of covariance showed that their proliferation rates were not significantly different ($p>0.05$).

Table 3. Proliferation of *Edwardsiella tarda* incubated at different temperature in the liver of Japanese eel reared at 25°C (mean Log CFU g/L liver±SD)*

Incubation temperature	Time			Linear regression slope
	24 h	48 h	72 h	
20°C	4.21±0.14	6.12±0.21	6.71±0.11	0.0743
25°C	3.88±0.32	5.61±0.21	6.28±0.25	0.0554
30°C	3.11±0.12	4.80±0.21	6.14±0.58	0.0734
37°C	2.97±0.76	3.95±0.56	5.92±0.86	0.0787

Analysis of covariance	F-value	P-value
	1.645	0.1<P<0.25

* *E. tarda* incubated at 15°C could not be isolated from the fish liver during the 72 h infection.

Discussion

It has been confirmed that *E. tarda* exists in the environment of eel farms even when the disease does not occur (Mamnur *et al.* 1994). Therefore, measures to establish or control the rearing environment to inhibit or reduce the pathogenicity of the pathogen are very important for aquaculture. In the present study, we looked that the pathogenicity of *E. tarda* is significantly influenced by incubation temperature and also the infection temperature. The pathogenicity of *E. tarda* was the highest at 25–37°C, and lowest at 15 and 20°C. This is accordance with the fact that edwardsiellosis has higher incidences when the water temperature is high. Statistical analysis revealed a significant interaction between the growth temperatures of pathogen and host. When the water temperature of fish culture increased, the LD50 values declined. Relatively higher temperature increased the proliferation rate of the pathogen. The pathogen incubated at 20°C could be isolated from fish infected at 20°C, but not be isolated from the fish infected at 25°C. This was likely due to the fact that the pathogen concentration used in the infection at 20°C was 3×108 CFU ml/L, but that used at 25°C was 3×107 CFU ml/L. This low concentration of the pathogen incubated at 20°C may not be able to proliferate fast enough to kill the fish. Additionally, higher temperature may also affect the immune response of the flounder. However, this requires further investigations to confirm. Considering the above conditions, it would be useful to maintain a relatively lower water temperature to alleviate edwardsiellosis as long as eel growth is not affected. Obviously, it is worth expanding study scope on the effects of environmental factors on pathogen pathogenicity to modify existing disease control strategies or to design new ones for aquaculture.

Lapaglia and Hartzell (1997) have been confirmed that environmental stresses can affect the physiological characters of microbes. *Archaeoglobus fulgidus*, an anaerobic marine hyperthermophile, can form a biofilm in response to environmental stress. *Deleya halophila* can produce several induced proteins in response to oxidative stress (Mylona and Katinakis 1992). Non-halophilic purple and green sulphur bacteria in response to osmotic stress can accumulate surrose and trehalose respectively (Welsh and Herbert 1993). Stephens *et al.* (1991) reported that growth temperature, dose size and route of infection affected the pathogenicity of *Listeria monocytogenes* strains to the mouse, and that some genes of this pathogen have been proved to be involved in stress response and pathogenicity (Cormac and Colin 1999).

Pathogen and host certainly interact with each other. The inner environment of fish is obviously different from that of the incubation medium. Its living environment also influences the immunity of fish. On the other hand, temperature may affect the production of some substances that contribute to the pathogenicity of *E. tarda*. This means that conditions that support fast proliferation of pathogens may not produce substances enhancing their pathogenicity. 10 strains of *E. tarda*, showing that neither 25 nor 35°C incubation temperature had any effect on the outer membrane protein (OMP) profiles of nine out of 10 *E. tarda* isolates (Darwish *et al.* 2001). In the present study, the conditions that produced lower LD50 values generally supported higher proliferation rates of this pathogen in the fish. It remains unknown, which contributes more to the pathogenicity of *E. tarda* for Japanese eel faster proliferation in fish tissue or some unknown secreted substances. From the study of Darwish *et al.* (2001) and the present one it can be seen that the OMP is possibly associated with the pathogenicity of *E. tarda*. OMP has been considered to be a factor related to the pathogenicity (Goullet *et al.* 1994).

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