ターボット由来Edwardsiella tarda株の病原性

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Pathogenic Potential of *Edwardsiella tarda* Strains Isolated from Turbot

Nuria Castro*, Beatriz Magariños, Soledad Núñez, Juan Luis Barja and Alicia E. Toranzo

Microbiology and Parasitology Department, Faculty of Biology-CIBUS and Aquaculture Institute, University of Santiago de Compostela, Santiago de Compostela, 15782 Spain

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ABSTRACT—In this study, pathogenicity of *Edwardsiella tarda* isolates from turbot *Scophthalmus maximus* as well as other strains from different hosts were tested against turbot, sole *Solea senegalensis* and sea bass *Dicentrarchus labrax*. In addition, the influence of challenge route and temperature in the pathogenic potential of *E. tarda* strains from turbot were also examined. The results obtained showed that *E. tarda* did not have host specificity in virulence and that the turbot isolates were highly virulent regardless of the inoculation route and temperature. Moreover, inoculation experiments performed in mice suggest that these isolates are virulent for mammals. All these findings suggest that this emergent turbot pathogen constitutes an important risk factor for the marine aquaculture.

Key words: *Edwardsiella tarda*, *Scophthalmus maximus*, turbot, virulence

In the last years, repeated outbreaks of edwardsiellosis in cultured turbot *Scophthalmus maximus*, caused by *Edwardsiella tarda*, have occurred in different geographical areas of Europe being a serious problem in the culturing of this fish species[1,2]. Moreover, this fish pathogen produced high mortalities in other cultured fish species worldwide[3].

Numerous studies report that *E. tarda* constitutes a biochemically homogeneous taxon with typical characteristics of an enteric bacterium[4,5]. With regard to the serological and genetic characteristics, the existence of antigenic[1,6] and genetic[7] heterogeneity has been described. However, recent studies in our laboratory indicated that all the *E. tarda* strains isolated from turbot constitute a serological homogeneous group[1] different from other *E. tarda* strains.

The route of infection of edwardsiellosis is still undefined. The marine environment can constitute an important reservoir of *E. tarda*, since it has been demonstrated that this pathogen remains in seawater and sediment in a dormant state[8–10] maintaining its pathogenic potential for fish. In addition, there is some evidence that *E. tarda* occurs in water within the vicinity of fish farms[4] and recently it was reported that cultured sole coexisting in a same rearing facility with turbot cultures, was infected by *E. tarda*[11].

Studies on pathogenicity of *E. tarda* employing different conditions or inoculation routes are mostly focused on fish species cultured in Asia as Japanese flounder *Paralichthys olivaceus*, yellowtail *Seriola quinqueridiata*, red sea bream *Pagrus major* or Japanese eel *Anguilla japonica*[12,13]. However, the determination of the pathogenic capacity and host specificity of *E. tarda* turbot strains could be of great importance for an integrated view of the edwardsiellosis in marine aquaculture. With this aim, challenge experiments in various economically important fish species (turbot, Senegalese sole *Solea senegalensis* and sea bass *Dicentrarchus labrax*) using different routes of infection and temperatures were performed. Moreover, the virulence of all these *E. tarda* strains for mammals was also examined employing a mouse assay model.

Materials and Methods

Bacterial strains

Nine strains of *E. tarda* isolated from fish with different origin and year of isolation were used in this study (Table 1). Their identification was confirmed by biochemical procedures[1] as well as specific PCR[14]. Four of them are representative isolates of outbreaks occurred in different turbot farms in Europe between 2004 and 2006[1]. *E. tarda* isolates from eel, tilapia, striped bass, flounder and red sea bream obtained from particular donors were also employed as comparison. Experimental cultures were prepared on Trypticase soy Agar supplemented with 1% of NaCl (TSA-1, Difco Laboratories) from stock cultures stored frozen at –80°C in Cryo-Billes (AES Laboratory).

In all the challenges, bacterial suspensions in sterile saline solution (NaCl at 0.85%) (SS) at an initial concentration adjusted by visual comparison to 1.2×10^9 CFU/mL (corresponding to tube No 4 of McFarland turbidity standards) were prepared. To the confirmation of the bacterial concentration, 10-fold dilutions of these suspensions were spread onto TSA-1 plates, incubated at 25°C, and colonies present in each plate were counted. The real concentrations employed in the challenges were expressed as CFU (colony forming units) / mL.

* Corresponding author
E-mail: nuria.castro@usc.es

Fish Challenge Experiments

Turbot coming from a marine farm and sole and sea bass from experimental facilities were selected as representative of economically important cultures of marine fish species. Prior of any inoculation, fish (10 g average weight) were analyzed by classical procedures[15] to determinate if they were free of any bacterial pathogen that could interfere in the results of the experimental infections. Moreover, fish were acclimated to the experimental conditions for 7 days between their arrival to the laboratory and the start of the inoculations. Three types of routes of infection were assayed: intraperitoneal injection (i.p.), bath challenge and cohabitation challenge. In all the fish challenges, faeces were siphoned off and fish behavior was observed daily. Moreover, dead fish were removed and mortalities were recorded daily over a 20 days period after the first dead and the degree of virulence (50% lethal dose; LD_{50}) was calculated by the method of Reed and Müench[16].

Intraperitoneal challenges were performed with turbot, sole and sea bass. According to the procedures of Toranzo *et al.*[17] three replicas of ten fish groups of each fish species were intraperitoneally inoculated with 0.1 mL of 10-fold dilutions ranging from 10^1 to 10^6 CFU/mL (doses of 10^0 to 10^5 CFU/g of fish) of each *E. tarda* strains tested. Control fish were inoculated with sterile SS. Inoculated and control fish were distributed in 5-liter aquaria with continuous aeration and maintained at 18°C with a heater.

Moreover, two additional experiments by i.p., employing the same procedures as describe above, were performed in order to evaluate the influence of temperature on the degree of virulence of the isolates and/or the kinetic of fish mortalities. These assays were conducted only in turbot and consisted in an assay at 15°C and other in which fish were subjected to a rapid water temperature increase. This heat shock experiment was conducted putting a heater in the aquarium 10 days after the fish inoculation, causing a raise of the water temperature from 15°C to 18°C. The dose employed in this assay was 1×10^4 CFU/g of fish. In addition, a control group consisted of inoculated fish with SS subjected to the same temperature increase, was employed.

In bath challenge, bacterial dilutions were prepared as mentioned above. Three replicas of ten fish groups (turbot and sole) were transferred to 5-liter aquaria, and exposed for 60 min at 18°C to different dilutions of each *E. tarda* strain with final concentrations ranging from 10^5 to 10^2 CFU/mL. Control fish were also subjected to the same operations with only SS added to the tanks. Each group of fish (infected and controls) were returned to their original tanks and maintained at 18°C with a heater.

In cohabitation experiments performed in turbot, the capability of *E. tarda* strains to multiply in the fish and infect others was evaluated. Three replica of 20 turbot per tank were employed for each strain. The 50% of each group (ten turbot) were inoculated via i.p. with a dose of 1×10^3 CFU/g of fish as described above and put into a 20-liter aquarium together with a 50% of not inoculated fish. Each aquarium was continuously aerated with an airstone and maintained at 18°C with a heater. Control groups were manipulated as described

Table 1. *E. tarda* strains used in this study

Strain	Host	Origin	Year
ACC35.1	Turbot	South Europe	2005
HL1.1	Turbot	North Europe	2005
RM288.1	Turbot	South Europe	2006
ACC69.1	Turbot	South Europe	2006
KGE7901	Tilapia	Japan	ND*
E-11-2	Eel	Japan	ND
9.8	Striped bass	USA	ND
WFE1	Flounder	Japan	2002
ET006	Red Sea Bream	Japan	2002

* no data

Table 2. LD_{50} values for *E. tarda* strains employed in this study

Strain	Turbot					Sole		Sea bass	Mouse
	i.p.			Bath	Cohabitation	i.p.	Bath	i.p.	i.p.
	at 18°C	at 15°C	Heat Shock						
ACC35.1	$2.6\pm0.2\times10^0$	$1.4\pm0.2\times10^2$	$1.6\pm0.2\times10^1$	$2.6\pm0.3\times10^3$	$1.6\pm0.1\times10^1$	$2.3\pm0.1\times10^1$	$3.1\pm0.2\times10^4$	$1.7\pm0.2\times10^2$	$<1.6\pm0.3\times10^6$
HL1.1	$2.4\pm0.1\times10^0$	$1.7\pm0.2\times10^2$	$1.1\pm0.1\times10^1$	$3.1\pm0.2\times10^3$	$1.1\pm0.2\times10^1$	$2.2\pm0.2\times10^1$	$3.4\pm0.2\times10^4$	$1.5\pm0.1\times10^2$	$<1.1\pm0.3\times10^6$
RM288.1	$2.3\pm0.2\times10^0$	$1.1\pm0.1\times10^2$	$3.1\pm0.3\times10^1$	$4.1\pm0.2\times10^3$	$3.1\pm0.2\times10^1$	$2.4\pm0.1\times10^1$	$4.1\pm0.3\times10^4$	$1.3\pm0.2\times10^2$	$<3.1\pm0.3\times10^6$
ACC69.1	$2.5\pm0.2\times10^0$	$1.6\pm0.2\times10^2$	$1.2\pm0.2\times10^1$	$2.0\pm0.2\times10^3$	$1.2\pm0.1\times10^1$	$2.7\pm0.2\times10^1$	$2.3\pm0.2\times10^4$	$1.6\pm0.2\times10^2$	$<1.2\pm0.3\times10^6$
KGE7901	$3.3\pm0.1\times10^2$	NT*	NT	NT	NT	$3.3\pm0.3\times10^2$	$7.3\pm0.2\times10^4$	NT	$<3.3\pm0.2\times10^6$
E-11-2	$4.5\pm0.1\times10^3$	NT	NT	$5.2\pm0.2\times10^3$	NT	$4.5\pm0.3\times10^3$	$3.6\pm0.2\times10^4$	NT	$<4.5\pm0.2\times10^6$
9.8	$2.2\pm0.1\times10^0$	$3.3\pm0.3\times10^2$	$3.2\pm0.2\times10^1$	$8.2\pm0.2\times10^3$	$1.2\pm0.1\times10^2$	$2.8\pm0.2\times10^1$	$4.2\pm0.2\times10^4$	$1.9\pm0.1\times10^2$	$<1.2\pm0.3\times10^6$
WFE1	$2.7\pm0.2\times10^2$	$5.7\pm0.1\times10^2$	NT	$8.7\pm0.2\times10^3$	$2.4\pm0.2\times10^2$	$2.7\pm0.2\times10^2$	$1.3\pm0.2\times10^4$	$2.2\pm0.2\times10^3$	$<1.7\pm0.3\times10^6$
ET006	$3.2\pm0.1\times10^0$	$4.2\pm0.1\times10^2$	$5.4\pm0.2\times10^1$	NT	NT	$3.3\pm0.2\times10^2$	$4.4\pm0.2\times10^4$	NT	$<3.2\pm0.3\times10^6$

Figures are expressed as CFU/g of fish or mouse in the i.p. and cohabitation challenges, and CFU/mL in the bath challenges.
* no test

above, but employing SS instead of the bacterial suspension.

Virulence for mice

BALB/c mice of 10 to 12 weeks old and 21 to 25 g were inoculated with doses of 10^6 to 10^8 CFU/ mouse of each *E. tarda* strain (five mice were employed by dose). In this assay, the *Photobacterium damselae* subsp. *damselae* RM71 strain, with a LD_{50} of 3×10^6 CFU/ mouse, was included as positive control[18]. Negative control animals were inoculated with SS. The LD_{50} was calculated by the method described by Reed and Müench[16].

Re-isolation of pathogen

Re-isolation of the pathogen from the kidney, liver and spleen of dead and moribund fish and kidney from mice was carried out in order to confirm the bacterial infection. In all cases, these internal organs were streaked on TSA-1 plates that were incubated at 25°C for 24 h. The strains isolated were subjected to taxonomical analysis by standard biochemical plate and tube test[19,20] and using the commercial API 20E system (Biomereux). In addition, slide agglutination was performed to confirm their identification as *E. tarda*.

Results and Discussion

The pathogenicity assays showed that *E. tarda* isolates employed in this work, regardless of their geographic origin and source of isolation, were able to cause mortality in all the fish species (Tables 1 and 2), demonstrating that *E. tarda* could represent a potential risk for these cultures. All dead and moribund fish showed cutaneous lesions as lack of pigmentation, small hemorrhages in the musculature and in the visceral region, and some of them presented tumefactions around the eyes. Internally, abundant ascitic fluid in the abdominal cavity and hemorrhagic kidney was observed. These diseased signs are coincident with those described in natural infections of edwardsiellosis in fish[1,2]. *E. tarda* was recovered in pure culture from all the internal organs of all dead and moribund fish.

In the i.p. challenges performed at 18°C, all turbot isolates presented the highest degree of virulence with LD_{50} values between 10^0 – 10^2 CFU/g, depending of the fish host (Table 2). In the i.p. assay performed at 18°C in turbot for the representative isolate ACC35.1, most of the doses tested (10^2 to 10^5 CFU/g of fish) caused a rapid evolution of mortalities reaching the 100% in a relative short time period (within 15 days post-inoculation) (data not shown).

Although edwardsiellosis caused by *E. tarda* is classically associated to warm-water temperature[5], our results obtained in the i.p. assays performed in turbot at 15°C (Table 2) confirmed the significant pathogenic potential of this bacterium even at this low temperature. However, as expected, temperature exerted influence in both the LD_{50} values and the evolution of mortalities (Table 2, Fig. 1). In fact, in this challenge, mortalities began 12 days after inoculation, even with the highest concentrations of inocula (10^3 to 10^4 CFU/g) and then, deads increased rapidly reaching the 100% in 3 or 4 days (at day 16 post-infection) (Fig. 1).

The results described in the i.p. assays at 15°C and 18°C are in agreement with those obtained in the temperature increase experiment. In fact, in this assay, mortalities began by day 11 (only 1 day after the heat stress) and continues for 4 days reaching 100% of mortalities (Fig. 1). These results confirm that temperature is closely related with the celerity of the mortalities caused by *E. tarda*. Similar findings were reported by Zheng *et al.*[21] who demonstrated that in Japanese flounder the virulence of *E. tarda* strains increased when the fish were reared at high temperature.

The prolonged immersion experiments showed that

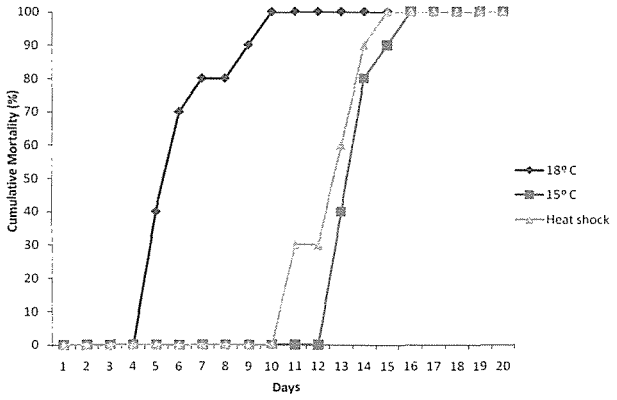

Fig. 1. Cumulative mortality (%) in the turbot inoculation experiments by i.p. at different temperatures employing the ACC35.1 strain at a dose of 1×10^4 CFU/g. Negative control groups showed 0% mortality during the experiment.

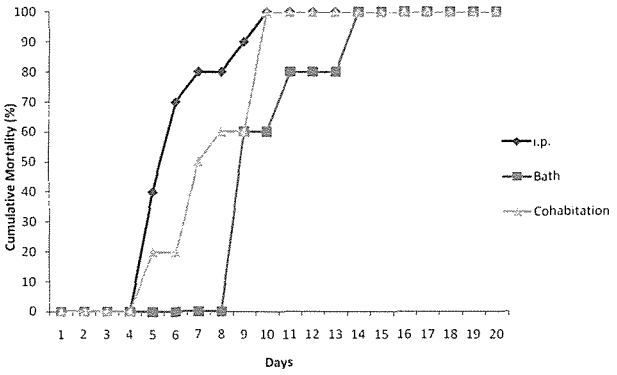

Fig. 2. Cumulative mortality (%) in the turbot experiments by different inoculation routes employing the ACC35.1 strain with a dose of 1×10^4 CFU/g (i.p. and cohabitation assays) or mL (bath experiments). Negative control groups showed 0% mortality during the experiment.

these *E. tarda* isolates were also able to infect turbot and sole by water route (Table 2). In this case, the LD_{50} values were around 10^3 CFU/mL for turbot and 10^4 CFU/mL for sole, and fish died between day 8 and 14 (Fig. 2). Therefore, as occurs in the majority of fish pathogens, the degrees of virulence by bath were always lower than those found when the fish were inoculated by intraperitoneal injection[22].

With regard to cohabitation assays, the results showed that whereas in the inoculated fish mortalities started in day 4, deaths in not inoculated fish began by day 6 and reached the 100% in 4 or 5 days (Fig. 2).

The results obtained in bath and cohabitation experiments indicates that *E. tarda* can remain in the aquatic environment maintaining its capacity to infect fish and that horizontal transmission play an important role in the development of the edwardsiellosis, especially in aquaculture industry where high population densities are common.

The demonstrated lack of host specificity for the turbot strains of *E. tarda* is in accordance with the data previously reported by Xiao *et al.*[23] employing Chinese turbot strains for swordtail fish *Xiphophorus helleri*. However, other previous studies described little or not pathogenicity of some *E. tarda* strains isolated from Japanese flounder, Japanese eel and tilapia to red sea bream[12].

In addition, the assays performed in mice demonstrated that all the *E. tarda* strains employed were pathogenic for mice, even with LD_{50} values lower than those showed for the control strain (Table 2). These results indicated that precautionary measures with the fish manipulation and other routine tasks in the aquaculture industry should be of great importance to avoid the possibility of zoonoses. In fact, Van Damme and Vandepitte[24], and Vandepitte *et al.*[25], reported that this pathogen could be transmitted to humans and cause infections from fishes or water.

With regard to the statistical analysis, significant differences ($p<0.05$) (Chi-square test) among different replica were not found in all the experiments developed.

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