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Sodium chloride-enhanced adherence of *Edwardsiella tarda* to HEp-2 cells

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ABSTRACT—The adherence of *Edwardsiella tarda* strains to HEp-2 cells was studied by the viable cell count and direct microscopic count methods. Three selected *E. tarda* strains having different hemagglutination activities against guinea pig erythrocytes were cultured in a peptone-yeast extract broth supplemented with 3% NaCl (3%-NaCl culture) or without NaCl (0%-NaCl culture). Strain FK1051 showed significantly higher adherence in the 3%-NaCl culture than in the 0%-NaCl culture. A similar adherence pattern was observed in strain KG8401, though the adherence of the 0%-NaCl culture was higher than that of FK1051. In contrast, both cultures of strain SU166 were low in the adherence. These adherence patterns to the HEp-2 cells correlated well with the previously reported hemagglutinating activities of the strains. *N*-acetylneuraminic acid markedly inhibited the adherence as well as the hemagglutination. This NaCl-enhanced adherence may be involved in pathogenesis of *E. tarda* infection particularly in marine fish.

Key words: *Edwardsiella tarda*, adherence, hemagglutination, HEp-2 cells, *N*-acetylneuraminic acid

Edwardsiella tarda, a member of family *Enterobacteriaceae*, causes infectious diseases in several species of cultured freshwater and marine fish (Hoshina, 1962; Wakabayashi and Egusa, 1973; Kusuda *et al*. 1977; Yasunaga *et al*., 1982; Nakatsugawa, 1983). The pathogenesis of *E. tarda* infection has not been fully understood, but it is thought that some virulence factors are involved in pathogenicity of the organism, i.e. the ability to invade epithelial cells (Janda *et al*., 1991; Ling *et al*., 2000), to resist serum- and phagocyte-mediated killing (Iida and Wakabayashi, 1993; Srinivasa Rao *et al*., 2001), and to produce siderophore (Kokubo *et al*., 1990; Igarashi *et al*., 2002), hemolysin (Janda and Abott, 1993), and toxins (Ullah and Arai, 1983; Suprapto *et al*., 1995).

Adherence of pathogenic bacteria to their hosts is regarded as an important step in disease initiation. *E. tarda* has the ability to adhere to gill and body surface of fish (Ling *et al*., 2001). Fimbriae (pili) or hemagglutinins are essential adherence factors usually found in pathogenic bacteria (Finlay and Falkow, 1997; Abraham *et al*., 1998). Recently, Sakai *et al*. (2003, 2004) reported that a fimbrial major subunit (19.3 kDa protein) is associated with the mannose-resistant hemagglutinating activity (HA) in *E. tarda*. Furthermore, our previous study revealed that the HA of *E. tarda* was induced by high (3%) NaCl concentration in the culture medium and *E. tarda* strains were classified into three types; type A showed HA only with the 3%-NaCl culture, type B exhibited HA in both 0%- and 3%-NaCl cultures, and type C demonstrated no HA in both the cultures (Yasunobu *et al*., 2006).

In the present study, using the representative strains among HA type A, type B, and type C described above, we investigated the influence of NaCl concentration on the adherence of *E. tarda* to HEp-2 cells, which were derived from human epidermoid larynx carcinoma. We also examined inhibition of the cell adherence by *N*-acetylneuraminic acid, which had been reported to interfere the HA of *E. tarda* (Sakai *et al*., 2003).

Materials and Methods

Bacterial strains and culture conditions

Three strains of *E. tarda*, representing the different types of HA described previously (Yasunobu *et al*. 2006), were used: strain FK1051 (type A) isolated from diseased Japanese flounder *Paralichthys olivaceus*, strain KG8401 (type B) from diseased Japanese eel

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Anguilla japonica, and a non-clinical strain SU166 (type C). These strains were pre-cultured on Trypto-Soya Agar (TSA, Nissui) at 30°C for 24 h.

According to the method described before (Yasunobu *et al*., 2006), each of the pre-cultured *E. tarda* strains 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 30°C overnight with shaking (100 rpm) and the bacterial cells were harvested by centrifugation (5,000 $\times$ *g*, 10 min). The bacterial cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and the cell concentration was adjusted to 1.0 at A_{530} (approximately 1-2 $\times$ 10^9 CFU/mL).

Adherence assay by the viable cell count

The adherence assay was performed as described before (Srinivasa Rao *et al*., 2001). HEp-2 cells were seeded into 24-well tissue culture plates and grown in RPMI 1640 (Sigma) containing 10% fetal calf serum at 37°C for 24 h under 5% CO_2 to reach completely confluent monolayer (5 $\times$ 10^5 cells/well). Then, 100 μL of the bacterial suspension was added to each well (1 $\times$ 10^8 CFU/well), and the plates were incubated at 37°C for 1 h under 5% CO_2. To measure the number of bacteria adhering to the monolayers, the plates were washed five times with PBS to remove the non-adhered bacteria. The epithelial cells were then lysed with 1% (v/v) Triton X-100 in PBS (200 μL/well) accompanied by vigorous agitation for 15 min. Thereafter, 800 μL of PBS was added to every well and pipetted thoroughly. The resulting bacterial suspension was 10-fold serially diluted and the adhered bacterial number was estimated by a spread plate method on TSA. This assay quantifies the total number of bacteria bound to the surface of the cells and bacteria internalized by the cells, as well as the bacteria non-specifically bound to the wells of the tissue culture plates. The number of the internalized bacteria was counted after incubating the infected HEp-2 cells with 100 μg/mL gentamicin at 37°C for 1 h under 5% CO_2. Also, the number of bacteria which non-specifically bound to the wells was counted after treated similarly using HEp-2 cell-free plates. The adherence assay was performed in triplicates in three independent experiments.

Microscopic count of adhered bacteria

This assay was conducted according to the method of Wang and Leung (2000). A sterile glass coverslip (13 mm in diameter) was placed in each well of the 24-well tissue culture plate, and the wells were seeded with HEp-2 cells and incubated at 37°C overnight under 5% CO_2 as described above till reaching semi-confluency (2 $\times$ 10^5 cells/well). The bacterial concentration was adjusted to 0.8 at A_{530} (approximately 1-2 $\times$ 10^8 CFU/mL). One hour after infection with 100 μL of bacterial suspension (2 $\times$ 10^7 CFU/well), the HEp-2 cells were washed five times with PBS to remove the non-adhered bacteria, then the cells were fixed with 70% methanol for 10 min and stained with 10% Giemsa stock solution for 30 min. After being washed three times with PBS, the stained coverslips were mounted on glass slides, examined by light microscope and photographed. The adhered bacteria to 50 epithelial cells were counted per coverslip, where two coverslips were counted in triplicate experiments. For every strain used, the mean number of adhered bacteria per cell was recorded.

Effect of N-acetylneuraminic acid on HA of E. tarda

The effect of a carbohydrate, *N*-acetylneuraminic acid (NANA) on HA was investigated in the manner described by Sakai *et al*. (2003). As a result of the preliminary experiments, NANA was used at a final concentration of 3 mM. The bacterial cells were harvested and washed twice with PBS and the bacterial concentration was adjusted to 1.0 at A_{530}. The bacterial suspension (2 $\times$ 10^9 CFU/mL) was mixed with an equal volume of NANA solution or PBS (control) and incubated at room temperature for 30 min. Using 96-well microtiter plates, serial 2-fold dilutions (20 μL/well) of the mixtures were prepared with the NANA solution or PBS and then 2% guinea pig erythrocyte suspension (20 μL/well) was added to each well. After mild agitation for 20 s, the plates were incubated at 25°C for 1 h and subsequently at 4°C overnight.

Effect of N-acetylneuraminic acid on adherence of E. tarda

According to the method of Nagayama *et al*. (1995) and Decostere *et al*. (1999), bacterial suspensions (2 $\times$

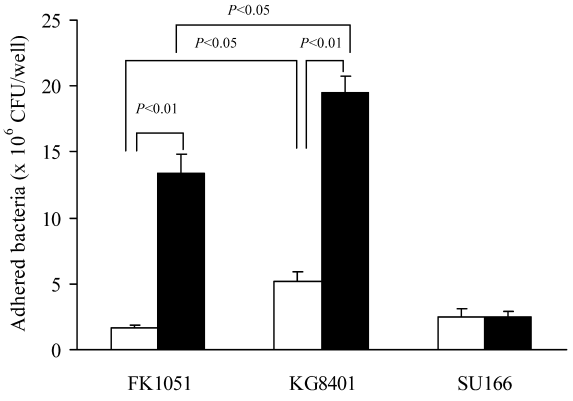

Fig. 1. Viable cell counts of *E. tarda* adhered to HEp-2 cells. HEp-2 cells were exposed to bacteria at a dose of 1 $\times$ 10^8 CFU/well. White bars represent 0%-NaCl cultures and black bars represent 3%-NaCl cultures.

10^9 CFU/mL) were mixed with equal volumes of either NANA solution (final concentration: 6 mM) or PBS as a control. These suspensions were kept at room temperature for 1 h. The bacterial cells in NANA suspension were harvested, washed twice in PBS and resuspended in PBS. Then 100 μL of NANA-treated or control bacterial suspension was added to each well of a triplicate set of 24-well plates containing confluent HEp-2 cells. After incubation at 37°C for 1 h under 5% CO_2, the cells were washed five times with PBS and processed for viable cell count as explained above. This assay was done in three separate experiments and the mean number of the adhered bacteria was calculated.

Statistical analysis

Student's *t* test was used to analyze the significance of differences between the results in adherence and adherence inhibition tests.

Results

Adherence assay by the viable cell count method

The internalized bacterial numbers in HEp-2 cells ranged from 4.9×10^3 to 2.0×10^4 CFU/well and from 6.5×10^3 to 7.1×10^4 CFU/well for the 0%-NaCl cultures and the 3%-NaCl cultures, respectively, where there were no significant differences among the strains. There were also no significant differences between the 0%-NaCl cultures and the 3%-NaCl cultures or among the strains in the bacterial numbers, which bound non-specifically to the wells, ranging from 3.3×10^3 to 7.9×10^3 CFU/well. These numbers of bacteria were fairly low com-

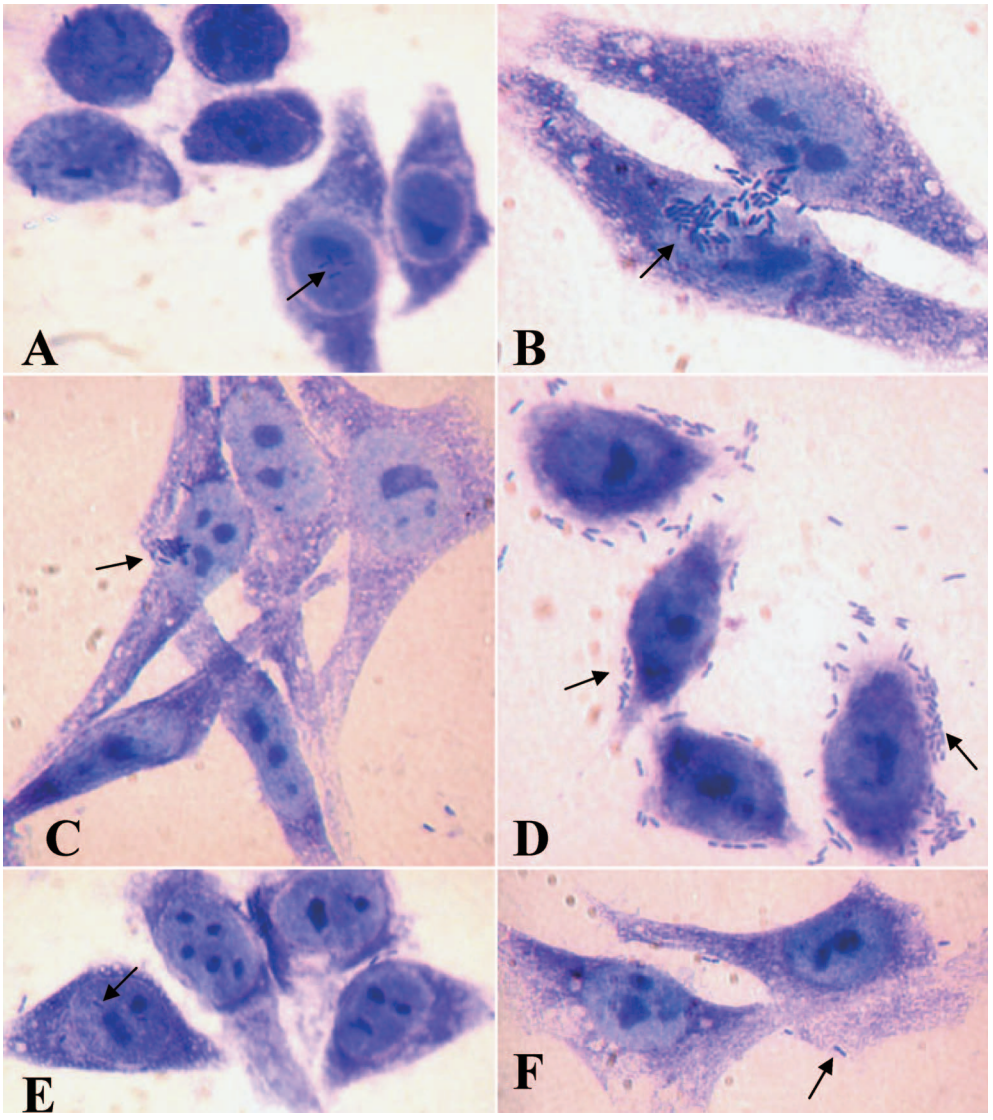

Fig. 2. Microscopic appearances (arrows) of *E. tarda* adhered to HEp-2 cells. HEp-2 cells were exposed to bacteria at a dose of 2×10^7 CFU/well and stained by Giemsa (× 1,000). A: 0%-NaCl culture of FK1051, B: 3%-NaCl culture of FK1051, C: 0%-NaCl culture of KG8401, D: 3%-NaCl culture of KG8401, E: 0%-NaCl culture of SU166, F: 3%-NaCl culture of SU166.

pared with those of the bacteria adhered to the cells, as shown below, and thus did not affect the overall adherence results.

The numbers of bacteria adhered to HEp-2 cells ranged from 1.7×10^6 to 1.9×10^7 CFU/well (Fig. 1). In strain FK1051, the number (1.3×10^7 CFU/well) of the 3%-NaCl culture was significantly higher than that (1.7×10^6 CFU/well) of the 0%-NaCl culture. Similarly, the viable cell count of adhered KG8401 significantly increased in the 3%-NaCl culture (1.9×10^7 CFU/well) in comparison with that of 0%-NaCl culture (5.2×10^6 CFU/well). When the numbers of the 0%-NaCl cultures were compared between strains FK1051 and KG8401, that of KG8401 was significantly higher. In contrast, both the 0%- and 3%-NaCl cultures of strain SU166 displayed low adherence (2.5×10^6 CFU/well).

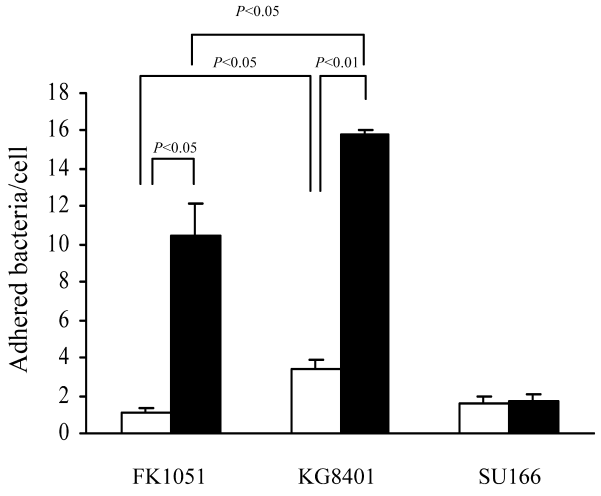

Fig. 3. Microscopic counts of *E. tarda* adhered to HEp-2 cells. HEp-2 cells were exposed to bacteria at a dose of 2×10^7 CFU/well and stained by Giemsa. White bars represent 0%-NaCl cultures and black bars represent 3%-NaCl cultures.

Microscopic count of adhered bacteria

Fig. 2 shows *E. tarda* cells adhered to HEp-2 cells. In both strains FK1051 and KG8401, the adhered bacterial number of the 0%-NaCl cultures (Fig. 2A, C) was

Table 1. Inhibition of hemagglutinating activity of *E. tarda* by *N*-acetylneuraminic acid (NANA)

Strain	NaCl conc. of the culture medium	Hemagglutination titer ($1:2^n$)[a]	
		Control	NANA -treated (3 mM)
FK1051	0%	< 0	< 0
	3%	5	0
KG8401	0%	4	0
	3%	6	1
SU166	0%	< 0	< 0
	3%	< 0	< 0

[a] Hemagglutination titer was expressed as the highest dilution of *E. tarda* suspension, which showed positive reaction against guinea pig erythrocytes. Titer 0 means initial bacterial suspension (1.0 at A_{530}, ca. 10^9 CFU/mL)

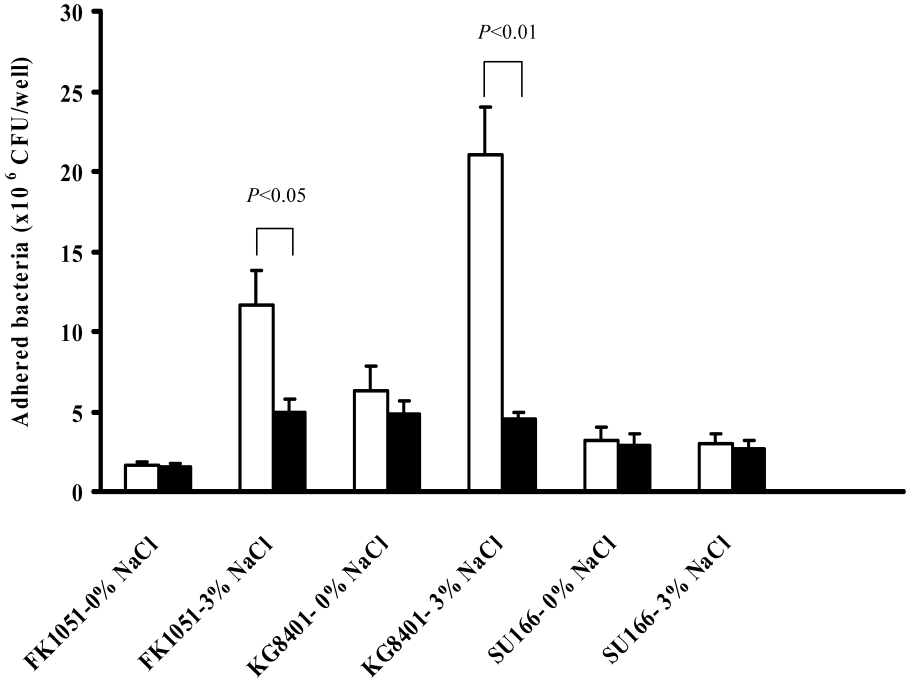

Fig. 4. Effect of *N*-acetylneuraminic acid (NANA) on adherence of *E. tarda* to HEp-2 cells. HEp-2 cells were exposed to bacteria at a dose of 1×10^8 CFU/well. White bars represent NANA-untreated bacteria (control) and black bars represent NANA-treated bacteria.

very few compared with those of the 3%-NaCl cultures (Fig. 2B, D). No apparent difference in the adhered bacterial numbers was seen between the 3%-NaCl and 0%-NaCl cultures of strain SU166 and the numbers were few in both cases (Fig. 2E, F).

The mean numbers of the adhered bacteria per cell are shown (Fig. 3). The numbers of the 3%-NaCl cultures of strains FK1051 (10.4 bacteria/cell) and KG8401 (15.8 bacteria/cell) were significantly higher than those (1.1 bacteria /cell for FK1051 and 3.4 bacteria /cell for KG8401) of the 0%-NaCl cultures. In addition, the number of the 0%-NaCl culture of KG8401 was significantly higher than that of the 0%-NaCl culture of FK1051. In strain SU166, no significant difference was found in the adhered bacterial numbers between the 0%-NaCl culture (1.6/cell) and 3%-NaCl culture (1.7/cell).

Effect of N-acetylneuraminic acid on HA of E. tarda

Preincubation of the 3%-NaCl culture of strain FK1051 with NANA caused a drop in the HA titer from $1 : 2^5$ in the untreated bacteria (control) to $1 : 2^0$ in the NANA-treated bacteria. No difference in HA between the control and NANA-treated bacteria was seen in case of the 0% NaCl-culture, which had no HA. In strain KG8401, preincubation with NANA resulted in a decrease of HA from $1 : 2^6$ in the control to $1 : 2^1$ in NANA-treated bacteria for the 3%-NaCl culture, while the 0%-NaCl culture exhibited HA of $1 : 2^4$ in the control and $1 : 2^0$ in NANA-treated bacteria. No HA was observed in SU166 cultures (Table 1).

Effect of N-acetylneuraminic acid on adherence of E. tarda

Viable cell counts of both NANA-treated and untreated (control) *E. tarda* strains revealed that the adherence of NANA-treated 3%-NaCl culture of strains FK1051 (4.9×10^6 CFU/well) and KG8401 (4.5×10^6 CFU/well) was significantly lower as compared with the controls (1.2×10^7 CFU/well for FK1051 and 2.1×10^7 CFU/well for KG8401), displaying an adherence inhibition of 59.2% for FK1051 and 78.6% for KG8401. In the other cases including the 0%-NaCl cultures, there were no significant differences in the number of adhered bacteria between NANA-treatment and control (Fig. 4).

Discussion

The ability to agglutinate erythrocytes, here mentioned as HA, has been widely used as a model for detecting the adhesive ability in a variety of bacterial species, including fish-pathogenic bacteria (Decostere *et al*., 1999; Moller *et al*., 2003). In the present study, we demonstrated that the adhesive patterns of *E. tarda* strains to HEp-2 cells well correlated with their HA patterns against guinea pig erythrocytes described previously, where the HA of *E. tarda* FK1051 and KG8401 was enhanced by the increase of NaCl concentration in the culture media (Yasunobu *et al*., 2006). The NaCl-induced adherence was also described in *Salmonella typhi* (Tartera and Metcalf, 1993), in which the adherence ability to Henle 407 epithelial cells increased under higher osmolarity (until 0.3 M NaCl) of the culture medium. Our previous infection experiments with *E. tarda* FK1051 showed that the 3%-NaCl culture was more virulent to Japanese founder than the 0%-NaCl culture, when fish were challenged orally, and thus we speculated that NaCl-induction of HA is important for adherence of *E. tarda* to the intestinal epithelial cells of fish (Yasunobu *et al*. 2006). The present result, i.e. a positive correlation between HA and adherence, supports this speculation. As high salt environment is not favorable for survival and growth of *E. tarda*, this NaCl-induced adherence might be essential for *E. tarda* to establish the infection particularly in marine fish. Further precise *in vivo* experiments about adherence to the epithelial cells of fish are required to demonstrate this hypothesis.

The tissue- and cell-specific adherence of bacteria is a key aspect of the bacterium-host cell interaction. Specific adherence to target cells is generally mediated by specific structures (adhesins) that exist on the bacterial cell surface. There are some differences among strains in hemagglutinins of *E. tarda* (Nowotarska and Mulczyk, 1977; Wong *et al*., 1989; Janda *et al*., 1991). Sakai *et al*. (2003, 2004) reported that a fimbrial protein (19.3-kDa protein) was associated with mannose-resistant HA of *E. tarda* and that the HA is inhibited by *N*-acethylneuraminic acid (monosaccharide) and fetuin (glycoprotein containing NANA). They suggested that the carbohydrate binding adhesion plays an important role in colonization of *E. tarda*. The present study revealed that the adherence, as well as the HA, of *E. tarda* strains FK1051 and KG8401 to HEp-2 cells was markedly inhibited by NANA. It was reported that virulent strains of *E. tarda* had the ability to adhere to the body mucus of blue gourami *Trichogaster trichopterus* (Ling *et al*., 2001). Since NANA is one of the most abundant carbohydrates in the fish mucus (Asakawa, 1983; Wolfe *et al*., 1998), NANA or related sugar moiety might be a receptor for *E. tarda* adhesin on the host cells. Future chemical identification of the cell receptor(s) is necessary for analysis of *E. tarda* adhesin (lectin-like substance), which leads to further understanding of the pathogenesis of *E. tarda* infection in fish.

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