Assiut Vet. Med. J. Vol. 61 No. 144 January 2015

INFLUENCE OF SALINITY ON *IN VIVO* ADHESION, PHAGOCYTOSIS AND PATHOGENICITY OF *EDWARDSIELLA TARDA*

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Received at:25/12/2014

Accepted: 18/1/2015

ABSTRACT

The effect of NaCl concentration on the adhesion of *Edwardsiella tarda* to goldfish intestine was studied by the viable cell count. Three strains of *E. tarda* with different hemagglutinating activities (HA) for guinea pig erythrocytes were cultured in a peptone-yeast extract broth containing 3% NaCl (3%-NaCl culture) and without NaCl (0%-NaCl culture). Both of the 3%-NaCl cultures of the strains FK1051 and KG8401 displayed significantly higher adhesion than their respective 0%-NaCl cultures. On the contrary, no significant difference in adhesion was found between the 0%- and 3%-Nacl cultures of the strain SU166, with low adhesion in both. Scanning electron microscopy of the bacteria adhered to the intestine confirmed the results of the viable cell count. Similarly, the time-course study on *E. tarda* infection showed significant increases in the bacterial numbers recovered from the kidney of fish infected with bacteria grown in high NaCl concentration in case of FK1051 and KG8401 strains. The adhesion and time-course results were consistent with the previous data for HA and *in vitro* adherence of these strains. The effect of NaCl on phagocytosis of *E. tarda* was studied using murine macrophages, revealing increased internalization of the bacteria in the 3%-NaCl culture. However, the total pathogenicity of *E. tarda* was not obviously altered by the increase of NaCl in the culture medium.

Key words: *Edwardsiella tarda, adhesion, time-course, phagocytosis, pathogenicity*

INTRODUCTION

Edwardsiella tarda is the causative agent of the disease known as edwardsiella septicemia (edwardsiellosis) which is responsible for great economic losses in freshwater fishes including Nile tilapia, *Oreochromis niloticus*, and marine fishes such as Japanese flounder *Paralichthys olivaceus* and other species (Kusuda and Salati, 1993; Thune *et al*., 1993; Muroga, 2001, Evance *et al*., 2011 and Ibrahem *et al*., 2011). The disease is multifactorial in nature (Leung *et al*., 2012), yet the precise pathophysiologic events involved in the infective process are not clearly revealed.

The ability to adhere to the host cells is considered to be the first step in disease initiation for many pathogenic enteric bacteria (Smith, 1977 and Benhamed *et al*., 2014). The adherence of bacteria to host cells is mediated by macromolecules known as adhesins (Smyth *et al*., 1996 and Niemann *et al*., 2004). Many fish bacteria, including *E. tarda*, adhere to intestine and disseminate to internal organs, where the intestine was reported as a portal of entry for those fish pathogens (Horne and Baxendale, 1983; Baldwin and Newton, 1993; Olsson *et al*., 1996, Ling *et al*., 2001; Smith *et al*., 2004; Jutfelt *et al*., 2006 and Harriff *et al*., 2007). *E. tarda* adherence to the human epithelial (HEp-2) cells, as well as its hemagglutinating activity (HA) was proved to be induced by the high NaCl concentration in the culture medium (Mahmoud *et al*., 2006 and Yasunobu *et al*., 2006). The expression of the putative major fimbrial subunit, 19.3-kDa protein, associated with HA of *E. tarda* (Sakai *et al*., 2003, 2004) was also augmented by high NaCl concentration (Yasunobu *et al*., 2006).

The objectives of the present study are to examine the effect of NaCl concentration on the *in vivo* adhesion of *E. tarda* to the intestine of goldfish *Carassius auratus* and on the resistance to phagocytosis, recovery from infacted fish kidney and pathogenicity of *E. tarda*.

MATERIALS and METHODS

Fish:

Goldfish *Carassius auratus* (6 ± 1 g) were purchased from a commercial fish farm. The fish were acclimated in 57 L aquaria for 2 weeks at least. The aquaria were supplied with dechlorinated water and

maintained at 25 ± 1°C. For the fish used for investigation of the adhesion of *E. tarda* to fish intestine, feeding was discontinued 1 week prior to experimental infection. Also, fish were not fed during the bacterial challenge according to the method of Suprapto *et al*. (1995).

Bacteria and culture conditions:
Three strains of *E. tarda* representing the different HA patterns (Yasunobu *et al*. 2006) were used. One strain isolated from diseased Japanese flounder (FK1051) representing type A (having HA at 3%-NaCl culture only), another strain (KG8401) isolated from diseased Japanese eel *Anguilla japonica* as one of type B (showing HA in both 0%- and 3%-cultures) and a non-clinical SU166 strain as type C (eliciting no HA in either cultures). Using Trypto-Soya Agar (TSA, Nissui, Japan), the strains were pre-cultured at 30°C for 24 h. Also, a type III secretion system (TTSS)-deficient *E. tarda* mutant, mET1229, prepared by Okuda *et al*. (2006) was grown on TSA containing kanamycin (50 µg/ mL) at 30°C for 24 h. Each of the pre-cultured *E. tarda* strains was inoculated into 10 mL of a liquid medium composed 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 (3%-NaCl culture) or without NaCl (0%-NaCl culture). Both 0%- and 3%-NaCl cultures of the mET1229 strain were provided with kanamycin (50 µg/ mL). The bacterial cultures were incubated at 30°C 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 1.0 at A_{530} (1-2 × 10^9 CFU/mL) as described by Mahmoud *et al*. (2006).

Adhesion of *E. tarda* to fish intestine:
The adhesion of *E. tarda* to the intestine of goldfish was determined quantitatively as described by Olsson *et al*. (1996) and Ling *et al*. (2001). Briefly, the fish were sedated using 100 ppm of tricane methane sulphonate (MS-222, Sankyo, Tokyo, Japan) for 2 min. Then, they were anally inoculated with 0.2 ml of the bacterial suspension in PBS (1-2 × 10^9 CFU/mL) from the 0%- and 3%-NaCl cultures of the used strains (Fig.1). One hour later, the fish were euthanized by decapitation and the intestine of each fish was dissected aseptically, washed five times in PBS to remove bacteria which were not firmly bound, and homogenized in 1 mL of PBS. Serial dilutions of the suspension obtained were then plated out on Salmonella Shigella (SS) agar (Nissui, Japan) and incubated for 24 h at 30°C. A control group of fish was inoculated anally with 0.2 ml of sterile PBS and examined in the same way as the treated group. Results were expressed as the number of bacteria recovered per gram of intestine. These results represent the means obtained after using six fish per each treatment.

Scanning electron microscopy of the bacteria adhered to intestine:
The processing for scanning electron microscopy was done according to the method of Knutton *et al*. (1987). The intestines of the infected fish were aseptically dissected, washed five times in PBS, then fixed in 3% phosphate-buffered (0.1 M, pH 7.4) gluteraldehyde, dehydrated through graded alcohol series and dried to the critical point. Specimens were mounted on stubs, coated with platinum and examined in the scanning electron microscope (JEOL, JSM-6390LV). Ten fields, at least, were examined for each specimen and the adhesion tendency of the 0%- and 3%-NaCl cultures was assessed by observing the mean adhered bacteria per field.

Effect of NaCl concentration on mRNA expression of *etfA* gene:
etfA is the gene encoding a 19.3-kDa protein that is associated with the possession HA of *E. tarda* (Sakai, *et al*., 2003). The transcript analysis of *etf*A was done by reverse transcription (RT)-PCR as described previously (Okuda *et al*., 2007). From the 0%- and 3%-NaCl cultures of the *E. tarda* strains, total RNAs were isolated with ISOGEN (Nippongene, Japan) following the instructions of the producer. The RNAs were further purified by treatment with RNase-free DNase I (Takara, Japan), and the amount and purity of the RNAs were assessed by measuring A_{260} and A_{280}. A total of 60 ng of the RNA was used to amplify the *etfA* transcript by RT-PCR. The RT-PCR was performed using the SuperScript One-Step RT-PCR with Platinum *Taq* system (Invitrogen, Japan). The primers used for *etf*A were those designed previously by Sakai *et al*. (2007), (F: 5′-CGGTAAAGTTGAGTTTACGGGTG-3′; R: 5′-TGTAACCGTGTTGGCGTAAG-3′). The RT-PCR was conducted with reverse transcription at 50°C for 30 min, then PCR with an initial denaturation step of at 94°C for 2 min, 30 cycles of denaturation (94°C for 15 s), annealing (50°C for 30 s) and extension (72°C for 1 min). The product (415bp) was visualized by ethidium bromide on 2% agarose gel electrophoresis.

Internalization and intracellular replication assay:
A murine macrophage-like cell line (J774 cells) derived from BALB/c mice was used in this experiment. It was maintained in RPMI1640 (Sigma) supplemented with 10% fetal calf serum (FCS). The internalization and the intracellular replication of *E. tarda* within the J774 cells was performed as described by Srinivasa Rao *et al*. (2001) and Okuda *et al*. (2006). Using 24-well tissue culture plates, 5 × 10^4 J774 cells/well were inoculated and incubated overnight at 37°C under 5% CO_2. The tissue culture was infected with 0%- and 3%-NaCl cultures of

FK1051 strain (wild-type) and mET1229 strain at a multiplicity of infection of one and incubated for 30 min. Then, the medium was replaced with pre-wormed RPMI1640 containing 200 µg/mL gentamicin. After 1.5-h incubation, the antibiotic-supplemented medium was removed, the cells were washed twice with PBS, and new tissue culture medium without gentamicin was added. The tissue culture was incubated for 0, 5, 12, and 22 h at 37°C under 5% CO_2. At each time point, the cells were washed twice with PBS and lysed with 1% (v/v) Triton X-100. The bacteria released after lysis of macrophages were 10-fold serially diluted and counted by plating on TSA.

Recovery of *E. tarda* from anally-infected fish kidney (Time-course):

Detection of the bacterial number in the kidney of fish, infected with 0%- and 3%-NaCl cultures, was performed following the method described previously (Ling *et al*., 2001). Briefly, the kidneys of the anally-infected fish were excised under aseptic condition at 6, 24 and 48 h post infection. The kidneys were weighed and homogenized in 1 mL of PBS/each kidney. Then, serial dilutions of the suspensions obtained were plated out on SS agar plates and incubated for 24 h at 30°C. Results were expressed as the mean number of bacteria recovered per gram of kidney, where five fish were employed for each NaCl concentration at each time point. Another five fish were inoculated with 0.2 ml of sterile PBS, similarly sampled and served as a control group.

Pathogenicity of *E. tarda* to goldfish:

The pathogenicity of 0%- and 3%-NaCl cultures of FK1051 was tested by intramuscular injection in goldfish. A dose of 50 µL/ fish from different concentrations of both cultures was used. The injected bacterial counts were 1.9×10^6, 10^5, and 10^4 CFU/fish for the 0%-NaCl culture and were 1.5×10^6, 10^5, and 10^4 CFU/fish for the 3%-NaCl culture. A control group was injected with sterile PBS. The mortalities were recorded over a period of 2 weeks and the survival rates were calculated.

Statistical analysis:

The data were analyzed with student's *t* test and probabilities lower than 0.05 ($P < 0.05$) were considered significant.

RESULTS

Adhesion of *E. tarda* to fish intestine:

The numbers of adhered bacteria at 0%- and 3%-NaCl cultures are illustrated in Fig. 2. The 3%-NaCl culture of FK1051 showed significantly higher number (5.1×10^7 CFU/g) than that of 0%-NaCl culture (1.2×10^7 CFU/g). Also, in case of KG8401, there was a significant increase in the adhered number of the 3%-NaCl culture (8.1×10^7 CFU/g) in comparison with that of the 0%-NaCl culture (3.3×10^7 CFU/g). The 0%-NaCl culture of KG8401 was significantly higher than that of FK1051, while no significant difference was found between the 3%-cultures of either strain. For SU166 strain, there was no significant difference between the adhered number of 3%-NaCl culture (1.7×10^7 CFU/g) and that (1.5×10^7 CFU/g) of the 0%-NaCl culture. No bacterial growth was found on the plates used for culturing of intestinal homogenates of the control group.

Scanning electron microscopy of the bacteria adhered to intestine:

The adhesion of the bacteria increased drastically by the increase of NaCl concentration. Many bacteria colonizing the intestinal epithelial surface were seen throughout the specimens of the 3%-NaCl cultures of FK1051 and KG8401, while less bacterial numbers colonized the 0%-Nacl culture of KG8401. On the other hand, very few bacteria were found adhered to the intestine in case of 0%-NaCl culture of FK1051 and both 0%- and 3%-NaCl cultures of SU166. Fig. 3 (A & B) shows the bacterial adhesion to representative 0%- and 3%-NaCl cultures of FK1051. Evaluation of the adhesion tendency of the different strains revealed an increase in the adhesion trend by the increase of the NaCl concentration in the culture medium, with the exception of SU166 which exhibited low trend in both 0%- and 3%-NaCl cultures (Table 1).

Effect of NaCl concentration on mRNA expression of *etfA* gene:

The RT-PCR revealed an increase in the expression of the *etfA* by the increase of NaCl in the culture medium. That gene was not expressed in either 0%- or 3%-NaCl cultures of SU166 strain (Fig. 4).

Internalization and intracellular replication assay:

At 0 h incubation time, the 3%-NaCl culture of the wild strain (FK1051) displayed a significantly higher number than that of 0%-NaCl culture (Table 2). The subsequent incubation periods showed that both 0%- and 3%-NaCl cultures of the wild strain could survive and replicate inside the macrophage along the time-course of the study, while the cultures of the mutant strain (mET1229) exhibited reduction in the bacterial count by time and the bacteria were almost killed by the 22 h (Fig. 5).

Recovery of *E. tarda* from infected fish kidney (Time-course):

Except for SU166, the bacterial numbers recovered from kidney increased by the increase of NaCl (3%-NaCl cultures) at every sampling time, from 6 h till 48 h post infection (Fig. 6). The bacterial load of the 0%-NaCl cultures of FK1051, throughout the time-course, ranged from 4.3×10^5 to 4.9×10^6 CFU/g of kidney, while the range was from 7.4×10^5 to 1.3×10^7 CFU/g of kidney for the 3%-NaCl cultures

exhibiting significant differences between 0%- and 3%- NaCl cultures at each time point ($P<0.05$). The same pattern was found in case of KG8401 which displayed a range of 5.4×10^5 to 4.9×10^6 CFU/g of kidney for the 0%-NaCl cultures and 8.9×10^5 to 1.6×10^7 CFU/g of kidney for the 3%-NaCl cultures, where each 3%-NaCl culture was significantly higher than its respective 0%- NaCl culture of the same time ($P<0.05$). On the other hand, no significant difference was detected between 0%- and 3%-NaCl cultures of SU166 at any time point, with low bacterial numbers at 6 h ($4.7\text{-}4.9 \times 10^5$ CFU/g of kidney) and declined by time pass reaching $1.7\text{-}2.1 \times 10^5$ CFU/g of kidney at 48 h post infection. The kidneys of the control group did not show bacterial growth on SS agar at any sampling time.

Pathogenicity of *E. tarda* to goldfish:

The mortality pattern after injection of the 3%-NaCl culture at a dose of 10^6 CFU/fish was faster than that of 0%-NaCl culture. However, after being injected with 10^5 CFU/fish, the course of mortalities induced by either 0%- or 3%-NaCl cultures was more or less similar. The injection of 10^4 CFU/fish from either culture caused no mortalities. Fig. 7 illustrates the survival rate after the intramuscular challenge.

Fig. 1: Anal inoculation of goldfish *Carassius auratus* with *E. tarda* using anal tube. The inoculated dose was 0.2 mL of bacterial suspension/fish ($1\text{-}2 \times 10^9$ CFU/mL).

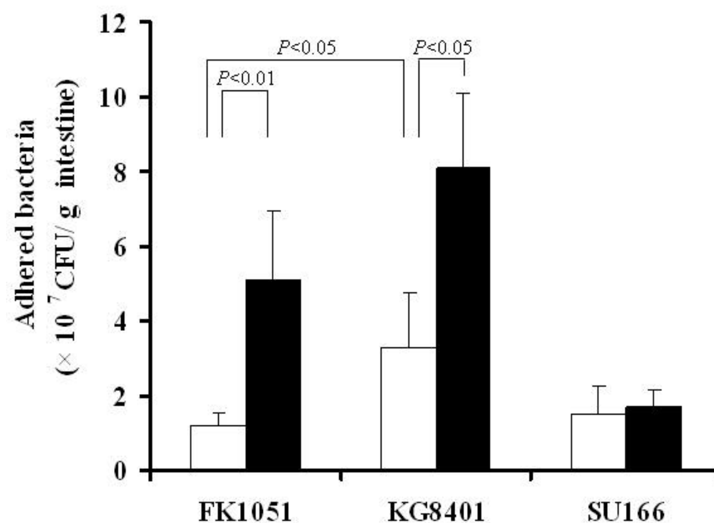

Fig. 2: Viable cell counts of *E. tarda* strains (FK1051, KG8401 and SU166) adhered to goldfish intestine. Fish were anally inoculated with 0.2 ml ($1\text{-}2 \times 10^9$ CFU/mL) of bacterial suspension/ fish. White bars refer to 0%-NaCl cultures and black bars refer to 3%-NaCl cultures.

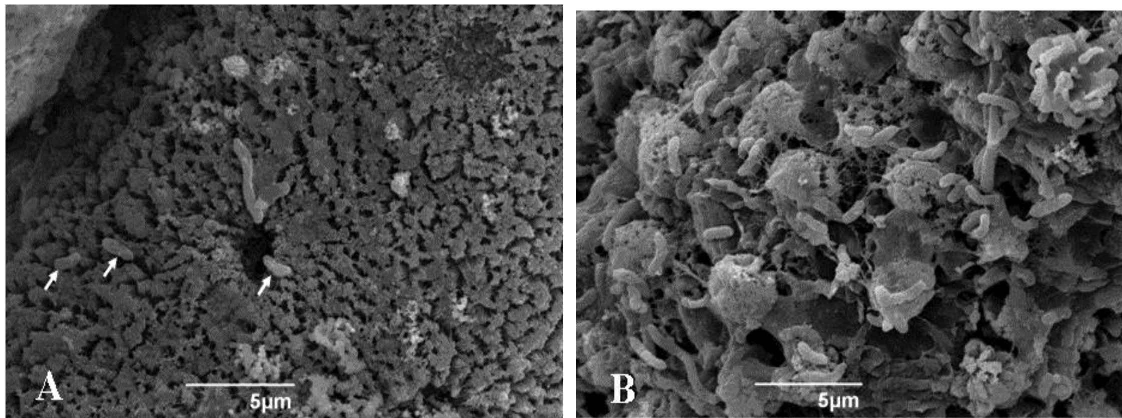

Fig. 3: Scanning electron microscopic appearance of *E. tarda* adhered to goldfish intestine. Fish were anally inoculated with 0.2 ml (1-2 × 10^9 CFU/mL) of bacterial suspension/ fish. A: 0%-NaCl culture (arrows), B: 3%-NaCl culture of FK1051.

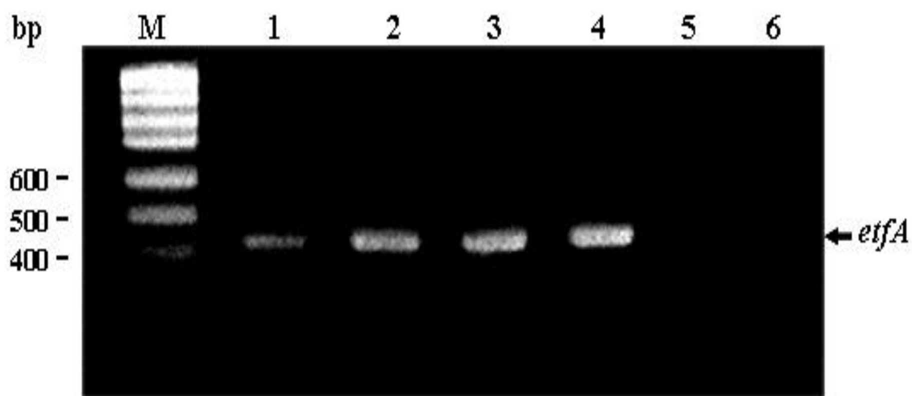

Fig. 4: Reverse transcription (RT)-PCR analysis of the *etfA* transcript levels (415bp). Lanes: M, 100 bp DNA ladder marker; 1, FK1051 (0%-NaCl culture); 2, FK1051 (3%-NaCl culture); 3, KG8401 (0%-NaCl culture); 4, KG8401 (3%-NaCl culture); 5, SU166 (0%-NaCl culture); 6, SU166 (3%-NaCl culture).

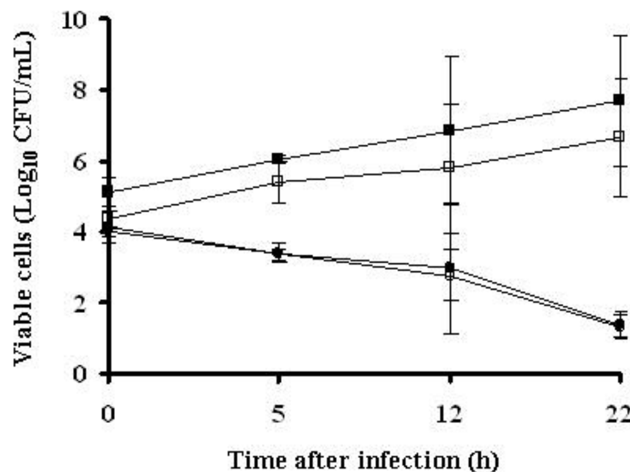

Fig. 5: Kinetics of Intracellular replication of *E. tarda* in murine macrophage (J774 cells). Wild-type (FK1051) 0%-NaCl culture (□), 3%-NaCl culture (■) and type III secretion system mutant (mET1229) 0%-NaCl culture (○), 3%-NaCl culture (●). Values represent the log numbers of the bacterial counts (mean ± S.D).

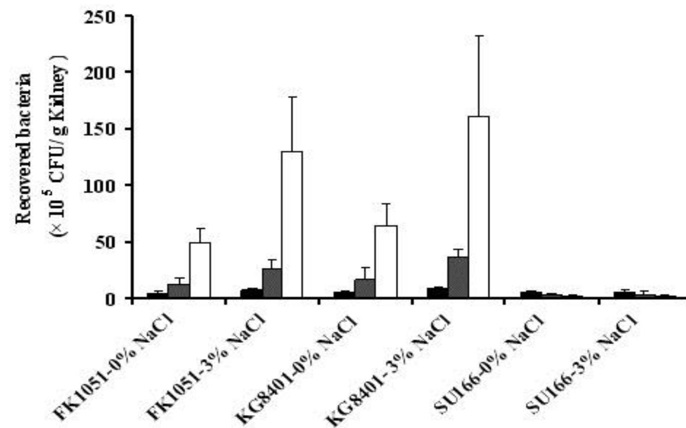

Fig. 6: Viable cell counts of *E. tarda* recovered from kidney of infected goldfish. Fish were anally inoculated with 0.2 ml (1-2 × 10^9 CFU/mL) of bacterial suspension/ fish. Black bars represent 6 h, grey bars represent 24 h and white bars represent 48 h post infection.

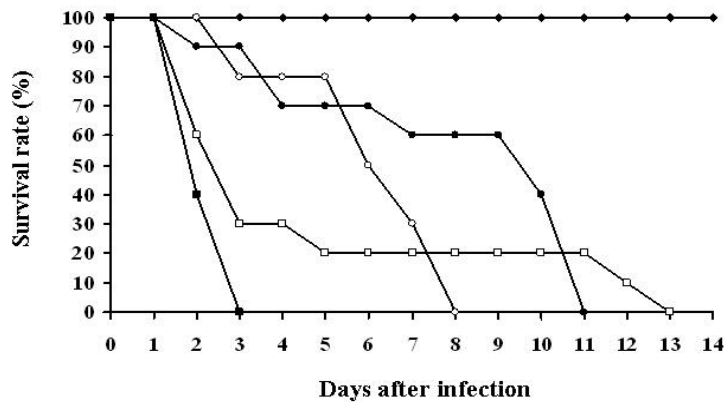

Fig. 7: Pathogenicity of 0%- and 3%-NaCl cultures *E. tarda* (FK1051) to goldfish. The survival rates (%) of the intramuscularly injected fish were recorded after 2 weeks of observation. The injected doses were 1.9 × 10^6 (□), 10^5 (○), and 10^4 (◊) CFU/fish for the 0%-NaCl culture and were 1.5 × 10^6 (■), 10^5 (●), and 10^4 (♦) CFU/fish for the 3%-NaCl culture.

Table 1: Adhesion tendency of *E. tarda* to goldfish intestine, as evaluated by scanning electron microscopy.

NaCl concentration of the culture medium	*E. tarda* strains		
	FK1051	KG8401	SU166
0%	-	++	-
3%	+++	+++	-

-, Less than 10 adhered bacteria/ field
+, 10-50 adhered bacteria/ field
++, 51-100 adhered bacteria/ field
+++, More than 100 adhered bacteria/ field

Table 2: Internalization of *E. tarda* to murine macrophage (J774 cells) at 0 h time incubation (1.5-h post infection).

Strain	Viable cell count (CFU/mL ± SD)	
	0%-NaCl culture	3%-NaCl culture
Wild-type (FK1051)	2.3 ± 0.36 × 10^4	1.3 ± 0.40 × 10^5*
Type III mutant (mET1229)	1.4 ± 0.16 × 10^4	1.6 ± 0.45 × 10^4

* P<0.05

DISCUSSION

Adhesion is a vital factor in bacterial pathogenicity as it enables the pathogen to resist cleaning mechanisms operating on the surfaces; also it induces the delivery of bacterial toxins and precedes penetration of the target cells by microorganisms. The use of the adhesion assay seems likely to yield accurate information towards determining the within-host ecology of the pathogen (Horne and Baxendale, 1983). The mucosal tissues of fish body comprise a large surface area and many pathogens invade through or colonize mucosal tissues, especially the intestinal tract (Schroers *et al*., 2008). The ascending infection through the anal opening and/or infectious materials in food that could pass the stomach without inactivation might lead to infection of fish by the intestine in natural conditions. The adhesion of fish bacteria to intestine was earlier investigated, where by the use of green fluorescent protein-tagged *E. tarda*, the role of intestine as a portal of entry of the bacteria in fish was highlighted (Ling *et al*., 2001). Schroers *et al*. (2008) reported that bacteria that showed a stronger adhesion to intestinal mucus were cytotoxic to cells *in vitro*. *Pasteurella piscicida* strains adhered strongly to the intestines from sea bream, sea bass and turbot in numbers ranging from 10^4 to 10^5 bacteria/g of intestine depending on bacterial isolate and the fish species employed (Magariños *et al*., 1996). The anal administration of *Piscirickettsia salmonis* was followed by adhesion and penetration of the bacteria to the intestine causing piscirickettsiosis in rainbow trout and coho salmon (Smith *et al*., 1999, 2004). The adhesion of *Lactococus garvieae* to the intestine of the yellow tail was demonstrated by Shima *et al*. (2006). *Aeromonas salmonicida* could translocate through the intestinal epithelium of rainbow trout, suggesting that the intestine is a possible route of infection (Jutfelt *et al*., 2006). The intestine was also considered as the primary route for mycobacterial infection of zebrafish (Harriff *et al*., 2007).

In this study, we examined the *in vivo* adhesion ability of 0%- and 3%-NaCl cultures of different strains of *E. tarda* to goldfish intestine. The promoted adhesion elicited by high concentration of NaCl (3%) was in accordance with our previous results regarding the *in vitro* adherence of those strains to HEp-2 cells and also their HA (Mahmoud *et al*., 2006 and Yasunobu *et al*., 2006). The enhancement of adhesion by NaCl was also illustrated previously (Tartera and Metcalf, 1993), where it was found that the adhesion of *Salmonella typhi* to the human epithelial cells (Henle 407) increased by the high osmolarity. The osmolarity has been noted as an environmental signal controlling virulence in several organisms (Mekalanos, 1992). Interestingly, adhesion ability of the *Flavobacterium columnare* correlated well with its virulence (Decostere *et al*., 1999). Thus the NaCl-induced adhesion of *E. tarda* may be one of the virulence factors. The scanning electron microscopy of the intestine-adhered bacteria confirmed the increase of adhesion by the high NaCl, where the 3%-NaCl cultures of both FK1051 and KG8401 showed numerous bacteria adhered to intestinal mucosa.

Fimbirae are known to mediate bacterial adhesion (Soto and Hultgren, 1999). Sakai *et al*. (2003, 2004) identified a gene, *etfA*, encoding a 19.3-kDa protein, a major fimbrial subunit, associated with the possession of HA among *E. tarda* strains. This gene was found in all tested pathogenic, but not in non-pathogenic *E. tarda*, and suggested to be used for rapid detection of the fish pathogenic *E. tarda* (Sakai *et al*., 2007). As long as the high NaCl increased the expression of the 19.3-kDa protein (Yasunobu *et al*., 2006), we investigated the effect of NaCl concentration in the culture medium on the expression of the gene (*etfA*) encoding that protein. The present results agree with our previous results where the *etfA* was up-regulated by the high NaCl, and also it was not expressed in the non clinical strain (SU166) which has no HA and low adhesion ability in both 0%- and 3%-NaCl cultures (Mahmoud *et al*., 2006).

After breaching the epithelium, the bacteria have to avoid or survive phagocytosis before reaching the circulation and being disseminated to the fish organs. Bacterial pathogens use various strategies to overcome phagocytosis. Some microorganisms secrete toxins to kill the phagocytes before they can be killed by them, for example the production of streptolysin by the group A *Streptococcus* (Datta *et al*., 2005). Other pathogens like *Streptococcus iniae* possess a capsule that promotes resistance to phagocytic killing (Locke *et al*., 2007). In addition, the intracellular bacteria use other mechanisms to survive within phagocytes, where *Listeria monocytogenes* escape from the phagosome into the cytoplasm (Speert, 1992). While, *Salmonella* spp. can withstand acidic environment within phagolysosomes and resist oxidative stress, insufficient nutrients, and antimicrobial peptides such as defensins (Foster and Spector, 1995). *Legionella pneumophilia* secretes a compound shown to inhibit the neutrophil oxidative burst (Miller and Britigan, 1997). *E. tarda*, as an intracellular pathogen, can resist phagocytic killing (Iida *et al*., 1993 and Iida and Wakabayashi, 1993).

Several mechanisms have been reported explaining the ability of *E. tarda* to persist within phagocytes including its resistance to reactive oxygen intermediates by production of superoxide dismutase and catalase (KatB) (Srinivasa Rao *et al*., 2001, 2003 and Han *et al*., 2006), and possessing type III secretion system (Tan *et al*., 2005 and Okuda *et al*., 2006). Data of the present study confirmed the ability of the wild strain (FK1051) to survive and replicate inside murine phagocytes, while the mutant deficient

in type III secretion system (mET1229) was killed 22 h post infection. The internalization of the 3%- NaCl culture of the wild strain (at 0 h post infection) was significantly higher than its 0%-NaCl culture. Auger and Ross (1992) stated that the adherence and phagocytosis are influenced by opsonization and cell surface structures of the pathogen, thus the high NaCl may induce some changes in the cell surface structure of *E. tarda* promoting its adherence and internalization to phagocytes. This also agrees with our previous results of the enhanced adherence to HEp-2 cells by NaCl (Mahmoud *et al*., 2006).

The present data of the recovery of *E. tarda* from the kidney (time-course) of infected fish were consistent with those of adhesion to intestine and the internalization to phagocytes, as the bacterial number of the 3%-NaCl cultures was significantly higher than that of 0%-NaCl cultures at the different time points, till 48 h post infection. The time when the bacteria were detected in the high number in tissues varies in different strains. The number of *Vibrio anguillarum* recovered from rainbow trout kidney remained high till 48 h after experimental infection (Horne and Baxendale, 1983). Also, *P. piscicida* remained viable within CHSE-214 cells till 48 h post infection (Magariños *et al*., 1996). However, this time may be longer as the case of *P. salmonis* which was detected in kidney smears up to 14 days after anal inoculation (Smith *et al*., 1999). The recovery of *E. tarda* from the kidney indicates its ability to invade intestinal epithelium and gain access to kidney.

The time-mediated decrease in the number of SU166 in the kidney may be due to the elimination of the bacteria through the clearance mechanisms of the host, indicating that this strain is non-pathogenic and could not multiply or even maintain its level within the fish tissue. On the reverse, in case of FK1051 and KG8401, the kidney tissues were overwhelmed by these strains which could survive and proliferate exponentially by time denoting their ability to cause the disease. Ling *et al*. (2001) also reported a sequential increase in the numbers of virulent *E. tarda* inside the hematopoietic organs such as kidney, liver and spleen, whereas the avirulent strain population decreased in the post infection period within these organs. As long as the bacterial pathogens may abuse phagocytes to invade and spread within the host system (Speert, 1992), therefore the enhanced uptake of the 3%-NaCl culture by phagocytes may be responsible for the increased number of the recovered bacteria from kidney.

Conclusively, the increase of the NaCl in the culture medium induced adhesion to intestine, internalization to macrophage and dissemination of the *E. tarda* to kidney, and this may be attributed to the NaCl-induced upregulation of adhesins on the pathogen surface leading to increased adhesion to intestinal cells and also to macrophages, with subsequent uptake by the macrophages which may help distribution of *E. tarda* to fish body and the increased number in fish kidney. However, the challenge of goldfish using 0%- and 3%-NaCl cultures demonstrated that the total virulence of *E. tarda* was not apparently affected by the high NaCl. This may refer to the role of the other assumed virulence factors of *E. tarda* in its overall pathogenicity. The recent researches demonstrated that hemolysin *EthA* is required for invasion abilities of *E. tarda*. These abilities are subjected to the control of a complicated and precisely regulated network primed for invasion, colonization and infection process in fish (Wang *et al*., 2010). Also, Hfq which is an RNA-binding protein has been reported to play an important role in many cellular processes including ability to replicate in host macrophages and affecting overall virulence and tissue dissemination and colonization capacity (Hu *et al*., 2014).

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تأثير الملوحة على الالتصاق في الجسم الحي، الالتهام والمرضية لميكروب الإدواردسيللا تاردا

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تم دراسة تأثير تركيز كلوريد الصوديوم على التصاق ميكروب الإدواردسيللا تاردا لأمعاء الأسماك الذهبية باستخدام طريقة عد خلايا البكتيريا الحية. ولقد استخدم لهذا الغرض ثلاثة سلالات من الإدواردسيللا تاردا تمثل ثلاثة أنماط مختلفة من قدرة هذا الميكروب على تلزن كرات الدم الحمراء لخنزير غينيا. وقد زرعت البكتيريا على بيئة تحتوى على ٣% كلوريد الصوديوم وأخرى لا تحتوى على كلوريد الصوديوم (صفر% كلوريد صوديوم). أظهرت النتائج زيادة معنوية فى معدل التصاق البكتيريا لسلالتى FK1051 و KG8401 المزروعتين على بيئة تحتوى على ٣% كلوريد الصوديوم عن نظيرتيهما المزروعتين على بيئة لا تحتوى على كلوريد الصوديوم. من ناحية أخرى، لم يكن هناك فرقاً معنوياً فى معدل الالتصاق لسلالة SU166 سواء فى وجود أو عدم وجود كلوريد الصوديوم فى البيئة المستخدمة، حيث كان الالتصاق فى كلا الحالتين منخفضاً. وباستخدام الميكرسكوب الالكترونى الماسح، تم تأكيد زيادة التصاق البكتيريا للأمعاء بزيادة تركيز كلوريد الصوديوم. وعلى نفس الوتيرة، جاءت نتائج عدوى الأسماك الذهبية بسلالات البكتيريا سالفة الذكر عن طريق فتحة الشرج. حيث تم عزل سلالتى FK1051 و KG8401 المزروعتين على بيئة تحتوى على ٣% كلوريد الصوديوم بنسب عالية وعلى فترات زمنية مختلفة من كُلى الأسماك المعرضة للعدوى. وقد جاءت نتائج الالتصاق متماشية مع ما تم الحصول عليه فى تجربة سابقة لاختبار تأثير تركيز كلوريد الصوديوم على قدرة نفس سلالات البكتريا المستخدمة على إحداث تلزن لكرات الدم الحمراء والالتصاق لخلايا مستزرعة. أيضاً تم دراسة تأثير تركيز كلوريد الصوديوم على التهام الخلايا الأكولة لميكروب الإدواردسيللا تاردا وذلك باستخدام خلايا أكولة معزولة من الفئران. وبالمثل زاد معدل التهام الخلايا الأكولة للسلالات المختلفة من البكتريا بزيادة تركيز كلوريد الصوديوم المستخدم فى بيئة الزرع. إلا أن الضراوة الكلية للسلالات المستخدمة لم تتأثر بصورة جلية بزيادة تركيز كلوريد الصوديوم المستخدم فى بيئة زرع هذا الميكروب.