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Research Paper

Adhesive and invasive capacities of *Edwarsiella tarda* isolated from South American sea lion

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Abstract

Edwarsiella tarda is a zoonotic bacterium that can be isolated from humans, animals and the environment. Although *E. tarda* is primarily considered a fish pathogen, it is the only species of its genus considered to be pathogenic for humans as well. A survey of zoonotic intestinal bacteria in fresh feces from South American sea lions (SASL) *Otaria flavescens*, reported *E. tarda* as the most frequently isolated species. In this study, we used HEp-2 cells to establish *in vitro* the adherence and invasive ability of 17 *E. tarda* strains isolated from SASL fecal material. All the strains were able to adhere and invade HEp-2 cells with adhesion and invasion percentages ranging from 56 to 100% and 21 to 74%, respectively. Despite the expression of these pathogenic factors, further investigation is needed to determine whether this bacterium could play a role as primary pathogen for this and other species of pinnipeds.

Key words: *Edwarsiella tarda*, adherence, invasion, *Otaria flavescens*.

Introduction

The South American sea lion [SASL] *Otaria flavescens* is a marine mammal found along the coasts of South America and the Falkland Islands. On the Pacific side, breeding colonies are found south of Zorritos (03°40' S), further down to Tierra del Fuego and, on the Atlantic side, they are found in Isla de Los Estados (54°45' S) in Argentina, and further up to Recife dos Tôrres (29°21' S) in Brazil. The type of sea habitat for SASL seems to be associated to the distribution and abundance of food. In general the species tends to feed in shallow waters in coastal areas, and around the continental slope, where it finds the greatest concentrations of pelagic fish and/or marine invertebrates. There are also records of the species entering river mouths (basin of the Uruguay River, basin of the Santa Lucía River, Arroyo Pando and Arroyo Solís in Uruguay) and Atlantic coastal lagunes. The sporadic presence of SASL individuals in the Valdivia River is a phenomenon observed through decades and scientifically documented since 1976 (González *et al.*, 2011). In the last decade, a colony of about 30 to 40 animals was established in a coastal area of the urban course of the Valdivia River (39°47'Southern latitude, 73°15' Western latitude), with the river being their feeding ground; whilst they use the sandy beach by the river, the lawn and the pavement of the street in the surrounding areas as resting ground. This prompted us to look for the presence of zoonotic intestinal bacteria in fresh sea lions feces placed on the pavement. During the course of this investigation, *Campylobacter insulaenigrae* (González *et al.*, 2011) and several species of the Family Enterobacteriaceae were isolated (González *et al.*, 2010), with *Edwardsiella tarda* being the most frequently found (73%) (González *et al.*, 2010). This is a zoonotic bacterium that has been isolated from humans, animals, and the environment and it is particularly considered a fish pathogen, being the only species of its genus that has been reported to be pathogenic to humans (Leung *et al.*, 2012).

In human beings it has been associated to infectious diarrhea, sepsis, and generalized infections predominantly in immunocompromised individuals (Marques *et al.*, 1984; Janda & Abbott, 1993; Nucci *et al.*, 2002; Leung *et al.*, 2012).

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Coles *et al.* (1978) recognize that marine animals are an important reservoir of this bacterium, with *E. tarda* being part of their microbiota. However, it could also be the etiological agent of several infectious diseases of freshwater and marine fish, including septicemia with extensive skin lesions, affecting internal organs such as liver, kidney, spleen and muscle, being pacu (*Myleus micans*) one of the affected fishes in their natural fresh water habitat. On the other hand, it could also be an experimental pathogen for tilapias (*Oreochromis* spp.) and common carp (*Cyprinus carpio*) (Lima *et al.*, 2008). Recently, it was associated to an outbreak of bacterial septicemia with high mortality in turbots (*Scophthalmus maximus*) from a mariculture farm (Xiao *et al.*, 2009).

The ability of *E. tarda* to invade fish epithelial cells and tissues has been reported as virulence factors (Marques *et al.*, 1984; Janda & Abbott 1993; Ling *et al.*, 2001). Despite these reports and the description of genes required to cause infection in fish and the role of type III secretion system in *E. tarda* virulence (Tan *et al.*, 2005), little is known about the pathogenic characteristics of *E. tarda* strains isolated from different sources, especially from marine mammals.

The aim of this study was to establish the ability of 17 *E. tarda* strains isolated from SASL fecal material to adhere to and invade HEp-2 cells *in vitro*.

Material and Methods

The *E. tarda* strains were isolated on MacConkey and XLD agars (Oxoid) and identified according to their biochemical characteristics with the assesment of the Vitek AutoMicrobic System (bioMérieux). All the adherence and invasion tests were carried out twice in duplicate.

Adhesiveness

The adhesive capacity was determined infecting HEp-2 cells with the strains under study following the protocol from Skaletsky *et al.* (1984). In brief, HEp-2 cells were cultured overnight on coverslips (20 x 8 mm), in Leighton tubes containing minimum essential medium with 10% fetal calf serum (MEM-10%), at 37 ºC under 5% CO_2 atmosphere. After being washed three times with phosphate-buffered saline (PBS), the medium was replaced with 1 mL *E. tarda* suspension (6 x 10^8 colony forming units) in MEM-10% and incubated for 30 min at 37 °C (infection period). The tubes were then washed with phosphate buffered saline six times, and 1 mL of MEM with 10% fetal calf serum was added to each tube. The tubes were then incubated for 3 h at 37 °C (multiplication period). After this period, cover slips were washed three times with PBS, and the cells were fixed with methanol and stained with May-Grünwald stain. The stained cover slips were washed with water, then removed from the Leighton tubes, dried, mounted on glass slides, and examined under a light microscope (x400 and x1,000). An adherent *Escherichia coli* isolate was included as positive control, whereas un-inoculated cell lines were used as negative controls. Adhesion results were expressed as percentage of HEp-2 cells (adhesion percentage) showing adhering bacteria and the number of bacteria (± SD) adhered to cells was determined. The adhesion percentage expressed the number of invaded cells/total cells examined x 100 and it was determined by counting at least 200 cells. Strains were considered as adherent if at least 20% of HEp-2 cells showed one or more adhered bacteria (Fernández *et al.*, 2010). Adherence was also demonstrated by scanning electronic microscopy.

Invasiveness

The acridine orange-crystal violet stain (Fernández *et al.*, 1997) was used to assess invasiveness. HEp-2 cells were cultured overnight on coverslips (20 x 8 mm), in Leighton tubes containing MEM-10%, at 37 °C in 5% CO_2 atmosphere. After washing three times with PBS, the medium was replaced with 1 mL *E. tarda* suspension (6 x 10^8 colony forming units) in MEM-10%. Cells were incubated for 2 h at 37 °C and 5% CO2 (infection period), washed 10 times in PBS and reincubated for 3 h with 1 mL MEM-10% (multiplication period). Then, coverslips were washed three times with PBS and, without fixing, stained with 0.01% acridine orange in Gey's solution for 45 s, rinsed with Hanks balanced salt solution and counterstained with 0.05% crystal violet in 0.15 N NaCl for 45 s, mounted on slides and sealed with colorless nail polish and then examined under epi-fluorescence microscopy at 400x magnification for screening and at 1000x magnification for quantitative evaluation. The invasion percentage (number of invaded cells/total cells examined x 100) was determined by counting at least 200 cells. The average number (± SD) of invading bacteria was estimated in a minimum of 35 invaded HEp-2 cells. Invasion was confirmed by using transmission electron microscopy as standard control.

Results and Discussion

All the strains were adherent, with adhesion percentages ranging from 56 to 100% and the number of adhered bacteria per cell ± SD ranging from 2.88 ± 2.46 to 20.5 ± 8.05 (Table 1). The adhesion pattern observed using light microscopy was similar to the enteroaggregative pattern described by Nucci *et al.* (2002) in *E. tarda* strains (data not shown). Scanning microscopy confirmed the actual adhesion of *E. tarda* to HEp-2 cells *in vitro* showing rounded ends and straight bacilli adhering to HEp-2 cells, creating an arrangement that resembled a microcolony (Figure 1).

E. tarda infects a broad range of freshwater and marine life including fish, amphibians, reptiles, birds, and mammals; while it is also the only species of the genus *Edwarsiella* that is pathogenic to humans (Leung *et al.*, 2012).

Table 1 - Adherence to and invasion of HEp-2 cells by *Edwarsiella tarda* studied with light microscopy and acridine orange-crystal violet staining and controlled by scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM), respectively.

Strains	Adhesion percentage (%)	N° of adhered bacteria/cell ± SD	SEM adherence control	Invasion percentage (%)	N° of invading bacteria/cell ± SD	TEM invasion control
1	70	2.88 ± 2.46	+	74	1.68 ± 1.30	+
3	56	3.76 ± 1.76	+	53	1.32 ± 1.07	+
4	86	6.6 ± 2.59	ND	58	2.92 ± 1.00	ND
7	92	5.38 ± 2.89	ND	47	2.76 ± 0.93	ND
8	78	5.26 ± 3.76	ND	54	2.24 ± 1.22	ND
9	93	12.1 ± 7.66	ND	37	3.62 ± 1.22	ND
10	98	17.22 ± 6.36	+	46	5.32 ± 1.71	+
11	96	9.4 ± 6.48	+	42	3.28 ± 1.75	+
15	94	8.22 ± 6.19	ND	48	3.94 ± 1.07	ND
17	100	9.52 ± 4.01	+	39	4.12 ± 1.72	+
20	99	13.66 ± 7.01	ND	30	4.42 ± 0.70	ND
22A	90	5.96 ± 3.12	ND	21	2.30 ± 0.81	ND
22B	93	14.62 ± 8.30	+	39	4.74 ± 1.19	+
23	98	20.5 ± 8.05	+	50	5.84 ± 1.33	+
25	96	15.5 ± 9.01	+	61	4.56 ± 1.38	+
26	94	8.08 ± 4.26	ND	59	2.12 ± 1.08	ND
27	90	7.84 ± 4.46	+	51	2.88 ± 1.11	+

SD = standard deviation; SEM = scanning electron microscopy; ND = not done.
TEM = transmission electron microscopy.

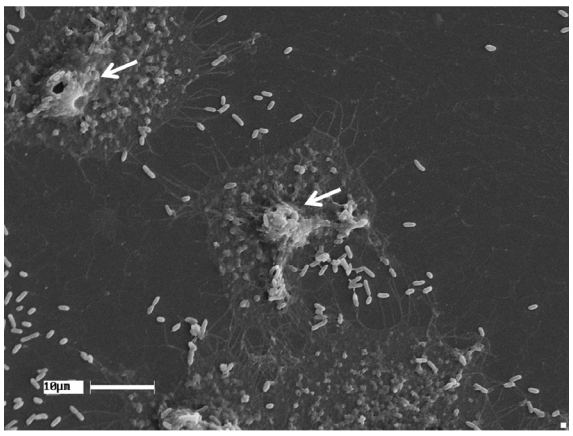

Figure 1 - Scanning electronic microphotography showing *E. tarda* adhered to HEp-2 cell and arrangements resembling microcolonies (arrows).

In order to cause an infection, *E. tarda* may attach to the host's epithelial cells. Fish and human cells models have been used to assess the adherence of *E. tarda*. Ling *et al.* (2001), demonstrated through a fish model that the main sites of attachment of *E. tarda* were the gastrointestinal tract epithelia, gills, and body surface. On the other hand, the adherence of an *E. tarda* strain isolated from a mariculture farm to an epithelioma papulosum cyprini cell line was determined by Xiao *et al.* (2009).

The adherence of *E. tarda* to human epithelial cells was previously described by Nucci *et al.* (2002). These authors found that all of the strains they studied were able to adhere to HeLa cells, independently of their human or fish origin. Moreover, they described for all their isolates an adherence pattern similar to enteroaggregative adherence, which had been previously described in *E. coli* by Scaletsky *et al.* (1984). This kind of adhesive pattern could probably be responsible for the microcolony-like arrangement that was observed on the HEp-2 cells through scanning electron microscopy. Similar arrangements have been described in adhesive *Arcobacter butzleri* strains (Fernández *et al.*, 2010). Since enteroaggregative *E. coli* forms biofilms on the intestinal mucosa (Sheikh *et al.*, 2001), it may be possible that the enteroaggregative pattern of adhesion observed in the *E. tarda* strains could be related to the capacity of this bacterium to form biofilm as described by He *et al.* (2011). After the attaching process, *E. tarda* can invade or can be internalized into the cells (Wang *et al.*, 2010) assisted by invasion-like proteins and/or the participation of a type III secretion system recently described (Tan *et al.*, 2005).

All the strains were able to invade HEp-2 cell with invasion percentages ranging from 21 to 74% while the number of internalized bacteria per cell ± SD ranged from 1.32 ± 1.07 (Table 1). Internalized bacteria were seen inside vacuoles through epi-fluorescence microscopy, this was later

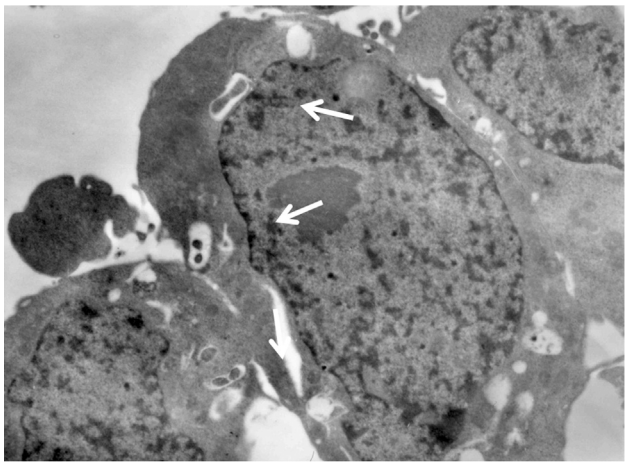

Figure 2 - Transmission electron microphotography of HEp-2 cells showing *E. tarda* enclosed within endocytic vacuoles (arrows).

corroborated by using transmission electron microscopy (Figure 2).

The *in vitro* invasive property of *E. tarda* was first described by Marques *et al.* (1984) in human strains and by Janda & Abbott (1993) in clinical, fish and environmental strains. However, the latter authors also found non-invasive strains. Later, Nucci *et al.* (2002) reported that invasion was a feature common to all isolates of *E. tarda* analyzed, independently of their source. Similarly to the findings reported by Marques *et al.* (1984) and Nucci *et al.* (2002), all our strains were able to invade HEp-2 cells with invasion percentages ranging from 21 to 74%. Examination by means of transmission electron microscopy allowed us to confirm the intracellular presence of *E. tarda*, with the bacteria being enclosed within endocytic vacuoles (Figure 2). Similar observations were previously reported by Marques *et al.* (1984) and Janda & Abbott (1993).

E. tarda seems to be a frequent inhabitant of the intestinal tract of the SASL (González *et al.*, 2010). As shown in this study, this bacteria species can express adherence and invasion in epithelial cells, properties that are considered as pathogenic factors. However, the role played by this bacterium as primary pathogen in this and in other species of pinnipeds is unknown. Several infectious diseases have been reported in different species of sea lions and other pinnipeds, but they were all secondary processes associated to previous predisposing clinical conditions like traumas, starvation, dystocia, neoplasia or primary infectious diseases (Coles *et al.*, 1978; Stroud and Roffe, 1979). These findings suggest that *E. tarda* could be an opportunistic invader in sick or injured marine mammals. Further studies are needed in order to clarify the real pathogenic role that *E. tarda* could play in these animals.

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