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RESEARCH ARTICLE

Invasion of differentiated intestinal Caco-2 cells is a sporadic property among atypical enteropathogenic *Escherichia coli* strains carrying common intimin subtypes

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Atypical enteropathogenic *Escherichia coli* (aEPEC) strains produce attaching–effacing (AE) lesions on enterocytes due to the interaction of the adhesin intimin with its translocated receptor. This study highlights the ability of an isolate with a uncommon intimin type to interact with the host cell and compares to more common intimin types.

Keywords

atypical enteropathogenic *Escherichia coli*; intimin subtype; invasion; intracellular persistence; Differentiated Caco-2 cells; Attaching and effacing lesion.

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Abstract

Atypical enteropathogenic *Escherichia coli* (aEPEC) strains produce attaching–effacing (AE) lesions on enterocytes due to the interaction of the adhesin intimin with its translocated receptor. aEPEC strain 1551-2 was previously shown to invade HeLa and T84 cells by means of the uncommon intimin subtype omicron. Other aEPEC strains carrying uncommon intimin subtypes have also been shown to invade differentiated T84 intestinal cells. In this study, seven aEPEC strains carrying the most common EPEC intimin subtypes (alpha, beta, and gamma) were evaluated regarding the ability to invade differentiated intestinal Caco-2 cells. Although all strains adhered to and promoted AE lesions, the numbers of cell-associated bacteria varied significantly between the different strains regardless of the intimin subtype ($P < 0.05$). Gentamicin protection assay and transmission electron microscopy analyses showed that in comparison with the invasive strain 1551-2, only one strain (aEPEC EC423/03, intimin beta) was invasive ($P = 0.05$). Although both strains persisted intracellularly until 48 h, the number of viable bacteria of EC423/03 decreased, whereas that of 1551-2 increased significantly up to 24 h and then decreased. In conclusion, invasiveness is a sporadic property among aEPEC strains carrying some common intimin subtypes.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) infections affect mainly very young children (Nataro & Kaper, 1998). Although acute diarrhea is the main clinical symptom, EPEC association with persistent diarrhea has also been reported (Levine & Edelman, 1984; Fagundes-Neto *et al.*, 1996).

The main characteristic of EPEC pathogenicity is the production of characteristic histopathological alterations termed attaching and effacing (AE) lesions. AE lesion production is also a property of enterohaemorrhagic *E. coli* (EHEC) (reviewed in Kaper *et al.*, 2004), *E. albertii* (Mora *et al.*, 2009), and *Citrobacter rodentium* (a murine pathogen) strains (Croxen & Finlay, 2010; Schmidt, 2010). The AE lesion is characterized by microvilli effacement and intimate bacterial adherence to the enterocyte membrane, sustained by a pedestal-like structure that is rich in actin and other cytoskeleton components (Moon *et al.*, 1983; Knutton *et al.*, 1989). Presumably, these structures favor a potent EPEC adherence to the cell surface, preventing its detach-

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ment and ensuring the later changes that elicit diarrhea (Vallance & Finlay, 2000).

A pathogenicity island named locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995) clusters many chromosomal genes that encode proteins that assemble a 'molecular needle' called the type 3 secretion system (T3SS) (Knutton *et al.*, 1998), an adhesin named intimin, transcriptional regulators, and translocated and effector proteins (Garmendia *et al.*, 2005). Altogether, these proteins alter the physiology and functions of the host cells, leading to the establishment of AE lesions.

Intimin is an outer membrane protein encoded by the *eae* gene that functions as an adherence factor with a central role in intestinal colonization (Jerse *et al.*, 1990; Frankel *et al.*, 2001). This protein mediates intimate adherence to the epithelial cells by interacting with another LEE-encoded protein, the translocated intimin receptor (Tir), which is injected into the host cell through the T3SS (Kenny *et al.*, 1997; Deibel *et al.*, 1998). The 5′ region of *eae* gene alleles is conserved, while the 3′ region is variable. This differentiation is an important tool for EHEC and EPEC typing, in the clinical diagnoses as well in pathogenesis, epidemiologic and immunologic studies. Although the intimin C-terminal portion interacts with Tir, it has been suggested that the different intimin subtypes may drive bacteria to different intestinal regions (Phillips & Frankel, 2000). Thus far, *eae* variant alleles encoding 33 different intimin subtypes have been established (reviewed in Schmidt, 2010), with subtypes alpha, beta, and gamma being the most common (Adu-Bobie *et al.*, 1998; Beutin *et al.*, 2003; Ramachandran *et al.*, 2003; Blanco *et al.*, 2006a, b; Abe *et al.*, 2009; Horcajo *et al.*, 2012).

Besides the LEE, some EPEC strains carry the 60-MDa EPEC adherence factor (EAF) plasmid (pEAF), which bears the *bfp* operon encoding a type IV fimbria named the bundle-forming *pilus* (BFP) as well as the transcriptional activator plasmid-encoded regulator (Per) that activates the *bfp* operon and many other LEE genes *in trans* (Bustamante *et al.*, 2011).

EPEC is subgrouped in typical (tEPEC) and atypical enteropathogenic *Escherichia coli* (aEPEC) based on the presence of pEAF in tEPEC and its absence in aEPEC (Kaper, 1996; Trabulsi *et al.*, 2002).

Previous studies have suggested that the frequency of tEPEC is decreasing (Rodrigues *et al.*, 2004; Ochoa *et al.*, 2008), while aEPEC have been detected more often among children and adults worldwide (Blanco *et al.*, 2006a; Trabulsi *et al.*, 2002; Gomes *et al.*, 2004; Bueris *et al.*, 2007; Hernandes *et al.*, 2009). Although tEPEC strains are seldom detected in nonhuman hosts, aEPEC strains may be found in different animal species (Carvalho *et al.*, 2003; Hernandes *et al.*, 2009; Mora *et al.*, 2009; Moura *et al.*, 2009).

tEPEC strains produce a BFP-mediated localized adherence (LA) pattern that is characterized by compact cluster formation on eukaryotic cell surfaces *in vitro* (HeLa and HEp-2 cells), after 3 h of infection (reviewed in Trabulsi *et al.*, 2002). On the other hand, most aEPEC strains produce a LA-like pattern characterized by looser clusters, which are visualized after longer assays (6 h) (Rodrigues *et al.*, 1996). We have previously shown that a few aEPEC strains produce compact LA in HeLa cells, despite the absence of BFP (Vieira *et al.*, 2001). Curiously, transmission electron microscopy (TEM) images of the interaction between one of these strains (aEPEC 1551-2, intimin subtype omicron) and HeLa cells revealed that the compact LA clusters in fact corresponded to bacteria contained in intracellular vacuoles, some of which were laying on pedestal-like structures (Hernandes *et al.*, 2008). In addition, it was verified that an *eae* mutant of aEPEC 1551-2 remained adherent despite losing its invasive capacity, thus suggesting that invasiveness was due to Tir–intimin interaction.

Besides aEPEC 1551-2, other aEPEC strains carrying uncommon intimin subtypes have also been shown to invade differentiated T84 and/or Caco-2 intestinal cells (Rosa *et al.*, 2001; Sampaio *et al.*, 2009; Yamamoto *et al.*, 2009). To further extend our knowledge on the frequency of the invasive capacity among aEPEC strains *in vitro*, in this study we evaluated the ability of aEPEC strains carrying the most common EPEC intimin subtypes (alpha, beta, and gamma) to invade differentiated intestinal Caco-2 cells, as well as the ability of the invasive strains to persist inside these cells. aEPEC 1551-2 bearing the uncommon intimin subtype omicron previously shown to invade HeLa and intestinal T84 cells was included in this study for comparisons.

Material and methods

Bacterial strains and cell culture conditions

Seven aEPEC strains carrying intimin subtypes alpha, beta, or gamma, which were isolated in Brazil and Spain from children or bovine diarrhea, were studied (Table 1). The invasive 1551-2 strain (intimin subtype omicron) was also included as a positive control of invasion (Hernandes *et al.*, 2008; Yamamoto *et al.*, 2009). JPN15, a spontaneously pEAF-cured strain derived from tEPEC prototype strain E2348/69 (Levine *et al.*, 1985), was included as a noninvasive control, because it could be used in the same incubation period (6 h) as the aEPEC strains, while the tEPEC E2348/69 strain would promote the detachment of most cells in such period. Strains were cultured statically in Luria–Bertani broth for 18 h at 37 °C to reach an OD 600 nm of 0.5–0.6. All strains were shown to be susceptible to $\leq$ 200 μg mL^{-1} of gentamicin and $\leq$ 30 μg mL^{-1} of amikacin prior to the invasion experiments. Caco-2 cells (10^5 cells) were cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen) supplemented with 10% bovine fetal serum (Gibco Invitrogen) and 1% antibiotics (Gibco Invitrogen) and were kept for 10 days at 37 °C and 5% CO_2 for differentiation.

Quantitative association and invasion assays

Quantitative assessment of bacterial association and invasion was performed as described previously (Luck *et al.*, 2005) with modifications. Briefly, differentiated Caco-2 cells were infected with 10^7 CFU of each aEPEC strain and JPN15 strain for 6 h. Thereafter, cell monolayers were washed three times with PBS and lysed in 1% Triton X-100

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Table 1 Atypical EPEC strains studied

Strain	Serotype	Intimin subtype	Origin	Reference
3121-6/85	O111:H9	Alpha	Child, Brazil	Gomes *et al.* (1989)
EC134/03	O111:H9	Alpha	Child, Brazil	Gomes *et al.* (2011)
7OR	O128:H2	Beta	Child, Brazil	Gomes *et al.* (2011)
FV10096	O177:H11	Beta	Bovine, Spain	Mora *et al.* (2009)
EC423/03	O119:H2	Beta	Child, Brazil	Gomes *et al.* (2011)
3041-1/85	O55:H7	Gamma	Child, Brazil	Gomes *et al.* (1989)
FV10106	O145:H28	Gamma	Child, Spain	Mora *et al.* (2009)
1551-2	ONT:H−	Omicron	Child, Brazil	Vieira *et al.*(2001)
JPN15	O127:H6	Alpha	USA	Jerse *et al.* (1990)
HB101	R:H−	–	USA	Boyer & Roulland-Dussoix (1969)

for 30 min at 37 °C. After cell lysis, bacteria were resuspended in PBS and quantified by plating serial dilutions onto MacConkey agar plates to obtain the total number of cell-associated bacteria (TB). To obtain the number of intracellular bacteria (IB), a second set of infected wells was washed five times and incubated in fresh media with 200 $\mu g\ mL^{-1}$ of gentamicin (Sigma) for 1 h at 37 °C. Following this incubation period, cells were washed, lysed with 1% Triton X-100 and resuspended in PBS for quantification by plating serial dilutions. The invasion indexes were calculated as the percentage of the TB that were located in the intracellular compartment (IB) after 6 h (IB × 100/TB) of infection. Assays were carried out in triplicate, and the results from at least three independent experiments were expressed as the percentage of invasion (mean ± SE).

Bacterial intracellular persistence

Monolayers were infected as described above and incubated in fresh media with 200 $\mu g\ mL^{-1}$ of gentamicin for 1 h at 37 °C. Following this incubation period, cells were washed and incubated in fresh media with 100 $\mu g\ mL^{-1}$ of gentamicin for additional 18 or 42 h at 37 °C to reach a total of 24 and 48 h of infection, respectively. Considering that higher gentamicin concentrations might penetrate in the host cell, some experiments were also performed with 250 $\mu g\ mL^{-1}$ of amikacin (1 h at 37 °C) followed by 25 $\mu g\ mL^{-1}$ of amikacin till 24 and 48 h in order to exclude death of IB induced by gentamicin. After these periods of time, monolayers were washed with PBS and lysed as described above. Intracellular persistence was calculated as the number of CFU recovered after 24 and 48 h. In parallel experiments, at the end of 24 and 48 h, cell monolayers were fixed for TEM.

TEM

Caco-2 cells were cultured in 6-well plate for 10 days and infected as described above. Then, they were washed 3 times (10 min each) with PBS (Sigma) and fixed with 2% glutaraldehyde (EMS) for at least 24 h at 4 °C. After fixation, cells were washed 3 times with PBS (10 min) and postfixed with 1% osmium tetroxide (EMS) in 0.1 M sodium cacodylate buffer for 30 min. After being washed for 3 times with distilled water, preparations were dehydrated through a graded ethanol series (50%, 75%, 85%, 95%, and 100%) and propylene oxide (100%). Preparations were then gradually embedded in Araldite and allowed to polymerize for 24–48 h at 60 °C. Ultrathin sections were applied onto 200-mesh copper grids and stained with 4% uranyl acetate (Merck, Germany) in water and Reynold's lead citrate (Merck). Grids were examined under TEM (LEO 906E) at 80 kV.

Statistical analyses

Differences in the percentages of all strains were assessed for significance by one-way ANOVA, while differences between each aEPEC strain and JPN15 were assessed for significance by using an unpaired, two-tailed *t*-test. Differences between aEPEC carrying the same intimin subtype were assessed for significance by using an unpaired, two-tailed *t*-test (for gamma subtype) or one-way ANOVA (for alpha and beta subtypes) (GRAPHPAD PRISM 5.0).

Results

aEPEC strains that associate efficiently with Caco-2 cells potentially produce AE lesions

Prior to the analyses of the invasive ability of the aEPEC strains studied, the bacterial capacity to adhere to and to aggregate actin filaments (a characteristic of AE lesions) was evaluated in polarized and differentiated Caco-2 cell monolayers. All strains adhered to and promoted actin accumulation as observed by DAPI staining and the fluorescent actin staining (FAS) assays (Supporting Information, Fig. S1).

In quantitative experiments, it was observed that the numbers of cell-associated bacteria varied significantly between the strains studied ($P < 0.05$; Fig. 1a). A growth curve did not show differences in growth rate between the strains (data not shown). In addition, significant differences in the efficiency of association with Caco-2 cells were observed between strains of the same intimin subtypes (alpha, beta, and gamma; $P < 0.05$).

Caco-2 cell invasion is a sporadic property among aEPEC strains carrying common intimin subtypes

The gentamicin protection assay was employed in order to quantify the invasion ability of aEPEC strains. Initially, as the ability of aEPEC 1551-2 to invade differentiated Caco-2 cells

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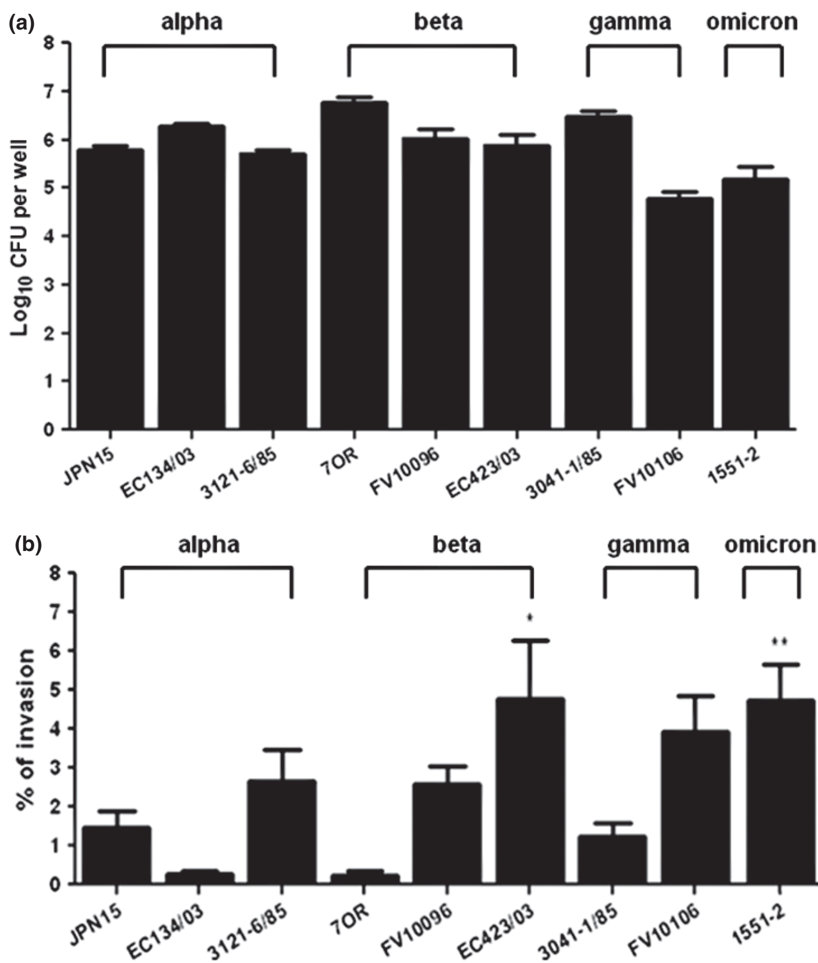

Fig. 1 Bacterial association with Caco-2 cell monolayers. (a) Total cell-associated bacteria. The number of associated bacteria varied significantly between all strains ($P < 0.05$). Comparison of the adherence indexes among strains carrying the same intimin subtype (alpha, beta, or gamma) showed significant differences ($P < 0.001$). Results represent the means ± SE from at least three independent experiments in triplicate wells ($P < 0.05$). (b) Invasion of Caco-2 cells by seven aEPEC strains, and positive and negative control strains (aEPEC 1551-2 and JPN15, respectively). Percentage of invasion is expressed as the percentage of cell-associated bacteria that resisted killing by gentamicin and are the means ± SE from at least three independent experiments in triplicate wells. (*) and (**) indicate significantly more invasive than JPN15 strain ($P < 0.05$ by an unpaired, two-tailed *t*-test).

was unknown, such property was evaluated in comparison with JPN15 (a spontaneous pEAF-segregant strain of tEPEC E2348/69, which is considered noninvasive). The invasion index of the 1551-2 strain was significantly higher than that of JPN15 (4.72% ± 0.95 and 1.57% ± 0.42, respectively) ($P < 0.05$; Fig. 1b). Among the aEPEC strains carrying common intimin subtypes, EC423/03 (subtype beta) had a significantly higher invasion rate (4.75% ± 1.50) when compared to JPN15 ($P < 0.05$). Comparison between aEPEC strains of the same intimin subtype demonstrated that the invasion indexes of strains with subtype gamma were not significantly different ($P > 0.05$), in contrast to the aEPEC strains carrying intimin subtype alpha or beta ($P < 0.05$), which were significantly different.

The invasion abilities of both aEPEC EC423/03 and 1551-2 were confirmed by TEM that showed bacteria-containing vacuoles, in consistency with the results obtained with the gentamicin protection assay (Fig. 2).

Invasive aEPEC strains survive within Caco-2 cells until 24 h

In order to investigate the ability of the invasive aEPEC strains to survive in the intracellular milieu, IB were quantified at prolonged incubation times (24 and 48 h) after gentamicin (or amikacin) treatment, and results were compared to those obtained after 6 h of infection. The number of intracellular CFU recovered from aEPEC EC423/03 gradually decreased at 24 and 48 h. Nevertheless, recovery of aEPEC 1551-2 increased significantly up to 24 h and then decreased in 48 h returning to the CFU number recovered at 6 h (Fig. 3a). Infected monolayers were also examined by TEM. Infection with strain 1551-2 showed that higher numbers of internalized bacteria could be observed inside vacuoles up to 24 h (Fig. 3b), when compared to those observed after 6 h of infection (Fig. 2), while smaller vacuoles containing a reduced number of bacteria were seen after 48 h (Fig. 3b), corroborating with the quantitative data (Fig. 3a). Presence of large vacuoles, but containing few bacteria, was also frequent after 48 h (data not shown). Smaller vacuoles containing bacteria were seen on EC423/03 at 24 h of infection (Fig. 3b). Interestingly, IB clusters appeared to be encircled by a cell membrane. Despite the presence of larger vacuoles at 48 h, the number of these structures was smaller than at 24 h, probably as a result of coalescence of small vacuoles and presence of internalized unviable bacteria, as no colonies grew on agar plates when lysates were cultured.

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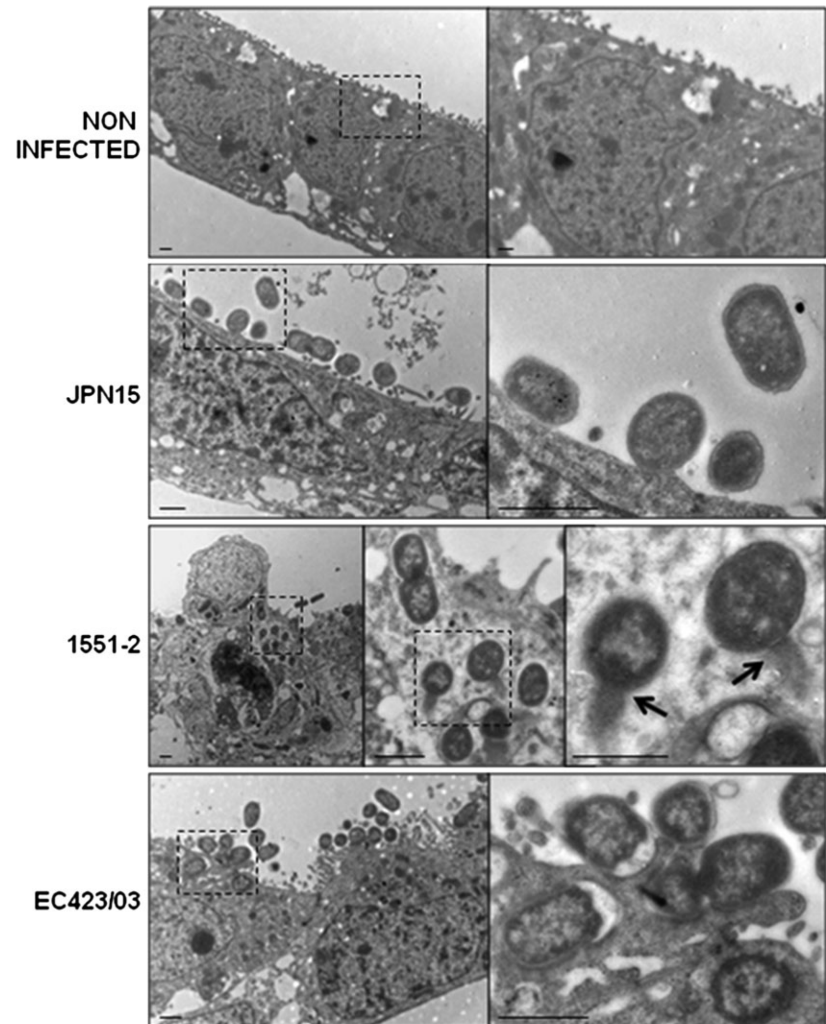

Fig. 2 TEM of aEPEC interaction with Caco-2 cells. Representative fields of differentiated Caco-2 cells, after 6 h of infection with JPN15 strain and different aEPEC strains. Bacteria are noted within individual vacuoles, some of which forming pedestals around the vacuoles (arrows). JPN15 strain presented fewer internalized bacteria in relation to 1551-2 and EC423/03, confirming the invasion indexes obtained in quantitative experiments. Black dashed areas, amplification. Magnification bars, 1 μm.

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Discussion

In two previous studies, we had demonstrated that the aEPEC strain 1551-2 carrying intimin subtype omicron and some aEPEC strains carrying uncommon intimin subtypes are able to invade HeLa and intestinal T84 cells (Hernandes *et al.*, 2008; Yamamoto *et al.*, 2009). In this study, we compared seven aEPEC strains carrying the most common intimin subtypes alpha, beta, and gamma (Hernandes *et al.*, 2009) and aEPEC 1551-2 regarding the capacity of invading Caco-2 cells.

To address this issue, we initially evaluated the adhesive ability of the strains in the Caco-2 lineage, because it has been demonstrated that a significant number of aEPEC strains do not adhere to epithelial cells *in vitro* (Abe *et al.*, 2009; Scaletsky *et al.*, 2010). All eight strains tested adhered to and promoted actin accumulation (a characteristic of the AE lesion) in Caco-2 cells as detected by FAS.

Because all strains were adherent, the gentamicin protection assay was performed to evaluate their invasive ability. As in previous studies, a comparison with tEPEC strain E2349/69 was desired, because tEPEC strains are considered noninvasive (Rosa *et al.*, 2001; Celli & Finlay, 2002; Sampaio *et al.*, 2009; Yamamoto *et al.*, 2009). However, as the E2349/69 strain adheres with higher efficiency than aEPEC strains due to BFP production and in order to be able to test the strains at the same incubation conditions (6 h) without harming the cells, we used the *bfp*-negative E2348/69 derivative strain JPN15. The data obtained showed that among aEPEC strains carrying common intimin subtypes (alpha, beta, and gamma), only one strain (EC423/03, intimin subtype beta) and the 1551-2 strain had a significant invasion index when compared to JPN15. Interestingly, TEM analyses of the invasive strains after 6 h of bacteria–cell interaction showed the presence of numerous vacuoles containing membrane-bound bacteria. These observations suggest an important participation of the pedestals formed in the AE lesions in bacterial uptake by Caco-2 cells.

Although FV10106 is apparently invasive, its invasion rate was not significantly different ($P > 0.05$) from that of the JPN15 strain due to large variations in the invasive

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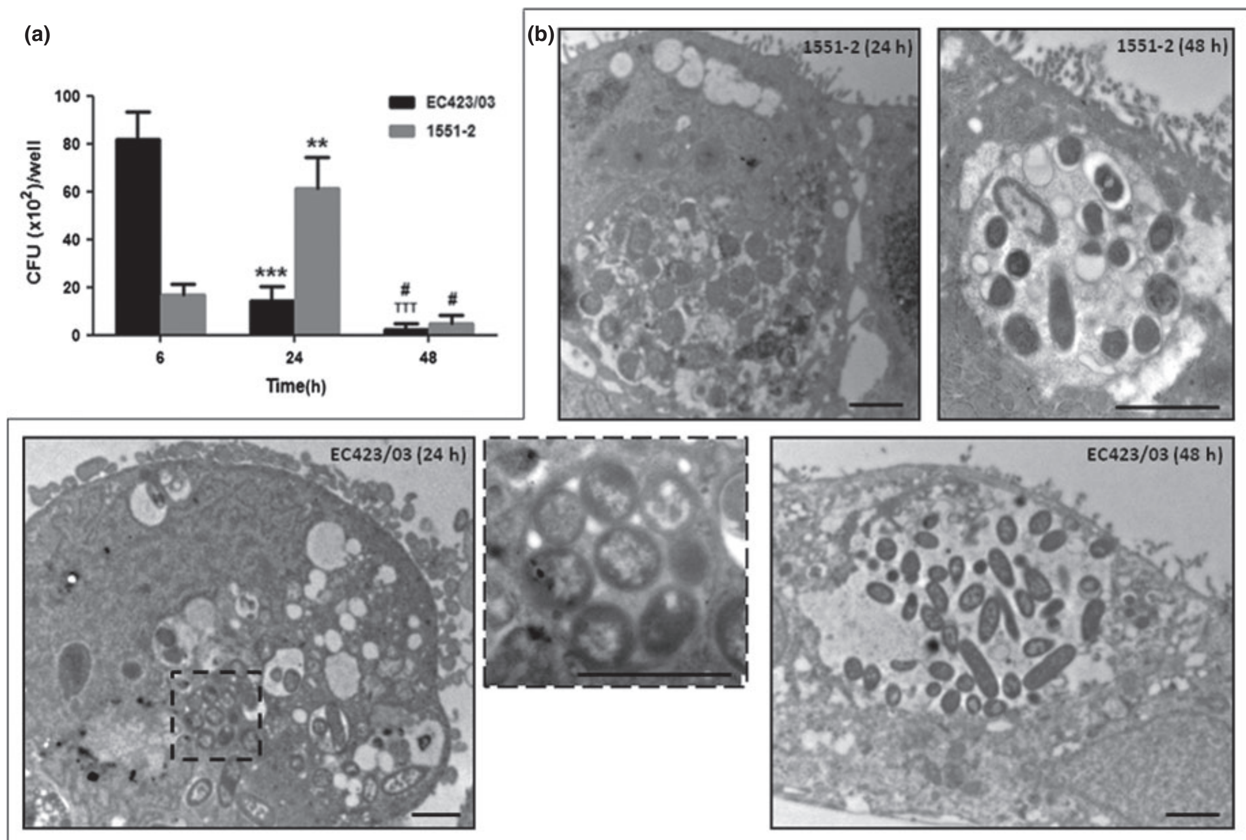

Fig. 3 Intracellular persistence of aEPEC strains EC423/03 and 1551-2 in differentiated Caco-2 cells after different periods of infection. (a) Numbers of CFU recovered at different incubation periods (6, 24, and 48 h) in the presence of gentamicin. (**) and (***) indicate a significant difference between 6 and 24 h; (#) indicates a significant difference between 24 and 48 h; (TTT) indicate a significant difference between 6 and 48 h. No statistical difference was found for aEPEC 1551-2 between 6 and 48 h. ($P < 0.05$ by an unpaired, two-tailed *t*-test). (b) TEM of aEPEC interaction with Caco-2 cells after 24 and 48 h of infection. Black dashed areas, IB cluster. Magnification bars, 2 μm.

percentages obtained in the different assays, suggesting that unknown factors might interfere in the invasion route. Additionally, some aEPEC strains (EC134/03 and 7OR) were clearly less invasive than JPN15.

It was also observed that aEPEC 1551-2 (intimin subtype omicron) is able to invade Caco-2 cells in a similar index (4.72% ± 0.95) as obtained previously in T84 cells (7.2% ± 1.4) (Yamamoto *et al.*, 2009). This result could suggest that this strain can invade both the small and large intestines, because Caco-2 and T84 cells, respectively, mimic these origins. Similar observations were obtained in our laboratory with the interaction between aEPEC strain O51:H40 (1711-4, intimin subtype theta) and these cell types (Sampaio *et al.*, 2011).

Our results also showed that the invasion efficiency differed between strains with the same intimin subtype tested, suggesting that the intimin subtype is not the exclusive factor that influences invasiveness, emphasizing the complexity of this event. In addition, Sheng *et al.* (2011) demonstrated that intimin–Tir interaction was not required for efficient EHEC O157:H7 invasion of primary bovine epithelial cells. Furthermore, other effector proteins were associated with EPEC invasion *in vitro*, such as Map (Jepson *et al.*, 2003), EspF (Weflen *et al.*, 2010), and EspT (Bulgin *et al.*, 2009a, b), that manipulate Rho-GTPase signaling pathways (reviewed in Wong *et al.*, 2011). All strains of the present study are *map* and *espF* genes positive (not shown), but, curiously, the noninvasive strains EC134/03 and 2932-2/89 carried the *espT* gene, while significantly invasive aEPEC strains from our collection (EC423/03 and 1551-2) lacked this gene (not shown). These observations suggest that in our experimental conditions, intimin–Tir interaction in association with injected effectors may trigger RhoA, Rac-1, and CDC42 activation resulting in bacterial uptake.

The destination of the internalized strains 1551-2 and EC423/69 was then evaluated by extending the incubation periods in the presence of gentamicin or amikacin (to impair re-infection). A distinct behavior was observed between the two strains. While EC423/03 was unable to survive inside Caco-2 cells, aEPEC 1551-2 survived and proliferated until 24 h. However, the CFU numbers of IB significantly decreased after 48 h, returning to similar numbers observed at the beginning of the invasion process (6 h). Besides

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allowing the escape from the host innate immune response, aEPEC invasion could be associated with persistent diarrhea as previously reported in the literature (Nguyen *et al.*, 2006) and discussed by Yamamoto *et al.*, 2009. However, considering the decrease in the numbers of IB after 48 h of infection, invasion could rather be a step for dissemination *in vivo* as recently demonstrated by experimental aEPEC translocation, from the gut to liver and spleen (Liberatore *et al.*, 2011).

Altogether, our data support the hypothesis that only some aEPEC strains can invade intestinal cells, with invasiveness being an uncommon virulence mechanism among strains carrying the most common intimin subtypes. Our results reinforce important differences in some aEPEC in regard to tEPEC that seems to comprise a more homogeneous group (Trabulsi *et al.*, 2002). In addition, our invasion data stress the higher potential to disseminate and/or escape from the immune system of some aEPEC strains that seem to localize in the intracellular compartment, including those persisting for at least 24 h in this environment.

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Authors' contribution

V.C.R.P. and D.Y. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Ability of aEPEC strains to adhere to and promote actin aggregation in differentiated Caco-2 cells *in vitro*.

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