Recent studies have shown that the effector repertoire of AE pathogens is much larger than previously thought and is not restricted to the LEE-encoded proteins. Following description of the first non-LEE-encoded effector NleA/EspI (Gruenheid et al., 2004; Mundy et al., 2004) many additional non-LEE effectors have been identified (Wong et al., 2011).

Enteropathogenic *Escherichia coli* (EPEC) are an important cause of gastroenteritis in children less than two years of age (Nataro and Kaper, 1998). The EPEC pathotype is sub-divided into typical EPEC (tEPEC), which carry the large virulence EAF (EPEC adherence factor) plasmid (pEAF), and atypical EPEC (aEPEC), which lack this plasmid (Kaper, 1996; Trabulsi et al., 2002; Hernandes et al., 2009). pEAF encodes the bundle forming pilus (BFP), which mediates a localized adherence (LA) pattern on HeLa/HEp-2 cells (Donnenberg et al., 1992).

The chromosomal pathogenicity island (PAI) termed locus of enterocyte effacement or LEE region, which is present in tEPEC and aEPEC strains, encodes several proteins that play a major role in their pathogenesis. The LEE region carries genes related with the attaching and effacing (AE) lesion, characterized by intimate adherence and several enterocyte modifications (Kaper et al., 2004). LEE-encoded proteins are also responsible for the assembly of the Type 3 Secretion System (T3SS), a molecular syringe used to inject an arsenal of virulence effectors into the host cells cytosol (Frankel et al., 1998).

In addition to EPEC, the AE pathogens family includes enterohemorrhagic *E. coli* (EHEC), which differs from EPEC due to Shiga toxin production and can cause severe kidney damage leading to the hemolytic-uremic syndrome (HUS), *Escherichia albertii*, and a number of animal pathogens such as the mouse-specific pathogen *Citrobacter rodentium* (Mundy et al., 2005; Schmidt, 2010; Ooka et al., 2012).

Besides the LEE region, other PAIs have been found on prophages and on integrative elements in the chromosome of AE pathogens (Perna et al., 2001; Iguchi et al., 2009; Petty et al., 2010). Recent studies have shown that the effector repertoire of AE pathogens is much larger than previously thought and is not restricted to the LEE-encoded proteins. Following description of the first non-LEE-encoded effector NleA/EspI (Gruenheid et al., 2004; Mundy et al., 2004) many additional non-LEE effectors have been identified (Wong et al., 2011).

The non-LEE effectors subvert various host cell processes including: inhibition of protein export from endoplasmic reticulum (EspI/NleA), inhibition of pro-inflammatory signaling (NleB, NleC, NleD, and NleE), inhibition of phagocytosis (EspJ and NleH), activation of Cdc42 and Rac1 that induces membrane ruffles and lamellipodia (EspT), microtubule disruption (EspG2), stress fiber formation (EspM), inhibition of cell detachment and modulation of cell death (NleD and Cif), which enable the bacteria...
to colonize, multiply and cause disease (Dean et al., 2009; Vossenkämper et al., 2011; Wong et al., 2011). However, studies regarding the frequency of these non-LEE effectors among tEPEC and aEPEC isolates are rare (Afset et al., 2006; Scaletsky et al., 2009; Vieira et al., 2010; Bugarel et al., 2011).

In the present study, we examined a total of 107 EPEC strains (44 typical and 63 atypical), isolated from 71 diarrheic and 36 non-diarrheic children in Brazil. The EPEC strains analyzed were isolated from 1989 to 1990, during an epidemiological study conducted in Sao Paulo city (Brazil) regarding the etiology of the diarrhea committing children up to one year of age (Gomes et al., 1989; Vieira et al., 2001). The presence of six non-LEE- genes (cif, espI/nleA, nleB, nleC, nleD, and nleE) was investigated by colony hybridization under stringent conditions, using as probes fragments of these genes obtained by PCR (Marchès et al., 2003; Mundy et al., 2004; Afset et al., 2006) and labeled with [32P]dCTP. The nle genes selected in this study are among the most prevalent non-LEE-encoded type 3 secretion system-dependent effectors in AE pathogens, and in some studies showing an epidemiological association with diarrheal diseases (Afset et al., 2006; Scaletsky et al., 2009; Vieira et al., 2010). Data were analyzed by two-tailed Fisher’s exact test and p values < 0.05 were considered statistically significant.

Regarding the prevalence of the non-LEE genes, nleC and espI/nleA were the most prevalent, followed by cif, nleB, nleE and nleD (Table 1). Three of the six non-LEE-genes examined (nleC, cif and nleB) were significantly more prevalent in tEPEC than in aEPEC strains.

The only gene found in association with diarrhea was espI/nleA in tEPEC (p value = 0.0130) (Table 1), in accordance with a previous report in which the espI/nleA gene was found more commonly in EPEC strains isolated from patients suffering from a more severe disease (Mundy et al., 2004). To note, none of the non-LEE- genes investigated was found in association with diarrhea among the aEPEC strains studied (Table 1).

The nleB and nleE genes are located in the PAI O122 (Perna et al., 2001; Karmali et al., 2003). This island also harbors genes that are very similar to pagC of Salmonella enterica serovar Typhimurium, sen that encodes a homologous Shigella flexneri enterotoxin, and efa1/lifA that encodes an EHEC adherence factor (Karmali et al., 2003). Genes located in the PAI O122 have been reported to be associated with diarrhea in aEPEC strains. For example, in Norwegian children, the efa1/lifA, sen, nleB and nleE were each found to be more prevalent in aEPEC strains isolated from patients than from controls (Afset et al., 2006). However, in a study performed in Japan efa1/lifA, but not nleB, was identified to be associated with diarrhea (Narimatsu et al., 2010).

In studies conducted in Brazil the nleB, nleE, sen and efa1/lifA genes, as observed in the present study for nleB and nleE, could not be individually detected in association with diarrhea (Scaletsky et al., 2009; Vieira et al., 2010). However, in one of these studies an association between the presence of a complete PAI O122 (efa1/lifA, sen, pagC, nleB and nleE) and diarrhea by aEPEC was detected, despite the occurrence of incomplete versions of PAI O122 in both tEPEC and aEPEC isolates (Vieira et al., 2010). In a recent study, the nleB gene was identified as the most conserved between the PAI O122 (O1-122) encoded genes, and suggested to be a suitable marker for genetic screening for the presence of the PAI O122 in EPEC and EHEC strains (Bugarel et al., 2011).

The cif gene, which encodes a protein responsible for cell cycle blocking at the G2/M and G1/S transitions (Marchès et al., 2003), was detected in 48.6% of all EPEC strains studied, being more prevalent among tEPEC strains (Table 1). In an earlier study, only about 2.3% of 5.049 E. coli strains of human, animal, and environmental origins carried the cif gene, with all cif-positive isolates also carrying the LEE region (Loukiadis et al., 2008). Interestingly, just

Table 1 - Prevalence of non-LEE genes in tEPEC and aEPEC strains from patients and controls.

| Non-LEE- genes | N (%) of positive strains | tEPECb | N (%) of strains: | aEPECc |
|----------------|--------------------------|--------|------------------|--------|
|                | Patients | Controls | Total | Patients | Controls | Total |
| nleC           | 84 (78.5) | 37 (97.4) | 6 (100.0) | 43 (97.4) | 20 (60.6) | 21 (70.0) | 41 (65.0) |
| espI/nleA      | 61 (57.0) | 28 (73.4) | 1 (16.7) | 29 (65.9) | 15 (45.4) | 17 (56.7) | 32 (50.8) |
| cif            | 52 (48.6) | 31 (81.6) | 3 (50.0) | 34 (77.3) | 10 (30.3) | 8 (26.7) | 18 (28.6) |
| nleB           | 50 (46.7) | 25 (65.8) | 3 (50.0) | 28 (63.6) | 12 (36.4) | 10 (33.3) | 22 (35.0) |
| nleE           | 45 (42.0) | 22 (57.9) | 3 (50.0) | 25 (56.8) | 10 (30.3) | 10 (33.3) | 20 (31.7) |
| nleD           | 25 (23.4) | 8 (21.0) | 2 (33.3) | 10 (22.7) | 10 (30.3) | 5 (16.7) | 15 (23.8) |

aTotal of EPEC strains studied: 107 (44 typical and 63 atypical).

bN of tEPEC strains studied: 38 from patients and 6 from controls.

cN of aEPEC strains studied: 33 from patients and 30 from controls.

p value = 0.0130, p value = 0.0001, p value = 0.0001, p value = 0.0056.
Table 2 - Prevalence of distinct genetic profiles of non-LEE genes in typical and atypical EPEC serotypes.

| Genetic profile | N° of EPEC strains (%) | Serotype (N° of strains and origin a) |
|-----------------|------------------------|--------------------------------------|
|                 |                        | tEPEC                                 |
| espI, cif, nleB, nleC, nleE | 19 (17.7)              | O88:H25 (4P)                          |
|                 |                        | O111:H2 (6P)                          |
|                 |                        | O111:H (5P)                           |
| espI, nleC      | 10 (9.3)               | O41:H (1P)                            |
|                 |                        | O55:H7 (1C)                           |
|                 |                        | O101:H33 (1C)                         |
|                 |                        | O109:H9 (1C)                          |
|                 |                        | O125:H6 (1P/1C)                       |
| espI, nleC      | 10 (9.3)               | ONT:H (2P/2C)                         |
| espI, cif, nleC | 9 (8.4)                | O86:H34 (1P)                          |
|                 |                        | O119:H6 (5P)                          |
| nleC            | 9 (8.4)                | O142:H6 (2P)                          |
|                 |                        | O142:H34 (1P/2C)                      |
| cif, nleB, nleC, nleE | 5 (4.7)              | O127:H40 (2C)                         |
| espI, cif, nleB, nleC, nleD, nleE | 4 (3.7)             | O55:H6 (1P/1C)                        |
|                 |                        | O145:H45 (1P)                         |
| espI, nleB, nleC, nleD, nleE | 4 (3.7)             | O55:H (1P)                            |
|                 |                        | O26:H (1P)                            |
|                 |                        | O55:H7 (1P/1C)                        |
| nleB, nleC, nleE | 4 (3.7)                | O111:H (1P)                           |
|                 |                        | O51:H (1P)                            |
| nleC, nleD      | 4 (3.7)                | O55:H (1C)                            |
|                 |                        | O98:H (1C)                            |
|                 |                        | O145:H45 (1P)                         |
| cif, nleB, nleC, nleD, nleE | 3 (2.8)             | O55:H6 (2P)                           |
|                 |                        | O127:H6 (1P)                          |
| espI, cif       | 3 (2.8)                | O119:H6 (1P)                          |
|                 |                        | O11:H6 (1P)                           |
|                 |                        | R:H11 (1C)                            |
| cif, nleB, nleD, nleE | 2 (1.8)              | O51:H40 (2P)                          |
| espI, cif, nleB, nleC | 2 (1.8)              | O88:H25 (2P)                          |
| espI, nleC, nleD | 2 (1.8)                | O145:H45 (1P)                         |
| cif, nleB, nleC | 2 (1.8)                | O55:H6 (1P)                           |
| espI, nleC, nleD | 2 (1.8)                | R:H40 (1P)                            |
|                 |                        | ONT:H (1P)                            |
|                 |                        | ONT:H19 (1P)                          |
| cif, nleC       | 2 (1.8)                | O86:H34 (1P)                          |
| nleB, nleE      | 2 (1.8)                | O129:H11 (1C)                         |
|                 |                        | O127:H6 (1P)                          |
|                 |                        | ONT:H34 (1P)                          |
|                 |                        | O49:H (1C)                            |
| espI            | 2 (1.8)                | O94:H (1C)                            |
|                 |                        | ONT:H8 (1C)                           |
| espI, nleB, nleD, nleE | 1 (0.9)              | O34:H (1P)                            |
| espI, nleB, nleC, nleD | 1 (0.9)              | O55:H7 (1P)                           |
| cif, nleB, nleC, nleE | 1 (0.9)              | O153:H7 (1C)                          |
| espI, nleC, nleD | 1 (0.9)                | O63:H6 (1P)                           |
| espI, nleD      | 1 (0.9)                | ONT:H (1P)                            |
| none            | 12 (11.2)              | O16:H (1P)                            |
|                 |                        | O104:H (1P)                           |
|                 |                        | O177:H (1P)                           |
|                 |                        | O111:H9 (1P)                          |
|                 |                        | O154:H9 (1P)                          |
|                 |                        | O162:H (1C)                           |
|                 |                        | ONT:H (2P/1C)                         |
|                 |                        | ONT:H11 (2C)                          |
|                 |                        | R:H28 (1P)                            |

*aP: patient, C: control.
44.0% of these cif-positive strains induced a typical Cif-related phenotype in eukaryotic cells (Loukiadis et al., 2008).

Even though the non-LEE- genes were more prevalent in tEPEC than in aEPEC (Table 1), a higher diversity of combinations of these genes (genetic profiles) was observed in aEPEC (Table 2). As suggested previously, the varied repertoire of the non-LEE effector-encoding genes found among distinct EPEC serotypes may suggest that different isolates can employ distinct infection strategies (Wong et al., 2011).

Except for some tEPEC serotypes (O111:H2, O127:H40, O142:H6 and O142:H34), and one aEPEC serotype (O125:H6), strains from a single serotype showed distinct genetic profiles (Table 2, data not shown). In five aEPEC strains from serotype O51:H40, three different combinations of non-LEE- genes were observed whereas among the five aEPEC strains of serotype O26:H1, four distinct genetic profiles were identified, and the four aEPEC strains of serotype O55:H7 showed three distinct genetic profiles. Similar findings could be observed among the tEPEC strains studied. These data strongly reflect the higher heterogeneity of the aEPEC strains (Gomes et al., 2004; Hernandes et al., 2006; Tennant et al., 2009). The high diversity of genes combinations found in the aEPEC strains studied is expected because virulence factor-encoding genes can be located on transmissible plasmids, PAIs, transposons or bacteriophages and thus they could have been transferred horizontally to these strains in the intestine and/or in the environment.

In conclusion, we observed that the non-LEE encoded T3SS-translocated effector genes are, in general, more prevalent among tEPEC than aEPEC strains. However a larger number of genes combinations are observed among the aEPEC isolates. The heterogeneity of aEPEC strains studied makes it difficult to identify the truly pathogenic isolates in the group bringing direct implications in the diagnostic strategies.

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