**Title:** Evaluation of the bacterial structures involved in the establishment of a hybrid localized/diffuse adherence in atypical enteropathogenic *Escherichia coli* of O2:H16 serotype

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**Abstract:**
The main virulence mechanism of enteropathogenic *Escherichia coli* (EPEC) is the capacity to cause a histopathological lesion on the intestinal mucosa, termed Attaching and Effacing (AE); characterized by intimate bacterial adherence, microvillus destruction and formation of F-actin rich pedestal-like structures, in infected enterocytes. Genes of the locus of enterocyte effacement (LEE region) encode all proteins necessary for AE lesion formation. EPEC are divided in typical (tEPEC) and atypical (aEPEC), based on the presence of the EPEC adherence factor plasmid in the former group. Previously, we reported a diarrheal outbreak due to aEPEC of the O2:H16 serotype, which was also isolated from sporadic cases of diarrhea in Brazil. From a collection of seven aEPEC O2:H16, obtained from the previously mentioned outbreak, and sporadic cases of diarrhea, we showed that five of them produced a hybrid localized/diffuse adherence (LA/DA) in HeLa cells. In this study, we selected one aEPEC isolate of this serotype (IAL5133) that produced the LA/DA pattern, to investigate the bacterial structures involved in its adhesive phenotype. For this purpose, aEPEC IAL5133 was mutagenized using the EZ::TN < R6Kyori/KAN-2 > Tnp transposome kit, generating a library of Tn5 insertions. These Tn5 insertion clones were screened for non-adherent or less adherent mutants, in assays performed in 6 h of incubation with HeLa cells. So far, among the 320 clones screened, nine were considered deficient in their ability to interact with epithelial cells, and four of them presented the Tn5 insertion in genes within the LEE region, such as: *tir*, *escV* and *grlR*. In order to confirm the role of intimin-tir interaction in the establishment of this hybrid phenotype, we constructed isogenic mutants in the *eae* and *escN* genes, which encode the adhesin intimin and the ATPase necessary for the T3SS (type III secretion system) assembly, respectively. The isogenic mutants generated in the intimin (*eae*) and T3SS translocon (*escN*), showed a decrease in the number of cell-associated bacteria ($p<0.05$), as compared to the wild-type strain. *In trans* complementation restored the ability of the *eae* and *escN* mutants to adhere efficiently to HeLa cells. In conclusion, we demonstrated that genes located in the LEE region are essential for the establishment of the hybrid LA/DA phenotype in aEPEC IAL5133.

**Keywords:** Atypical enteropathogenic *Escherichia coli*, diarrheal outbreak, hybrid adherence pattern.

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