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Pathogenicity Island O-122 in enteropathogenic *Escherichia coli* strains is associated with diarrhea severity in children from Lima Peru

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ABSTRACT

EPEC is an attaching and effacing diarrheal pathogen that carries a large pathogenicity island, locus for enterocyte effacement (LEE). Recently, the pathogenicity island PAI O-122 was described among non-LEE effectors and found to be associated with diarrhea among atypical EPEC strains. It is unknown if incomplete PAI O-122 could be associated with diarrhea duration and severity. To identify these virulence determinants we analyzed 379 EPEC strains isolated from Peruvian children. EPEC was diagnosed by PCR(eae+, stx-) and classified as typical(t-EPEC) or atypical(a-EPEC). To characterize PAI O-122 we amplified three modules by PCR: Module 1(*pagC*), Module 2(*senA*, *nleB* and *nleE*) and Module 3(*lifA/efa-1*). To characterize the large ORF *lifA/efa-1* we amplified the regions known as efa-N, efa-M and efa-C. Clinical information was obtained from the cohort study. A total of 379 EPEC strains were able to analyze PAI O-122 genes, 128 (10.4%) EPEC strains were isolated from 1235 diarrhea episodes and 251(9.2%) from 2734 healthy controls. t-EPEC strains were isolated from 14.8% (19/128) of children with diarrhea and 25/251(10.0%) from healthy controls. The most frequent PAI O-122 genes were *nleE*(37.7%), *senA*(34.6%) and *nleB*(37.5%), with similar prevalence among diarrhea and control samples. However, *lifA/efa-1* was more common among diarrhea cases than healthy control cases (30.5% vs. 21.1%, $p < 0.05$). The presence of complete PAI O-122 was associated with diarrhea episodes of higher severity among single pathogen infection (33.3% vs. 1.8%, $p < 0.05$) mainly due to the presence of a complete *lifA/efa*-1 gene. In summary, the gene *lifA/efa-1* is significantly associated with diarrheal episodes of higher severity, suggesting to be an important virulent factor.

1. Introduction

Diarrhea is the third most common cause of death worldwide in children under the age of five, with the majority of cases occurring in developing countries (Liu et al., 2012). A series of pathogens have been associated with diarrhea, however, their relative importance varies both in relation to geographical distribution and severity of the illness (Lanata et al., 2013; Kotloff et al., 2013). In a recent multicenter diarrhea study (GEMS), EPEC has been associated with increased risk of death in young children (Donnenberg and Finlay, 2013; Kotloff et al., 2013).

Multiple genetic determinants encode virulent factors that underlie the ability of EPEC to cause illness. Among the main genetic determinants are (1) EAF-plasmid (EPEC adherence factor) which mediates localized adherence to intestinal cells (Kaper et al., 2004) and discriminates typical EPEC (t-EPEC) strains (*eae+, bfpA* +) from atypical EPEC (a-EPEC) strains (*eae+, bfpA*-); (2) the "locus of enterocyte effacement" pathogenicity island (PAI-LEE) located on the bacterial chromosome which encodes more than 40 proteins for the type III secretion system and effector proteins necessary for the attaching and effacing (A/E) lesion in the intestinal epithelial surface (Nougayrède et al., 2003); and (3) the non-LEE genes including the O-122 pathogenicity island (PAI O-122), which comprises a series of putative virulence genes such as *pagC*, *senA*, *nleB*, *nleE* and *lifA/efa-1* (Karmali et al., 2003).

The *lifA/efa-1* (lymphocyte inhibitory factor A/EHEC factor for adherence-1) gene encodes lymphostatin, a very large surface protein that inhibits proliferation of mitogen activated lymphocytes

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and the synthesis of pro-inflammatory cytokines (Klapproth et al., 2000). *lifA/efa-1* has also been implicated in the attachment of a-EPEC strains to host cells (Badea et al., 2003) and is critical for intestinal colonization by *Citrobacter rodentium,* an A/E lesion-producing bacterial murine pathogen (Klapproth et al., 2005). However, its association with diarrhea is controversial (Afset et al., 2006). Vieira et al., have shown that typical and atypical EPEC strains may harbor a complete (*pagC*, *senA*, *nleB*, *nleE* and *lifA/efa-1* all together) or incomplete PAI O-122; a strong association between the presence of a complete PAI O-122 and diarrhea was observed only in a-EPEC strains (Vieira et al., 2010). It is unknown if incomplete PAI O-122 could also be associated with diarrhea.

Thus, the aim of this study was to determine the prevalence of PAI O-122 genes, their distribution pattern among EPEC strains, and their correlations with clinical characteristics in strains isolated from Peruvian Children with and without diarrhea.

2. Materials and methods

2.1. Patients and controls

Specimens analyzed in this study were obtained from a previous community-based, randomized, double blind placebo-controlled diarrhea trial, conducted from January 2008 through May 2011, in children from peri-urban areas in Lima, Peru. Since no difference in EPEC prevalence and severity with the study intervention (lactoferrin) was observed, we worked with all samples collected in the study (Ochoa et al., 2013). Diarrhea was defined as three or more liquid or semi-liquid stools within 24 h or a single bloody semi-liquid stool within 24 h. We have also included stool samples from healthy "control" children without diarrhea one week before and one week after the sample collection period. Samples were evaluated for common enteric pathogens (*Shigella*, *Salmonella*, *Vibrio*, *Campylobacter*, adenovirus, rotavirus, *Giardia lamblia, Cryptosporidium* and other parasite) by conventional methods (Ochoa et al., 2013). Five lactose positive colonies were isolated from MacConkey plates and tested by a multiplex real time PCR with specific primers to detect virulence factors associated with diarrheagenic *Escherichia coli* (Guion et al., 2008).

2.2. Strains and DNA extraction

A total of 379 EPEC strains, stored at −70 °C in Trypticase Soy Broth (TSB) containing 15% glycerol, were included in this study; one colony per sample was used. All EPEC strains were cultured on Mac Conkey agar plates for overnight grow at 37 °C. A single colony from the plates was suspended in 50 uL of PCR water for DNA extraction by boiling method (Guion et al., 2008). Two microliters of DNA were used as a template in a 25 ul total PCR volume or stored at −20 °C until use.

2.3. PCR analysis

2.3.1. PCR for determination of typical and atypical EPEC

To discriminate t-EPEC from a-EPEC strains, a duplex conventional PCR assay was performed, using primers for *eae* and *bfpA* genes (Table 1). PCR reaction mix (Promega Corp., Madison, WI) included: 1x GoTaq Flexi Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, 0.75 U of GoTaq DNA Polymerase and 2 μl of DNA template adjusted to 25 μl per reaction with PCR water. The duplex conventional PCR reaction was programmed as follows: pre-denature at 94 °C for 5 min; 30 cycles at 94 °C for 20 s, 58 °C for 20 s, 72 °C for 30 s, and post-annealing at 72 °C for 5 min in an Applied Biosystems 2720 Thermal Cycler.

2.3.2. PCR for PAI O-122 characterization

To characterize the PAI O-122, we searched five genes distributed in three modules (Konczy et al., 2008): Module 1 (*pagC*), Module 2 (*senA*, *nleB* and *nleE*) and Module 3 (*lifA/efa-1*) (Fig. 1), using primers and specific annealing temperatures for each gene as previously described (Table 1). The complete (all five PAI O-122 genes present) or Incomplete PAI O-122 (at least one but not all five PAI O-122 genes present) was consider for analyze.

To characterize a large open reading frame (ORF) spanning of 9669 bp, which is designate *lifA/efa-1* (module 3), we used a set of primers to amplify three regions: 5′ known as efa-N; central, efa-M and 3′, efa-C (9). The PCR amplification of each region was performed with the same reagents used for PAI O-122, under the following conditions: 94 °C per 2 min, 30 cycles of 94 °C per 15 s, a specific annealing temperature for each amplicon (Table 1) per 20 s, and 72 °C per 90 s and final extension of 72 °C per 5 min.

The PCR products were analyzed by agarose electrophoresis (Invitrogen, Carlsbad, CA) using 12 uL of the amplicon. For the determination of typical and atypical EPEC and PAI O-122 characterization we use a 2% gel, and 1% for *lifA/efa-1* characterization. The gels were stained with ethidium bromide then visualized and analyzed under ultraviolet light (Bio- Rad transilluminator). The product length was determined using GeneRuler 100 bp plus DNA Ladder (Thermo Scientific, USA).

2.4. Clinical data

Clinical information about diarrheal episodes was obtained from the clinical trial. Children were followed for 6 months with daily home visits. Diarrhea duration was recorded until the last day an unformed stool was produced. Diarrhea duration was classified as acute (<7days) and prolonged (≥7days) (Moore et al., 2010). We used a Modified Ruuska-Vesikari Score (MRV) to determine the severity of the EPEC episodes, as previously described (Ochoa et al., 2009). The scored included: duration of diarrhea in days (0–3 points), maximum number of stools per day during the episode (1–3), presence of vomiting (0–1), maximum number of emesis per day during the episode (0–3), fever (0–1), dehydration (0–1), and treatment (0–2). The maximum possible score was 19; a score > 8 was considered moderate/severe.

2.5. Statistical analysis

Differences between isolation rates, clinical characteristics, and presence of the PAI-O122 gene among EPEC strains isolated from diarrhea and control samples were evaluated by chi-square or Fisher exact tests. All statistical analyses were performed using free Epi Info version 7. The significance level was set at a p value of<0.05.

3. Results

3.1. EPEC prevalence

One hundred twenty eight (10.4%) EPEC strains isolated from 1235 diarrhea episodes and 251 (9.2%) EPEC strains isolated from 2734 healthy controls were analyzed. The presence of t-EPEC (*bfpA* +) was found in 19/128 strains isolated from children with diarrhea (14.8%) and 25/251 from healthy controls (10.0%). a-EPEC was found in 109/128 (85.2%) of diarrhea samples and 226/251 (90.0%) of healthy controls. Additionally, all EPEC strains have been checked for *Stx1 and Stx2* to exclude Shiga toxin producing *E. coli* (STEC).