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# Non-diarrheagenic and diarrheagenic *E. coli* carrying supplementary virulence genes (SVG) are associated with diarrhea in children from Mexico

Uriel A. Angulo-Zamudio [a, 1], Javier Gutiérrez-Jiménez [a, b, 1], Luis Monroy-Higuera [a, c, 1], Hector Flores-Villaseñor [a, d], Nidia Leon-Sicairos [a, e], Jorge Velazquez-Roman [a], Jorge E. Vidal [f], Gabriela Tapia-Pastrana [g], Adrian Canizalez-Roman [a, h, *]

[a] CIASaP, School of Medicine, Autonomous University of Sinaloa, 80246, Culiacan, Sinaloa, Mexico
[b] Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Tuxtla, Gutiérrez, Mexico
[c] Programa de Maestría en Ciencias en Biomedicina Molecular, UAS, 80246, Culiacan, Sinaloa, Mexico
[d] The Sinaloa State Public Health Laboratory, Secretariat of Health, 80020, Culiacan, Sinaloa, Mexico
[e] Pediatric Hospital of Sinaloa, 80200, Culiacan, Sinaloa, Mexico
[f] Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, MS, USA
[g] Laboratorio de Investigación Biomédica, Hospital Regional de Alta Especialidad de Oaxaca, Oaxaca, 71256, Mexico
[h] The Women's Hospital, Secretariat of Health, 80127, Culiacan, Sinaloa, Mexico

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ABSTRACT

*Escherichia coli* strains, including diarrheagenic *E. coli* (DEC), are among the most important causes of childhood diarrhea in developing countries. Since these strains also colonize healthy children, additional factors leading to diarrhea remains to be discovered. We therefore conducted a comprehensive study to investigate if supplementary virulence genes (SVG) carried by DEC strains and non-DEC strains, contribute to diarrhea in Mexican children. *E. coli* strains were isolated from $n = 317$ children between 6 and 12 years, $n = 114$ with diarrhea and $n = 203$ asymptomatic children from Northwestern Mexico, PCR was used to identify SVG, then virulence score and cytotoxic assay in HT-29 cells were performed to evaluate virulence of *E. coli* strains. DEC prevalence was 18.6% and its presence was significantly associated with diarrhea cases. aEPEC, tEAEC, ETEC, DAEC, aEAEC, tEPEC, and EIEC pathotypes were identified. aEPEC strains were significantly associated with asymptomatic children, whereas ETEC was only identified in children with diarrhea. *E. coli* strains carrying colonization-related SVG and/or proteolysis-related SVG were significantly associated with diarrhea. DEC strains were associated to diarrhea if strains carried SVG *ehaC, kps, nleB,* and/or *espC*. Virulence score was significantly higher in *E. coli* from diarrhea cases than asymptomatic. In addition, DEC strains carrying SVG$^+$ were more virulent, followed by non-DEC SVG$^+$ strains, and correlated with the cytotoxicity assay. Nearly 50% of DEC strains were MDR, and ~10% were XDR. In conclusion the findings of this work provide evidence that the presence of *E. coli* strains (regardless if strains are DEC or non-DEC) with SVG were associated with diarrhea in Mexican children.

## 1. Introduction

Diarrhea is a major cause of morbidity and mortality in children worldwide. The incidence of this illness is estimated to be ~1731 billion cases per year causing ~711,800 deaths in children [1]. In 2016 diarrhea was the 8th cause of deaths in population of all ages and the 5th in children less than 5 years old [2]. As in other developing countries in Mexico diarrhea is still a serious problem. During the last two decades intestinal infections have been the 2nd most common cause of death in children, with more than 5.5 million of diarrhea cases reported in Mexico in 2010, most of them in the vulnerable population of young children [3,4]. Whereas there are many etiologic agents that cause diarrhea, diarrheagenic *Escherichia coli* (DEC) strains are a common cause of diarrhea in developing countries.

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* Corresponding author. Autonomous University of Sinaloa, School of Medicine, 80246, Culiacan, Sinaloa, Mexico.
*E-mail address:* canizalez@uas.edu.mx (A. Canizalez-Roman).

[1] Authors contributed equally to the works.

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DEC strains are classified based on the production of a number of virulence factors (e.g., adhesion patterns, capsule, and invasiveness) and more recently using genetic approaches that identify genes encoding these virulence factors [5]. Using these phenotypic and genotypic approaches, along with clinical presentation, and epidemiology, DEC strains belongs to one of the six different categories recognized to date: *i)* Enteropathogenic *E. coli* (EPEC) strains are characterized by the presence of a pathogenicity island known as the "locus of enterocyte effacement" (LEE). The LEE Island carries the *eaeA* gene that encodes an outer membrane adhesive protein known as intimin. EPEC strains also carry a plasmid called EPEC adherence factor (EAF). EPEC strains can be subtyped in typical and atypical strains. Atypical EPEC carries LEE and therefore presents the A/E phenotype but does not possess the EAF plasmid. *ii)* Enterotoxigenic *E. coli* (ETEC) strains are defined by the production of enterotoxins including the heat labile and heat-stable toxins (LT and ST) [5,6]. *iii)* Enteroinvasive *E. coli* (EIEC) strains invade the epithelium of the colon using the transcription activator *virF*, and a type three secretion system (T3SS) [7]. *iv)* Enteroaggregative *E. coli* (EAEC) strains present a characteristic aggregative adherence (AA) pattern on different cell lines that is associated to a 60-MDa plasmid. EAEC strains carrying the AggR regulon are classified as typical EAEC [8,9]. *v)* Diffuse adherent *E. coli* (DAEC) strains are characterized by the presence of genes encoding afimbrial (Afa), or fimbrial (Dr), adhesins [10]. *vi)* Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC) are terms used in the developed world for pathotypes that cause enteric *E. coli* infections associated to production of toxins and carries shiga toxin genes *stx1 and*/or *stx2*. Enterohemorrhagic *E. coli* (EHEC) is the most prevalent subgroup of VTEC/STEC that in addition to shiga toxin genes also carry the *eaeA* gene encoding for intimin, a protein responsible for attaching and effacing lesions [6,10]. All of these categories (EPEC, EIEC, EHEC, ETEC and EAEC serotypes) have been associated to diarrheal illness, including outbreaks of gastrointestinal disease [11–13].

The capacity of DEC to cause diarrhea has been demonstrated in different parts of the world, but some pathotypes have been associated to specific geographic zones. For example, the majority of DEC cases reported in Mexico, Colombia, and Nicaragua are associated to ETEC strains while diarrheal cases caused by EAEC have been more prevalent in Brazil, Paraguay and Peru. On the other hand in Venezuela, Chile, Argentina and Uruguay EPEC is the most prevalent pathotype and EHEC has been also detected frequently in these South American countries [14–16].

In addition to the known virulence factors encoded and produced by every DEC pathotype, there are many genes called supplementary virulence genes (SVG). These SVGs apparently lead to a higher virulence phenotype of the DEC strain that carry them, and non-DEC strains turn virulent if they acquire those genes [17]. SVGs encode proteins with a demonstrated role, *in vitro,* within three different phenotypes associated to virulence, *i)* colonization, *ii)* cytotoxicity, and *iii)* proteolysis. SVGs with a potential role in colonization include genes of putative adhesins (e.g., *aida-I, tibA, ehaA, ehaB, ehaC* and *ehaD*), or that involve in biofilm formation (*sab)* and accessory adhesion genes require for the attachment to host cells (*agg4A and efa*) [18–24]. For those associated to cytotoxicity, their encoded proteins caused damage of *in-vitro* cultured cells, and showed enterotoxic activity, through a mechanism leading to the degradation of the cytoskeletal protein spectrin, pepsin, and factor V (*espC* and *pet*) or by targeting the tight junctions (*sat*) [25–27]. Those with proteolytic activity (but not necessarily showing enterotoxic activity) include proteins degrading mucin, spectrin, pepsin, or proteins of the plasma (*espL, espP* and *pic*) [28–31]. The phenotype investigated *in vitro* suggests a role in pathogenesis but the role in SVGs inducing, or exacerbating diarrheal disease has not been yet clarified.

The occurrence of antibiotic-resistant bacteria is very common worldwide, due to the indiscriminate use of antibiotics and the genomic plasticity of *E. coli*. Recent studies reported a high prevalence of antibiotic resistance in DEC strains isolated from patients with diarrhea, and those isolated from asymptomatic people, thereby DEC strains might become a public health concern [32].

Despite that diarrheal illness is a major cause of death and morbidity in Mexican children; few molecular epidemiology studies have been conducted to evaluate the prevalence of DEC strains, or non-DEC strains, carrying SVGs in children with diarrhea, and asymptomatic children. Moreover, the role of SVGs in cases of diarrhea have not been studied at the population level. The prevalence of DEC strains has been studied in water, food, and diarrhea cases in populations of all ages in Mexico [15,33,34]. In this comprehensive study, we investigated the prevalence of DEC strains and non-DEC strains in children 6–12 years of age with diarrhea and asymptomatic controls, and examined the prevalence of *E. coli* strains and DEC strains carrying SVG. The association of SVGs with diarrhea cases was also investigated. Finally, we investigated the antibiotic resistance profiles of DEC strains.

## 2. Materials and methods

### 2.1. Bacterial strains

DEC reference strains utilized in this study belong to our laboratory collection and include EPEC E2348/69 (*eaeA* + and *bfpA*+), ETEC (*lt*+ and *st*-), EIEC (*ipaH*+ and *virF*-), EHEC O157:H7 EDL933 (*eaeA* +, *hlyA*+, *stx*1+, and *stx*2+), DAEC (*daaE*+), EAEC O42 (*aggR*+, *aap*+, *pCVD*432+, and *aafII*+) and *E. coli* DH5α [35]. DNA from the following reference strains carrying SVG were utilized as a control in PCR reactions: *Shigella flexneri* (*sat,* [36]), *E. coli* K12 (*aida-I* [37]), EHEC O157:H7 (*cah, ehaABCD, espI* and *espP* [19,29,30,38]), ETEC H10407 (*eatA* and *tibA* [24,39]), EAEC (*pet, kps, pic* and *agg4A* [23,26,31,40]), aEPEC (*efa1/lifA* and *nleB* [41]), EPEC E2348/69 (*espC* [25]), and STEC O113:H21 (*sab* [20]), were used. Bacteria were routinely grown overnight in Luria-Bertani (LB) broth (0.5% yeast extract, 1% tryptone and 0.5% NaCl) and incubated at 37 °C in a shaker incubator (Thermo Scientific, Waltham, Massachusetts USA).

### 2.2. Study population

Stools were collected from children with diarrhea and asymptomatic children. Specimens from children with diarrhea were collected from N = 114 children that came to the primary health center with acute diarrhea, with at least three liquid stools a day during the last three days. Children were 6–12 years of age and belonged to 17 municipalities of the Sinaloa state. Asymptomatic children: samples were taken from 203 children 6–12 years of age from the same municipalities of the Sinaloa state from where diarrhea cases were collected. Children of the asymptomatic control group met the following inclusion criteria: absence of vomiting, diarrhea, fever, loss of appetite or abdominal pain; not be under antibiotic treatment, have the informed consent signed by the parents to participate in the project (The study was approved by the Ethics Committee of The Women's Hospital, Secretariat of Health No. 202006-04). Clinical and epidemiological information was recorded through questionnaires.

### 2.3. Isolation and identification of E. coli strains

*E. coli* was identified in feces from children with diarrhea and asymptomatic. Five g of feces were collected from asymptomatic children while the feces of children with diarrhea were collected with rectal swabs and placed in Cary-Blair transport medium. All samples were transferred (in a time not exceeding 2 h) in a cooler with ice packs (~4 °C) to the laboratory for processing. The samples were seeded on MacConkey agar for the selection of lactose fermenting colonies (presumptive *E. coli*). Five colonies were taken, and individually analyzed

for biochemical identification, the API 20E® system (Biomeriux, USA) was used following the manufacturer's instructions. To molecular identification, DNA extraction by boiling method of presumptive colonies to *E. coli* was made and strains were confirmed by PCR according to the protocol described by Tsen et al. [42]. All stool samples collected in Cary-Blair transport medium were cultured on selective and differential agar media by standard laboratory procedures, and were negative for *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, and *Campylobacter* spp. Parasitology techniques were utilized to discard intestinal parasitosis, all specimens were negative for *Entamoeba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Giardia lamblia* [43,44].

### 2.4. Sequential multiplex PCR, scheme to identify DEC strains

DEC strains among *E. coli* isolates were further identified using a scheme of sequential multiplex, duplex, and singleplex PCR reactions, as previously reported [35]. These sequential multiplex PCR reactions identify the most prevalent DEC groups in decreasing order; reaction #1 identified EPEC strains and negative samples were further used as template for reactions #2 and #3 to identify EAEC strains, and so on. Multiplex PCR reaction #1 contained primers to amplify the intimin gene (*eaeA*) and the structural subunit of the bundle-forming pilus (Bfp) gene (*bfp*A) and detected both typical (*eaeA* + and *bfp*A+) and atypical (*eaeA* + and *bfp*A-) EPEC strains. Multiplex PCR reaction #2 detected EAEC strains with primers amplifying a gene encoding a regulator protein (*aggR*) and a fimbriae gene (*aafII)*. Multiplex PCR reaction #3 contained primers to amplify the gene coding a protein dispersin (*aap*) and the AA pattern associated plasmid (*pCVD432*) to identify typical (*pCVD432*+ and *aggR* + and/or *aafII* + *aap*+) and atypical (*pCVD432*+ and *aggR*- and/or *aafII* + *aap*+) EAEC strains. Duplex PCR reaction #4 detected ETEC strains by amplifying the gene encoding the heat labile toxin (*Lt*) and/or that encoding the heat stable toxin (*stII*). PCR reaction #5 targeted DAEC strains and contained primers that amplified the structural subunit gene of the F1845 fimbria (*daaE*). Detection of EIEC strains was performed with reaction #6, this reaction contained primers to amplify the invasion genes (*virF*) and (*ipaH)*. Negative strains and DEC *eaeA* + strains (from reaction #1) were screened in multiplex PCR reaction #7 to detect possible EHEC strains. This reaction amplified genes *stx1* and/or *stx2,* EHEC strains encode Shiga toxin genes and the *eaeA* gene whereas STEC strains were *eaeA*-. Finally, multiplex PCR reaction #8 contained primers that amplified the hemolysin gene (*hlyA*), the *rfbE* gene coding for production of the lipopolysaccharide O of *E. coli* O157 and the *fliC* gene which encodes the *E. coli* flagellum H7 serotype and so detecting EHEC O157:H7 strains. Primer sequences, PCR conditions, and product sizes were reported by Canizalez-Roman et al. [35].

### 2.5. Detection of SVG by PCR

The supplementary virulence genes were identified using nineteen singleplex PCR reactions. SVG targets were divided in three groups depending on the putative role in virulence of proteins encoded by each gene. The SVGs related to colonization were: *aida-1, Cah*, *ehaA*, *ehaB*, *ehaC*, *ehaD*, *tibA, efa/lifA*, *Kps*, *nleB*, *agg4A* and *Sab*. Those related to cytotoxicity: *espC*, *pet* and *sat* while *eatA*, *espI*, *espP* and *Pic* are related with proteolysis. Primer sequences, and the size of PCR products, are show in Supplemental Table S1.

After the identification of SVGs in *E. coli* strains, a virulence score was calculated for each strain using a published formula/approach as follows: ("x" SVGs carried ÷ total SVGs screened) x 10 [45].

### 2.6. Cytotoxicity assay

Cytotoxicity was evaluated in strains randomly selected of groups DECs and non-DECs without SVGs whereas those with SVGs were selected based on similar SVG gene profiles in DEC strains and non-DEC strains. Colorectal human cells (HT-29 cells, ATCC® HTB-38™) were cultivated in 1X McCoy 5A modified with L-glutamine (Gibco, Massachusetts, United States), supplemented with 10% of fetal bovine serum and antibiotics [penicillin 5000 U, streptomycin 5 mg, and neomycin 10 mg (Sigma, Missouri, United States)]; cells were incubated in an atmosphere of 5% $CO_2$, at 37 °C until get 80–90% of confluence. HT-29 cells were detached with 0.25% trypsin, 2.21 mM EDTA and 1X sodium bicarbonate (Corning, New York, United States), and seeded in 96-well plates at a density of 20,000 cells/mL and grown until the monolayer was confluent using the above incubation conditions. Then $1 \times 10^7$ CFU/mL of *E. coli* were used to infect cells in McCoy media during 12 h in atmosphere of 5% $CO_2$, at 37 °C. *E. coli* HB101 was used as a negative control of cytotoxicity while EHEC O157:H7 was used as a positive control. Cytotoxicity was quantified using a Pierce LDH Cytotoxicity assay kit (ThermoFisher, Massachusetts, United States) following the manufacture instructions.

### 2.7. Antimicrobial susceptibility testing

Antibiotic susceptibility testing of pathogenic isolates was performed by the Kirby-Bauer disk diffusion method [46] following the guidelines developed by the Clinical Laboratory Standard Institute (CLSI) [47]. Suspensions of *E. coli* strains were prepared in LB at a turbidity of 0.5 using the McFarland standard. Then, Mueller-Hinton agar plates were swabbed with these cultures and antibiotic disks (BD BBL, Franklin Lakes, NJ), were placed aseptically on the inoculated agar. Antibiotics tested were: gentamicin, ciprofloxacin, nalidixic acid, sulfamethoxazole-trimethoprim, tetracycline, ampicillin, ceftazidime, cefotaxime and chloramphenicol. The plates were incubated at 37 °C for 18–20 h. The diameters (in millimeters) of clear zones of growth inhibition around the antimicrobial agent disks were measured using a precision digital caliper (Absolute, Mitutoyo, Japan). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 obtained from the American Type Culture Collection (ATCC) were used as control. Recommendations by the National Antimicrobial Resistance Monitoring System for *E. coli* were utilized to define breakpoints of antibiotics and thus categorize the isolates as resistant, intermediate, or sensitive [48]. Isolates that showed resistant to ≥3 different categories of antibiotics were classified as Multidrug-resistant (MDR) and Extremely drug resistant (XDR) those resistant to ≥ 6 different categories of antibiotics [49]. Antibiotics were selected based on their use to treat human infection by Gram negative bacteria [50] and represent different classes of antimicrobial agents that are available to treat these infections in Mexico.

### 2.8. Statistical analysis

The Kolmogorov-Smirnoff test was used to evaluate the normality of the sample and the Mann-Whitney *U* test to compare the means of age. Associations between nominal variables were analyzed with Fisher's exact test and/or chi square. Differences in virulence score and cytotoxicity level of *E. coli* strains were determined by one-way analysis of variance. Statistical significance was determined when $p \leq 0.05$; analyzes were made with the IBM® SPSS® Statistics program version 20 (New York, United State); the charts were built with the SigmaPlot version 12 program (CA, United States).

## 3. Results

### 3.1. Prevalence of non-DEC strains, and DEC pathotypes in children with diarrhea and in the asymptomatic control group

We isolated $n = 317$ *E. coli* strains from children of which $n = 114$ (35.9%) were isolated from diarrhea cases and $n = 203$ (64%) from the control group. Of these $n = 317$ *E. coli* strains, 81% (258/317) were classified within the non-DEC group due to the absence of DEC-associated virulence factors, as investigated by PCR, while 18.6% (59/317) were classified as DEC strains (Table 1). As expected, the distribution of non-DEC between children with diarrhea or the asymptomatic control group was similar, while the prevalence of DEC strains was significantly higher in children with diarrhea, compared to the asymptomatic control group (*p*: 0.003). Moreover, the relative odds of isolating a DEC strain from a child with diarrhea was higher compared to isolating DEC from a healthy child, in this age group, Table 1.

**Table 1**
Distribution of *E. coli,* non-DEC, and DEC pathotypes in relation with clinical condition of children.

| Type of *E. coli* | Total (n = 317) | Diarrhea (n = 114) | Asymptomatic (n = 203) | *p* value | OR | 95%, CI |
|---|---|---|---|---|---|---|
| | n (%) | n (%) | n (%) | | | |
| Non-DEC | 258 (81.4) | 83 (72.8) | 175 (86.2) | 0.003 | 2.33 | 1.3–4.1 |
| DEC | 59 (18.6) | **31 (27.2)*** | 28 (13.8) | | | |
| DECs pathotypes | | | | | | |
| tEAEC | 13 (22.1) | 8 (25.8) | 5 (17.9) | 0.46 | 1.6 | 0.45–5.6 |
| aEAEC | 7 (11.8) | 3 (9.7) | 4 (14.3) | 0.69 | 0.64 | 0.13–3.1 |
| tEPEC | 2 (3.4) | 2 (6.5) | 0 (0.0) | 0.49 | – | – |
| aEPEC | 18 (30.5) | 4 (12.9) | **14 (50.0)*** | 0.002 | 0.14 | 0.04–0.53 |
| ETEC | 10 (16.9) | **10 (32.3)*** | 0 (0.0) | 0.001 | – | – |
| DAEC | 8 (13.6) | 3 (9.7) | 5 (17.9) | 0.45 | 0.49 | 0.1–2.2 |
| EIEC | 1 (1.7) | 1 (3.2) | 0 (0.0) | 1 | – | – |

DEC: Diarrheagenic *Escherichia coli;* OR: Odds Ratio; CI: Confidence Index. Fisher's Exact test was carried out to get statistical significance. Comparison between *E. coli* isolated from diarrhea vs asymptomatic. *: *p*: $^{<}$0.05.

Diarrheagenic *E. coli* pathotypes identified in the two groups, from the most prevalent to the least, were aEPEC (30.5%), tEAEC (22.1%), ETEC (16.9%), DAEC (13.6%), aEAEC (11.8%), tEPEC (3.4%), and EIEC (1.7%). aEPEC was the pathotype significantly most prevalent in asymptomatic children, and with a lower odds of being isolated from children with diarrhea (OR: 0.14, CI: 0.04–0.53). ETEC was only isolated from children with diarrhea (*p*: 0.001), Table 1. All other DEC pathotypes were similarly isolated from diarrhea cases and asymptomatic children.

### 3.2. Supplementary virulence genes (SVG) carry by E. coli strains

The proportion of strains with a positive PCR reaction for any SVG, out of $n = 317$ non-DEC, was significantly higher for strains isolated from diarrhea cases than those from the asymptomatic control group (Table 2); whereas all DEC strains carried SVG regardless the source of isolation. The distribution of SVG carried by strains was wide-ranging with some *E. coli* strains carrying only one gene (13.2%) and strains yielding a positive PCR reaction for ≥5 SVGs [40.7% (Table 2),]. We found statistical difference when DEC strains isolated from asymptomatic children were compared against those from children with diarrhea, only in those strains with seven genes (associated with diarrhea cases), the rest of strains with SVG did not show differences, perhaps because there was a limited number of strains in each category (Table 2). However, when strains were grouped into those carrying one to four SVG or those carrying five to 11 genes, we demonstrated that carrying fewer SVG (1–4) was associated with DECs isolated from asymptomatic children, while carrying five to 11 SVG was associated with strains isolated from diarrhea cases (Table 2). Concerning the distribution of SVG in non-DEC, strains carrying no SVG, carrying only one SVG was significantly associated to strains isolated from asymptomatic children than from those of the diarrhea group, whereas strains with 5 SVG were associated with diarrhea (Table 2). There was not significant association in individual analyses of strains carrying 6, 7, 8, 9, 10 and 11 SVG with diarrhea or asymptomatic cases (Table 2). We identified, however, that non-DEC strains carrying one to 4 SVG were associated with strains isolated from asymptomatic children whereas those carrying five to 11 SVG were associated with children with diarrhea in comparison with those isolated from asymptomatic children, Table 2. In 10.4% of the $n = 317$ strains isolated in this study we did not identify any SVG. Together these results indicate that the diar-

**Table 2**
Distribution of SVGs in non-DEC and DEC isolated from asymptomatic children or from children with diarrhea.

| SVG (N = 20) | *E. coli* | DEC N = 59 | | | | | Non-DEC N = 258 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Diarrhea | Asymptomatic | *p* value | Odds ratio | 95%, CI | Diarrhea | Asymptomatic | *p* value | Odds ratio | 95%, CI |
| | N = 317 (%) | N = 31 (%) | N = 28 (%) | | | | N = 83 (%) | N = 175 (%) | | | |
| 0 | 33 (10.4) | 0 (0.0) | 0 (0.0) | ND | – | – | 1 (1.2) | **32 (18.3)*** | <0.001 | 0.05 | 0.007–0.40 |
| Any gene | 284 (89.6) | 31 (100) | 28 (100) | ND | – | – | **82 (98.8)*** | 143 (81.7) | <0.001 | 18.3 | 2.46–136.7 |
| 1 | 42 (13.2) | 0 (0.0) | 3 (10.7) | 0.1 | – | – | 1 (1.2) | 38 (21.7)* | <0.001 | 0.04 | 0.005–0.32 |
| 2 | 34 (10.7) | 1 (3.2) | 1 (3.6) | 0.73 | 0.93 | 0.05–15.6 | 10 (12.0) | 22 (12.6) | 0.92 | 0.95 | 0.42–2.11 |
| 3 | 39 (12.3) | 2 (6.5) | 1 (3.6) | 0.52 | 1.86 | 0.15–21.7 | 15 (18.1) | 21 (12.0) | 0.18 | 1.61 | 0.78–3.32 |
| 4 | 40 (12.6) | 0 (0.0) | 4 (14.3) | 0.06 | – | – | 11 (13.3) | 25 (14.3) | 0.82 | 0.91 | 0.42–1.96 |
| 5 | 56 (17.7) | 5 (16.1) | 9 (32.1) | 0.14 | 0.41 | 0.12–1.4 | **25 (30.1)*** | 17 (9.7) | <0.001 | 4.3 | 2.2–8.5 |
| 6 | 34 (10.7) | 6 (19.4) | 8 (28.6) | 0.4 | 0.5 | 0.14–1.7 | 8 (9.6) | 12 (6.8) | 0.46 | 1.4 | 0.55–3.5 |
| 7 | 19 (5.9) | **8 (25.8)*** | 0 (0.0) | 0.003 | – | – | 5 (6.2) | 6 (3.4) | 0.33 | 1.8 | 0.53–6.1 |
| 8 | 12 (3.8) | 5 (16.1) | 1 (3.6) | 0.11 | 5 | 0.57–47.5 | 4 (4.8) | 2 (1.1) | 0.06 | 4.3 | 0.79–24.4 |
| 9 | 6 (1.9) | 3 (9.7) | 1 (3.6) | 0.35 | 2.8 | 0.28–29.5 | 2 (2.4) | 0 (0.0) | 0.03 | – | – |
| 10 | 2 (0.6) | 1 (3.2) | 0 (0.0) | 0.33 | – | – | 1 (1.2) | 0 (0.0) | 0.14 | – | – |
| 11 | 0 (0.0) | 0 (0.0) | 0 (0.0) | – | – | – | 0 (0.0) | 0 (0.0) | – | – | – |
| 1 to 4 | 155 (48.8) | 3 (9.06) | **9 (32.1)*** | 0.03 | 0.23 | 0.05–0.95 | 37 (42.5) | **106 (60.5)*** | 0.01 | 0.52 | 0.31–0.89 |
| 5 to 11 | 129 (40.7) | **28 (90.3)*** | 19 (67.9) | 0.03 | 4.26 | 1.05–18.4 | **45 (54.2)*** | 37 (21.1) | <0.001 | 4.41 | 2.51–7.76 |

SVG: Supplementary Virulence Genes. ND, not done because of the limited number of strains in each of these categories. Fisher's Exact test was carried out to investigate statistical significance. Comparison between *E. coli* isolated from diarrhea vs asymptomatic. Any gene: *E. coli* strains positive to at least one gene. *: p: $^{<}$0.05.

rheagenic potential of *E. coli* strains (DEC, or non-DEC) might be associated with the number of SVGs that bacteria carry.

To investigate what genes were associated with diarrhea we analyzed each individual gene belonging to one of the following three categories, (A) genes encoding putative proteins involved in colonization (i.e., *in vitro*), (B) those related to cytotoxicity, and (C) genes whose encoded proteins have proteolytic activity (Table 3). The most prevalent colonization-related SVGs were *ehaD, ehaA* and *kps*, whereas *sab* was not detected by PCR in any strain assessed. The presence of the following colonization-related SVGs in *E.* coli strains was significantly associated with diarrhea cases, in comparison with asymptomatic children, *ehaA*, *ehaB, ehaD, efa/lifA, kps*, *nleB*, and *agg4A*, Table 3. In general, *E. coli* strains isolated from diarrhea cases carried a higher number of colonization-related SVGs than that carried by strains from asymptomatic children. As per the analysis of overall SVGs, carrying no colonization-related genes, or carrying only one, was associated to strains isolated from the asymptomatic control group, Table 3. By the number of SVGs, those related to colonization carried by *E. coli* isolated from diarrhea were significantly higher if strains carrying 4, 5 or those that have 4 to 7 of these genes in comparison to strains isolated from asymptomatic children, (Table 3).

SVGs implicated in cytotoxicity such as *espC, pet* and *sat* were also assessed. Of these genes, *espC* was the most prevalent with 33.4% of *E. coli* strains carrying the gene, followed by *sat* (19.6%) and *pet* (11.4%). Surprisingly, none of these cytotoxicity-related SVGs was associated to diarrhea, since the prevalence of each gene was similar in both groups (Table 3).

Regarding SVGs implicated in proteolysis, the presence of *eatA, espI, espP, pic* and *in E. coli* strains was statistically significantly associated with diarrhea cases in comparison with asymptomatic children, Table

**Table 3**
Distribution of SVG in *E. coli* isolated from children with diarrhea or asymptomatic.

| SVG implied on: | *E. coli* (n = 317) | Diarrhea (n = 114) | Asymptomatic (n = 203) | *p* value | Odds ratio | 95%, CI |
|---|---|---|---|---|---|---|
| | n (%) | n (%) | n (%) | | | |
| **Colonization** | | | | | | |
| ehaD | 174 (54.9) | **72 (63.2)*** | 102 (50.2) | 0.034 | 1.69 | 1–2.7 |
| ehaA | 153 (48.3) | **73 (64.0)*** | 80 (39.4) | 0.000038 | 2.73 | 1.70–4.40 |
| kps | 144 (45.9) | **74 (64.9)*** | 70 (34.5) | 0.000064 | 3.51 | 2.17–5.6 |
| ehaC | 140 (44.2) | 56 (49.1) | 84 (41.4) | 0.196 | 1.36 | 0.86–2.16 |
| ehaB | 117 (36.9) | **74 (64.9)*** | 43 (21.2) | 0.000001 | 6.88 | 4.12–11.4 |
| nleB | 60 (19.1) | **30 (26.3)*** | 30 (14.8) | 0.016 | 2.05 | 1.16–3.63 |
| cah | 25 (7.9) | 12 (10.5) | 13 (6.4) | 0.199 | 1.71 | 0.75–3.9 |
| tibA | 10 (3.2) | 3 (2.6) | 7 (3.4) | 0.487 | 0.75 | 0.19–2.98 |
| efa/lifA | 8 (2.5) | **6 (5.3)*** | 2 (1.0) | 0.027 | 5.58 | 1.10–28.1 |
| agg4A | 6 (1.9) | **5 (4.4)*** | 1 (0.5) | 0.024 | 9.26 | 1.06–80.3 |
| aida-1 | 4 (1.3) | 0 (0.0) | 4 (2.0) | 0.301 | – | – |
| sab | 0 (0.0) | 0 (0.0) | 0 (0.0) | – | – | – |
| by number of gene | | | | | | |
| 0 | 42 (13.2) | 2 (1.7) | **40 (19.3)*** | <0.001 | 0.07 | 0.01–0.3 |
| Any gene | 275 (86.8) | **112 (98.2)*** | 163 (80.29) | <0.001 | 13.7 | 3.2–58.0 |
| 1 | 57 (18.0) | 12 (10.5) | **45 (22.1)*** | 0.01 | 0.41 | 0.2–0.81 |
| 2 | 48 (15.1) | 18 (15.7) | 30 (14.7) | 0.81 | 1.08 | 0.5–2.04 |
| 3 | 59 (18.6) | 16 (14) | 43 (21.8) | 0.11 | 0.6 | 0.32–1.13 |
| 4 | 60 (18.9) | **30 (26.3)*** | 30 (14.7) | 0.01 | 2.05 | 1.16–3.63 |
| 5 | 41 (12.9) | **29 (25.4)*** | 12 (5.9) | <0.001 | 5.45 | 2.64–11.1 |
| 6 | 8 (2.5) | 5 (4.3) | 3 (1.4) | 0.14 | 3.05 | 0.71–13.0 |
| 7 | 2 (0.6) | 2 (1.7) | 0 (0.0) | 0.12 | – | – |
| 4 to 7 | 101 (31.8) | **66 (57.8)*** | 45 (22.1) | 0.01 | 4.82 | 2.93–7.94 |
| **Cytotoxicity** | | | | | | |
| espC | 106 (33.4) | 41 (36.0) | 65 (32.0) | 0.535 | 1.19 | 0.73–1.93 |
| pet | 36 (11.4) | 9 (7.9) | 27 (13.3) | 0.196 | 0.55 | 0.25–1.23 |
| sat | 62 (19.6) | 22 (19.3) | 40 (19.7) | 1 | 0.97 | 0.54–1.73 |
| by number of gene | | | | | | |
| 0 | 164 (51.7) | 58 (50.8) | 106 (52.2) | 0.81 | 0.71 | 0.45–1.13 |
| Any gene | 153 (48.3) | 56 (49.2) | 97 (47.7) | 0.81 | 1.05 | 0.66–1.66 |
| 1 | 110 (34.7) | 42 (36.8) | 68 (33.4) | 0.54 | 1.15 | 0.74–1.87 |
| 2 | 35 (11.0) | 12 (10.5) | 23 (11.3) | 1 | 0.92 | 0.43–1.92 |
| 3 | 8 (2.5) | 2 (1.7) | 6 (2.9) | 0.71 | 0.58 | 0.11–2.95 |
| **Proteolysis** | | | | | | |
| eatA | 44 (13.9) | **40 (35.1)*** | 4 (2.0) | <0.001 | 54.3 | 12.8–230 |
| espI | 23 (7.3) | **20 (17.5)*** | 3 (1.5) | <0.001 | 14.18 | 4.11–48.9 |
| espP | 22 (6.9) | **18 (15.8)*** | 4 (2.0) | 0.000007 | 7.53 | 2.47–23.3 |
| pic | 45 (14.3) | **26 (22.8)*** | 19 (9.4) | 0.001 | 2.86 | 1.50–5.44 |
| by number of gene | | | | | | |
| 0 | 205 (64.7) | 28 (24.5) | **177 (87.1)*** | <0.001 | 0.04 | 0.02–0.08 |
| Any gene | 112 (35.3) | **86 (75.4)*** | 26 (12.8) | <0.001 | 20.9 | 11.5–37.8 |
| 1 | 93 (29.3) | **71 (62.8)*** | 22 (10.8) | <0.001 | 13.5 | 7.58–24.3 |
| 2 | 16 (5.0) | **12 (10.5)*** | 4 (1.8) | 0.002 | 5.85 | 1.84–18.6 |
| 3 | 3 (0.9) | **3 (2.6)*** | 0 (0.0) | 0.04 | – | – |

SVG: Supplementary Virulence Genes; CI: confidence index; 34 strains non-DEC were negative to SVG, 33 belonged to asymptomatic group and 1 to children with diarrhea. Fisher's Exact test was carried out to get statistical significance. Comparison between *E. coli* isolated from diarrhea vs asymptomatic. Any gene: *E. coli* strains positive to at least one gene. *: p: $^{<}$0.05.

3. *E. coli* carrying any gene, one, two, or three SVGs were associated to those strains isolated from children with diarrhea vs asymptomatic, Table 3.

### 3.3. Supplementary virulence genes in DEC strains

A potential increased virulence might occur if DEC strains carry additional virulence genes such as SVGs. To assess this hypothesis, we investigated if carrying SVGs was associated to DEC strains isolated from children with diarrhea. Of those SVGs involve in colonization, the *ehaA* gene was the most prevalent in DEC strains whereas *ehaC, kps,* and *nleB* were associated with diarrhea; the genes *sab* and *tibA* gene were not identified in this study. In the case of EAEC, only the genes *kps* and *nleB* were associated with EAEC isolated from children with diarrhea (Table 4). In EPEC all SVG were more prevalent in strains isolated from children with diarrhea but there was not statistical difference compared to the prevalence observed in asymptomatic children. Similarly, not association of carrying SVGs was observed in DAEC strains isolated from diarrhea compared to the control group. The majority of ETEC and EIEC strains, which were only isolated from diarrhea cases, carried SVGs (data no shown).

SVG related to cytotoxicity were identified in DEC strains, of which 81.4% were positive for at least one gene of this group. Only carrying *espC* was associated with DEC strains isolated from diarrhea, and specifically associated to EAEC strains from children with diarrhea, Table 4. Regarding SVG related to proteolysis, 50.8% DEC strains were positive to at least one gene of this group, the most prevalent gene in DEC strains was *pic,* but none of them were associated to DEC strains isolated from diarrhea cases, Table 4.

### 3.4. Virulence score between groups of E. coli (DEC and non-DEC)

To continue the characterization of these *E. coli* isolates, we utilized a previously established a virulence score based on the number of SVGs strains carry [45]. This arbitrary score was obtained by dividing the number of supplementary virulence genes present in each strain over the number of SVG screened ($n = 20$). We grouped strains in four categories taking into account whether strains were isolated from diarrhea cases, or controls, and whether the strain was a DEC isolate, or a non-DEC strain (Fig. 1). The highest SVG-related score was that from DEC strains isolated from children with diarrhea (media: 3.43) which was statistically significant different than that of all other categories, [($p$: $<$0.05), Fig. 1]. In contrast, the SVG-related virulence score was statistically similar when we compared DEC strains obtained from asymptomatic children (media: 2.52) with non-DEC strains isolated from cases of diarrhea (media: 2.40). The lowest virulence score was obtained for non-DEC *E. coli* strains isolated from asymptomatic children (media: 1.38) and it was statistically significant compared to all others.

### 3.5. Cytotoxicity of DEC and non-DEC strains on human intestinal HT29 cells

To assess the virulence potential of these *E. coli* strains, we challenged human intestinal HT-29 cells with a selected group of strains, and cytotoxicity was evaluated using the LDH assay. We selected representative DEC and non-DEC *E. coli* strains carrying a combination of SVG. To best assess the potential cytotoxicity of strains against human intestinal cells, strains were selected to carry similar adhesin genes (i.e., providing similar adhesion capacity) but with a different combination of enterotoxin genes, i.e., strains carrying *espC, pet* and *sat,* (EPEC S1), *espC* and *pet* (EPEC S2), or *espC* and *sat* EAEC S3, etc. *E. coli* strains were selected from the following groups: DEC strains from diarrhea cases or asymptomatic controls, or non-DEC strains from diarrhea cases or asymptomatic controls (Fig. 2). *E. coli* HB101, which does not encode virulence genes, and *E. coli* strains isolated from asymptomatic children and for whom virulence genes were not detected in this study, were utilized to establish the basal level of LDH release (i.e., no cytotoxicity). EHEC O157:H7 was utilized as a positive control of cytotoxicity (i.e., 100% cytotoxicity). DEC strains isolated from children with diarrhea, or isolated from the asymptomatic control group, produced the highest LDH release, comparable to the positive control EHEC O157:H7 (Fig. 2). On the other hand, non-DEC isolated from diarrhea cases or asymptomatic control induced a statistically significant lower LDH release than the positive control, and certainly a lower LDH release than the majority of DEC strains no matter from where the DEC strain was isolated (i.e., diarrhea cases or from the control group). No difference was observed when LDH released was quantified from the supernatant of intestinal HT29 cells infected with a non-DEC strain isolated from chil-

**Table 4**
Distribution of SVGs in DEC strains isolated from children in this study.

| SVG involved on: | Total DEC | | | *E. coli* pathotype n (%) | | |
|---|---|---|---|---|---|---|
| | | | | EAEC 20 (33.9) | | |
| | **D** | **A** | **Total** | **D** | **A** | **Total** |
| | **31** | **28** | **59** | **11** | **9** | **20** |
| **Colonization** | | | | | | |
| aida-1 | 0 (0.0) | 1 (3.6) | 1 (1.7) | 0 (0.0) | 1 (11.1) | 1 (5.0) |
| cah | 3 (9.7) | 3 (10.7) | 6 (10.2) | 2 (18.2) | 2 (66.7) | 4 (20.0) |
| ehaA | 26 (83.9) | 20 (71.4) | 46 (78.0) | 8 (72.7) | 8 (88.9) | 16 (80.0) |
| ehaB | 14 (45.2) | 7 (25.0) | 21 (35.6) | 3 (27.3) | 2 (22.2) | 5 (25.0) |
| ehaC | **28 (90.3)*** | 17 (60.7) | 45 (76.3) | 9 (81.8) | 6 (66.7) | 15 (75.0) |
| ehaD | 18 (58.1) | 22 (78.6) | 40 (67.8) | 6 (54.5) | 9 (100) | 15 (75.0) |
| sab | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| tibA | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| efa/lifA | 6 (19.4) | 0 (0.0) | 6 (10.2) | 4 (36.4) | 0 (0.0) | 4 (20.0) |
| kps | **24 (77.4)*** | 12 (42.9) | 36 (61.0) | **10 (90.9)*** | 4 (44.4) | 14 (70.0) |
| nleB | **23 (90.3)*** | 9 (32.1) | 32 (62.7) | **10 (90.9)*** | 2 (22.2) | 12 (60.0) |
| agg4A | 4 (12.9) | 1 (3.6) | 5 (8.5) | 2 (18.2) | 1 (11.1) | 3 (15.0) |
| Total | 31 (100) | 28 (100) | 59 (100) | 11 (100) | 9 (100) | 20 (100) |
| **Citotoxicity** | | | | | | |
| espC | **26 (83.9)*** | 7 (21.4) | 32 (54.2) | **9 (81.8)*** | 2 (22.2) | 11 (55.5) |
| pet | 3 (9.7) | **12 (42.9)*** | 15 (25.4) | 0 (0.0) | 5 (55.6) | 5 (25.0) |
| sat | 5 (16.1) | 11 (39.3) | 16 (27.1) | 1 (9.1) | 3 (33.3) | 4 (20.0) |
| Total | 27 (87.1) | 21 (75.0) | 48 (81.4) | 9 (81.8) | 7 (77.8) | 16 (80.0) |
| **Proteolysis** | | | | | | |
| eatA | 8 (25.8) | 4 (14.3) | 12 (20.3) | 2 (18.2) | 2 (22.2) | 4 (20.0) |
| espI | 2 (6.5) | 2 (7.1) | 4 (6.8) | 0 (0.0) | 2 (22.2) | 2 (10.0) |
| espP | 0 (0.0) | 2 (7.1) | 2 (3.4) | 0 (0.0) | 1 (11.1) | 1 (5.0) |
| pic | 12 (38.7) | 6 (21.4) | 18 (30.5) | 9 (81.8) | 3 (33.3) | 12 (60.0) |
| Total | 19 (61.3) | 11 (39.3) | 30 (50.8) | 10 (90.9) | 6 (66.7) | 16 (80.0) |

D: Diarrhea; A: Asymptomatic. The remaining pathotypes (i.e., strains) did not present significant differences.*: $p$ $<$0.05. Fisher's Exact test was carried out to get statistical significance DEC isolated from diarrhea vs asymptomatic.

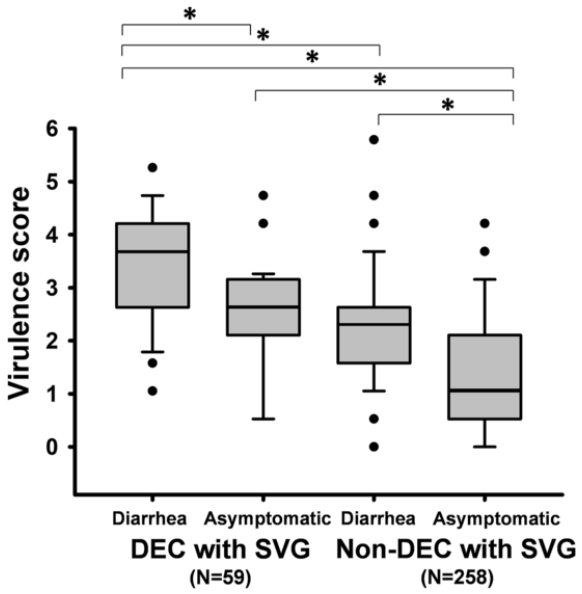


**Fig. 1. Comparison of virulence score between groups of *E. coli* isolated from children with diarrhea and asymptomatic.** Virulence score was calculated dividing the number of supplementary virulence genes present in strains, over the 19 tested x 10. The differences were compared between DEC and non-DEC with SVG isolated from children asymptomatic or with diarrhea. Asterisks indicate statistical significance calculated using one-way ANOVA based in ranges. 34 strains non-DEC were negative to SVG, 33 belonged to asymptomatic group and 1 to children with diarrhea. *: $p$: $^{<}$0.05 compared to DEC with SVG against non-DEC with SVG and non-DEC without SVG.

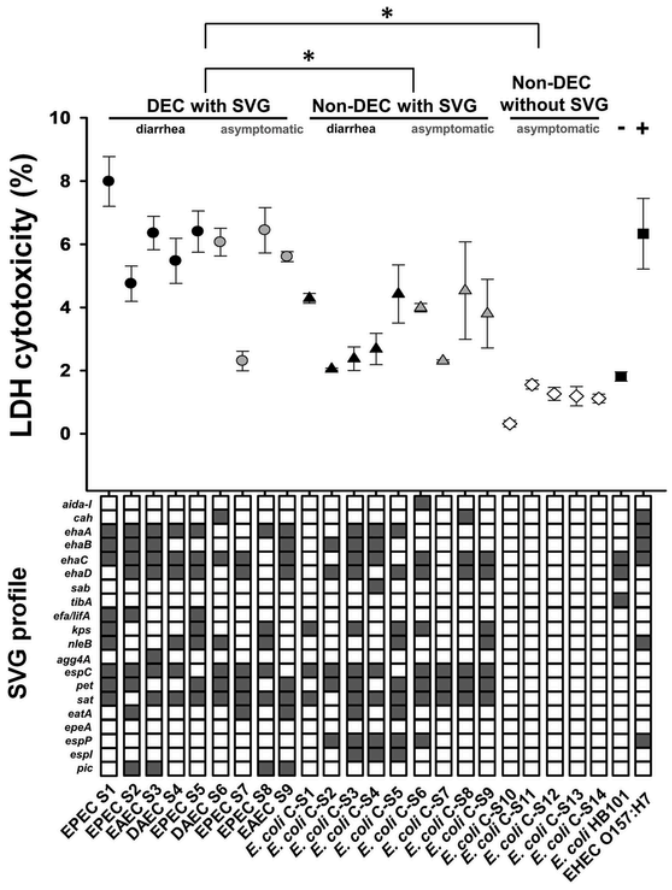


**Fig. 2. Relation between cytotoxicity and score virulence of groups of *E. coli* isolated from children with diarrhea and asymptomatic.** The cytotoxicity of *E. coli* strains was evaluated. From the group of children with diarrhea it was analyzed 5 DEC with SVG (gray circles) and 5 non-DEC with SVG (black triangles), while 4 non-DEC with SVG (gray triangles) and 5 non-DEC without SVG (white diamonds) both belonged from asymptomatic children. *E. coli* HB101 and EHEC O157:H7 were used as controls. The SVG genes in strains (X axis) are represented in cadres as presence (Gray) or absence (white). *E. coli* strains (1 × 107 CFU/ml) were inoculated with HT-29 cells during 12 h at 37 °C at 5% $CO_2$. Then cytotoxic activities of *E. coli* strains were quantified using Pierce LDH Cytotoxicity assay kit, in the end the supernatants were quantified at 490 nm. The results were represented as LDH percentage. *: $p$: $^{<}$0.05.

dren with diarrhea compared to non-DEC strains from the asymptomatic control group (Fig. 2).

### 3.6. Antimicrobial resistance of diarrheagenic E. coli strains isolated from children with diarrhea and asymptomatic

The antibiotic resistance information of DEC strains is shown in Table 5. Overall >50% of DEC strains whether isolated from diarrhea cases or the asymptomatic control group were resistant to nalidixic acid, tetracycline, trimethoprim sulfamethoxazole, ampicillin, and cefotaxime. DEC strains isolated from children with diarrhea were significantly more resistant to tetracycline and ampicillin ($p$: $^{<}$0.05). Regarding each DEC category, strains isolated from children with diarrhea presented higher resistant to most of those antibiotics mentioned in comparison with strains isolated from asymptomatic children, excepting to nalidixic acid and cefotaxime (Table 5). On the other hand, DEC strains presented low resistant to gentamicin (22%), ciprofloxacin (8.5%), ceftazidime (13.5%) and chloramphenicol (15.2%).

The majority of DEC strains were resistant to at least one antibiotic (91.5%). The proportion of DEC strains from the diarrhea group and bearing resistance to at least one antibiotic was higher but not statistically significant than those strains isolated from asymptomatic children (96.4% vs 87.1%). Similarly, the proportion of DEC strains with a MDR phenotype was higher, but not significant in those isolated from diarrhea cases than those strains from asymptomatic children (54.8% vs 39.3%, respectively). Strains with a XDR phenotype had a similar prevalence in both groups (10.7% vs 6.5%). It is worth mentioning that more that 40% of DEC regardless of the pathotype were MDR (Table 5).

When the number antibiotics that the strains were resistant to was analyzed, we observed that 10.2% of DEC strains were resistant to one antibiotic, 18.6% bear resistance to two antibiotics, 16.9% were resistant to three different antibiotics, 18% to four, but 27.12% DEC strains were resistant to five or more antibiotics. In proportion, ETEC (data not presented) and EPEC strains were the pathotypes which showed resistance to more antibiotics (Table 5).

## 4. Discussion

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children worldwide but particularly in children from developing countries. *E. coli* strains involved in diarrheal diseases are one of the most important of the various etiological agents of diarrhea in young children [51]. It has been estimated that 30–40% of diarrhea cases in children are caused by DEC strains [52]. DEC strains, however, have been isolated from healthy children and also non-DEC strains have the ability to produce diarrhea. In this study we demonstrated that DEC, and non-DEC, carrying SVG were significantly associated to diarrhea cases, suggesting than in children from developing countries such as Mexico, DEC strains have adapted to cause disease by acquiring a number of other virulence genes. It was evident that strains no carrying SVG or carrying 1–2 genes were associated to strains isolated from healthy children whereas those strains isolated from diarrhea cases carry five or more SVG. Sur-

**Table 5**
Antibiotic resistance among diarrheagenic *Escherichia coli* strains isolated from children.

| Class and antimicrobial | Total DEC | | | *E. coli* pathotype n (%) | | |
|---|---|---|---|---|---|---|
| | | | | EPEC 20 (33.9) | | |
| | D | A | Total | D | A | Total |
| | 31 | 28 | 59 | 6 | 14 | 20 |
| **Aminoglycosides** | | | | | | |
| *Gentamicin* | 6 (19.3) | 7 (25.0) | 13 (22.0) | 2 (33.3) | 5 (35.7) | 7 (35.0) |
| **Quinolones and fluoroquinolones** | | | | | | |
| *Ciprofloxacin* | 3 (9.6) | 2 (7.1) | 5 (8.5) | 1 (16.7) | 2 (14.3) | 3 (15.0) |
| *Nalidixic acid* | 13 (41.9) | 19 (67.8) | 32 (54.2) | 2 (33.3) | 12 (85.7) | 14 (70.0) |
| **Sulfonamides and potentiated sulfonamides** | | | | | | |
| *Trimethoprim-Sulfamethoxazole* | 18 (58.8) | 13 (46.4) | 31 (52.5) | 5 (83.3) | 4 (28.6) | 9 (45.0) |
| **Tetracyclines** | | | | | | |
| *Tetracycline* | **24 (77.4)*** | 11 (39.2) | 35 (59.3) | **6 (100)*** | 4 (28.6) | 10 (50.0) |
| **Beta lactams** | | | | | | |
| *Ampicillin* | **21 (67.7)*** | 9 (32.1) | 30 (50.8) | **6 (100)*** | 3 (21.4) | 9 (45.0) |
| **Cephalosporins** | | | | | | |
| *Ceftazidime* | 2 (6.4) | 6 (21.4) | 8 (13.5) | 2 (33.3) | 4 (28.6) | 6 (30.0) |
| *Cefotaxime* | 11 (35.4) | **19 (67.8)*** | 30 (50.8) | 4 (66.7) | 9 (64.3) | 13 (65.0) |
| **Phenolics** | | | | | | |
| *Chloramphenicol* | 6 (19.3) | 3 (10.7) | 9 (15.2) | 1 (16.7) | 1 (7.1) | 2 (10.0) |
| **Category** | | | | | | |
| Susceptible | 4 (12.9) | 1 (3.6) | 5 (8.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Resistant to any antibiotic | 27 (87.1) | 27 (96.4) | 54 (91.5) | 6 (100) | 14 (100) | 20 (100) |
| MDR | 17 (54.8) | 11 (39.3) | 28 (47.5) | 5 (83.3) | 4 (28.6) | 9 (45.0) |
| XDR | 2 (6.5) | 3 (10.7) | 5 (8.5) | 0 (0.0) | 1 (7.1) | 1 (5.0) |
| **Number of antibiotic resistant to** | | | | | | |
| 0 | 4 (12.9) | 1 (3.6) | 5 (8.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| 1 | 2 (6.5) | 4 (14.3) | 6 (10.2) | 0 (0.0) | 1 (7.1) | 1 (5.0) |
| 2 | 5 (16.1) | 6 (21.4) | 11 (18.6) | 0 (0.0) | 6 (42.9) | 6 (30.0) |
| 3 | 3 (9.7) | 7 (25.0) | 10 (16.9) | 1 (16.7) | 3 (21.4) | 4 (20.0) |
| 4 | 7 (22.6) | 4 (14.3) | 11 (18.6) | 2 (33.3) | 2 (14.3) | 4 (20.0) |
| 5 | 7 (22.6) | 2 (7.1) | 9 (15.3) | 2 (33.3) | 0 (0.0) | 6 (30.0) |
| 6 | 2 (6.5) | 3 (10.7) | 5 (8.5) | 0 (0.0) | 1 (7.1) | 1 (5.0) |
| 7 | 1 (3.2) | 0 (0.0) | 1 (1.7) | 1 (16.7) | 0 (0.0) | 1 (5.0) |
| 8 | 0 (0.0) | 1 (3.6) | 1 (1.7) | 0 (0.0) | 1 (7.1) | 1 (5.0) |

D = Diarrhea; A = Asymptomatic; ant = antibiotic. *p < 0.05 with respect to DEC strains of asymptomatic children; MDR = Multidrug-resistant, resistant ≥3 different categories of antibiotics; XDR = Extremely drug. Resistant ≥6 different categories of antibiotics. P values were calculated with Fisher's Exact test. The remaining pathotypes (i.e., strains) did not present significant differences.

prisingly, only those SVG related colonization and proteolysis were statistically associated with diarrhea cases, but not those with a proven *in vitro* cytotoxic activity such as *pet*, the gene encoding for the plasmid encoded toxin (pet) or *sat*, encoding for autotransporter toxins.

The detection of a significantly higher prevalence of DEC strains in children with diarrhea compared to asymptomatic children confirms a role of DEC strains as the etiologic agent of diarrhea in children studied in this work. Bueris V. et al. (2007), also found higher proportion of DEC in children with diarrhea in comparison with asymptomatic [53]. On the other hand the prevalence of DEC found in this work was similar than that found by Patzi-Vargas S et al. (2015) in southeast Mexico (28%) for strains isolated from children 6 months old to one year of age. In developed countries such as the US, investigators have demonstrated a lower prevalence of DEC strains than the prevalence observed in developing countries. For example, 5.5% of strains isolated in the US from children with diarrhea were DEC and the most prevalent pathotypes were EAEC and EPEC [54]. This is in contrast with the almost 30% diarrhea cases caused by DEC strains demonstrated in the current study and elsewhere [17].

Whereas the overall prevalence of DEC was significantly associated to diarrhea, the most prevalent categories EPEC, and EAEC were similarly isolated from cases and controls. Moreover, aEPEC strains were significantly more prevalent in the asymptomatic control group. Ochoa, T.J et al. (2011), also found a high prevalence of EPEC in asymptomatic children [55]. We hypothesize that the high isolation of aEPEC strains demonstrated in the current study, from asymptomatic children, is related to the adaptive immune response because these strains have been circulating in this particular population for years. Therefore, for DEC strains to cause disease they now have to acquire new, accessory, and virulence factors to overcome this adaptation. Results within this study indicating that DEC strains carrying more than five accessory virulence-related genes were significantly more prevalent in diarrhea cases, supports this hypothesis. Furthermore, ETEC strains were only isolated from children with diarrhea and >60% carried SVG related to colonization (data not shown) and, surprisingly, the majority of ETEC strains have acquired an additional enterotoxin *espC* gene. ETEC strains are responsible for 13% of diarrhea cases in Mexican children and it is estimate that this pathotype causes 325,000 deaths in children less 5 years [56]. Additionally, the density of DEC strains in the intestine rather than its presence or absence can also be a factor to trigger diarrhea [57,58].

We found that 98.2% of *E. coli* strains isolated from diarrhea had SVG related to colonization. It is known that the proteins encoded by genes such as *eahA* and *ehaB* are important for colonization *in vitro* and biofilm formation [19]. While the *ehaA* and *ehaB* genes encode proteins commonly found in highly virulent *E. coli* O157:H7 strains, the current study detected both genes in all pathotypes at a similar proportion (data no shown) [19]. *efa1/lifA* and *nleB* are other SVG of colonization which were related to diarrhea (Table 3). The proteins encode by these two genes participate in the adhesion process, and pathogenicity of *E. coli*; for example a deletion of *efa1/lifA* affected the ability of *E. coli* to cause diarrhea. These genes have been detected mainly in aEPEC strains and in the current study *efa1/lifA* was found in EPEC (10%) and EAEC (20%) and *nleB* in almost all pathotypes (50–60%) except EIEC [41,59–61]. The last SVG of colonization associated with diarrhea were *kps* and *agg4A* (Table 3). The proteins encoded by these genes have different function. For example, *kps* participate in capsular structure of *E. coli,* while *agg4A* is part of the AAF/IV fimbrial subunit. The genes are amplified more frequently from strains isolated from diarrhea cases caused by EAEC [40,62,63]. In compassion with our results, the gene *kps* was amplified in all pathotypes (40–100%), while *Agg4A* only in EAEC (15%) and ETEC (20%) strains (data not shown).

Surprisingly, none of those SVG related to cytotoxicity (*espC, pet* and *sat*) were associated with *E. coli* strains obtained from diarrhea cases; these genes were detected in a similar proportion among strains

isolated from children with diarrhea and asymptomatic children (Table 3). However, carrying *espC* was significantly higher in DEC strains isolated from diarrhea than from the asymptomatic control group, which appears to associate production of this autotransporter toxin with diarrhea in children. More intriguingly, *espC* was more prevalent (>80%) in DEC pathotypes (i.e., EAEC, DAEC, ETEC, EIEC) other than EPEC strains (66.7%). The enterotoxin gene *espC* was first identified in EPEC strains and the EspC toxin has been purified and characterized from EPEC strains [64]. In developed countries such as Japan, Narimatsu H et al. (2010), did not find an association between DEC strains carrying *espC* from strains isolated from diarrhea cases compared to those isolated from healthy individuals. These authors also indicated that *espC* was more frequent in EPEC strains, while we identified the enterotoxin *espC* gene in all pathotypes (55–100%) (data no shown) [41]. Although premature, these differences observed in DEC strains isolated from developed nations versus less developed countries might suggest that DEC strains colonizing children from developing countries have acquired additional accessory genes to cause diarrhea. The role that *sat* may have in diarrhea requires of more studies. In a study conducted in western China, ~50% of DAEC and EAEC strains isolated from diarrhea cases carried *sat*. However in the current study the prevalence of *sat* in non-DEC strains was similar whether the strains was isolated from diarrhea or from healthy children. The prevalence of *sat* in DEC strains was reversed with the highest prevalence of *sat* observed in DEC strains isolated from healthy children (data no shown) [65].

All SVG related to proteolysis were significantly more prevalent in *E. coli* strains of the diarrhea group suggesting these SVG could had an important participation in diarrhea development. To the best of our knowledge, other studies investigating the prevalence of SVG had investigated these genes in DEC strains. The presence of SVG related to proteolysis in *E. coli* that cause diarrhea was reported by Andrade et al. (2017), in a study that they identified *pic*, *espI*, *espP* and *eatA* in DEC isolated from diarrhea [66]. Patel et al. (2004), demonstrated that carrying the protease *eatA* gene was associated to diarrhea caused by ETEC strains [67]. Similarly, *pic* had been associated with acute diarrhea, and chronic diarrhea, caused by EAEC strains in Peruvian children [68]. How non-DEC and DEC strains acquire accessory genes and what specific set of genes would allow bacteria to cause diarrhea remain to be investigated. It is thought that the exchange of genes occurs while colonizing the intestine, contaminating food, water, or inside of biofilms [69–71].

Another insightful evidence that in these Mexican children diarrhea was associated to carrying SVG was the calculation of the virulence score. Using this score, those DEC strains carrying SVG and non-DEC *E. coli* carrying SVG obtained the higher virulence score when strains were isolated from diarrhea cases compared to those strains from the control group (Fig. 1). Lefort A et al. (2011), determined the virulence score in *E. coli* using genes encoding 18 virulence factors (adhesins, toxins, iron capture, and protectins, genomic islands also called pathogenicity-associated islands, and resistance to antibiotics). Authors demonstrated that *E. coli* strains isolated from the urinary tract had higher score than those isolated from intestine (10 vs 5) [45]. Another work that estimated the virulence score of *E. coli* found that strains with a strong capacity to form biofilms yielded the higher virulence score [72]. Regarding to cytotoxicity activity of *E. coli*, the addition of SVG to some strains as EPEC helped to this bacterium to present higher cytotoxic activity in intestine cells than EHEC, the *E. coli* pathotype cataloged as the most dangerous to human by cause diarrhea, hemorrhagic colitis and the hemolytic uremic syndrome [73].

As expected given our experience typing *E. coli* strains from diarrhea cases or contaminating food [35], the strains isolated in the current study were resistant to multiple antibiotics. As observed in other similar studies [65,67], >50% of strains DEC strains isolated in the current work from both groups presented resistance to tetracycline, ampicillin, nalidixic acid, trimethoprim-sulfamethoxazole and cefotaxime. Resistance to tetracycline and ampicillin was significantly different in DEC strain isolated from children with diarrhea. The high prevalence of antibiotic-resistant DEC strains in the study children is undoubtedly the indiscriminate use of antibiotics that for years have been going on in Mexico [74–76].

## 5. Conclusions

This study found an overall highest prevalence of DEC strain in children with diarrhea than in children of the control (healthy) group. A surprisingly significant more prevalence of non-DEC *E. coli* strains carrying SVG in diarrhea cases was observed suggesting carrying SVG is sufficient for commensal *E coli* strains to cause diarrhea in this population of Mexican children. While non-DEC *E. coli* strains were more prevalent in diarrhea cases if they carried five or more SVG, DEC strains were associated to diarrhea if strains carried *kps*, *nleB*, and/or *espC*. Moreover, carrying appears to be sufficient for any DEC pathotype identified in this study to be associated to diarrhea in children.

## Author contribution

Uriel A. Angulo-Zamudio: Data curation, Writing - original draft preparation, conceptualization; Javier Gutiérrez-Jiménez and Luis Monroy-Higuera: Visualization, Investigation, Methodology; Hector Flores-Villaseñor and Jorge Velazquez-Roman: Formal analysis; Jorge E. Vidal: Writing-reviewing & editing; Nidia Leon-Sicairos: Supervision and Reviewing; Gabriela Tapia-Pastrana: Software, Validation and Reviewing; Adrian Canizalez-Roman: Writing - original draft preparation, Conceptualization, Project administration, Funding acquisition.

## Author statement

We declare that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

### *Ethics approval*

The study was approved by the Ethics Committee of The Women's Hospital, Secretariat of Health No. 202006-04.

## Consent to participate and for publication

All participants were informed about the project and gave verbal and writing consent (parents) to participate.

## Data availability

Manuscript has data included as electronic supplementary material (Supplemental Table S1).

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## Declaration of competing interest

None of the authors have any proprietary interests or conflicts of interest related to this submission.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2021.104994.

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