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Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern Mexico

Adrian Canizalez-Roman [a,b,*], Edgar Gonzalez-Nuñez [a], Jorge E. Vidal [c], Héctor Flores-Villaseñor [a], Nidia León-Sicairos [a,d]

[a] School of Medicine, Autonomous University of Sinaloa, 80246 Culiacan Sinaloa, Mexico
[b] The Sinaloa State Public Health Laboratory, Secretariat of Health, 80020 Culiacan Sinaloa, Mexico
[c] Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA
[d] Pediatric Hospital of Sinaloa, 80200 Culiacan Sinaloa, Mexico

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ABSTRACT

Diarrheogenic *Escherichia coli* (DEC) strains are an important cause of intestinal syndromes in the developing world mainly affecting children. DEC strains often infect tourists from developed countries traveling to Mexico, causing so-called "traveler diarrhea". DEC strains are typically transmitted by contaminated food and water; however, the prevalence of these strains in food items that are produced, consumed and sometimes exported in northwestern Mexico has not been evaluated. In this study, we conducted a large microbiological survey of DEC strains in 5162 food items and beverages consumed throughout Sinaloa state during 2008 and 2009. We developed a panel of eight sequential PCR reactions that detected the presence of all DEC categories, including typical or atypical variants. Thermotolerant coliforms (also known as fecal coliforms) and *E. coli* were detected by conventional bacteriology in 13.4% (692/5162) and 7.92% (409/5162) of food items, respectively. Among 409 *E. coli* isolates, 13.6% (56/409) belonged to DEC strains. Dairy products (2.8%) were the most contaminated with DEC, while DEC strains were not detected in beverages and ice samples. The pathogenic type that was most commonly isolated was EPEC (78.5%), followed by EAEC (10.7%), STEC (8.9%) and ETEC (1.7%). EHEC, DAEC and EIEC strains were not detected. Approximately 80% of EPEC and EAEC strains were classified as atypical variants; they did not adhere to a culture of HEp-2 cell. Of the isolated DEC strains, 66% showed resistance to at least one commonly prescribed antibiotic. In conclusion, the presence of DEC strains in food items and beverages available in northwestern Mexico is low and may not represent a threat for the general population or those traveling to tourist areas.

1. Introduction

Diarrheogenic Escherichia coli (DEC) strains can cause mild to severe diarrhea and other symptoms in humans. These strains constitute a heterogeneous group of organisms with different virulence properties, epidemiology, and disease associations (Kaper et al., 2004). DEC strains are among the most common etiologic agents of diarrhea. Based on their specific virulence factors and phenotypic traits, DEC strains are divided into six pathogenic types: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC) and Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC), which includes the well-known subgroup enterohaemorrhagic *E. coli* (EHEC) (Kaper et al., 2004).

The identification of DEC strains involves the detection of encoded genes and phenotypic characteristics (e.g. production of toxins and adhesion patterns on epithelial cells). Thus, typical EPEC strains contain *eae* and *bfp*A, which are genes encoding the proteins intimin and bundle-forming pilus (BFP) that are implicated in the localized adherence phenotype (LA) on cell cultures. EAEC is commonly associated with traveler's diarrhea in developing countries. EAEC strains are distinguished by their typical adherence to cultured cells, i.e., an aggregative adherence pattern due to a variety of virulence factors (fimbriae gene, *aaf*; promoter of colonization, dispersin; and plasmid, *pCVD432*) regulated by the aggregative factor (AggR). ETEC strains are defined by the presence of one or two plasmid-encoded enterotoxins, the thermostable toxin (encoded by *st*) and the thermolabile toxin (encoded by *lt*). ETEC strains are the most common cause of childhood diarrhea among all *E. coli* pathotypes and the most frequent cause of diarrhea in travelers to developing countries. DAEC is defined by a diffuse pattern of adherence in HEp-2 cell culture assays. A fimbrial adhesin known as F1845 (encoded by *daaE*) mediates the adherence of DAEC to epithelial cells. EIEC shows pathogenic,

* Corresponding author at: Autonomous University of Sinaloa, School of Medicine, 80246 Culiacan Sinaloa, Mexico. Tel.: +52 6677538802; fax: +52 6677538801.
E-mail address: canizalez@uas.edu.mx (A. Canizalez-Roman).

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phenotypic and genetic similarities with *Shigella* strains. EIEC can be identified among other criteria, by their epithelial cell invasiveness (mediated in part by the *ipaH* and *virF* genes) and association with dysentery. EHEC is associated with bloody diarrhea and hemolytic uremic syndrome. EHEC contains the locus of the enterocyte effacement pathogenicity island, which is also present in EPEC, and expresses one or two shiga-like toxin-encoding genes (*stx1* and *stx2*). The most common serotype associated with outbreaks in the United States and Europe is the serotype O157:H7 (Kaper et al., 2004).

DEC strains include several emerging pathogens of worldwide public health importance, since they have been associated with outbreaks and travelers' diarrhea around the world in recent years (Bradley et al., 2012; Dallman et al., 2012; Frank et al., 2011; Guiral et al., 2011), including in Mexico (Cortes-Ortiz et al., 2002; Ouyang-Latimer et al., 2010). This explosive epidemic behavior is in part due to an increase in international travel and trade globalization (WHO). Thus, individuals traveling to countries where some DEC-associated diseases are prevalent have an increased risk of not only acquiring these diseases but also transmitting these DEC strains upon their return to their home country. Furthermore, in recent years, there has been a discussion about the potential for strains in developing countries that are resistant to antibiotics to be spread to more industrialized ones (Guiral et al., 2011). This highlights the need for microbiological surveillance of food and beverages, such as the one conducted in this study, of antibiotic resistant strains that can be potentially circulating in places with uncontrolled prescription of antibiotics and visited by tourists and entrepreneurs from developed countries.

Today, food safety is an increasingly important public health issue. Governments all over the world are intensifying their efforts to improve food safety mainly because there has been an increase in food-related diseases and outbreaks, which have raised consumer concerns and public health awareness (Langiano et al., 2012; Taylor et al., 2012). Finding the source of contamination has also become imperative, as this information would help to stop further spread of disease outbreaks. For example, several outbreaks of deadly *E. coli* O157:H7 infections have occurred recently, and the investigated source has been identified as contaminated food products as diverse as vegetables, cookie dough or raw milk (CDC, 2006; Frank et al., 2011; Gaulin et al., 2012; McCollum et al., 2012; Neil et al., 2012; Xiong et al., 2012).

Outbreaks caused by DEC strains have been closely linked to consumption of contaminated food and water. In developed countries, outbreaks caused by EHEC have been associated with hamburgers (O'Brien et al., 1993) sausages, unpasteurized milk, lettuce (Ackers et al., 1998; Hilborn et al., 1999), radish sprouts and spinach (CDC, 2006; Michino et al., 1999). EPEC, EIEC and ETEC have been isolated mainly from sources of water and other foods (Bengtsson et al., 1966; Black, 1990; Lanyi et al., 1959; Nakajima et al., 2005; Valentini et al., 1992), whereas sources of infection of DAEC strains are still unknown.

Active surveillance of drinking water and food sources is imperative as new DEC strains have emerged in recent years. An outbreak reported in May 2011 in Germany was due to a new serotype of EHEC, O104:H4. This new strain caused more than 3816 disease cases, with 845 cases of severe hemolytic uremic syndrome (HUS) leading to 54 deaths within three months (Frank et al., 2011). After a thorough investigation of possible sources of infection, it was determined that this strain originated in contaminated sprouts. The outbreak extended to 15 countries, including Denmark, France, Greece, the United Kingdom, Netherlands, Norway, Austria, Spain, the Czech Republic, Luxembourg, Poland, Sweden, Switzerland, Canada, and the United States (WHO).

In Mexico, only few studies have examined the presence of DEC strains in specific food items and persons. For example ETEC, EPEC, EAEC, STEC and EIEC have been identified in some food items (salads, taco dressings, chili sauces, chili peppers, acid-fermented foods and desserts) (Adachi et al., 2002; Castro-Rosas et al., 2012; Cerna-Cortes et al., 2012; Estrada-Garcia et al., 2002; Lopez-Saucedo et al., 2010; Sainz et al., 2001; Vigil et al., 2009) and particularly in children (Cortes-Ortiz et al., 2002; Cravioto et al., 1985; Estrada-Garcia et al., 2005a; Lopez-Saucedo et al., 2010) and US visitors (Paredes-Paredes et al., 2011) who developed diarrhea. During the last few years, Sinaloa, a state located in northwestern Mexico, has had an increase in cases of gastrointestinal diseases where the etiologic agent has not been identified. This may be mainly due to a lack of epidemiological data about reemerging pathogens (Velazquez-Roman et al., 2012), such as DEC strains. Furthermore, active surveillance of emerging and reemerging pathogens is necessary, as Mazatlan and Culiacan, both located in Sinaloa, are world-class tourist destinations. Sinaloa is also a source of food exports to the US and other countries.

Therefore, the aim of this study was to investigate the presence and prevalence of DEC strains in beverages and food items (N = 5,162) meant for human consumption in the state of Sinaloa, for example, dairy and meat products, seafood and fish, beverages and prepared foods. These strains were identified phenotypically by using a newly developed panel of PCR reactions. The adhesion potential of these strains to human epithelial HEp-2 cells as well as their antibiotic resistance profile was investigated. Although the prevalence of these strains was low in the studied items, antibiotic resistance of the isolated strains was high. To our knowledge, this is the first report of the prevalence of DEC strains isolated from diverse food items and beverages in Mexico.

2. Materials and Methods

2.1. Bacterial Strains

DEC reference strains utilized in this study belong to our laboratory collection and included EPEC E2348/69 (*eae*+ and *bfpA*+) (Flores-Villasenor et al., 2012a), ETEC (*lt*+ and *st*−), EIEC (*ipaH*+ and *virF*−), EHEC O157:H7 EDL933 (*eae*+, *hlyA*+, *stx*1+ and *stx*2+) (Flores-Villasenor et al., 2012b), DAEC (*daaE*+), EAEC O42 (*aggR*+, *aap*+, *pCVD*432+ and *aafII*+) and *E. coli* DH5α (Invitrogen). 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. Sample Collection

Food samples were collected from 10 different municipalities in the Sinaloa State located in northwestern Mexico between January 2008 and December 2009. A total of 5162 samples were collected from food service establishment and retail food samples. The sampling sites included, but were not limited to bakeries, bars, bed-and-breakfast operations, coffee shops, convenience stores, fairs, food banks, grocery stores, meal services for home-bound persons, mobile food carts, restaurants, vending machine operators and street food vendors. The food items that were analyzed consisted of prepared foods (n = 1594), including broths and soups, beef, pork, goat, lamb meat, poultry, fruits, salads, hamburgers, sandwiches, eggs, sausages, ham, rice, sauces, beans, chilaquiles, desserts, mayonnaise, hard pork sausages and tortillas. Beverages and ice (n = 1597) samples included, drinking water fountains, tap water, tea, fruit-flavored water and ice. Dairy products (n = 669) consisted of cream, buttermilk, milk, butter, cheese and yogurt. Fish and seafood (n = 656) samples included raw products, seasoned shrimp, clams, squid, mixed seafood, shrimp, stingrays, oysters, octopus and fish. Meat and meat products (n = 646) included mammalian meat and poultry, sausages, ham and hard pork sausage (Table 2). Approximately 15 g or 15 ml of a sample was placed in a sterile plastic bag and laid directly into a

wide-mouth thermos containing wet ice. Samples collected during the day were processed within 4 h, whereas those collected during the evening were refrigerated at 4 °C until processing the next morning.

2.3. Bacteriological Analyses

Samples were first processed for two indicator bacteria: fecal coliform and *E. coli*, as described in the Mexican Official Standard and Bacteriological Analytical Manual of the Food and Drug Administration Chapter 4 (BAM, 2002). Positive tubes from the EC broth assay were streaked on MacConkey agar, and presumptive *E. coli* were transferred to eosin-methylene blue (EMB) agar and violet red bile agar. The colonies were further phenotypically (biochemical, API 20E; bioMerieux) and genotypically identified (PCR, described below).

2.4. Preparation of Template DNA

Five colonies of *E. coli* per sample were grown in 3 mL of LB broth for 18 h to reach stationary phase. The cells were pelleted by centrifugation at 10,000 ×*g* for 10 min and then resuspended in 0.3 ml of distilled water. Pellets were heated at 100 °C for 10 min, vortexed for 10 s, and centrifuged again at 12,000 ×g for 3 min. DNA-containing supernatants were transferred to 0.5 mL microfuge tubes and stored at −20 °C until use.

2.5. Sequential Multiplex PCR, Protocol to Identify DEC Strains

PCR reactions to confirm *E. coli* (Supplementary data, Fig. S1) at the species level were carried out according to the protocol described by Tsen et al. (1998). To further identify DEC strains within our *E. coli* isolates, a protocol of sequential multiplex, duplex and single PCR reactions was designed and validated using reference strains. These reactions amplified a set of genes encoded by each individual DEC category. Our protocol of sequential reactions was based on previous reports from Mexico and other Latino American countries (Araujo et al., 2007; Cortes-Ortiz et al., 2002; Cravioto et al., 1985; Estrada-Garcia et al., 2005b; Lopez-Saucedo et al., 2010). These sequential multiplex PCR reactions identified the most prevalent DEC groups in decreasing order. The first reaction identified EPEC strains. Negative samples were advanced to reaction #2 to identify EAEC strains, and so on (Table 1 and Fig. 1).

Multiplex PCR reaction #1 contained primers to amplify the intimin gene (*eae*) and the structural subunit of the bundle-forming pilus (Bfp) gene (*bfp*A). Reaction #1 detected both typical (eae^+ and $bfpA^+$) and atypical (eae^+ and $bfpA^-$) EPEC strains. Multiplex PCR reaction #2 contained primers that detected a gene encoding a regulator protein (*aggR*) and a fimbriae gene (*aafII*), and multiplex PCR reaction #3 contained primers to amplify the gene encoding the protein dispersin (*aap*) and the aggregative adherence pattern associated plasmid (*pCVD432*) to identify typical ($aggR^+$ and $pCVD432^+$ and/or $aafII^+$ aap^+) and atypical ($aggR^-$ and $pCVD432^+$ and/or $aafII^+$ aap^+) EAEC strains. Duplex PCR reaction #4 amplified both the gene encoding the heat labile toxin (*Lt*) and that encoding the heat stable toxin (*stII*) present in ETEC strains. PCR reaction #5 contained primers that amplified the structural subunit gene of the F1845 fimbria (*daaE*) to detect DAEC strains. 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 eae^+ 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 both Shiga toxin genes and the *eae* gene, whereas STEC strains were eae^-. Finally, multiplex PCR reaction #8 contained primers that amplified the hemolysin gene (*hlyA*), the *rfbE* gene coding for the production of the lipopolysaccharide O of *E. coli* O157 and the *fliC* gene that encodes the *E. coli* flagellum H7 serotype. Reaction #8 detected EHEC O157:H7 strains (Fig. 1). Primer sequences, PCR conditions and product sizes are shown in Table 1 and the supporting material (Supplementary data, Fig. S2).

Table 1
Oligonucleotide primers for the detection of diarrheagenic *E. coli* strains by PCR.

Primer mix	Primer sequence (5′–3′)	Gene target	PCR product (bp)	Condition	DEC	Reference
M1	TTATTTTAAATTGGGTTCGGAT AATTTAATGCCTTGTCATCGG	*eae*	365	95 °C 45 s, 54 °C 45 s,72 °C 45 s 30 Cycles	EPEC	This study
	TTTACTACCAGTCTGCGTCT ATGCCGCTTTATCCAACCTG	*bfp*	282			
M2	CACAGGCAACTGAAATAAGTCTGG ATTCCCATGATGTCAAGCACTTC	*aafII*	378	94 °C 30s, 56 °C 30s,72 °C 30s 30 Cycles	EAEC	Maricel Vidal, 2005
	GTATACAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	*aggR*	254			Claudia Toma, 2003
M3	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	*Pcvd432*	630	94 °C 50s, 54 °C 50s,72 °C 50s 30 Cycles	EAEC	Jamal Mo., 2006
	ATGAAAAAAATTAAGTTTGTTATCTT TTATTTAACCCATTCGGTTAGAGC	*aap*	351			
M4	GCACACGGAGCTCCTCAGTC TCCTTCCTTTCAATGGCTTT	*lt*	218	94 °C 30s, 60 °C 30s,72 °C 30s 30 Cycles	ETEC	Maricel Vidal, 2005
	AAAGGAGAGCTTCGTCACATTTT AATGTCCGTCTTGCGTTAGGAC	*stII*	129			
M5	GAACGTTGGTTAATGTGGGGTAA TATTCACCGGTCGGTTATCAGT	*daaE*	542	94 °C 1 min, 60 °C 1 min,72 °C 1 min 30 Cycles	DAEC	Maricel Vidal, 2005
M6	AGCTCAGGCAATGAAACTTTGAC TGGGCTTGATATTCCGATAAGTC	*virF*	618	94 °C 1.5 min, 60 °C 1.5 min,72 °C 1.5 min 35 Cycles	EIEC	Maricel Vidal, 2005
	CTCGGCACGTTTTAATAGTCTGG GTGGAGAGCTGAAGTTTCTCTGC	*ipaH*	933			
M7	CAAAGACGTATGTAGATTCGC TTCGTTCAACAATAAGCCGTA	*stx1*	192	95 °C 45 s, 54 °C 45 s,72 °C 45 s 30 Cycles	EHEC or STEC	This study
	TATTATTTAAATGGGTACTGTGC CATAACTTTGTTGGGTCGAAA	*stx2*	*96*			
M8	AGCTGCAAGTGCGGGTCTG TACGGGTTATGCCTGCAAGTTCAC	*hlyA*	569	95 °C 40s, 60 °C 40s,72 °C 40s 30 Cycles	EHEC	Gehua Wang, 2002
	CTACAGGTGAAGGTGGAATGG ATTCCTCTCTTTCCTCTGCGG	*rfbEO157*	327			
	TACCATCGCAAAAGCAACTCC GTCGGCAACGTTAGTGATACC	*fliCH7*	247			

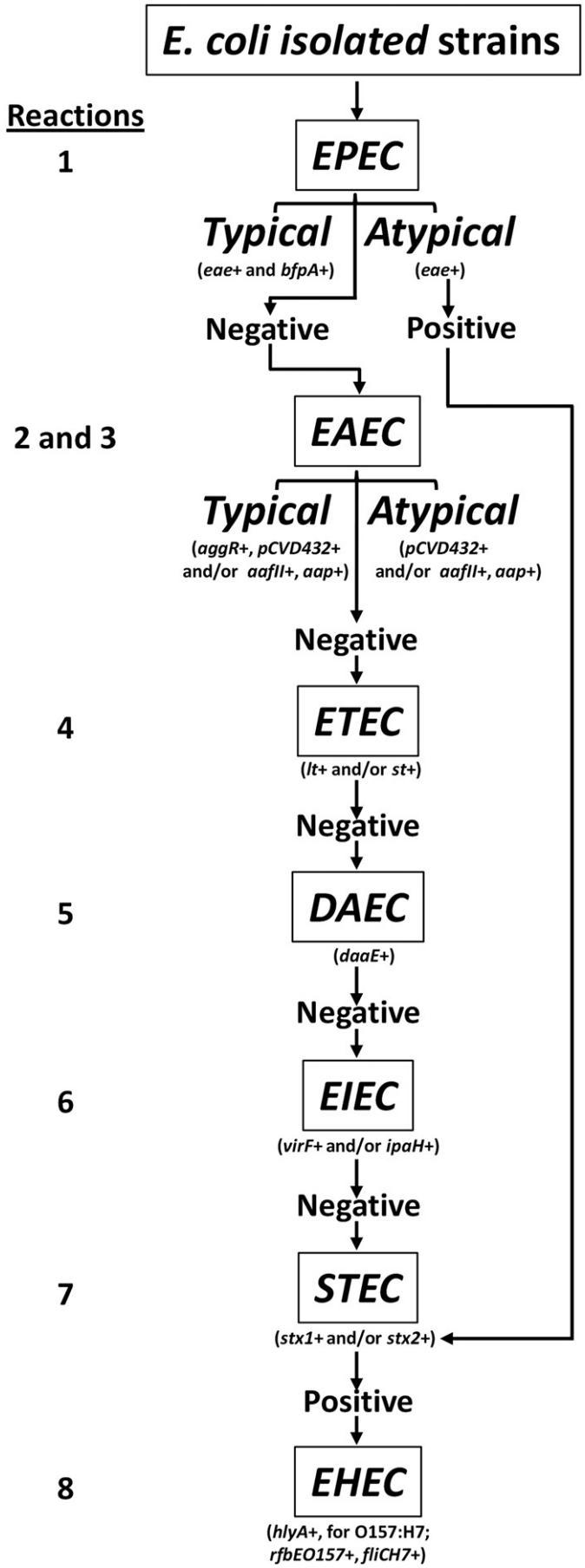

Fig. 1. Flowchart of the protocol used in this study to detect diarrheagenic *E. coli* strains by PCR.

PCR amplification was performed in a 25 μL volume consisting of 1x GoTaq Green Master Mix (Promega), forward and reverse primers 1 μmol and 2 μl of DNA template, with the remaining volume consisting of molecular biology grade water. PCR reactions were carried out in a Thermal Cycler C1000 (BIO-RAD Laboratories, Hercules, California). PCR products were separated by electrophoresis in a 2% agarose gel. Gels were stained with ethidium bromide (0.5 mg/mL) and photographed using a digital imaging system (Kodak, Model E1 logia 100 imaging system).

2.6. Adherence Assay on HEp-2 cells

The adhesion assay on HEp-2 cells was performed essentially as described by Cravioto et al. (1979). Briefly, HEp-2 cells grown in Eagle's minimal essential medium (EMEM, GIBCO-BRL, Gaithers-burg, MD), with 10% fetal calf serum and antibiotics were seeded onto coverslips that had been placed in the wells of 24-well plates (Costar, Cambridge, MA) and incubated until reaching 70–80% confluence (2–3 days). Washed HEp-2 cells were fed with 1 ml of fresh MEM medium without fetal bovine serum or antibiotics, but with 0.5% (wt/vol) of D-mannose before being inoculated.

One day before the experiment, *E. coli* strains were grown overnight in 5 ml of peptone water with 3% D-mannose. Cultures were then centrifuged, washed twice with sterile PBS and resuspended in MEM with no additives. One hundred microliters of the bacterial suspension (~1.5×10^8 CFU) was inoculated onto HEp-2 cell cultures. Infected cells were incubated for 3 h at 37 °C under a 5% CO_2 atmosphere and then washed three times with sterile PBS, fixed with 70% methanol, and stained with Giemsa. Adherence patterns were assigned according to the description of Scaletsky et al. (1984) and Nataro et al. (1987) (Nataro et al., 1987; Scaletsky et al., 1984). The EPEC reference strain (E2349/68) showing localized adherence (LA), EAEC 042 showing aggregative adherence and *E. coli* K12 that does not attach to HEP-2 cells were included as controls in every experiment.

2.7. Serotyping O157:H7

Serotypes of strains positive for at least one EHEC/STEC virulence factor (*stx1*, *stx2 or HlyA*) were investigated using rabbit antisera obtained from the National Institute of Epidemiological Reference (InDRE), Mexico, and prepared against the 157 somatic (O) and the 7 flagellar (H) antigens. The microagglutination test was used to determine the serogroup O157:H7 (Orskov et al., 1984).

2.8. Antimicrobial Agent Susceptibility Testing

The Kirby–Bauer disk diffusion method was used to determine sensitivity or resistance to antimicrobial agents, according to guidelines developed by the Clinical Laboratory Standard Institute (CLSI, 2011). Recommendations of 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 (Supplementary data, Table S2). These antibiotics were chosen because of their importance in treating human (Amabile-Cuevas, 2010) or animal *E. coli* infections and their use as feed additives to promote growth in animals. They represent different classes of antimicrobial agents that are available to treat gram-negative infection in Mexico. The protocol was performed as follows: fresh cultures were inoculated into LB broth and incubated until they reached a turbidity of 0.5 using the McFarland standard. Mueller–Hinton agar plates were swabbed with these cultures, and antibiotic disks (BD BBL, Franklin Lakes, NJ) were placed onto inoculated plates in a sterile environment. The plates were incubated at 37 °C for 18 to 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 (American Type Culture Collection, Manassas, VA) was used as negative control, this strain bear no resistance to the selected antibiotics. *E. coli* strain ATCC 35218 was used as positive control for β-lactam/β-lactamase inhibitor combinations.

3. Results

3.1. Detection of E. coli in Food Items Consumed in Northwestern Mexico

Since water- and food-borne DEC strains, such as EPEC and ETEC, have been isolated from food items produced in different regions of Mexico (Adachi et al., 2002; Castro-Rosas et al., 2012; Cerna-Cortes et al., 2012; Estrada-Garcia et al., 2002; Lopez-Saucedo et al., 2010; Sainz et al., 2001; Vigil et al., 2009) and shiga-toxin-producing EHEC has recently been implicated in an outbreak that infected more than 3000 people and killed ~50 in Europe (Frank et al., 2011), we performed an epidemiological surveillance of the most common food items and sources of water (N = 5162) consumed in northwestern Mexico to evaluate the presence of DEC strains, including EHEC strains.

The geographical area covered by our sampling is home to approximately 87% of Sinaloa's state population. A total of 5162 samples, including dairy products, meat products, seafood and fish, prepared foods, beverages and ice, were analyzed (Table 2). Our first screening for fecal contamination detected coliform bacteria above permissive levels for human consumption in 696 samples (13.4%), whereas *E. coli* was isolated in 409 food items (7.92%) (Table 2). Further molecular analyses by PCR targeting the 16SrRNA gene confirmed the identity of these isolates (Supplementary data, Fig. S1). Our study found that dairy products were the food items that were most frequently contaminated with *E. coli* (21.3%), followed by meat products (17%) (Table 2). The presence of coliform bacteria and *E. coli* in beverage samples and ice was very low, 3.38% and 0.93%, respectively (Table 2).

3.2. Prevalence of DEC Strains in Food Items

To identify the DEC group of our *E. coli* isolates, a protocol of eight sequential (multiplex, duplex, and single) PCR reactions was developed in this study (Fig. 1 and Table 1). These reactions amplified a set of genes encoded by each individual DEC category (see Material and Methods). The optimization of duplex PCR was performed with DNA extracted from reference DEC strains (e.g. EHEC, EPEC, EAEC, EIEC, STEC and DAEC). We confirmed that our PCR identified only the targeted pathogenic group (Supplementary data, Fig. S2). Our multiplex PCR studies revealed that 56 food products (13.69%) contained DEC strains (Table 2). The food items that were most often contaminated with DEC were dairy products, 19 samples [33.92% (19/56)], while beverages and ice samples were not contaminated with DEC (Table 2).

In regard to individual DEC categories, our protocol of multiplex PCR reactions found, as expected, that EPEC was the most common (78.57%) DEC isolated (Table 2). The prevalence of EPEC strains was similar among the different food items, ranging from 68% through to 84% (Table 2). EAEC was isolated in only 10% of DEC-positive food samples, while STEC strains were found in 8.92% of DEC isolates (Table 2). Our PCR studies also revealed the presence of ETEC in a dairy product, representing the 1.78% of all DEC. ETEC strains isolated from Mexico have been associated with traveler's diarrhea, infecting mainly American tourists (Paredes-Paredes et al., 2011). These results indicate that the food items and beverages that are available in Sinaloa may not be a threat for those traveling to tourist areas of Sinaloa, such as Mazatlan. Moreover, no EHEC strains or DAEC strains could be isolated from any of the 5162 food items that were evaluated.

3.3. Presence of Typical and Atypical DEC

An emerging epidemiological trait of DEC isolates is the appearance of atypical variants that have lost virulence genes but have increasingly been implicated in cases of human diarrhea. Thus, our multiplex PCR reactions 1, 2 and 3 included primers that differentiated between typical and atypical variants of EPEC or EAEC. Supplementary data, Table S1 shows that most of the EPEC isolates belonged to atypical strains (n = 35) containing only the gene for the intimin (*eae*) but not for *bfp*A. The remainder of the EPEC strains (n = 9) contained both *eae* and *bfp*A and therefore were considered typical strains (Supplementary data, Table S1). Only two typical EAEC strains (containing the *agg*R gene) were identified, while four atypical EAEC isolates were identified. As reported elsewhere (Weintraub, 2007), all typical or atypical EAEC strains contained the aggregative adherence pattern-associated plasmid (*pCVD*432) (Supplementary data, Table S1).

3.4. Adherence Patterns of DEC Isolated from Food Items

Adhesion properties of EPEC and EAEC to *in vitro*-cultured HEp-2 epithelial cells have been associated with the virulence of these strains (Vidal et al., 2007; Weintraub, 2007). Thus, typical EPEC can attach to cell cultures in a pattern called localized adherence (LA) because the bacteria form a microcolony-like structure on the cell surface (Fig. 2A). As shown in Fig. 2D and Supplementary data, Table S1, all typical EPEC isolates showed the LA phenotype. Moreover, only one atypical EPEC showed the LA phenotype, whereas all others (n = 34) did not attach to HEp-2 cells (Supplementary data, Table S1). The typical EAEC, containing the aggregative adherence fimbriae *agg*R gene, forms a stacked-brick adherence pattern, also called aggregative adherence (AggA), when incubated for 3 h with HEp-2 cells (Fig. 2B). Our studies found that all typical EAEC that were isolated from the food samples showed the AggA phenotype, whereas the atypical EAEC did not attach to cultured HEp-2 cells (Fig. 2E). All other DEC strains (i.e., STEC and ETEC) that were isolated from the food items did not adhere to HEp-2 cells within the 3 h incubation period (Fig. 2E and Supplementary data, Table S1).

3.5. Antibiotic Resistance Profiles of DEC Strains

The antibiotic resistance of all DEC isolates (N = 56) was investigated. We found that 66% of DEC strains were resistant to at least one

Table 2
Presence of thermotolerant coliforms, *E. coli* and DEC in food and water samples.

Food samples	Fecal coliforms (%)	*E. coli* (%)	*Diarrheogenic E. coli*				
			Total	EPEC (%)*	EAEC (%)*	STEC (%)*	ETEC (%)*
Dairy products (n = 669)	190 (28.4)	143 (21.3)	19 (2.84)	13 (68.42)	2 (10.52)	3 (15.78)	1 (5.26)
Meat products (n = 646)	156 (24)	110 (17)	12 (0.75)	10 (83.33)	2 (16.66)	0	0
Seafood and fish (n = 656)	117 (17)	71 (10.8)	13 (1.98)	11 (84.61)	2 (15.38)	0	0
Prepared foods (n = 1594)	175 (10.97)	70 (4.3)	12 (0.75)	10 (83.33)	0	2 (16.66)	0
Beverages and ice (n = 1597)	54 (3.38)	15 (0.93)	0	0	0	0	0
Total (N = 5162)	692 (13.40)	409 (7.92)	56 (1.08)	44 (78.57)	6 (10.71)	5 (8.92)	1 (1.78)

(%)* Percentage according total *diarrheogenic E. coli* detected in each food samples.

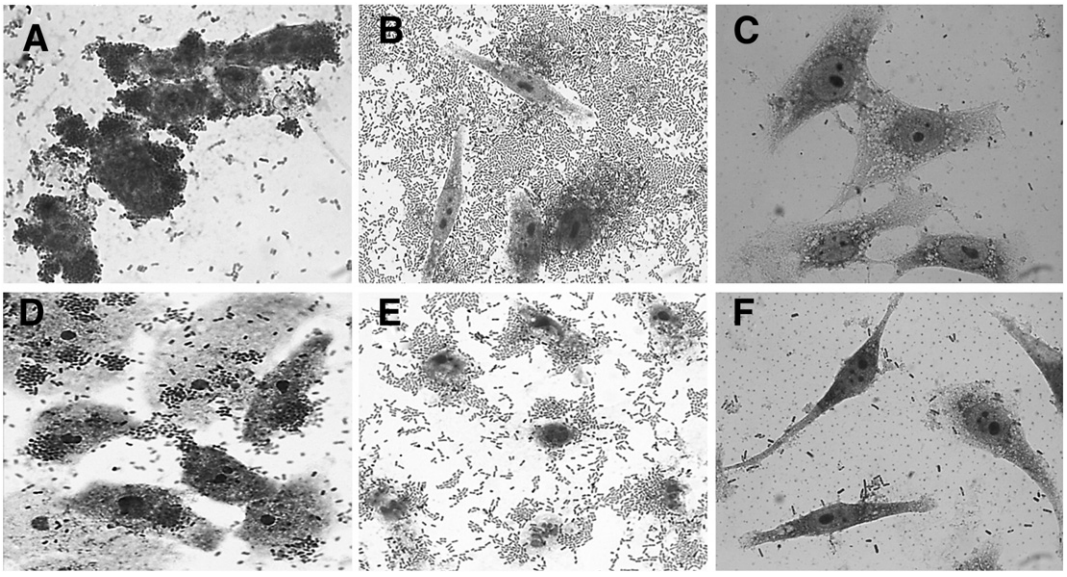

Fig. 2. Adherence phenotype of DEC on *in vitro*-cultured HEp-2 cells. DEC cultures were used to infect HEp-2 cells for 3 h at 37 °C, 5% CO_2. Strains shown are A) EPEC E2348/69, B) EAEC O42, C) DH5α, D) EPEC isolated from fish, E) typical EAEC isolated from raw milk and F) ETEC isolated from salad. Cells were stained by Geimsa and photographed using a 40X objective.

of the commonly prescribed antibiotics in Mexico. Of these, 39.2% of strains were resistant to two or more drugs. Resistance to tetracycline (34%), cefotaxime (30%) and ampicillin (29%) was common (Table 3). Almost 60% of EPEC strains were resistant to at least one antibiotic; they were frequently resistant to cefotaxime (34%) and tetracycline (23%). Of great interest, all EAEC strains were resistant to ampicillin, and 83.3% were resistant to two or more antibiotics, On the other hand, STEC strains were commonly resistant to tetracycline (60%) and gentamicin (40%) (Table 3). Very low resistance (EPEC strains) or sensitivity (EAEC and STEC strains) was observed for ciprofloxacin, nalidixic acid and ceftazidime.

The percentage of resistant bacteria was different for the two years of the study. For DEC strains isolated in 2008 (n = 10), 70% were found to be resistant to tetracycline, 50% to ampicillin, 30% to sulfamethoxazole-trimethoprim, 20% to gentamicin and 10% to cefotaxime. In 2009, the percentage of DEC strains resistant to tetracycline, ampicillin and sulfamethoxazole–trimethropin decreased to 26%, 24% and 11%, respectively. The percentage of strains resistant to gentamicin in 2009 showed a significant increase from 20% to 35% (Table 3).

Table 3
Antibiotic resistance among *E. coli* strains isolated from food sources during 2008 and 2009.

Class and antimicrobial	Total (n = 56)	Phenotype and % of resistance		
		EPEC (n = 44)	EAEC (n = 6)	STEC (n = 5)
Aminoglycoside				
Gentamicin	5/56 (9%)	3/44 (7%)	0/6	2/5 (40%)
Quinolones and Fluoroquinolones				
Ciprofloxacin	3/56 (5%)	3/44 (7%)	0/6	0/5
Nalidixic acid	4 (7%)	4 (9%)	0/6	0/5
Sulfonamides and potentiated sulfonamides				
Sulfamethoxazole-trimethoprim	8/56 (14%)	4/44 (9%)	4/6 (67%)	0/5
Tetracyclines				
Tetracycline	19/56 (34%)	10/44 (23%)	5/6 (83%)	3/5 (60%)
Beta lactams				
Ampicillin	16/56 (29%)	10/44 (23%)	6/6 (100%)	0/5
Cephalosporins				
Ceftazidime	2/56 (4%)	2/44 (5%)	0/6	0/5
Cefotaxime	17/56 (30%)	15/44 (34%)	1/6 (17%)	1/5 (20%)
Phenicols				
Chloranphenicol	3/56 (5%)	3/44 (7%)	1/6 (17%)	0/5
Number of drugs resistant to:				
0	19 (33.9%)	18 (40.9%)	0	1 (20%)
1	15 (26.8%)	12 (27.3%)	1 (16.7%)	2 (40%)
2	10 (17.8%)	6 (13.6%)	1 (16.7%)	2 (40%)
3	8 (14.2%)	4 (9.1%)	4 (66.6%)	0
4	2 (3.6%)	2 (4.5%)	0	0
5	2 (3.6%)	2 (4.5%)	0	0

Note: One *E. coli* strain was identify as ETEC with the following antibiotic resistance pattern (Ampicillin = Sensible; *Sulfamethoxazole-trimethoprim* = Sensible; *Chloranphenicol* = Sensible; Gentamicin = Sensible; Tetracyclin = Resistant; Cefotaxime = Resistant; Ceftazidime = Sensible; Ciprofloxacin = Sensible and Nalidixic acid = Sensible). Strains with Resistance to 6, 7, 8 and 9 drugs were not detected.

4. Discussion

Virtually nothing was known about the presence of DEC strains in food products or water consumed at northwestern Mexico, until this study demonstrated a very low prevalence (1%). Infectious diseases transmitted by foods have become a major public health concern in recent years (Frank et al., 2011; Langiano et al., 2012; Taylor et al., 2012). It is imperative to evaluate the quality of food and water sources, especially in places where tourists and non-residents can be exposed. Sinaloa is a strategic region where food products are produced and exported to the US and other countries. Moreover, the port of Mazatlan receives more than one million tourists every year. Our study demonstrated that 1% of the 5162 food samples collected during 2008 and 2009 were contaminated with DEC strains. We also investigated the antibiotic resistance profiles and found that these strains were sensitive to ceftazidime or showed low resistance to nalidixic acid or ciprofloxacin. To our knowledge, this is the first report of DEC isolation from several food items in Mexico.

To evaluate the presence of DEC strains, we have developed a protocol of sequential multiplex, duplex and single PCR reactions. To our knowledge, this is the first protocol with sequential reactions aimed at identifying all known DEC strains and their variants. Identifying these strains and their variants was particularly important, as our study included an analysis of 409 *E. coli* strains. As demonstrated, our scheme first identified EPEC strains in 10.7% (n = 44) of *E. coli* isolates, and we then reducing the number of strains (n = 365) to

be classified by subsequent reactions. An earlier published set of reactions targeted only EPEC, EIEC, EHEC and ETEC strains, but not EAEC or EPEC and EAEC typical and atypical variants (Rappelli et al., 2001). There is also an available multiplex PCR protocol of two reactions, but the limited number of targets within these two reactions fails to identify typical and atypical variants of EPEC and EAEC or epidemic strains of EHEC O157:H7 (Fujioka et al., 2009). On the other hand, our approach to detecting DEC's screened 5 individual *E. coli* colonies per sample; this approach increased the likelihood of detecting positive i.e. DEC strains or negative, i.e. normal flora *E. coli*.

The low prevalence of DEC strains (1.08%) demonstrated in our studies is similar to that reported in a recent study that analyzed more than 3000 samples of beef, poultry, and pork in Korea, and detected ~1.3% prevalence of DEC strains (Lee et al., 2009).

E. coli and DEC strains were identified in all food groups analyzed; therefore, there was no association with a particular food product or water sample. Nevertheless, dairy products were the samples that were most frequently contaminated by DEC bacteria, followed by meat derivatives and seafood and fish. It is therefore reasonable to speculate that the source of contamination could be the result of human fecal contamination of those products. For example, the cheese industry in Sinaloa is "artisanal", so producers regularly manipulate their products with their bare hands, or maybe the same pair of gloves, from the beginning of the production process until the final product is shipped. Accordingly, 13.4% of samples were contaminated with coliform bacteria at levels not permissive for human consumption. Although less likely, DEC strains from the normal flora of animals (i.e., cattle) can be a source of contamination (Nataro and Kaper, 1998; Stella et al., 2012).

The most prevalent DEC strain was EPEC, followed by EAEC and STEC, and only one ETEC strain was isolated. This higher prevalence of EPEC may suggest that 1) local people may carry EPEC with no apparent signs of disease or that 2) EPEC strains are a possible cause of diarrheal disease in Sinaloa. The presence of EPEC in diarrheal cases in Sinaloa has been suggested (Canizalez-Roman et al., in preparation). EPEC strains are commonly isolated from children with diarrhea in Mexico (Paniagua et al., 2007) and other developing countries including India, Peru and Brazil (Ghosh and Ali, 2010; Nunes Mdo et al., 2012; Ochoa et al., 2009a).

The EPEC pathotype was classified as typical if the strain possessed the *E. coli* adherence plasmid (EAF) and the *bfp*A gene encoding the structural subunit of BFP. *E. coli* strains that do not possess the EAF plasmid or *bfp*A gene are classified as atypical EPEC (Trabulsi et al., 2002). Our genotypic and phenotypic characterization demonstrated that 35 strains (80%) were atypical variants of EPEC, whereas the remaining 9 strains (20%), were typical. Until the 1990s, typical EPEC strains were more commonly isolated than atypical EPEC strains, though recent studies have indicated that the prevalence of typical EPEC strains has decreased and that they are gradually being replaced by atypical EPEC (Trabulsi et al., 2002). This bacterial burden in Sinaloa's food samples may be related to the potential of atypical EPEC strains to cause human disease. For example, a recent paper by Estrada-Garcia et al. (2009) reported that the prevalence of atypical and typical EPEC strains isolated from cases of diarrhea in children from Mexico was 44.5% and 10%, respectively (Estrada-Garcia et al., 2009). Similarly, studies in Peru (Contreras et al., 2010), Brazil (Nunes Mdo et al., 2012), Japan (Yatsuyanagi et al., 2003) and Australia (Nguyen et al., 2006) have reported an increase in diarrheal cases due to atypical EPEC, suggesting that atypical EPEC is an emerging diarrheagenic pathogen, not only in developing countries, but also in industrialized countries.

The second most prevalent DEC strain that was detected was EAEC; EAEC strains were also further classified as typical and atypical. The term typical or atypical EAEC refers to strains harboring or lacking the gene encoding the transcriptional regulator AggR, respectively. Some studies have demonstrated an association of typical EAEC with diarrhea (Estrada-Garcia et al., 2009; Huang et al., 2006). While the pathogenicity of atypical EAEC has not been fully elucidated, these strains have been associated with food-borne outbreaks (Cobeljic et al., 1996) and clearly have been associated with a massive outbreak of gastrointestinal illness in school children from Japan (Itoh et al., 1997), Brazil (Regua-Mangia et al., 2009), and other developing countries (Bangar and Mamatha, 2008; Scavia et al., 2008).

Another important DEC that was evaluated in this work was enterohemorrhagic *E. coli* (EHEC), also known as Shiga-toxin producing *E. coli* (STEC). EHEC is a group of well-recognized pathogens that are responsible for serious human infections such as hemorrhagic colitis and hemolytic-uremic syndrome (Law, 2000; Nataro and Kaper, 1998). Production of Shiga toxins (encoded by the *stx*1 and *stx*2 genes) is a key feature of most STEC. Other virulence-associated factors include the pO157 plasmid, which encodes hemolysin (*hlyA*) among other genes (Schmidt et al., 1994). Although outbreaks of O157 STEC have been associated with the consumption of raw vegetables (Watanabe et al., 1999), little is known about the presence of non-O157 STEC (Balague et al., 2006) in food items worldwide. Our findings showed that STEC (serotype O157 negative) was the third most common DEC identified in our study (5/56). Similarly, in another study in Mexico, STEC (serotype O157 negative) was the most prevalent pathotype found in street-vended taco dressings (coriander, onion and red chili sauce) (Lopez-Saucedo et al., 2010). In this study, we detected STEC mainly in vegetables salad, fresh cheese and Strawberry pie, suggesting that these food items could be a potential vehicle of transmission for STEC strains in Mexico.

The potential of STEC strains to cause diarrhea in Mexico has been previously studied by Rosas et al. (2006) and Cortes-Ortiz et al. (2002), who reported the presence of STEC (22% and 0.08% respectively. The first study was conducted in environmental samples and the second reported an outbreak of diarrhea associated with the overflow of sewage water in "Valle the Chalco", Mexico (Cortes-Ortiz et al., 2002).

Another important strain is EHEC serotype O157:H7. This serotype is the most frequent EHEC that is implicated in food-borne outbreaks worldwide (Mead and Griffin, 1998). Our studies did not detect O157:H7 in our food and water samples. To the best of our knowledge, the presence of *E. coli* O157:H7 in food samples has not been previously reported in Mexico. This strain, however, has been isolated from beef carcasses at a slaughter plant in Mexico [2.7% (7/258)] (Varela-Hernandez et al., 2007). Moreover, between 2009 and 2010 six cases of disease produced by *E. coli* O157:H7, reported by the Public Health Agency of Canada (PHAC), affected Canadian travellers whom had recently been in Mexico; the implicated food was not detected (PHAC, 2009, 2010). The presence of STEC (*stx1*) strains in our samples, the geographical proximity of Sinaloa to the U.S. border, and outbreaks reported by the FDA in 2006 in California warrant permanent epidemic surveillance for STEC and EHEC strains in food and water items, as well as in cases of DEC-associated food-related disease.

ETEC was only isolated from one food item (milk-derivative product). ETEC strains are associated with two major clinical syndromes: weanling diarrhea among children in the developing world, and traveler's diarrhea. Traveler's diarrhea is usually contracted from contaminated food and water (Black, 1990; Mattila, 1994). Several studies have shown the importance of ETEC in infections among the tourists visiting Asia, Africa and South America (Adachi et al., 2001; Daniels, 2006; Daniels et al., 2000), and as a cause of diarrhea among Mexican adults and US travelers in Mexico (Bouckenooghe et al., 2002). Therefore, our results suggest that food and drinking water that are available in Sinaloa may not be a risk for ETEC-associated traveler's diarrhea for tourists visiting Sinaloa and the world-class tourist areas of Mazatlan.

While the prevalence of DEC strains was low, they can still either cause isolated cases of DEC-related diseases or produce outbreaks in child care centers, schools or resorts. Our studies have identified

antibiotic therapies that can be utilized in such an outbreak. Out of a panel of antibiotics, we observed very low resistance (EPEC strains) or sensitivity (EAEC and STEC strains) to ciprofloxacin, nalidixic acid and ceftazidime. On the other hand, an alarming 66% of DEC isolates were resistant to antibiotics. This high resistance rate is clearly the result of the uncontrolled self-prescription of antibiotics that has occurred in Mexico for several decades. Fortunately, a new law has been approved by the Health Secretary that finally regulate the sale of antibiotics and other controlled drugs in Mexican drugstores and pharmacies.

Strains of EAEC showed resistance to more antibiotics than the other DEC strains. In our population, EAEC strains might be more exposed to antimicrobials, as these strains can cause persistent diarrhea and are often carried asymptomatically, thus increasing the chances of exposure to multiple antibiotics. In support of our argument, Estrada-Garcia et al. (2005a, 2005b) found, in a study of Mexican children, that among all of the DEC categories, EAEC was significantly more resistant to ampicillin and trimethoprim–sulfamethoxazole (Estrada-Garcia et al., 2005b). In a recently published study carried out in Peru, the prevalence of resistance to ampicillin, clotrimoxazole, tetracycline and nalidixic acid was significantly higher in EAEC than in EPEC and ETEC (Ochoa et al., 2009b).

In conclusion, our work represents the first comprehensive study that evaluated the distribution, prevalence, and detection of virulence factors in DEC strains in more than 5000 samples of food and drinking water utilized for human consumption in Mexico. To the best of our knowledge, there has not been any outbreak of food borne DEC in Sinaloa for at least the last ten years. The protocol for evaluating DEC strains in food and water samples, as well as in *E. coli* strains isolated from cases of diarrhea, that was designed and validated in this study, is currently in use by our laboratory to monitor for the presence of these strains.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.03.020.

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