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Original Article

Multi-Typing of Enterobacteria Harboring LT and ST Enterotoxin Genes Isolated from Mexican Children

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SUMMARY: Enterotoxigenic *Escherichia coli* is the most common cause of diarrhea in children younger than 5 years in the developing world. We used 16S rRNA gene sequencing, the Biolog® system, and an Amplified Ribosomal DNA Restriction Analysis (ARDRA) to identify 69 enterobacteria isolated from the feces of healthy children up to 12 years old and 54 enterobacteria isolated from stool samples obtained from children up to 5 years old with diarrhea from Morelia, Michoacán, Mexico. In the diarrheic group, 18 isolates belonged to the enterotoxigenic pathotype, 1 isolate had both LT (heat labile toxin) gene and ST (heat stable toxin) gene, and 17 had the ST gene. The identity of most of the strains harboring the ST gene was *E. coli*, and 3 of the strains were identified as *Morganella morganii*. The ST toxin gene of one of the strains identified as *M. morganii* showed 100% identity with an ST toxin gene of *E. coli*. The ARDRA was a very useful tool to differentiate between *E. coli* and *M. morganii*. The phenotypic and genetic analyses of the isolates using the Biolog® system and Random Amplified Polymorphic DNA, respectively, showed physiological variation among the studied strains and genetic differences between subgroups.

INTRODUCTION

Escherichia coli comprise non-pathogenic commensal isolates and diarrheagenic *E. coli* (DEC), classified into enteroaggregative *E. coli* (EAggEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli*, enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC) (1). According to the World Health Organization (2), ETEC causes 280–400 million diarrheal episodes annually in children under 5 years old in the developing world and an additional 100 million episodes in children aged 5–14 years. The same organization declared ETEC as the most common cause of travelers' diarrhea, responsible for one-third to one-half of all diarrheal episodes in travelers to Africa, Asia, and Latin America. The Institute of Diagnostics and Epidemiologic Reference in Mexico has reported ETEC as the most frequent pathotype, and in Morelia, Michoacán (a central state in México) this is the only pathotype that has been found in children under 5 years old (3).

Although 16S rRNA gene sequence analyses (4) and the Amplified Ribosomal DNA Restriction Analysis (ARDRA) (5) are molecular techniques for enterobacteria species determination, the ETEC pathotype is still currently diagnosed based on the PCR detection of genes associated with LT (heat labile toxin) and ST (heat stable toxin) and other genes, such as *astA*, which encodes an EAggEC heat-stable enterotoxin (EAST-i), an adhesin involved in diffuse adherence that is widely distributed in DEC, including ETEC (6,7). However, the ability to transfer LT enterotoxin plasmids in vitro into other enterobacteria was documented long ago (8). An alternative method to confirm the identity of these strains is Biolog Phenotype Microarrays (PMs), which have revealed that differences between pathogenic and non-pathogenic *E. coli* strains extend beyond the additional virulence factors carried by pathogenic strains and include central metabolic functions, carried by some, but lost by others (9).

Polyphasic approaches involving phenotypic and molecular methods have been used to analyze several organisms that belong to different species, but exhibit similar patterns according to their morphological, physiological, and/or other phenotypic features, allowing the identification of closely related species (4). However, it has not been used to analyze strains that belong to the same pathotype.

E. coli populations have a clonal structure with low levels of recombination (10). ETEC strains sharing the same serotype and virulence-associated factors may be clonally related, as demonstrated by RAPD analyses (11–14). The structures of several pathogenic and commensal populations have been extensively studied in developed countries (15,16). However, little is known about populations in Mexico (17,18).

To this end, in the current study, a multi-typing approach was used to establish accurate identifications of several enterobacteria isolated from children in Morelia, Michoacán and to determine if the *E. coli* strains

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present in the samples are clonally related.

MATERIALS AND METHODS

Sample collection and reference strains: This study included 69 fecal samples from healthy children between 1 and 12 years old and 54 stool samples from children up to 5 years old that were admitted to the emergency department of the ''Eva Sámano de López Mateos'' Children's Hospital in the city of Morelia, Michoacán, Mexico, between September and October 2002 with acute watery diarrhea (acute diarrhea was defined as 3 or more aqueous stools in 24 h). No child had taken antibiotics within 4 months prior to sampling.

The pathotypes ETEC-ST (25611), ETEC-LT-ST (H10407), EPEC (O111), and EIEC (E11) were used as reference strains.

***E. coli* isolation:** Each sample (feces or stool) was streaked on Sorbitol-MacConkey (DIFCO, Sparks, MD, USA) and incubated at 37°C for 24 h. Sorbitol-fermenting colonies (small red/pink) with the *E. coli* morphology were inoculated in tryptic soy slants, incubated for 24 h at 37°C, and then stored at 4°C. The *E. coli* isolates were identified by phenotypic characterization and conventional biochemical activities (Gram, catalase, oxidase, indol, methyl red/Voges-Proskauer, citrate, and urease) (19). One *E. coli* isolate was obtained from each sample. The 69 isolates obtained from healthy children were named VGC isolates and the 54 isolates obtained from stool samples were named VGP isolates. All isolates were processed for DNA extraction and conserved in stab agar (US Biological Life Technologies, Salem, MA, USA)

Identification of the bacterial isolates using the Biolog® system: The 69 VGC isolates and the VGP isolates that harbored the LT or ST toxin genes determined by the methodology described below were typed using Biolog GN plates for gram-negative aerobic bacteria according to the manufacturer's instructions (Biolog, Hayward, CA, USA). Plates were read using a Biolog Microstation and assays were performed in triplicate. The isolates were analyzed for their carbon substrate oxidation pattern (PMs) using the same methodology described in the results section.

Biolog GN and GP database software version 3.5 was used. A similarity index of ≥ 0.500 indicated a good species match. When 3 similarity indices of ≥ 0.500 were recorded after 6 h and 24 h of incubation, the highest similarity index was used (20).

DNA extraction: Genomic DNA was extracted from the isolates using the phenol-chloroform method and the quality of DNA obtained was assessed according to standard procedures (21).

PCR amplification: Primers are shown in Table 1. The reaction mixture to amplify the *E. coli* 16S rRNA gene consisted of 25 ng of DNA, 10 mM pH 9.0 Tris-HCl, 1.0 mM $MgCl_2$, 0.2 mM each triphosphate deoxynucleoside (dATP, dCTP, dGTP, and dTTP) added independently, 0.5 U of Taq DNA recombinant polymerase (Invitrogen, Life Technologies, Waltham, MA, USA), and 1 μM each primer (ECA75F/ECR619R) in a final 25 μL volume. The same reaction mixture was used for PCR to detect virulence genes. The primers LT-IF/LT-2R (Labile toxin) and ST-1F/ST-2R (Stable toxin) were used to identify ETEC-LT and ETEC-ST and the primers EI-1F/EI-2R (*ipaH*) and EP-1F/EP/2R (*BFP*) were used to identify EIEC and EPEC, respectively. The amplification program was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles (45 s at 94°C, 1 min at 60°C, and 1 min at 72°C for each cycle), with a final extension of 72°C for 7 min. For the RAPD assays, the reaction mixture in a total volume of 25 μL consisted of the following: 25 ng of DNA, 10 mM pH 8.5 Tris-HCl, 1.5 mM $MgCl_2$, 0.5 mM each triphosphate deoxynucleoside (dATP, dCTP, dGTP, and dTTP) added independently, 0.7 μM primer, and 1.0 U of Taq DNA recombinant polymerase (Invitrogen, Life Technologies). The amplification program consisted of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, with a final extension of 72°C for 7 min. Assays were carried out in triplicate. All amplification assays were performed using the GeneAmp® PCR System 2700 (Applied Biosystems, Waltham, MA, USA) and the amplification products were assessed on 1.5% agarose gels stained with ethidium bromide (21).

Amplification products of the *E. coli* 16S rRNA gene were sequenced by Macrogen (Rockville, MD, USA) with a Life Technologies AB 3730XL DNA Sequencing Analyzer using the Sanger method. After quality trimming and vector removal using the LUCY2 program (25), the sequences were compared with sequences in

Table 1. Primers used in this study

Gene	Target	Primer	Sequence (5′→3′)	Fragment length (bp)	Reference
16S rRNA	*Enterobacteria*	ECA75F	GGAAGAAGCTTGCTTCTTTGCTGAC	544	(22)
		ECR619R	AGCCCGGGGATTTCACATCTGACTTA		
Labile toxin	ETEC-LT	LT-1F	GCGACAAATTATACCGTGCT	708	(23)
		LT-2R	CCGAATTCTGTTATATATGT		
Stable toxin	ETEC-ST	ST-1F	CTGTATTTGTCTTTTTCACCT	182	(23)
		ST-2R	GCACCCGGTACAAGCAGGAT		
*ipa*H	EIEC	EI-1F	GCTGGAAAAACTCAGTGCCT	424	(23)
		EI-2R	CCAGTCCGTAAATTCATTCT		
BFP	EPEC	EP-1F	CAATGGTGCTTGCGCTTGCT	324	(23)
		EP-2R	GCCGCTTTATCCAACCTGGT		
	E. coli	Ecorapd 1252	GCGGAAATAG		(24)
	E. coli	Ecorapd 1254	CCGCAGCCAA		(24)
	E. coli	Ecorapd 1290	GTGGATGCGA		(24)

GenBank using the Blast algorithm. Additionally, the sequences were submitted to GenBank. The accession numbers assigned to the submitted sequences are KC165745 to KC165762 for VGP1-VGP18 isolates; KC165763 to KC165832 for VGC1-VGC11, VGC14-VGC27, VGC30-VGC31, VGC35-VGC43, VGC45-VGC47, VGC49-VGC54, VGC60-VGC67, VGC69, VGC71-VGC73, VGC75-VGC77, and VGC80-VGC87.

Amplified ribosomal DNA restriction analysis (ARDRA): The in vitro digestion of amplified fragments from ribosomal DNA was carried out in a 15 μL volume containing 10 μg of DNA (amplified fragment), 5 U of restriction enzyme (*Bgl*II, *Eco*RI, *Hind*III, *Hae*III, or *Mbo*I), and 1.5 μL of 10 × buffer. The digestion mixture was incubated for 2 hours at 37°C and the results were assessed on 1.5% agarose gels stained with ethidium bromide (21).

Dendrogram construction: Results obtained from the Biolog® system were registered as 0 when there was no color change in a well and as 1 when there was a color change. A similar dichotomous matrix was built using the bands generated in the RAPD analysis, with values of 1 or 0 indicating band presence or absence, respectively. These matrices were used to calculate Euclidian distances among strains and the values were used to construct dendrograms with the Ward algorithm; these analyses were performed using the Past software (26). Major clusters were defined at an 85% similarity level, and strains with a similarity of at least 95% were included in the same subtype. The numerical index of discrimination, based on the Simpson's index (D) of diversity (27), was used to assess the capacity of the RAPD and Biolog assays to differentiate isolates.

RESULTS

Selection of ETEC isolates: A duplex PCR assay was performed to detect the LT and ST toxin genes in the 54 VGP isolates. The assay produced an expected pattern for the reference strains 25611 and H10407 (Fig. 1A). The duplex PCR analysis revealed that 17 out of 54 VGP isolates showed an amplification product for the ST toxin gene (Fig. 1B) and the isolate VGP8 (Fig. 1B) showed an amplification pattern for both LT and ST toxin genes.

A duplex PCR assay conducted for EPEC and EIEC pathotypes produced the expected pattern for the reference strains EPEC and EIEC (Fig. 1A). No amplicons were generated by duplex PCR for the *BFP* and *ipaH* genes for any of the 54 VGP isolates. The same PCR assays were performed with the 69 VGC isolates obtained from healthy children and none generated an amplification product using specific primers for the LT and ST genes, as well as for the *ipaH* and *BFP* genes.

Typing of isolates: Identification Using Biolog® GN Plates: The species determination using Biolog plates was carried out with 87 isolates, including 18 VGP isolates positive for LT and ST toxin genes and 69 VGC isolates. We found strains of the genus *Citrobacter* (7.9%) and the species *Morganella morganii* (5.6%), *Klebsiella oxytoca* (1.1%), and *E. coli* (85.4%). Among the 18 VGP isolates, the VGP7 isolate was identified as *Citrobacter braakii* and the isolates VGP6, VGP10, and VGP14 were identified as *M. morganii* subsp. *morganii*; the other VGP isolates were identified as *E. coli*. Using the Biolog identification system for the isolates obtained from healthy children, isolates VGC3 and VGC43 belonged to *Citrobacter freundii* (2.3%); VGC66, VGC80, VGC86, and VGC88 belonged to *C. werkmanii* (1.1%), *C. rodentium* (1.1%), *C. gillenii* (1.1%), and *C. braakii* (1.1%), respectively. The isolate VGC73 was *M. morganii* and isolate VGC40 was *K. oxytoca*. The remaining isolates were identified as *E. coli*. In vitro ARDRA assays: The Biolog® system identified isolates VGP6, VGP10, and VGP14 as belonging to the genus *Morganella* and the VGP7 isolate as *C. braakii*. To determine whether these metabolic typing results coincided with the genetic typing results, in vitro ARDRA was performed using different nucleases to digest the 540-bp fragment obtained by the amplification of the 16S rRNA gene with the ECA75F/ECR619R primers. First, for all samples, the expected

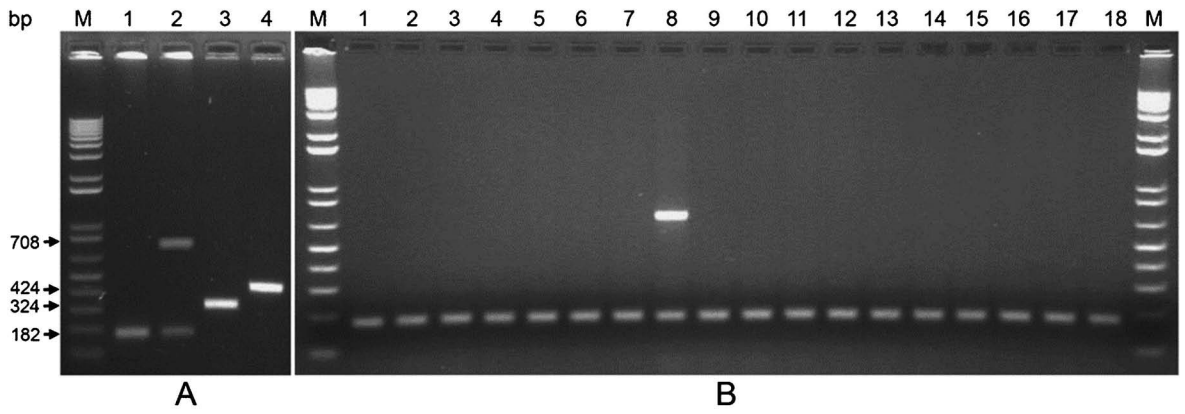

Fig. 1. Pathotype determination by duplex PCR of reference strains and clinical isolates. Agarose gels (1.5%) stained with ethidium bromide showing the amplification products obtained by duplex PCR from the DNA of: (A) The reference strains of *E. coli*. Lanes: M. 1 Kb plus DNA Ladder; 1, 25611 strain (ETEC-ST) with primers LT-1F/LT-2R and ST-1F/ST-2R; 2, H10407 strain (ETEC-LT-ST) with primers LT-1F/LT-2R and ST-1F/ST-2R; 3, O111 strain (EPEC) with primers EI-1F/EI2R and EP-1F/EP-2R; 4, E11 strain (EIEC) with primers EI-1F/EI-2R, and EP-1F/EP-2R. (B) The clinical isolates and the primers LT-1F/LT-2R and ST-1F/ST-2R: Lanes: M, 1 Kb plus DNA Ladder; 1 to 18, isolates VGP 1–18 respectively.

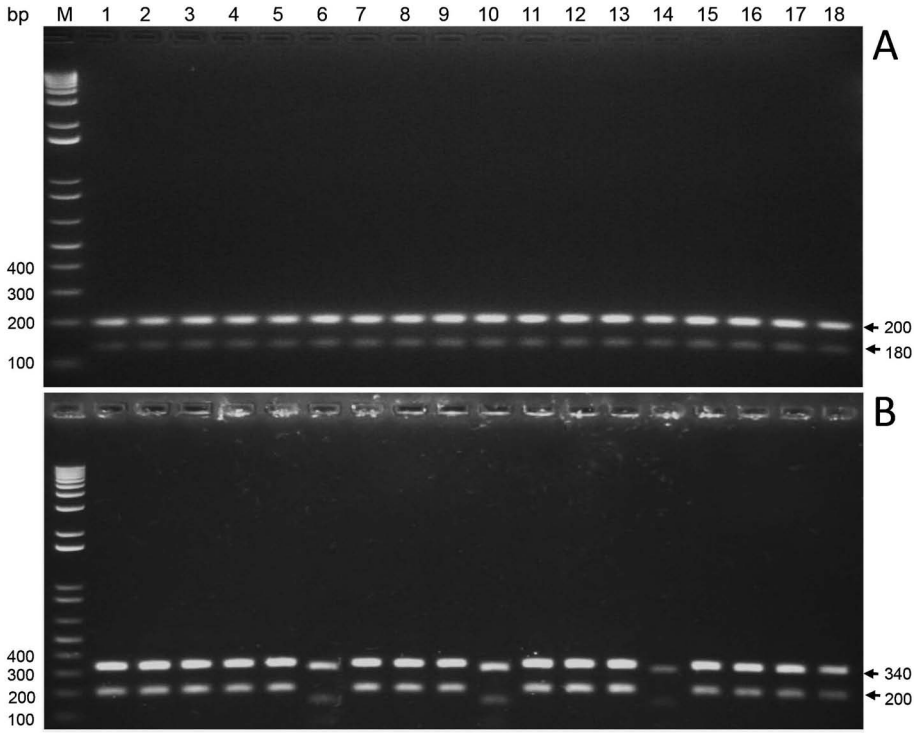

Fig. 2. In vitro ARDRA patterns for the VGP isolates with positive amplification to LT and ST toxin genes. 1.5% agarose gel stained with ethidium bromide that shows the digestion products with: (A) *Hae*III and (B) *Mbo*I from the amplified fragment with primers ECA 75F and ECR 619R, for the 16S rRNA gene, using DNA from the bacterial isolates. Lanes: M, 1 Kb Plus DNA Ladder; 1 to 18, isolates VGP 1–18 respectively.

540-bp product was observed (data not shown). Second, none of the VGP isolates showed a restriction site for *Bgl*II, *Eco*RI, and *Hind*III enzymes (data not shown). Digestion with *Hae*III produced 2 restriction products of approximately 200 and 180 bp in all VGP isolates (Fig. 2A). The sum of the 2 fragment lengths did not account for the expected value of 540 bp, and this could be explained by the generation of small fragments that could not be detected by electrophoresis. The enzyme *Mbo*I generated 2 restriction products of approximately 340 and 200 bp for 15 isolates, but for VGP6, VGP10, and VGP14, 2 bands of 320 and 180 bp were observed (Fig. 2B). 16S rRNA gene sequence: To corroborate the taxon identification results, we obtained the partial sequence (475 bp on average) of the 16S rRNA gene. The species identity was confirmed for all of the studied isolates that were identified as *E. coli* and isolates VGP6, VGP10, VGP14, and VGC68, identified as *M. morganii*, using the Biolog® system, except for VGC73, which was identified as *K. oxytoca*. However, the identity was not the same for strains identified using the Biolog system as *Citrobacter* and *K. oxytoca*; based on the sequencing results, the VGP7, VGC3, VGC43, VGC80, and VGC86 isolates were identified as *E. coli* and VGC66 and VGC40 were identified as *M. morganii* and *K. oxytoca*, respectively.

However, the sequence of the amplification product for the ST toxin gene of the VGP10 isolate, identified as *M. morganii*, showed 100% identity with the ST toxin gene of *E. coli* (GenBank accession number KM186149).

Genetic diversity: The RAPD assay was used to compare the genetic diversity between 87 isolates, including the 18 strains with positive amplification results for LT/ST genes and 69 strains isolated from healthy children. Fig. 3 shows the banding pattern obtained for 18 out of 87 isolates based on RAPD. The analysis of the banding pattern of the 87 isolates revealed the following. For primer 1252, 16 loci were obtained with a simple banding pattern, including a minimum of 1 and a maximum of 5 bands per isolate ranging from 0.45 to 5 Kb (Fig. 3A). In the case of primer 1254, the band patterns were more complex and 2 isolates presented up to 10 bands ranging from 0.37 to 3.77 Kb (Fig. 3B). Using primer 1290, we obtained a total of 16 loci with a minimum of 1 and a maximum of 7 bands ranging from 0.39 to 3.43 Kb (Fig. 3C).

The dendrogram generated based on the RAPD results (Fig. 4) showed, at an 85% similarity cut-off, that the strains are distributed in 4 groups. Group I included 25 VGC strains. Group III included 13 out of 18 pathogenic strains, the VGC65 strain, and the reference strains E11 and H10407. Group II included 28 VGC strains and VGP16. Finally, group IV included the remaining 5 pathogenic strains and 13 VGC strains and the reference strains 0111 and 25611. Interestingly, the latter group included all pathogenic and VGC strains belonging to *Morganella* and *Citrobacter* based on the 16S rRNA gene sequence and the Biolog analysis. Additionally, at a 95% similarity cut-off, 21 RAPD types (R) were observed. Surprisingly, we observed that R12 and R15 included all *E. coli* reference strains, pathogenic and non-pathogenic, and R18 and R19 included all strains typed as *Morganella*, pathogenic and non-pathogenic, respectively. The numerical index of discrimination (D) of the RAPD assay calculated for all of

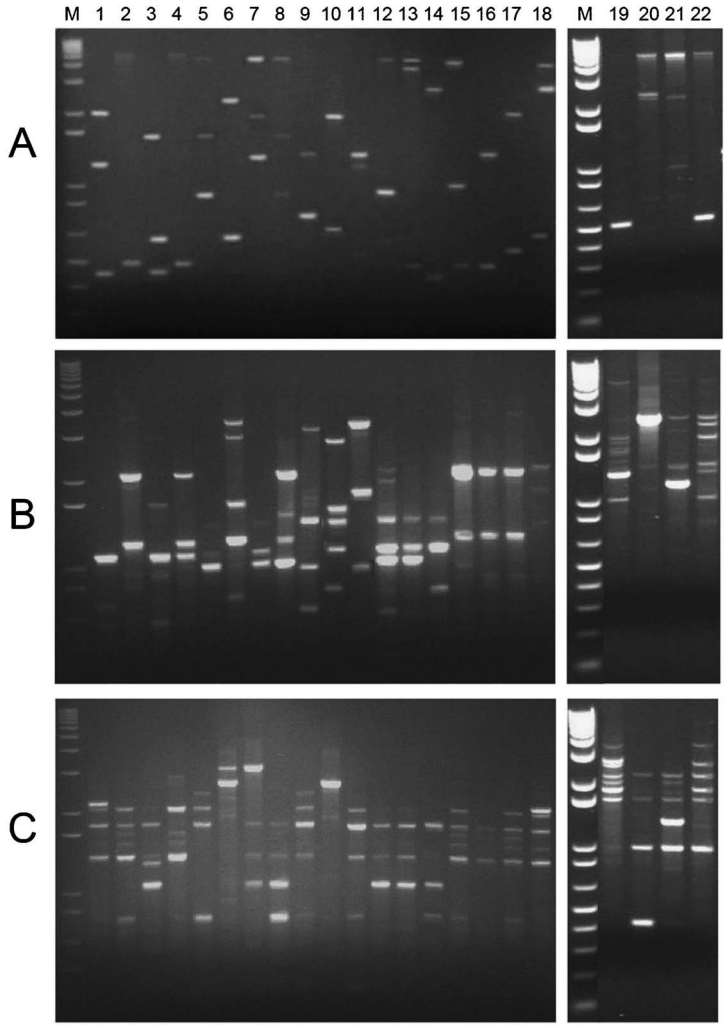

Fig. 3. Banding pattern obtained for 18 out of 87 isolates analyzed by RAPD. Agarose gel (2%) stained with ethidium bromide that shows the RAPD amplifications from bacterial DNA isolates with primers: (A) 1252; (B) 1254; and (C) 1290. Lanes: M, 1 Kb Plus DNA Ladder; 1 to 18, isolates VGC 4, 5, 6, 10, 13, 14, 16, 18, 20, 23, 24, 26, 52, 53, 54, 55, 56, and 57 respectively; 19, EIEC; 20, EPEC; 21, ETEC-ST; 22, ETEC-LT.

the isolated strains was 0.96.

Metabolic profile: An analysis of the Biolog PMs was performed on 68 strains recovered after 3 years from stab agar, 14 strains with positive amplification results for LT/ST genes (VGP1-VGPVGP11, VGP14-VGP16, and VGP18), and 54 isolates from healthy children (VGC1-VGC11, VGC18, VGC20-VGC26, VGC37, VGC399VGC49, VGC51, VGC34-VGC54, VGC60-VGC67, VGC69, VGC71-VGC73, VGC81-VGC83, VGC85-VGC88). The carbon sources were metabolized in a differential pattern by the clinical isolates. *N*-Acetyl-D-glucosamine D-galactose, D-mannose, D-gluconic acid, uridin, α-D-glucose-1-phosphate, and D-glucose-6 phosphate were metabolized by all of the strains. All VGP isolates were able to metabolize, in addition to the sources described above, L-fucose, α-D-glucose, D-mannose, D-psicose, and pyruvic acid methyl ester. VGC strains were able to metabolize from 17 to 61 sources, whereas pathogenic strains metabolized from 28 to 45. In general, more than 50% of the isolates metabolized compounds, such as amino acids, simple carbohydrates, and their derivatives (Table 2). Carbon sources that were metabolized by few or none of the isolates included mainly organic acids. In sum, only 7 (7.3%) out of 95 available carbon sources were metabolized by all of the isolates and 14 (14.7%) were not metabolized by any of the isolates. Using these results, a dichotomous matrix was built and used to obtain a grouping pattern (Fig. 5).

The tree clearly shows 4 clusters, at an 85% similarity cut-off. Cluster A included 16 VGC isolates: 21, 47, 25, 63, 6, 53, 5, 23, 22, 11, 41, 49, 88, 80, 3 and 54, the non-pathogenic reference strain of *E. coli*, the EPEC (O111) and EIEC (E11) reference strains. Group B included strains VGP6, 10, and 14, and VGC73, typed as *Morganella* either using Biolog or by sequencing the 16S rRNA gene. Cluster C included VGP18 and 16 VGC strains, 10, 83, 64, 65, 66, 67, 86, 1, 20, 71, 85, 87, 81, 82, 45, and 46. Cluster D included 21 VGC strains, 62, 40, 8, 24, 60, 4, 26, 69, 7, 9, 18, 37, 44, 48, 43, 2, 61, 39, 42, and 51, as well as the remaining 8 pathogenic strains, 16, 11, 5, 9, 15, 2, 3, and 4; 25611 and H10407 were also included in this cluster. The corresponding dendrogram also showed that, at a 95% similarity cut-off, 19 Biolog types formed groups. All *Morganella* strains grouped in B6 and all *Citrobacter*

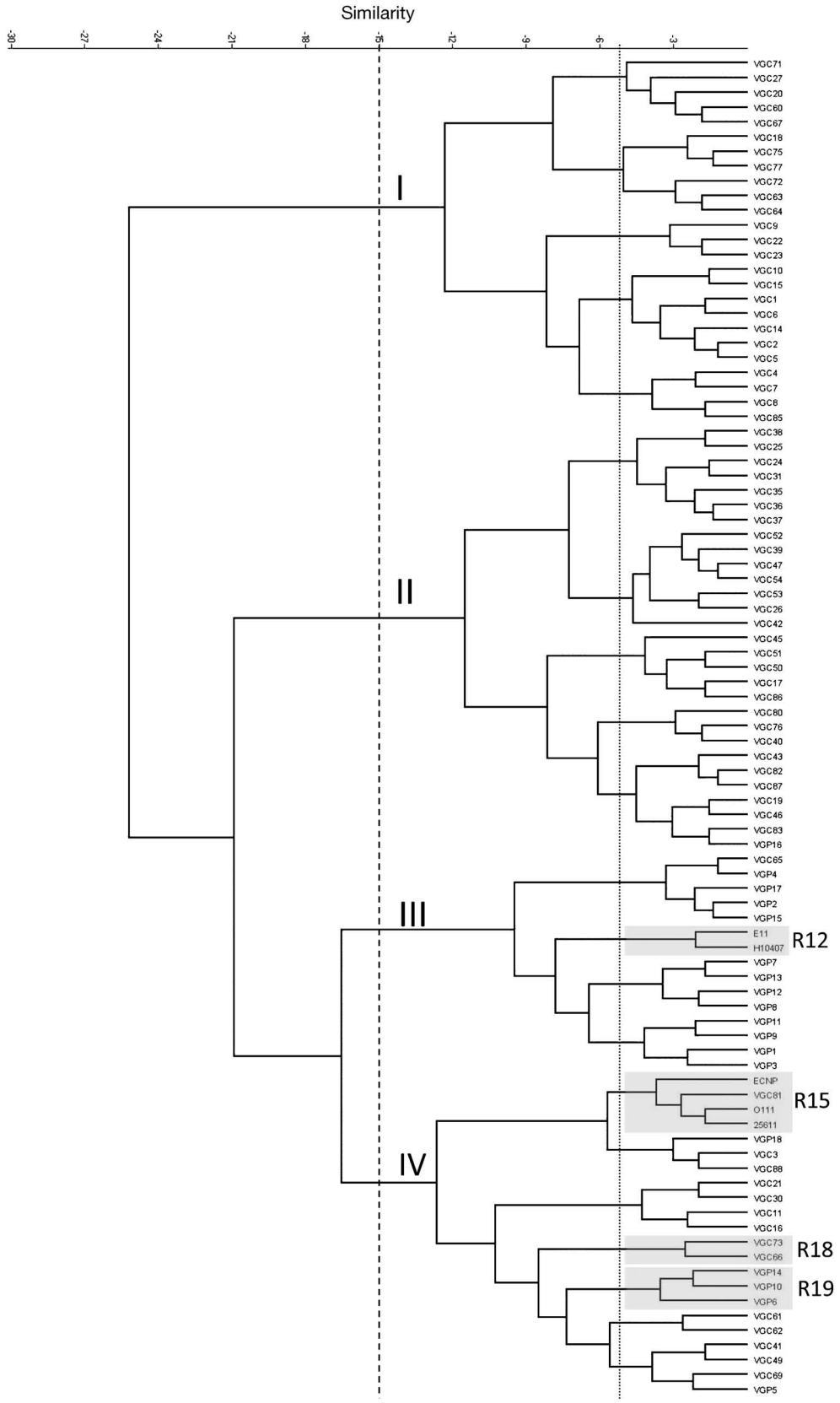

Fig. 4. Dendrogram generated with the results from the RAPD assays. Patterns were generated by calculating the genetic differences through the Euclidian distances calculus and with the values obtained by the grouping pattern using the Ward algorithm. Dashed line indicates an 85% of similarity cut off. Dotted line indicates a 95% of similarity cut off. I to IV clusters obtained at 85% of similarity cut off. R, RAPD types obtained at 95% of similarity cut off.

Table 2. Carbon sources used by 14 VGP strains and 54 VGC strains

C source	% isolates using them VGC[1)]–VGP[1)]	C source	% isolates using them VGC[1)]–VGP[1)]
α-ciclodextrin	neither	ORGANIC ACIDS	
Dextrin	94 (51)–79 (11)	Itaconic acid	neither
Glycogen	33 (18)–7 (1)	α-Keto Butyric Acid	19 (10)–14 (2)
Tween 40	19 (10)–0	α-Keto Glutaric Acid	15 (8)–14 (2)
Tween 80	39 (21)–0	α-Keto Valeric Acid	2 (1)–0
SACCHARIDES		D,L-Lactic Acid	85 (46)–100 (14)
N-Acetyl-D-Galactosamine	85 (46)–86 (12)	Malonic acid	2 (1)–0
N-Acetyl-D-Glucosamine	all	Propionic acid	30 (16)–21 (3)
Adonitol	17 (9)–7 (1)	Quinic acid	2 (1)–0
L-Arabinose	94 (51)–86 (12)	D-Saccharic Acid	11 (6)–0
D-Arabitol	17 (9)–7 (1)	Sebacic Acid	neither
D-Cellobiose	6 (3)–7 (1)	Succinic Acid	65 (35)–93 (13)
i-Erythritol	neither	Bromosuccinic Acid	48 (26)–43 (6)
D-Fructose	98 (53)–100 (14)	Succinamic Acid	2 (1)–0
L-Fucose	91 (49)–57 (8)	Glucuronamide	94 (51)–71 (10)
D-Galactose	all	AMINOACIDS	
Gentiobiose	11 (6)–0	L-Alaninamide	20 (11)–21 (3)
α-D-Glucose	98 (53)–100 (14)	D-Alanine	44 (24)–64 (9)
m-Inositol	4 (2)–0	L-Alanine	63 (34)–71 (10)
α-D-Lactose	93 (50)–71 (10)	L-Alanyl-glycine	69 (37)–71 (10)
Lactulose	48 (26)–36 (5)	L-Asparagine	80 (43)–86 (12)
Maltose	94 (51)–71 (10)	L-Aspartic Acid	76 (41)–79 (11)
D-Mannitol	96 (52)–71 (10)	L-Glutamic Acid	13 (7)–21 (3)
D-Mannose	all	Glycyl-L-Aspartic Acid	67 (36)–93 (13)
D-Melibiose	89 (48)–71 (10)	Glycyl-L-Glutamic Acid	35 (19)–21 (3)
β-Methyl-D-Glucoside	76 (41)–43 (6)	L-Histidine	neither
D-Psicose	74 (40)–100 (14)	Hydroxy-L-Proline	neither
D-Raffinose	41 (22)–36 (5)	L-Leucine	0–21 (3)
L-Rhamnose	76 (41)–57 (8)	L-Ornithine	neither
D-Sorbitol	94 (51)–71 (10)	L-Phenylalanine	0–21 (3)
Sucrose	41 (22)–50 (7)	L-Proline	13 (7)–21 (3)
D-Trehalose	98 (53)–71 (10)	L-Pyroglutamic Acid	neither
Turanose	7 (4)–7 (1)	D-Serine	70 (38)–57 (8)
Xylitol	6 (3)–21 (3)	L-Serine	80 (43)–86 (12)
CARBOXILIC ACIDS		L-Threonine	35 (19)–36 (5)
Piruvic Acid Methyl Ester	96 (52)–100 (14)	OTHER COMPOUNDS	
Succinic Acid Mono-Methyl-Ester	54 (29)–86 (12)	D,L-Carnitine	2 (1)–0
Acetic acid	56 (30)–7 (1)	γ-Amino Butyric Acid	neither
Cis-Aconitic Acid	2 (1)–0	Urocanic acid	neither
Citric acid	4 (2)–0	Inosine	96 (52)–100 (14)
Formic acid	9 (5)–79 (11)	Uridine	all
ORGANIC ACIDS		Thymidine	96 (52)–93 (13)
D-Galactonic Acid Lactone		Phenylethyl-amine	neither
D-Galacturonic Acid	95 (51)–79 (11)	Putrescine	neither
D-Gluconic Acid	all	2-Aminoethanol	2 (1)–0
D-Glucosaminic Acid	4 (2)–0	2,3-Butanediol	neither
D-Galacturonic Acid	96 (52)–86 (12)	Glycerol	87 (47)–100 (14)
α-Hydroxybutyric Acid	24 (13)–29 (4)	D,L-α-Glycerol Phosphate	72 (39)–79 (11)
β-Hydroxybutyric Acid	neither	α-D-Glucose-1-Phosphate	all
γ-Hydroxybutyric Acid	neither	D-Glucose-6-Phosphate	all
p-Hydroxy Phenylacetic Acid	22 (12)–7 (1)		

[1)]: Number of isolates.

strains grouped in B4. The reference strains were assigned to various Biolog types: B3, B5, B16, and B19. The numerical index of discrimination (D) of the PMs calculated for all of the isolated strains was 0.965. The only relationship shared between dendrograms generated by Biolog and RAPD was the branch including VGP isolates 6, 10, and 14, found in both trees.

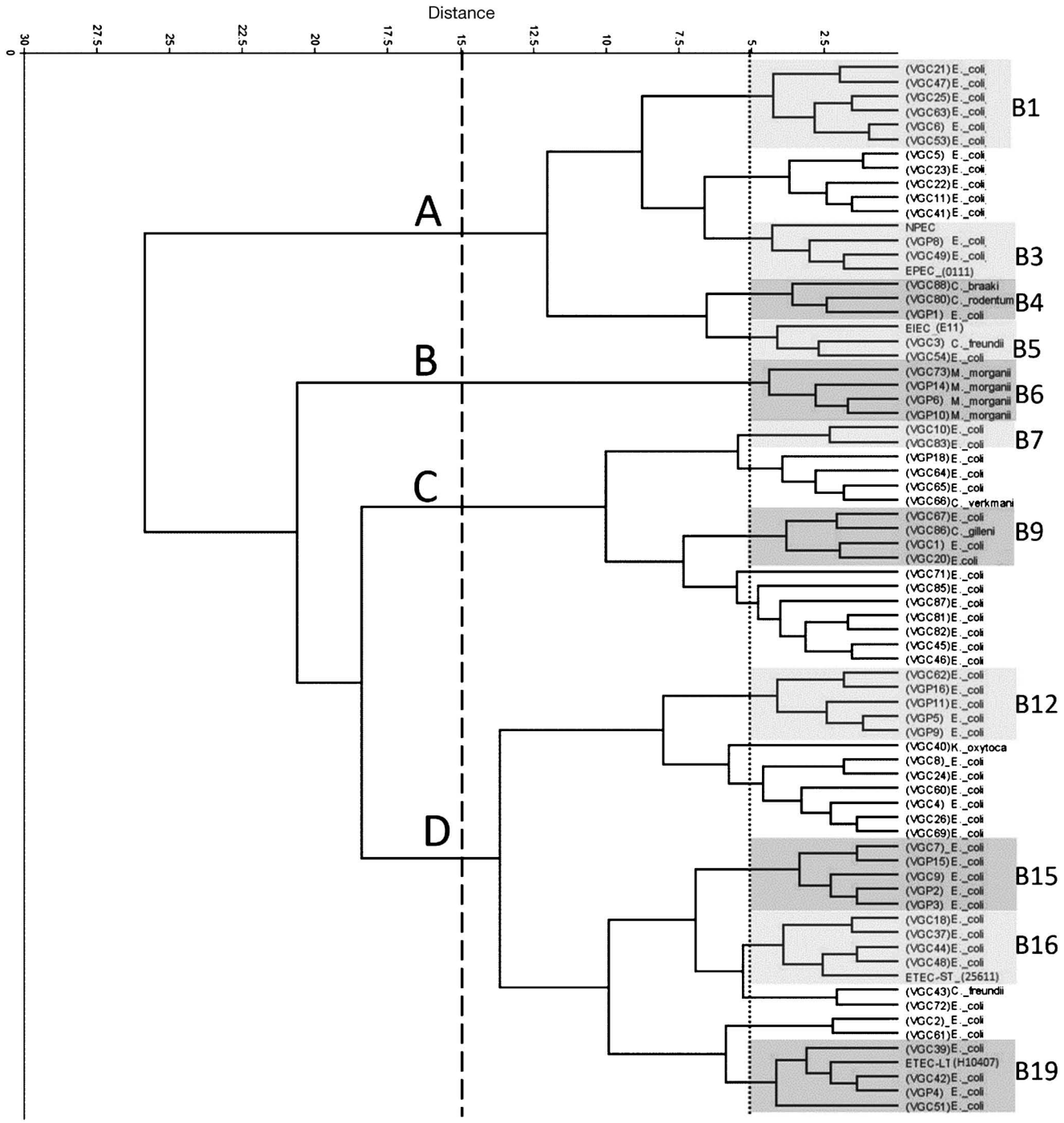

Fig. 5. Dendrogram generated with the carbon source utilization results using the Biolog® system. Patterns were generated by calculating the metabolic differences through the Euclidian distances calculus and with the values obtained by the grouping pattern using the Ward algorithm. Dashed line indicates an 85% of similarity cut off. Dotted line indicates a 95% of similarity cut off. A to D, clusters obtained at 85% of similarity cut off. B, Biolog types obtained at 95% of similarity cut off.

DISCUSSION

Biochemical tests used in this study to identify isolates are specific for *E. coli*; however it has been reported that approximately 0.1% of isolates in the genera *Citrobacter*, *Morganella*, and *Klebsiella* can exhibit the same reactions as *E. coli* (19). To corroborate the taxonomic assignments of the strains isolated in this study, we combined the results obtained using the Biolog® System and by sequencing a hypervariable region of the *E. coli* 16S rRNA gene, which is useful for discriminating *E. coli* from other enteric bacteria, including its closest relative (22). VGP6, VGP10, and VGP14 isolates were typed as *Morganella* both molecularly and metabolically. Using Biolog, the VGP7 isolate was identified as *Citrobacter* and the VGC40 isolate as *K. oxytoca*, but both isolates were typed as *Escherichia* using molecular methods. These results suggest that these bacteria have very similar or identical genetic sequences, which is not unusual since *Citrobacter* and

Klebsiella belong to the Enterobacteriaceae family and are found in the human gut as commensal microbiota. For VGP7 and VGC40, the 16S rRNA gene must be completely sequenced to differentiate between *Citrobacter*, *Klebsiella*, and *E. coli* owing to the assay approach.

The ARDRA patterns obtained with *Mbo*I for all VGP strains identified as *E. coli*, either by Biolog® or based on the 16S rRNA gene sequences, were the same, but were different for strains identified as *Morganella*. Therefore, in vitro ARDRA could be used to detect *Morganella* strains among enterotoxigenic bacteria prior to confirmation by another typing method.

The VGP8 isolate was positive for the LT and ST genes. The presence of these 2 genes in the same strain has been reported previously in hospitalized children with acute diarrhea in northern Jordan (28) and in patients with traveler's diarrhea acquired in Guadalajara, Mexico (29). The enterotoxin genes are associated with a plasmid that can be easily transferred between strains of *E. coli* and to other organisms (30,31). The ability to transfer LT enterotoxin plasmids in vitro to other species of enterobacteria, such as *Salmonella* Typhimurium has long been known (8). Three strains typed in this work as *Morganella* showed the presence of the ST gene. The presence of genes encoding the LT toxin was previously reported in *M. morganii* obtained from stool samples of travelers with diarrhea acquired in Guadalajara and Cuernavaca, Mexico (18); however, this is the first report of the presence of the ST gene in *Morganella*. Our results suggest an interspecific transfer of plasmid(s) containing genes involved in the synthesis of the ST toxin in *E. coli* to *M. morganii*.

In this work, some strains were typed as a particular species using the Biolog® system and as another species based on a comparison of the 16S rRNA gene with the GenBank database using Blast. In this way, VGC66 was identified as *M. morganii* based on the 16S rRNA gene and the dendrogram based on RAPD data indicated that this strain was closely related to other *Morganella* strains; however, Biolog typed this strain as *Citrobacter*. This Biolog misassignment of the identity of VGC66 is not surprising because previous results have indicated that strains belonging to *M. morganii* may differ in the use of carbon sources on Biolog GN plates (32). Considering these results, VGC66 is a phenotypic variant of *M. morganii*, with a carbon source utilization pattern similar to that of *Citrobacter*. This conclusion is consistent with the subdivision of *M. morganii* subspecies in different biotypes based on biochemical differences (33).

The high numerical index of discrimination (D) of the RAPD assay and PMs calculated for all of the isolated strains indicates that the markers allowed the adequate isolate discrimination. The primers used in the RAPD assays have been successfully used to obtain *E. coli* fingerprints (34). RAPD assays are robust enough to reveal genetic diversity between ETEC strains in a restricted geographical area (11,13). In this work, samples were collected in Morelia, which is less than 1,200 km^2; however, the RAPD assays revealed high genetic diversity among the isolated samples. Nevertheless, the grouping patterns suggested that some strains could be considered clones. Eleven out of 18 *E. coli* strains harboring LT and ST toxin genes, VGP1–VGP4, VGP7, VGP9, VGP11–VGP13, VGP15, and VGP17; all *Morganella* strains, pathogenic and non-pathogenic, were grouped in R18 and R19, 2 very close branches in cluster IV. The dendrogram generated based on the results of the RAPD assays allocated the isolates into 4 clusters. This array partially matches the dendrogram generated based on metabolic diversity, i.e., only some isolates maintained the same position in both analyses. This information indicates that the genetic differences are not associated with genes related to metabolic activities. Additionally, the RAPD amplification results obtained in this study show that there is genetic heterogeneity among pathogenic isolates from children with acute diarrhea as well as among VGC strains. This genetic variation can be explained by the fact that patients that donated samples came from different areas of the same city.

Phenotypic tests were used to study ETEC isolates in children with diarrhea, and this was of great aid to study the clonal composition (35). The Biolog® system has been used for bacterial typing (36), ecological and evolutionary studies in *E. coli* (37,38), and to estimate correlations between genes and specific functions (39). The Biolog® system has also been used to study *E. coli* pathotype O157:H7 in order to examine their ability to use carbon sources with respect to that of non-pathogenic strains (40), and showed that non-pathogenic strains have high metabolic diversity than pathotype O157:H7. On the other hand, the genome of an *E. coli* commensal strain possesses more genes involved in the metabolism of carbohydrates than a pathogenic strain. These genes are associated with the uptake of available nutrients, allowing *E. coli* to survive in the intestinal tract, which is rich in oligo and polysaccharides (41) and explaining the adaptation of the commensal *E. coli* strain to the human gut habitat (42).

Although this approach has been adapted to characterize the functional potential of microbial communities, these patterns have not been utilized to compare the metabolic diversity among clinical isolates from the same species; instead, these comparisons have only been performed between different isolates or species from environmental microbial communities (43,44). It is important to identify these patterns in isolates from the same species because environmental perturbations, such as patient characteristics, e.g., diet, age, and even gender, can change the behavior of microorganisms (45). The results obtained using clinical isolates in this study indicated that there is variation in the utilization of various carbon sources. Such variation depended on the genus, although there was also variation among isolates within the same species, i.e., *E. coli*. Utilization patterns of carbon sources on Biolog® plates showed a wide variety of metabolic abilities in the studied strains. These results showed that particular strains did not preferentially use a specific carbon source, with a certain chemical structure. Some acids could be used by most strains, whereas other acids were only used by 1 or 2 strains. This metabolic variability may be related to the feeding patterns of the patients who donated the sample. In this work, the VGP isolates were capable of metabolizing several compounds that were not metabo-

lized by VGC. In contrast, other non-pathogenic strains had a wider metabolic diversity than that of pathotype O157:H7 (40), and this can probably be attributed to a previous observation that the genome of an *E. coli* commensal strain possesses more genes involved in the metabolism of carbohydrates than a pathogenic strain (42).

The increased ability of VGP isolates to take up certain compounds may explain why these isolates are capable of metabolizing several compounds that VGC cannot. The normal pathway for the uptake of small molecules is via porins (46). According to a previous study, a pathogenic strain of *E. coli* possesses more porin genes than a commensal strain (9). It would be interesting to investigate if such differences exist in our strains.

The analysis of the RAPD and Biolog® dendrograms suggests a distribution of clonal groups. Furthermore, it was possible to detect patterns of genetic and phenotypic diversity.

To the best of our knowledge, this is the first report in which the Biolog® system and molecular techniques are used to type and to analyze metabolic and genetic diversity of enterotoxigenic enterobacteria from samples of children suffering from diarrhea and *E. coli* obtained from healthy children. It is also the first report of the presence of the ST gene from *E. coli* in a *Morganella* strain. Although a multiplex RT-PCR assay has been used to identify diarrheagenic *E. coli* in clinical and public health laboratories (47), our results reveal the importance of a multi-typing approach for the accurate identification of bacterial isolates of clinical relevance, especially for studies of the Enterobacteriaceae family, as an effective means to establish epidemiological surveillance programs to detect and characterize other bacterial taxa in this geographic region.

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Conflict of interest None to declare.

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