Virulence-associated genes diversity in *Escherichia coli* O128 strains isolated in São Paulo, Brazil

Diversidade dos genes associados à virulência em cepas de *Escherichia coli* O128 isoladas em São Paulo, Brasil

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ABSTRACT

Hundred-three *Escherichia coli* strains of serogroup O128 were serotyped and examined for virulence-associated genes**.** The 59 representative strains of all serotypes were submitted to ribotyping. The serotypes O128:H35 (41.7%), O128:H2 (14.6%), O128:H$^-$ (8.7%) and O128:H8 (6.8%) were the most frequently found. Serotype O128ab:H2 strains only carried the *eae* and *bfpA* sequences. These strains exhibited the LA-like adhesion pattern**.** Serotype O128ab:H8 strains and some non-motile strains reacted only with the *eae* probe**,** and they were classified as atypical EPEC. The O128:H35 strains corresponded to the enteroaggregative category. Enterotoxigenic strains were found among O128ac:H7, O128ac:H21, O128ac:H27 and O128ac:H$^-$ strains. Clonal group A comprised the majority of virulence markers - harbouring strains, while clonal group B included mainly those strains devoid of any virulence marker.

Key words. *Escherichia coli*, Enteropathogenic *E.coli*, EPEC, Serotypes O128.

RESUMO

Foram estudadas 103 cepas de *Escherichia coli* do sorogrupo O128, quanto às características fenotípicas e genotípicas associadas à virulência. Cinqüenta e nove cepas representantes de todos os sorotipos foram submetidas a ribotipagem .Os sorotipos mais freqüentes foram O128:H35 (41,7%), O128:H2 (14,6%), O128:H$^-$ (8,7%) e O128:H8 (6,8%). Diferentes grupos enteropatogênicos foram identificados. Somente as cepas do sorotipo O128:H2 foram positivas para as sondas *eae* and *bfpA* e apresentaram o padrão de adesão AL-like. Cepas do sorotipo O128:H8 e algumas imóveis reagiram apenas com a sonda *eae* e foram classificadas como EPEC atípicas. As cepas O128:H35 corresponderam à categoria enteroagregativa e os sorotipos O128:H7, O128:H21, O128:H27 e as cepas imóveis foram classificadas como enterotoxigênicas. Todas as cepas que apresentaram marcadores de virulência pertenciam ao grupo clonal A**,** enquanto que no grupo clonal B estavam incluídas as cepas desprovidas dos fatores de virulência pesquisados.

Palavras-chave. *Escherchia* coli, *E.coli* enteropatogênica, EPEC, Sorotipos O128.

INTRODUCTION

During the 1940's and 1950´s, many outbreaks of infantile diarrhoea were epidemiologically linked to *Escherichia coli* throughout the world [1]. Although such outbreaks have become substantially uncommon in developed countries, enteropathogenic *E.coli* (EPEC) is still the leading cause of acute childhood diarrhoea in developing countries. EPEC are recovered from more than 30% of acute diarrhoea affecting mainly infants under one year old in large Brazilian urban centres[2,3].

In addition to certain *E. coli* strains historically associated to outbreaks of diarrhoea, several other strains have also been incriminated as capable of causing diarrhoeal diseases, as demonstrated by further investigations carried out in many countries [4,5,6]. In 1987 [7], the World Health Organization recognized as EPEC 12 *E. coli* serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158.

Over the past several years, the use of HEp-2 and HeLa cells in culture and molecular techniques for studying the virulence characteristics of *E. coli* associated with diarrhoeal diseases led to the establishment of six diarrheogenic categories, and more recently of another one designated atypical EPEC[8,9]. The main characteristics of these categories are: EPEC, localised adherence pattern (LA), and presence of the *eae* sequence and the EAF plasmid; Atypical EPEC, presence of *eae* and absence of EAF; Enterohemorrhagic *E.coli* (EHEC), production of Shiga toxins I or II or both; Enterotoxigenic *E. coli* (ETEC), production of heat-labile (LT) or heat-stable (ST) toxins or both; Enteroinvasive *E .coli* (EIEC), invasiveness (INV)and keratoconjunctivitis in guinea-pig; Enteroaggregative *E. coli* (EAEC), aggregative adherence pattern (AA), and diffusely adherent *E.coli* (DAEC), diffuse adherence pattern (DA) and gene *daaC*. The pathogenicity of DAEC strains however is not well established.

In 1953, Taylor & Charter[10] identified the first strain belonging to serogroup O128, which was isolated from a severely ill baby (Cigleris) and from several cases of infantile diarrhoea in the United Kingdom. Since then, strains of this serogroup have been isolated in association with outbreaks or sporadic cases of diarrhoea in many countries[11]. Furthermore, it has been reported that O128 strains may have different virulence properties[12,13,14,15]. In England and other European countries, Shiga toxin-producing strains are relatively frequent in this serogroup[13,14]. The main purpose of this study was to determine the virulence properties and the association between serotypes and ribotypes of O128 strains isolated in São Paulo, Brazil.

MATERIAL AND METHODS

Bacterial strains

A total of 103 *E. coli* strains of serogroup O128 was analysed. All strains were isolated from patients with diarrhoea at Instituto Adolfo Lutz (Central and Regional Public Health Laboratories), São Paulo, Brazil, between 1977-1993. Of the 103 strains, 90 (87.4%) were isolated from 1 to 5 year old children. The strains were stored on nutrient agar slants and kept at room temperature. Strain Cigleris (serotype O128:H2), the prototype strain of serogroup O128, also included in this study, was provided by the International Reference Center for *E. coli* and *Klebsiella*, Copenhagen, Denmark.

Serotyping

Strains were grown on nutrient broth and streaked onto nutrient agar plates. Selected smooth colonies were tested for serogroup confirmation and subgroup O128ab and O128ac determination. H-antigens were determined after several passages on semi-solid medium. All antigens were determined following standard methods[16] by using antisera prepared with type strains in the Enteric Section of Instituto Adolfo Lutz.

Hybridisation with DNA probes

All strains were tested by colony hybridisation as described by Maas [17] using the following isotopically (α-^{32}P[dATP]) labelled DNA probes: *eae* (*E. coli* attaching and effacing gene); *bfpA* (bundle-forming pilus); EAF (EPEC adherence factor); EHEC (EHEC hemolysin); Stx1 (Shiga toxin 1); Stx2 (Shiga toxin 2); EAEC (enteroaggregative *E. coli* adherence plasmid); AAF/I and AAF/II (aggregative adherence fimbriae I and II); *astA* (enteroagregative *E. coli* heat-stable enterotoxin); *daaC* (diffuselly adhering *E. coli*); INV (*E. coli* invasiveness); LT-I (*E. coli* heat-labile enterotoxin type I); LT-II (*E. coli* heat-labile enterotoxin type II); ST-Ih (heat-stable enterotoxin type I from *E.coli* of human origin); ST-Ip (heat-stable enterotoxin type I from *E. coli* of porcine origin), and *cdt* (cytolethal distending toxin)[18].

Search for colonisation factors (CFA/I and CFA/II)

Was performed with bacteria cultivated in Casamino Acids-yeast extract medium and were determined following standard methods [19].

β-phenylpropionic acid reaction (hydrocinnamic acid)

All strains were tested for hydrocinnamic acid degradation according to Ewing [20]. Cultures were inoculated on the surface of a medium containing 0.02g of β-phenylpropionic acid (PPA) (Sigma, ST Louis, MO, USA), incubated at 37°C and examined daily for three days for development of a pink colour (positive reaction) in the medium.

Hemolysin production

Hemolysin production was searched for according to Beutin et al.[21]. *E. coli* U4 / 41 (serotype O4:K3:H5), producing α-hemolysin and *E. coli* E-Hly$^+$ (serotype O157:H7), producing the EHEC hemolysin, were used as positive controls.

Heat-stable (ST) enterotoxin production assay

The infant mice assay described by Dean et al.[22] was used for detection of ST-I in culture supernatants.

Search for cytolethal distending toxin

Strains were statically grown in Brain Hearth Infusion Broth (Difco, Michigan) at 37°C for 48h. Supernatants were filtered through 0.22µm membranes (Millipore products, Bedford, Mass, USA) and diluted 1:4 in MEM (Modified Eagle Medium, Gibco) for inoculation of HeLa cells monolayers. Changes in cell morphology were surveyed every 24h for 5 days. All strains were tested in two experiments. Two positive control strains (*E. coli* 86-6136 and 741-4) and a negative control strain (*E. coli* C600) were used in all assays[23].

HeLa cells adhesion assay

Strains were tested for the ability to adhere to HeLa cells in the presence of D-mannose as previously described, using the 3 and 6 h assays[24]. Adherence patterns were determined by comparison with those of strains *E. coli* E2348/69, C1845, and O42, which are prototypes for localised adherence (LA), diffuse adherence (DA), and aggregative adherence (AA), respectively [25,26,27]. The adherence patterns were defined after the strains had been tested at least in two assays.

Ribotyping and clonal analysis

Chromosomal DNA (2µg) extracted and purified according to Brenner et al.[28] was digested with *Bgl*I and *Hin*dIII (Amersham Pharmacia Biotech) following the instructions of the manufacturer. Electrophoresis of digested DNA samples and transfer to nylon membranes were done as described elsewhere[29]. Hybridization of membranes with 16 + 23S cDNA probe was done according to Popovic et al.[30]. Fragment sizes of hybridized DNA were estimated as described by Dalla-Costa et al.[31]. A similarity matrix was contructed by visually scoring the presence or absence of each fragment (0, absence of a fragment; 1, presence of a fragment for each ribotype) and the dendogram was contructed as previously described[18,32].

RESULTS

Serotyping

Among the 103 strains, 70 (67.9%) were of subgroup O128ab and 33(32,1%) of subgroup O128ac. Nine strains were non-motile (H-) and the remaining harboured one of the following H-antigens: H2, H5, H7, H8, H12, H21, H25, H27, and H35. Some serotypes occurred with O128ab, others with O128ac and others with both O antigenic varieties. The most prevalent serotype found in the study period was O128ab:H35 (38.8%). The frequencies in which the different associations between H-types and ab and ac antigens occurred are presented in Table 1.

Virulence properties

None of the 103 strains produced hemolysin or reacted with the EAF, *daaC*, EHEC, Stx1, Stx2, LT-I, LT-II, ST-Ip, INV, AAF/I, and AAF/II probes. The results obtained with the remaining probes as well as the toxin production and adherence patterns are summarised in Table 1, according to the ab and ac O subgroups and H-types. All strains of serotypes O128ab:H5, O128ac:H8 and O128ac:H25 were devoid of any of the virulence properties tested and presented an indefinite pattern of adherence (IN) to HeLa cells. Strains carrying antigens H7, H8, H12, and non-motile strains of sub-group ab presented virulence properties distinct from those presented by strains of sub-group ac of the same serotype. The *eae* sequence was found only in strains of serotypes O128ab:H2 (86.6% of the strains), O128ab:H8 (50.0%), and O128ab:H- (100%). The majority of strains of the former serotype that carried *eae* also carried *bfpA*. 68.2% of the strains presenting *eae* produced the localised adherence-like pattern of adherence (LAL) described by Rodrigues et al.[18] , which was characterised by loose clusters of few bacteria detected only in the prolonged 6h assay (Figure 1A). The ST-Ih sequence was found only in strains belonging to the ac subgroup (in one of 3 strains of O128ac:H7, in all strains of O128ac:H- , in all strains of O128ac:H21 and 3 of 10 strains of O128ac:H27), and all of them expressed ST-I in the infant mouse assay. Moreover, the 2 strains of serotype O128ac:H7, 5 strains of serotype O128ac:H12 and 7 strains of serotype O128ac:H27 that lacked the ST-Ih sequence were also positive in this assay at the time of isolation. Among the ST-Ih producing strains, one (serotype O128ac:H7) produced CFA/I and two (serotype O128ac:H21) produced CFA/II. All O128ac:H- and O128ac:H21 strains that produced ST-Ih displayed DA (Figure 1C). The EAEC sequence was found only in serotype O128ab:H35 (15% of these strains), but most of the strains of this serotype (82.5%) presented AA to HeLa cells (Figure1B). This pattern was also detected in the two strains of serotype O128ab:H12 and one of 3 strains of serotype O128ac:H35. The AA pattern in all O128 strains could only be determined in the 6h assay. The *cdt* sequence was only found in one O128ab:H- strain which did not express the toxin; in the same serotype, another strain produced CDT but did not react with the *cdt* probe used. Most of the strains of serotypes O128ab:H2 (73.3%), O128ab:H35 (72.5%), and O128ac:H35 (66.6%) carried sequences homologous to *astA.*

β-phenylpropionic acid reaction

Only strains belonging to serotypes O128ab:H2 (13 strains) and O128ab:H$^-$ (4 strains) and bearing the *eae* gene were positive in this test.

Ribotypes

For ribotyping analysis we selected 59 strains that represented all serotypes and subgroups identified in this study. Using DNA digested with *Bgl*I and *Hin*dIII, 8 banding patterns (ribotypes R1 to R8) were found among these strains

(Figure 2). The distribution of strains according to the ribotypes associated with serotypes and their virulence properties is shown in Figure 3.

DISCUSSION

The results of this study show that *E.coli* serogroup O128 include strains with the characteristics of atypical EPEC (*eae*+), enterotoxigenic *E.coli* (ST-Ih+), and enteroaggregative *E.coli* (AA adherence pattern). This serogroup also includes strains as those of serotype O128:H2 (eae+, bfp+, and EAF-) which can not be placed in any of the diarrheogenic *E.coli* categories. In fact O128:H2 strains are very similar to O119:H2[33] strains and were classified as atypical EPEC[34]. Most strains of this serotype carried the *astA* gene. Surprisingly enough we have not found among our O128:H2 strains either Stx or EAF positive strains. Although the number of strains studied is not

Table 1. H-antigens , O subgroups and virulence characteristics of 103 O128 *E. coli* isolated in São Paulo, Brazil, between 1977 and 1993.

H-types	O subgroup	Virulence properties	Adherence pattern
H2 (15)[a]	ab (15)	*eae bfpA astA* (11)	LAL (9) ; IN (2)
		eae bfpA (1)	LAL (1)
		eae (1)	LAL (1)
		# (2)	IN (2)
H5 (1)	ab(1)		
		#(1)	IN (1)
H7 (4)	ab (1)	# (1)	IN(1)
	ac (3)	ST-Ih [c](1)[*]	IN (1)
		# (2)	IN (2)
H8 (6)	ab (5)	*eae* (3)	LAL (1) ; IN (2)
		# (2)	LAL(1) ; NA (1)
	ac (1)	# (1)	IN (1)
H12 (7)	ab (2)	# (2)	AA (2)
	ac (5)	# (5)	IN (4) ; NA (1)
H21 (6)	ac (6)	ST-Ih[c] (6)[**]	DA (6)
H25 (2)	ac (2)	# (2)	IN (2)
H27 (10)	ac (10)	ST-Ih[c] (3)	NA (3)
		# (7)	NA (7)
H35 (43)	ab (40)	EAEC (4)	AA (4)
		EAEC *astA* (2)	AA (2)
		astA (27)	AA (21) ; IN (6)
		# (7)	AA (6); IN (1)
	ac (3)	*astA* (2)	AA (1); IN (1)
		# (1)	IN (1)
H-[b] (9)	ab (6)	*eae* (4)	LAL (3); IN (1)
		eae cdt (1)	IN (1)
		eae astA CDT (1)	IN (1)
	ac (3)	ST-Ih[c] (3)	DA (3)

[a] (number of strains); [b] (non-motile);[c] (all strains which reacted with this probe were positive for ST(Dean test); [*] (positive for CFA/ I, colonization factor antigen I) ; [**] (two strains were positive for CFA/II, colonization factor antigen II); *eae* (EPEC attaching and effacing); *bfpA* (bundle forming pilus); *ast-A* (enteroaggregative heat-stable enterotoxin 1 gene); ST-Ih (heat-stable enterotoxin I from *E.coli* of human origin); EAEC (enteroaggregative *E.coli*); *cdt* (cytolethal distending toxin gene) ; CDT (cytolethal distending toxin); # (negative with all probes); LAL (localized adhesion like) ; AA (aggregative adhesion); DA (diffuse adhesion) ; IN (indefinite adhesion) ; NA (non-adherent)

large this suggests that the Brazilian O128:H2 strains are different from the strains isolated in the UK and other European countries where strains of this serotype frequently produce Stx and occasionally bear the EAF plasmid[13,14].

An interesting result of this study was the close association found between antigenic characteristics and virulence properties. Strains carrying antigens H7, H8, H12, H35 and some non-motile strains of sub-groups ab presented virulence factors distinct from those presented by strains of sub-group ac of the same serotype. Still interesting yet, strains bearing the *eae* and EAEC sequences and those displaying AA belonged to sub-group ab, while strains which reacted with the ST-Ih probe or produced this toxin were of sub-group ac. Similar results were reported by Campos et al.[32] with serogroup O111 where all EPEC (typical and atypical) and all aggregative strains had the ab antigen and all STEC strains had ac antigens.

The subdivision of O groups in ab and ac subgroups, introduced by Ewing et al. [35] in the 50's, may be more significant than accepted currently not only for virulence but also in epidemiological studies[4]. In regard to these phenotypical characteristics, it should be emphasized that the phenyl-propionic acid test, could separate the O128:H2 strains from all other strains suggesting that this serotype can be identified by this property.

The use of the adherence assay in this study was crucial for the characterisation of the strains since it demonstrated the existence of a great number of EAEC strains which could not be detected by the EAEC DNA probe currently available [36,37] and also because it allowed us to confirm that strains lacking the EAF region exhibit regularly the LAL adherence pattern. But it should be noted that the results of the assay for both adherence patterns are consistent only after a 6 hour incubation period.

Clonal analysis based on ribotyping data showed that O128 strains are organized in two main groups comprising some clusters. The great majority of strains bearing virulence markers were assigned to clonal group A. Our finding that R2 included serotypes H2, H27 and H35 is similar to the data reported by Whittam et al.[38] who showed that these serotypes belonged to a single electrophoretic type (ET). Although one cluster typically included ETEC strains of serotypes O128:H7,

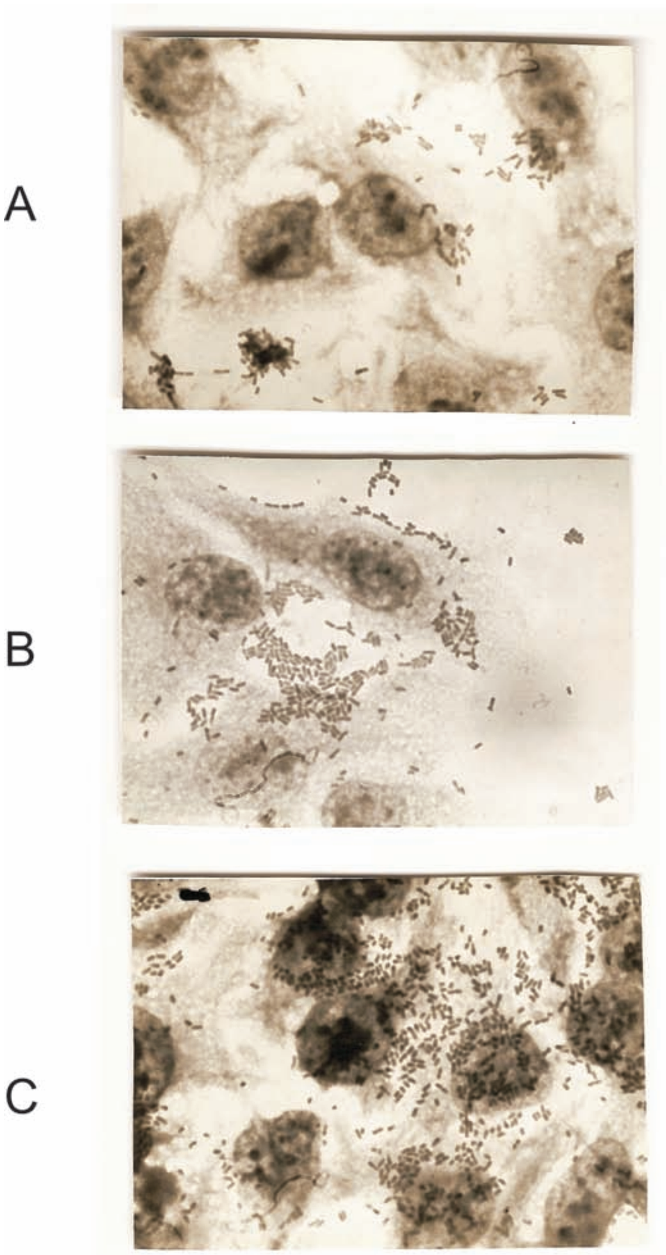

Figure 1. Adherence patterns of O128 strains after 6h incubation. (A) Localized adherence–like pattern (LAL) of O128:H2 strains; (B) AA pattern displayed by O128:H35 strains; (C) DA pattern of O128ac:H21 strains.

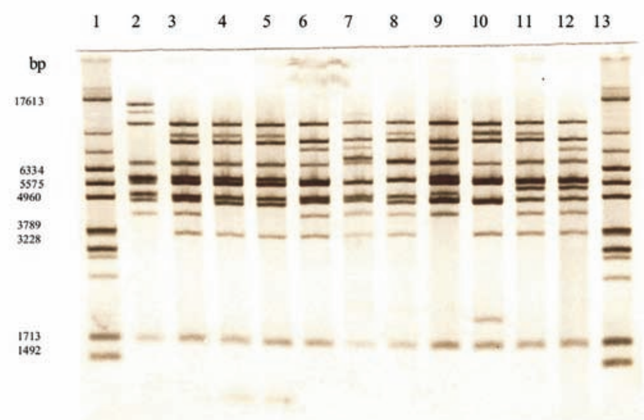

Figure 2. Banding patterns (ribotypes) of 59 O128 strains obtained with *Bgl*I. 1 and 13, molecular marker, *H.aegyptius Eco*RI DNA digest; 2, rib otype 1 (Cigleris,H2);3, ribotype 2 (H2); 4 and 5, ribotype 3 (H-) ;6, ribotype 4 (H7);7, ribotype 4a (H7); 8, ribotype 5 (H8); 9, ribotype 6 (H12); 10, ribotype7 (H25; 11, ribotype 8 (H21;12, ribotype 8a (H21)

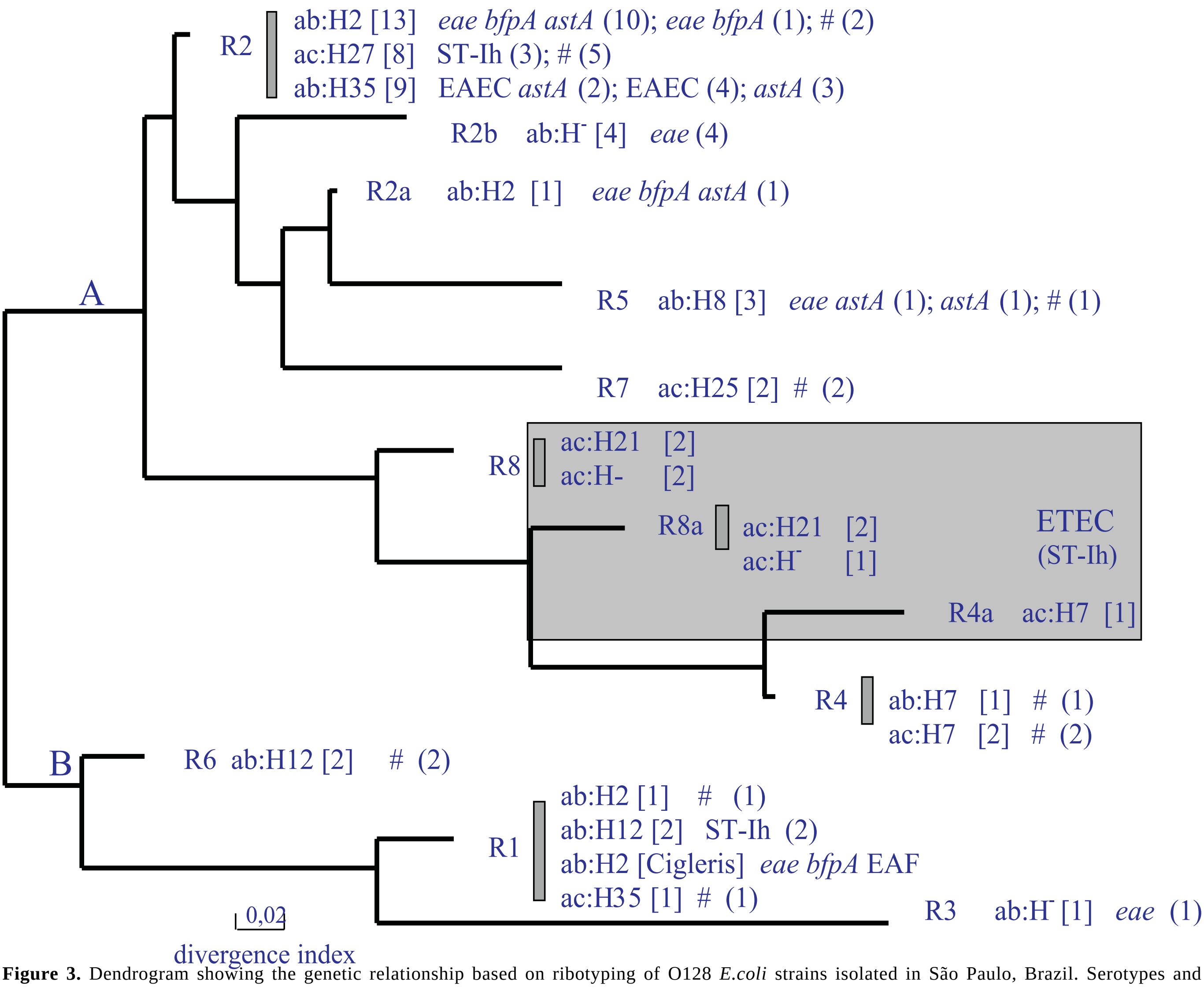

Figure 3. Dendrogram showing the genetic relationship based on ribotyping of O128 *E.coli* strains isolated in São Paulo, Brazil. Serotypes and characteristic virulence determinants are indicated on the right of each ribotype.When multiple strains were analysed the number of strains are indicated in parentheses following the virulence markers. # indicates absence of any virulence marker.

O128:H21 and non-motile strains, other enterotoxigenic serotypes as O128ac:H27 and O128ac:H12 were classified in other clusters, suggesting that strains belonging to the same diarrhoeagenic category are not monophyletic[39]. Clonal group B included mainly strains devoid of any virulence marker except for one non-motile strains carrying the *eae* sequence and two O128ac:H12 strains that were originally ST-Ih positive, but lost this virulence marker possibly during storage. The prototype strain "Cigleris" (O128:H2 *eae bfpA* EAF) was also assigned in this group suggesting its substantial genotypic difference compared with our H2 strains, and supporting that strain Cigleris, a O128:H2 strain isolated in England many years ago, likely belongs to a different lineage or clone. Genetic diversity among O128:H2 strains has already been reported by Whittam et al.[39] and Orskov et al.[40] based on electrophoretic profiles. Similar distribution based on multilocus enzymes electrophoresis (MLEE) has been reported in other EPEC serogroups [14,16,29], showing a close relationship among electrophoretic types, serotypes and virulence properties. Dalla-Costa et al.[31] also reported an association between ETs and ribotypes in many EPEC serogroups.

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