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Original Article

Molecular characterization of *Escherichia coli* O157:H7 strains isolated from different sources and geographic regions

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***Escherichia* (*E.*) *coli* serotype O157:H7 is a globally distributed human enteropathogen and is comprised of microorganisms with closely related genotypes. The main reservoir for this group is bovine bowels, and infection mainly occurs after ingestion of contaminated water and food. Virulence genetic markers of 28 O157:H7 strains were investigated and multilocus enzyme electrophoresis (MLEE) was used to evaluate the clonal structure. O157:H7 strains from several countries were isolated from food, human and bovine feces. According to MLEE, O157:H7 strains clustered into two main clonal groups designated A and B. Subcluster A1 included 82% of the O157:H7 strains exhibiting identical MLEE pattern. Most enterohemorrhagic *E. coli* (EHEC) O157:H7 strains from Brazil and Argentina were in the same MLEE subgroup. Bovine and food strains carried virulence genes associated with EHEC pathogenicity in humans.**

Keywords: enterohemorrhagic *Escherichia coli,* molecular characterization, O157:H7, virulence

Introduction

Enterohemorrhagic *Escherichia* (*E.*) *coli* (EHEC) belong to a subset of Shiga toxin (Stx)-producing *E. coli* (STEC) serotypes that are associated with bloody diarrhea and hemolytic uremic syndrome [23]. Epidemiologically, O157:H7 is considered a highly pathogenic serotype responsible for severe human diseases, usually occurring as outbreaks, and as a prototypical EHEC [8,9,23]. Besides expressing Stx, EHEC produces a variety of virulence factors encoded by lysogenic toxigenic bacteriophages along with virulence chromosomal pathogenicity islands and plasmids [2-4,11,23]. Unlike other *E. coli* pathotypes, cattle are the main reservoirs of EHEC, and are the major source of direct and indirect transmission [23].

Genotypic and phenotypic methods have been employed to investigate the clonal relationship and virulence properties of O157:H7 strains. Epidemiological and phylogenetic studies have revealed that *E. coli* O157:H7 strains form a clonal complex of related genotypes found worldwide and exhibit differences in virulence characteristics or transmissibility between lineages [10,13,16,21,25,26,28]. Among the molecular systems used, isoenzyme electrophoresis known as multilocus enzyme electrophoresis (MLEE) has been traditionally used for phylogenetic studies of *E. coli* and provides important information about the virulence background of particular lineages [16,18-20,24].

Isoenzymes play an essential role in housekeeping metabolic activities which make them useful for conducting evolutionary studies of many bacterial populations, particularly among strains belonging to *E. coli* pathotypes. Isoenzymes also participate in specific steps of the infection processes of many pathogens, including colonization, persistency, and invasion of the host tissue [14]. In the present study, isoenzymatic type and virulence potential were examined in order to evaluate the diversity and clonal relationships of a set of *E. coli* O157:H7 strains isolated during distinct periods and from different sources. Of particular interest was the comparison between O157:H7 strains recovered from healthy cattle in Brazil and strains isolated from humans.

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Materials and Methods

Bacterial strains

The study included 28 *E. coli* O157:H7 strains isolated from diverse geographic regions. These included Argentina (n = 4), UK (n = 2), USA (n = 2), and three Brazilian states [São Paulo (n = 1), Rio Grande do Sul (n = 8), and Rio de Janeiro (n = 11)]. Among the total number of strains, two were isolated from food, five from patients, and 21 from bovine feces (Table 1). Brazilian bovine strains were isolated between 1999 and 2001 from distinct farms located in 13 rural counties. Only one strain from each farm was included in this analysis. EHEC strains from Argentina, the UK, and USA were kindly donated by Dr. Marta Rivas (Administración Nacional de Laboratorios e Institutos de Salud, Argentina) and Dr. Sylvia Scotland (Central Public Health Laboratory, UK). EPEC strain 116I (O157:H-, sorbitol fermenting, *stx*-, *eae*+, EAF+, *bfpA*+) was included in this study for comparison [17] .

Detection of virulence genes

Multiplex PCR assays were used to detect Shiga toxin (*stx*), intimin (*eae*), and enterohemolysin (E-*hlyA*) genes as previously described [5,25].

Table 1. General characteristics of *Escherichia coli* O157 strains

Pathotype (strain)	Geographic area	Brazilian locations* /year isolated	Serotype	Zymovar	MLEE group (subgroup)	Virulence genotype†	Source of isolation
EHEC							
YB20	Brazil	SP/1997	O157:H7	5	B (B2)	*stx1, stx2c, eae γ, ehlyA*	Bovine
B1/1	Brazil	RJ/1997	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
B18/1	Brazil	RJ/1997	O157:H7	3	A (A3)	*stx2c, eae γ, ehlyA*	Bovine
GC148	Brazil	RJ/1997	O157:H7	6	B (B2)	*stx2, eae γ, ehlyA*	Bovine
581/1	Brazil	RJ/1999	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
579//3	Brazil	RJ/1999	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
691/1	Brazil	RJ/1999	O157:H7	2	A (A2)	*stx2c, eae γ, ehlyA,*	Bovine
902/1	Brazil	RJ/1999	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
1728/1	Brazil	RJ/2000	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
2004/1	Brazil	RJ/2000	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
1770/1	Brazil	RJ/2000	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
2228/1	Brazil	RJ/2000	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
111/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
440/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
678/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
685/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
872/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx1, stx2c, eae γ, ehlyA*	Bovine
869/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx1, stx2c, eae γ, ehlyA*	Bovine
956/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
356/1	Brazil	RS/2001	O157:H7	1	A (A1)	*stx1, stx2, eae γ, ehlyA*	Bovine
ME18/96	Argentina	1996	O157:H7	1	A (A1)	*stx2, stx2c, eae γ, ehlyA*	Human
TRA 71/96	Argentina	1996	O157:H7	1	A (A1)	*stx2, stx2c, eae γ, ehlyA*	Food
145/98	Argentina	1998	O157:H7	1	A (A1)	*stx2c, eae γ, ehlyA*	Bovine
ME135/99	Argentina	1999	O157:H7	1	A (A1)	*stx1, stx2, stx2c, eae γ, ehlyA*	Human
E30138	UK		O157:H7	1	A (A1)	*stx2, stx2c, eae γ, ehlyA*	Human
E40705	UK		O157:H7	1	A (A1)	*stx1, eae γ, ehlyA*	Human
EDL931	USA		O157:H7	1	A (A1)	*stx1, stx2, eae γ, ehlyA*	Human
EDL933	USA		O157:H7	4	B (B1)	*stx1, stx2, eae γ, ehlyA*	Food
EPEC							
116I	Brazil	RS	O157:H-	7	C	*eae α*	Human

*RJ: Rio de Janeiro, SP: São Paulo, RS: Rio Grande do Sul. †*stx1*: Shiga toxin type 1, *stx2*: Shiga toxin type 2, *stx2c*: Shiga toxin type 2c, *eae γ*: gamma intimin, *eae α*: alpha intimin, *ehlyA*: enterohemolysin. MLEE: multilocus enzyme electrophoresis, EHEC: enterohemorrhagic *E. coli*, EPEC: enteropathogenic *E. coli.*

Isoenzyme electrophoresis

Bacterial cells were typed according to variation of eleven isoenzymes [16]. The following enzymatic isoenzymes were evaluated: malate dehydrogenase (MDH), aconitase (ACO), adenylate kinase (ADK), isocitrate dehydrogenase (IDH), leucyl amino peptidase (PEP-2), alcohol dehydrogenase (ADH), glucose phosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM) and malic enzyme (ME). Cluster analysis was performed using the unweight pair group average method, UPGMA of the NTSYS pc software package (ver. 2.0; Exeter Software, USA). Isolates lacking detectable enzyme activity were designated as being in a null state at the locus in question. Electrophoretic types (ETs) were defined based on combinations of isoenzymic markers. A numerical index measuring the discriminatory ability of MLEE typing method was calculated with the Simpson's Index of Diversity equation [22].

Results

Multiplex PCR revealed that out of the 28 O157:H7 strains, 20 carried *stx2*, seven strains were positive for both *stx1* and *stx2*, and one strain of human origin was positive for *stx1* alone. EHEC O157:H7 carrying the *stx2* sequence was obtained from bovines (21 strains), humans (four strains), and food (two strains). Strains carrying both *stx1*

Table 2. Numerical representation of electromorphs and zymovars according to MLEE analysis of *E. coli* O157 strains

Strain	MLEE electromorphs*											Zymovars
	IDH	PGM	MDH	GPI	ACO	PEP-2	PGD	G6PDH	ADH	ADK	ME	
581/1	1	1	1	1	1	1	1	1	1	1	1	1
579//3	1	1	1	1	1	1	1	1	1	1	1	1
691/1	1	1	1	1	1	1	2	1	1	1	1	2
872/1	1	1	1	1	1	1	1	1	1	1	1	1
2004/1	1	1	1	1	1	1	1	1	1	1	1	1
1770/1	1	1	1	1	1	1	1	1	1	1	1	1
902/1	1	1	1	1	1	1	1	1	1	1	1	1
2228/1	1	1	1	1	1	1	1	1	1	1	1	1
145/98	1	1	1	1	1	1	1	1	1	1	1	1
B18/1	1	2	1	2	1	1	1	1	1	1	1	3
B1/1	1	1	1	1	1	1	1	1	1	1	1	1
E30138	1	1	1	1	1	1	1	1	1	1	1	1
E40705	1	1	1	1	1	1	1	1	1	1	1	1
EDL931	1	1	1	1	1	1	1	1	1	1	1	1
EDL933	2	2	1	1	2	2	1	1	1	1	1	4
YB20	0	2	1	3	0	0	1	1	1	1	1	5
ME135/99	1	1	1	1	1	1	1	1	1	1	1	1
ME18/96	1	1	1	1	1	1	1	1	1	1	1	1
GC148	1	2	1	3	1	2	1	1	1	1	1	6
TRA71/96	1	1	1	1	1	1	1	1	1	1	1	1
111/1	1	1	1	1	1	1	1	1	1	1	1	1
440/1	1	1	1	1	1	1	1	1	1	1	1	1
678/1	1	1	1	1	1	1	1	1	1	1	1	1
685/1	1	1	1	1	1	1	1	1	1	1	1	1
872/1	1	1	1	1	1	1	1	1	1	1	1	1
869/1	1	1	1	1	1	1	1	1	1	1	1	1
956/1	1	1	1	1	1	1	1	1	1	1	1	1
356/1	1	1	1	1	1	1	1	1	1	1	1	1
116I	2	2	2	4	2	1	3	2	2	2	2	7

*IDH: isocitrate dehydrogenase, PGM: phosphoglucomutase, MDH: malate dehydrogenase, GPI: glucose phosphate isomerase, ACO: aconitase, PEP-2: leucyl amino peptidase, PGD: 6-phosphogluconate dehydrogenase, G6PDH: glucose-6-phosphate dehydrogenase, ADH: alcohol dehydrogenase, ADK: adenylate kinase, ME: malic enzyme.

and *stx2* were isolated from bovines (four strains), humans (two strains) and from food (one strain). The enterohemolysin (E-*hly*A) gene was detected in all EHEC O157:H7 strains that also carried the *eae* γ gene variant. The control EPEC strain 116I (O157:H-) carried only the *eae* α gene (Table 1).

MLEE typing based on 11 isoenzymatic systems identified six distinct ETs in the EHEC O157:H7 population that was analyzed (Table 2). Enzymes G6PDH, ME, MDH, ADH, and ADK were monomorphic. The others were polymorphic with the number of electromorphs ranging from two (PEP-2, ACO, PGM, PGD and IDH) to three (GPI). Specific isoenzymatic variants of MDH, GPI, PGD, G6PDH, ADH, ADK, and ME were observed in the EPEC O157 strain alone. MLEE typing had a discriminatory index of 0.3768. The dendrogram based on isoenzymatic markers segregated the O157:H7 strains into two main clonal groups, designated A and B, exhibiting a 75% similarity. Cluster A included strains more closely related and was subdivided into subgroups A1～A3 while cluster B into B1～B2 subgroups (Fig. 1). Subgroup A1 included 86% of the studied strains that exhibited similar isoenzymatic types. These O157:H7 strains were isolated from cattle and humans, and were obtained from different Brazilian localities (Rio de Janeiro and Rio Grande do Sul) and other geographic regions (Argentina, UK, and USA). Subgroups A2 and A3 each had a single representative strain of bovine origin isolated from Rio de Janeiro. Group B included only three strains which were subdivided into subgroup B1, including the O157:H7 prototype strain EDL933 isolated from food, and subgroup B2, composed of strains recovered from cattle in Brazil. MLEE clustering was not associated with the period of isolation. The outgroup, represented by the non-motile O157 EPEC strain 116I, was found to be distantly related to the EHEC O157:H7 strains.

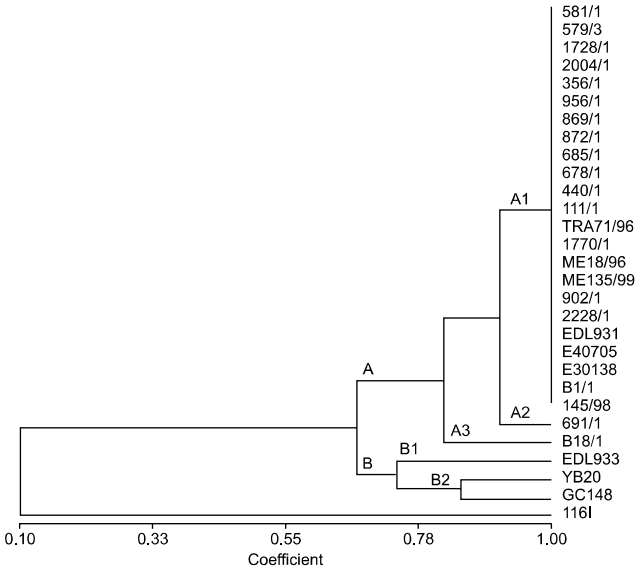

Fig. 1. Isoenzymatic relationships among the *Escherichia coli* O157 strains.

Discussion

In order to determine the clonal relationships and potential virulence of the EHEC O157:H7, strains isolated from distinct sources and geographic regions were submitted for isoenzymatic typing and virulence genotyping. Several basic criteria such as typeability, reproducibility, discriminatory power, availability, cost, and technical requirements must be taken into account for typing procedures. Genetic approaches are used worldwide due to their higher discriminatory power and standardized global data. Unlike some genetic methods for bacterial typing, isoenzymatic characterization relies on the analysis of housekeeping enzymes which can indicate distinct rates of diversification within a complex evolutionary process; this procedure may thus have lower discriminatory potential [7]. Currently, genotyping techniques are more frequently performed and better appreciated than phenotypical approaches typically used for epidemiology and evolutionary studies [7,24]. MLEE is the exception and has historically been the technique of choice for *E. coli* characterization. This method provides relevant information about the metabolic background of potentially virulent bacterial populations [14,16,21]. This methodology is strongly recommended for long-term epidemiological studies due to the conserved nature of the molecular targets [21,24].

In the present investigation, the bacterial population we analyzed included epidemiologically independent strains representing differences in time of isolation, geographical origin, sources, and possibly distinct evolutionary lineages. Isoenzymatic typing detected variability within the O157:H7 serotype that divided the strains into subgroups. Identical isoenzymatic electrophoretic profiles were shared by O157:H7 strains presenting distinct ecological and epidemiological features. In order to achieve greater accuracy and determine the precise relationship of such strains, additional enzymatic systems could be used. Subtyping genetic methods based on the analysis of nucleotide polymorphisms have also demonstrated diversity within the O157:H7 serotype. Additionally, specific variants and distinct lineages were found to be more commonly associated with particular disease phenotypes [4,6,21,25,28]. Genomic divergence from an ancestral form and the occurrence of genetic events, mostly related to lateral gene transfer, are thought to have promoted the evolution of O157:H7 lineages [12,15,21,27]. Despite its limited discriminatory power, the methodology employed in the present study has also been used in molecular studies conducted on *E. coli* epidemiology and strongly correlated MLEE data and pulsed-field gel electrophoresis (PFGE)

genotypes. PFGE is a DNA typing methodology considered to be a reference system for evaluating genetic variability and the overall chromosomal relatedness of several microorganisms, including *E. coli* populations [18,19].

Comparison of virulence and isoenzymatic profiles of strains isolated in Argentina and Brazil represents a particularly important approach. This is because O157:H7 is the most prevalent EHEC serotype associated with cattle and human diseases in Argentina whereas O157:H7 strains were rarely found to be associated with human disease in Brazil [8,23]. An early PFGE analysis of Argentinean and Brazilian O157:H7 strains isolated before 1999 (YB20, B1/1, B18/1, and GC148) showed that only one strain (YB20) is related to the Argentinean strains [7]. Most EHEC O157:H7 strains isolated from Brazil and Argentina after 1999 belong to the same MLEE subgroup, A1. However, the first O157:H7 isolates from Brazilian locations obtained in 1997 exhibited distinct MLEE types (B2, A3, and A2), suggesting the circulation and transmission of a new bacterial lineage, possibly of Argentinean origin, among Brazilian cattle reservoirs.

When considering virulence potential, bovine and food strains carry virulence genes encoding Shiga toxin, intimin, and enterohemolysin that are associated with EHEC pathogenicity in humans [1,2,8,23]. However, most Argentinean O157:H7 strains isolated from animals, food [8], and humans [11] are of the *stx2*/*stx2c* genotype. In contrast, most Brazilian strains are of the *stx2c* genotypes alone or carry *stx1*. STEC strains carrying only *stx2c* are less virulent in humans than strains carrying *stx2* or *stx2*/*stx2c* genes [6,11].

Despite the relatively small number of strains analyzed in the present study, our results reinforce previous observations by detecting diversification within the serotype. We also identified bacterial clonal dissemination of potential virulent strains in distinct geographic regions, especially among different locations in Brazil. These findings underscore the need for more effective surveillance of bovine EHEC O157:H7 isolates in Brazil, especially considering the pathogenicity attributed to these microorganisms. Studies of additional enzymatic systems as well as a broader set of virulence genes is recommended for determining precise lineage specificities.

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