

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

HeLa-Cell Adherence Patterns of Enteropathogenic *Escherichia coli* Strains Isolated from Faecal Samples

^{1,3}Mohammad Yousef Alikhani, ¹Akbar Mirsalehian and ²Mohammad Mehdi Aslani

¹Department of Microbiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

²Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran

³Department of Microbiology, Faculty of Medicine,
Hammadan University of Medical Sciences, Hamadan, Iran

Abstract: *Escherichia coli* strains that cause nonbloody diarrhea in infants are known to present four distinct patterns of adherence to epithelial cells, namely, Localized (LA), Localized Adherence Like (LAL), Diffuse (DA) and Aggregative (AA) adherence. Strains with LA and AA are well recognized as a cause of diarrhea, but the role of strains with DA is controversial and strains with LAL have been more frequently isolated in diarrheal than asymptomatic cases. To determine the distribution of the different types of enteropathogenic *E. coli* (EPEC) adherence patterns in diarrhea, we studied 191 EPEC strains isolated from infants less than 5 years of age with and without diarrhea in Iran. Totally 131 (68.5%) strains adhered to HeLa cells and 60 (31.5%) isolates did not. The results revealed that Localized Adherence (LA) was manifested by 30 of 111 (86%) strains isolated from diarrheal cases of which the most belonged to serogroups O86 and O55. Localized Adherence like (LAL) was exhibited by 16 and Aggregative Adherence (AA) by 9 strains isolated from patients. However, Diffuse Adherence (DA) was exhibited by 8 strains equally distributed between both diarrheal and healthy persons. Undefined Pattern (UDP) was observed in 24 strains. Overall, the results showed that LA, LAL and AA adherence significantly associated with diarrhea ($p < 0.05$).

Key words: *Escherichia coli*, EPEC, HeLa cell, adherence, diarrhea

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea in developing countries. In industrialized countries, the frequency of these organisms has decreased, but they continue to be an important cause of diarrhea (Nataro and Kaper, 1998, Trabulsi *et al.*, 2002). The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E), which is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment. *Escherichia coli* strains that cause nonbloody diarrhea in infants are known to present three distinct patterns of adherence to epithelial cells, namely, (i) Localized Adherence (LA), where micro colonies attach to one or two small areas on the cells (HeLa or HEP-2); (ii) Diffuse Adherence (DA), where bacteria cover the cells uniformly (Scaletsky *et al.*, 1984, 2005) and (iii) enteroadherent-aggregative adherence (AA), where the

bacteria have a characteristic stacked-brick-like arrangement on the surface of the cells and on the glass slide free from the cells (Nataro *et al.*, 1987). The LA pattern is significantly associated with diarrhea production (Nunes *et al.*, 2003) and has been shown to be an important property of enteropathogenic *E. coli* (EPEC). The LA phenotype is associated with the induction of the attaching and effacing lesions (A/E) (Moon *et al.*, 1983). The pathogenic role of *E. coli* showing a DA pattern (DAEC) in the etiology of diarrheal disease is controversial (Arikawa *et al.*, 2005; Gunzburg *et al.*, 1993; Jallat *et al.*, 1993). The role of *E. coli* giving an aggregative pattern of attachment to tissue culture cells (EAEC) in diarrhea has been more frequently related to persistent and pediatric diarrhea (Harrington *et al.*, 2006; Cravioto *et al.*, 1991). EAEC strains possess a plasmid of 60 MDa which is necessary for the expression of the aggregative phenotype (Moon *et al.*, 2005). A new adherence pattern called the Localized Adherence-like (LAL) pattern has described by Scaletsky *et al.* (1996, 1999). This pattern is characterized by the presence of

Corresponding Author: Dr. Mohammad Mehdi Aslani, Associate Professor, Head of Enterobacteriaceae Laboratory,
Address: No. 69, Pasteur Ave, Department of Microbiology, Institute Pasteur of Iran, Tehran, Iran
Fax: 009821-664645132

less-compact microcolonies or clusters of bacteria in a few cells observed only in tests with prolonged incubation periods (6 h). The mechanism of this adherence pattern is unknown. The role of *E. coli* showing an LAL pattern as an agent of diarrhea is not established yet (Nataro and Kaper, 1998). Studies evaluating the epidemiological importance of HeLa cell adherence pattern of EPEC in diarrheal diseases are required particularly in developing countries. For this reason, we determine the prevalence of the different types of EPEC adherence patterns in strains isolated from diarrheal and asymptomatic children.

MATERIALS AND METHODS

Bacterial strains: A total of 191 EPEC strains were studied for determine the distribution of the different types of adherence patterns. The 111 EPEC strains were isolated from children with diarrhea and 80 strains from children without any gastrointestinal symptoms.

EPEC serogrouping: *E. coli* strains were isolated on MacConkey plates. Four separate lactose-fermenting and two non-lactose-fermenting colonies of each distinct morphologic type, presumed to be *E. coli* by colony morphology, were cultivated for biochemical confirmation of species. *E. coli* colonies were submitted to slide agglutination with polyvalent and monovalent antisera (Bio-Rad Co), against O antigens of EPEC serogroups. The serogroups considered were as follows: O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128 and O142. When two or more strains of identical serogroup were isolated from the same infant, only one strain have used in this study.

HeLa cell adherence assay: EPEC colonies were characterized by the pattern of adherence to HeLa cells (National cell Bank of Iran, Institute Pasteur of Iran) in the presence of D-mannose as assayed by the method described by Scaletsky *et al.* (1984). Three to five individual isolates from each case were assessed for adherence to HeLa cells. Briefly, monolayer HeLa cells infected with bacteria were grown to 50% on coverslips in Leighton tubes in presence of 1% mannose for 3 h at 37°C in 5% CO₂. The infected monolayers were washed with sterile phosphate-buffered saline, fixed with 70% methanol, stained with 10% Giemsa stain and examined for LA, LAL, DA, or AA patterns under a light microscope. A number of strains adhered in non-specific manner and were put in a different category as Undefined Pattern (UDP). When the adherence pattern was weak or negative, a new preparation was made and examined after a 6 h incubation period. Prototype EPEC strain E2348/69

(serotype O127: H6), *E. coli* strain E17-2 (serotype O3:H2) and *E. coli* strain C1845 (serotype O75: NM) showing LA, AA and DA, respectively, was used as positive controls in adherence to HeLa cells assays and *E. coli* K12 as a negative control.

Statistical analysis: The χ^2 test and Fisher's exact test were used for statistical analysis.

RESULTS

The prevalence of enteropathogenic *E. coli* serogroups identified in the stool cultures of patients and controls is presented in Table 1. Only the EPEC strains isolated from the stools as a single potential enteropathogenic agent were studied. EPEC serogroups was isolated as sole pathogen from 58.1% (111 of 191) of children with diarrhea, compared with 41.9% (80 of 191) of those without diarrhea ($p < 0.05$). EPEC strains belonged to O26, O55, O86, O111, O114, O119, O125, O126, O127, O128 and O142 serogroups (Table 1).

Totally 131 (68.5%) strains adhered to HeLa cells and 60 (31.5%) isolates did not. Four distinct patterns of adherence were distinguished: LA, DA, AA and the LAL pattern, which was observed only in strains incubated for 6 h (Fig. 1). LA was the most frequent pattern among isolates (18.3%), followed by LAL (14.1%), AA (7.9%) and DA (4.2%) (Table 2).

Table 1: Prevalence of EPEC serogroups in infants with diarrhea and healthy cases

EPEC serogroups	No. (%) of positive infants		
	Diarrhea	Without diarrhea	Total
O26	4 (3.6)	2(2.5)	6(3.1)
O55	10 (9)	4(5)	14(7.3)
O86	17 (15.3)	6(7.4)	23(12.0)
O111	22 (19.8)	32(40)	54(28.3)
O114	1 (0.9)	2(2.5)	3(1.6)
O119	4 (3.6)	4(5)	8(4.2)
O125	2 (1.8)	1(1.3)	3(1.6)
O126	7 (6.3)	3(3.8)	10(5.2)
O127	20 (18)	13(16.2)	33(17.2)
O128	3 (2.7)	3(3.8)	6(3.1)
O142	21 (18.9)	10(12.5)	31(16.2)
Total	111	80	191(100.0)

Table 2: Adhesion pattern of EPEC strains isolated from patients and healthy cases

Adhesion pattern	No. (%) of cases		
	Diarrhea	Without diarrhea	Total
LA	30(86)	5(14)	35(18.3)
LAL	16(59)	11(41)	27(14.1)
DA	4(50)	4(50)	8(4.2)
AA	9(60)	6(40)	15(7.9)
UDP	31(67.4)	15(32.61)	46(24.0)
NA	21(35)	39(65)	60(31.4)
Total	111(58.1)	80(41.9)	191(100.0)

UDP = Undefined Pattern, NA = Non Adherence

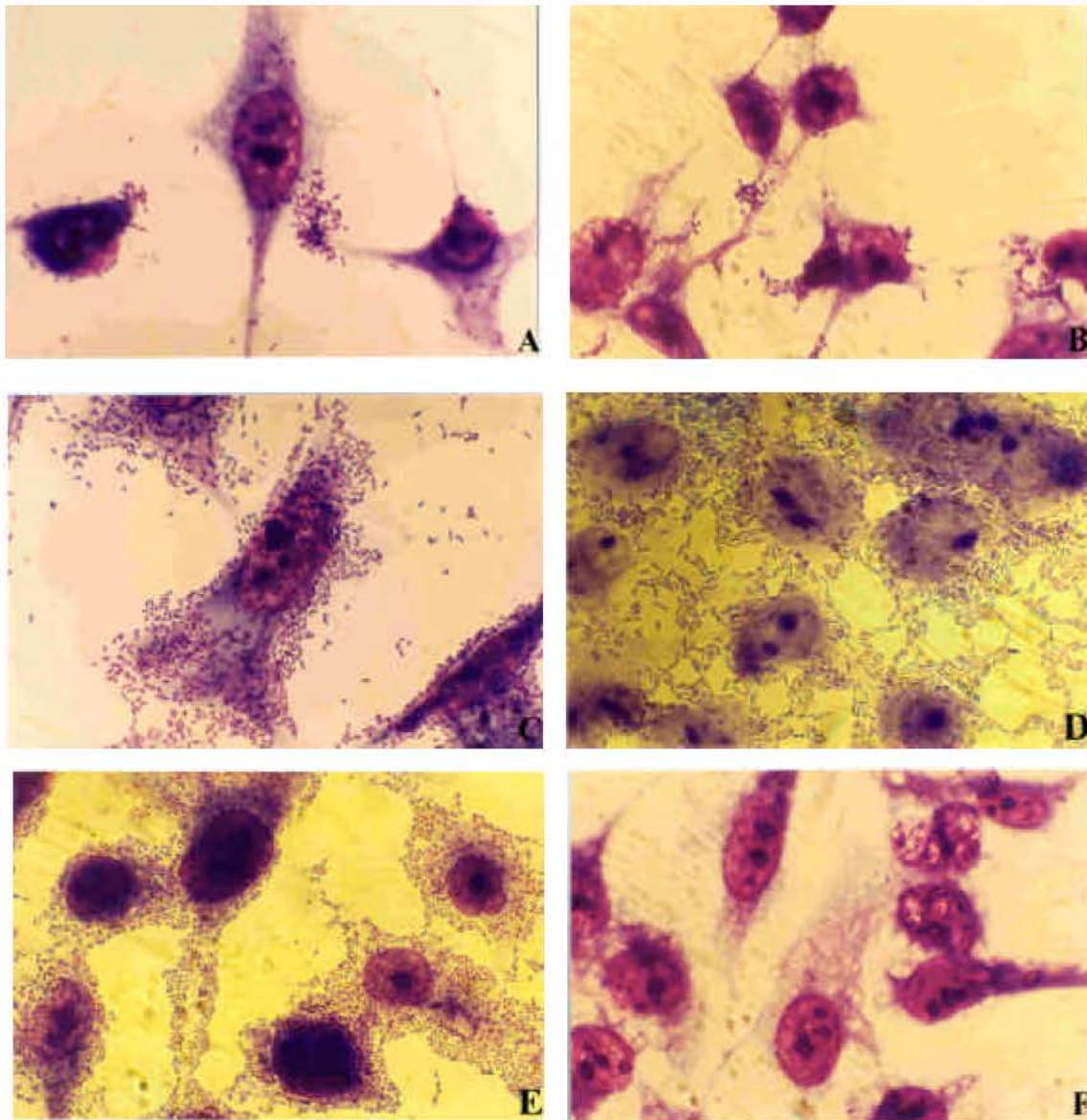


Fig. 1: HeLa cell adherence patterns. A: Localized adherence (LA), B: Localized adherence-Like (LAL), C: Diffuse adherence (DA), D: Aggregative adherence (AA), E: Undefined adherence (UDP), F: Non-adherence (NA)

Strains with LA were significantly associated with diarrhea (30 of 35 [86%] versus 5 of 35 [14%] in healthy persons; $p < 0.05$). Out of 111 strains isolated from patients, 57 strains (51.3%) were adhered to HeLa cell with a defined pattern (LA, LAL, DA, AA) and 33 (29.7%) showed an undefined pattern (UDP) and the remaining 21 (18.9%) were non-adherent (NA) (Table 2). Among adherence patterns, LA, LAL and AA found to be significantly associated with diarrhea ($p < 0.05$).

Out of the 80 strains isolates from healthy persons, 28 (35%) strains were adherent with a defined pattern and

39 (48.8%) strains were non-adherent. UDP was shown by 13 strains (Table 2).

DISCUSSION

Adherence to HeLa and HEP-2 tissue culture cells have been used as a marker of virulence of EPEC strains (Nakazato *et al.*, 2004; Forestier *et al.*, 1996; Gonzalez *et al.*, 1997). The present results support the evidence from prospective case control studies showing an association between strains with the LA pattern and

Table 3: Distribution of different patterns of adherence among EPEC serogroups

Serogroups	Adherence patterns						Total
	LA	LAL	DA	AA	UDP	NA	
O26	1	0	0	1	2	2	6(3.1)
O55	9	0	0	1	1	3	14(7.3)
O86	10	2	0	1	1	9	23(12.0)
O111	5	12	4	3	9	21	54(28.3)
O114	0	1	0	0	1	1	3(1.6)
O119	1	0	0	1	2	4	8(4.2)
O125	0	0	0	3	0	0	3(1.6)
O126	0	0	1	0	5	4	10(5.2)
O127	4	7	3	3	7	9	33(17.3)
O128	0	0	0	2	4	0	6(3.1)
O142	5	5	0	0	14	7	31(16.2)
Total	35(18.3)	27(14.1)	8(4.2)	15(7.9)	46(24)	60(31.4)	191(100.0)

diarrhea (Cravioto *et al.*, 1991; Gonzalez *et al.*, 1997). Present study and several case-control investigations have showed that LA-positive EPEC were more often isolated from diarrheal cases (Nataro *et al.*, 1985; Baudry *et al.*, 1990). The LA adherence pattern was associated with some EPEC serogroups (Scaletsky *et al.*, 1999; Gomes *et al.*, 1989). In present study, the most common serogroups with LA patterns were O86 and O55 (Table 3), which also was found in other studies (Cravioto *et al.*, 1991; Levine *et al.*, 1988). There was a significant association between strains with LAL pattern and diarrhea in our study ($p < 0.05$). The LAL pattern was found in strains belonging to O114, O111 and O127 classical serogroups (Table 3).

Several studies have implicated DAEC strains as agents of diarrhea (Germani *et al.*, 1996; Scaletsky *et al.*, 2002), while other studies have not recovered DAEC strains more frequently from diarrheal patients than asymptomatic controls (Gunzburg *et al.*, 1993; Germani *et al.*, 1996). In this study, there was no correlation between DA pattern and diarrhea.

E. coli strains that exhibited AA pattern were strongly associated with enteric diseases in Iran and other developing countries (Bouzari *et al.*, 1994; Aslani *et al.*, 1999; Beutin *et al.*, 2003). In our study, *E. coli* showing an AA pattern was found more frequently in patients (60%) than healthy cases (40%) ($p < 0.05$). The AA isolates belonged to the different EPEC serogroups (Table 3), a finding that has been demonstrated by other authors (Nataro and Kaper, 1998; Scaletsky *et al.*, 2002).

In conclusion, this study showed the importance of adherence virulence factor in EPEC with LA, LAL and AA patterns. There was a significant correlation between these patterns and diarrhea ($p < 0.05$). The existence of the LAL adherence pattern observed in patients with diarrhea may indicate a new mechanism of production of the disease. Further studies are necessary to confirm the present observation as well as to elucidate the

pathogenesis of this new type of enteric infection. Present study indicated that EPEC strains with adherence property are more related to diarrhea than the non-adherence ones.

ACKNOWLEDGMENTS

We thanks Dr. H. Shojaei and Dr. S. Bouzari for useful discussion. Mr. H. Khamse and Mr. N. Islami are acknowledged for their technical help.

REFERENCES

- Arikawa, K., I.M. Meraz, Y. Nishikawa, J. Ogasawara and A. Hase, 2005. Interleukin-8 secretion by epithelial cells infected with diffusely adherent *Escherichia coli* possessing Afa adhesin-coding genes. *Microbiol. Immunol.*, 49: 493-503.
- Aslani, M.M., N. Badami and S. Bouzari, 1999. Adherence patterns of Verotoxigenic *Escherichia coli* (VTEC) Non-O157 strains isolated from faecal samples in Iran. *Iran Biomed. J.*, 3: 71-75.
- Baudry, B., S.J. Savarino, P. Vial, J.B. Kaper and M.M. Levine, 1990. A sensitive and specific DNA probe to identify enteroaggregative *E. coli*, a recently discovered diarrheal pathogen. *J. Infect. Dis.*, 161:1249-1251.
- Beutin, L., O. Marches and K.A. Bettelheim *et al.*, 2003. Hep2- Cell Adherence, actin aggregation and intimin types of attaching and effacing *Escherichia coli* strains isolated from healthy infants in Germany and Australia. *Infect. Immun.*, pp: 3995-4002.
- Bouzari, S., A. Jafari, A.A. Farhoudi-Moghaddam, F. Shokouhi and M. Parsi, 1994. Adherence of non-enteropathogenic *Escherichia coli* to HeLa cells. *J. Med. Microbiol.*, 40: 95-97.
- Cravioto, A., A. Tello, A. Navarro, J. Ruiz, H. Villafan, F. Uribe and C. Eslava, 1991. Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhea. *Lancet*, 337: 262-264.
- Forestier, C., M. Meyer, S. Favre-Bonte, C. Rich, G. Malpuech, C. Le Bouguenec, J. Sirot, B. Joly and C. De Champs, 1996. Enteroadherent *Escherichia coli* and diarrhea in children: A prospective case-control study. *J. Clin. Microbiol.*, 34: 2897-2903.
- Germani, Y., E. Begaud, P. Duval and C. Le Bouguenec, 1996. Prevalence of enteropathogenic, enteroaggregative and diffusely adherent *Escherichia coli* among isolates from children with diarrhea in New Caledonia. *J. Infect. Dis.*, 174: 1124-1126.

- Gomes, T.A.T., M. Vieira, I.K. Wachsmuth, P.A. Blake and L.R. Trabulsi, 1989. Serotype-specific prevalence of *Escherichia coli* strains with EPEC adherence factor genes in infants with and without diarrhea in Sao Paulo, Brazil. *J. Infect. Dis.*, 160: 131-135.
- Gonzalez, R., M. Marino, R. Cloralt, M. Pequenez and I.P. Schael, 1997. Age-specific prevalence of *Escherichia coli* with localized and aggregative adherence in Venezuelan infants with acute diarrhea. *J. Clin. Microbiol.*, 35: 1103-1107.
- Gunzburg, S.T., B.J. Chang, S.J. Elliot, V. Burke and M. Gracey, 1993. Diffuse and enteroaggregative patterns of adherence of enteric *Escherichia coli* isolated from aboriginal children from the Kimberley region of Western Australia. *J. Infect. Dis.*, 167: 755-758.
- Harrington, S.M., E.G. Dudley and J.P. Nataro, 2006. Pathogenesis of enteroaggregative *Escherichia coli* infection. *FEMS. Microbiol. Lett.*, 254: 12-18.
- Jallat, C., V. Livrelli, A. Darfeuille-Michaud, C. Rich and B. Joly, 1993. *Escherichia coli* strains involved in diarrhea in France: High prevalence and heterogeneity of diffusely adhering strains. *J. Clin. Microbiol.*, 31: 2031-2037.
- Levine, M.M., V. Prado, R.M. Robins-Browne, H. Lior, J.B. Kaper, S.L. Moseley, K. Gicquelais, J.P. Nataro, P. Vial and B. Tall, 1988. Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli*. *J. Infect. Dis.*, 158: 224-228.
- Moon, J.Y., J.H. Park and Y.B. Kim, 2005. Molecular epidemiological characteristics of virulence factors on enteroaggregative *E. coli*. *FEMS. Microbiol. Lett.*, 253: 215-220.
- Moon, H.W., S.C. Whipp, R.A. Argenzio, M.M. Levine and R.A. Giannella, 1983. Attaching and effacing of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect. Immun.*, 41: 1340-1351.
- Nakazato, G., C. Gyles, K. Ziebell, R. Kell, L.R. Trabulsi, T.A.T. Gomes, K. Irino, W.D.D. Silveira and A.F.P. Castro, 2004. Attaching and effacing *Escherichia coli* isolated from dogs in Brazil: Characteristics and serotypic relationship to human enteropathogenic *E. coli* (EPEC). *Vet. Microbiol.*, 101: 269-277.
- Nataro, J.P., M.M. Baldini, J.B. Kaper, R.E. Black, N. Bravo and M.M. Levine, 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. *J. Infect. Dis.*, 152: 560-565.
- Nataro, J.P. and J.B. Kaper, 1998. Diarrheogenic *Escherichia coli*. *Clin Microbiol. Rev.*, 11: 142-201.
- Nataro, J.P., J.B. Kaper, R. Robins-Browne, V. Prado, P. Vial and M.M. Levine, 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Paediatr. Infect. Dis. J.*, 6: 829-831.
- Nunes, E.B., H.O. Saridakis, K. Irino and J.S. Pelayo, 2003. Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from children with and without diarrhoea in Londrina, Brazil. *J. Med. Microbiol.*, 52: 499-504.
- Scaletsky, I.C.A., S.H. Fabbri, S.O.C. Silva, M.B. Morais and U. Fagundes-Neto, 2002. HEp-2-Adherent *Escherichia coli* strains associated with acute infantile diarrhea, Sao Paulo, Brazil. *Emerging. Infect. Dis.*, 8: 855-858.
- Scaletsky, I.C.A., J. Michalski, A.G. Torres, M.V. Dulguer and J.B. Kaper, 2005. Identification and characterization of the locus for diffuse adherence, which encodes a novel afimbrial adhesin found in atypical enteropathogenic *Escherichia coli*. *Infect. Immun.*, 73: 4753-4765.
- Scaletsky, I.C.A., M.Z. Pedroso, C.A.G. Oliva, R.L.B. Carvalho, M.B. Morais and U.A. Fagundes-Neto, 1999. Localized adherence-like pattern as a second pattern of adherence of classic enteropathogenic *Escherichia coli* to HEp-2 cells that is associated with infantile diarrhea. *Infect. Immun.*, 67: 3410-3415.
- Scaletsky, I.C.A., J.S. Pelayo, R. Giraldo, J. Rodrigues, M.Z. Pedroso and L.R. Trabulsi 1996. EPEC adherence to HEp-2 cells. *Rev. Microbiol.*, 27: 58-62.
- Scaletsky, I.C.A., M.L.M. Silva and L.R. Trabulsi, 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. *Infect. Immun.*, 45: 534-536.
- Trabulsi, L.R., K. Rogéria and T.A.T. Gomes, 2002. Typical and atypical enteropathogenic *Escherichia coli*. *Emerging. Infect. Dis.*, 8: 508-513.