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CAPTURE IMMUNOASSAY FOR LT DETECTION PRODUCED BY ENTEROTOXIGENIC *ESCHERICHIA COLI* IN BACTERIAL ISOLATES

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ABSTRACT

A capture enzyme-linked immunosorbent assay (ELISA), which detects LT-I toxin produced by enterotoxigenic *Escherichia coli* strains, has been developed. This capture assay was performed using the IgG enriched fraction of anti-LT-I antiserum and IgG2b anti-LT-I monoclonal antibody and allowed a clear distinction between *E. coli* LT-I - producing and non-producing strains. The estimated accuracy of the assay is 78% for sensitivity, 94% for specificity and 92% for efficiency. Thus, the capture immunoassay is a sensitive tool for detection of *E. coli*, which produces heat-labile enterotoxin, and is suitable for use in clinical laboratories and epidemiological surveys in developing world.

Key words: detection, polyclonal antibody, monoclonal antibody, thermo-labile toxin, *E. coli.*

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a common cause of infectious diarrhea, especially in tropical climates, where uncontaminated water is not readily available. ETEC strains are an important cause of diarrhea among children in developing countries and are estimated to cause 700,000 deaths annually. ETEC are furthermore the main cause of traveler's diarrhea and, in Brazil, these pathogens are responsible for 7 to 20% of cases of infantile diarrhea (1,2,6). The ability of these strains to colonize the small intestine is mediated by appendages called colonization factors (CFs). In addition, the most important characteristic of them is the production of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST), which are responsible for the intestine's fluids loss. For detection of the LT of enterotoxigenic *E. coli*, various assays methods have been developed, namely, GM1 – ELISA, modified Elek test, staphylococcal agglutination test and RPLA (5). However, some of these assay methods are unsuitable for routine diagnosis. Molecular methods have been used to detect LT genes, however, they cannot demonstrate toxin production. Thus, the aim of this study was the development of a capture assay method using a polyclonal rabbit and a monoclonal mouse IgG2b anti LT antibodies (Mabs) for detection ETEC LT producing strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All *E. coli* strains used in this study were isolated from stools of children with and without diarrhea from sporadic cases in Brazil (1,7). All bacterial strains were grown in LB broth at

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37ºC for 18h under constant shaking (200 rpm). The bacterial growth was centrifuged at 5000 X g and the supernatant was filtered through a 0.45 mm membrane. These conditions were employed in all experiments.

LT antiserum

Rabbits were immunized subcutaneously with 100 µg LT (Sigma Chemical Co. St. Louis, MA, USA) in complete Freund's adjuvant and the IgG-enriched fractions were obtained from rabbit antisera after being submitted to caprylic acid and ammonium sulfate precipitation (4).

IgG2b monoclonal antibody

MAbs were produced as described by Köhler and Milstein (3) with some modifications. Popliteal lymphnode cells from BALB/c mice immunized with 2 µg of LT in complete Freund's adjuvant were fused with SP2-O cells (2:1) using polyethyleneglycol 4000 (Sigma Chemical Co. St. Louis, MA, USA). Hybrids were selected in HAT-RPMI 1640 medium (Sigma Chemical Co. St. Louis, MA, USA) containing 10% FCS (Cultilab, Campinas, SP, BR).

LT GM1 ELISA

Microtiter plates (C96 Maxisorp - NUNC) were incubated at 37ºC for 16h with 1.25 µg/mL ganglioside GM1 in phosphate-buffered saline, pH 7.2 (PBS). And reaction was developed according to Svennerholm and Holmgren (8).

Capture ELISA immunoassay

Microtiter plates (C96 Polysorp - NUNC) were incubated at 4ºC for 16h with 25 µg/mL of anti-rabbit LT IgG enriched fraction in carbonate-bicarbonate-buffered, pH 9.6. Supernatant of bacterial cultures were incubated for 1 hour at room temperature. Unbound toxin was removed by washing them three times with PBS. Toxin bound to IgG enriched fraction was then detected with 2 µg/mL of IgG2b monoclonal antibody followed by anti-mouse IgG peroxidase (Zymed, 1:10,000). All experiments were carried out in quadruplicate and results correspond to three independent experiments. Differences were considered significant when the probability of equality was less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Expression of LT toxin can be used as a practical way to detect enterotoxigenic *E. coli*. Thus detection of toxin using phenotypic methods is more accurate and allows to incriminate the real pathogen responsible for diarrheal. Based up on this facts we obtained a polyclonal rabbit and a mouse monoclonal IgG2b anti-LT antibodies for standardization of a capture assay. The capture assay was performed using *E. coli* strains, from which virulence factors were previously determined by molecular methods. Capture assay statistical analysis suggests that the difference between the LT producing and non-producing strains by mean and standard deviation were considered extremely significant ($p<0.0001$) (Fig. 1). The found values are 78% of sensitivity, 94% of specificity and 92% of efficiency. The capture assay was 3 fold more sensitive than GM1 ELISA. Thus this capture assay showed to be an excellent tool for LT producing strains detection and could be employed in the diagnosis of diarrhea caused by LT.

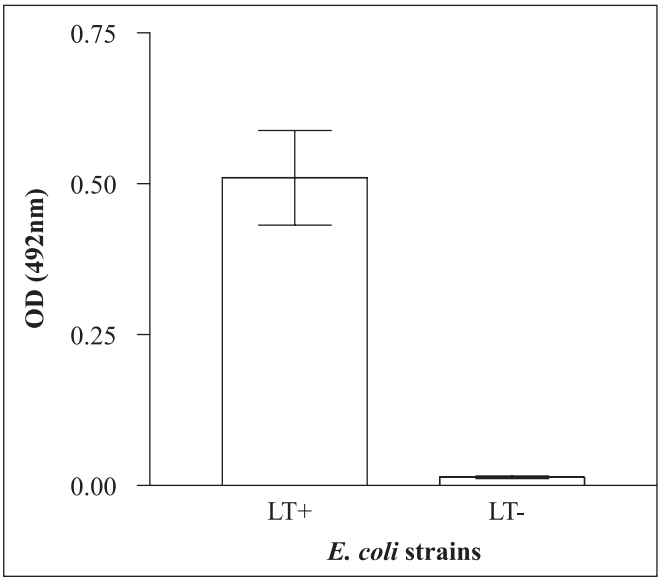

Figure 1. Distribution of LT producing and non-producing strains by capture assay. The reaction was detected with IgG enriched fraction of rabbit polyclonal and IgG2b monoclonal anti-LT. Goat anti-mouse IgG peroxidase labeled (1:10,000) and freshly prepared solution of OPD plus H_2O_2 recording of the A_{492} on a Multiskan EX ELISA reader. Bars represent 95% confidence interval.

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RESUMO

Imunoensaio de captura para detecção da toxina LT produzida por *Escherichia coli* enterotoxigênica em isolados bacterianos

O objetivo do presente trabalho foi a padronização de um imunoensaio de captura para detecção de amostras de *E. coli* produtoras da toxina LT-I. Este ensaio de captura foi desenvolvido utilizando-se a fração enriquecida em IgG do

anticorpo policlonal anti-LT e um anticorpo monoclonal caracterizado como IgG2b. Através deste método verificou-se uma clara distinção entre cepas de *E. coli* produtoras e não produtoras da toxina ($p< 0,0001$), sendo a sensibilidade do método de 78%, a especificidade de 94% e a eficiência de 92%. Assim, o imunoensaio de captura mostrou-se como uma ferramenta sensível para a detecção de amostras de *E. coli* que produzem a enterotoxina termo-lábil, podendo ser aplicado em laboratórios clínicos e inquéritos epidemiológicos em paises em desenvolvimento.

Palavras-chave: detecção, anticorpo policlonal, anticorpo monoclonal, toxina termo-lábil, *E. coli.*

REFERENCES

1. Fernandes-Filho, A.; Montemor, L.P.G.; Gomes, T.A.T.; Santos Filho, L.; Trabulsi, L.R.; Martinez, M.B. Enteropathogens associated with acute diarrheal disease in urban infants in João Pessoa, Brazil. ASM 103rd General Meeting, Washington, D.C. pp. 139, 2003.
2. Gomes, T.A.T.; Rassi, V.; MacDonald, K.L.; Ramos, S.R.T.S.; Trabulsi, L.R.; Vieira, M.A.M.; Guth, B.E.C.; Candeias, J.A.N.; Ivey, C.; Toledo, M.R.F.; Blake, P.A. Enteropathogens associated with acute diarrheal disease in urbans infants in São Paulo. *J. Infect. Dis.,* 164:331-337, 1991.
3. Köhler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256:495-497, 1975.
4. McKinney, M.M.; Parkinson, A. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. *J. Immunol. Methods*, 96:271-278, 1987.
5. Nataro, J.P.; Kaper, J.B. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.,* 11:142-201, 1998.
6. Reis, M.H.L.; Guth, B.E.C.; Gomes, T.A.T.; Murachovschi, J.; Trabulsi, L.R. Frequency of *Escherichia coli* strains producing heat-labile toxin and heat-stable toxin or both in children with and without diarrhea in São Paulo. *J. Clin. Microbiol.,* 15:1062-1064, 1982.
7. Souza, E.C.; Martinez, M.B.; Taddei, C.R.; Mukai, L.; Gilio, A.E.; Racz, M.L.; Silva, L.; Ejzenberg, B.; Okay, Y. Perfil etiológico das diarréias agudas de crianças atendidas em São Paulo. *J. Pediatr.,* 78(1):31-38, 2002.
8. Svennerholm, A.M.; Holmgren, J. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioiside immunosorbent assay (GM1-ELISA). *Current Microbiol*., 1:19-23, 1978.