EVALUATION OF A RAPID SCREENING ASSAY FOR BACTERIAL IDENTIFICATION (DOT-ELISA) IN FECAL SAMPLES FROM CHILDREN

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SUMMARY

With the objective of standardizing a Dot Enzyme-Linked Immunosorbent Assay (Dot-ELISA) to detect antigens of fecal bacterial enteropathogens, 250 children, aged under 36 months and of both sexes, were studied; of which 162 had acute gastroenteritis. The efficacy of a rapid screening assay for bacterial enteropathogens (enteropathogenic *Escherichia coli* "EPEC", enteroinvasive *Escherichia coli* "EIEC", *Salmonella* spp. and *Shigella* spp.) was evaluated. The fecal samples were also submitted to a traditional method of stool culture for comparison. The concordance index between the two techniques, calculated using the Kappa (k) index for the above mentioned bacterial strains was 0.8859, 0.9055, 0.7932 and 0.7829 respectively. These values express an almost perfect degree of concordance for the first two and substantial concordance for the latter two, thus enabling this technique to be applied in the early diagnosis of diarrhea in infants. With a view to increasing the sensitivity and specificity of this immunological test, a study was made of the antigenic preparations obtained from two types of treatment: 1) deproteinization by heating; 2) precipitation and concentration of the lipopolysaccharide antigen (LPS) using an ethanol-acetone solution, which was then heated in the presence of sodium EDTA.

KEYWORDS: Infantile diarrhea; Enteropathogens; Fecal antigens; Stool culture; Dot-ELISA.

INTRODUCTION

The possibilities of etiologic diagnosis of diarrheal disease have increased since 1970, following a series of technological advances. Especially after the identification of the *rotavirus*[5] and the *Campylobacter* spp.[41]; the development of diagnostic tests for enterotoxigenic *Escherichia coli* (ETEC)[28] and *Clostridium difficile*[27]; and the detection by electron microscopy of several enteric viruses[23]. Studies in Molecular Biology[6, 14, 40] have provided significant new data for the microbiologists, principally for epidemiological research.

The isolation of bacterial enteropathogens through stool culture is the classical method to identify an infection, enabling what is considered to be a definitive dignosis. However, this method has certain drawbacks; a considerable period of time for growth and bacterial identification is needed before the results from the culture can be obtained. Furthermore, identification of

the microorganism is not always possible, especially if the patients received antibiotic therapy before the fecal sample was collected[42].

Thus detection of antigens in body fluids[3,8,9,42] represents a new path ahead for microbiological diagnosis, enabling rapid results, irrespective of the viability of the microorganisms or their growth rate. Among the methods developed for this, the immunoenzymatic Dot-ELISA test as described by PAPPAS *et al.* (1983)[34], (1984)[35], derived from the Dot-Immunobinding Assay presented by HAWKES *et al.* (1982)[16], with a modification to the Dot-hybridization of KAFATOS *et al.* (1979)[19], to test monoclonal antibodies offers an extensive applicability to the laboratory diagnosis of different types of microorganisms[1,30,34,45]. Particularly taking into consideration that it can be employed to detect a wide variety of substances, such as: soluble proteins,

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nucleic acids, membranes, various organelle, fungi, protozoa, bacteria and viruses[7]. The present study has the following objectives: 1) The standardization and evaluation of the possibility of applying the immunoenzymatic Dot-ELISA assay using stool samples for the detection of antigens of the most frequent bacterial enteropathogens, i.e. enteropathogenic *Escherichia coli* (EPEC), enteroinvasive *Escherichia coli* (EIEC), *Salmonella* spp. and *Shigella* spp; 2) Verify the efficacy of the method used to treat stool samples to make clear the lipopolysaccharide; 3) To establish the degree of sensitivity, specificity and concordance of this technique compared with the classical stool culture methodology.

MATERIALS AND METHODS

Biological Samples: Fecal samples from 250 children of both sexes aged up to 36 months were obtained from the Instituto da Criança do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, (Brazil) during the period from August 1994 to November 1995. Of these, 162 samples were from children clinically diagnosed as having acute gastroenteritis and 88 samples from children with no gastrointestinal symptoms. These samples were collected in Cary & Blair medium, stored at 37°C for posterior stool culture and at 1:3 (weight/volume) in PBS (0.01 M phosphate buffered saline solution pH 7.2) and kept at 4°C, for later preparation and use in Dot-ELISA at the Laboratório de Investigação Médica de Bacteriologia (LIM-54) da Faculdade de Medicina da Universidade de São Paulo. On a parallel basis, the same samples were checked for the presence of specific viral, bacterial and parasitic agents (cysts and trophozoites of protozoa as well as helminth eggs and larvae), at the Instituto Adolfo Lutz in São Paulo.

Microbiological Tests: the bacteriological studies of the fecal samples were performed according to the methodology described by EDWARDS & EWING (1986)[10].

Immunological Test: Dot-ELISA
Stock Antigens:
1 – Bacterial strains used for the standardization of the assays were supplied by the Laboratório Probac do Brasil Produtos Bacteriológicos Ltda.: enteropathogenic *Escherichia coli* O111, O125, O126; enteroinvasive *Escherichia coli* O112, O28; enterohemorrhagic *Escherichia coli* O157, *Shigella dysenteriae* 1, 9; *Shigella boydii* 4, 7, 8; *Shigella flexneri* var. x; *Shigella sonnei*; *Salmonella typhimurium*; *Yersinia* enterocolitica O3; *Vibrio cholerae* O1.

Preparation of the Antigen:
1) The antigens were obtained from the above-mentioned bacterial strains, according to the methodology used by CHOI *et al.* (1990)[7].

2) Fecal samples were submitted to two types of treatment:

- Heating for deproteinization, at 100°C for 30 minutes, according to DOSKELAND & BERDAL (1980)[9].

- Treatment for the precipitation and concentration of the lipopolysaccharide antigen (LPS) according to

SUWANAGOOL *et al.* (1986)[42] and REQUEJO *et al.* (1991)[36].

Hyperimmune sera: Polyvalent sera, prepared in rabbits and supplied by PROBAC do Brasil Produtos Bacteriológicos Ltda., were used in the Dot-ELISA assays, as listed below:

Anti enteropathogenic *E. coli* (EPEC) serum; anti enteroinvasive *E. coli* (EIEC) serum; *anti Shigella* spp. serum and anti somatic *Salmonella* spp. serum. In compliance with the manufacturer's recommendations, these were stored in a refrigerator at 4°C until used.

Absorption of antisera with heterologous antigens: this was effected following the techniques described by JONGH-LEUVENINK *et al.* (1985)[18] and EDWARDS & EWING (1986)[10]. An initial test was made to ascertain with which antigen (obtained from the strains mentioned in the section "Immunological tests") each antiserum presented a cross-reaction. The selection of the bacterial antigens to be used in a pool for the absorption of the antisera at the proportion of 3×10^9 bacteria/ml per bacterial strain was based on these tests.

Titration of the antisera: after absorption, doubling dilutions of the antisera were prepared in TBS (20 mM TRISMA, 500 mM NaCl pH 7.5) from 1:50 up to 1:12,800. The titration was effected using the Dot-ELISA technique and the stock antigens obtained from the bacterial strains responsible for the production of the antisera. These were pre-heated for 15 minutes[10] and diluted in ratio 2 up to 1:128, according to the example shown in Figure 1.

Horseradish peroxidase goat anti-rabbit IgG conjugate (IgG-HRP): a goat anti-rabbit IgG labelled with peroxidase (Sigma Chemicals Co., St. Louis, USA, code number A-0545), stored at $-20°C$ in 0.1 ml aliquots.

Titration of the conjugate (IgG-HRP): Conjugate dilutions of 1:2,000, 1:5,000, 1:10,000; 1:15,000 and 1:20,000 against a fixed dilution of antigen 3×10^9 bacteria/ml (absorbance 1 at 540 nm) and the non-labelled antibody at 1:100. The titer of the conjugate corresponded to the greatest dilution that was still capable of giving maximum reactivity in the absence of any reaction in the negative control test (normal rabbit serum).

Test conditions: Several experiments were necessary to find the optimal conditions for each stage of the test. The optimal volume of antigen was 2µl of a solution corresponding to 3×10^9 bacteria/ml (absorbance 1 at 540 nm). The optimum dilutions were: 1:10,000 for the conjugate and 1:100 for the antisera of the various bacterial strains. The reaction was assessed visually and a positive result was established as equal to or greater than the cut-off value 1+.

Based on the consulted literature[7,16,24,30,35,36] and a series of titrations and assays, appropriate values were established for that enabled a standardization of the Dot-ELISA immunoenzymatic assay. The materials used were reference reagents together with
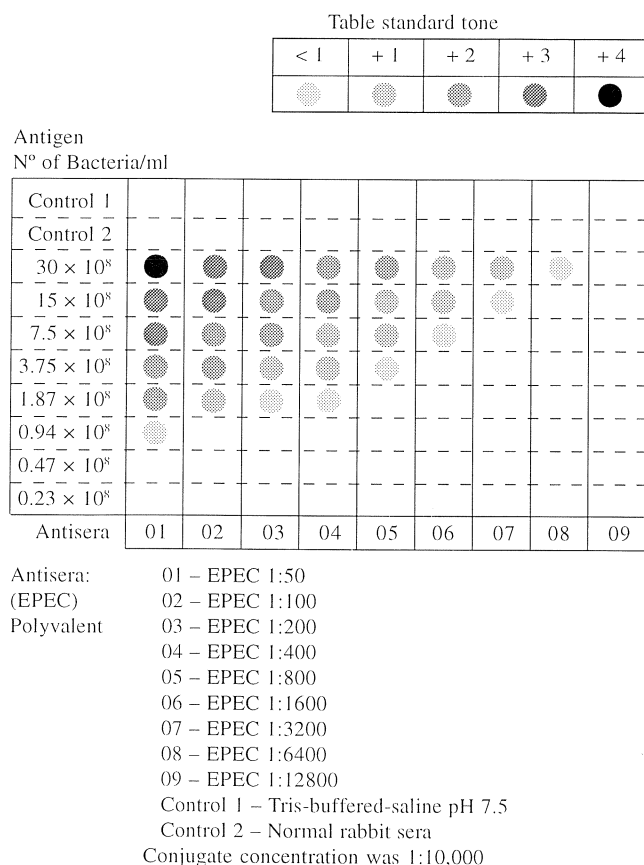
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< 1	+ 1	+ 2	+ 3	+ 4
○	◌	●	●	●

Antigen
N° of Bacteria/ml

	01	02	03	04	05	06	07	08	09
Control 1									
Control 2									
30 × 10⁸	●	●	●	●	●	●	●	●	
15 × 10⁸	●	●	●	●	●				
7.5 × 10⁸	●	●	●	●	●	●			
3.75 × 10⁸	●	●	●	●	●				
1.87 × 10⁸	●	●	●	●					
0.94 × 10⁸	●								
0.47 × 10⁸									
0.23 × 10⁸									
Antisera	01	02	03	04	05	06	07	08	09

Antisera: 01 – EPEC 1:50
(EPEC) 02 – EPEC 1:100
Polyvalent 03 – EPEC 1:200
04 – EPEC 1:400
05 – EPEC 1:800
06 – EPEC 1:1600
07 – EPEC 1:3200
08 – EPEC 1:6400
09 – EPEC 1:12800
Control 1 – Tris-buffered-saline pH 7.5
Control 2 – Normal rabbit sera
Conjugate concentration was 1:10,000

Fig. 1 – Standardization of Dot-ELISA technique through enteropathogenic *Escherichia coli* strain (EPEC-O111).

Antigen linked to nitrocellulose strips
↓
Blocking
↓
Washing
↓
Antibody Incubation
↓
Washing
↓
Anti IgG-peroxidase
↓
Washing
↓
Substrate
↓
Color reaction

Fig. 2 – Procedure used in Dot-ELISA technique.

Washing: The nitrocellulose strips in the acrylic tray were washed 3 times with Tris-buffered-saline (TBS pH 7.5) for 10 minutes while under constant shaking, as shown in Figure 3.

Incubation with Hyperimmune sera: Antisera against the EPEC, EIEC, *Salmonella* spp. and *Shigella* spp. antigens, supplied by PROBAC do Brasil, were diluted at 1:100 in Tris-buffered-saline (TBS pH 7.5) containing 1% of skimmed milk and incubated at 37°C for one hour while under constant shaking.

positive and negative control antigens obtained by treatment of the bacterial strains and stool samples.

Dot-ELISA technique

The tests were performed using the methods of HAWKES *et al.* (1982)[16] and PAPPAS *et al.* (1983)[34] and (1984)[35], though with some modifications. As a support for the technique a nitrocellulose membrane (0.22µm pore size) (Schleicher & Schuell, BA83) was used. This was cut into 7 × 0.5 cm strips and carefully placed in the canals of an acrylic tray in accordance with BENNETT & YEOMAN (1983)[2]. Prior to this, areas of 0.5 × 0.5 cm were marked off on the strips and onto each of these squares samples obtained from fecal material antigen or pure cultures of bacterial strains were applied with a micropipette (2µl). Then the antigen was fixed in an oven at 37°C for 15 minutes. The test was then carried out as shown in the schematic diagram in Figure 2.

Blocking: This was achieved by adding a blocking solution selected through pre-testing: (gelatin at 5% in Tris-buffered-saline, pH 7.5) for 60 minutes at 37°C while under constant shaking, with mild movements over a stirring plate.
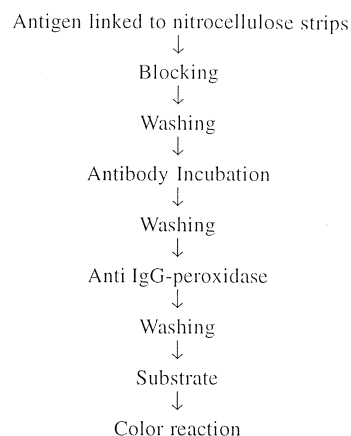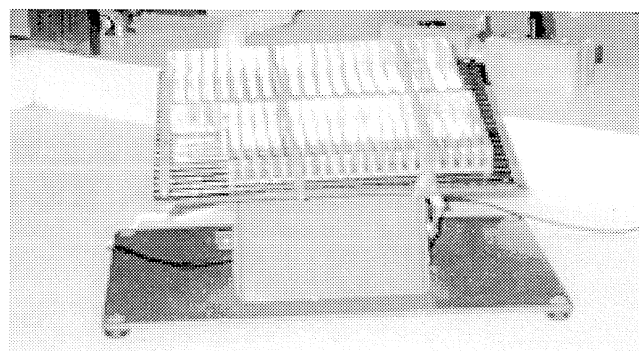

Fig. 3 – Nitrocellulose membrane cut into strips and laid into the canals of an acrylic tray on agitator.

Incubation with anti-rabbit IgG peroxidase conjugate (IgG-HRP): This was effected in a dilution of 1:10,000 in Tris-buffered-saline (TBS pH 7.5) and incubated at 37°C for one hour, while under constant shaking.

Detection of immunoreaction: This was achieved by immersing the strips of nitrocellulose in a chromogenous revealing solution containing 4-chloro-1 naphthol (Sigma Chemical Co., St. Louis, USA), for 10 minutes, according to HAWKES et al. (1982)[16]. The reaction was then interrupted by rinsing several times with distilled water. The membranes were dried between filter paper sheets. Positive reactions were demonstrated by the appearance of round stains with a blue tone rated by crosses (1+ to 4+) and the negative reaction by light tones, below 1+, or absence of spots.

Statistical Analysis: Tables were prepared comparing the results obtained from the two techniques (Dot-ELISA and stool culture). Using the computer program S.A.S. – Statistical Analysis System[38]. The evaluation indexes of sensitivity, specificity, efficiency and degree of agreement were calculated as described by FLETCHER et al. (1983)[12].

RESULTS

Using the traditional stool culture method it was ascertained that fifty eight (23.2%) of the 250 children's fecal samples (0-3 years) revealed the presence of bacterial enteropathogens distributed as shown in Table 1. In the group which presented diarrhea, 22 samples (13.5%) presented parasites and 32 viral agents (19.8%), stressing that rotavirus was present in 26 samples (16%) whereas in the control group 20 patients (22.7%) were parasite carriers and 5 (5.7%) viral carriers.

TABLE 1

Bacterial enteropathogens found in 250 fecal samples, obtained from children aged up to 36 months, grouped according to those with or without diarrhea.

Bacterial Enteropathogens	Patients with diarrhea (162)*		Patients without diarrhea (88)*	
	N°	%	N°	%
EPEC[a]	19	11.7	2	2.3
ETEC[b]	4	2.5	0	0.0
EIEC[c]	5	3.1	0	0.0
Shigella spp	11	6.8	2	2.3
Salmonella spp	6	3.7	1	1.1
Campylobacter jejuni	4	2.5	2	2.3
Clostridium difficile	2	1.2	0	0.0
TOTAL	51	31.5	7	8.0

* Number of samples.
[a]EPEC enteropathogenic Escherichia coli
[b]ETEC enterotoxigenic Escherichia coli
[c]EIEC enteroinvasive Escherichia coli

In search of means to improve the sensitivity of the Dot-ELISA technique, two procedures were used to treat the samples[9, 36], with some modifications, the results of which are presented in Tables 2 and 3. It was observed that very few samples would react before being submitted to treatment for deproteinization and antigen concentration.

Comparison of the efficacy between stool culture and Dot-ELISA immunoenzymatic technique: Tables 2 and 3 show the results obtained from bacterial tests on the stool samples. These were compared with the values from the Dot-ELISA assays. In the samples from the group of children with diarrhea, it was observed that the degree of antigenic identification improved significantly after performing antisera absorption several times (3 to 8) with heterologous antigens, thus eliminating most of the non-specific reactions; this was verified through cross-reaction, according to BECH-NIELSEN et al. (1992)[1].

TABLE 2

Comparison of the techniques applied for detection of bacterial enteropathogens in stool samples from the control group of 88 children without diarrhea.

Bacteria	Culture	Dot-ELISA*	Dot-ELISA (LPS)**
EPEC[a]	2 (2.3)+	2 (2.3)	3 (3.4)
EIEC[b]	0 (0.0)	0 (0.0)	1 (1.1)
Salmonella spp.	1 (1.1)	0 (0.0)	1 (1.1)
Shigella spp.	2 (2.3)	2 (2.3)	4 (4.5)
TOTAL	5 (5.7)	4 (4.6)	9 (10.2)

+ Number (%)
* Treatment for deproteinization
** Treatment for lipopolysaccharide concentration and precipitation
[a] EPEC enteropathogenic Escherichia coli
[b] EIEC enteroinvasive Escherichia coli

TABLE 3

Comparison of the techniques applied for detection of bacterial enteropathogens in stool samples from 162 children with diarrhea.

Bacteria	Culture	Dot-ELISA*	Dot-ELISA (LPS)**
EPEC[a]	19 (11.7)+	17 (10.5)	20 (12.3)
EIEC[b]	5 (3.1)	4 (2.5)	6 (3.7)
Salmonella spp.	6 (3.7)	3 (1.9)	4 (2.5)
Shigella spp.	11 (6.8)	9 (5.6)	13 (8.0)
TOTAL	41 (25.3)	33 (20.4)	43 (26.5)

+ Number (%)
* Treatment for deproteinization
** Treatment for lipopolysaccharide concentration and precipitation
[a] EPEC enteropathogenic Escherichia coli
[b] EIEC enteroinvasive Escherichia coli

Table 4 shows the calculations of sensitivity, specificity and prognostic value.

DISCUSSION

The Dot-ELISA technique was used to perform simultaneous tests for antigens in stool samples from children with diarrhea.

TABLE 4
Evaluation of the Dot-ELISA, compared with stool culture, in 162 children with diarrhea.

Bacterial enteropathogens	Sensitivity %	Specificity %	Efficiency %	Kappa Index	Classification according to FLEISS
EPEC[a]	94.7	97.9	97.5	0.8859	almost perfect
EIEC[b]	100.0	99.4	99.4	0.9055	almost perfect
Salmonella spp	66.7	100.0	98.7	0.7932	significant
Shigella spp	90.9	97.3	96.9	0.7829	significant

[a] EPEC enteropathogenic *Escherichia coli.*
[b] EIEC enteroinvasive *Escherichia coli.*
Obs.: These rates were calculated as described for FLETCHER et al. (1983)[12].

This methodology enables the use of polyvalent antisera or other antibodies[16]. The choice of polyvalent antisera against the antigen "O" of the bacterial enteropathogens in the study was determined by the antibody's heterogeneous affinity[26, 33, 43]. According to NISONOFF & PRESSMAN (1958)[33], research into the combination of antibodies with antigens or haptens almost always involved serum "pools", due to the greater reactivity of the "antibodies induced by antigens" and "antibodies produced spontaneously" against the different types of antigenic determinants. Furthermore, the amount of reagents, conjugate, chromogen solution and time necessary are reduced, moreover the test does not require sophisticated apparatus: thus facilitating its application in field work, epidemiological research and laboratorial routine[30, 45].

The use of nitrocellulose membrane, Schleicher & Schuell (BA-83, 0.22 µm) provides excellent sensitivity, avoiding the loss of polipeptides smaller than 20,000 daltons[24].

It was observed that the use of a heated blocking solution gelatin at 5% in Tris-buffered-saline (TBS, pH 7.5) enabled the retention of the antigen in the nitrocellulose membrane, thus avoiding loss during the washing stages as well as filling the remaining pore spaces, as described by LIN & KASAMATSU (1983)[24].

The secondary reactions were diminished when the antibodies used were diluted in a lactic solution (1% skimmed milk in Tris-buffered-saline, pH 7.5) as described by HAWKES et al. (1982)[16].

The antisera used for the bacterial strains, initially presented titers ranging from 400 to 6400. However, after repeated absorptions and standardization, the titer used corresponded to 100, inline with other studies related to various microorganisms (viruses, bacteria, fungi and parasites)[11, 17, 20, 22, 30, 39]. From the literature we observed that this fact was due to a molar proportion, between the antibody and the antigen or hapten, responsible for the equivalence point at which the values are approximately optimal for the antigenic determinants to interact with the antibodies present in the antiserum, thus determining the average affinity expressed by the equilibrium constant "K"[43]. This fact confirmed by MINDEN et al. (1969)[29] in their research to detect the antibody's primary link to the antigen using seven types of procedure (quantitative and qualitative) with human and rabbit antisera. They demonstrated that a dilution of the antiserum to approximately 1:100 led to a greater affinity of the antibody "population" to the antigen. However, the effect of this dilution generally depends on the time between the initial exposure of the animal to the antigen and the extraction of the antiserum, the fourteenth day being the ideal time.

The main bacterial enteropathogens were found to be prevalent in children with diarrhea in São Paulo (see Table 2), according to GOMES et al. (1991)[15].

The results shown in tables 2 and 3 demonstrate that, the use of the procedures detailed in the present study, enable a greater specificity and sensibility in the detection of bacterial antigens in body fluids, via the deproteinization and concentration before the test is performed[3, 9, 36]. This results in the liberation of complex antigens with antibodies, reducing the non-specific interferences, according to DOSKELAND & BERDAL (1980)[9] and LYERLY et al. (1988)[27]. MORRISON & LEIVE (1975)[31] suggested that the heterogeneity[13, 18, 25, 32] of the outer membrane structure containing lipopolysaccharides, with different molecular weights and configurations (LPS-fraction I and II) covering the cellular surface, enables a greater stability for the antigens.

Several modifications were made to improve the test, despite these, non-specific reactions still persist in patients with diarrhea (9.9%) as well as in patients considered to be clinically normal (5.7%). These were evaluated through the observation of simultaneous positive reactions (≥ 1+) for antigens of the various enteropathogens tested. REQUEJO et al. (1992)[37] cited that cross reactivity occurs frequently in immunological tests, due to the existence of many groups of antigenic determinants, occurring particularly in polysaccharides; these being distributed in many microorganisms, as well as animal and plant tissue[4]. TURK (1959)[44] studied the immunoadherence of antibodies found in human serum, guinea pig and rabbit, verifying their reactivity with particles of starch (in a similar way to the milk casein and gelatine used in this study) and the different bacteria, including *Salmonella typhi, Shigella flexneri, Erysipelothrox rhusiopathiae* and *Staphylococcus aureus.* The conclusion was reached that the total activity against these antigens detectable in normal serum

was due to the combined effects of specific and non specific factors, the former associated with the "euglobulin" fraction, and the latter with the "pseudoglobulin".

However in the present research the bacterial antigens were specifically recognized by the homologous antibody, thereby proving the high degree of specificity achieved by this technique.

Despite the relatively low number of samples tested per enteropathogen, the diagnostic indexes obtained (see Table 4), demonstrate that this technique can be applied viably.

The Dot-ELISA technique should not substitute the bacterial culture method, but is an alternative for the diagnosis of intestinal infections, principally in cases where the patient has already been treated with antibiotics.

Future studies are needed to collect information related to the detection of antigens of enteropathogenic bacteria in fecal material in order to make further comparison of the data.

RESUMO

Avaliação de um teste rápido de identificação bacteriana (Dot-ELISA) em amostras fecais de crianças

Com o objetivo de padronizar um Dot Enzyme-Linked Immunosorbent Assay (Dot-ELISA) para a detecção de antígenos de enteropatógenos bacterianos fecais, estudaram-se 250 crianças, abaixo de 36 meses de idade, de ambos os sexos, 162 portadoras de gastroenterite aguda. Avaliou-se a eficácia de um teste rápido para bactérias enteropatógenas (*Escherichia coli* enteropatogênica "EPEC", *Escherichia coli* enteroinvasora "EIEC", *Salmonella* spp. e *Shigella* spp.). As amostras fecais foram também submetidas à metodologia tradicional de coprocultura para comparação. Os índices de concordância entre as 2 técnicas, calculado através do índice Kappa (k) para as cepas bacterianas foram 0,8859; 0,9055; 0,7932 e 0,7829 respectivamente. Estes valores expressam um grau de concordância quase perfeito nos 2 primeiros casos e significante para os 2 últimos, o que permite que a técnica seja aplicada no diagnóstico precoce das diarréias infantis. Procurando-se aumentar a sensibilidade e especificidade deste teste imunológico, efetuou-se um estudo de preparações antigênicas obtidas através de 2 tipos de tratamentos: 1) desproteinização por aquecimento; 2) precipitação e concentração do antígeno lipopolissacáride (LPS) através de uma solução de etanol-acetona, com posterior aquecimento na presença de EDTA sódico.

ACKNOWLEDGMENTS

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