DETECTION OF LABILE ENTEROTOXIN OF *E. COLI* BY THE BIKEN ASSAY AND THE GM_1-ELISA

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SYNOPSIS

One hundred and twelve *E. coli* strains from diarrhoeic children were tested for labile toxin production (LT) by the Biken assay kit (Institute Virion) and a GM_1-ELISA (monosialoganglioside GM_1). Ninety four of the 112 strains agreed on both assays — 93 being classified as negative and one strain being classified as positive. A further 18 strains were identified as positive by the GM_1-ELISA. With a specificity of 97:5%, the GM_1-ELISA was more sensitive than the Biken assay kit. However, the GM_1-ELISA is not suitable for routine screening of *E. coli* isolates but an improved and more sensitive test similar to the Biken kit may be appropriate.

SING MED J. 1988; 29:17–19

INTRODUCTION

A non-invasive secretory diarrhoea is produced by enterotoxigenic *E. coli* (ETEC) via the elaboration of a heat stable (ST) or a heat labile (LT) enterotoxin or both. ST is a low molecular weight, non-immunogenic polypeptide; LT however is a high molecular weight immunogenic polypeptide (1). In addition, LT is similar to cholera toxin (CT) in structure, function and immunogenicity and cross-reacts with anti-CT (2).

Current detection methods established for ETEC both bacteriological and immunological, are dependent on the demonstration of production of ST or LT, or both. The established assay for ST to date is the infant mouse assay (3). The progress of developing an *in vitro* assay for ST has been hindered by the non-immunogenicity of ST.

The development of *in vitro* assays for LT has been more rapid, and sequential stages of development have paralleled those for cholera toxin. Classically animal models were used (4) followed by tissue culture systems (5,6,7). The immunogenic property of LT now allows the detection of LT in immunological assays using antisera raised to LT or CT. These assays include the ELISA (8), Biken assay (9), coagglutination assays (10,11) and radioimmunoassay (12). The ELISA and coagglutination assays require the preparation of culture extracts which are used to assay for the presence of LT. In the Biken and radioimmunoassay, bacterial colonies grown on antiserum incorporated agar are lysed *in situ* to release LT, which then forms antigen-antibody complexes.

In this communication, we report the comparison of the Biken assay kit (Institute Virion) for the detection of LT with a GM_1-ELISA (monosialoganglioside GM_1) (8) in *E. coli* strains isolated from diarrhoeic stools of Malaysian children, to establish the feasibility of the Biken kit for detection of ETEC in a routine diagnostic laboratory.

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MATERIALS AND METHODS

Bacterial strains A total of 112 *E. coli* strains as defined by biochemical testing at the Department of Medical Microbiology, University of Malaya, from diarrhoeic stools of children aged newborn to five years were included in this study. None of the strains belonged to the classical enteropathogenic *E. coli* serotypes. *E. coli* strain 487475 (ICDDR, Bangladesh) of clinical origin and *E. coli* WF5 (University of Surrey, U.K.) of environmental origin, were used as the LT positive and negative control strains respectively. All strains were stored on nutrient agar slopes at + 4C° for the duration of this study.

GM_1-ELISA Cell-free culture supernatants were prepared as described in WHO Manual (13) for use in the ELISA. Each *E. coli* strain was cultured in 5 mls of tryptone soy broth (Oxoid, U.K.), plus 1.2% yeast extract (Oxoid, U.K.). These were incubated on a slant, with loose caps for maximum aeration at 37°C for 48 hours. The broth cultures were centrifuged at 12000 × g for 15 minutes to pellet the bacterial cells and the supernatant harvested. The ELISA was carried out as described by Sack (8) and modified by Miller (14). Culture supernatant, as prepared above, was added to GM_1 (Radley-Superchem U.K.) adsorbed to 96-well Linbro microtiter plates (Flow Labs, U.K.), followed by the addition of anti-LT rabbit (WHO, Switzerland). To this, peroxidase-labelled swine anti-rabbit immunoglobulin (Dako-Patt, U.K.) was added. Substrate, orthophenylene diamine (Sigma, U.K.), was then added and the reaction stopped after 20 minutes by the addition of 8N H_2SO_4. The plates were read spectrophotometrically at 492nm by a Flow Multiskan ELISA reader. Commercially produced cholera toxin (Sigma, U.K.) was used at dilutions of 10^{-6} to 10^{-9}.

A negative: positive cut-off value was calculated from the optical density (OD) values obtained in the wells containing culture supernatants of control negative strain WF5. This calculated value was equal to the negative OD value + 2 standard deviation, corresponding to a specificity at 97.5%.

Biken assay The Biken assay was carried out as per instructions of the Biken kit. In brief four *E. coli* strains, two test and two control strains were inoculated on to Biken agar as per template provided. Following incubation at 37°C for 48 hours, polymyxin B discs (500 IU) were placed on the colonies and at the same time a 4 mm central well was punched in the agar. Following further incubation for six hours at 37°C, 20ul of anti-LT was added to the central well. The plates were further incubated at 37°C and observed for lines of precipitation between the bacterial colonies and the central well after 24 and 48 hours.

TABLE 1
COMPARISON OF THE BIKEN ASSAY WITH GM$_1$-ELISA FOR THE DETECTION OF THE HEAT-LABILE ENTEROTOXIN OF *E. COLI*

LT ASSAY METHODS			TOTAL NO. OF STRAINS
GM$_1$-ELISA	BIKEN 24 hrs	BIKEN 48 hrs	
+	+	+	1
+	+	−	0
+	−	+	0
+	−	−	18
−	+	+	0
−	+	−	0
−	−	+	0
−	−	−	93

RESULTS

One hundred and twelve *E. coli* strains from diarrhoeic stools of children aged newborn to five years were used to compare the Biken assay kit with GM$_1$-ELISA. No other enteric bacterial pathogen had been identified by standard bacteriological methods in these stools.

The results (Table 1) indicate that 94 out of 112 strains agreed on both assays, 93 strains were negative for ETEC by both assays and one strain was positive. The GM$_1$-ELISA identified a further 18 strains as being positive which the Biken assay failed to identify. In the Biken assay there was no difference in classification of strains as positive or negative when the plates were read after 48 hours instead of 24 hours. However the lines of precipitation were thicker after 48 hours as compared to 24 hours.

DISCUSSION

Acute diarrhoea is one of the leading causes of mortality in children under five years of age and morbidity in adults in developing countries. About 10 years ago, bacteriological studies of diarrhoea in different geographic areas showed that the majority of infections (60–80%) were not associated with any known enteropathogen. As a result of improved diagnostic methods and increased surveillance, rotavirus and enterotoxigenic *E. coli* are now known to be very significant and at present about 20% of cases remain undiagnosed (15).

The detection of ETEC from diarrhoeic stools in routine microbiological laboratories has been greatly hindered by the non-availability of rapid, inexpensive and simple assays. With the development of the Biken assay, the possibility of overcoming these obstacles seemed promising. Hence we undertook to compare the performance of the Biken Virion kit and GM$_1$-ELISA The GM$_1$-ELISA was chosen as the standard assay because it is as sensitive and specific as the radioimmunoassay (16) and Y1 adrenal cells assay (8) for detecting *E. coli* LT.

Both assays agreed on 94 out of 112 strains, giving a concordance rate of 84%, of which 93 strains were identified as non-toxigenic and only one strain as positive. A further 18 strains were positive for ETEC by the GM$_1$-ELISA but negative by the Biken assay. The findings of this study indicate that the Biken assay was less sensitive than the GM$_1$-ELISA in detecting LT. This was expected as gel diffusion assays are generally biologically less sensitive and may therefore not detect low levels of toxins. Another important consideration is that, in our study, the Biken assay had a propensity of identifying strains as negative. This may have been due to the titre of anti-LT used. The amount of precipitate formed varies with the levels of toxin and anti-LT available. Any immunological complex formed in the presence of a low anti-LT titre would not have precipitated. This may have been the case with the Biken assay kit that we used and therefore the kit should perhaps be re-evaluated with another batch of anti-LT.

When comparing both assays, the ease and rapidity of performance and equipment outlay are important. Results of the GM$_1$-ELISA may be obtained within 72 hours after primary subculture as the ELISA may be performed over 24 hours. However, both assays are easy to perform and read. Automation is required to read results accurately for the GM$_1$-ELISA although strong positives can be read visually. The initial cost to set up the GM$_1$-ELISA may be more expensive than the Biken kit but the GM$_1$-ELISA has proven to be more sensitive than the Biken assay.

In conclusion, although attempts have been made to develop simple and rapid assays for the routine diagnosis of LT-producing toxigenic *E. coli* from diarrhoeic stools, the reliability of the Biken assay is yet to be proven in routine diagnostic laboratories. Although the assay itself requires simple media and is easy to perform, a total time of five working days is required to obtain results from the time the stool sample is first obtained, which is not rapid enough for clinical decision-making. The Biken assay may perhaps be an important initiating point for "simpler" LT assays but this is still a long way from reality.

ACKNOWLEDGEMENTS

This work was supported by University of Malaya Research Grants F56/83 and F66/86.

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