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THE BIKEN TEST FOR DETECTION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* PRODUCING HEAT-LABILE ENTEROTOXIN (LT)
A LABORATORY MANUAL

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November 1981

Special Publication No. 16

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PREFACE

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is an autonomous, international, philanthropic and non-profit centre for research, education and training as well as clinical service. The Centre is derived from the Cholera Research Laboratory (CRL). The activities of the institution are to undertake and promote study, research and dissemination of knowledge in diarrhoeal diseases and directly related subjects of nutrition and fertility with a view to develop improved methods of health care and for the prevention and control of diarrhoeal diseases and improvement of public health programmes with special relevance to developing countries. ICDDR,B issues two types of papers: scientific reports and working papers which demonstrate the type of research activity currently in progress at ICDDR,B. The views expressed in these papers are those of authors and do not necessarily represent views of International Centre for Diarrhoeal Disease Research, Bangladesh. They should not be quoted without the permission of the authors.

INTRODUCTION

Enterotoxigenic *Escherichia coli* produces two distinct enterotoxins: one is heat labile (LT), of high molecular weight, and antigenic; the other is heat stable (ST), of low molecular weight, and hypoantigenic. Both have been considered to be responsible for diarrhoea in humans and cattle. For detection of LT, various assay methods have been developed (Table I). Many of these assays are unsuitable for routine use because they require a large number of animals, and stocks of special tissue culture cells, or radioisotopes, and appropriate techniques.

This manual describes the Biken test, a new, simple method for detection of LT in clinical laboratories. The test requires no special materials or techniques and can be performed on petri dishes with commercially available reagents by laboratory staff with a minimum of training.

THE PRINCIPLE OF THE BIKEN TEST

The Biken test was first developed for the detection of extracellular toxins produced by *Corynebacterium diptheriae* and *Staphylococcus aureus* using antitoxin antisera (13). It was later applied to the detection of the enterotoxins (14) of *Vibrio cholerae* and *Clostridium perfringens* as well as other bacterial products. The original test known as Elek test with these organisms was technically difficult to perform and the special media embedded with antitoxin containing paper could not be stored. To eliminate these difficulties and to increase the sensitivity of the assay, we have modified the classical Elek test using techniques from the Ouchterlony test. This modified Elek test was first applied successfully to the detection of Kanagawa phenomenon of *Vibrio parahaemolyticus* (15).

The modified Elek test is based on an antigen-antibody reaction which takes place between an LT-producing colony and an LT-specific antiserum. As seen in Figs.1&2, LT toxin, the antigen, elaborated by the *Escherichia coli* colony diffuse into the agar and reacts with LT-specific antisera put into a well at the centre of a cluster of colonies. If the colony produces LT, a precipitin line forms between the LT-producing colony and the well.

Three important techniques are used to increase the production of LT and thereby improve the sensitivity of the test; 1) a special agar preparation, Biken agar 2, has been formulated to increase the yield of LT; 2) lincomycin, has been added to the medium to further stimulate LT production (16); and 3) each colony has been treated with polymyxin B to promote the release of LT from the cell before antiserum is added.

Since the method is based on an antigen-antibody reaction, the result should be very specific for LT production in contrast to biological assays and the immune hemolysis assay where hemolysins and other cytotoxic substances may interfere with the tests.

METHODS OF BIKEN TEST

1. Methods for Preparing Biken Agar 2:

Table II lists the ingredients of Biken agar 2 which should be prepared as follows:

i) Prepare the Biken Agar 2 medium containing all components except lincomycin and Noble agar.

ii) Adjust pH to 7.5 with IN NaOH.

iii) Add Noble agar to the medium.

iv) Boil the medium until the agar melts.

v) Autoclave the medium at 121°C for 15 mins.

vi) Cool until the temperature reaches 50°C.

vii) Add lincomycin to the medium to a final concentration of 90 mcg/ml. Mix well.

viii) Pour 15 ml* of this medium into the petri dish (90 x 15 mm).

ix) Store these plates in a refrigerator at 4°C. They can be kept for 2 - 3 weeks.

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* This is a very important point. When the plate is too thick (more than 20 ml/plate), the LT will diffuse more widely, producing a fainter, less noticeable precipitin line.

2. Antitoxin Serum:

The procedure to prepare antisera against Lt (or, if LT is not available, against CT) is summarized in Table III. The antisera must be checked first for its titre against LT with the Ouchterlony agar gel diffusion test. Conduct this test as follows:

1) Prepare a solution containing 1 gm% Noble agar in 100 ml PBS with 0.25% NaN_3 (sodium azide).

2) Mix solution and boil until the agar melts.

3) Pour plates.

4) Cut wells in the plate in the configuration shown in Figure 3.

5) Place purified LT or CT (2.5 mcg) in the centre well and two fold serial dilutions of serum in the 6 surrounding wells. Incubate the plate overnight at 37°C.

6) Read precipitin lines after 12 - 14 hours.

7) Report the highest dilution which gives a positive line as the serum antitoxin titre (e.g. titre 1:8 in Figure 3). Antiserum for the Biken test should have a titre of 1:1.5. If for example, the initial titre is 1:8, then dilute 1 cc of this serum with 5 cc of diluent (PBS).

   If serum with a higher or a lower titre is used, the sensitivity of this test will decrease, or only a faint precipitin line may be observed.

3. Inoculation of Strains of *E.coli* to be Tested:

Inoculate *E.coli* freshly isolated on McConkey agar or from stocks from blood agar base on nutrient agar or Dorset agar directly onto Biken agar 2. Twelve to sixteen strains can be inoculated on a single petri dish, as shown in the configuration in Figure 1.A. The size of the inoculum should be similar to those in Figure 1, since small spots will not yield good results. We usually inoculate a strain on 2 separate plates to ensure consistent results, and if either is positive, the result is considered to be positive since no false reactions are expected. Incubate for 48 - 72 hours at 37°C.

4. Treatment with Polymyxin B and Antisera:

After proper incubation, saturate a Whatman AA disc in a polymyxin B solution containing 20,000 I.U./ml (approximately 40 mcg) and place on each growth. Punch a well in the centre of the cluster of colonies (Figure 1). Cut the agar with a 4 - 5 mm glass or metal rod and remove the agar with a pin. Be careful not to crack the agar. After incubating the plate for another 5 hours at 37°C, put 1 drop (25 µl) of the antisera into each centre well. Incubate again for 24 hours or longer at 37°C.

5. Results - Reading:

After incubating the plates with antiserum for 24 hours the precipitin line would be read. To best observe the precipitin line, examine the plate against a dark background and let light from a bright, shaded bulb diffuse through the agar from the side. If the *E.coli* strain makes LT toxin, a precipitin line should form between the colony and the well, as shown in Figures 1 and 2. After reading the results, keep the plate for another day and read again to allow those colonies with a weak precipitin line more time to react.

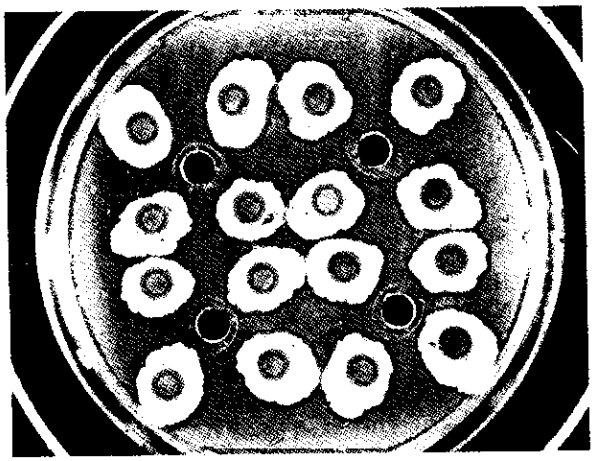
Summary of Comparative Studies of the Biken Test:

Few studies have compared the results of the Biken test with the standard Chinese hamster ovary (CHO) cell assay. In both studies (12, 17) the sensitivity and specificity of the Biken test was at least 98%. Both anti-LT and anti-CT sera have been used, and while the anti-LT serum gives a sharper, bright precipitin line, both are equally reliable. [Appendices A & B]

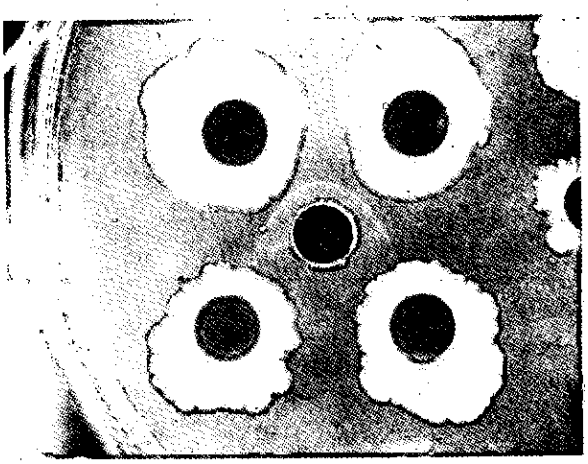
TABLE I--METHODS FOR DETECTING *ESCHERICHIA COLI* HEAT LABILE ENTEROTOXIN (LT)

Biological methods
Ileal loop test (1)
Vascular permeability (PF) test (2)
Suckling animals (mouse, rat, hamster) assay (3)
CHO cell assay (4)
Y_1 adrenal cell assay (5)
S49 lymphosarcoma cell assay (6)
Immunological methods
Passive immune hemolysis test (7)
Reversed passive hemagglutination test (8)
Staphylococcal coagglutination test (9)
Radioimmunoassay (10)
Enzyme-linked immunosorbent assay (11)
Biken test (modified Elek test) (12)

FIGURE 1

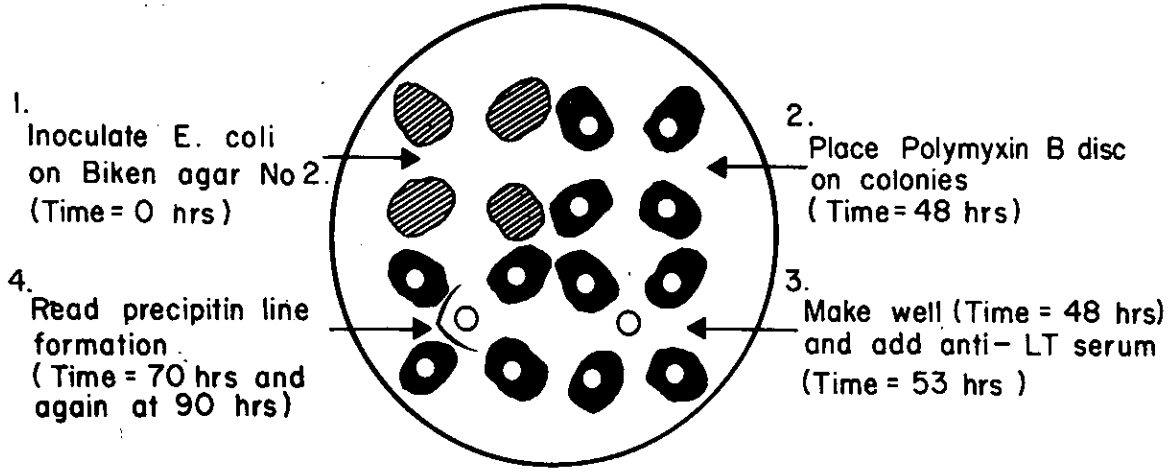


Low magnification of Biken plate showing 7 LT positive and 9 LT negative *E.coli* isolates (A).



High magnification of Biken plate showing 2 LT positive and 2 LT negative isolates (B).

Figure 2



TESTING E. COLI. STRAINS FOR LT TOXIN
USING BIKEN TEST

TABLE II--COMPOSITION OF BIKEN AGAR 2

Component	Concentration (%)
Casamino acid	2
Yeast extract	1
NaCl	0.25
K_2HPO_4	1.5
Glucose	0.5
Trace salts*	0.05 (vol %)
Noble agar	1.5
Lincomycin	90 (mcg/ml)
pH	7.5

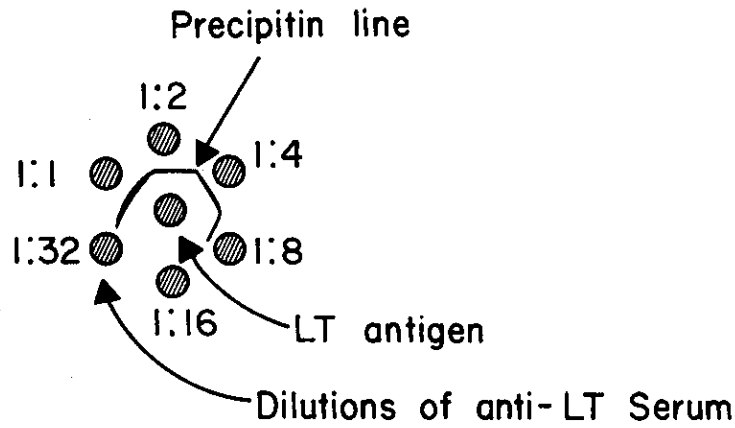
* 5% $MgSO_4$, 0.5% $FeCl_3$ and 2% $CoCl_2 \cdot 6H_20$ (cobalt chloride).

TABLE III--TECHNIQUE FOR PREPARATION OF ANTISERA IN RABBITS FOR USE IN BIKEN TEST

PROCEDURE - RABBIT IMMUNIZATION SCHEDULE

Day 0 -	Collect preimmune serum (4 ml) from each rabbit. Test serum by Biken test against *E.coli* to insure that non-specific or undesirable reactions do not occur.
Day 1 -	Priming - Inject i.m. a mixture containing 50 mcg (1 ml) of LT (or if LT not available) and Freund's complete adjuvant (1 ml).
Day 30 -	Booster - Inject i.m. a mixture containing 50 mcg of LT (or CT) and 1 ml of Freund's incomplete adjuvant.
Day 60 -	Booster Infect i.m. mixture containing 50 mcg of LT (or CT) and 0.5 ml Freund's complete and 0.5 ml incomplete adjuvant.
Day 70 -	Test bleeding - bleed 5 ml from each rabbit and check antibody titre by Ouchterlony method against pure homologous antigen (2.5 mcg). When the titre is more than 1:16, bleed the animal. If the titre is lower than 1:16, continue with a further booster.
Day 90 -	Additional booster - as on day 60.
Day 100 -	Test bleeding as on day 70.

Figure 3



OUCHTERLONY TECHNIQUE FOR TESTING TITER OF ANTI-LT SERUM: TITER IN FIGURE IS 1:8

APPENDICES (A & B)- A

Comparison of Biken Test with CHO Cell Assay for Detection of LT Producing E. coli from Patients with Diarrhoea and Selected Stocked Isolates[17]

Assay Methods			Selected Stocked
CHO	BIKEN	Patients	Isolates*
+	+	13	160
-	-	258	345
+	-	1	3
-	+	1	8
	Total	273	516

* Selected stock includes both LT positive and negative isolates.

All 13 discrepant cases have been repeated and found mixed cultures of LT positive and negative isolates.

B

Results of assays of 164 strains of E. coli[12]

Group[a]	Assay method			No. of strains
	Elek	CHO[d]	PIH[e]	
I	+	+	+	73
II	+	+	-	0
III	+	-	+	0
IV	-	+	+	0
V	+	-	-	2
VI	-	+	-	0
VII	-	-	+	1
VIII	-	-	-	88

a Grouping was based on the results of the assays.

d Chinese hamster ovary cell assay

e Passive immune hemolysis

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