BIKEN JOURNAL Vol. 29 73–75, 1986

SHORT COMMUNICATION

APPLICATION OF BIKEN TEST (MODIFIED ELEK TEST) FOR SAMPLING OF HEAT-STABLE ENTEROTOXIN OF *ESCHERICHIA COLI* ISOLATED IN BANGLADESH

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The usefulness of Biken Agar 2 as a source of heat-stable toxin for the suckling mouse assay was examined. *Escherichia coli* (*E. coli*) strains isolated in Bangladesh from patients with gastroenteritis found to produce heat-stable toxin (n=152), both heat-stable and heat-labile toxin (n=60) and not to produce heat-stable or heat-labile toxin (n=25) by standard suckling mice assay using broth culture were tested. Sampling from Biken Agar 2 gave comparable results to those obtained using standard broth cultures. This is the first field survey of evaluation of the Biken test for sampling heat-stable toxin of *E. coli*. The result further clarifies the applicability of the Biken test for sampling heat-stable toxin, and the usefulness of the Biken test for detections of heat-labile and heat-stable toxin produced by *E. coli*.

Escherichia coli strains producing heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) are major causes of diarrhoea in Bangladesh. Various biological and immunological methods are available for the detection of heat-labile enterotoxin of *E. coli* and recently a very simple, inexpensive, reliable and reproducible method, the Biken test, that shows 98% sensitivity and 99% specificity has been established. The Biken test was developed by combining the principles of the Elek and Ouchterlony tests. The sensitivity of the Biken test has also been evaluated in Bangladesh. Honda et al., Takeda et al. also reported use of the Biken test for sampling of heat-stable enterotoxin. This test was used in this study for examining strains isolated in Bangladesh.

The strains studied were all isolated from patients with diarrhoea in the hospital of the International Center for Diarrhoeal Disease Research, Bangladesh. Freshly isolated strains and stock strains were used. In all 237 isolates were tested. Biken Agar 2 plates were prepared from 2% casamino acid, 1% yeast extract, 0.25% NaCl, 1.5% K_2HPO4, %0.5 glucose, 0.05 (vol. %) trace salts (5% $MgSO_4$, 0.5% $FeCl_3$ and 2% $CoCl_2 \cdot 6H_2O$), 1.5% Noble agar (agar) and 90 μg lincomycin per ml of medium as described previously. The surface of the agar plates was dried before their use to avoid swarming of *E. coli* cells. Primary cultures or subcultures of test isolates in MacConkey's agar were usued. Inocula-

tions of single *E. coli* colonies of 4 test strains on a Biken Agar 2 plate were made in a manner that ensured a fairly large area of confluent growth, taking care that the strains did not touch each other when they grew. Plates were incubated for 48 h at 37 C. Drying of the agar plates was prevented by enclosing 10–15 plates with a damp paper towel to maintain saturation humidity in a polyetheylene bag during incubation. After incubation for 48 h, a polymyxin B disc was placed on top of each confluently growing colony. After 5 h, 4 agar plugs, each 7 mm in diameter, were punched out to the full depth of the agar from just outside the periphery of each colony. ST was extracted from the 4 agar plugs overnight at 4–8 C was 0.7 ml of sterile phosphate buffered saline (PBS; 0.01 m*M*; pH 7.0–7.5). With this volume of sterile PBS, about 0.45 ml of removable fluid was obtained, which was enough to inoculate 4 mice. When the volume was insufficient, the agar plugs were re-extracted with the minimum volume of PBS required. Extracts were usually used immediately after the overnight extraction, though they could be stored at 4 C for several weeks without loss of activity. The extracts were inoculated into suckling mice intragastrically. After the incluation, the mice were kept at 25 C for 4 h, and then ST activity was measured as the ratio of gut weight/body weight. A positive result was taken as a ratio of 0.083 or more and a negative result as a ratio of 0.082 or less. The supernatants of liquid cultures (standard broth cultures), grown aerobically in trypticase soy broth with 0.6% yeast extract in a roller drum were subjected to ST assay in parallel by the same method. Four mice were treated with each isolate.

Of the samples tested, 152 isolates produced only ST, as shown by suckling mice assay with standard broth cultures. Of the 152 isolates, 150 (98.68%) produced ST when tested using samples from Biken Agar 2. Sixty *E. coli* isolates producing both LT and ST detected by conventional methods were retested for ST using both the supernatants of standard broth cultures and extracts from Biken Agar 2. Similar results were obtained in the two sets of samples. With 95% of the isolates, positive results were obtained by inoculation of Biken Agar 2 extracts into mice. Of the isolates from diarrhoea patients known not to produce LT or ST, 25% were also subjected to both sets of experiments as negative controls. These samples all gave negative results in both tests (Table 1).

TABLE 1. *Assay in suckling mice of ST using culture supernatats and Biken Agar 2 extracts*

No. of strains	Toxin characterization	ST-positive samples from culture supernatants	ST-positive samples from Biken Agar 2 extracts
152	ST	152	150
60	LT/ST	60	57
25	0	0	0

In this work of Biken Agar 2 extracts which have been used for detection of LT production by *E. coll* was also found to be very effective for the detection of ST. Strains producing ST gave similar results on assay by inoculation of culture supernatants and Biken Agar 2 extracts into suckling mice. The Biken test has been well established and evaluated to be reasonably efficient, and development of a technique for sampling of ST has made it more applicable. In this study we confirmned the applicability of this test using *E. coll* isolates recovered from patients with diarrhoea in Bangladesh. Of the isolates known to produce ST (98.68%) gave positive results when evaluated using Biken Agar 2 extracts. Similar sensitivity (95%) was observed in isolates known to produce both ST and LT enterotoxins. When a Biken plate is used for LT detection, a separate broth culture and special incubation conditions are necessary for inoculation of suckling mice and detection of ST. But our finding that phosphate-buffered-saline extracts of ST from Biken Agar 2 gave similar results to those

with broth culture showed that the same plate can routinely be used for assays of both LT and ST.

ACKNOWLEDGMENT

This research was financed from the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). ICDDR, B is supported by countries and agencies that share its concern about the impact of diarrhoeal diseases on the developing world. Current donors giving assistance to ICDDR, B includes: AGFUND, Australia, Bangladesh, Japan, Saudi Arabia, Sweden, Switzerland, the United Kingdom and USAID. The author thanks the Microbiology Branch of the ICDDR, B for support and providing facilities for work. The author also thanks Dr. T. Takeda, Tokyo University, Japan, for reviewing the manuscript.

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