SIMPLE SEROLOGICAL TESTS FOR DETECTING CLASSICAL HEAT LABILE ENTEROTOXIN (LT-I) OF *ESCHERICHIA COLI*

WANPEN CHAICUMPA, SURASAK SAE ER, MANAS CHONGSA-NGUAN and PETER ECHEVERRIA*

Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, and *Armed Forces Research Institute of Medical Sciences, U.S. Component, Bangkok 10400, Thailand.

INTRODUCTION

Enterotoxigenic *E. coli* (ETEC) are known to produce four types of enterotoxin. These are : 1) the classical heat labile toxin (LT-I); 2) the LT-like toxin (Green *et al.*, 1983) or type II heat labile toxin (LT-II) (Pickett *et al.*, 1986); 3) the heat stable toxin I (STa) and 4) the heat stable toxin II. The LT-I is a complex protein which has a molecular weight of approximately 91,000 daltons (Clements *et al.*, 1980) and comprises of two non-covalently associated subunits A and B (Clements and Finkelstein, 1979). The subunit A is synthesized as a single polypeptide chain (MW 25,000 - 29,000) (Clements *et al.*, 1980). This peptide is cleaved by trypsin into A1 and A2 fragments. An enzymatically active A1 fragment (MW 21,000) links by disulphide bond to an A2 chain (Clements and Finkelstein, 1979). The B protomer, a MW of 59,000 (Clements *et al.*, 1980) consists of five identical polypeptide chains linking non-covalently to each other (MW 11,800) (Gill *et al.*, 1981). The B and A1 chains of LT-I and cholera toxin (CT) have 80% amino acid homology (Spicer *et al.*, 1981), whereas the homology of A2 chains of both toxins is only 31-55% (Spicer *et al.*, 1981). LT-I and CT also resemble in their mode of action.

ETEC and non-ETEC including normal flora *E. coli* are morphologically identical and produce the same biochemical reactions. At present, no selective media are available for their differentiation. Thus, the way to recognize ETEC is to demonstrate their released enterotoxin(s).

There are varieties of *in vivo* as well as *in vitro* assays which have been developed to demonstrate the ETEC enterotoxins. The *in vivo* bioassays which detect living tissue reactions or measure the biological changes after exposing to the toxin(s) include the infant rabbit test (Gorbach and Khurana, 1972), the suckling mouse test (Dean *et al.*, 1972), the ligated ileal loop test and the skin permeability test (Evans *et al.*, 1973). These techniques involve complicated pretreatment and/or surgical operation of laboratory animals which are costly, time consuming and labourious. The *in vitro* bioassays which deal with isolation and cultivation of isolated living tissues in synthetic medium e.g. Y1-adrenal cell assay (Sack and Sack, 1975), Chinese hamster ovary cell assay (Guerrant *et al.*, 1974) and Vero cell assay (Edelman and Levine, 1983) require sterile tissue culture techniques, expensive media and experienced hands which may not be available in developing countries where ETEC diarrhoea is highly prevalent.

Solid phase radioimmunoassay (Ceska *et al.*, 1978), DNA hybridization (Moseley *et al.*, 1983) and Gm1 ganglioside enzyme-linked

immunosorbent assay (Czerkinky and Svenerholm, 1983) have been found to be very sensitive in detecting ETEC enterotoxins. Nevertheless, these techniques require sophisticated instruments and reagents which can only be performed in a well-equipped laboratory. The immune hemolysis test (Castro *et al.*, 1980), although is simple, has a major disadvantage in its false positive reaction due to the fact that some strains of *E. coli* produce hemolysin. The lysis inhibition test (Evans and Evans, 1977) is sensitive and specific in differentiating between LT producing ETEC and non-ETEC but one obvious drawback is the requirement of large quantities of "pure" LT as crude LT was proved to be inappropriate in this assay. The counter immunoelectrophoresis is not sensitive and three dilutions of LT have to be included in each test (Morgan *et al.*, 1983).

The serological techniques which are cheap, uncomplicated and may be performed in a remote health centre would be the Biken test (BT) (Brill *et al.*, 1979), the staphylococcal coagglutination test (SCOAG) (Kudoh *et al.*, 1979) and the reverse passive haemagglutination test (RPHA) (Kudoh *et al.*, 1979). This paper reports the sensitivity, specificity and accuracy of the BT, SCOAG and RPHA using locally produced reagents in comparison with the commercially available Biken test kits, DNA hybridization and Y1-adrenal cell assay in detecting classical LT-I from 100 strains of *E. coli* provided by the World Health Organization.

MATERIALS AND METHODS

One hundred *E. coli* strains in Dorset slants labelled nos. 1 to 100 were provided by WHO. They were either LT-I producers or non-producers. The bacteria were subcultured to blood agar (BA) 4-5 hours before inoculating into suitable medium for BT, SCOAG or RPHA. *E. coli* strains B2C and XACK12 were used as LT-I producer and non-producer controls, respectively. The B2C (the strong LT producer) released LT-I and formed precipitin band within 12 hours after adding anti-LT serum in the standard BT using commercial Biken kit. During the experiments it was found that the WHO *E. coli* no. 5 produced band at about 60 hours after adding the anti-LT. Thus, the strain was used as a control, weak LT-I producer in the subsequent experiments.

Rabbit anti-CT-B subunit serum was prepared by injecting a rabbit intramuscularly with CT-B subunits (Sigma). The first dose was 30 µg of the antigen in 1 ml of 0.01 M PBS pH 7.0 emulsified in equal volume of Freunds complete adjuvant. The booster doses were 30 and 90 µg of the same antigen mixed with equal volumes of Freunds incomplete adjuvant. The injections were given at 14 day intervals. Six weeks after the last intramuscular inoculation, the rabbit was given an intravenous booster of 60 µg of CT-B subunits in 1 ml of PBS pH 7.0. The animal was bled from heart 7 days after the last injection. The anti-CT-B subunit serum was collected, heat inactivated at 56°C for 30 minutes and kept in small aliquots at -20°C. The serum was used in the BT and the SCOAG. Immunoglobulins of the antiserum was also prepared by ammonium sulphate precipitation and were used to sensitize sheep red blood cells in the RPHA.

Stock colistin solution (500,000 units/ml) was prepared by dissolving sodium colistimethate (Dumex, 1,000,000 units/vial) in 2 ml of sterile triple distilled water. The working colistin solution (20,000 units/ml) was made by adding 0.4 ml of the stock colistin into 9.6 ml of sterile normal saline solution (NSS). Colistin discs were prepared by ap-

plying 25 µl of the working colistin solution onto a steriled and completely dried standard disc (Whatman no. AA). Lincomycin solution (Sigma) was used at 90 µg/ml of sterile NSS.

Biken II agar (B2A) was prepared according to the method described by Honda *et al.*, (1981). The surface of the medium was dried just before use. The Biken broth (B2B) was prepared in the same manner as the B2A but the special noble agar was omitted.

Determination of optimum dilution of anti-CT-B subunit serum for Biken test: The B2C and *E. coli* sample no. 5 (strong and weak LT producers, respectively) were grown on BA for 4 hours and were then inoculated onto B2A in circles of about 8 mm in diameter (Fig. 1). The B2A plate was incubated at 37°C for 48 hours. A colistin disc was placed on top of each *E. coli* colony and incubation was continued for further 6 hours. Four wells (4 mm in diameter) were made in the agar around each colony at a distance of 4 mm away from the margin of the colony (Fig. 1). Various dilutions of the anti-CT-B subunit serum in 30 µl volume were added to

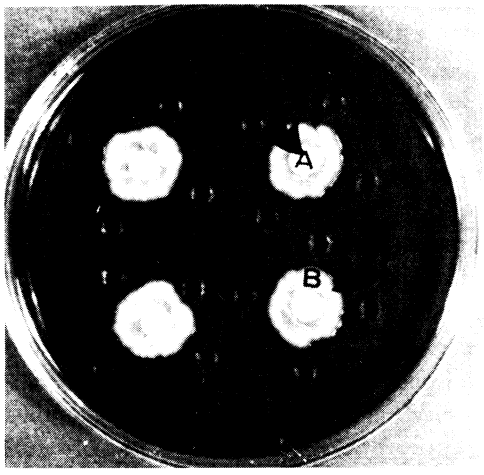

Fig. 1–Biken test for determining optimum dilution of anti-CT-B subunit serum, A = colistin disc, B = *E. coli* colony, C = well of anti-CT-B serum.

appropriate wells. The plate was then incubated at 37°C for 3 days with intermittent observation. The optimum concentration of the antiserum was the concentration which reacted with the LT released from the B2C and *E. coli* sample no. 5 and yielded sharp precipitin lines against both strains. This dilution of serum was subsequently used in all the BT. The dilution was also titrated against various amount of CT-B subunits in double diffusion assay to determine the smallest amount of the CT-B subunits which could be detected by the antiserum.

Biken test : *E. coli* samples to be tested were grown in BA for 4 hours then every two strains were streaked on B2A in straight lines of about 4 mm thick and 11 mm long. The lines of streaking were perpendicular to each other. Six pairs of *E. coli* were streaked on the peripheral area of the B2A plate. At the centre of the plate, the control positive LT producer (B2C) and the control negative (K12) were grown (Fig. 2). After 48 hours of incubation at 37°C, a colistin disc was placed on top of each line of the bacterial growth and the incubation was continued for 6 hours. A well (4 mm in diameter) was made in the B2A medium at a distance of 4 mm from each pair of the samples. The optimum dilution of the anti-CT-B subunit serum (30 µl) was added to each well. The plate was incubated and checked for precipitin lines at 12, 24, 48, 60 and 72 hours after adding the serum. Examples of precipitin lines are shown in Fig. 3.

For preparing protein A rich-staphylococcal cells, the methods of Brill *et al.*, (1979) were followed with modifications. *Stapylococcus aureus* strain Cowan I (NCTC 8532) was streaked onto a BA plate. A colony with complete hemolysis was picked and inoculated into a 10 ml trypticase soy broth (TSB) and incubated at 37°C for 6

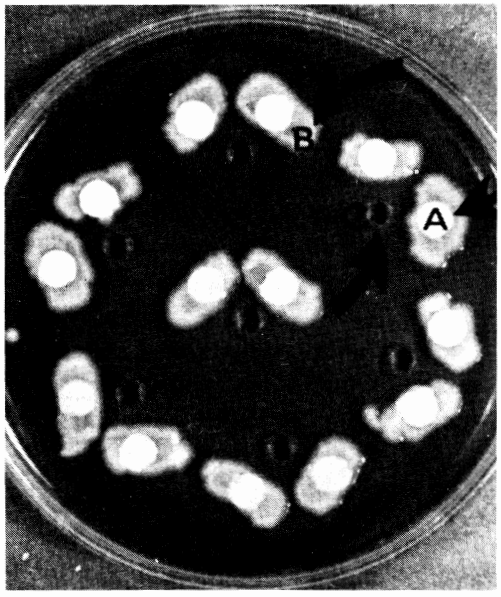

Fig. 2–Example of a plate of Biken test for detecting LT of unknown *E. coli* strains, A = colistin disc, B = *E. coli* growth, C = well of anti–CT–B serum.

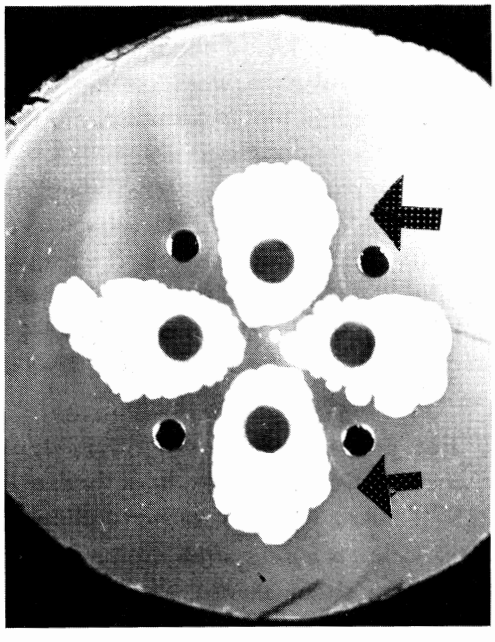

Fig. 3–Precipitin bands formed between LT released from the ETEC and antiserum (arrows).

hours with rotary shaking at 100 rpm per minute. The culture was then transferred to a 2,000 ml flask containing 500 ml of TSB + 1% yeast extract (TSB – Y medium) and incubated at 37°C with aeration for 18 hours. The cells were harvested and washed three times with PBS pH 7.0 by centrifuging at 6,000 rpm for 25 minutes. Finally the cells were suspended in 0.5% formaldehyde in PBS pH 7.0 to make 10% cell suspension (v/v). The preparation was incubated at 25°C for 3 hours with occasional shaking. Three washes with PBS pH 7.0 were done at the end of the incubation period. The cells were resuspended to 10% in a prewarmed PBS pH 7.0 and incubated at 80°C for 8 minutes with occasional swirling. At the end of the heat-fixed process, the preparation was cooled down immediately under running tap water. Two washes were done and the cells were resuspended to 10% suspension in PBS pH 7.0 containing 0.1% NaN_3 and 0.5 mg % of lactose. Aliquots of 2 ml were lyophilized. These preserved cells were found to be stable many months later.

Sensitization of the preserved staphylococci by anti–CT–B subunit serum was performed by adding 2 ml of distilled water to each aliquot of the lyophilized cells. The cells were kept at 25°C for 20 minutes to allow clumps to settle. One millilitre of the homogeneous 10% cell suspension was mixed with a volume of anti–CT–B subunit serum and the mixture was incubated at room temperature for 1 hour with occasional and gentle agitation. At the end of the incubation, the cells were washed by adding 10 volumes of PBS pH 7.0 and centrifuged at 2,000 rpm for 15 minutes. The cells were resuspended to 2% suspension in PBS containing 0.5% bovine serum albumin, 0.0005% tween 80 and 0.01% gelatin (PBS–BTG) (sensitized staphylococci were

prepared).

Preparation of *E. coli* lysate for SCOAG: LT-I preparation was prepared by the methods of Honda *et al.*, (1983) with modifications. *E. coli* were grown in BA for 3-4 hours and large amounts were streaked onto BA-Lin medium (BA containing 0.009% lincomycin). The plate was incubated at 37°C for 8 hours. A loopful of *E. coli* was homogeneously suspended in 75 µl of working colistin (20,000 units/ml) in microcentrifuge tube. The tube was incubated at 37°C for 1 hour then 15 µl of 1% triton x-100 were added. The cells were incubated 15 minutes further then were packed by centrifugation. The supernatants (lysates) were collected.

SCOAG : Ten microlitres of the lysate were mixed with equal volume of 2% sensitized staphylococci on a glass slide. The reactants were mixed thoroughly by rocking the slide to and fro for 2 minutes and the agglutination was examined.

RPHA : Sheep red blood cells kept in Alsever's solution were washed three times with NSS by centrifugation at 400xg for 5 minutes. The washed cells were suspended in PBS pH 7.2 to a 2.5% concentration and were cooled down in ice-bath. Equal volume of freshly prepared 1:20,000 tannic acid was gradually added to the chilled cell suspension. The preparation was kept in the ice-bath for 10 minutes. Occasional swirling was made to mix the suspension thoroughly. At the end of the tanning process, cells were washed once with PBS. The packed cells were resuspended in PBS pH 6.4 to the 2.5% concentration. One volume of the optimum concentration of the anti-CT-B immunoglobulins in PBS pH 6.4 was mixed with four volumes of the 2.5% tanned red blood cells. The mixture was kept at 37°C for 30 minutes with occasional and gentle mixing. At the end of the sensitization, three washes were done with 1% heat-inactivated normal rabbit serum (preabsorbed with packed sheep red blood cells) in NSS. The washed cells were resuspended in 0.066 M PBS pH 7.0 containing 0.4% BSA and 0.1% tween 80 (PBS-BT) to 1% suspension.

E. coli were grown in BA-Lin medium for 8 hours. Two loopfuls of the bacterial cells were suspended in 150 µl of working colistin solution and incubated at 37°C for 1 hour. Cells were packed by centrifugation and 25 µl aliquots of each supernatant were added to wells no. 1 and 2 of the microtitre haemagglutination tray. Two fold serial dilutions of the supernatant were, then, made to well no. 10. Wells no. 11 and 12 of each row contained supernatant of LT-I producer and non-producer *E. coli*, respectively. To each well, 25 µl of the sensitized red blood cells was added. The content in the wells was mixed and the tray was kept at 25°C for 2 hours. Agglutination of the sensitized cells indicated the presence of the LT-I in the *E. coli* supernatant when the positive and negative control wells showed typical appearances.

RESULTS

Determination of anti-CT-B titres in rabbit sera : serum samples of the rabbit at pre- and post-immunization with CT-B subunits were assessed for anti-CT-B titres by double diffusion tests against 3 µg of CT-B subunits (30 µg/ml). The undiluted serum sample of the rabbit before immunization did not give precipitin band when reacted with the antigen. After immunization, a single precipitin band was formed between the immune serum and the antigen. The highest dilution (titre) of the serum which gave positive reaction was 1:32 (Fig. 4).

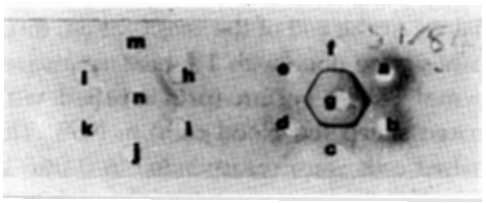

Fig. 4–Titre of the anti–CT–B subunit serum on reaction with 3 μg CT–B subunits (wells g and n) in double diffusion assay; well of undiluted serum (a) and two fold dilutions (1:2 – 1:2,048) (wells b to m); the titre was 1:32 (well f).

Optimum dilution of the anti–CT–B serum to be used in the BT : Various dilutions (1:2, 1:4, 1:8, 1:10 and 1:16) of the serum were tested in the Biken reaction with LT released from B2C and *E. coli* strain no. 5. The experimental plate of this determination is shown in Fig. 1. However, the precipitin lines could not be seen in the photograph although they appeared in the plate when observed with naked eyes. When tested with the B2C (strong LT–I producer) at dilution 1:16, the precipitin band was formed too near to the well containing antibody. When the concentrations of the antibody were higher (1:2, 1:4, 1:8 and 1:10), the precipitin bands occurred midway between the antiserum wells and the bacterial colony. At dilutions 1:8, 1:10 and 1:16 the precipitin bands were formed halfway between colony of the weak LT producer (strain no. 5) and the antiserum wells. The reaction occurred too near to the bacterial colony or sometimes underneath the colony when the concentrations of the antibody were higher (1:2 and 1:4). Thus too high or too low concentrations of the serum seemed to interfere with the locations of the precipitin bands formed; with the more effect to the reaction of the weak LT–I producer. The 1:10 dilution of the serum was, therefore, chosen for the subsequent BT. With this dilution it was found that the maximum amount of the CT–B subunits which gave sharp precipitin band was 20 μg (10 μl of 200 μg/ml) and the minimum amount was 0.3 μg (10 μl of 30 μg/ml), respectively.

Information concerning LT phenotypes of the 100 reference strains of *E. coli* were given by WHO. Fifty strains were LT–I^+ phenotype as detected by Y1-adrenal cell assay and DNA hybridization. These strains were nos. 1, 5, 6, 8, 9, 10, 14, 15, 16, 17, 18, 24, 25, 26, 27, 28, 35, 36, 42, 43, 44, 45, 53, 57, 58, 63, 64, 67, 68, 69, 71, 72, 73, 75, 76, 77, 78, 81, 82, 83, 85, 86, 89, 90, 91, 93, 94, 96, 99 and 100. The other 50 strains were LT–I^- phenotype. These informations were obtained after all of them had been tested by the BT, SCOAG and RPHA using the locally made reagents.

Biken test: In performing the BT to detect LT producer by the 100 reference strains of *E. coli*, the commercial Biken kits were also used along with the reagents prepared locally. The results obtained from the use of the commercial Biken kits (Meguto Institute Co. Ltd.) were that 46 strains were LT–1 positive, 48 strains were LT–I negative with 2 falsely positive strains (nos. 13 and 14) and 4 falsely negative strains (nos. 43, 57, 58 and 73). Thus, sensitivity, specificity and accuracy of the test were 92%, 96% and 94%, respectively while the positive and negative predictive values were 96% and 92%, respectively.

When the locally made reagents were used in the BT, it was found that there were 46 LT–I positive strains, 50 LT–I negative strains and 4 false negative strains (nos. 16, 64, 69 and 73). No false positive one was found. Thus, the sensitivity, specificity and accuracy of the BT using locally made reagents were 92%, 100% and 96% respectively while the positive and negative predic-

tive values were 100% and 93%, respectively.

It was, however, noted that the precipitin bands formed by the LT produced by ETEC with the commercial Biken reagents were clearer than those formed with locally made reagents. The LT was detected from strong LT producers between 12–24 hours after adding the serum while 24–48 hours and 48–72 hours were required for the moderate and weak LT producers, respectively.

SCOAG : Varying amounts of the anti-CT-B subunit serum (0.05, 0.1 and 0.15 ml) were used to sensitize each ml of the 10% staphylococci. The cells sensitized with 0.1 ml or 0.15 ml of the antiserum could detect as low as 5 ng of CT-B subunits by the SCOAG while as high as 100 ng of the antigen was required to produce agglutination with the cells sensitized with 0.05 ml of the antiserum. Thus, 0.1 ml of the anti-CT-B subunit serum was used for sensitizing each ml of the preserved staphylococci in the subsequent SCOAG.

Strong agglutination of freshly prepared sensitized cells occurred in the presence of CT-B subunits ranging from 50 to 200 ng (10 μl of 5 to 20 μg/ml). The reaction was seen as large clumps of the agglutinated cells within few seconds after mixing the sensitized cells with the antigen. The minimum amount of CT-B subunits which could be detected by the freshly prepared sensitized cells was 5 ng (10 μl of 0.5 μg/ml). However, it took about 2 minutes before the agglutination appeared (Fig. 5).

After storage of the cells sensitized with 0.1 ml anti-CT-B serum at 4°C for 30 and 73 days, the sensitivity of the test was lowered to 40 ng and 50 ng of the CT-B subunits, respectively.

The formalinized, heat-fixed *Staphylococcus aureus* in lyophilized form was kept for 22 months. These cells when sensitized with 0.1 ml of the anti-CT-B subunits serum were still able to detect as low as 10 ng of CT-B subunits by SCOAG.

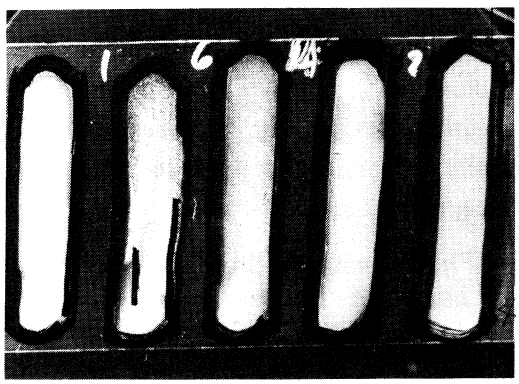

Fig. 5–Staphylococcal co-agglutination test
CT : large clumps of agglutinated sensitized *Staphylococcus aureus* in the presence of CT
1 : medium sized clumps of the sensitized cells in the presence of LT-I released from strong producer *E. coli*
6 : fine granules of the sensitized cells in the presence of LT-I released from weak producer *E. coli*
14 : minute granules of sensitized cells in the presence of LT-I released from very weak producer (ambiguous result)
2 : homogeneous sensitized *Staphylococcus aureus* when mixed with lysate of LT-I nonproducer (negative result).

Lysates of the 100 *E. coli* strains were prepared and checked for the presence of LT-I by SCOAG using freshly sensitized staphylococci. It was found that 47 strains were true positive, 50 strains were true negative and three strains gave ambiguous (±) results. These three strains (nos. 69, 82 and 83) did not give confluent growth in the culture medium. However, if these strains were read as weak LT-I producers, the sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the test would be 100%. If they were

classified as LT−I negative strains, the results would be false negative and the test would be 94%, 100%, 97%, 100% and 94% sensitivity, specificity, accuracy, positive predictive value and negative predictive value, respectively.

RPHA : It was found that the tanned sheep red blood cells sensitized with the anti−CT−B immunoglobulins ranging from 600 to 1,000 ng/ml could equally detected 10−20 ng/ml of the CT−B subunits by the RPHA. The smaller amounts of the immunoglobulins (400, 300 and 200 ng/ml) yielded less sensitive sensitized cells which could not detect CT−B subunit in the amount less than 20 ng/ml. Thus the subsequent RPHA was performed using tanned cells sensitized with 600 ng/ml of the anti−CT−B immunoglobulins.

However, the RPHA gave unsatisfactory results on the detection of LT−I from the 100 reference *E. coli* strains. Only 24 positive and 31 negative strains were detected while the rest 38 strains gave ambiguous agglutination of which the results could not be interpreted.

DISCUSSION

One of the most important reagents in the serological assays for detecting LT−I enterotoxin of *E. coli* is the antibody to the LT. However, the LT−I has been reported to have immunological cross-reactivity with cholera toxin (CT) and the CT−B subunits are commercially available. Thus, the anti−CT−B subunit serum was prepared locally by immunizing a rabbit with the CT−B subunits. The serum was used in the Biken test, staphylococcal co-agglutination test and reverse passive haemagglutination test. The rabbit anti−CT−B subunit serum at dilution 1:32 could react and formed sharp precipitin band with 3 μg of CT−B subunits in double diffusion test.

The modified CAYE medium (B2A) was prepared locally and used in the BT. The optimum dilution (1:10) of anti−CT−B serum which could detect LT released from both weak and strong producers had sensitivity at 2 μg when tested with the CT−B subunits. This level of sensitivity was in the same order as the results reported by Honda *et al.*, (1981) whose purified anti−CT−B subunit serum had sensitivity at 2.5 μg of CT−B subunits.

Although the commercial Biken test kits (using purified anti−LT) gave sharper precipitin bands when tested against the 100 reference strains of *E. coli* than the locally made anti−CT−B subunit serum, the specificity, accuracy and the positive predictive value of the BT using locally made reagents were slightly better than the tests used commercially available reagents, while the sensitivity and negative predictive value of the tests using either reagents were not different.

The staphylococcal co-agglutination test (SCOAG) using locally made reagents gave 100% sensitivity, specificity, accuracy, positive predictive value and negative predictive value. The 0.1 ml volume of the anti−CT−B subunit serum was optimum for sensitizing each ml of the preserved 10% staphylococci. The sensitized cells could detect as low as 5 ng of the CT−B subunits which was comparable to the results reported previously by Honda *et al.*, (1983) whose sensitized cells had the sensitivity at 10 ng of the CT. The advantage of our anti−CT−B subunit serum over that of Honda *et al.*, (1983) was that the whole serum could be used directly to sensitize the bacterial cells while the immunoglobulin fractions of the antiserum was required by Honda's method. Our sensitized cells could be kept at 4°C for at least 73 days

with very slight decrease in the sensitivity (longer time intervals were not studied). Brill *et al.*, (1979) reported that the bacterial cells prepared similarly were stable for 3 months. In this study, the formalinized, heat-fixed *Staphylococcus aureus* in lyophilized form could be stored for at least 22 months with slight decrease in the sensitivity (could detect 10 ng of CT).

In comparison between the BT and SCOAG, the two tests had equal specificity and positive predictive value. If the ambiguous results of the SCOAG were classified as positive, the sensitivity, accuracy and negative predictive value of the test would be 8%, 4% and 7% higher than those of the BT, respectively. If the ambiguous results were read as negative, the sensitivity, accuracy and the negative predictive value of the agglutination test were 2%, 1% and 1% higher than the BT, respectively. Moreover, the time required for SCOAG was 1 day while 2 days and 4 days were needed for the BT to detect strong and weak LT-I producers, respectively. The medium used in SCOAG was blood agar which is a common medium of microbiology laboratories. Lincomycin was used for enhancing LT-I synthesis by *E. coli* (Levner *et al.*, 1977); colistin, polymixin B or polymixin E and triton x-100 help in breaking down the bacterial cell wall to release the LT-I (Evans *et al.*, 1974). These reagents are commercially available in most of the less developed areas. It is our opinion that the SCOAG is the most suitable method for LT-I detection in the poorly equipped laboratories because of its simplicity, sensitivity and specificity.

SUMMARY

Diarrhoea caused by enterotoxigenic *Escherichia coli* (ETEC) remains a problem in Southeast Asia. At present, no routine laboratories as yet are available for ETEC detection. In this study, attempts were made to produce reagents for use in simple serological tests for detecting LT. The serological methods were the Biken, the staphylococcal coagglutination and the reverse passive hemagglutination tests.

For the Biken test, medium was prepared locally by mixing constituents as described previously by Honda *et al.*, (1981). Anti-CT-B subunit was prepared by immunizing a rabbit with commercial CT-B subunits (Sigma). Other chemical reagents e.g. colistin, lincomycin etc. were obtained from the local supplies. Using the locally made reagents to detect LT from 100 WHO reference strains of *E. coli* by the Biken test, it was found that the test had 100%, 92%, 96%, 100% and 92.5% of specificity, sensitivity, accuracy, positive predictive value and negative predictive value, respectively.

Protein A rich *Staphylococcus aureus* from the stock culture of the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University were grown in suitable medium i.e. blood agar containing lincomycin (BA-Lin). Suitable amount of the rabbit anti CT-B subunit (0.1 ml) was used to sensitize each ml of the formalinized, heat-fixed bacteria. The sensitized bacteria were used for detecting LT in the lysates of the 100 *E. coli* reference strains. The lysates were prepared by growing the *E. coli* strains on BA-Lin medium for 8 hours, then a loopful of each strain was inoculated into colistin solution (20,000 unit/ml). After incubating for 1 hour, the cell debris were removed and the lysate was tested. The staphylococcal coagglutination test was found to have 100% specificity, sensitivity, accuracy, positive predictive value and negative predictive value.

Reverse passive hemagglutination test us-

ing anti CT–B immunoglobulins sensitized tanned sheep red cells were used for detecting LT in the *E. coli* lysates prepared as described above. The test was found to give inconclusive results.

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