WORKING MANUAL FOR ASSAY OF E. COLI ENTEROTOXIN AND ELISA ASSAY FOR ROTA VIRUS ANTIGEN

M. I. Huq
D. A. Sack
R. E. Black

INTERNATIONAL CENTRE FOR
DIARRHOEAL DISEASE RESEARCH, BANGLADESH
Dacca, Bangladesh
April, 1979

Special Publication No. 3

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M.I. Huq[1]
D.A. Sack[2]
R. E. Black[2]

INTERNATIONAL CENTRE FOR
DIARRHOEAL DISEASE RESEARCH, BANGLADESH
G.P.O. Box 128, Dacca - 2
Bangladesh

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1 Head, Microbiology Branch
2 Investigator

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.

E.COLI ENTEROTOXIN ASSAY

Escherichia coli, a gram negative bacilli was considered in the past to be the non-pathogenic normal habitant of the gut. Most of the literature of the late 1950's regarding E.coli diarrhoea has concerned enteropathogenic strains which were diagnosed only by slide agglutinating with the commercially available antisera. Various studies done in the past 10 years have clearly indicated that entero-pathogenic Escherichia coli causes diarrhoeal disease either by invasion of the intestinal mucosa or by the elaboration of enterotoxins (1,2,3). Though available microbiological procedures can detect three main different types of Escherichia coli such as a) Entero-pathogenic b) Enterotoxigenic and c) Enteroinvasive; the clinical criterion and pathogenic manifestation of the disease separates them into two latter groups (b) & (c).

Enteropathogenic Escherichia coli.

As have already been mentioned the slide agglutination with the commercially available antisera has been and still the only method to detect the enteropathogenic serotypes. As the antiserum available is limited to these few serotypes, the laboratory methods used now may not reflect the total number of cases during the epidemic and may give misleading informations when studying an epidemic of enteropathogenic Escherichia coli.

Most enterotoxigenic E.coli from Bangladesh patients with severe diarrhoea fall into a few serotypes. The correlation between serotype and toxin production is most marked with strains which produce toxin(s). These serotypes are not the classical enteropathogenic serotypes which are detected in many clinical laboratories. Detecting these serotypes which when isolated from patients with diarrhoea, have a high likelihood of being enterotoxigenic is useful in epidemiologic studies. It should also be noted that the "enterotoxigenic serotypes" have some geographic specificity so that a different set of enterotoxigenic E.coli may be more common elsewhere.

Enterotoxigenic Escherichia coli.

In the late 1960's it was first recognized that certain strains of Escherichia coli produced an enterotoxin similar to cholera enterotoxin which causes fluid accumulation in ligated ileal segments of adult rabbits and some other animals. It was further determined that the enterotoxigenic strains of Escerichia coli elaborate one or both of two plasmid mediated enterotoxins

1) high molecular weight heat labile enterotoxin (LT) similar to cholera toxin with acts by the stimulation of adenylate cyclase and (2) a low molecular weight heat stable enterotoxin (ST) which acts by the stimulation of guanylate cyclase (4,5). The Chinese hamster ovary cell assay and the Y_1 Adrenal cells assay are convenient and sensitive methods for the detection of LT. The infant mouse assay has been found to be a reliable and convenient method for the detection of ST.

A. Chinese Hamster ovary cell assay and the infant mouse assay.

1. Selection of strains of E.coli

Stool cultures from patients are plated onto MacConkey Agar media along with other diagnostic media. Individual E. coli colonies, well isolated, are picked and stocked in a Blood Agar base slant in 1 dram screw cap culture vial and the same colony transferred onto K 1 Agar and MIU medium for confirmation of E.coli. Two to 5 colonies from each patients along with a pool of 10 colonies are picked and stocked on separate slants. The slant well closed will keep the culture viable for about a year. Most subsequent tests can be carried out from this slant.

2. Media and cultural conditions: Various media have been tried by different workers for the production of toxin for infant mouse assay. Dean et al. (6) used T.S.B. (BBL) in flasks and inoculated that with broth cultures of the E.coli and incubated at 37°C in a rotary shaker bath at 200 r.p.m. Giannella (7) used four E.coli strains and three different media, Trypticase Soybroth, Brain Heart Infusion broth and Casamino acid with yeast Extract medium. They found that Casamino acid Y.E. medium is the best for the production of toxin by E.coli cultures and shaking condition in a roller drum gives the maximum toxin in the broth. Wachsmuth et al. (unpublished data) worked with different media (Heart infusion broth, syncase broth, casamino acid plus 0.6% yeast Extract and Trypticase Soybroth plus 0.6% yeast Extract at different incubation time (18, 24 & 48 hours) and different conditions (roller, shaker or stationery). Using strains from different geographic location she found that TSB with yeast Extract is as good as and in some cases better than Casamino Acid Yeast Extract medium.

We have worked at CRL with isolates of E.coli using four different media Trypticase Soybroth, Heart infusion broth, Trypticase Soybroth plus 0.6% yeast Extract and Casamino

acid with 0.6% yeast extract. In our hand Trypticase Soybroth plus yeast extract gave the best toxin production in roller drum at 22 r.p.m. of 37°C and Heart infusion broth gave the least. On this basis TSB with 0.6% Y.E. has been chosen to be the media for toxin production of E.coli.

3. Preparation of crude filtrate

Crude culture filtrate containing toxin for both ST and LT assay are prepared at the same time in the same culture tube. E.coli strains to be tested are inoculated into 2.5 ml. of Trypticase Soybroth + 0.6% yeast Extract medium already sterilized in tubes suitable to fit in a roller drum. For each run two control Toxin positive and a control Toxin negative strains are inoculated. Cultures are incubated at 37°C in a roller drum running at a speed of about 22-25 circles per minute.

After about 20-22 hours incubation the tubes are taken out and centrifuged in a Sorvall centrifuge using SM-24 type head and plastic tubes. The supernate liquid is divided equally in two one dram vials. In one vial a drop of gentamycin solution (containing 5 mcg gentamycin sulphate) is added which will be used for ST assay. The other tube is kept frozen at 60°C and is used for Chinese hamster overy cell assay.

4. Infant mouse assay

Newborn Swiss albino suckling mice (2-4 days old) are separated from their mother immediately before use and randomly divided into groups of two. In these mice the stomach can easily be seen from outside as this contains milk. Each mouse is inoculated (intragastric, by percutaneous injection) with 0.1 ml. of crude culture supernate containing 2 drops of 2% Even's blue per ml. as described by Dean et al. (6). The mouse is incubated at 25°C for 4 hours. It has been found by Giannella (7) that incubation at 25°C gave more positive result than at 37°C and the best incubation time ranges from 2-4 hours. After 2-4 hours incubation at 25°C the mice are killed by cervical dislocation, the abdomen is opened by a forcep and the entire intestine is removed with the forcep. The weight of the gut and of the remaining carcass of the two mice is taken. The ratio of gut

weight to remaining carcass weight is calculated for each mouse and the result averaged. The animals which do not show dye in the intestine or with dye within the peritoneal cavity at autopsy are discarded. A ratio of less than 0.070 is considered negative, 0.070-0.085 is considered borderline and should be repeated while those having a ratio over 0.085 are considered positive.

5. Chinese hamster ovary (CHO) cell assay for LT

Guerrant et al. (8) while working on the effect of enterotoxins of E.coli and V.cholerae found that the toxin can alter the morphology of the Chinese hamster ovary cell when kept as a monolayer in a specific medium. The test has been found to be reproducible and can be done with minimum equipment.

The assay is done as follows: A monolayer of Chinese hamster ovary cell is grown in a 25 CM^2 tissue culture flask under 4.5 ml. of F_{12} medium with 10% Fetal calf serum (FCS) and antibiotic (100 units/ml. Penicillin G and 100 mcg/ml Streptomycin). When a measured quantity of the prepared toxin is added to the cells suspension in a microtitre plate and incubated overnight, a toxin positive preparation will cause elongation of the cells.

a. Materials

1. F - 12 medium
2. Flat bottom tissue culture plate
3. Fetal calf serum
4. Antibiotic: Penicillin G and Streptomycin
5. Tissue culture flask
6. Inverted Microscope

b. Preparation of F - 12 medium

1. Take 1000 ml. of distilled water and adjust pH to 6.8
2. Add 10.6 gms of F-12 dehydrated media and then HEPES buffer 5.955 gram.
3. Add 100 mcg/ml. of Streptomycin and 100 unit/ml. of Penicillin G. Mix well.
4. Filter with 0.22μ Millipore membrane filter.
5. Aseptically, aliquot 100 ml. amounts into 150 ml. milk dilution bottles and store at $4^{o}C$.

c. Propagation of CHO cells

One to two drop of trypsinised cell suspension is added to a new tissue culture flask containing 4.5 ml. of F12 medium with 10% FCS and antibiotics. A monolayer of CHO cells should spread all over the floor of the tissue culture flask within 3-4 days which can be seen under the inverted microscope. Cells must be passaged atleast once a week and two flasks must always be maintained.

d. Trypsinisation of CHO cells

1. Decant gently F12 medium from the flask after it is grown in the medium atleast 3-4 days and add 2.0 ml. trypsin.
2. Tilt the flask 10-12 times gently within 5-6 seconds and decant the trypsin.
3. Repeat process (2)
4. Now put the flask in the incubator at 37°C for 20 minutes with the monolayer side of the flask up.

e. Preparation for inocula for new flask inoculation and for assay

1. From the step (4) described above take the flasks out and suck back the remaining trypsin carefully from the flask.
2. Add 2.0 ml. of F12 medium added with 10% FCS.
3. Agitate gently to get the cells released from the flask wall and break off any clot present.
4. Take 0.1 and 0.05 ml. of the cell suspension and inoculate into 4.5 ml. of medium with 10% FCS in duplicate.
5. Replace 4.5 ml. of F12 media 10% FCS after 4 hours inoculation.
6. The remaining suspension from step (3) is used for CHO cell assay.

f. CHO tissue culture assay for enterotoxin

1. Add 0.02 ml. of prepared toxin into the well of the tissue culture plate in duplicate.
2. Add to it 0.2 ml. of cells suspended in F12 medium with 1% FCS. (cell suspension from one flask is suspended to 100 ml. of F12 + 1% FCS).
3. Incubate at 37°C.

4. Read assay at 20-24 hours (no longer) Pour off all fluid, prior to reading fix the cells in 10% buffered formalin and 100% methanol for 5 minutes then stain with Giemsa (1:40) for 20 minutes. Laboratory test chamber slides are dissembled and treated as ordinary slides i.e. stained into copland Jar.
5. The number of elongated cells seen under the inverted microscope in each 100 cells may be counted and recorded. Elongation is defined as bipolar and longer than wide.

Result Interpretation

A negative is equal to or less than 9.3 elongated cells/100 cells and a positive is equal to or more than 13.6 elongated cells/100 cells. Tests with 9.3 to 13.6 elongated cells/100 cells are border line and should be repeated.

INFANT MOUSE & CHO CELL ASSAY

EQUIPMENT & MEDIA REFERENCE

1. Inoculation tube: 9 ml. screw cap tube Kimax Cat. No. 14 - 930 - 10 - A (Fisher Scientific)

2. Roller Drum: Belco Roller Drum, Bellco Glass Inc. Vineland, New Jersey

3. Centrifuge: Sorvall Superspeed. Automatic Refrigerated Centrifuge Mod. RC2 -- B, Head: SORVALL Type SM - 24

4. Plastic tubes: NALGEN, Sybron Crop. Nalgen glass wan division. Centrifuge table round P.C. Cat. No. 3117-0035.

5. Tissue Culture flask: 30 ml. tissue culture flask FALCON - 3012. Cat. No. 10-126-2B (Fisher Scientific)

6. Milk dilution Bottle: PYREX 160 ml. Screw cap Milk dilution Bottle Cat. No. 2-943-5 (Fisher Scientific)

7. Inverted Microscope: CK OLYMPUS TOKYO, JAPAN P-120 V. 50,60 C/S. 6 V, 2 A Made in Japan

8. Millipore filter: Millipore Membrane filter Assemblies. Conical flask, Filtering head, pump, membrane filter 0.22 μ pore size S X 00 - 025 - 00 Swinese 25, filter holders GSWP - 025 - 00 MF - Millipore Filter, Millipore corporation, Bedford, Mass. 01730

9. 62406 - 106 Tissue culture plate, 96 well. drop VWR Scientific, 6601 Amberton Drive Baltimore, Md. 21224

10. Ham's F - 12 (IF - D83A), Flow Laboratories Rock Ville MD. 20852

11. Fetal Calf Serum (No. 614)
Grand Island Biological Co.
3175 Staley Road
Grand Island, New York 14072

12. Trypsin (No. 505)
Grand Island Biological Co.
3175 Staley Road
Grand Island, New York 14072

13. Giemsa Blood Stain
Harleco (No. 620-16)
Scientific products
P.O. Box - 1470
New York, N.J. 07101

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Y_1 ADRENAL CELL ASSAY FOR E.COLI HEAT LABILE TOXIN

INTRODUCTION

Certain strains of E.coli produce a heat labile enterotoxin which may be detected in the Y_1 adrenal cell assay. This assay is based on a morphologic change in the cells which occurs when the toxin is added to a monolayer of Y_1 adrenal cells. This morphologic change (and an associated change stimulation of steroid production) is due to stimulation of adenylate cyclase with the resulting increase in intracellular cyclic AMP, and is specific for this type of stimulation.[1] Donta, et al., first demonstrated that picogram quantities of the purified cholera enterotoxin were capable of inducing these morphologic changes and steroidogenesis in adrenal cells,[2] and he subsequently showed that culture filtrates from enterotoxigenic E.coli could also cause these same changes; whereas, culture filtrates from non-toxigenic E.coli did not. Sack, et al., modified the technique for enterotoxigenic testing by using Y_1 adrenal cells grown in miniculture plates.

Adrenal Cell Morphology

Y_1 adrenal cells when growing in monolayer appear flatened. Following exposure to enterotoxin they change their morphology to a round cell which can be appreciated under light or phase-contrast microscopy. This morphologic change is specific for adenyl cyclase stimulation and does not occur with prostaglandin stimulation, heat-stable toxin from E.coli or other toxins, and therefore is the basis for the morphologic assay for enterotoxin.

Growth of Cells

Normally, when being subcultured, and in suspension, the cells are round due to trypsinization. They quickly fall to the bottom of the new tissue culture dish and attach to the dish, assuming the flattened appearance. Once attached, they begin proliferating; each cell (or group of cells) forming an island. As the culture grows, the islands become confluent and occasionally "heap-up" with cells growing on top of each other. With further growth, the cells eventually loose their attachment and begin coming off the plate.

The cells are most reliable for assay when the islands are well developed but prior to complete confluence, and should not

be used when they are heaped up, or coming off the plate. The optimal time for assay is two days after subculturing.

Subculturing of cells should be done when the cells are confluent but before they start lifting off the plate (7-10 days). The media should be changed every 2-4 days.

METHOD OF READING ROUNDING

After stimulation with E.coli heat-labile toxin or cholera toxin, the cells change their shape, assuming a round appearance. Depending on the dose of toxin, this change occurs in 4-8 hours, occasionally earlier, but is most reliable when examined the next morning. The individual cells and cells growing on the edge of an island tend to change first; however, individual cells can be misleading, so that it is best to look of cells. A positive cell will generally appear lighter and refractile in appearance, often with a dark center and will stay in focus when focusing up and down while flat cells are darker and go quickly out of focus when focusing up and down. Positive cells also have projections which connect to other cells.

False negatives - These generally occur only if the monolayer is too light, i.e., either the culture was used too early before islands were formed, or the plate was seeded too lightly.

False positives - Old senescent cells, especially ones which are heaped up occasionally become rounded. Non-specific toxins which damage cells also cause a type of rounding. This rounding however can be differentiated because of the absence of projections extending from the cell, by a darker appearance to the cell, with loss of detail, and often by excess cellular debris in the medium. This non-specific toxic effect can be induced by undiluted serum, stool filtrate, chemicals such as 2ME, and by tissue culture plates which have been improperly treated by the manufacturer. They are rare however when following the protocol given.

Another false positive is "over reading" rounding. Normally there are a few cells which are rounded, and the percentage of "background rounding" varies between different strains of Y_1 cells and media used. Negative, and positive controls need to be included therefore for comparison. Since bacterial filtrates contain an excess of toxin, if it is present at all, this will be no problem in testing strains of E.coli for toxigenicity, but

is a problem with antitoxin titration since one is working with minimal quantities of toxin, and minimal rounding.

Media, Procedures and Equipment

Hams F_{10} media supplemented with horse serum 12.5% and fetal calf serum 2.5% has been used as the traditional media. MEM supplemented with fetal calf serum 10% has also been successfully used. Gentamicin (40 µg/ml) and penicillin (1000 units/ml) has been added in our laboratory to help prevent contamination, especially since we often put live bacteria into the tissue culture. The phenol red indicator in the media is useful in telling when to change the media since, with cell metabolism, the media pH changes toward acid.

Basic tissue culture equipment is needed including a sterile hood (UV or laminar flow), a 5% CO_2, 95% air, humidified 37°C incubator, an inverted (phase contrast preferably) microscope, a table top centrifuge, and the usual sterile glassware and pipettes. All steps in handling and subculturing cells must be done using strict aseptic technique.

Nutrient Media F_{10} (Ham's)

Obtained from Gibco as dry powder in 1 liter packets.

Ingredients for 4 liter batches:

1.	4 packets F_{10}	
2.	Horse Serum	600 ml.
3.	Fetal Calf	100 ml.
4.	Gentamicin	4 ml. (40 µg/ml.)
5.	Penicillin	4 million units
6.	Distilled water	3300 ml.
7.	$NaHCO_3$	4 grams - adjust pH to 7.2

Procedure:

1. Mix all ingredients 1 through 6 into millipore container. Add $NaHCO_3$ dissolved in small quantity H_2O to adjust pH to 7.2.
2. Filter sterilize with pressure sterilization - dispense into 100 or 500 ml. bottles.
3. Bacterial quality control.
4. Freeze media.

Subculturing and Maintaining Y_1 Cells in Flasks

Adrenal cells can be grown on tissue culture petri dishes, and flasks of any size. Cells are subcultured when they have reached near confluence usually in approximately 7 days. This growth can be determined grossly by looking at the bottom of the culture plate. Old cells begin coming off the plate, so subculturing needs to be accomplished before this occurs.

Ingredients and Equipment Needed:

Mature cell culture on flask
PBS pH 7.4
Trypsin 0.125% (Catalog # 505 Gibco)
Tissue Culture Media
Sterile Centrifuge tubes, pipettes
Counter-top Centrifuge
Suction apparatus

Procedure:

1. Suction old media from mature culture.
2. Wash culture once with PBS and suction off PBS.
3. Add trypsin to plate - enough to cover culture (2 ml. for large flask).
4. Let trypsin incubate with cell culture until cells begin to loosen from plate (usually 5-10 min.) at $37^{o}C$.
5. Add 5 ml. of tissue culture media to culture to neutralize trypsin.
6. Suspend cells in media by agitation.
7. Transfer suspended cells in media to sterile centrifuge tube.
8. Centrifuge at 2000 RPM x 5 min.
9. Suction off supernatant in centrifuge tube and leave button of cells in tube.
10. Resuspend button in 5 ml. of fresh media.
11. Dispense drops of cell suspension into dishes or flasks to which you have added fresh media (3-4 drops into flask).
12. Place new cultures back into incubator.

Subculturing Y_1 Cell onto Miniplates

Ingredients and Equipment Needed:

Mature cell culture
Fresh tissue culture media
PBS pH 7.4
Trypsin 0.125 (Gibco Catalog #505)
Sterile centrifuge tubes, pipettes
Centrifuge
Suction apparatus
Miniculture plates (Cooke) (96 well)

Procedure:

1. Wash, trypsinize cells, centrifuge, decant, and resuspend cells as described for subculturing cells onto plates and flasks.
2. Dilute the cell suspension approximately 1:100 in tissue culture media, and dispense 0.2 ml. of dilute cell suspension into each well. (The dilution factor can be varied according to the quantity of cells in the button.)
3. Cover the plate with plate sealer or sterile plastic cover (Cooke) and place in incubator.

MAINTENANCE

Cultures are always kept in 5% CO_2 humidified incubator except when being handled. Tissue culture media is changed every 2-4 days. The cultures may be used for assay when a good growth of cells has been formed, usually in 2 days or may be kept up to 7 days if they are to be used as stock cultures (with media changes as indicated). The phenol red indicator will turn the media yellow in acid conditions and this can be used as a guide to how often the media needs to be changed.

Cell Freezing and Storage

Ingredients and Equipment Needed:

Same as for subculturing cells. In addition:

Sterile freezing vials

Tissue culture media with 10% glycerol or 5-10% Dimethyl Sulfoxide (freezing media)

Procedure:

1. Remove media, wash with PBS, Trypsinize, centrifuge and decant supernatant as for subculturing cells.
2. Add 1 ml. freezing media to button in centrifuge tube, suspend cells in this media, and transfer to freezing vial. Seal freezing vial with 0_2 flame using the pull method.
3. Freeze the vials gradually ($1^{o}C$/minute drop in temp) and maintain in liquid nitrogen.
4. Thaw rapidly when needed, transfer to centrifuge tube, add 4 ml. fresh media, centrifuge for 5 min. at 2000 RPM. Decant supernatant, resuspend cells in 5 ml. of fresh media and plate entire enoculum of cells onto one dish or flask.
5. Maintain cells as described previously.

Toxin Assay - Morphologic

Ingredients Needed:

Test substance (usually culture or culture filtrate but may be stool, liquified tissue, gut washing, etc.).

Adrenal cell tissue culture which has attached and grown.

Pasteur pipettes.

Transfer plate (Cooke) if mini-culture used.

Procedure:

1. Inspect tissue culture to assure that it has attached and grown into islands of cells. Cultures which are scanty, or too old are not reliable.

2. Add test substance to tissue culture (0.05 ml. for each mini culture well).

3. After 5-10 minutes, remove with suction the tissue culture media containing test substance.

4. Wash with PBS and aspirate PBS. For sterile substances (filtrates, sera, etc.) this step is not necessary.

5. Replace fresh tissue culture media and return plate to incubator.

6. After 6 hours and/or overnight - observe cells for presence of rounding.

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SERENY TEST FOR ENTEROINVASIVE ESCHERICHIA COLI

It has been observed by many authors that many organisms, like shigellae and other dysentery bacilli, after their entry into the gut penetrate and replicate within the intestinal mucosa which results in gross and microscopic intestinal lesions. The invasiveness of these shigellae organisms has been studied by investigators in animal models (1-2) especially in guineapig eye.

Some E.coli strains has also been found to cause a dysentery - like illness with diarrhea, fever and abdominal cramps but rarely bloody stools. The E.coli associated with this illness produces Keratoconjunctivitis in the guineapig, (1-3). The importance of these organisms as a cause of disease is unknown, but they have been isolated from cases of diarrhea from several studies in the U.S.A., Bangladesh and many other countries of the world.

Enteroinvasiveness is tested by dropping the test bacteria into guineapig eye. The invasive strains cause Keratoconjunctivitis in the eye.

Animals Adult Albino guineapigs are used for the experiment. Prior to infection the animal eyes are carefully examined and those with ocular alteration are excluded. After inoculation, the animals are kept in individual cages.

Bacterial culture and inoculation of animals

All the cultures were identified and confirmed by inoculating onto Kligler Iron Agar slant, Motility Indole Urea medium and citrate medium. Fresh smooth strains are stocked onto Blood agar base slant.

For making culture suspension, 4-6 hours grown cultures are spread onto a Blood Agar plate and the lawn of bacteria in the next days is suspended into sterile normal saline. A suspension containing approximately 10^9 cells/per ml. is made and 0.05 ml. of this suspension is instilled in one of the guineapig eyes after lifting the upper and lower lids, and both lids then gently massaged to ensure distribution of the organisms over the eyeball. One guineapig is used for testing each strain. Control positive and negative strains are always used. The ED_{50} for most strains of invasive bacteria is approximately 10^6 per eye so the inoculum used will give consistent results.

Observations

Following inoculation, the animal eyes are inspected every 24 hours for 7 days for signs of Keratoconjunctivitis. If signs of infection were not present after 7 days of the inoculation, the culture were recorded negative. Nearly all positive guineapigs are positive within 5 days.

Note: When inoculating a single eye, we have not seen cross infection to the opposite eye. This means that each eye may be used for a different test organism. Also, if an eye is infected it may develop immunity to a homologous rechallenge; therefore, guineapigs with a positive test cannot be reused. Guineapigs may be reused however if they did not develop Keratoconjunctivitis.

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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF ROTAVIRUS ANTIGEN

Antibodies and antigens labelled with flourescent dyes or isotopes have been used extensively for the immunodiagnosis for over two decades. However they do have some disadvantages. Immunoflorescence and Radioimmunoassay are subjective, requires expensive equipment and are thus restricted to central laboratory facilities. The use of enzyme labelled reactants in the ELISA methods yields objective results, with same sensitivity as radio-immunoassay but is cheaper and the test can be carried out in laboratories with limited facilities. (1-3). The basic Elisa test depends on two assumptions a) that antigen and antibody can be attached to a solid phase support yet retain immunological activity and b) that either antigen or antibody can be linked to an enzyme and the complex retain both immunological, enzymatic activity. It has been shown that antibodies and many antigens can be readily attached to paper discus or to plastic surfaces such as polyvinyl or polystyrene, either chemically or by passive adsorption and still retain their activity.

Competitive assays utilizing a known enzyme labelled reagent, as in most radioimmunoassay can be used. However it has been found that non-competetive method - the "double antibody sandwitch" are the most useful in practice for the detection of viral antigen, the basis of which are as follows:

a. The wells in polysterene plates are coated with immunoglobulins containing specific antibody to the antigen. Plates are washed.

b. The test solutions thought to contain antigens are incubated in the sensitized wells. Washing removes unreacted material and any antigen remains attached to the unmobilised antibody on the plastic surface.

c. The conjugate consisting of enzyme labelled specific antibody is then incubated in each well. This will react with any antigen already captured by the antibody on the well surface. A further washing removes excess conjugates.

d. Finally the enzyme substrate is added. Its rate of degridation depends on the amount of enzyme labelled antibody present and that, in turn depends on the amount of antigen in the test sample. The enzyme

substrate is chosen to give a colour change upon degridation, and this can be assessed visually or measured in a spectrophotometer. Yolken et al (5) used this method for the first time with success for the detection of human Reovirus-like agents (Rota virus) from cases of infantile gastroenteritis. Yolken et al modified the method by using higher detection of guineapig antirotavirus antibody (5).

A. Collection of Specimen:

Stool specimens or rectal swabs are collected from patients suspected to be suffering from gastroenteritis. The specimens are transferred to Phosphate Buffer Saline and sent back to the laboratory for assay for the detection of Rotavirus antigens. The swabs in PBS can be kept frozen for years at-70^{o}C.

B. Materials:

Suggested materials and supplies required for rotavirus antigen detection.

1. Goat anti guineapig serum
2. Alkaline Phosphatase type VII.
3. PBS and Tween 0.05% + 1% fetal calf + 1% normal goat serum.
4. Goat antirotavirus serum.
5. Guineapig antirotavirus serum.
6. Alkaline phosphatase labelled goat anti-guineapig serum.
7. Normal fetal calf serum.
8. Normal fetal goat serum.

Procedures:

1. Dilute goat antirotavirus 1:100,000 (1:20,000 of a 1:5 dilution) in carbonate buffer. Add 100 µlto well and store at 4^{o}C unit use (store atleast 24 hours).
2. Just prior to use wash three times with PBS tween.

3. Add 50 µl. of Tween - 1% FCS - 1% goat serum and add 50 µl. of stool suspension (2-10%). Incubate overnight at 4°C.

4. Suck all the stool from the plate and wash three times with PBS tween buffer.

5. Add 100 µl. of guineapig anti-rotavirus serum diluted 1:4000 in PBS - tween (1:800 of a 1:5 dilution) + 1% faetal calf serum and normal goat serum. Incubate 1 hour at 37°C.

6. Suck and wash three times with PBS tween.

7. Add 1:800 dilution alkaline phosphate labelled anti G.P. serum (0.1 µl.) quantity.

8. Incubate 1 hour at 37°C and wash three times with PBS tween and add 0.1 µl. of substrate (1 tablet of sigma 104 substrate for each 5 ml. dielhanolamine.).

9. Incubate plates 5-20 minutes (depending on the room temperature) and read plate. Positive control diluted and undiluted and negative control should always be given on a plate.

10. Block the colour before taking the reading with 3 molar sodium hydroxide.

A positive control, a highly diluted but still positive dilution of a positive control, and a negative control is given in each plate run. A good yellow positive colour is compared with weakly positive standard of diluted positive pool.

EQUIPMENTS AND CHEMICALS FOR ELISA

Titertek - 8 channel pipette 100 ml.

Titertek - 8 channel pipette 25 ml.

Poly vinyl micro titre plates

Finnipipette 1 - 5 ml.
5 - 50 ml.
50 - 250 ml.

Wash bottle

Suction Pump

Pasteur Pipette

Tips

Chemicals

Alkaline phosphatase type VII
1000 units/ml.

P-nitrophenyl phosphate
(5 mg. tablet)

Tween 20

Diethanolamine

and other chemicals to make the buffers

Buffers

a. Carbonate - bicarbonate buffer pH 9.6

Sodium Carbonate	-	1.59 gms.
Sodium Bi-carbonate	-	2.93 "
Sodium Azide	-	0.2 "

Make up to 1 litre with distilled water. Store at $4^{o}C$.

b. PBS - Tween pH - 7.4

Sodium chloride	-	8.0 gms
Monopot. Dihydrogen phosphate		0.2 "
Disod. hydrogen phosphate		1.15 "
Pot. chloride		0.2 "
Sodium Azide		0.2 "
Tween 20		0.5 "

Make up to 1 litre with distilled water.

c. Substrate - Each tablet dissolved in 5 ml. of DEA (Diethanolamine) buffer
Diethanolamine buffer

DEA	-	97 ml.
Magnesium chloride $6,H_2O$	-	0.2 gms
Distilled water	-	0.5 to make litre after adjustment of pH

Add 1 normal Hydrochloric acid to make pH 9.8

ELISA

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ACKNOWLEDGEMENT

The authors are grateful to Dr. M.M. Rahaman and Dr. W.B. Greenough, III for their helpful suggestions and to Dr. A. Al. Mahmood, Mr. A. Alim, Mr. Golam Kibryia, Mr. P.K. B. Neogy, Mr. S. Huda, Mr. Abdul Huq and Mr. M.U. Khandkar for their technical support in doing the laboratory works and to Mr. Nurul Huda for getting this manual published.

ICDDR,B (CRL) publications can be obtained from Publications Unit, International Centre for Diarrhoeal Disease Research, Bangladesh, G.P.O. Box 128, Dacca - 2, Bangladesh.

List of current publications available:

A. CRL Annual Report 1976.

CRL Annual Report 1977.

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