ENTEROVIRUSES AND BACTERIA IN RELATION TO SEWAGE TREATMENT EFFLUENTS

by

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SUMMARY

This project was begun in 1968 when the isolation of enteric viruses from the effluents of sewage treatment was not a routine procedure. After a number of preliminary experiments, a method of enteric virus isolation was developed for use with South Australian effluents. Volumes of filtered effluent in cell culture medium were inoculated directly onto monolayers of primary monkey kidney cells for viral adsorption.

Preliminary work included the effect of serum on the recovery of poliovirus after membrane filtration of a distilled water suspension. The recovery of poliovirus from suspension in Bolivar stabilisation pond effluent was also investigated. The serum was shown to prevent loss of poliovirus from the distilled water suspension during filtration, although recovery from stabilisation pond effluent was low. The low recovery was apparently caused by adsorption of viruses to solids which were lost on centrifugation to remove bacterial and fungal contaminants.

One experiment showed that plaque forming units of virus could be recovered from aluminium hydroxide floc after flocculation of screened sewage. Development of reliable methods to concentrate virus from water is necessary for further work with waters such as

chlorinated effluents and public water supplies.

Numbers of the indicator organism *E.coli* in stabilisation pond effluent from Bolivar were estimated weekly over a four year period. It was shown that there were significant differences between *E.coli* estimations between months and between years over the four years. The variance between months was attributed to seasonal variations, and that between years to an increase in stabilisation pond area in April 1970. No significant difference was found in *E.coli* estimations derived from the direct 44°C examination compared with the confirmed 37°C test, using stabilisation pond effluent.

A Spearman rank correlation of + 0.75 showed a significant correlation between the isolation of *Salmonella* and the indicator organism *E.coli*, validating the use of *E.coli* as an index of the presence of *Salmonella* in Bolivar stabilisation pond effluent. Similar treatment of the enterovirus isolations gave a Spearman rank correlation of + 0.39 with *E.coli*, showing that this organism was not suitable as an index of enteroviruses in stabilisation pond effluent.

In examinations of effluents from other sources, enteroviruses were found in 4 out of 23 one litre samples from metropolitan activated sludge effluent (Glenelg Treatment Works), 5 out of 9

from country stabilisation pond effluent samples, compared with 11 out of 44 from Bolivar effluent, suggesting that stabilisation ponds were less effective in virus removal than activated sludge treatment. A possible further disadvantage of stabilisation ponds is their potential to become breeding grounds for vectors of a number of viral diseases.

DECLARATION

I declare that this thesis is of my own composition and that it is a record of original work conducted during the years 1967 to 1971 in the Engineering and Water Supply Department, Water and Water Pollution Control Laboratories, Bolivar. The work described herein has not been submitted for any other degree, award or diploma.

Maurice Lloyd Goss

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PART A

INTRODUCTION

PART A

INTRODUCTION

This thesis is based on work done during investigations of the possible extensive reuse of treated effluent from Bolivar Sewage Treatment Works. Reuse of such water can be "considered an additional water resource, and its planned reuse for lower grade purposes than drinking can result in large savings of clean water supplies" (World Health Organisation 1973). The volume of sewage treated in this stabilisation pond plant was 36,500 mega-litres in the year 1971/72, which, with suitable reuse, could save an equivalent quantity of higher grade water.

However the quantification of human enteric viral and bacterial pathogens and determination of the association of these with the indicator organism *Escherichia coli* (Tables 18, 20) was necessary to permit the evaluation of the effluent as a possible vehicle in the transmission of disease in any particular form of reuse. The assessment of risk of human infection is an epidemiological problem and outside the scope of this thesis.

Estimates were also made of the number of human enteric virus

particles present in the effluent of Glenelg Treatment Works (Table 21), an activated sludge plant, and a number of small country treatment works (Table 22).

It is difficult to define animal viruses concisely. WILSON and MILES (1965) gave a tentative definition of pathogenic viruses as being "Particles of smaller diameter than 0.3 microns, those less than 0.2 microns being invisible by ordinary microscopic methods, which pass through filters impermeable to bacteria, which reproduce only within susceptible living cells after adsorption and an eclipse period, which are of variable complexity but consist essentially of protein, lipid and either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), which may invade one or more species of host, which sometimes give rise to characteristic inclusion bodies in the tissues, and which cause a latent or overt infection often followed by a lasting immunity".

The majority of human faecal or enteric viruses found in water and sewage are enterovirus members of the picornavirus group. The enteroviruses were defined (Report 1962) as being small (23-30 nm) RNA viruses resistant to diethyl ether. They were also stabilised against heat by the presence of magnesium ions (WALLIS and MELNICK, 1962). Over 100 enteric viruses may be excreted in human faeces

and therefore present in sewage and sewage contaminated waters. These are the enteroviruses (polio, coxsackie and echoviruses), the reoviruses, adenoviruses and the agent of infectious hepatitis, as well as at least 2 agents of viral gastro enteritis. The enteric viruses are listed in Table 1 with a brief summary of their associated diseases (GRABOW, 1968; GELDREICH and CLARKE, 1971).

GRABOW (1968) and NUPEN (1970) reviewed the isolation and epidemiology of viruses from sewage and effluents, and concluded that of the enteric viruses only the agent of infectious hepatitis has been unequivocally transmitted by water contaminated with sewage. This is the only viral disease agent to have met beyond doubt Maxcy's hypothesis (MAXCY and HOWE, 1943) that before a disease is classified as waterborne "it must be convincingly shown that the behaviour of a disease in human communities is such as would be expected if the causative parasitic microorganisms were dependent for continued dissemination in part or at times, upon the medium of contaminated water." The most convincing epidemiological evidence according to Maxcy is the explosive outbreak in which the only common medium of dissemination is a water subject to human faecal contamination. All work on the epidemiology and survival of infectious hepatitis has had to rely on its cultivation in human volunteers (NEEFE and STOKES, 1945) as no other culture

TABLE 1. Potential waterborne viral disease agents and associated diseases.

Virus	Number of Types	Disease
Infectious Hepatitis	1 (?)	Infectious hepatitis
Coxsackie A	26	Herpangina, aseptic meningitis, paralysis
Coxsackie B	6	Bornholm disease, aseptic meningitis, acute infantile myocarditis
Reoviruses	3	Fever, respiratory infections and diarrhoea
Echoviruses	34	Aseptic meningitis, rash and fever, diarrhoeal disease, respiratory illnesses
Adenoviruses	32	Respiratory and eye infections
Polioviruses	3	Paralytic poliomyelitis, aseptic meningitis
Gastroenteritis Virus	?	
Diarrhoea Virus	?	

(Table 1 adapted from Grabow, 1968, and Geldreich and Clarke, 1971)

system is at present available. The largest recorded waterborne outbreak occurred at Delhi in 1955-56 with an estimated number of 29,300 icteric cases (DENNIS, 1959).

However BERG (1967) felt that regular ingestion of small quantities of virus even by small numbers of people could result in asymptomatic carriers who could then spread the infection by normal person to person contact, which could, because of the higher virus concentrations, result in a greater rate of overt disease. This would then appear as a higher illness rate rather than as transmission by water.

Since *E.coli* or *Bacillus coli communis* (SMITH, 1895) was introduced as an index of water quality, bacteriological standards have been based largely on this organism. Other faecal organisms such as the coliform group, faecal streptococci and *Clostridium perfringens* (Anon., 1969) have been used as indicators of contamination with human excrement and sewage.

Organisms implicated in human waterborne disease in USA listed by CRAUN and McCABE (1973) included the agent of infectious hepatitis, *Salmonella*, *Shigella*, enteropathogenic *E.coli*, the flagellated protozoan *Giardia lamblia*, *Entamoeba histolytica* (amoebic dysentery),

Pseudomonas (infections of premature babies) and flavobacteria (systemic infections following extensive surgery). McKEE and WOLF (1963) also included *Vibrio comma* (cholera), mycobacteria *Brucella*, *Bacillus anthracis*, *Pasteurella tularensis* and *Leptospira* as human waterborne diseases.

SCHWIMMER and SCHWIMMER (1964) suggested that some clinically diagnosed cases of waterborne "infectious hepatitis" and other viral diseases were due to intoxication with blue-green algae. The first recorded domestic animal deaths caused by blue-green algae (Lake Alexandrina S.A., Francis 1878) were apparently due to drinking of lake water containing *Nodularia*. The blue-green algae therefore could be of public health importance in water reuse if toxic blooms occur in stabilisation ponds.

The use of *E.coli* and coliforms in water was regarded by GELDREICH and CLARKE (1971) as a reliable indicator of the adequacy of water treatment processes as far as pathogenic bacteria were concerned, but further studies were required to assure the reliability of *E.coli* and coliforms as satisfactory indicators of the presence of enteric viruses.

HILL, AKIN and BENTON (1971) reviewed methods of detection of viruses in water, and described various concentration methods

including adsorption to membrane filters or inorganic flocs such as aluminium phosphate, aluminium or ferric hydroxides, iron oxide particles or polyelectrolytes; aqueous polymer two-phase separation, soluble alginate filtration, ultrafiltration, ultracentrifugation, electrophoresis, electroosmosis, polyethylene glycol hydro-extraction and gauze sampling.

In the present work, although one experiment was carried out by the aluminium hydroxide flocculation method of WALLIS and MELNICK (1967), all other enteric virus isolations were made by directly inoculating volumes of filtered effluent in the cell culture medium and allowing viral adsorption onto a monolayer of primary monkey kidney cells (PMK) in a similar way to that suggested by BERG, BERMAN, CHANG and CLARKE (1966).

The object of the present work was to estimate the numbers of enteroviruses of possible public health significance present in metropolitan stabilisation pond effluent, to study their relationship to the indicator organism *Escherichia coli*, and to investigate the association of the latter organism with *Salmonella;* also to compare isolation rates of enteroviruses from metropolitan sewage treatment plants, both stabilisation pond and activated sludge, with those from country treatment works.

PART B

MATERIALS AND METHODS

PART B

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Cell Culture - Introduction

Viruses are obligate intracellular parasites and cannot be grown on any non-living medium. A few human viruses have not been cultivated in any other host apart from some related primates. The majority of human viruses, however, may be grown in 'media' such as embryonated eggs, laboratory animals or suitable cultured cells.

ENDERS, WELLER and ROBBINS (1949) showed that poliomyelitis virus could be grown in cultured non-neural cells (human foetal skin, muscle and connective tissue) with the production of characteristic histological changes, or cytopathic effects. Previously the use of experimental animals was mandatory, preventing rapid quantitative work. Since 1949, many previously unknown viruses, e.g. the adenoviruses and the echoviruses, have been isolated and characterised directly as a result of the use of cultured cells.

Cell culture was defined by FELL (1959) as "the cultivation of actively multiplying cells in a histologically undifferentiated

state", in contrast with organ culture as "the cultivation of tissue in a differentiated functional state similar to that of the corresponding tissue in the body". These definitions overcome the confusion from the use of the term "tissue culture". In this thesis the term "cell culture" has been used in preference to "tissue culture".

Cell cultures are cultivated by allowing a suspension of cells prepared by disaggregation of a tissue or cell monolayer with a proteolytic enzyme to settle out from a nutrient medium onto a glass or plastic surface. The cells rapidly adhere to the surface, and divide about once a day until a confluent monolayer of cells forms. It may be necessary to change the nutrient medium before confluence is reached because of depletion of nutrients or lowered pH.

Type of cell culture

Primary

These are cells taken directly from a suitable animal, and are usually capable of only limited growth *in vitro*. A number of cell types are present, and as they have been freshly removed from the body, there has not been opportunity for them to undergo marked selection or phenotypic alterations. Primary cultures permit the

growth of a wide spectrum of viruses.

Primary cell cultures used in this work included cynomolgus monkey kidney (for enteric virus isolations) and human amnion (for propagation of stock virus cultures).

Diploid semi-continuous cell strains

Diploid cells of a single type established from embryos which may be grown for a limited number of subcultures before reaching a 'senescent' stage after about a year of continuous passage (HAYFLICK and MOOREHEAD, 1961), are termed semi-continuous strains.

These cells are sensitive to a wide range of human viruses.

Human foetal tongue cells were used in this work as a possible replacement for cynomolgus monkey kidney.

Continuous cell lines

Aneuploid cells of a single type capable of indefinite *in vitro* cultivation are termed "continuous lines".

Lines grown were H.Ep.-2 and BS-C-1.

Enterovirus spectra and characteristics of cell cultures

TABLE 2. Enterovirus spectrum of Primary H.Ep.-2 and PMK cells (Wenner, 1964).

Virus	Type	Susceptibility to enteroviruses	
		Primary H.Ep.-2	Cynomolgus kidney (PMK)
Poliovirus	1-3	+	+
Coxsackie A	7	-	+
	9	-	+
	14	-	+
	16	-	+
	21	+	+
	23	-	+
Coxsackie B	1-6	+	+
Echovirus		-	+

Table 2 from WENNER (1964) lists enteroviruses which may give a cytopathic effect (CPE) with primary cynomolgus monkey kidney cells (PMK) (Figs. 2 and 4) and H.Ep.-2 continuous line cells.

Enterovirus CPE with any particular cell was described by Wenner as in many cases being "inconstant", varying with both virus strain and intratypic strains. The range of viral susceptibility of most continuous lines is less than those of primary or diploid cultures (SCHMIDT, 1969), and the virus spectrum of the 'same' continuous cell line may differ between laboratories. For example, although echoviruses were listed in Table 2 as not capable of producing a CPE in primary H.Ep.-2 cells, STAMP and FERRIS (1970) used a H.Ep.-2 line to isolate echovirus from effluents.

It was intended that the restricted spectrum of the continuous cell lines (particularly H.Ep.-2) could be used to select for polio and coxsackie B viruses (Table 2) on subculture of presumptive enterovirus isolates in H.Ep.-2 coverslip cell cultures.

However the H.Ep.-2 cells, although readily grown and subcultured, would not stay on maintenance medium for more than 3-5 days without the cell sheet degenerating. In spite of treatment with kanamycin to remove a possible mycoplasma contaminant, the cells remained difficult to handle on maintenance, and they were not used further.

The BS-C-1 cells required selected foetal calf serum for their

growth, and as this was not always obtainable commercially, this cell line was also abandoned.

Two diploid cell cultures of Human Foetal Tongue (HFT) were obtained commercially. HAYFLICK and MOOREHEAD (1961) stated that diploid human foetal cells were capable of up to 50 subcultures before deteriorating; however these two diploid cell strain cultures were lost at passage 17 and 19.

The HFT cells were intended as a replacement for PMK cells in isolation procedures, having a wide virus spectrum and the advantage that low passage numbers could be stored in liquid nitrogen to provide an independant source of cells. STAMP and FERRIS (1970) abandoned use of a semi-continuous cell line of human foetal lung because its viral sensitivity was said to vary. The time involved in handling these delicate cells, the cost of media, and low yield using a 1:2 subculture (Table 23), made use of these cells uneconomic. However at low passage numbers, HFT gave clean cell monolayers without the problems of PMK cells such as contamination with simian viruses e.g. "foamy agent". The monolayers stayed in good condition on maintenance medium for 10-14 days, but experience showed that enterovirus isolation attempts involving direct cell contact with effluents was not feasible because of toxic effects on the cells.

Sources of cell cultures

Primary monkey kidney (PMK) cells were purchased from the Commonwealth Serum Laboratories (CSL) as a 1 in 10 dilution of the packed cell volume. These were delivered on ice by air freight on a weekly basis.

Placentae for the preparation of primary human amnion (PHA) were obtained from the Queen Victoria Maternity Hospital, Rose Park.

Milk dilution (MD) bottle cultures of the semi-continuous human foetal tongue (HFT) and the continuous cell lines, H.Ep.-2 and BS-C-1 were originally obtained from CSL. In contrast with the primary cells, these continuous and semi-continuous cells could be propagated further, indefinitely in the case of the continuous cell lines.

Media for cell culture

Chemically defined media were used for the growth and maintenance of cell cultures. All the cells but the human semi-continuous cell strain HFT were grown and maintained on MORGAN, MORTON and PARKER's (1950) medium 199. This was one of the earliest and most complex of the attempts to produce a complete synthetic

medium. The product marketed by CSL is based on Hanks' balanced salt solution (BSS) and contains a total of 66 components, which apart from the BSS, includes various amino acids, vitamins, purines, pyrimidines and other defined nutrients as well as antibiotics and the non-toxic indicator phenol red.

However, the medium has to be supplemented with serum to initiate cell growth, although capable of maintaining the viability of cells for extended periods without sera.

Work by EAGLE (1955) to develop a less complex synthetic medium led to the introduction of Eagle's basal medium (BME). This is far less complicated, the CSL product based on Earle's BSS having only 31 components. BME, in contrast with 199, will allow the prolonged growth of human diploid cells. Again, addition of serum is essential for the growth of the diploid cells. Preparation of both media from 10 fold concentrate is described in Table 32.

Both media require the adjustment of pH with sodium bicarbonate solution which was also purchased from CSL as a 2.8%(w/v) aqueous solution. The bicarbonate/CO_2 buffer system demands sealed cell culture containers to avoid loss of CO_2 and a lethal increase in pH. The volumes of bicarbonate solution used are set out in Table 23. The pH was maintained at 7.2 - 7.6 by changing the medium, or

adding extra bicarbonate solution to the cell culture container.

Preparation of cell cultures

Mammalian cells are readily grown as monolayers on glass or plastic. The procedures are essentially similar whether the cells are newly isolated from the body or long accustomed to continuous culture. Cell culture begins with a suspension of cells, either purchased in that form e.g. PMK, or as in this study, obtained by a trypsinisation method from human amnion (PHA) or a cell monolayer (BS-C-1, H.Ep.-2 or HFT).

Primary Human Amnion (PHA) Cells

Primary human amnion cells were obtained from placentae from the Queen Victoria Hospital, Rose Park, using a method similar to that described by SCHMIDT (1969). A heat sterilised stainless steel dressing container was left at the hospital for the collection of an aseptically delivered placenta. These were available from caesarian section.

The placenta was transferred aseptically to a sterile stainless steel tray, and the amnion stripped from the chorion using two flamed forceps. Three rinses with warm Dulbecco phosphate buffered saline (DULBECCO and VOGHT, 1954) removed most adherent blood and

mucus. After the first wash in warm PBS the amnion was cut into about 10 pieces for easy handling and removal of blood clots. Instruments used during these procedures were 'sterilised' in a stainless steel beaker of boiling water and repeatedly alcohol flamed.

Five serial trypsinisations in calcium and magnesium free Dulbecco PBS containing 0.25%(w/v) trypsin (Difco 1:250 trypsin) were carried out at 37^{o}C in wide mouth Corning MD bottles each containing 100 ml of PBS-trypsin solution. The amnion pieces were transferred to a bottle of fresh PBS-trypsin every half hour. The products of trypsinisation in the first bottle were discarded due to the presence of toxic materials such as blood and mucus not completely removed in the preliminary washings. The 400 ml of cell suspension collected after trypsinisation were centrifuged at not more than 1000 rpm for 5 minutes, and the pellets resuspended in a growth medium consisting of medium 199 (MORGAN, MORTON and PARKER, 1950) containing 3%(v/v) of 2.8%(w/v) sodium bicarbonate and 15%(v/v) of bovine serum. After pooling and further centrifugation, the cells were suspended in the growth medium at about 0.5 x 10^{6} cells per ml, based on the stained cell count (using a haemocytometer) of a cell suspension in 0.1%(w/v) trypan blue.

Lipid granules in the cells usually disappeared within 48 hours of seeding, and the medium was changed at this time with the same growth medium apart from an extra 2%(v/v) i.e 5%(v/v) of sodium bicarbonate solution. Confluence was reached after about 5 to 10 days largely by an increase in cell size rather than cell division. The cells were maintained with Medium 199 containing 7%(v/v) sodium bicarbonate solution and 2%(v/v) bovine serum.

Propagation of the continuous cell line H.Ep.-2

Production of continuous line cultures may be illustrated by the methods applied to H.Ep.-2 cells. Variations between the cell culture types are set out in Table 23, but the H.EP.-2 cell is typical of the semi-continuous and continuous cultured cells.

A H.Ep.-2 monolayer culture in a milk dilution (MD) bottle was originally received by air freight from CSL together with a bottle of growth medium. The cells were shipped with only a few ml of medium in the bottle, and this was removed, and replaced by 12.5 ml of the medium supplied.

After overnight incubation, the medium was completely removed by pipette, and the monolayer washed with 5 ml of warm sterile

Dulbecco calcium magnesium free PBS to remove traces of serum, which contains trypsin inhibitor. One ml of trypsin-versene (Table 29) was introduced into the bottle, rinsed over the monolayer and then pipetted out again. The bottle was incubated inverted (monolayer uppermost), at 37°C for several minutes, and then the culture bottle was examined with the inverted microscope (Carl Zeiss) to see if the monolayer was sloughing from the glass surface. After the cells began to dislodge, 4 ml of growth medium (Medium 199 with 20%(v/v) bovine serum and 6%(v/v) sodium bicarbonate solution) was pipetted over the surface of the disintegrating monolayer to give a cell suspension. The cells could be counted, but with practice a suitable 'split' on subculture could be judged. Usually a 1:4 to a 1:6 subculture could be used. When starting a culture it was advisable not to seed the cells at too low a concentration, and a 1:4 subculture was reasonable. This dilution gave at least 10^5 cells/ml. One ml of the suspension was placed in each of 4 MD bottles, growth medium added to give a final volume of 12.5 ml, and the bottles incubated on their sides at 37°C. The cells were handled on a Monday-Thursday schedule, being subcultured on the Monday, the growth medium being changed on Thursday (at a higher bicarbonate level) and then usually reaching confluence the following Monday.

The cell cultures could then be placed on maintenance medium (medium 199 with 5% bovine serum and 8% sodium bicarbonate solution) ready to use, or subcultured again.

TABLE 3. Preparation of cell cultures : volumes of cell suspension inoculated.

Culture vessel	Volumes of cell suspension inoculated	Volumes of subsequent medium changes
Culture tube 16x150 mm	0.5 ml	1.0 ml
Leighton tube 16x150 mm	1.0 ml	1.0 ml
Milk dilution bottle	12.5 ml	12.5 ml
Petri dish	10.0 ml	10.0 ml

Tube cell cultures

Tube cultures were prepared by adding 0.5 ml of cell suspension in growth medium to 16 x 150 mm pyrex screw cap tubes. These were incubated at 37^{o}C in stationary racks at an angle of about 5-7^{o}.

Care was taken to ensure that the tubes were not disturbed until the cells became attached to the surface of the glass. After attachment, the medium was replaced with 1.0 ml of fresh growth medium, and the tube cultures rolled at 37°C in a roll drum apparatus at 7 revolutions per hour. After confluence had been reached, the cells were placed on maintenance medium ready for use. These tube cultures were used for the passage of MD bottle cell cultures during the isolation of enteric viruses (Part B, "Method of enteric virus isolation"). Tubes were used for this purpose in preference to larger culture containers to reduce the amounts of expensive PMK cells and medium required (Table 3).

Coverslip cell cultures

"Assistent" No. 1 22 x 9 mm coverslips were cut with a diamond pencil to 22 x 7 mm, as the untrimmed coverslips were too wide to insert into leighton tubes, and stored individually in bijou bottles of 70%(v/v) ethanol until required.

Corning leighton tubes were seeded with 1.0 ml of PMK cell suspension in growth medium after a sterile coverslip prepared as described had been placed on the optical flat with the aid of flamed forceps. No contamination problems were experienced with this

procedure. The cells were then handled as shown in Table 23.

After confluence of the cell monolayer on the coverslip was reached, the cultures were ready for the presumptive identification of enteric virus isolates (Part B, "Presumptive identification of entero viruses).

Petri dish cell cultures

Primary cynomolgus monkey kidney 10% suspension (PMK) was diluted 1 in 50 with growth medium (Medium 199 with 3%(v/v) of 2.8%(w/v) sodium bicarbonate and 2%(v/v) foetal calf serum) to give a cell suspension of about 2×10^5 cells per ml. This was dispensed, care being taken to keep the cells in suspension, in 10 ml volumes into "Camelec" 90 mm disposable plastic petri dishes.

The petri dishes were rapidly transferred, after swirling to distribute the cells evenly, to an air-CO_2 incubator based on a design by HAMPTON, THAYER and HOWES (1969). The 5% CO_2 mixture with air was supplied from premium grade CO_2 and medical air cylinders through pressure reducing valves to needle valves. The gas flow was controlled to give the correct mixture by reference to rotameters mounted above the needle valves. The sources of materials and apparatus used have been set out in Table 31.

The petri dishes were examined daily at 25 times magnification with the inverted microscope to monitor cell growth. As soon as the pH dropped to about 7.0, usually after 3 or 4 days, the medium was removed by sterile pipette and replaced by fresh growth medium with a higher bicarbonate concentration. Care had to be taken to avoid damaging the cell sheet by vigorous pipetting of medium or by touch. Confluence was usually reached after about 7 days, and the petri dish cell cultures were then ready for use in plaque assays (Part B, "Estimation of enterovirus numbers : plaque assay").

Collection of sewage effluent samples

All samples for virological or bacteriological examination were 'grab' samples.

Sterile sample bottles were held in a flamed stainless steel sampling rod, and lowered into the sewage liquor until the bottle almost filled. All sample bottle closures were ground glass dust-proof stoppers. Before each sample was processed in the laboratory, it was vigorously shaken to evenly distribute any sediment throughout the sample volume. All samples were held at 2-5^{o}C, and processed as soon as possible after collection.

Methods of estimating *E.coli* numbers in metropolitan stabilisation pond effluent

E.coli numbers from 'grab' samples were estimated by the multiple tube method (Anon., 1957) i.e. using 5 replicates of a series of 10 fold dilutions, and were defined as those organisms which produce acid and gas from lactose in MacConkey broth (Oxoid CM5a) within 48 hours at 44°C. The results were expressed as a most probable number (MPN) of *E.coli* per 100 ml of water.

This 'direct' 44°C method was used on samples dating from 2/9/69. Prior to this date, a confirmed test (Anon., 1969) using the MACKENZIE, TAYLOR and GILBERT (1948) method was followed. This involved inoculation, using wooden probes (McGUIRE, 1964), of presumptive positive formate lactose glutamate tubes (GRAY, 1964) which had been incubated at 37°C ± 0.1 for up to 48 hours, into a tube of brilliant green bile broth (Oxoid CM31) at 44°C for 24 hours. The change in method would not have affected MPN values significantly (Table 6).

At the same time a tube of peptone water was inoculated to test for the production of indole at 44°C after 24 hours incubation. Ehrlich's reagent (Anon., 1969) was used to demonstrate the production of indole. *E.coli* produced gas in the brilliant green

bile broth at 44°C as well as indole from tryptophane in the peptone water within 24 hours.

The water baths were controlled by mercury contact thermometers to give a temperature of 44°C ± 0.1. Anhydric incubators were not used because of the difficulties involved in maintaining this tolerance.

Method of estimating *Salmonella* numbers and serotypes from metropolitan stabilisation pond effluent

One litre grab samples of stabilisation pond effluent were examined by membrane filtration, 10 replicates of 10 or 100 ml aliquots being filtered through a 47 mm 0.45 micrometre pore size membrane using a Whatman GF/C 4.25 cm prefilter in a sterile Millipore filter apparatus, the filter combination then being immersed in 10 ml of Rappaport medium (RAPPAPORT, KONFORTI and NAVON, 1956) in a one ounce wide mouth McCartney bottle.

Swabs (MOORE, 1948) were not used because the isolations from these are qualitative, and although useful in detecting the presence of *Salmonella*, would not give as much information as the grab samples.

Samples which filtered with difficulty were centrifuged in the chosen volumes at 5,000 rpm for 20 minutes, and the centrifugate suspended in 10 ml of Rappaport medium. The supernatant was then filtered as described above. As a result, 20 wide mouth one ounce McCartney bottles of enrichment medium were used in these cases. Centrifugation before filtration was required as too much organic material resulted in overnight reduction of the malachite green in the Rappaport medium which, based on previous experience, rendered the medium less selective for *Salmonella*.

Following incubation of the enrichment medium at 37°C for 24 hours, a sterile cotton bud (Johnson and Johnson) was used to heavily inoculate a section of 2 Brilliant Green Agar plates (Oxoid CM 263). A 6" sterile wooden probe was then used to streak over each plate. After 18-24 hours incubation at 37°C, 2 pink convex circular colonies (presumptive *Salmonella*) if present, were selected from each Brilliant Green Agar plate (BGA) for restreaking onto fresh BGA plates using a platinum wire. A well separated colony from each of these plates was then grown overnight in Christensen's urea broth. Urease negative cultures were then subcultured onto agar (as a stock culture) and agglutination agar (Table 28).

The growth on the agglutination agar slope was washed off with formol-saline next day, and slide agglutinations carried out to establish the serotype present. Commercially available agglutinating antisera (Burroughs Wellcome and CSL) were used.

Fewer difficulties arose through the use of Burroughs Wellcome agglutinating antisera than the CSL product. The CSL antisera exhibited a greater tendency to cross react.

BGA gave good colour differentiation from other gram negative organisms growing in the BGA plates with the exception of pseudomonads, which, although the colonies had the same colour, had a completely different colonial morphology and a characteristic odour.

Difficulties have been regularly encountered through contamination of cultures by *Bacillus subtilis*. These impure cultures were readily recognised by their utilisation of sucrose, and the typical spreading white growth on the agar slopes.

The number of tubes from which *Salmonella* were isolated was then used to determine the MPN per litre. This was a 'crude' MPN because in some cases more than one serotype may have been isolated from the one aliquot. However as an estimate of *Salmonella* was required, the sum of the MPN of all serotypes could not be used.

This is why the number of serotypes isolated may exceed the MPN recorded for that sample.

Table 4 (McCOY, 1962) gives values of the MPN per 100 ml and approximate 5% (0.05) confidence limits for a test using 10 replicate tubes each containing 0.1 ml of sample.

TABLE 4. Most probable number (MPN) figures and approximate 5% confidence limits for a dilution test of ten tubes each containing 0.1 ml of sample.

No. of positive tubes	MPN/100 ml	Limits (approximate 5%) Lower	Upper
0	-	-	369
1	105	15	749
2	223	56	895
3	357	114	1,110
4	511	190	1,380
5	693	283	1,690
6	916	400	2,100
7	1,200	548	2,640
8	1,610	743	3,490
9	2,300	1,030	5,160
10	-	1,180	-

Table 4 may be expanded for larger or smaller inocula, and this has been done for occasions when 10 ml or other volumes have been used. Table 5 gives the MPN per litre for a series of 10 tubes each containing the bacteria derived from 100 ml. After any proportional adjustment of the MPN figure necessary to compensate for volumes other than 10 or 100 ml in the 10 aliquots, the MPN was rounded to the nearest whole number.

TABLE 5. Most probable number (MPN) figures for a test of ten tubes each containing the bacteria derived from 100 ml of sample.

No. of positive tubes	MPN/litre
0	not detected
1	1.05
2	2.23
3	3.57
4	5.11
5	6.93
6	9.16
7	12.00
8	16.10
9	23.00
10	23 +

A culture of each serotype isolated in any examination was sent to the Salmonella Reference Laboratory, Institute of Medical & Veterinary Science, Adelaide, for verification.

Preparation of effluent samples for enteric virus isolations

Foetal calf serum (2%v/v), penicillin G, and streptomycin sulphate (1,000 units and 1,000 micrograms per ml respectively) were added to litre samples of effluent before filtration in 50 ml volumes through a Whatman GF/C 4.25 cm glass fibre pre-filter over a 0.45 micrometre Sartorius 47 mm membrane in a Millipore all glass filter apparatus. Filtering small volumes was necessary due to the small volume of effluent which could be passed through a membrane before blockage occurred. The prefilter-filter combination was replaced after each 50 ml, and then the one litre of combined filtrates passed through another sterile membrane filter in a new sterile all glass filter apparatus.

This treatment removed organisms such as yeasts, moulds and bacteria which could grow in the cell culture medium and prevent virus isolations, but would allow the passage of the enteric viruses. The filtered effluent has been referred to throughout the text as Membrane Filtered Effluent (MFE). The MFE free of these contaminants was then used to dilute 10 fold concentrated medium 199 back

to single strength Effluent Medium 199 (EM 199, Table 32) suitable for inoculation onto cell cultures. The filtrate was divided into 100 ml volumes in ten sterile Ehrlenmeyer flasks, and 11 ml of 10 fold concentrated medium 199 was added to each flask. Sufficient amphotericin B (Squibb) was added to give a concentration of 2 micrograms per ml in the EM 199 to prevent breakthrough of possible mould contaminants. The concentrations of the antibiotics used were not cytotoxic for primary cynomolgus monkey kidney cells (TOMKINS and FERGUSON, 1965). The EM 199 was then ready for adsorption to PMK cells after adjustment of pH with sodium bicarbonate.

Method of enteric virus isolation

EM 199 with 2%(v/v) of 2.8%(w/v) $NaHCO_3$ solution added to adjust the pH to about 7.5, was placed in 30 ml volumes on each of ten confluent PMK cell monolayers in MD bottles to allow viral adsorption to proceed for 48 hours at 37°C. This medium was then decanted and replaced with a second 30 ml of warm EM 199 for another 48 hours.

Bicarbonate was added immediately before the first adsorption to minimise loss of CO_2 through any faulty screw caps while the EM 199 was stored in the refrigerator. The high pH after CO_2 loss

would inactivate enteric viruses present in the EM 199.

After a third 30 ml of EM 199 was adsorbed on each of the 10 monolayers for 48 hours, a final 2 day adsorption period was given for the remaining 23 ml per bottle. Each monolayer was then placed on maintenance medium (Table 23).

An uninoculated control cell culture was incubated at the same time, and the 11 cell cultures examined daily at 25 times magnification during the following 14 days for typical cytopathic effects (CPE).

When a suspected enteric virus CPE appeared (Fig. 2), the affected culture was frozen, complete with medium to assist release of virus from infected cells. After thawing, one ml of cell culture medium containing the disrupted cells was adsorbed on a PMK tube cell culture for 1 hour at 37°C before changing onto maintenance medium. This subculture tube, or 'passage' to confirm the presence of enteric virus, was placed on a roll apparatus, rotated at 7 revolutions per hour, and examined daily at 25 times magnification for a further 14 days at 37°C. All negative cultures including the uninoculated control were 'blind' passaged in the same way. Sources of glassware and apparatus are listed in Table 31.

One ml of each of the original isolation bottle cultures was stored in a glass screw-cap vial in the deep freeze as a stock culture, and the remainder discarded. Cell cultures which were positive on passage were inoculated onto coverslip cultures of PMK cells. After cytopathic effects appeared, the coverslips were fixed and stained by the May-Grunwald Giemsa method to obtain presumptive identification of the virus isolate (Figs. 4, 7).

Presumptive identification of enteric viruses

All cell cultures used in isolation work which on passage showed cytopathic effects apparently of enteric virus origin, were examined by inoculation of the original cell culture fluid onto a PMK coverslip cell culture.

Confluent PMK cultures prepared in leighton tubes were drained of medium, and 0.5 ml of the original isolation cell culture fluid adsorbed onto individual monolayers for one hour at 37°C. The leighton tubes were incubated at 37°C with fresh maintenance medium, and examined daily at 25 times magnification for a total of 14 days or until degeneration of the cell sheet began. Coverslips were then washed three times in the leighton tube with warm PBS, fixed with methanol, stained by the May-Grunwald Giemsa method (Table 30) described by MERCHANT, KAHN and MURPHY (1964), and examined micro-

scopically after removal of the coverslip from the leighton tube and attachment to a microscope slide with Canada balsam in xylol.

Estimation of enterovirus numbers

Plaque assay (PA) technique

DULBECCO (1952) introduced a modification of the bacteriophage plaque assay that revolutionised the quantitation of animal viruses.

The method used in this thesis was to remove the growth medium from the petri dish PMK cell cultures before washing twice with Dulbecco phosphate buffered saline. Medium was readily removed by tilting the petri dish and using a sterile pasteur pipette connected by rubber tubing via a collection bottle to a source of vacuum. The inoculum usually of 0.5 ml was then distributed carefully over each monolayer (HOWES, 1969a), and the cell cultures left to adsorb on a level glass sheet for one hour at room temperature. The inoculum was swirled over the cells every 5 minutes during the hour to facilitate even virus adsorption.

The cultures were overlaid with 10 ml of T5L agar medium at about 44^{o}C (HOWES, 1969b), prepared as described in Table 26. The overlaid cultures were swirled to mix the 0.5 ml of inoculum with

the overlay before being incubated inverted at 37°C in the humidified 5% CO_2-air incubator.

The T5L overlay was developed specifically for plaque estimations of poliovirus on PMK cells, and the plaque characteristics with other enteric viruses were not known. The petri dishes were examined daily for up to 7 days, and the plaques resulting from focal cell destruction were marked with a felt pen as they appeared (Fig. 3). The neutral red vital dye incorporated in the medium stained living cells but was not retained in infected cells. Plaque assays were used to estimate virus recoveries from various isolation procedures. The plaque assay method is referred to throughout the text as the PA technique, and number of plaques as plaque forming units or PFU.

Multiple tube (MPN) method

Multiple tube methods consisting of ten replicate PMK bottle cell cultures each with the same inoculum of effluent as EM 199 were used in addition to the plaque technique in quantitative work. The most probable number (MPN) was found by reference to Table 5. This method could be used to estimate the numbers of any virus which produced a recognisable CPE, and also had the advantage of allowing 'passage' or subculture into fresh cell cultures to increase the number of isolations.

Flocculation and plaque assay of enterovirus with aluminium hydroxide

Aluminium hydroxide floc was prepared by the method described by WALLIS and MELNICK (1967). The details of the preparation have been set out in Table 26. Five ml of aluminium hydroxide floc suspended in pH 6 phosphate buffer (Table 26) were added to each litre of virus suspension. The mixture was gently stirred with a magnetic stirrer in a sterile flask for one hour at room temperature i.e. approximately 25^{o}C. WALLIS and MELNICK (1967) filtered the floc by negative pressure through a Millipore type HA 47 mm diameter 0.45 micrometre pore size membrane filter, and removed the precipitate from the membrane with a platinum spatula. Removal of the floc with a spatula was found by experience to be difficult, and centrifugation at 5,000 rpm for 10 minutes was substituted for filtration. Each precipitate was to be made up to 1 ml with physiological saline, and inoculated in 0.5 ml volumes onto petri dish PMK cell cultures. The plaque assay method (Part B, "Estimation of enterovirus numbers : plaque assay") gave results expressed as plaque forming units (PFU) per ml.

Glassware decontamination and preparation

Cell cultures

Cell culture demands the highest standards of cleanliness in apparatus such as pipettes and culture vessels.

Immediate and total immersion of discarded glassware in a "Paramount" stainless steel food container 10" x 22" x 6" deep, of detergent (Haemosol) in distilled water prior to autoclaving prevented the deposition of media components such as serum on the glassware on decontamination.

All cell cultures and associated media were regarded as being potentially infective, and all glassware such as pipettes in contact with any cell culture were similarly treated. The lids of the containers were secured with pressure sensitive autoclave tape, and the containers sterilised by autoclaving for 30 minutes at 20 lb pressure.

Pipettes, after removal of the cotton wool plug, were placed in a clean "Paramount" stainless steel food container in distilled water-Haemosol solution, steamed for one hour to remove the last residues and allowed to cool overnight. The pipettes were rinsed in a pipette washer for 30 changes of cold tap water and 10 rinses in distilled water. After drying at 60^{o}C cotton plugs were inserted, and pipettes packed in aluminium bins, and sterilised by hot air at 175^{o}C for 2 hours.

Other glassware such as MD bottles was left to soak overnight

in Haemosol detergent and hot tap water after autoclaving. The individual pieces were thoroughly scrubbed next day with a strong bristle brush. Screw cap culture tubes were cleaned individually by using a small brush attached to an electric cake mixer on a heavy base. A Heinicke laboratory glassware washer was used to rinse the glassware before sterilisation. The all-glass membrane filter apparatus was washed disassembled, the parts then individually wrapped in aluminium foil, and cotton wool plugs inserted in the flask openings before autoclaving.

Washed MD bottles were individually closed with nonabsorbent cotton wool plugs covered with aluminium foil before autoclaving at 15 lb for 20 minutes. The plug was covered in fine gauze to prevent the shedding of cotton wool fibres into the culture vessels. Screw caps for MD bottles and tubes were packed in glass beakers in layers separated by heavy sheets of vegetable parchment paper, and then autoclaved at 15 lb for 20 minutes. After autoclaving, baskets of glassware and caps were immediately placed in the 60^{o}C drying oven to prevent condensation. Glass culture tubes were sterilised by hot air at 175^{o}C for 2 hours in "Paramount" stainless steel dressing containers. The tubes were packed vertically with the open end down. The culture vessels i.e. tubes and bottles, were aseptically capped just before seeding cell cultures.

Bacteriology

Bottles and tubes containing media used for *Salmonella* isolations were autoclaved in "Paramount" stainless steel food containers containing tap water and detergent. Disposable 90 mm plastic petri dishes of selective medium streaked during isolation work were also autoclaved in the "Paramount" containers. The glassware pieces were scrubbed with a bristle brush, rinsed in the Heinicke washer, filled with medium and sterilised for further isolation procedures.

Similar cleaning and sterilisation methods were used with bottles and tubes for coliform and *E.coli* estimations, except that the glassware was not decontaminated before being washed. Rigorous cleaning methods similar to those used in cell culture work was not required.

PART C

ESTIMATION OF ENTERIC BACTERIA IN METROPOLITAN STABILISATION POND EFFLUENT

PART C

ESTIMATION OF ENTERIC BACTERIA IN METROPOLITAN STABILISATION POND EFFLUENT

Comparison of direct and confirmed *E.coli* MPN

Current English and American practice for the examination of water for *E.coli*, (Anon. 1969, American Public Health Association, 1971) as well as the World Health Organisation (1971) standard, delete the direct 44°C multiple tube method for the estimation of *E.coli* completely. This is surprising when the delay of up to 3 days involved in completing the confirmed 37°C test for *E.coli* is compared with the 48 hours required for the direct examination. This delay could be important from the public health viewpoint, even if the direct 44°C examination may give lower results.

The question whether there is a significant difference between these two methods was tested statistically. Paired samples of metropolitan stabilisation pond effluent collected daily, except weekends, were examined using the 44°C direct and the 37°C confirmed tests.

The results of the comparison between the two methods are set out in Table 6, and the test of significance follows:

TABLE 6. Comparison of direct and confirmed *E.coli* MPN

The most probable number of *E.coli* per 100 ml			
Gray's Medium at 37°C	McConkey's Medium at 44°C		
Confirmed x_1	Unconfirmed x_2	Difference D= $x_1 - x_2$	D^2
900	170	730	
17,000	5,500	11,500	
8,000	5,500	2,500	
5,000	3,500	1,500	
35,000	5,500	29,500	
25,000	3,500	21,500	
8,000	5,500	2,500	
3,500	2,500	1,000	
11,000	25,000	-14,000	
3,500	900	2,600	
17,000	7,000	10,000	
9,000	9,000	0	
11,000	9,000	2,000	
11,000	17,000	- 6,000	
55,000	3,500	51,500	
25,000	35,000	-10,000	
17,000	27,500	-10,500	
11,000	5,500	5,500	
55,000	35,000	20,000	
25,000	25,000	0	
25,000	11,000	14,000	
90,000	27,500	62,500	
80,000	160,000	-80,000	
160,000	25,000	135,000	
160,000	35,000	125,000	
55,000	25,000	30,000	
27,500	55,000	-27,500	
35,000	22,500	12,500	
$\Sigma x_1 = 985,400$	$\Sigma x_2 = 592,070$	$\Sigma D = 393,330$	
$N = 28$	$(\Sigma D)^2 = 154708488900$	$\Sigma D^2 = 51281292900$	

To find whether there is a significant difference between the means of these two sets of data, the arithmetic mean of the differences between sample pairs was obtained and tested to see if this differs significantly from 0, the null hypothesis being that $\bar{x}_D$ was the mean of a random sample from a population of differences with a mean of zero (CROXTON, 1959).

$$\bar{x}_D = \frac{\Sigma D}{N} = 14{,}041$$

$$\sigma_D = \sqrt{\frac{\Sigma D^2}{N-1} - \frac{(\Sigma D)^2}{N(N-1)}} = 41{,}166$$

$$\sigma_{\bar{x}_D} = \frac{\sigma_D}{\sqrt{N}} = \frac{41{,}166}{\sqrt{28}} = 7{,}782$$

The value of t is given by

$$t = \frac{\bar{x}_D - 0}{\sigma_{\bar{x}_D}} = \frac{14041}{7782} = 1.8$$

Since there were 27 degrees of freedom, inspection of the t tables (FISHER and YATES, 1970) shows that the probability lies between 0.05 and 0.1.

A significant difference between the methods was therefore not demonstrated at the 0.05 level.

Estimations of *E.coli* in metropolitan stabilisation pond effluent with 5 week weighted running means

A five week weighted running mean (CROXTON, COWDEN and KLEIN, 1968) was calculated for the four years of weekly *E.coli* MPN values set out in chronological order in Table 7. This smoothing technique was required to remove irregular fluctuations to show seasonal trends clearly, and to provide a means of interpolating *E.coli* numbers in association with enterovirus isolations (Tables 19, 20). The MPN values were weighted 1, 2, 4, 2, 1 which gives greatest weight to the centre week and least to the end weeks. This weight pattern totals 10, allowing calculation without using an adding machine. On those occasions where an *E.coli* MPN value was missing, the next *E.coli* MPN was used to give the 5 values necessary for calculating the running mean.

Table 7 lists the calculated running means with the logarithm of the mean from which the curve of Fig. 9 and Fig. 10 was plotted.

TABLE 7. Estimations of *E.coli* in metropolitan stabilisation pond effluent with 5 week weighted running means.

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1967							
July 5	130,000	-	-	Oct. 4	140	557	2.75
" 12	35,000	-	-	" 11	900	545	2.74
" 19	35,000	48,800	4.69	" 18	110	292	2.47
" 26	70,000	65,100	4.81	" 25	250	229.5	2.36
Aug. 2	8,000	70,950	4.85	Nov. 1	40	131	2.12
" 10	250,000	110,200	5.04	" 6	95	176	2.25
" 16	2,500	54,350	4.73	" 13	350	268	2.43
" 23	11,000	34,100	4.53	" 20	350	297.5	2.47
" 30	3,500	11,050	4.04	" 27	350	288.5	2.46
Sept. 6	35,000	16,290	4.21	Dec. 4	80	217	2.34
" 14	2,000	8,440	3.93	" 11	275	213.5	2.33
" 20	900	4,494	3.65	" 18	250	418	2.62
" 27	1,100	938	2.97	" 25	-	-	-

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1968							
Jan. 3	25	637.5	2.80	Apr. 17	130	492	2.69
" 9	2,500	1,147	2.17	" 24	350	601	2.78
" 17	500	742.5	2.87	May 1	1,600	916	2.96
" 24	170	465	2.67	" 8	900	754	2.88
" 31	60	181.5	2.26	" 15	130	1,118	3.05
Feb. 7	350	181	2.26	" 22	130	2,368	3.37
" 14	35	135	2.13	" 29	7,000	4,989	3.70
" 21	50	212	2.33	June 5	8,000	7,463	3.87
" 28	350	328.5	2.52	" 12	5,500	8,600	3.93
Mar. 6	800	442	2.65	" 19	17,500	10,600	4.03
" 13	150	449	2.65	" 26	6,000	8,250	3.92
" 20	170	523	2.72	July 3	5,000	6,640	3.82
" 27	1,600	752	2.88	" 10	8,000	5,880	3.77
Apr. 3	250	498	2.70	" 17	900	4,660	3.67
" 10	-	-	-	" 24	9,000	7,380	3.87

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1968							
July 31	4,000	11,990	4.08	Nov. 6	-	-	-
Aug. 7	20,000	24,200	4.38	Nov. 13	50,000	38,800	4.59
" 14	45,000	45,400	4.66	" 20	35,000	38,050	4.58
" 21	55,000	58,300	4.77	" 27	55,000	35,260	4.55
" 28	120,000	83,100	4.92	Dec. 4	5,500	17,075	4.23
Sept. 4	13,000	68,870	4.84	" 11	1,600	7,353.5	3.87
" 12	170,000	88,440	4.95	" 18	550	1,102	3.04
" 18	1,700	71,980	4.85	" 24	35	308	2.49
" 25	55,000	89,740	4.95	" 31	50	145	2.16
Oct. 2	250,000	115,970	5.06	1969			
" 9	4,000	66,400	4.82	Jan. 8	140	156.5	2.19
" 16	40,000	46,900	4.67	" 15	350	210.5	2.32
" 23	13,000	23,600	4.37	" 22	170	214	2.33
" 30	25,000	30,100	4.48	" 29	35	228	2.36

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1969							
Feb. 5	550	331	2.52	May 14	6,500	5,930	3.77
" 12	350	295.5	2.47	" 21	5,000	7,300	3.86
" 19	170	289	2.46	" 28	17,000	34,150	4.53
" 26	80	331	2.52	June 3	3,500	55,650	4.74
Mar. 5	800	528	2.72	" 10	250,000	105,600	5.04
" 12	700	868	2.94	" 17	3,500	62,250	4.79
" 19	350	1,105	3.04	" 24	25,000	81,700	4.91
" 26	3,500	1,655	3.22	July 1	55,000	110,350	5.04
Apr. 2	450	1,135	3.05	" 8	350,000	180,900	5.26
" 9	250	925	2.97	" 15	130,000	131,000	5.12
" 16	1,700	995	3.00	" 22	14,000	69,100	4.84
" 23	450	1,055	3.02	" 29	7,000	34,800	4.54
" 30	1,300	1,930	3.29	Aug. 5	11,000	36,300	4.56
May 7	2,500	3,105	3.49	" 12	140,000	61,350	4.79

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1969							
Aug. 19	11,000	35,600	4.55	Nov. 25	200	2,170	3.34
" 26	2,500	20,950	4.32	Dec. 2	5,500	3,245	3.51
Sept. 2	16,000	9,650	3.98	" 9	4,000	2,807	3.45
" 9	5,500	7,100	3.85	" 16	350	1,559	3.19
" 16	5,500	5,690	3.76	" 23	170	768	2.89
" 23	3,500	3,247	3.51	" 30	350	554	2.74
" 30	-	-	-	1970			
Oct. 7	900	1,689	3.23	Jan. 6	1,600	1,127	3.05
" 14	-	-	-	" 13	250	1,200	3.08
" 21	170	938	2.97	" 20	3,500	1,735	3.24
" 28	450	1,054	3.02	" 27	450	981.5	2.99
Nov. 4	2,500	1,777	3.25	Feb. 3	350	601	2.78
" 11	2,500	1,905	3.28	" 10	65	327	2.51
" 18	1,700	2,020	3.31	" 17	80	422.5	2.63

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1970							
Feb. 24	1,700	718.5	2.86	June 2	1,200	2,635	3.42
Mar. 3	25	405	2.61	" 9	2,500	4,490	3.65
" 10	110	304	2.48	" 16	9,000	6,870	3.84
" 17	250	202.5	2.31	" 23	11,000	8,450	3.93
" 24	350	230	2.36	" 30	4,500	7,260	3.86
" 31	80	157.5	2.20	July 7	11,000	7,270	3.86
Apr. 7	130	1,212	3.08	" 14	1,600	4,640	3.67
" 14	45	5,752	3.76	" 21	5,500	5,020	3.70
" 21	-	-	-	" 29	2,500	4,410	3.64
" 28	-	-	-	Aug. 4	9,000	5,900	3.77
May 6	11,000	11,477	4.06	" 11	3,500	6,150	3.79
" 12	35,000	16,664.5	4.22	" 18	5,500	7,250	3.86
" 20	550	9,140	3.96	" 25	16,000	8,420	3.93
" 26	3,500	5,500	3.74	Sept. 1	2,500	6,490	3.81

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1970							
Sept. 8	700	5,780	3.76	Dec. 15	300	298	2.47
" 15	16,000	7,540	3.88	" 22	350	294.5	2.47
" 22	2,000	4,805	3.68	" 29	-	-	-
" 29	3,500	3,478	3.54	1971			
Oct. 5	350	1,081	3.03	Jan. 5	250	224	2.35
" 13	80	519	2.72	" 12	95	138	2.14
" 20	250	202	2.31	" 19	50	87	1.94
" 27	170	180	2.26	" 26	50	70.5	1.85
Nov. 5	170	192	2.28	Feb. 2	130	90	1.95
" 10	200	198	2.30	" 9	50	79.5	1.90
" 17	250	204	2.31	" 16	130	82.5	1.92
" 24	170	199	2.30	" 23	25	63	1.80
Dec. 1	130	211	2.32	Mar. 2	25	87	1.94
" 8	350	278	2.44	" 9	170	150.5	2.18

TABLE 7 (contd.)

Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean	Date	*E.coli* MPN/100ml	5 week weighted running mean	log of mean
1971							
Mar. 16	250	194.5	2.20	June 15	3,500	4,790	3.68
" 23	250	238	2.38	" 22	5,500	6,365	3.80
" 30	80	272	2.43	" 29	16,000	8,130	3.91
Apr. 6	550	426	2.63	July 6	950	-	-
" 13	550	608	2.78	" 13	900	-	-
" 20	550	885+	2.95+				
" 27	1,600	1,345+	3.13+				
May 4	1,800+	1,615+	3.21+				
" 11	1,800+	1,740+	3.24+				
" 18	1,600	1,890+	3.28+				
" 25	1,800+	2,090+	3.32+				
June 1	3,500	2,610+	3.42+				
" 8	1,700	2,810+	3.45+				

Analysis of variance of *E.coli* estimations over a four year period in metropolitan stabilisation pond effluent

The four years' weekly *E.coli* estimations in Table 7 were subjected to analysis of variance to see if there were significant differences in *E.coli* numbers between months and between years.

The *E.coli* MPN figures from Table 7 are set out again in full in Table 8 together with preliminary calculations. It is assumed that the *E.coli* values in the individual months (boxes) are normally distributed, and that the variances within boxes are the same. Due to the small numbers of results within months, these assumptions could not be tested rigorously.

TABLE 8. Analysis of variance of *E.coli* numbers (MPN/100 ml) in metropolitan stabilisation pond effluent. Estimations of *E.coli* made weekly over a four year period.

From the data in Table 8

$$\text{Total variation} = \Sigma x^2 - \frac{(\Sigma x)^2}{N}$$

(Where grand total $(\Sigma x)^2 = 2933220^2$

and grand total N = 201)

$$= 441708837900 - 42804873475$$

$$= 398903964425$$

Variation between (column) month means

$$= \sum\left[\frac{(\Sigma x)^2}{N}\right] - \frac{(\Sigma x)^2}{N}$$

$$= 95840994096 - 42804873475$$

$$= 53036120621$$

Variation between (row) year means

$$= \sum\left[\frac{(\Sigma x)^2}{N}\right] - \frac{(\Sigma x)^2}{N}$$

$$= 57204352097 - 42804873475$$

$$= 14399478622$$

Variation within months (boxes)

$$= \Sigma x^2 - \sum\left[\frac{(\Sigma x)^2}{N}\right]$$

$$= 441708877900 - 182097216540$$

$$= 259611621360$$

Table 9 gives the analysis of variance.

TABLE 9. Summary of computations for analysis of variance of *E.coli* estimations.

Source of variation	Variation	Degrees of freedom	Estimated Variance
Between (columns) month means	53036120621	11	4821465511
Between (rows) year means	14399478622	3	4799826207
Interaction (calculated by difference)	71856743822	33	2177477086
Within boxes	259611621360	153	1696807983
TOTAL	398903964425	200	-

Testing interaction for significance by dividing the estimated variance of the interaction by the estimated variance within boxes, the variance ratio F, is given by

$$F = \frac{2177477086}{1696807983}$$

$$= 1.28 \text{ with } 33 \text{ (numerator) and } 153$$

(denominator) degrees of freedom.

The interaction is not significantly greater than the estimated variance within boxes at this value of F, and therefore the estimated variance of the column and row means is tested against an estimated variance obtained by combining the variation within boxes with the variation attributable to interaction, and dividing by their combined degrees of freedom (CROXTON, COWDEN and KLEIN, 1968). This gives 331469517535 as the variation, with a total of 186 degrees of freedom. The estimated variance is then 1782094180.

Testing (column) month means for significance,

$$F = \frac{4821465511}{1782094180}$$

= 2.71 with 11 and 186 degrees of freedom, which indicates a significant difference at the 0.05 level (FISHER and YATES, 1970). (It is still significant at the 0.01 level).

Similarly testing (row) year means for significance,

$$F = \frac{4799826207}{1782094180}$$

= 2.69 with 3 and 186 degrees of freedom which is significant at the 0.05 level (FISHER and YATES, 1970).

Estimations of *Salmonella* in metropolitan stabilisation pond effluent with serotypes isolated

The first MPN estimate of *Salmonella* in metropolitan stabilisation pond effluent was conducted on 7/9/67 as set out in Table 10.

On that occasion the effluent was not centrifuged first, but filtered in 10 x 100 ml aliquots, and the pre-filter/membrane filter combination from each 100 ml was incubated for 24 hours in 10 ml of Rappaport medium (RAPPAPORT, KONFORTI and NAVON, 1956) at 37°C and then streaked onto 2 BGA plates. After 18-24 hours, 2 presumptive *Salmonella* colonies from each primary BGA plate were restreaked onto fresh BGA plates. After incubation, a well separated colony from each plate was then subcultured into Christensen's urea broth and incubated overnight at 37°C. A total of 4 presumptive *Salmonella* colonies were examined from those isolated from each of the ten bottles of enrichment medium, except for the two bottles from which no presumptive colonies appeared on BGA.

All these cultures were urease negative next day, and the urea broth was used to inoculate with a pasteur pipette a stock nutrient agar slope as well as an agglutination agar slope. At the same time Andrade peptone water bijou bottles of glucose, sucrose, mannitol and lactose were inoculated for overnight incubation. All the

cultures were + - + - respectively for these sugars. The overnight growth on the agglutination agar was washed off with formol-saline and examined by slide agglutination.

The serotypes isolated on that occasion are summarised in Table 10 below.

TABLE 10. Example of the estimation of *Salmonella* numbers and serotypes in metropolitan stabilisation pond effluent.

Bottle No.	*Salmonella* serotypes isolated
1	havana, typhimurium
2	typhimurium, paratyphi B
3	havana
4	typhimurium
5	havana
6	havana
7	no isolates
8	havana, typhimurium, adelaide, derby
9	havana
10	no isolates

Eight out of ten bottles were positive for *Salmonella*, and reference to Table 5 gives a 'crude' MPN of 16 *Salmonella* per litre. All subsequent MPN values were derived in a similar way.

In Table 11 the MPN values have been adjusted if the ten replicates have not been of 100 mls each. In the case of 10 x 50 ml volumes, for example, the MPN value from Table 5 has been multiplied by 2, and so on.

The limited number of colonies examined from the primary plates would result in under-estimation of the number of serotypes present. McCOY (1962) streaked from enrichment medium over a period of four days to give a quantitative result. However an incubation period of one day was used for isolations in this thesis. As all the examinations were made in a similar fashion, the isolations gave a valid indication of the numbers of *Salmonella* present. A similar method of deriving an MPN from a series of 4 tenfold dilutions of polluted water in triplicate was described by CHENG, BOYLE and GOEPFERT (1971).

TABLE 11. Estimations of *Salmonella* in metropolitan stabilisation pond effluent, with serotypes isolated.

Date	No. of serotypes detected	Serotype	Volume Examined	Crude MPN per litre
1967				
Sept.7	5	adelaide, havana paratyphi B, typhimurium, derby	10 x 100 ml	16
Oct.18	none	-	10 x 100 ml	0
25	none	-	10 x 100 ml	0
Nov. 1	none	-	10 x 100 ml	0
23	none	-	10 x 100 ml	0

TABLE 11 (contd.).

Date	No. of serotypes detected	Serotype	Volume Examined	Crude MPN per litre
Nov.28	1	bovis morbificans	10 x 100 ml	1
Dec. 6	1	bovis morbificans	10 x 100 ml	2
13	1	bareilly	10 x 100 ml	2
18	1	bareilly	10 x 100 ml	5
1968				
Jan.2	none	-	10 x 100 ml	0
10	none	-	10 x 100 ml	0
31	1	chester	10 x 100 ml	7
Feb.14	none	-	10 x 100 ml	0
21	none	-	10 x 100 ml	0
Mar.13	none	-	10 x 100 ml	0
20	none	-	10 x 100 ml	0
27	none	-	10 x 100 ml	0
Apr.10	none	-	10 x 100 ml	0
23	6	adelaide, anatum, derby, havana, typhimurium, orion	10 x 100 ml	5
May 15	none	-	10 x 100 ml	0
28	8	anatum, derby, havana, kottbus, infantis, orion, paratyphi B, tennessee	10 x 100 ml	7

TABLE 11 (contd.).

Date	No.of serotypes detected	Serotype	Volume Examined	Crude MPN per litre
1968				
June 26	4	bovis morbificans, derby, havana, typhimurium	10 x 100 ml	5
July 10	5	anatum, bovis morbificans, derby, paratyphi B, typhimurium	10 x 100 ml	16
24	6	adelaide, bovis morbificans, derby, havana, singapore, typhimurium	10 x 100 ml	12
Aug. 14	10	adelaide, anatum, bovis morbificans, derby, havana, orion, potsdam, saint-paul, senftenberg, typhimurium	10 x 100 ml	>23
Sept.16	7	adelaide, bovis morbificans, chester, derby, havana, litchfield, typhimurium	10 x 100 ml	>23
Oct. 30	6	adelaide, derby, give, paratyphi B, tennessee, saint-paul	10 x 90 ml	14
Nov. 27	4	adelaide, anatum bovis morbificans, saint-paul	10 x 10 ml	36

TABLE 11 (contd.).

Date	No. of serotypes detected	Serotype	Volume Examined	Crude MPN per litre
1968				
Dec. 11	1	adelaide (an *Arizona* also isolated)	10 x 10 ml	10
18	none	-	10 x 100 ml	0
1969				
Jan. 8	1	saint-paul	10 x 100 ml	1
Feb. 5	none	-	10 x 100 ml	0
Feb. 10	1	senftenberg	10 x 100 ml	1
Mar. 5	3	anatum, oranienburg, typhimurium	10 x 100 ml	9
Apr. 9	none	-	10 x 100 ml	0
May 7	2	adelaide, typhimurium	10 x 90 ml	3
June 3	3	derby, typhimurium, potsdam	10 x 100 ml	5
July 8	11	adelaide, anatum, bovis morbificans, chester, derby, havana, orion, oranienburg, senftenberg, typhimurium, singapore	10 x 100 ml	>23

TABLE 11 (contd.).

Date	No. of serotypes detected	Serotype	Volume Examined	Crude MPN per litre
1969				
Aug. 5	9	adelaide, bovis morbificans, derby, give, new-brunswick, orion, paratyphi B, senftenberg, typhimurium	10 x 100 ml	>23
Sept. 2	3	anatum, derby, orion	10 x 100 ml	9
Oct. 7	2	anatum, typhimurium	10 x 50 ml	4
Nov. 4	1	potsdam	10 x 100 ml	1
Dec. 2	1	bovis morbificans	10 x 100 ml	1
1970				
Apr. 7	1	havana	10 x 100 ml	1
May 5	5	muenchen, typhimurium bareilly, adelaide, oranienburg	10 x 100 ml	7
19	none	-	10 x 100 ml	0
June 2	1	newport	10 x 100 ml	1
July 7	1	adelaide	10 x 100 ml	1
Aug. 4	none	-	10 x 100 ml	0
Sept. 8	2	derby, saint-paul	10 x 90 ml	3

TABLE 11 (contd.)

Date	No. of Serotypes detected	Serotype	Volume Examined	Crude MPN per litre
1970				
Sept. 14	2	derby, typhimurium	10 x 90 ml	3
Oct. 5	none	-	10 x 100 ml	0
Nov. 3	1	adelaide	10 x 100 ml	1
Dec. 8	none	-	10 x 100 ml	0
1971				
Jan. 5	none	-	10 x 100 ml	0
Feb. 2	none	-	10 x 100 ml	0
Mar. 1	none	-	10 x 100 ml	0
Apr. 6	none	-	10 x 100 ml	0

PART D

IDENTIFICATION OF VIRUS ISOLATES

PART D

IDENTIFICATION OF VIRUS ISOLATES

Identification of enteric virus isolates

Presumptive identification of enterovirus

The cytopathic effect of the enterovirus on coverslip PMK cell cultures developed early after inoculation, leading to rapid and complete cell destruction. In contrast, the reovirus CPE developed slowly, usually taking 4-5 days to appear after inoculation, and not leading to rapid cell death. The enterovirus infected cells rounded up as shown in unstained live cells (Fig. 2), retracting and becoming refractile. The preparation of prototype echovirus I in PMK cells stained by the May-Grunwald Giemsa method (Fig. 4) shows the characteristic nuclear pyknosis, cell swelling, and deep blue stained cytoplasm of infected cells. Since May-Grunwald Giemsa stains DNA red and RNA blue, the unaffected cells in Fig. 4 have a red nucleus containing one or two blue nucleoli and the cytoplasm stains a faint blue. The infected cells normally lysed after 2 or 3 days with complete destruction of the cell sheet.

This method of presumptive identification by cytopathic changes

Fig. 1 Normal milk dilution bottle control culture of primary monkey kidney cells (PMK) in uninfected culture that has not been fixed or stained.
Bright field at approx. 30 x magnification.

Fig. 2 MD bottle culture of PMK cells 24 hours after inoculation with prototype echovirus I showing early cytopathic effect, i.e. swelling of infected cells (IC).
Bright field at approx. 30 x magnification.

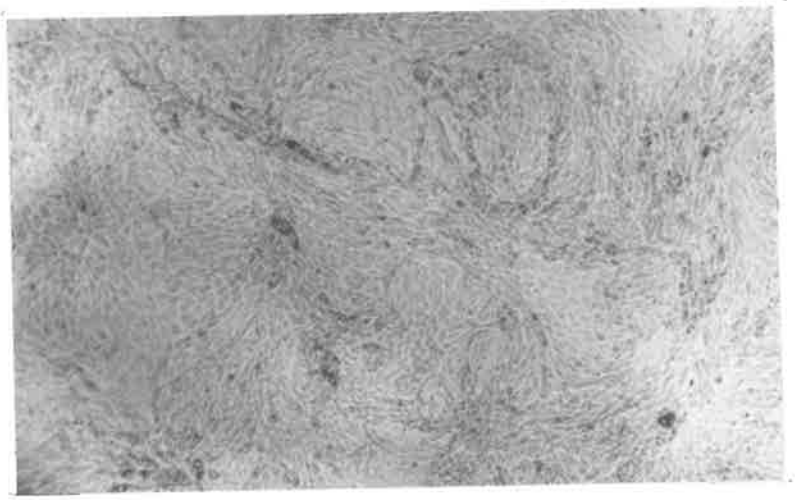

Fig. 1

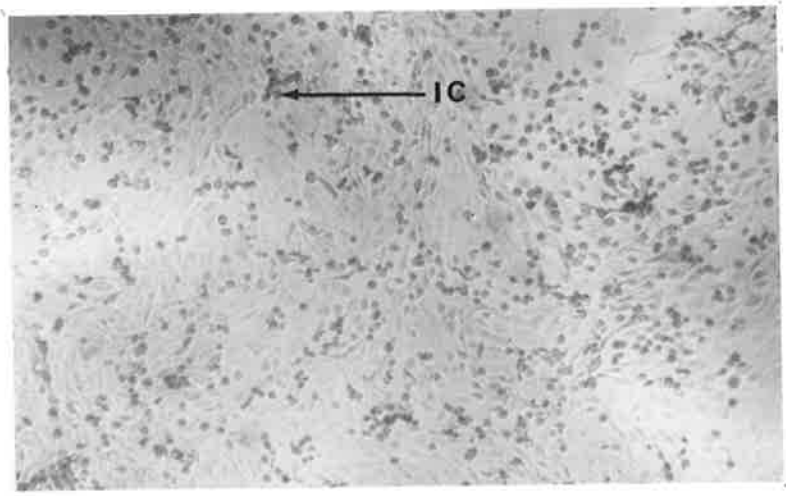

Fig. 2

confirmed with stained coverslip cultures was used for both the enteroviruses and reoviruses by MALHERBE and STRICKLAND-CHOLMLEY (1967). Thes authors stated that the serological identification of viral types is not necessary except when the concentration of a particular agent is required.

Five MD bottle isolates of enterovirus identified by stained CPE in PMK coverslip cell cultures were confirmed as poliovirus by Mr. A. Murphy, The Institute of Clinical Pathology and Medical Research, Lidcombe. The origin of these isolates and the types identified are shown in Table 12.

TABLE 12. Presumptive enterovirus isolates confirmed by Reference Laboratory.

Sample	Date	No. of confirmed CPE enterovirus positive MD bottles out of 10	Isolates referred to Lidcombe	Enterovirus types identified at Lidcombe
Metropolitan stabilisation pond	23/10/68	3/10	Bottle No.4 Bottle No.8	poliovirus 3 poliovirus 3
Berri South stabilisation pond	11/2/69	10/10	Bottle No.4 Bottle No.6 Bottle No.8	poliovirus 3 poliovirus 3 poliovirus 1 2 & 3

The poliovirus strains isolated were probably the Sabin oral vaccine strains which were introduced by South Australian health authorities during July 1967 (Report 1970).

It would have been possible to characterise these isolates further by using a method such as McBRIDE's (1959) antigenic analysis by the kinetics of neutralisation with a number of reference antisera.

Although this type of analysis is expensive and time consuming, identification of poliovirus isolates from sewage treatment works as wild type or vaccine strains could be useful in estimating the level of infection of each vaccine strain serotype in the population, and also of detecting the presence of neurovirulent wild types.

Presumptive identification of reovirus

Many PMK cell cultures being examined during enterovirus isolations from effluents showed a form of degeneration slightly different from the non-specific degeneration seen in an old cell culture. Examined at 25 times magnification in the culture bottle, the cells appeared granular and eventually became partially detached from the glass, fluttering when the bottle was agitated during

Fig. 3 Plaque assay of plaque forming units following $Al(OH)_3$ flocculation of membrane filtered sewage. 29 plaques (PS) in neutral red stained PMK cell sheet marked with black felt pen four days after inoculation with floc.
(Kodachrome II, approximate magnification 1 x, Print prepared by Adelaide Colour Laboratory).

Fig. 4 May-Grunwald Giemsa stained PMK coverslip cell culture 24 hours after infection with prototype echovirus I. Uninfected cells show red stained nucleus (NS) containing nucleoli (NI) with faint blue stained cytoplasm (CM).
Two groups of enterovirus infected cells (IC) show rounding and nuclear pycnosis (NP)
(Kodachrome II Bright field at about 500 x magnification).

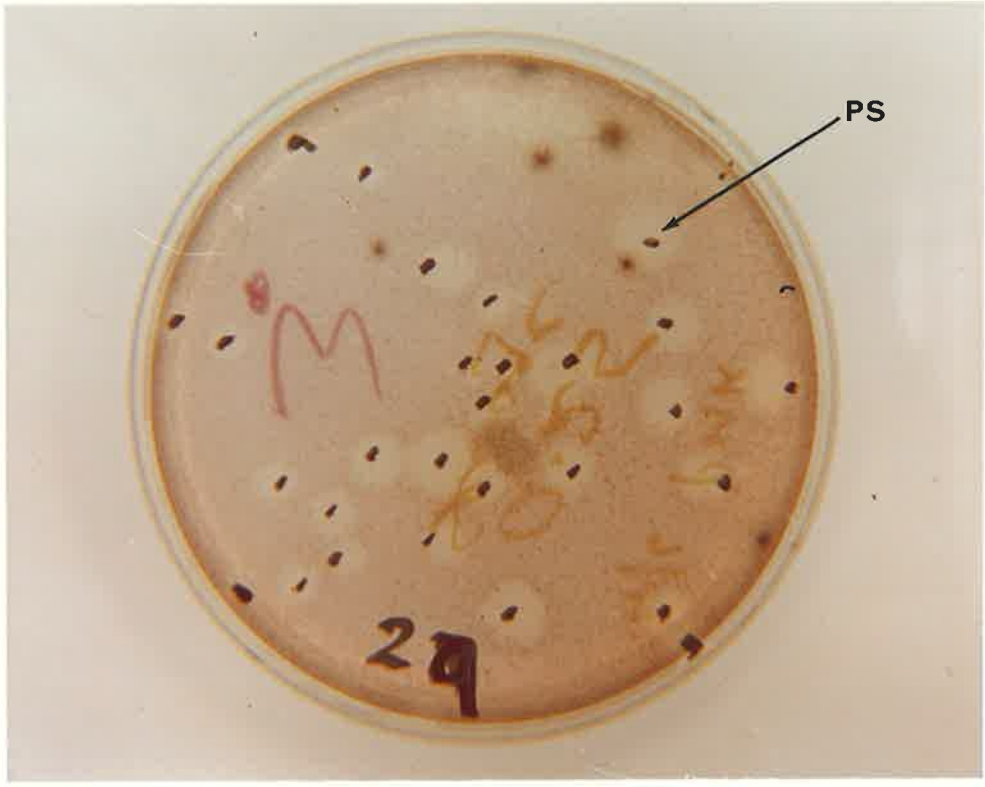

Fig.3

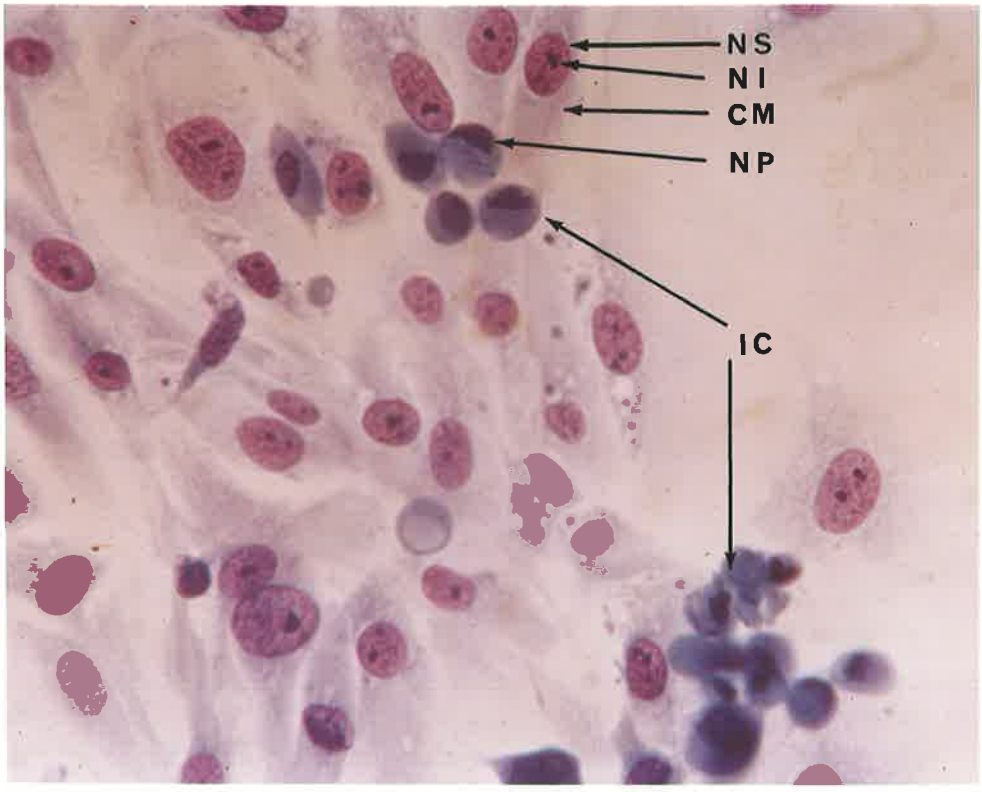

Fig.4

examination. The unstained cytopathic effects were undramatic and slow to appear, often developing only after blind passage.

However, coverslip PMK cultures inoculated with cell culture fluids from such bottles and their uninoculated control showed striking differences after staining. The coverslips were washed with warm PBS, fixed with methanol and stained with May-Grunwald Giemsa (Table 30).

The nuclei of cells showing the atypical degeneration were surrounded by a mass of deeply stained inclusion body (Fig. 7). Similar inclusion bodies could be seen in the positive control of reovirus type 1 (Fig. 6) obtained from Professor N.F. Stanley, but were absent from the coverslip culture inoculated with the negative PMK control from the isolation (Fig. 5). The perinuclear inclusion bodies seen in infected stained cells are pathognomonic (FENNER and WHITE, 1970).

The presumptive reovirus and negative control used to show these effects were obtained from an examination of metropolitan activated sludge effluent sampled on 29/7/69 (Table 21).

Fig. 5 May-Grunwald Giemsa stained coverslip culture of primary monkey kidney cells in uninfected control culture. Nucleus (NS) containing darker nucleoli (NI) with lightly stained cytoplasm (CM). Bright field approx. 350 x magnification. Plus X pan. film.

Fig. 6 May-Grunwald Giemsa stained coverslip culture of primary monkey kidney cells 4 days after inoculation with prototype reovirus I. Perinuclear inclusions (PI) and sparse cell sheet. Bright field approx. 350 x magnification. Plus X pan. film.

Fig. 7 May-Grunwald Giemsa stained coverslip culture of primary monkey kidney cells 4 days after inoculation with cell culture fluid from suspected reovirus isolate (Table 21). Perinuclear inclusions (PI) in sparse cell sheet similar to positive control Fig. 6. Bright field approx. 350 x magnification. Plus X pan. film.

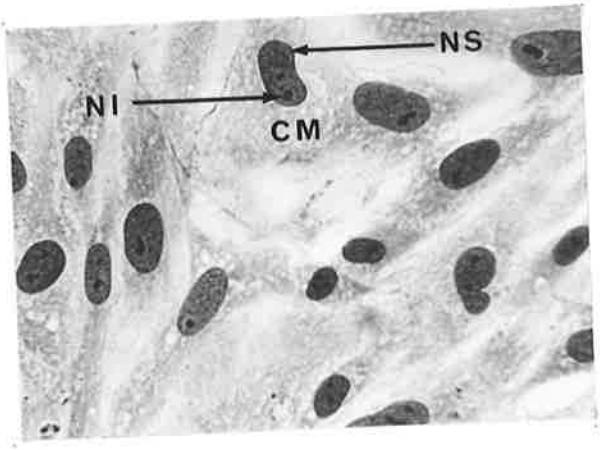

Fig. 5

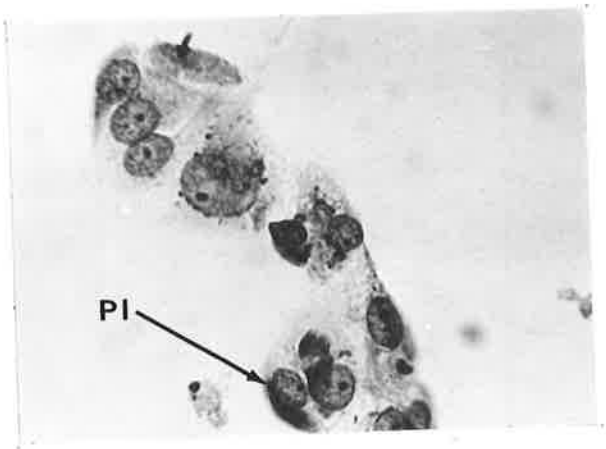

Fig. 6

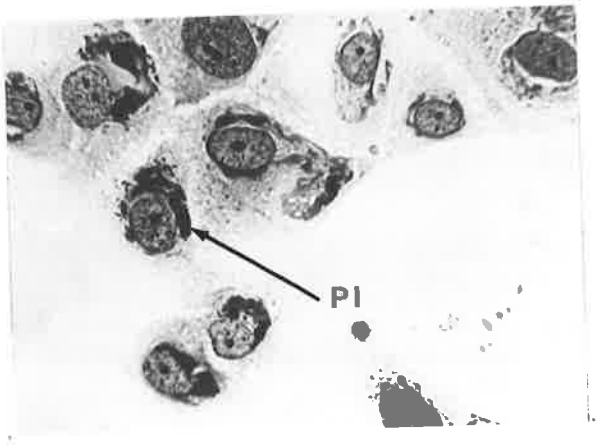

Fig. 7

PART E

ISOLATION METHODS FOR ENTEROVIRUSES IN WATER, SEWAGE AND STABILISATION POND EFFLUENT

PART E

ISOLATION METHODS FOR ENTEROVIRUSES IN WATER, SEWAGE AND STABILISATION POND EFFLUENT

To establish the effect of various treatments, distilled water and stabilisation pond effluent were each seeded with poliovirus 1.

An aluminium hydroxide flocculation experiment was also conducted on unseeded metropolitan sewage as a preliminary experiment in the concentration of virus from sewage and effluents.

A stock culture of Sabin Poliovirus 1 was prepared in PHA cells from virus supplied by Dr. J. Hampton, National Biological Standards Laboratory, Melbourne. The number of PFU in the freeze-thawed centrifuged cell culture fluid were counted by the PA technique (Part B, "Estimation of enterovirus numbers : plaque assay") using serial tenfold dilutions in tubes of PBS containing 0.1% inactivated calf serum (HOWES, 1969a) as a stabilising agent. Half ml volumes of the 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions were adsorbed for one hour at room temperature on duplicate PMK petri dish cell cultures, overlaid with T5L medium (Table 26), and incubated in the 5% CO_2/air incubator at $37^{o}C$, the plaques being marked with a felt pen as they appeared (Table 13).

TABLE 13 Plaque assay (PA) of stock Sabin 1 poliovirus (plaques counted separately each day - no more appeared after 3 days).

Dilution	Inoculum/ plate	Days incubated 1	2	3 (plaques counted on each day)	PFU/plate	PFU/ml
10^{-5}	0.5 ml	0	258	-	-	-
	"	0	not countable	-	-	-
10^{-6}	"	0	60	38	98)	178
	"	0	43	37	80)	
10^{-7}	"	0	4	6	10)	18
	"	0	3	5	8)	
10^{-8}	"	0	0	2	2)	2
	"	0	0	0	0)	

A problem with the PA method is that as the plaques develop they tend to overlap, leading to underestimation of the number of PFU present. COOPER (1967) stated that as a guide, 100 plaques per plate would not give significant overlap bias if the internal diameter of the plate was at least 25 times the average plaque diameter.

Accordingly, plates with counts in excess of 100 have been disregarded, giving an estimate of 180 PFU/ml in the 10^{-6} dilution of the stock culture of poliovirus 1. One ml volumes were sealed in glass ampoules and stored in the deep freeze.

Effect of the presence of serum during membrane filtration of Sabin poliovirus 1 suspended in distilled water

A suspension of about 180 PFU/ml of poliovirus 1 was prepared by adding 1.0 ml of a fresh 10^{-3} dilution of the stock poliovirus (Table 13) to one litre of sterile double distilled water in a screw cap erhlenmeyer flask, swirling well to mix.

The contents of the flask was divided into two 500 ml volumes and 10 ml of foetal calf serum was added to one flask, and 10 ml of distilled water to the other. Each nominal 500 ml volume was then further divided into two 250 ml aliquots. The four 250 ml volumes were marked A, B, C & D, flasks C & D containing 2%(v/v) foetal calf serum.

Leaving flasks B & D on the bench the 250 ml in A & C were filtered at the same time through a prefilter and 0.45 micrometre Sartorius membrane filter combination as described in the preparation of EM199 (Table 32), in separate sterile all glass Millipore filter apparatus.

The number of PFU/ml in each of the flasks B & D, as well as the 2 filter flasks A & C, was then estimated by the PA method (Part B, "Estimation of enterovirus numbers : plaque assay"). A 0.5 ml volume of the 10^{-6} dilution of stock Sabin 1 poliovirus was also examined as a control.

TABLE 14. Effect of the presence of 2%(v/v) foetal calf serum on recovery of Sabin strain poliovirus type 1 during membrane filtration in a distilled water suspension.

Flask	Treatment	Inoculum/ plate	Days incubated (plaques counted) 1	2	3	PFU/ plate	PFU/ml
A	Filter	0.5 ml	0	15	10	25)	53
	"	"	0	21	7	28)	
B	No Filter	"	0	35	18	53)	140
	"	"	0	67	20	87)	
C	Filter with serum	"	0	58	15	73)	141
	" "	"	0	42	26	68)	
D	No Filter with serum	"	0	35	21	56)	142
	" "	"	0	63	23	86)	
Stock poliovirus control 10^{-6} dilution		"	0	41	23	64	128

The presence of serum prevented loss of poliovirus 1 during filtration through the 0.45 micrometre pore size membrane filter/ prefilter combination described in the preparation of EM199 (Part B, "Preparation of effluent samples for enteric virus isolations").

Effect of the presence of serum during filtration of Sabin poliovirus type 1 suspended in stabilisation pond effluent

The results in Table 14 showed that foetal calf serum prevented loss of poliovirus during filtration in a distilled water suspension. To establish whether a similar suspension of poliovirus in stabilisation pond effluent behaved in the same way, 1.0 ml of a fresh 10^{-3} dilution of the stock Sabin poliovirus 1 was added to one litre of stabilisation pond effluent. Penicillin, streptomycin and amphotericin B were added to give the same concentrations as in the preparation of EM199 (Table 32) i.e. 1,000 units, 1,000 micrograms and 2 micrograms per ml respectively.

After mixing these components well, the antibiotic treated stabilisation pond effluent was centrifuged in 6 x 90 ml volumes at 18,000 rpm for 30 minutes at 4^{o}C in an MSE High Speed 18 centrifuge to remove contaminating micro-organisms. The supernatant was carefully pipetted off and divided into four 100 ml volumes in flasks which were marked A, B, C & D.

Two ml of foetal calf serum was added to each of flasks C & D containing 100 ml volumes of the virus seeded centrifuged stabilisation pond effluent, and 2 ml of distilled water to each of the other two flasks A & B.

Leaving flasks B & D on the bench, the 100 ml in A & C were filtered at the same time through a prefilter and 0.45 micrometre Sartorius membrane filter combination as described in the preparation of EM199, in separate all glass Millipore filter apparatus.

The number of PFU/ml in each of the flasks B & D as well as the 2 filter flasks A & C, was then estimated by the PA method. A 10^{-6} dilution of stock Sabin poliovirus 1 was included as a control (Table 13).

While the results were analogous to those obtained with distilled water (Table 14), the recovery of poliovirus was low. However, the presence of serum during filtration gave a higher recovery of poliovirus PFU than filtration without serum (Table 15).

TABLE 15. Effect of the presence of 2%(v/v) foetal calf serum on recovery of Sabin strain poliovirus type 1 during membrane filtration in a metropolitan stabilisation pond effluent suspension.

Flask	Treatment	Inoculum/ plate	Days incubated 1 (plaques	2	3 counted)	PFU/ plate	PFU/ml
A	Filter	0.5 ml	1	2	0	3))	3
	"	"	0	0	0	0)	
B	No Filter	"	5	3	1	9))	18
	"	"	5	2	2	9)	
C	Filter with serum	"	6	3	0	9))	16
	" "	"	5	2	0	7)	
D	No filter with serum	"	5	5	3	13))	26
	" "	"	4	7	2	13)	
Stock poliovirus control 10^{-6} dilution		"	25	35	21	81	162

Enumeration of plaque forming units from metropolitan sewage by flocculation with aluminium hydroxide

One experiment to concentrate plaque forming viruses from sewage by flocculation with aluminium hydroxide is described in detail below. Sewage was used rather than effluent because of the higher number of PFU potentially present.

Extension of concentration methods to effluents would simplify enumeration of plaque forming viruses and allow greater accuracy than when the quantal MPN assay was used.

A sample of metropolitan screened sewage was centrifuged at 5,000 rpm for 10 minutes to remove gross debris. A sterile Millipore GS 47 mm diameter membrane (0.22 micrometre pore size) and prefilter were pretreated by passing through 50 ml of BME containing 10% foetal calf serum and then washed with 50 ml of sterile distilled water in a procedure similar to that described by MOORE, LUDOVICI and JETER (1970). These authors stated that this treatment inhibited adsorption of the enteroviruses to this type of membrane, and reported an average loss of Sabin 1 poliovirus of 31% after filtration through BME-10% foetal calf treated Millipore GS 0.22 micrometre membranes, compared with a loss of 76% without serum pretreatment.

It was shown (Tables 13 & 14) that Sartorius 0.45 micrometre pore size membrane filters do not remove Sabin 1 poliovirus in the presence of serum.

After discarding the flask containing the BME, another sterile flask was fitted to the Millipore all glass filter apparatus, and 100 ml of the supernatant sewage after centrifugation was filtered through pretreated filters. Antibiotics were not used because 1,000 micrograms per ml of streptomycin raised the pH of sewage the order of 0.5 of a pH unit. This could affect the adsorption of virus to the floc, and to ensure bacteriological sterility, a 0.22 micrometre membrane filter was used without antibiotic cover.

The filtrate was stirred for one hour at room temperature with a sterile magnetic stirrer rotor with 0.5 ml of aluminium hydroxide floc in pH 6.0 buffer (WALLIS and MELNICK, 1967).

The floc was not collected on a membrane filter, but centrifuged in two 50 ml volumes because of the problem of quantitative floc removal from the membrane. One centrifuge tube was broken. The remaining yellowish floc was resuspended in 0.5 ml of saline, and 0.25 ml was placed in each of 2 PBS washed PMK petri dish cell cultures on a flat glass plate together with an uninoculated control culture. The number of plaque forming units was then estimated

by the PA technique (Part B, "Estimation of enterovirus numbers : plaque assay").

Plaques began to appear on the third day and the count completed on the fourth. The delay when compared with the Sabin poliovirus 1 plaques (Table 13) which appeared within 3 days could be due to a different type of enterovirus, or toxicity of the aluminium hydroxide slowing plaque development. A positive control could not be put down because of lack of cell cultures. The uninoculated control did not develop any plaques.

One plate gave 29 plaques (Fig. 3) and the other 15. The difference was probably due to the difficulty of pipetting floc before sedimentation occurred. The number of PFU was therefore estimated as 88 per 100 ml.

PART F

ESTIMATION OF ENTERIC VIRUSES IN STABILISATION POND EFFLUENT

PART F

ESTIMATIONS OF ENTEROVIRUS IN METROPOLITAN STABILISATION POND EFFLUENT

Enterovirus isolations were made by the EM199 adsorption method (Part B, "Method of enterovirus isolation") from grab samples obtained from metropolitan stabilisation pond effluent. The presumptive identification was made on the cytopathic effect on primary cynomolgus monkey kidney cell cultures (MALHERBE and STRICKLAND-CHOLMLEY, 1967) also set out in Part B, "Presumptive identification of enteroviruses". From the 44 samples examined a total of 11, or 25%, were positive. Presumptive reovirus were similarly identified on their characteristic cytopathic effect (Fig. 6).

Table 16 gives the MPN figures for entero and reovirus from these examinations, obtained by reference to Table 5. Table 16 also includes for convenience the enteric bacteria *E.coli* and *Salmonella* isolated from this effluent. The bacteria are separately tabulated in Tables 7 and 11 respectively.

These are included to give a concise summary of the isolation work carried out on this effluent. Graphical representations of

the relationship between *E.coli* and *Salmonella* and enteroviruses are given in Fig. 9 and Fig. 10 respectively. Following these Figures, a statistical approach to the relationship between the numbers of *E.coli* and *Salmonella* and enteroviruses is given.

TABLE 16. Summary of *E.coli*, *Salmonella*, enterovirus and reovirus estimations from metropolitan stabilisation pond effluent.

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1967					
July 5	130,000				
12	35,000				
19	35,000				
26	70,000				
Aug. 2	8,000				
10	250,000				
16	2,500				
23	11,000				
30	3,500				
Sept.6	35,000				
7		5	16		
14	2,000				
20	900				
27	1,100				
Oct. 4	140				
11	900				

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1967					
Oct.18	110	0	0		
25	250	0	0		
Nov. 1	40	0	0		
6	95				
13	350				
20	350				
23		0	0		
27	350				
28		1	1		
Dec. 4	80				
6		1	2		
11	275				
13		1	2		
18	250	1	5		
25	-				
1968					
Jan. 2		0	0		
3	25				
9	2,500				
10		0	0		
17	500				
24	170				
31	60	1	7		

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1968					
Feb. 7	350				
14	35	0	0		
21	50	0	0		
28	350				
Mar. 6	800				
13	150	0	0		
20	170	0	0		
27	1,600	0	0		
Apr. 3	250				
10	-	0	0		
17	130				
23		6	5	0	-
24	350				
May 1	1,600				
8	900				
15	130	0	0		
22	130				
28		8	7	0	-
29	7,000				
June 5	8,000			0	-
12	5,500				
19	17,500				
26	6,000	4	5		

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1968					
July 3	5,000			>23	-
10	8,000	5	16		
17	900				
24	9,000	6	12	0	-
31	4,000				
Aug. 7	20,000				
14	45,000	10	>23		
21	55,000				
27				7	-
28	120,000				
Sep. 4	13,000				
12	170,000				
16		7	>23	0	-
18	1,700				
25	55,000				
Oct. 2	250,000				
9	4,000				
16	40,000				
23	13,000			4*	-
30	25,000	6	14		
Nov. 5				0	-
6	-				

*See Table 12 for enterovirus types isolated

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1968					
Nov.13	50,000				
20	35,000				
27	55,000	4	36		
Dec. 2				0	-
4	5,500				
11	1,600	1	10		
18	550	0	0		
24	35				
31	50				
1969					
Jan. 8	140	1	1		
15	350				
22	170				
29	35				
Feb. 5	550	0	0		
10		1	1		
12	350				
19	170				
26	80				
Mar. 5	800	3	9		
12	700				
19	350				
26	3,500				

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1969					
Apr. 2	450				
9	250	0	0		
15				0	-
16	1,700				
23	450				
30	1,300				
May 7	2,500	2	3		
14	6,500				
16				0	-
21	5,000				
28	17,000				
June 3	3,500	3	5	4	7
10	250,000				
17	3,500				
18				0	>23
24	25,000				
July 1	55,000				
8	350,000	11	>23	>23	0
15	130,000				
22	14,000			0	>23
29	7,000				
Aug. 5	11,000	9	>23	0	>23
12	140,000				

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1969					
Aug.19	11,000			0	>23
26	2,500				
Sept.2	16,000	3	9	0	>23
9	5,500				
16	5,500			0	1
23	3,500				
30	-				
Oct. 7	900	2	4	0	2
14	-				
21	170			0	12
28	450				
Nov. 4	2,500	1	1		
11	2,500				
18	1,700			Cell cultures contaminated	
25	200				
Dec. 2	5,500	1	1	0	2
9	4,000				
16	350			0	0
23	170				
30	350				
1970					
Jan. 6	1,600				
13	250				
20	3,500				

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1970					
Jan.27	450				
Feb. 3	350			0	0
10	65				
17	80			0	2
24	1,700			0	0
Mar. 3	25				
10	110				
17	250			0	1
24	350				
31	80				
Apr. 7	130	1	1	0	2
14	45				
21	-			4	1
28	-				
May 5		5	7	0	>23
6	11,000				
12	35,000				
19		0	0		
20	550				
21				Cell cultures contaminated	
26	3,500			Cell cultures contaminated	
June 2	1,200	1	1		
9	2,500				
16	9,000			5	0

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1970					
June 23	11,000				
26				0	0
30	4,500				
July 7	11,000	1	1		
14	1,600				
21	5,500			0	0
29	2,500				
Aug. 4	9,000	0	0		
7				2	0
11	3,500				
18	5,500				
25	16,000				
Sept. 1	2,500				
8	700	2	3	5	0
14		2	3		
15	16,000				
22	2,000			5	1
29	3,500			0	0
Oct. 5	350	0	0		
13	80				
20	250			>23	0
27	170			0	0
Nov. 3		1	1	0	0
5	170				

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1970					
Nov.10	200				
17	250			0	0
24	170				
Dec. 1	130			0	0
8	350	0	0		
15	300				
22	350			Cell cultures contaminated	
29	-				
1971					
Jan. 5	250	0	0		
12	95				
19	50				
26	50				
Feb. 2	130	0	0		
9	50				
16	130			0	0
23	25				
Mar. 1		0	0		
2	25				
9	170				
16	250				
23	250				
30	80				
Apr. 6	550	0	0		

TABLE 16 (contd.)

Date	*E.coli* MPN per 100 ml	*Salmonella* No.of Serotypes	*Salmonella* MPN per litre	Enterovirus MPN per litre	Reovirus MPN per litre
1971					
Apr.13	550				
20	550				
27	1,600				
May 4	1,800 +				
11	1,800 +				
18	1,600				
25	1,800 +				
June 1	3,500				
8	1,700				
15	3,500				
22	5,500				
29	16,000				

PART G

ASSOCIATION OF *E.COLI* WITH *SALMONELLA* AND ENTEROVIRUSES IN METROPOLITAN STABILISATION POND EFFLUENT

PART G

ASSOCIATION OF *E.COLI* WITH *SALMONELLA* AND ENTEROVIRUSES IN METROPOLITAN STABILISATION POND EFFLUENT

Association of *E.coli* and *Salmonella* in metropolitan stabilisation pond effluent

The relationship between *Salmonella* isolations and the smoothed *E.coli* curve is illustrated graphically in Fig. 9. Most determinations of *E.coli*, *Salmonella* and enteroviruses summarised in Table 16 were obtained from 'unpaired' samples. However, sufficient of these were paired to allow the direct calculation of a regression equation and a correlation coefficient to show the association between *E.coli* and *Salmonella*.

The following Table 17 gives values of these observations of paired *Salmonella* and *E.coli* samples extracted from Table 16. The results which are included in the original data as >23 have been arbitrarily accepted as 23. The most probable number of *Salmonella* is shown as well as the actual number of serotypes isolated from each positive sample.

A Canola 164P electronic calculator was used for the computations, and therefore the individual values for various steps have not been given in all cases.

TABLE 17. Association of *E.coli* and *Salmonella* in metropolitan stabilisation pond effluent. Paired data.

E.coli MPN per 100 ml	Crude *Salmonella* MPN per litre		Number of *Salmonella* serotypes isolated	
x	y	xy	z	xz
110	0		0	
250	0		0	
40	0		0	
250	5		1	
60	7		1	
35	0		0	
50	0		0	
150	0		0	
170	0		0	
1600	0		0	
130	0		0	
6000	5		4	
8000	16		5	
9000	12		6	
45000	23		10	
25000	14		6	
55000	36		4	
1600	10		1	
550	0		0	
140	1		1	
550	0		0	
800	9		3	
250	0		0	
2500	3		2	

TABLE 17 (contd.)

E.coli MPN per 100 ml	Crude *Salmonella* MPN per litre		Number of *Salmonella* serotypes isolated	
x	y	xy	z	xz
3500	5		3	
350000	23		11	
11000	23		9	
16000	9		3	
900	4		2	
2500	1		1	
5500	1		1	
130	1		1	
1200	1		1	
11000	1		1	
9000	0		0	
700	3		2	
350	0		0	
350	0		0	
250	0		0	
130	0		0	
550	0		0	
$\Sigma x = 570295$	$\Sigma y = 213$	$\Sigma xy = 12154040$	$\Sigma z = 79$	$\Sigma xz = 4978480$

The problem of zero values of y was overcome by using ranked data. The *E.coli* estimates listed in Table 17 were rearranged to give an ascending order, and each corresponding *Salmonella* MPN, y, entered alongside, in Table 18.

The rank value is found by dividing the 'tied' rank total by the number of tied values in the following way:

35	1
40	2
50	3
60	4
110	5
130	6)
130	7) $\frac{6 + 7 + 8}{3} = 7$
130	8)
140	9
150	10 etc.

The rank of the three 130 values is 7. The ranking is then carried to 140, of rank 9 and so on (MORONEY, 1970).

Similary for values of y ranks are calculated. There are 18 zero values. The rank of these 'tied' zero values is given by

$$\frac{1 + 2 + 3 + 4 \ldots\ldots + 18}{18} = 9.5.$$

The next value of y is 1. The rank is given by 19 + 20 + 21 + 22 + 23 + 24 (since there are six values of 1) divided by 6 = 21.5. This process has been applied to all 41 of the paired results.

TABLE 18. Association of *E.coli* and *Salmonella* in metropolitan stabilisation pond effluent. Ranked paired data.

E.coli MPN/100 ml x	Crude *Salmonella* MPN/litre y	Rank x	Rank y
35	0	1	9.5
40	0	2	9.5
50	0	3	9.5
60	7	4	31
110	0	5	9.5
130)	0	7	9.5
130)	0	7	9.5
130)	1	7	21.5
140	1	9	21.5
150	0	10	9.5
170	0	11	9.5
250)	5	13.5	29
250)	0	13.5	9.5
250)	0	13.5	9.5
250)	0	13.5	9.5
350)	0	16.5	9.5
350)	0	16.5	9.5
550)	0	19	9.5
550)	0	19	9.5
550)	0	19	9.5

TABLE 18 (contd.)

E.coli MPN/100 ml x	Crude *Salmonella* MPN/litre y	Rank x	Rank y
700	3	21	25.5
800	9	22	32.5
900	4	23	27
1200	1	24	21.5
1600)	0	25.5	9.5
1600)	10	25.5	34
2500)	3	27.5	25.5
2500)	1	27.5	21.5
3500	5	29	29
5500	1	30	21.5
6000	5	31	29
8000	16	32	37
9000)	12	33.5	35
9000)	0	33.5	9.5
11000)	23	35.5	39
11000)	1	35.5	21.5
16000	9	37	32.5
25000	14	38	36
45000	23	39	39
55000	36	40	41
350000	23	41	39

(brackets indicate tied values of x)

To calculate the Spearman rank correlation, the sum of the differences in ranks squared is given by $(1 - 9.5)^2 + (2 - 9.5)^2$ etc. = 3338.5.

The correction for 'tied' values of x is

$$\frac{(41)^3 - 41}{12} - \left[\frac{3^3 - 3}{41} + \frac{4^3 - 4}{41} + \frac{2^3 - 2}{41} + \frac{3^3 - 3}{41} + \frac{2^3 - 2}{41} + \frac{2^3 - 2}{41} + \frac{2^3 - 2}{41} + \frac{2^3 - 2}{41} \right] \text{ giving } \Sigma x^2_t = 5736.6341$$

Similarly the correction for 'tied' values of y is

$$\frac{(41)^3 - 41}{12} - \left[\frac{18^3 - 18}{41} + \frac{6^3 - 6}{41} + \frac{2^3 - 2}{41} + \frac{3^3 - 3}{41} + \frac{2^3 - 2}{41} + \frac{3^3 - 3}{41} \right] \text{ giving } \Sigma y^2_t = 5591.6098$$

The Spearman rank correlation coefficient is given by

$$r_s = \frac{5736.6341 + 5591.6098 - 3338.5}{2 \times \sqrt{5736.6341 \times 5591.6098}} = \frac{7989.7439}{11327.3156}$$

$$= +0.7535 \text{ (corrected for ties)}$$

To test for significance the t test was used

$$t = r_s \sqrt{\frac{n - 2}{1 - r_s^2}}$$

$$= 0.7535 \sqrt{\frac{41 - 2}{1 - 0.7243^2}}$$

= 6.8248 at 39 degrees of freedom, which is significant at the 0.05 level. The Spearman rank correlation of +0.75 was

significant, and indicated a positive correlation between the isolation of *Salmonella* and the indicator organism *E.coli* in stabilisation pond effluent.

To show the relation between the crude MPN of *Salmonella* and *E.coli*, a regression line was calculated in the form $y = mx + c$ by the method of the sum of least squares.

The value of m is given by

$$m = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

$$= \frac{12154040 - \frac{(570295)(213)}{41}}{129000045225 - \frac{325236387025}{41}}$$

$$= \frac{9191288}{121067450420}$$

$$= 0.000076$$

and that of c by

$$c = \frac{(\Sigma x)(\Sigma xy) - (\Sigma y)(\Sigma x^2)}{(\Sigma x)^2 - N(\Sigma x^2)}$$

$$= \frac{(570295)(12154040) - (213)(129000045225)}{(325236387025) - 41(129000045225}$$

$$= \frac{-20545621391125}{-4663765467200}$$

$$= \underline{4.14}$$

Substitution of the values of m and c obtained from the data contained in Table 17 in $y = mx + c$ gives a regression equation of $y = 0.000076x + 4.14$.

Although it is apparent on a linear scatter diagram (not included because of the physical size of the Figure), that the relation between the crude *Salmonella* and *E.coli* results is linear, and would approximate the form $y = mx + c$, there is a bias near the origin due to a cluster of *Salmonella* isolations associated with low *E.coli* counts. The problem is increased by the presence of 3 *Salmonella* counts of greater than 23. These have been treated as though they are values of 23, although they could be of greater magnitude.

The combination of a cluster of *Salmonella* isolations at low *E.coli* levels and a low trend (due to not reaching extinction), in 3 of the high *Salmonella* results, biasses the regression line, decreasing its value as a means of predicting the presence of *Salmonella* from *E.coli* numbers. The regression line, if taken literally, implies that 4 *Salmonella* will be present per litre, even when *E.coli* is absent (Fig. 8).

Similarly a regression equation was calculated for $z = mx + c$. In this case, the number of *Salmonella* serotypes z, was used instead of the crude MPN.

Fig. 8 Scatter diagram of *Salmonella* (MPN/litre) estimations against the logarithm of the *E.coli* (MPN/100 ml) estimations with linear regression line superimposed.

Calculation for m and c gives a regression equation of

$$z = 0.000032x + 1.48$$

$$m = \frac{\Sigma xz - \frac{(\Sigma x)(\Sigma z)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

$$= \frac{4978480 - \frac{(570295)(79)}{41}}{129000045225 - \frac{(325236387025)}{41}}$$

$$= \frac{3879619}{121067450420}$$

$$m = \underline{0.000032}$$

$$c = \frac{(\Sigma x)(\Sigma xz) - (\Sigma z)(\Sigma x^2)}{(\Sigma x)^2 - N(\Sigma x^2)}$$

$$= \frac{(570295)(4978480) - (79)(129000045225)}{(325236387025 - 41(129000045225)}$$

$$c = \frac{-7351801321175}{-4663765467200}$$

$$c = \underline{1.48}$$

This is also a poor mathematical model because 1.48 *Salmonella* serotypes are predicted at zero *E.coli* MPN.

The correlation coefficient between *E.coli* and the number of *Salmonella* serotypes isolated (x and z respectively, Table 18) is given by

$$r = \frac{\frac{1}{N}\ \Sigma xz - \bar{x}\bar{z}}{\sigma x \sigma z}$$

$$= +0.62$$

Calculating

$$t = \frac{r\sqrt{N-2}}{\sqrt{1-r^2}}$$

$$t = 4.96$$

This value of t at 40 degrees of freedom exceeds that value given by a probability of 0.05 (FISHER and YATES, 1970), and the correlation coefficient is significant.

Fig. 9. Comparison of *E.coli* (5 week weighted running mean of MPN/100 ml) in metropolitan stabilisation pond effluent with *Salmonella* isolations (MPN/litre). Smoothed *E.coli* curve showed marked seasonal fluctuations with an associated variation in *Salmonella* isolations.

Association of *E.coli* and enterovirus in metropolitan stabilisation pond effluent

From inspection of Table 16 it can be seen that there were 30 instances where *E.coli* and enterovirus isolations were attempted on paired samples. Eight of these 30 samples yielded enteroviruses. These numbers were too low to allow reasonable calculation of the association of *E.coli* and enteroviruses.

The *E.coli* figures have been interpolated from the graph in Figure 10 at that date on which each of the 44 enterovirus isolation attempts was made, the interpolated figures being set out in Table 19, together with the appropriate enterovirus titre. A Spearman rank correlation coefficient has been derived from the ranked data in Table 20 in a similar fashion to that previously calculated for *E.coli* and *Salmonella* from Table 18.

a

Fig. 10 Comparison of *E.coli* (5 week weighted running mean of MPN/100 ml) in metropolitan stabilisation pond effluent with enterovirus isolations (MPN/litre). Smoothed *E.coli* curve showed marked seasonal fluctuations with an associated variation in enterovirus isolations.
Values of *E.coli* (Table 19) for calculation of the association between *E.coli* and enteroviruses were interpolated from this Figure.

TABLE 19. MPN estimations of enterovirus (and any contemporaneous *E.coli* estimations) in metropolitan stabilisation pond effluent, with interpolated *E.coli* MPN's.

Date	Enterovirus MPN per litre	Interpolated *E.coli* Numbers from Fig.10		Actual *E.coli* MPN
		log MPN	MPN	
1968				
Apr. 23	0	2.76	580	-
May 28	0	3.65	4,500	-
June 5	0	3.88	7,600	8,000
July 3	>23	3.82	6,600	5,000
24	0	3.86	7,300	9,000
Aug. 27	7	4.89	78,000	-
Sep. 16	0	4.88	76,000	-
Oct. 23	4*	4.36	23,000	13,000
Nov. 5	0	4.52	33,000	-
Dec. 2	0	4.35	22,000	-
1969				
Apr. 15	0	2.99	980	-
May 16	0	3.80	6,300	-
June 3	4	4.74	55,000	3,500
18	0	4.92	83,000	-
July 8	>23	5.26	180,000	350,000
22	0	4.84	69,000	14,000
Aug. 5	0	4.56	36,000	11,000
19	0	4.55	36,000	11,000
Sep. 2	0	4.02	10,000	16,000

*See Table 12 for enterovirus types isolated

TABLE 19 (contd.)

Date	Enterovirus MPN per litre	Interpolated *E.coli* Numbers from Fig.10		Actual *E.coli* MPN
		log MPN	MPN	
1969				
Sep. 16	0	3.77	5,900	5,500
Oct. 7	0	3.24	1,700	900
21	0	2.97	930	170
Nov. 18	Cell cultures contaminated			1,700
Dec. 2	0	3.50	3,200	5,500
16	0	2.95	890	350
1970				
Feb. 3	0	2.80	630	350
17	0	2.63	430	80
24	0	2.87	740	1,700
Mar. 17	0	2.32	210	250
Apr. 7	0	3.08	1,200	130
21	4	3.86	7,300	-
May 5	0	4.05	11,000	-
21	Cell cultures contaminated			-
26	Cell cultures contaminated			3,500
June 16	5	3.82	6,600	9,000
26	0	3.90	7,900	-
July 21	0	3.70	5,000	5,500
Aug. 7	2	3.77	5,900	-
Sept. 8	5	3.77	5,900	700
22	5	3.69	4,900	2,000
29	0	3.54	3,500	3,500

TABLE 19 (contd.)

Date	Enterovirus MPN per litre	Interpolated *E.coli* Numbers from Fig.10		Actual *E.coli* MPN
		log MPN	MPN	
1970				
Oct. 20	>23	2.82	660	250
27	0	2.76	580	170
Nov. 3	0	2.78	600	-
17	0	2.81	650	250
Dec. 1	0	2.82	660	130
22	Cell cultures contaminated			350
1971				
Feb. 16	0	1.92	83	130

TABLE 20. Association of (interpolated) *E.coli* and enterovirus in metropolitan stabilisation pond effluent. Ranked, paired data.

E.coli MPN/100 ml (Interpolated)	Enterovirus MPN/litre	Rank	
x	y	x	y
83	0	1	17
210	0	2	17
430	0	3	17
580)	0	4.5	17
580)	0	4.5	17
600	0	6	17
630	0	7	17
650	0	8	17
660)	0	9.5	17
660)	>23	9.5	43
740	0	11	17
890	0	12	17
930	0	13	17
980	0	14	17
1,200	0	15	17
1,700	0	16	17
3,200	0	17	17
3,500	0	18	17
4,500	0	19	17
4,900	5	20	39
5,000	0	21	17
5,900)	0	23	17
5,900)	2	23	34
5,900)	5	23	39

TABLE 20 (contd.)

E.coli MPN/100 ml (interpolated) x	Enterovirus MPN/litre y	Rank x	Rank y
6,300	0	25	17
6,600)	>23	26.5	43
6,600)	5	26.5	39
7,300)	0	28.5	17
7,300)	4	28.5	36
7,600	0	30	17
7,900	0	31	17
10,000	0	32	17
11,000	0	33	17
22,000	0	34	17
23,000	4	35	36
33,000	0	36	17
36,000)	0	37.5	17
36,000)	0	37.5	17
55,000	4	39	36
69,000	0	40	17
76,000	0	41	17
78,000	7	42	41
83,000	0	43	17
180,000	>23	44	43

The sum of the difference in ranks squared is given by

$$(x_1 - y_1)^2 + (x_2 - y_2)^2 + \text{----}(x_n - y_n)^2 = 8166.5$$

The correction for 'tied' values of x is given by

$$\frac{44^3 - 44}{12} - \left[\frac{2^3 - 2}{44} + \frac{2^3 - 2}{44} + \frac{3^3 - 3}{44} + \frac{2^3 - 2}{44} + \frac{2^3 - 2}{44} + \frac{2^3 - 2}{44} \right]$$

$$= 7095.00 - 1.2275$$

$$= 7093.7725$$

Similarly the correction for 'tied' values of y

$$\frac{44^3 - 44}{12} - \left[\frac{33^3 - 33}{44} + \frac{3^3 - 3}{44} + \frac{3^3 - 3}{44} + \frac{3^3 - 3}{44} \right]$$

$$= 7095.00 - 814.3635$$

$$= 6280.6365$$

The Spearman rank correlation coefficient is given by

$$r_s = \frac{7093.7725 + 6280.6365 - 8166.50}{2\sqrt{7093.7725 \times 6280.6365}}$$

$$= \frac{5207.909}{13349.6676}$$

$$r_s = +0.3902$$

Testing for significance

$$t = r_s \sqrt{\frac{n - 2}{1 - r_s^2}}$$

$$= 0.3902 \sqrt{\frac{42}{0.8477}}$$

$$\therefore t_s = 2.7465$$

This value of t at 42 degrees of freedom exceeds that value given by a probability of 0.05 (it also exceeds 0.01, FISHER and YATES, 1970). The observed correlation of +0.39 is therefore significant.

This conclusion may be criticised due to the interpolation used to derive the *E.coli* MPN values. However this is a reasonable way to obtain estimates of the expected *E.coli* MPN. The majority of these values were close to the actual observed *E.coli* MPN which are recorded, where available, with the interpolated figures in Table 19.

With a correlation of only +0.39, *E.coli* does not represent a good index of the presence of enterovirus in stabilisation pond effluents with the methods used. This result is confirmed by inspection of Fig. 10 which shows that while most enterovirus positive samples were in the winter months, and associated with *E.coli* peaks, many enterovirus examinations during these *E.coli* peaks were negative.

The overall conclusion is that while the *E.coli* MPN is a good indicator of the presence of *Salmonella* in stabilisation pond effluents, it is not sufficiently correlated with enterovirus isolations to be acceptable as an index of the latter.

PART H

COMPARISON OF ENTEROVIRUS ESTIMATIONS IN STABILISATION POND AND ACTIVATED SLUDGE EFFLUENTS

PART H

COMPARISON OF ENTEROVIRUS ESTIMATIONS IN STABILISATION POND AND ACTIVATED SLUDGE EFFLUENTS

Estimations of enterovirus and reovirus in metropolitan activated sludge effluent

Grab samples of metropolitan activated sludge effluent were adsorbed onto primary cynomolgus monkey kidney cell cultures. The method used was the same as that employed for both country and metropolitan stabilisation ponds (Part B, "Method of enterovirus isolation").

The results of these examinations and the presumptive enterovirus and reovirus MPN figures are shown in Table 21. The MPN values were again derived from Table 5.

Four positive enterovirus samples were found in 23 isolation attempts i.e. 17% of the examinations yielded enterovirus, suggesting that activated sludge removed enterovirus more efficiently than metropolitan stabilisation pond treatment. The latter yielded 11 out of 44, or 25% positive samples.

TABLE 21. Estimations of enterovirus and reovirus in metropolitan activated sludge effluent.

Date	Enterovirus MPN per litre	Reovirus MPN per litre	Date	Enterovirus MPN per litre	Reovirus MPN per litre
1969					
May 28	1	7	Mar.10	0	9
June 10	0	>23	26	5	0
24	0	>23	Apr.30	0	0
July 15	0	>23	May 12	0	>23
29*	0	>23	June 9	cell cultures contaminated	
Aug. 12	0	>23	30	0	0
Sept.12	0	>23	Aug.26	0	0
23	1	23	Oct.13	>23	0
Oct. 14	0	0	Nov.24	0	0
28	0	0	Dec.15	cell cultures contaminated	
Nov. 25	0	16	1971		
Dec. 9	0	>23	Feb.10	0	0
1970			Mar. 9	0	0
Feb. 10	0	0			

*The reovirus giving the CPE in Fig. 7 was isolated in MD bottle number 6 from this examination.

Estimations of enterovirus in country stabilisation pond effluent

Effluent samples from country stabilisation ponds were examined in primary cynomolgus monkey kidney cell cultures as described in Part B, "Method of enterovirus isolation".

In cases where the volume examined was not 1.0 litre, the MPN was not changed significantly if each of the ten bottles was adsorbed with 80 ml instead of 100 ml of effluent. The MPN values were again derived from Table 5.

The results of these examinations are set out below in Table 22. There were 9 examinations made of which 5, or 55% were positive. This was a higher yield of positives than from either type of metropolitan treatment.

TABLE 22. Enterovirus estimations from country stabilisation ponds.

Location	Date	Volume of Effluent	Enterovirus MPN per litre	Reovirus MPN per litre
Angaston	25/1/68	0.8 litre	0	not examined
	26/7/70	1.0 litre	16	"
Myponga	25/1/68	0.8 litre	0	"
	26/2/69	1.0 litre	0	"
Pinnaroo (Railway Pond)	22/2/68	1.0 litre	2	"
Pinnaroo (No.2 Pond)	22/2/68	0.9 litre	4	"
Whyalla	2/12/68	1.0 litre	0	"
Berri South	11/2/69	1.0 litre	>23*	"
Barmera	11/2/69	1.0 litre	>23	"

*See Table 12 for enterovirus types isolated.

Relation between type of sewage treatment and enterovirus isolation from effluents

Of the three different classes of sewage effluents that were examined for the presence of enteroviruses, namely metropolitan stabilisation ponds, country stabilisation ponds, and the effluent of a metropolitan activated sludge plant, there was a higher percentage of positive samples from the smaller country plants (25% positive samples from the metropolitan stabilisation pond, 55% from country stabilisation ponds and 17% from the activated sludge plant effluent). It is unlikely that the populations of country towns excreted a greater number of enteroviruses per person than those of the metropolitan areas.

Berri and Barmera lagoons were 'common effluent' systems with primary treatment in the household septic tank. Pinnaroo had a similar common effluent scheme with 2 separate lagoons. Angaston and Myponga were plants which combined Imhoff tanks, which have the same effect as a septic tank, followed by stabilisation ponds. Whyalla had a series of anaerobic and aerobic stabilisation ponds. Other stabilisation ponds mentioned throughout this thesis were aerobic.

However treatment was not as well controlled and virus removal

probably less effective in the country plants than in the large metropolitan treatment works with close process control. Flows in the small plants were subject to wide variation because the pumping of sewage liquors through the plant was not usually continuous. These surge effects could lead to less efficient treatment with increased enterovirus isolations. In addition, dilution of country sewage with trade wastes was negligible.

PART I

DISCUSSION

PART I

DISCUSSION

Introduction

Work for this thesis was begun in 1968 when the isolation of enteric viruses from waters such as sewage and effluents was not routine. Very little guidance was available in the literature, and Australian work in this area was apparently unpublished until STAMP and FERRIS (1970) laid out am empirical procedure for a "standard test" for enteric virus in effluents. Basic experimental work justifying their method was not done.

A method of enteric virus isolation for use with local effluents had to be developed, and this was supported by a number of preliminary experiments (Part E, "Isolation methods for enteroviruses in water, sewage and stabilisation pond effluent").

Australian and overseas workers have concentrated on the isolation of enteric viruses, and few serious attempts have been made to relate these virus isolations to indicator organisms such as *E.coli*.

No study comparable with the work presented in this thesis appears to have been published. In the present work a correlation coefficient was calculated between *E.coli* and *Salmonella* (Table 18). Similar methods were used to calculate a correlation coefficient between *E.coli* and enteroviruses (Table 20). Previous workers relied on mean numbers and isolation ratios of dubious significance and adequate statistics were not used.

This thesis confirmed the validity of the use of *E.coli* as an indicator for *Salmonella* in stabilisation pond effluent under South Australian conditions, but showed that *E.coli* was not suitable as an indicator of the presence of enteroviruses in that effluent. This observation is important because present microbiological standards applied to water are based largely on *E.coli* and the coliforms (World Health Organisation 1971; American Public Health Association 1971; Anon.,1959). Further efforts to find a better indicator for the presence of viruses are essential.

Development of reliable methods to concentrate viruses from waters with low virus numbers such as chlorinated effluents and public water supplies is necessary to allow evaluation of the relationship between enteric viruses and various indicators.

E.coli may prove to be an unsuitable index for enteroviruses in these waters as well as in stabilisation pond effluents.

The *E.coli* index

SMITH in 1895 introduced the examination of drinking water for *Bacillus coli communis*, which later would be termed "bacteria of the coli-aerogenes group of faecal origin", in place of a direct examination for pathogenic enteric bacteria, which presented formidable difficulties at that time.

Reliable methods for the detection of enteric pathogens such as *Salmonella* and some enteric viruses in waters are presently available, but a properly devised test for indicator organisms retains its importance for the following reasons:

(1) the agent of infectious hepatitis cannot be detected by any other means than inoculation of human volunteers,

(2) even when enteric pathogens are not isolated in a particular water sample, the failure to detect them is of small significance when compared with the repeatedly established absence, or limited presence, of an indicator organism from a succession of samples, virtually eliminating the risk of a dangerously contaminated water. These comments (DRION and MOSSEL, 1972) referred to food processing, but are equally applicable to water.

GELDREICH and CLARKE (1971) concluded that while the absence of coliforms in a treated water indicated a bacteriologically safe water, it was yet to be determined whether coliforms were acceptable indicators for viruses. The validity of the coliform examination as an indicator of the presence of viruses has not been adequately examined, but as BERG (1972) remarked, "reflections continue".

Current English and American methods for the examination of water for *E.coli* (Anon., 1969; American Public Health Association, 1971) as well as the World Health Organisation (1971) standard, do not include the direct 44°C multiple tube method for the estimation of *E.coli*.

The direct examination has the advantage that it is completed within 48 hours, compared with 3 days for the confirmed 37°C test.

The results (Table 6) of a comparison between the direct 44°C MacConkey multiple tube method for estimating the MPN of *E.coli* (Anon., 1957) and the confirmed 37°C examination in Gray's medium (Anon., 1969) showed that there was no significant difference at the 0.05 level in the MPN values obtained in Bolivar stabilisation pond effluent (Part C, "Comparison of direct and confirmed *E.coli* Most Probable Number (MPN)").

The conclusion that there is no significant difference between the MPN values derived at 37°C and 44°C was not shared by the Metropolitan Water Board, London (1939), but their treatment of the difference between the methods was based on total numbers of positive tubes, and statistics were not used to analyse the data presented.

CLEGG (1941) used the binomial distribution in a comparison between 353 pairs of *E.coli* counts at the two temperatures. His use of the binomial, a non-parametric technique, to show a significant difference could not be re-examined by the paired t test because the results of Clegg's paired examinations were not published. The binomial is not as rigorous as the paired t test because the extent of the difference between the members of each pair is not taken into account. Another source (Anon., 1957) referred to the work of ALLEN, PASLEY and PIERCE (1952) as justification for abandoning the direct 44°C test. However, the latter used MacConkey agar for roll tube counts, and did not compare the performance of liquid media at the two temperatures.

The 10 fold dilution MPN has been used for *E.coli* estimation throughout this project because it is widely accepted, and gives the order of magnitude of the numbers of the organism present, allowing comparisons to be made with MPN determinations of *Salmonella* and enteroviruses.

E.coli in Bolivar effluent

Results of MPN estimations of *E.coli* in Bolivar stabilisation pond effluent over a 4 year period (Table 7) were subjected to analysis of variance (Table 8) which showed significant differences at the 0.05 level between the *E.coli* results accumulated for each month over the four years examined. A significant difference was also demonstrated between years in the 4 years examined (Table 9).

The smoothed *E.coli* curve, which may be inspected in both Figures 9 and 10, showed seasonal fluctuations, the stabilisation pond effluent giving lower MPN estimations during the summer months. The variation between months was largely due to these seasonal fluctuations. However the difference between years was probably due to an increase of 56% in stabilisation pond area in April 1970. Following this increase, the winter *E.coli* peak (Figs. 9 and 10) of 1970 was truncated.

Preliminary virus isolation experiments

CLIVER (1965) showed that adsorption of poliovirus 1 and Coxsackie B2 to membrane filters could be minimised by the incorporation of serum in the virus suspension, or by pretreating the membrane with serum or a gelatin solution.

Any loss of virus during removal of contaminants by membrane filtration (Part B, "Preparation of effluent samples for entero-virus isolations") is, as pointed out by STAMP and FERRIS (1970), important. These authors concluded that filtration through a Millipore 0.45 micrometre pore size membrane was the most suitable method for the removal of contaminants before the inoculation of cell cultures, but they did not investigate the possibility of virus loss on filtration.

It was shown in a preliminary experiment (Table 14) that the addition of 2%(v/v) foetal calf serum prevented loss of Sabin poliovirus 1 during membrane filtration of a distilled water suspension through Sartorius 0.45 micrometre filters.

Stabilisation pond effluent was also seeded with Sabin poliovirus 1, and treated with penicillin, streptomycin and amphotericin B. The antibiotic concentrations were the same as in EM199 (Table 32). After centrifugation at 18,000 rpm (40,000g) for 30 minutes at $4^{o}C$ to remove contaminants, the recovery of plaque forming units was only about 10% of the positive control (Table 15). This loss was probably due to adsorption of the poliovirus to solids which were then removed by centrifugation. The presence of 2%(v/v) foetal

calf serum on filtration, gave a recovery of 16 PFU/ml compared with 3 PFU/ml on filtration without serum (Table 15). The serum apparently prevented some loss of virus on filtration, but recoveries were poor compared with the positive control of 162 PFU/ml.

This suggests that the enterovirus MPN values obtained in this thesis could be underestimates of the actual enterovirus present. However as all enterovirus estimations were made in the same way, comparisons between virus isolations from various effluents and the indicator organism *E.coli* were valid.

Similar loss of poliovirus in seeded stabilisation pond liquor was described by SOBSEY and COOPER (1973) following centrifugation at 1000g for 5 minutes to remove solids. It was stated that although they found up to about half of the total virus sedimented with the solids, the association of virus with the solids was reversible. Further work on obtaining maximum recoveries of virus is required.

Virus concentration

HILL, AKIN and BENTON (1971) in their review of methods of virus detection in waters, described various concentration techniques such as adsorption to membrane filters, inorganic flocs

such as aluminium hydroxide, iron oxide, or polyelectrolytes; aqueous polymer two-phase separation, soluble alginate filtration, ultra filtration, ultra centrifugation, electrophoresis, polyethylene glycol hydroextraction and gauze sampling. Freeze concentration (RUBENSTEIN et al., 1973) has also been used.

In the present work, one experiment was carried out by the aluminium hydroxide flocculation method. Aluminium hydroxide prepared as described by WALLIS and MELNICK (1967) was used to flocculate screened sewage from which contaminants had been removed by filtration through serum treated membrane filters (MOORE, LUDOVICI and JETER, 1970). The number of PFU in the sewage was estimated at 88 per 100 ml (Part E, "Enumeration of plaque forming units from metropolitan sewage by flocculation with aluminium hydroxide" : Fig. 3).

All other enteric virus isolations were made by directly inoculating volumes of filtered effluent in the cell culture medium and allowing viral adsorption onto a monolayer of primary monkey kidney cells (PMK) in a similar way to that suggested by BERG, BERMAN, CHANG and CLARKE (1966).

Correlation of *E.coli* and *Salmonella*

GELDREICH (1972) said that bacterial indicators of pollution "must not only detect the occurrence and magnitude of fecal contamination, but also exhibit survival patterns in water which closely parallel the expected persistence of some co-existing pathogen". The usually accepted indicator of faecal pollution is *E.coli* or "fecal coliforms".

The term "fecal coliform" has been applied largely by American authors to bacteria which produce acid and gas from lactose within 48 hours at 35°C and then produce gas from lactose within 24 hours after subculture at 44.5°C. The media and method for this MPN procedure were described by the American Public Health Association (1971), although there have been many modifications in the literature. A direct, unconfirmed membrane filter colony count method was also laid down.

Different methods make comparisons between authors difficult but in this thesis "fecal coliform" has been regarded as equivalent to *E.coli*.

These organisms have been used as indicators of faecal contamination in a variety of situations. For example VAN DONSEL and

GELDREICH (1971) found that *Salmonella* were isolated from 19% of bottom mud samples in stream and lake sediments when the fecal coliform density in the overlying waters was between 1 and 200 organisms per 100 ml, 50% between 201 and 2,000 and from 90% of sediments when the waters contained greater than 2,000 fecal coliforms per 100 ml. The evidence for fecal coliforms being a good indicator for *Salmonella* is therefore based in this instance on association, and not directly on survival characteristics. SMITH and TWEDT (1971) found the geometric mean ratios of *Salmonella* to fecal coliforms were 1:2737 in the Sabine River and 1:300 in the Huron River. These ratios were also based on isolations. COETZEE and FOURIE (1965) showed that in a stabilisation pond, *S.typhi* survived better than *E.coli*, and concluded that *E.coli* was not the infallible indicator organism often suggested. GALLAGHER and SPINO (1968) reviewed a number of studies which showed little correlation of the levels of fecal and total coliforms with *Salmonella* isolations in streams throughout U.S.A., and presented data from laboratory model experiments which showed greater persistence of *Salmonella* than fecal coliforms. The authors concluded that while the fecal coliform examination was valuable, low levels of this indicator did not guarantee bacteriological safety. Enteritis due to *Salmonella montevideo* was traced by SELIGMANN and REITLER (1965) to surface

drinking waters, which although low in *E.coli*, contained both *Salmonella* and *Arizona* organisms, apparently derived from dried faecal material. It was assumed that the pathogens had survived drying better than the *E.coli*.

GELDREICH's (1972) definition of the ideal indicator does not therefore always apply to *E.coli*, and if this organism is to be used as an indicator of the bacteriological safety of a water, it must show an association with bacterial enteric pathogens in that particular water.

E.coli and *Salmonella* isolations from stabilisation pond effluent at Bolivar (Table 16) showed a significant Spearman rank correlation of +0.75 (Table 18) between these organisms.

E.coli was therefore found to be an adequate indicator of the presence of *Salmonella* in stabilisation pond effluent.

An attempt to estimate from a linear regression line (Fig. 8) an *E.coli* MPN above which the majority of *Salmonella* isolations would lie was not successful. The regression equation gave a *Salmonella* MPN of 4 when the *E.coli* MPN was zero. A similar regression equation of *Salmonella* serotypes isolated against *E.coli*

MPN also failed as a predictive model. The equation gave one *Salmonella* serotype when the *E.coli* MPN was zero.

The *E.coli* MPN gave a significant correlation of +0.62 with the number of *Salmonella* serotypes isolated from metropolitan stabilisation pond effluent. The correlation could be calculated in a ranked form, but inspection of the data (Table 11) shows that this was not warranted.

Some selection for particular serotypes was inherent in using only one medium, Rappaport, for enrichment (RAPPAPORT, KONFORTI and NAVON, 1956). The limited number of colonies (2) selected from each primary BGA plate probably resulted in underestimation of the number of serotypes present.

Other possible factors affecting numbers isolated could include periodic variation of serotypes present in sewage, and any differences in survival between these serotypes in stabilisation ponds.

E.coli was said to be "undoubtedly of faecal origin" (Anon., 1969) but caution should be used when applying *E.coli* as an indicator organism of faecal origin in some situations. For example, significant multiplication of *E.coli* in trade waste (pea haulm) challenged the specificity of the *E.coli* test for faecal pollution

of animal origin (ROBERTSON, CROLL, JAMES and GAY, 1966). Similar growth of *E.coli* was shown in piles of weed, commonly *Cladophora* (Metropolitan Water Board, 1971) in England. Fermentation of heaps of this alga left on sand filter bands used for the filtration of public water supplies resulted in the detection of *E.coli* in the filtered water. Laboratory experiments showed that *E.coli* could multiply rapidly in the liquid expressed from the fermenting weeds. Attention should be drawn to the risk of multiplication of faecal borne pathogens such as *Salmonella* under similar circumstances.

In spite of these documented instances of environments in which *E.coli* can multiply even with the competition of other organisms, *E.coli* still represents the best available warning of human or animal faecal pollution and therefore of potential dangers from enteric pathogens.

Correlation of *E.coli* and enterovirus

The Spearman rank correlation (Table 20) of +0.39 between enterovirus and *E.coli* showed that *E.coli* was not suitable as an indicator of the presence of enteroviruses in stabilisation pond effluent.

Out of 44 one litre samples examined, 11 yielded enterovirus isolates. The estimated number present in the positive samples ranged from 2 to greater than 23 per litre (Table 16), much lower than figures presented by other workers. For example KOTT (1973) stated that in more than 20 samples from stabilisation pond effluents in Israel, enterovirus numbers ranged from 30 to 290 per 100 ml. However the "fecal coliform" numbers ranged from 490,000 to 1,300,000 per 100 ml. Inspection of Table 8 shows that Bolivar effluent was of higher quality, having a range of 25 to 350,000 *E.coli* per 100 ml during the 4 years examined.

STAMP and FERRIS (1970) used a direct "effluent medium" isolation method similar to that used in this thesis, and gave MPN estimates of up to 600 viruses per 100 ml in Braeside (Victoria) stabilisation pond effluent. However these figures included reo and adenoviruses as well as the enteroviruses, and *E.coli* numbers were not presented, making comparison with Bolivar impossible. These authors did not attempt to correlate virus isolations with any indicator organism, although the effect of chlorination on viruses in effluent was investigated.

Reovirus type 1 has occasionally been found as a latent infection of PMK cells (FENNER and WHITE, 1970). Estimates of reovirus

MPN are set out in Tables 16 and 21, but have not been used because of this uncertainty, although evidence of reovirus infection was not found in passaged PMK negative control cultures.

Comparison of stabilisation pond and activated sludge treatment

BERG (1971) stated that viruses were removed from sewage by activated sludge treatment more efficiently than by any other means of biological treatment. Virus removal in stabilisation ponds was said to be "highly variable and responsive to factors that are as yet not well defined".

Enterovirus isolations from South Australian effluents in the present study support these conclusions. Enteroviruses were found in 11 out of 44 samples of Bolivar stabilisation pond effluent, 5 out of 9 from country stabilisation ponds and 4 out of 23 activated sludge effluent samples (Glenelg Treatment Works).

Stabilisation ponds have become a widely accepted method of sewage treatment, particularly in country areas where land is readily available and cheap. However the possibility of arbovirus transmission by arthropods resident in these ponds should not be forgotten. Activated sludge plants would not give arthropods similar opportunity for breeding.

The arboviruses are maintained in a continuous cycle through transmission by an arthropod vector to a vertebrate, producing a viraemia which is infectious to another arthropod taking a blood meal. Murray Valley encephalitis (MVE) is an example of a mosquito borne arbovirus apparently maintained in birds. Mosquitoes transmit the virus to man after feeding from a viraemic bird.

ANDERSON and EAGLE (1953) postulated that the southerly spread of MVE from Queensland could follow increases in the area of natural waters in the Murray Valley, allowing multiplication of the suspected vector, *Culex annulirostris*.

It has been suggested (McANALTY, 1964) that stabilisation ponds, particularly in tropical areas, could provide a source of mosquitoes capable of transmitting arboviruses. The incidence of recognised human MVE and other arbovirus infections in South Australia is low, but could be affected by the presence of mosquitoes from stabilisation ponds.

Mosquito transmission of the enterovirus coxsackie A6 has been demonstrated from viraemic to uninfected mice if the mosquitoes had a second blood meal from the uninfected animal within 13 days of the initial feeding (MAGUIRE, 1970). Reovirus 3 (FENNER and WHITE, 1970)

has been isolated from mosquitoes, although the carriage of this virus was also said to be purely mechanical.

The possibility of transfer of enteric virus infection by arthropods to humans appears to have been ignored, but the viral status of South Australian mosquitoes should be given more attention.

PART J

APPENDIX

TABLE 23 Handling of cell cultures.

Cell	Type of Culture	Origin	Abbre-viation	Type of cell	Seeding conc. cells/ml	Base medium	MEDIA Growth Serum v/v	Growth 2.8%(w/v) $NaHCO_3$ v/v	Maintenance Serum v/v	Maintenance 2.8%(w/v) $NaHCO_3$ v/v	Handling Schedule	Days to reach confluence
Cynomolgus monkey kidney	Primary	*Macaca irus* kidney	PMK	largely epithelial	10^5/ml	199	2% foetal calf	3%	½% foetal calf	6%	Medium changed at 3-4 days	7-10
Human amnion	Primary	Human placenta	PHA	"	$0.5x10^6$/ml	"	15% bovine	"	2% bovine	7%	Medium changed 48 hours after seeding	5-10
Human foetal tongue	Semi-continuous strain	Human foetus	HFT	fibroblast	10^5/ml	Eagles Basal Medium (BME)	10% foetal calf	2%	2% foetal	3%	Subculture 1:2* every 3-4 days	3-4
H.Ep.-2	Continuous line	Human carcinoma larynx	H.Ep.-2	epithelial	10^5/ml	199	20% bovine	6%	5% bovine	8%	Medium changed 2 x per week Subculture 1:5	7**
BS-C-1 African green monkey kidney	Continuous line	*Cercepithecus aethiops* kidney	BS-C-1	fibroblast	10^5/ml	199	20% foetal*** calf	3%	2% foetal calf	4-6%	Medium changed 3 x per week Subculture 1:2	5-7

* Cells should be kept in log. phase

** Changed to maintenance at 95% confluence to avoid sloughing of monolayer

*** Foetal calf serum should be tested for suitability. Some batches are toxic for this cell, while suitable for others

TABLE 24.

Preparation of $Al(OH)_3$ floc (WALLIS and MELNICK, 1967)

Three ml of 2M Na_2CO_3 were added to 100 ml of 25 mM $AlCl_3$. The mixture was stirred both during and for 15 minutes after preparation. After centrifugation at 2,000 rpm for 15 minutes, the centrifugate was re-suspended in 0.15 M NaCl and re-centrifuged. The sediment was washed again with saline by centrifugation, then re-suspended in saline and autoclaved at 121°C for 15 minutes. The cooled centrifuged sediment of $Al(OH)_3$ was re-suspended in 110 ml of saline and stored at 4°C.

Preparation of $Al(OH)_3$ for flocculation

Immediately before use, the $Al(OH)_3$ floc was centrifuged at 2,000 rpm for 15 minutes, and 0.25 ml of the packed precipitate re-suspended in 5.0 ml of pH 6.0 buffer (described below). This was sufficient to flocculate one litre of sample as described in Part B, "Flocculation and plaque assay of enterovirus with aluminium hydroxide".

Preparation of 0.1 M pH 6.0 phosphate buffer

Sterile phosphate buffer of pH 6.0 was prepared by combining one litre of a 0.1 M solution of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$, 15.601 gm/1) with 232 ml of a 0.1 M solution of disodium hydrogen orthophosphate ($Na_2H\ PO_4$, 14.196 gm/1) and autoclaving in 100 ml volumes.

The pH was checked with a Beckman pH meter.

TABLE 25.

Dulbecco phosphate buffered saline (PBS), calcium magnesium free 10 times concentrate

NaCl	80 gm
KCl	2 gm
KH_2PO_4	2 gm
Na_2HPO_4	11.5 gm
H_2O	to 1 litre

Autoclave at 15 lb for 15 minutes

Dulbecco phosphate buffered saline, calcium magnesium free (single strength)

10X PBS calcium magnesium free	100 ml
Penicillin Streptomycin (P-S) (contained 10^5 units and 10^5 micrograms per ml respectively)	1 ml
H_2O (sterile)	to 1 litre

TABLE 26.

T5L overlay

Two times concentrated overlay		Sterilisation
Sterile glass distilled water	58.3 ml	autoclave
Earle's BSS 10X A	16.0 ml	autoclave
Earle's BSS 10X B	4.0 ml	autoclave
Tris/HCl buffer 2.0M pH 7.3	2.0 ml	autoclave
Yeast extract 10% (Oxoid)	2.0 ml	filter
Magnesium chloride 2.0M	2.5 ml	autoclave
Calf serum, inhibitor free, 50% in PBS	8.0 ml	purchased sterile
P-S	0.2 ml	purchased sterile
Sodium bicarbonate 2.8% without phenol red	10.0 ml	filter
1/1000 neutral red in double distilled water	5.0 ml	autoclave
	108.0 ml	

(2M Tris/HCl buffer prepared by adding concentrated HCl to solution of tris (hydroxy methyl)-amino methane (Sigma) to pH 7.3, and making up to desired volume with distilled water).

Two times concentrated agar

Ionagar 2% (Oxoid) was made up in double distilled water and autoclaved at 15 lb for 15 minutes.

Preparation of T5L overlay

The two times T5L was warmed to 45°C, saturated with carbon dioxide using a sterile pipette and mixed with an equal volume of melted 2% sterile Ionagar, also at 45°C. Ten ml volumes were then overlaid on the petri dish cell cultures.

TABLE 27.

Earle's (1943) Balanced Salt Solution (tenfold concentration) as used in T5L medium.

Solution A	NaCl	68.0 gm
	KCl	4.0 gm
	$CaCl_2$	2.0 gm
	$MgSO_4.7H_2O$	2.0 gm
	(or $MgCl_2.6H_2O$ = 1.7 gm)	
	Distilled water	to 800 ml
Solution B	$NaH_2PO_4.H_2O$	1.4 gm
	(or $NaH_2PO_4.2H_2O$ = 1.6 gm)	
	Glucose	10.0 gm
	Distilled water	to 200 ml

Solutions A and B were dispensed in 160 and 40 ml volumes respectively, and sterilised by autoclaving at 121°C for 15 minutes.

TABLE 28.

Agglutination Agar

Yeast extract	3 gm
Soya peptone	5 gm
Tryptone	15 gm
Sodium chloride	5 gm
Agar Oxoid No. 3	15 gm
Distilled water	1 litre

pH = 7.3 before adding agar.
The medium was sloped in approximately 10 ml volumes in 1 oz. narrow mouth McCartney bottles.

TABLE 29.

Trypsin versene

Versene 10 times concentrate

NaCl		80 gm
KCl		2 gm
KH_2PO_4		2 gm
Na_2HPO_4		11.5 gm
Versene, disodium salt		2 gm
NaOH		0.67 gm
H_2O	to	1 litre

Sterilised by autoclaving, 15 lb for 15 minutes.

Trypsin versene (single strength)

10X Versene		100 ml
12.5% trypsin (w/v)		8 ml
P-S		1 ml
H_2O	to	1 litre

TABLE 30.

May Grunwald Giemsa Stain*

Materials

Stock May-Grunwald stain: 2.5 gm of BDH May-Grunwald stain was dissolved in absolute methanol and made up to 1 litre. The solution was aged one month and filtered (Whatman No. 1) before use.

Stock Giemsa: 1.0 gm of BDH Giemsa stain was dissolved in 66 ml of glycerol at 55-60°C for 1.5 - 2.0 hours, before 66 ml of absolute methanol was added.

Procedures

Coverslip cultures were -

1. Washed three times in warm PBS.
2. Fixed by shaking with absolute methanol for 5 minutes.
3. Stained for 10 minutes in filtered stock May-Grunwald solution.
4. Stained for 20 minutes in dilute Giemsa solution (diluted 1:15 in distilled water just before use).
5. Rinsed for 10-20 seconds in distilled water.
6. Quickly rinsed in 2 changes of acetone to dehydrate the coverslip culture, care being taken not to let the coverslip dry.
7. Cleared by rinsing 3 times in acetone-xylol (2:1), 3 times in acetone-xylol (1:2), and 10 minutes in fresh xylol.
8. Mounted on a slide with neutral Canada balsam in xylol.

*MERCHANT, KAHN and MURPHY (1964)

TABLE 31.

Sources of apparatus, glassware, cells and media

Air, Medical (for use in CO_2/air incubator)

Commonwealth Industrial Gases,
90 Jervois St.,
Torrensville, S.A. 5031.

Antibiotics

Amphotericin B	Squibb
Kanamycin	Sigma
Penicillin G	CSL
Streptomycin sulphate	Glaxo

Carbon dioxide/air incubator

Scientific Equipment Manufacturers,
2 Uren St.,
Magill, S.A. 5072.
(Hampton, Thayer and Howes, 1969)

Carbon dioxide, premium grade (for use in CO_2/air incubator)

Carba Aust. Ltd.
98 Jervois St.,
Torrensville, S.A. 5031.

Coverslips, Assistent No. 1

DHA - Anax Pty. Ltd.
279 Rundle Street,
Adelaide, S.A. 5000.

Cell cultures

Primary cynomolgus monkey	kidney
Human foetal tongue (HFT)	semi-continuous strain
H.Ep.-2	continuous cell line
BS-C-1	continuous cell line

Commonwealth Serum Laboratories,
45 Poplar Avenue,
Parkville, Victoria, 3052

TABLE 31 (contd.)

Culture tubes and bottles

Corning screw cap pyrex culture tubes 16 x 150 mm
Cat. No. 9825
Corning screw cap pyrex leighton tubes 16 x 150 mm
Cat. No. 9831
Corning narrow mouth dilution bottles 160 ml cap.
Cat. No. 1367
Corning wide mouth dilution bottles 160 ml cap.
Cat. No. 1368

"Haemosol" detergent

H.B. Selby Pty. Ltd.
Adelaide, S.A. 5000.

Media

Medium 199, Eagles Basal Medium (BME),
bovine and foetal calf serum (FCS)

Commonwealth Serum Laboratories,
45 Poplar Avenue,
Parkville, Victoria, 3052.

Membrane filters

Sartorius 0.45 micrometre Cat. No. 11406

Oliphant Pty. Ltd.,
11 Shepley Avenue,
Panorama, S.A. 5041.

Millipore 0.22 micrometre GS.

H.B. Selby Pty. Ltd.,
Adelaide, S.A. 5000.

Membrane filter apparatus

Millipore all glass membrane filter apparatus
Cat. No. XX 15 047 00

H.B. Selby Pty. Ltd.,
Adelaide, S.A. 5000.

TABLE 31 (contd.)

"Paramount" stainless steel food container

F.L. Rungie & Sons,
Adelaide, S.A. 5000.

Petri dishes

Camelec 90 mm sterile disposable plastic suitable for cell culture, Cat. No. PDS 350(H).

Camelec Medical Plastics Division,
60 Patricia Avenue,
Camden Park, S.A. 5038.

Stationary racks

(designed to hold 16 x 150 mm tube cell cultures at an angle of 5-7°)

Paton Industries Ltd.,
35 Henry St.,
Stepney, 5069.

Roll tube apparatus

Scientific Equipment Manufacturers,
2 Uren St.,
Magill, S.A. 5072.

Vials, glass (plastic autoclavable cap)

Johnson and Jorgenson
Cat. No. 5/H/9001

DHA - Anax Pty. Ltd.
279 Rundle Street,
Adelaide, 5000.

TABLE 32

Media preparation

Medium 199

for cell culture (suitable for cell growth and maintenance)

Medium 199 10x concentrate	10	ml
Solution D-G-P	0.1	ml
Sodium Bicarbonate (2.8%w/v)	3	ml
P-S	0.1	ml
Distilled water (double glass) to	100	ml
	100	ml

Eagle's (Basal) Medium (BME)

for diploid semi-continuous strains

Eagle's (Basal) Medium 10x concentrate	10	ml
Glutamine solution (1.46%) for BME	2	ml
P-S	0.1	ml
Sodium Bicarbonate 2.8%(w/v)	as required	
Distilled water (double glass) to	100	ml
	100	ml

Effluent Medium 199 (EM 199)

for enteric virus isolations

Medium 199 10x concentrate	10	ml
Solution D-G-P	0.1	ml
Sodium Bicarbonate (2.8%w/v)	3	ml
Penicillin G	100,000	units
Streptomycin sulphate	100,000	micrograms
Membrane filtered effluent (MFE)	100	ml
	113	ml

Serum and bicarbonate levels for various cell cultures are given in Table 23.

PART K

BIBLIOGRAPHY

BIBLIOGRAPHY

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PART L

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ABBREVIATIONS

Abbreviation	Meaning	First used on page
BGA	Brilliant Green Agar	26
BME	Eagles Basal Medium	15
BSS	Balanced Salt Solution	15
CPE	Cytopathic Effect	11
CSL	Commonwealth Serum Laboratories	14
DNA	Deoxyribose Nucleic Acid	2
EM 199	Effluent Medium 199	31
HFT	Human Foetal Tongue	13
MD bottle	Milk Dilution bottle	14
MFE	Membrane Filtered Effluent	30
MPN	Most Probable Number	24
MVE	Murray Valley Encephalitis	138
PA	Plaque Assay	34
PBS	Phosphate Buffered Saline	17
PFU	Plaque Forming Unit	35
PHA	Primary Human Amnion	14
PMK	Primary Monkey Kidney	7
P-S	Penicillin-Streptomycin	142
RNA	Ribose Nucleic Acid	2

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