IDENTIFICATION OF ENTERIC VIRUSES AND SALMONELLAE FROM SEWAGE

By

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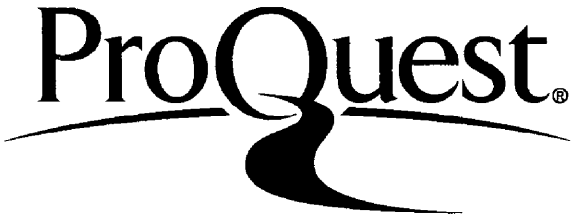

ABSTRACT

The utilization of improved tissue culture techniques has led to the finding of many viral agents in the excreta of man and animal. A new group of viruses, enteric cytopathogenic human orphan (Echo), has been isolated in tissue culture, along with the polio and some of the Coxsackie viruses. Some of the Echo viruses have been incriminated as the etiological agents for a number of diseases, including aseptic meningitis, summer diarrhea of infants and mild paralysis. The ease of detecting these enteric viruses in sewage would greatly aid in epidemiological investigations.

Along with the viruses, salmonellae are also present in sewage. New techniques, such as the gauze pad sample and new media, offer the prospect of better evaluation of these organisms in sewage.

When it was determined that tetrathionate enrichment broth was not ideally suited for the isolation of salmonellae from sewage (32 of 142 samples positive), selenite brilliant green enrichment medium was used. The isolations of salmonellae increased dramatically (62 of 92 samples positive) with the employment of SBG medium.

A total of 1018 sewage samples were tested in monkey kidney tissue culture and/or suckling mice to detect enteric viruses. Viral agents were isolated on 186 occasions. Thirty-five of the isolates were identified as Echo or polioviruses, 76 were identified as Coxsackie viruses, 36 samples were positive both in tissue culture and in suckling mice, an additional 17 viruses were not identified, and 22 tissue culture cytopathogenic agents were lost in passage or storage. Echo 1 and/or 13 was identified on 10 occasions and was the Echo

virus most often isolated.

Samples were collected from 7 plant locations to determine the effect of sewage treatment on the viability of viruses and salmonellae. Enteric viruses and salmonellae were found in all stages of sewage treatment tested, including final effluent.

Sewage samples provide a source of material from which the enteric viral flora of a population may be determined. In addition, the prevalence of salmonellae in a community may be estimated by the testing of appropriate sewage samples.

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INTRODUCTION

The isolation of viral agents has become more commonplace since the refinement of tissue culture methods, within the past ten years. Epidemiological studies of the viral flora of selected groups have been made possible through these new techniques.

The purpose of this study was to identify a number of viruses that were isolated from samples collected at two sewage plants. It was necessary to limit the study to a particular group of viruses since sewage represents such a heterogeneous mixture of human and animal waste products, that may contain a variety of viral entities. The enteric virus group was selected as the focal point of the study. This group consists of the polioviruses, Coxsackie viruses and the Echo viruses (enteric cytopathogenic human orphan). In addition to the virus identification, attempts were made to isolate salmonellae from similar types of sewage specimens. Sewage can serve as a source for epidemiological information in attempting to determine the presence of disease-producing bacteria and viruses in a community at any given time, since mass rectal swabbing is impractical. Information was also sought on the effect sewage disposal treatment had in removing the viruses and salmonellae present in the sewage prior to discharge from the plant.

REVIEW OF THE LITERATURE

Poliovirus was first isolated from sewage by Paul et al., (1939), although the virus had been isolated from stool samples by Sawyer (1915) and Harmon (1937). A series of three papers followed in which the recovery of poliovirus from stools and from sewage was discussed.

Trask et al., (1940) reported that the ease of detection of the virus was related directly to the non-paralytic type of poliomyelitis and inversely to age. An important contribution made in this paper was that at least one infective monkey dose per gram could be extracted from one stool, indicating that relatively large quantities of virus were present in stool specimens from patients. The method of extracting the virus from the stools was relatively simple. The stools were diluted 5 to 10 times by weight in cold distilled water and after mixing were allowed to settle 2 to 10 hours in the cold. A portion was shaken with 10 per cent ether by volume and was again held overnight in a refrigerator. The supernatant fluid was inoculated into monkeys after centrifugation at 1000 rpm for 25 minutes.

The second paper by Paul et al., (1940) was an extension of the work presented in the first paper. Since they had shown that poliovirus was present in stools of patients, they were interested in detecting the virus in the sewage from the hospital. During two of three large urban epidemics of poliomyelitis the virus was isolated from samples of sewage. To avoid the dilution effect of the sewage system, the samples were collected in the immediate vicinity of the isolation hospitals.

The third paper in the series dealt with methods of detecting the virus in sewage and stools. Gard (1940) described the precipitation of the virus from the specimens with 50 per cent ammonium sulphate followed by dialysing the precipitate. Basically, this method was employed by Melnick (1947) with the addition of an ultracentrifugation procedure in an effort to concentrate further the virus. After this basic knowledge of poliovirus was presented, several new thoughts on the pathogenesis of poliomyelitis were brought forth in the literature. Paul and Trask (1942) placed poliovirus among the intestinal diseases since the virus could be recovered from the feces of a convalescent patient for as long as two or three weeks following a severe or mild attack of the disease. To demonstrate the foresight of these workers a quotation is taken from the paper: "From the epidemiological standpoint, from the engineering standpoint, and from the public health standpoint, the intestinal tract seems like a dangerous place for this virus to be." Further recoveries of the poliovirus from sewage followed, including one from New York City reported by Trask and Paul (1942). The largest single obstacle in the path of these experimental procedures was the absolute dependence upon the monkey as a test animal. The very high cost of using this animal was instrumental in the appearance of reports based on a single positive isolation from relatively few samples. The earlier work of Paul and Trask was later confirmed by Kling et al., (1942) and still later by Melnick (1947). Horstmann and Melnick (1946) also reinforced the findings that poliovirus persisted in the stools after acute infections.

The Coxsackie virus group was first described by Dalldorf (1948) and since the isolation of the virus depended on the utilization of suckling mice rather than the more expensive monkey, attempts were made immediately to isolate this group of viruses from sewage. Melnick et al., (1949) recovered the virus from sewage in six different cities during the summer and fall of 1948. No isolations were reported during the winter months. Clark et al., (1951) recovered the virus from sewage in Canadian cities during a summer season when there was a low incidence of reported poliomyelitis cases. In an attempt to improve the methods of isolation Kelly (1953) described a method which consisted of virus adsorption on, and elution from, ion-exchange resins.

Aside from the difficulty involved in isolating the virus from the sample per se, an additional problem further complicates the procedure. The actual sampling method must be taken into consideration. A dip sample, of approximately 100 ml, normally taken for routine physical, chemical and standard biological tests, is at best a random sample of the contents of the flow through a sewage system and there is a good chance that particular viruses or bacteria are not present in a given sample. To increase the probability of collecting samples containing virus, larger quantities of sewage were collected and processed. Melnick (1947) used samples of two to three gallons. The use of a gauze pad sampling procedure was described by Moore (1948) for the detection of the typhoid bacillus in sewage. A detailed description of this method is presented in the materials and methods section of this thesis. Kelly (1953) employed this sampling

method for monitoring sewage over a 24 to 48 hour period and reported that the number of Coxsackie isolations was increased consistently over the isolations obtained using the dip samples. Melnick et al., (1954) also reported that the gauze pad method of obtaining sewage samples increased the number of Coxsackie isolations considerably over the dip sample method of collection.

Since the gauze pad was suspended in the sewage flow for long periods of time (up to seven days), the viability of the viruses in this environment was of considerable importance. The work of Rhodes et al., (1950) revealed that poliovirus survived, in experimentally contaminated river water, at least 188 days when the stored water was kept at 4 C and the stool diluted 1 to 200. In a second study, (Kelly et al., 1955) the seasonal distribution of Coxsackie virus in the sewage of Albany, New York, area was determined. Coxsackie viruses were present continuously between June and November and only sporadically during the remainder of the year. The effect of sewage plant treatment on the viability of Coxsackie virus was also investigated. Coxsackie viruses were recovered regularly from the effluent as well as from the influent of the plant during the summer and fall, and therefore, sewage does not appear to affect enteric viruses to any great extent.

With the advent of the wide-spread use of roller-tube tissue culture technique as applied to the isolation and identification of polioviruses by Robbins et al., (1952), it became practicable, from an economic standpoint, to conduct extensive clinical and epidemiological investigations of poliomyelitis. Tissue culture techniques

were refined to such an extent that other viruses besides polioviruses were isolated and recognized from fecal material. A number of types of Coxsackie viruses were isolated and identified in the HeLa strain of human cancer cells by Sickels et al., (1955) and Crowell and Syverton (1955) as well as other viruses which are occasionally found in the feces, such as herpes simplex (Weller, 1953), mumps (Henle et al., 1954) and the adenoviruses (Rowe et al., 1957). At times, more than one virus was found in a stool specimen (Kibrick, 1955). With the development of a procedure by Dulbecco and Vogt (1954) for the preparation of cell suspensions of monkey kidney by the use of a trypsinization procedure, an important step in tissue culture methodology was achieved. This technique, further improved by Younger (1954), provided a method for the preparation of a monolayer of cells grown on any glass surface in such containers as Petri dishes, test tubes or bottles held in a stationary position.

Comparative studies by Melnick et al., (1954) demonstrated that epithelial monolayer cultures from monkey kidneys are more sensitive for primary isolation of poliovirus than suspended fragments of kidney tissue or of fibroblast cultures from monkey testis. Wenner and Miller (1954) also reported that for primary isolation of poliovirus, monkey kidney epithelial cells proved superior to testicular cultures and also to HeLa cultures with which they were compared.

Melnick (1955a) has shown that poliovirus can be isolated at about twice the frequency from stools as from rectal swabs. While many individuals yielded positive stools but negative swabs, the reverse situation was a rare occurrence. During tissue culture

investigations using stools from suspected poliomyelitis patients viruses were isolated that were not identifiable as poliovirus or Coxsackie virus by serological or suckling mice virulence tests. Melnick (1955b) tentatively placed them in a group which he called "orphan viruses," since there was so little known about their relationship to disease. These agents have been isolated in the United States and in other countries. In this country, Robbins et al., (1951), Riordan et al., (1952), Kibrick et al., (1953), Melnick et al., (1953) and Steigman et al., (1954) have all isolated a number of these viruses, chiefly from the stools of patients with aseptic meningitis or non-paralytic poliomyelitis. In Egypt they were isolated from the blood, throat and rectal swabs of young children brought to a public clinic with various non-differentiated clinical symptoms (Horstmann, 1955). Melnick and Agren (1952) also isolated an "orphan" virus from the stool of a normal Egyptian child.

A study was undertaken by Ramos-Alvarez and Sabin (1954) to determine if avirulent poliomyelitis viruses occurred in nature. Rectal swabs were taken of 1566 healthy children in Ohio. In addition to five strains of poliomyelitis virus and one strain of Coxsackie virus that were isolated, 25 other enteric viruses were found. Twenty-two of these viruses were unrelated to three serologic types previously isolated by other workers.

It can readily be seen that a problem existed in classifying these "orphan viruses." In an attempt to solve this dilemma, the National Foundation for Infantile Paralysis formed a committee to help resolve this matter of nomenclature. The committee's title was

the "Committee on the ECHO Viruses" (Enteric Cytopathogenic Human Orphan Viruses). The first report of the committee (1955) contained a list of the 13 strains that were designated prototype strains of the Echo group of viruses. These strains did not reveal any high degree of cross-reactions among one another and were considered to be separate and distinct viral entities.

An additional 1771 stool specimens were obtained from healthy children in Mexico by Ramos-Alvarez and Sabin (1956) and 261 of the samples were positive for viruses other than poliovirus and Coxsackie virus. Cross-neutralization tests between the strains isolated from healthy children in Ohio and healthy children in Mexico revealed that Echo 7 was not present in any of the Mexican isolates, while it was present in 32 per cent of the strains isolated in Ohio. Echo 2, with an incidence of 44 per cent in Ohio, constituted only eight per cent of the Mexican strains. Approximately 90 per cent of the Mexican strains were not neutralized by Echo strains 1 to 13.

Gelfand et al., (1957) studied a group of 150 young children in Louisiana during a two-year sampling period in an attempt to determine their normal enteric viral flora over a fairly long period of time. Stool specimens were obtained monthly and viral isolation was accomplished in monkey kidney cell cultures. A marked seasonal effect, with a predominance of positive specimens in the summer and fall months was observed, with parallelism between the polio and non-polio viruses.

Ormsbee and Melnick (1957) classified 30 Echo viruses from 1558 fecal samples taken during a 29-month period, from 136 children of

preschool age in Charleston, West Virginia. Twenty of the 30 were typed Echo 3, 6, 7, 9, 11, 14, or a new type, designated Echo 15. A previous paper by Honig and Melnick (1956) on this same group of children reported that there was a repeatable seasonal incidence noted in the Echo virus isolations, with over 90 per cent isolated from June to October.

The Committee on the Echo viruses, in its second report (1957), stated in part: "recognizing that the poliomyelitis, Coxsackie and ECHO viruses all inhabit the alimentary tract as well as share other properties, has changed the name of its Committee on the ECHO Viruses to the Committee on the Enteroviruses." Six new antigenically distinct members of the Echo group were listed; Echo 14 to 19. Since the publication of the Committe's first report (1955), it was discovered that the Echo 13 prototype strain (Hamphill) actually was a mixture of Echo 1 and Echo 13 strains, or was serologically related to Echo 1.

With the refinement in tissue culture methods Kelly et al., (1957) were able to approach the problem of enteric viruses in sewage on an expanded scale. In addition to the Coxsackie viruses which she and her group searched for in 1955, an attempt was made, using suckling mice and tissue culture, to detect other enteric viruses which might be present in sewage. Three hundred and eight sewage samples were tested for pathogenic agents in newborn mice. Two hundred and eight of them were tested for cytopathogenic agents in HeLa cell cultures which were described by Scherer et al., (1953) and the remaining 100 in monkey kidney epithelium cultures.

Coxsackie viruses were isolated on 119 occasions, polioviruses were isolated 97 times and unknown agents were encountered on five occasions. The authors reported that they did not observe great differences in sensitivity between HeLa cell and monkey kidney cell cultures.

To isolate and classify these Echo viruses was in itself a significant advancement, a task that is continuing. However, until it can be ascertained to what extent these viruses function as disease producing agents, much work remains. The Committee on Enteroviruses (1957) published a summary of available information showing the association of enteroviruses with human disease and it is presented here:

Enteroviruses	Associated Disease
Poliovirus	Paralysis (complete to slight muscle weakness) Aseptic meningitis Undifferentiated febrile illness particularly during the summer
Coxsackie viruses, Group A	Herpangina Undifferentiated febrile illness particularly during the summer Aseptic meningitis (Types A7, A9)
Coxsackie viruses, Group B	Aseptic meningitis Pleurodynia Undifferentiated febrile illness with pharyngitis Myocarditis or encephalomyocarditis during neonatal period and early childhood Mild paralysis (?)
Echo viruses	Aseptic meningitis (Types 2, 3, 4, 5, 6, 14, 16) Summer rash (Types 4, 9, 16) Summer febrile illness Mild paralysis (?) (Type 6) Summer diarrhea of infants and children (Type 18 and others)

According to Rivers (1948), the clinical picture of poliomyelitis is one of a biphasic nature, with both phases not seen in all cases. Phase one may simulate a mild upper respiratory infection, with headache or a nonexudative pharyngitis, or it can be ushered in by what appears to be a simple gastro-intestinal disturbance with nausea and vomiting. The second phase involves a disturbance of the central nervous system (CNS) and may appear immediately following phase one or several days after the remission of phase one symptoms. The symptoms seen at the onset of CNS disturbances are: muscle tenderness and stiffness, rigidity of the neck and back with pain when an attempt is made to place the chin on the chest. Lumbar puncture reveals an abnormal number of granulocytes and lymphocytes in the cerebrospinal fluid (CSF). Later when paralysis is apparent, a mononuclear pleocytosis is nearly always present, ranging from 15 to 200 cells per cu. mm. Increased protein is usually demonstrable in the CSF and persists for some weeks after the cell count has returned to normal. Paralysis usually reaches its maximum extent before the fifth day following its appearance.

The aseptic meningitis syndrome has been described by Kibrick (1957). The most prominent clinical features of this disease complex are an abrupt onset in most of the cases characterized by headache, stiff neck, stiff back and vomiting. Sore throat, abdominal pain and myalgia were reported from a smaller number of patients. The majority of the patients were febrile after admission and the temperature remained elevated from one to four days, ranging from 100 to 102 F. In the CSF the number of cells rarely exceeded 500

per cu. mm. and the levels of protein were normal or slightly elevated. Muscle weakness was consistently of a mild or moderate degree and in most instances tended to disappear with the passage of time.

Karzon (1957) reported a clinical syndrome, resembling aseptic meningitis, occurring in epidemic form in western New York State during the summer of 1955. The features of this disease, from which Echo 6 was isolated from 75 of the 84 hospitalized patients, included an acute onset with severe frontal headache, nausea and vomiting, pains in the abdomen, limbs or chest, and signs of meningeal irritation. Transient muscle weakness, especially of the anterior neck muscles, occurred in some of the cases, but there was no residual paralysis. It was of interest to note that Echo 6 virus was isolated from the CSF from seven of the patients.

Svedmyr (1957) claimed that the etiological role of Echo 6 virus in aseptic meningitis appeared to be quite evident in the material studied during an epidemic in Sweden. In a review on the etiologic role of Coxsackie B and Echo viruses in meningitis, Rhodes and Beale (1957) cite numerous references on the isolation of these viruses from feces and CSF of patients clinically classified as showing the aseptic meningitis syndrome.

Lehan et al., (1957) isolated Echo 4 virus from stools of aseptic meningitis patients during an epidemic in Iowa. A significant rise in titer against Echo 4 virus was reported in the majority of the paired serum specimens collected.

A report, by Nihoul, (1957), incriminated Echo 9 virus as the agent responsible for an outbreak of aseptic meningitis in Belgium.

Echo 9 virus was isolated from 28 CSF samples and 122 stool specimens.

From the previous information it may be concluded that the aseptic meningitis syndrome may be caused by various viral agents that produce a similar clinical appearance.

Group A Coxsackie viruses have been associated with herpangina. Herpangina, is mainly a disease of children occurring in the summer months. It is characterized by fever, pharyngitis and small ulcerative lesions on the base of the tongue and the tonsils (Parrott, 1957).

Group B Coxsackie viruses are recognized as the important etiological agents of pleurodynia or Bornholm disease, which was first clearly described by Sylvest (1932). This condition is characterized by fever, severe attacks of muscle pain, usually affecting the lower thorax and abdomen, and pharyngitis. Lazarus et al., (1952) described an outbreak of pleurodynia in Washington in 1950 from which Coxsackie B virus was isolated and incriminated as the etiological agent.

Enders (1957) has reviewed the findings of a number of workers concerning acute aseptic myocarditis of infants. This disease has been found in young infants and clinical symptoms are those of anorexia, vomiting, cyanosis and dyspnea, together with a tachycardia. In fatal cases, the heart was dilatated and sometimes hypertrophic. No association with pathogenic bacteria could be demonstrated. Coxsackie B virus has been isolated on at least two occasions from the myocardial tissue of infants, diagnosed as acute aseptic myocarditis cases.

A study by Ramos-Alvarez (1957) included stool samples from 100 infants and children, up to the age of four years, coming to a clinic for the treatment of diarrhea. His findings indicated that various types of Echo viruses were associated significantly with the disease. Since these fecal specimens were collected only during the summer months this syndrome has been termed "summer diarrhea."

Since many of the "orphan" viruses have been suspected as the etiological agents for various diseases and doubtless many others will be thought to be the causes of other maladies, a set of guidelines has been offered by Huebner (1957). "In order that a virus be regarded as an established cause of a specific human illness, the following conditions seem to be necessary:

1. Virus as "real" entity.
2. Origin of virus.
3. Antibody response.
4. Characterization and comparison with known agents.
5. Constant association with known illness.
6. Studies with human volunteers.
7. Epidemiologic studies.
8. Prevention by specific vaccination.
9. Financial support."

A review of the literature on the occurrence and survival of salmonellae in sewage was made by Rudolfs et al., (1950). One of the highlights of this review article was Gray's (1929) seven isolations of paratyphoid organisms from 20 samples of Edinburgh, Scotland, sewage. All isolations were from a district which had suffered an outbreak of the disease two years previously. More than a year later, Begbie (1930) reported seven isolations of the organisms from 58 samples of Edinburgh sewage. Bruns and Sierp (1927) noted that Salmonella paratyphoid B was still found in experimentally inoculated

sludge samples even after prolonged aeration. In a search for S. typhi, Hajna (1935) found six samples positive out of 22 tested from five different plants. In the raw sludge, three out of seven samples from two plants showed positive results. Plant effluents showed no positive results from nine samples tested. The presence of salmonellae in irrigation waters was reported by Dunlop et al., (1951). Using tetrathionate enrichment broth followed by streaking on bismuth sulfite agar plates, 23 of 113 samples were positive for salmonellae comprising 13 species. Four of 21 one-ml samples, five of 12 ten-ml samples and 14 of 66 one-hundred-ml samples were also positive for salmonellae. In a later paper Dunlop et al., (1952) compared recoveries of Salmonella from naturally-contaminated irrigation water with tetrathionate broth and with Galton's modification (1950). Galton's modification consisted of the addition of sodium-sulfathiazole to Kauffmann's brilliant-green tetrathionate broth. This modified medium resulted in higher recovery rates than did the regular tetrathionate broth. In this study, the volume of irrigation water sampled ranged from 333 ml to 3000 ml and the material was concentrated ten-fold, using a continuous centrifuge, before inoculation into the enrichment media.

Stokes and Osborne (1955) described a selenite brilliant green (SBG) enrichment medium for the isolation of Salmonella. This liquid primary enrichment medium was reported to be more effective than the more commonly used selenite F broth. In addition to preventing excess growth of non-salmonella organisms, SBG medium supported abundant growth of salmonellae from very small inocula. Thirteen

smooth stock strains of Salmonella were tested by Kenner et al., (1957) utilizing SBG medium in conjunction with the membrane filter. Recovery rates of 40 to 60 per cent were obtained with the SBG medium as compared to 10 per cent or less using various formulations of SS medium (Salmonella-Shigella) with the membrane filter procedures.

Moore (1950) described a method of sampling sewage for salmonellae over a long period of time, by using a gauze swab suspended in the sewage. This method was used by Moore (1951) for the detection of typhoid carriers. By suspending these gauze pads at various points along a sewerage system, it was possible to locate the dwellings, from which S. typhi had been excreted into the system. Follow-up rectal swab studies of the occupants revealed the carrier. This method has been used with success by Moore et al., (1952), Kelly et al., (1955) and Greenberg et al., (1957). It was observed by Kelly (1955) that S. montevideo was isolated five times from the final effluent of a sewage plant in New York State.

MATERIALS AND METHODS

Collection of Samples

From September 1955 through August 1957 biweekly samples of sewage were collected from the East Lansing and Lansing sewage plants. During the latter part of August and the months of September and October 1957 daily samples were obtained from seven sampling points located in the East Lansing plant.

A pumping station, serving the northside and eastside sections of Lansing, was the location from which samples representing the Lansing area were obtained. Lansing is a highly industrialized city with a population of 92,129 (1950). The flow of sewage through the pumping station contained both residential and industrial wastes.

The sewage plant in East Lansing serves a population of 40,000 when the university is in session. The final effluent was not chlorinated when it was passed into the Red Cedar River.

Three types of sewage samples were obtained from the East Lansing plant. The samples collected were 100 ml dip samples, gauze pad samples and raw sludge samples. The gauze pads were similar to those described by Moore (1950) and consisted of adsorbent cotton placed between two layers of cheese cloth, 4 inches by 4 inches square, which were sewed together around the periphery. The pads were placed directly into the flow and remained in contact, at the various sampling stations, from 24 to 72 hours. Approximately 150 ml of raw sludge were collected from an outlet on a sludge pump and represented a 24 hour sample, as the raw sludge was kept in a holding tank and pumped to the digestors once daily. At the Lansing pumping

station only dip samples and gauze pad samples were collected.

Specimens were processed in the laboratory within one hour of collection in the case of salmonellae examination or were frozen and held at -20 C until they could be processed for subsequent virus isolation techniques.

Processing of the Samples:

Virus Isolation

For the isolation of viruses, the fluid was extracted from the gauze pad samples by placing the pad in the barrel of a 100 cc syringe and forcing the liquid through by exerting pressure with the plunger. This was followed by washing the pad with approximately 40 ml of distilled water. The liquid was then centrifuged at 2400 rpm for 5 minutes to remove the larger particles. The supernatant fluid was placed in two 30 ml lusteroid ultracentrifuge tubes and placed in a Rotor B for ultracentrifugation. The samples were ultracentrifuged at 42,040 rpm (114,610 g) for one hour in a Spinco Model E Ultracentrifuge. The sediment, in the form of a pellet, was resuspended in 3 ml of supernatant fluid and the remainder was discarded. To the 3 ml, 1500 units of penicillin per ml and 4 mg of streptomycin per ml were added to eliminate bacteria. The specimens were then held for 24 to 48 hours at 5 C before they were tested for sterility by inoculating a loopful of the specimen into a tube of brain heart infusion broth containing one per cent agar. The specimen was considered to be free of contaminating bacteria if the appearance of the brain heart infusion broth indicated no bacterial activity at the end of a 72 hour incubation period at 37 C. If,

however, the specimen proved to be contaminated, an additional step was included in an effort to prepare the specimen for tissue culture inoculation. The contaminated specimen was transferred to a 7 ml Pyrex glass tube which was placed in an angle-head rotor and centrifuged in an International Refrigerated Centrifuge, Model PR-1. The specimen was centrifuged at 9,000 rpm for 30 minutes while the temperature was kept at 4 C. The supernatant fluid was removed and 3000 units of penicillin per ml and 12 mg of streptomycin per ml were added. The sediment was discarded. A sterility test was made again and the specimen was discarded if still found to be contaminated. Virus was recovered from specimens receiving the second high speed centrifugation, indicating that the method used in eliminating contaminating organisms was effective and did not destroy or remove the virus.

The outlined procedures for the processing of the gauze pad samples also apply to the dip and sludge samples, with the exception that there was no need of the extraction procedure.

Virus Strains

The strains of Echo viruses used in the study are listed in Table I. The titers obtained in monkey kidney cultures are presented along with those reported by Wenner et al., (1955), Ormsbee and Melnick (1957), Microbiological Associates Inc. (1957) and Stulberg et al., (1958) for comparison. The titers, from the Michigan State University laboratory, represent the average of three titrations. With Echo viruses 3, 4, 5, and 14, there appears to be a wide variation in titer among the three laboratories. This range in titers

was not unexpected, since each shipment of tissue culture represented the cells of a different monkey. Slight variations in viral susceptibility within the same species can be expected.

Dr. C. S. Stulberg generously provided Echo types 7 through 13 and Echo types 1 - 6 and 14 were purchased from the American type Culture Collection. Polio Type I (Mahoney) and Polio Type III (Saukett) strains were kindly supplied by the School of Public Health, University of Michigan. Polio Type II (Lansing) has been maintained in this laboratory for a number of years. The titers of these polio strains, in monkey kidney cells, representing an average of three titrations, were $10^{8.0}$ TCD50 per ml for Type I, $10^{7.9}$ TCD50 per ml for Type II, and Type III was $10^{7.7}$ TCD50 per ml.

TABLE I

ECHO VIRUS STRAINS USED IN THE PREPARATION OF ANTISERA

Echo virus type	Prototype strain	Source of strain	TDC50 per ml*				
			MSU	Stulberg (1958)	Micro. Assoc. (1957)	Archetti (1955)	Ormsbee (1957)
1	Farouk	ATCC**	6.8	9.2	7.0	7.7	7.3
2	Cornelis	ATCC	6.3	8.8	6.1	5.8	6.5
3	Morrisey	ATCC	5.8	8.2	6.2	7.5	6.0
4	Pesascek	ATCC	6.5	9.5	5.8	4.5	5.8
5	Noyce	ATCC	6.5	9.5	7.7	8.8	6.5
6	D'Amori	ATCC	8.4	8.8	8.3	8.4	6.8
7	Wallace	Stulberg	8.4	8.6	8.2	8.5	6.5
8	Bryson	Stulberg	6.4	7.6	6.5	7.8	6.5
9	Hill	Stulberg	6.8	6.5	7.4	7.2	5.8
10	Lang	Stulberg	5.6	6.5	6.2	7.4	6.3
11	Gregory	Stulberg	7.1	7.7	6.9	7.0	7.3
12	Travis	Stulberg	8.2	8.5	8.3	8.8	7.3
13	Hamphill	Stulberg	7.1	8.4	6.7	8.1	7.3
14	Tow	ATCC	6.7	9.4	5.7		

*Expressed as the log of the TCD50.

**American Type Culture Collection, Washington, D. C.

Mice

A colony of Swiss strain mice was maintained to supply the suckling mice necessary for the detection of Coxsackie viruses present in the sewage specimens.

One to three-day old mice were used. Six-hundredths ml of the specimen was inoculated intraperitoneally into all but two suckling mice of a litter. The uninoculated two were held as controls and were marked by cutting off the tips of their tails. All of the mice were held for a 21 day period and observed for signs of Coxsackie infection, manifested by paralysis of the hind-limbs and occasional deformity in the fore-limbs, with death occurring within a few hours after onset of symptoms. Mice dying within 24 hours of inoculation were not considered "specific" deaths.

The brains from dead and paralyzed mice were harvested. A 20 per cent suspension was prepared in nutrient broth, for two additional passages in suckling mice. The harvest from the third passage was injected intracerebrally in 21-day old mice, 0.03 cc per mouse, to rule out the presence of a virus pathogenic for older mice.

Tissue Culture

Monkey kidney cells were purchased from Microbiological Associates, Bethesda, Maryland. Round, screw cap tubes, 16 x 125 mm, containing a confluent monolayer of monkey kidney epithelial cells on one side of the tube were shipped weekly by air express. The growth medium used by the company contained calf serum two per cent, lactalbumin hydrolysate 0.5 per cent and Hank's Balanced Salt Solution 97.5 per cent. Upon arrival the 0.5 ml of growth medium was

replaced with 0.5 ml of Mixture 199, developed by Morgan et al., (1950) containing two per cent inactivated horse serum buffered to pH 7.2 with 2.8 per cent $NaHCO_3$. The monkey kidney cells were held 24 hours at 37 C after arrival before they were inoculated. After inoculation the medium was changed every third day in original isolation techniques and on the fifth day following inoculation in the neutralization tests.

Each of three tubes of monkey kidney cells was inoculated with 0.03 ml of the processed sewage specimen. The cells were observed every other day for signs of cytopathogenic effect (CPE). The CPE was progressive in character. The first visible sign of viral activity was the degeneration of the epithelial cells shown by small clusters of cells becoming rounded and transparent. In this early stage, a partial release of some of the cells from the glass surface of the tubes could be observed. As viral multiplication continued some of the cells became spindle shaped and more of the cell population showed the characteristic rounding of the epithelial cells. As the infection progressed there was a sloughing of the cells from the glass surface which greatly interrupted the continuity of the monolayer. The tissue culture was harvested when the majority of the cells were released from the glass surface. On primary isolation, the cell cultures were observed over a 20 day period. CPE was usually observed anytime from the second to the twelfth day after inoculation with sewage specimens. In many cases, only one or two of the three inoculated tubes were suspected as containing a virus infection. Only tubes demonstrating CPE were harvested and passed. The first

passage, after the original isolation, consisted of inoculating each of three tubes of monkey kidney cells with 0.1 ml of undiluted tissue culture fluid. The CPE observed usually occurred much sooner and affected all three tubes. During the early part of the study all suspected viral isolates were passed three times after original isolation before a virus was considered ready for identification procedures. In the latter part of the study it was found that the titer of the virus was, in most cases, directly related to the time of onset of complete CPE. This differentiation was also used by Melnick (1957) in studying unknown viruses, thought to belong to the enteroviruses group, isolated in West Virginia. An arbitraty method was chosen to determine whether the virus was passed more than once; on first passage if marked CPE was observed in less than four days following inoculation no further passages were made.

The tissue culture fluid from all positive tubes was pooled, harvested and stored at -20 C.

Preparation of Antisera

The antisera were prepared by inoculating rabbits with the strains listed in Table I. Undiluted tissue culture fluids served as antigens. Rabbits received three injections each of 0.25 ml, 0.5 ml and 1.0 ml, given intravenously, on successive weeks. This was followed by booster injections of 1.0 ml given at various intervals. In Table II are presented the serum neutralization titers obtained and the total volume of antigen each rabbit received.

The method of producing specific antiserum of high titer differed from Sabin and Ramos-Alvarez (1954), Melnick (1957), and

TABLE II

SERUM NEUTRALIZATION TITERS AGAINST ECHO PROTOTYPE STRAINS PREPARED IN RABBITS

Virus	Volume of TC Antigen Injected**	Serum Neutralization Index*		
		MSU	Microbiological Associates (1957)	Melnick and Kanda (1957)
Echo 1	9.0 ml	400	340	1000
2	11.0	600	1900	2000
3	8.5	1800	270	800
4	8.5	100	11	25
5	12.0	2800	1150	1000
6	16.0	1700	3100	1600
7	19.0	5700	900	2000
8	20.0	1600	490	800
9	9.5	5300	2800	3200
10	7.0	690	3400	800
11	11.5	2200	600	650
12	12.5	2600	1600	4000
13	12.0	1600	650	3200
14	8.0	800	335	400

*Titers are listed as 50 per cent end point per 0.1 ml serum against 100 TCD50 of virus.

**Divided among a basic series consisting of 13 injections, totaling 6.5 ml, and booster injections given at various intervals thereafter.

Microbiological Associates Inc. (1957) because the initial volumes injected in this laboratory were smaller and the period of time in which the animals were inoculated was longer. However, the total volume injected into each rabbit was the same or greater than that used by the other investigators and it was felt that the longer period of injection would help to produce high titers in the antisera.

Antisera against poliovirus types I, II, and III, prepared in monkeys, was kindly supplied by Dr. H. A. Wenner.

Neutralization Tests

The 14 Echo prototype specific rabbit antisera were divided into four pools designated A, B, C and D according to the work of Melnick (1955). Pool A contained antisera 2, 3, 5, and 6. Pool B consisted of Echo antisera 1, 12 and 13, and Pool C was composed of Echo antisera 7, 8, 9, 10, and 11. Pool D was comprised of Echo antisera 4 and 14. The composition of these pools was based on the geographical distribution of the original prototype strain isolations, with the exception of Pool D which was made up of the two poorest antiserum-producing members of the group.

The pools were changed when it appeared that only seven members of the Echo virus group were being identified from the unknown isolates. A pool containing Echo virus antisera 1, 3, 13 and 14 was designated E and a pool containing 6, 7, and 9 was labeled F.

Viruses to be identified, which were non-infectious for suckling mice, were titrated in monkey kidney cell cultures using 10-fold dilutions made in Medium-199. The TCD50, for each unknown virus, was

calculated according to the method of Reed and Muench (1938). These titers are shown in Table III and represent the highest dilution in which 50 per cent of all inoculated tissue culture tubes showed demonstrable CPE. The unknown viruses were mixed, at a dilution containing 200 TCD50, with equal volumes of the pooled antisera. Each antiserum in the pool contained from 10 to 40 units of antiviral activity. One unit was equivalent to the highest dilution of antiserum neitralizing 100 TCD50 of homologous virus. The serum-virus mixtures were allowed to incubate at room temperature for one hour. One tenth ml of the mixture was placed in each of three monkey kidney tissue culture tubes. Two control series were included in each test. One set of tubes contained 100 TCD50's of virus in normal rabbit serum and one group of tubes was uninoculated. The tubes were incubated at 37 C and were observed daily over a 10 day period for evidence of viral activity.

In most of the identification procedures either Pool E or Pool F antisera neutralized the unknown virus. The next step was to perform the neutralization test against each of the component members of the pool. If Pool E or Pool F did not neutralize the unknown virus, a series of neutralization tests, using rabbit antiserum prepared against each of the poliovirus types was performed. In the event that the unknown virus specimen contained more than one type of poliovirus, a mixture composed of equal volumes of all three types of poliovirus antiserum was included. The same number of units of virus and antiserum as described above was used in this screening procedure. Failure of the unknown virus to be neutralized in the

poliovirus screen resulted in the screening of the unknown virus against the remaining members of the Echo group. Pool G contained Echo antiserum 2, 4, and 5 while Pool H contained Echo antiserum 8, 10, 11, and 12.

When the unknown virus was not neutralized by pools G and H, it was designated as an "Unknown" and subjected to further tests in suckling and 21-day old mice to detect the presence of Coxsackie B virus.

Salmonella Isolations

For the isolation of species of Salmonella, only gauze pad and sludge samples were utilized. The extraction of the liquid from the pads was similar to the procedure outlined in the section on virus isolation. In many instances, the same pad or sludge sample served as a source both for virus and salmonellae isolation techniques. Approximately one ml of material served as an inoculum for 150 ml of enrichment medium*. The enrichment media used in the study were Difco tetrathionate broth and Selenite Brilliant Green (SBG) developed by Stokes and Osborne (1955). The formulation of Stokes' and Osborne's medium is as follows:

	Per cent
Peptone	0.5
Yeast extract	0.5
Mannitol	0.5
Sodium selenite	0.4
Sodium taurocholate	0.1
Brilliant green	0.0005
Phosphate buffer , pH 7.0	0.025 molar

*Later work has shown that 25 ml of SBG medium is sufficient for one ml of inoculation.

"In the preparation of this medium, the first five ingredients were dissolved in somewhat less than the required amount of water and adjusted to pH 7.0 by the addition of a few drops of 5N HCL. The phosphate buffer and brilliant green were then added and the volume of the medium was adjusted to the required level with water.

The phosphate buffer was prepared by mixing appropriate quantities of 0.25M solutions of KH_2PO_4 to give a solution of pH 7.0 and this was added to the medium in a ration of one part of buffer to ten parts of medium.

The brilliant green had a dye content of 93 per cent. One ml of a 0.1 per cent aqueous solution of the dye was added to each 200 ml of medium. Since the dye solutions lost considerable color on standing overnight in the laboratory, a fresh solution was prepared on each day that a new batch of medium was to be made."

The inoculated enrichment media were incubated for 18 to 24 hours at 37 C. One large loopful of material was then streaked onto a Difco bismuth sulfite agar plate which was incubated at 37 C for 24 to 48 hours. Typical salmonellae-like colonies were picked and inoculated into triple sugar iron agar tubes. At least four suspicious colonies were picked from each plate when possible. Transfers into tubes of Stuart's urea broth, Simmon's citrate agar, nutrient agar and one per cent solutions of lactose, sucrose, salicin and mannite, made up in a purple broth base, were made from tubes of triple sugar iron agar showing salmonella-like reactions. Final serological typing was generously performed by the Salmonella Typing Station of the Michigan Department of Health Laboratories.

RESULTS

During 27 consecutive months of sampling, a total of 951 sewage specimens were tested in monkey kidney tissue culture. In Table III are presented the composite data. Sewage specimens from East Lansing accounted for 585 of the samples tested and the remaining 366 were collected from Lansing. The per cent positive isolations of enteric viruses accomplished in East Lansing was 14.2 per cent (83 of 585), which was exactly twice the 7.1 per cent recovery rate from Lansing (26 of 366). An analysis of the differences in the recovery rates from the two locations revealed that the number of positive dip samples was approximately equal; 10 of 189 samples were positive from Lansing and 7 of 179 samples were positive from East Lansing. The gauze pad samples in East Lansing yielded a much higher number of viruses, 45 of 285, than did similar types of samples from Lansing, 16 of 177. The fact that raw sludge samples were only collected from the East Lansing sewage plant must also be taken into consideration. However, even though the sludge samples accounted for 20.7 per cent of the total number of samples and 11.0 per cent of the positive samples, the differences between the two locations was still significant: 11 per cent of the specimens tested were positive from East Lansing as opposed to 7.1 per cent from Lansing, after excluding the sludge samples from the calculations.

Table III also includes a summary of the mouse inoculation tests carried out in parallel with the tissue culture studies. A total of 859 sewage specimens from East Lansing and Lansing were tested in infant mice. Lansing contributed 303 specimens of which six were

positive (2.0 per cent). Seventy of the 556 specimens from East Lansing were positive (12.6 per cent). The dip samples from Lansing were comparable to those of East Lansing in that 5 of 155 (3.2 per cent) were positive in mice as opposed to seven of 141 samples (5.0 per cent that were positive in the East Lansing series. There was a dramatic difference in the number of positive isolations obtained from the gauze pad samples. Only one of 148 samples was positive from Lansing, while there were 38 of 310 (12.3 per cent) positive samples from East Lansing. The raw sludge specimens yielded positive results in 25 of 105 attempts (23.8 per cent).

The information presented in Table III does not take into consideration the number of duplicate isolations obtained from the same specimen in tissue culture and mouse inoculation tests. From the 951 tissue culture isolation procedures, 110 tissue culture cytopathogenic agents were found (11.6 per cent). An infectious agent was demonstrated in 76 of 859 specimens tested in infant mice (8.8 per cent), and since there is no evidence in the literature to indicate that all paralyzed suckling mice were not infected with either Coxsackie A or Coxsackie B virus, these were recorded as Coxsackie viruses. The number of sewage specimens tested in mice on a monthly basis during the course of the study is presented in Table IV. During the last four months of 1955, 161 specimens were processed and three samples contained Coxsackie viruses. During the 12 months of 1956, 26 of 345 specimens tested were positive in mice (7.5 per cent). During the 10 months of 1957 in which 353 samples were collected, the number of isolations obtained in mice was 26 (11.0 per cent).

TABLE III

VIRUSES ISOLATED FROM SEWAGE BY TISSUE CULTURE AND MOUSE INOCULATIONS

Sample	Location	Tissue Culture			Mouse Inoculations		
		Number tested	Number positive	Per cent positive	Number tested	Number positive	Per cent positive
Gauze pad	Lansing	177	16	9.0	148	1	0.7
	East Lansing	285	45	15.8	310	38	12.3
Dip	Lansing	189	10	5.3	155	5	3.2
	East Lansing	179	7	3.9	141	7	5.0
Sludge	East Lansing	121	32	26.4	105	25	23.8
Totals	Lansing	366	26	7.1	303	6	2.0
	East Lansing	585	83	14.2	556	70	12.6
Grand Total		951	110	11.6	859	76	8.8

TABLE IV

VIRUS ISOLATIONS IN SUCKLING MICE FROM SEWAGE SPECIMENS

Month of specimen collection	1955		1956		1957		1955-1957	
	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive
January			27	3.7	27	3.7	54	3.7
February			29	0	0	0	29	0
March			21	0	3	0	24	0
April			30	3.3	2	0	32	0
May			5	0	22	4.5	27	3.4
June			31	3.2	35	2.9	66	3.0
July			40	10.0	43	7.0*	83	8.4
August			30	30.0	70	18.6*	100	21.0
September	65	3.1	33	15.2	89	12.4*	187	10.7
October	38	2.7	34	11.8	53	26.4*	125	15.2
November	31	0	33	3.0	9	22.4	73	4.1
December	27	0	32	0	-	-	59	0
Totals	161	1.9	345	7.5	353	11.0	859	8.8

*Includes a total of 17 specimens which were negative when originally inoculated into suckling mice, but subsequent tissue culture passage was positive in mice.

By combining the number of samples tested in each month of each year (Table IV, 1955-1957) of the study, a pattern of the seasonal occurrence of Coxsackie virus was indicated. The months of the lowest incidence of recoveries were December through June. In July there was a noticeable increase in the recovery of Coxsackie viruses (8.4 per cent). The peak occurrence was in August when 21 of 100 samples were positive in infant mice. A decrease from this peak of 21 per cent positive specimens was seen during the month of September when the incidence fell to 10.7 per cent. A slight rise was seen in October over the previous month, but was still considerably below the peak incidence in the month of August, when 19 of the 125 samples (15.2 per cent) were positive in mice.

The number of specimens tested in monkey kidney cell cultures during the study is shown in Table V. During the last four months of 1955, 172 specimens were processed and yielded 11 tissue culture cytopathogenic agents (6.4 per cent). For the year 1956 in which 359 samples were tested, 43 positive isolations were accomplished (13.3 per cent) and in 1957, 56 of the 420 specimens were positive (13.3 per cent).

By combining the number of samples tested in each month of each year (Table V, 1955-1957) of the study, a pattern of the seasonal occurrence of tissue culture cytopathogenic agents from sewage was obtained. These agents were isolated during each month, with the peak incidence occurring during the month of October, when 18.9 per cent of the 111 samples tested were positive. Since the differences in per cent recovery between the months of August, September, and

TABLE V

VIRUS ISOLATIONS IN TISSUE CULTURE FROM SEWAGE

Month of specimen collection	1955		1956		1957		1955-1957	
	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive
January			28	10.7	38	2.6	66	6.1
February			34	11.8	28	0	62	6.5
March			21	9.5	30	6.7	51	7.8
April			31	3.2	27	7.4	58	5.2
May			5	0	31	3.2	36	2.8
June			31	9.7	35	5.7	66	7.6
July			40	7.5	42	14.3	82	11.0
August			32	18.8	68	14.7	100	16.0
September	72	6.9	34	23.5	84	23.8	190	17.4
October	38	5.3	38	21.1	35	31.4	111	18.9
November	32	9.4	33	9.1	2	50.0	67	16.4
December	30	3.3	32	6.3			62	4.8
Total	172	6.4	359	9.7	420	13.3	951	11.6

November did not vary more than three per cent (16.0, 17.4, and 16.4 per cent respectively) lower than the October peak, the results indicated that there was a long period of high incidence. Marked reduction in incidence was noted during December (4.8 per cent), January (6.1 per cent), February (6.5 per cent), March (7.8 per cent), and April (5.2 per cent). The small number of isolations obtained during the month of May in both years of the study (2.8 per cent) may be attributed to the smaller number of samples tested (36) or in part due to the heavy rainfall in the area. The recovery rate of cytopathogenic agents was increased to 7.6 per cent during the month of June and was further elevated during the month of July, in which 11 per cent of the specimens tested in tissue culture yielded cytopathogenic agents.

Serum neutralization tests in monkey kidney culture tubes were used to identify 35 of the viruses isolated from sewage in tissue culture. Nine of the 14 Echo types included in the identification schema were present (Table VI). Echo 1 or 13 virus was identified most frequently and occurred on 10 occasions. Since the prototype Echo 13 virus has been reported by the Committee on the Enteroviruses (1957) to contain an Echo 1 contaminant, these two strains were grouped together, since cross-reactions were seen in most cases. Echo 7 virus was identified from six of the samples. Next, listed in the order of their most frequent appearance were Echo 6 (four isolations), Echo 8 (four isolations), Echo 3 (three isolations), Echo 14 (two isolations), and Echo 9 (one isolation). In addition to the Echo viruses that were identified, four samples yielded poliovirus Type

TABLE VI

IDENTITY OF VIRUSES ISOLATED
IN TISSUE CULTURE

Location	Echo 1 and/or 13*	Echo 3	Echo 6	Echo 7	Echo 8	Echo 9	Echo 14	Polio type 1	Polio type 3
East Lansing	10	2	1	3	4		3		4
Lansing			3	3		1		1	
Total	35								

*Echo 1 and 13 grouped together since cross reactions were so frequently encountered that all Echo 1 strains may be Echo 13 and vice versa. This has also been reported by the Committee on the Enteroviruses (1957).

III and one sample contained poliovirus Type I. In Table VII is presented information on the source from which the viruses were isolated, the titer and growth rate in tissue culture, and the month and year of isolation.

Sewage specimens from East Lansing accounted for 27 of the viruses that were identified as Echo or polioviruses. However, by comparing the number of isolations obtained from the gauze pad and dip samples from the two cities, during similar periods of collections, little difference was noted between them. Ten Echo virus identifications were made from East Lansing and eight from the Lansing samples. It can be seen from Table VI that Echo virus types 1 or 13, 3, 8, and 14 were isolated only from East Lansing. In Lansing one specimen yielded Echo virus type 9, while this type was not found in East Lansing. Poliovirus Type I was found once in Lansing and poliovirus Type III was found only in East Lansing and on four occasions. Echo virus types 6 and 7 were found in both locations.

The seasonal incidence of these enteric viruses was again noted, with two isolations obtained during the month of January, one Echo 9 isolated in April and one polio Type I virus was found in a sample collected in March. The remainder of the isolations was accomplished during the period June through November with a concentration during the peak months of September and October.

In Table VIII are listed the cytopathogenic agents that were not identified as being members of the Echo 1-14 group, poliovirus group or the Coxsackie group of viruses. Of the 17 isolates that composed this "unknown" group, 12 had a growth rate classified as being

TABLE VII

ANTIGENIC TYPES AND SOME BIOLOGIC CHARACTERISTICS OF VIRUSES ISOLATED IN TISSUE CULTURE FROM SEWAGE SAMPLES

Sample number	Source*	Echo type	Titer** TCD50 per ml	Growth rate***	Month and year isolated
439	ELP	1 or 13	9.3	Fast	9-56
527	ELS	1 or 13	9.5	Fast	11-56
536	ELS	1 or 13	9.0	Fast	12-56
571	ELS	1 or 13	7.5	Fast	1-57
904	ELS	1 or 13	9.0	Fast	9-57
906	ELP	1 or 13	8.5	Fast	9-57
933	ELP	1 or 13	9.5	Fast	9-57
1016	ELP	1 or 13	8.5	Fast	9-57
1063	ELP	1 or 13	8.5	Fast	10-57
1114	ELS	1 or 13	6.8	Fast	10-57
407	ELS	3	8.0	Moderate	8-56
490	ELP	3	8.3	Fast	10-56
126	LD	6	8.3	Fast	11-55
157	LP	6	9.5	Fast	12-55
409	LD	6	9.6	Fast	8-56
436	ELP	6	9.5	Moderate	9-56
74	LP	7	8.6	Fast	11-55
446	LP	7	8.5	Fast	9-56
462	LP	7	9.6	Fast	9-56
750	ELS	7	8.5	Fast	6-57
801	ELP	7	9.5	Moderate	7-57
845	ELP	7	7.8	Fast	8-57
205	ELP	8	8.5	Slow	2-56
476	ELP	8	4.2	Slow	10-56
897	ELS	8	8.0	Fast	9-57
926	ELP	8	7.5	Fast	9-57
675	LP	9	7.2	Fast	4-57
191	ELS	14	7.6	Moderate	1-56
487	ELS	14	8.5	Slow	10-56
924	ELS	14	8.5	Slow	9-57
799	LP	Polio I	8.6	Fast	7-57

TABLE VII (continued)

Sample number	Source*	Echo type	Titer ** TCD50 per ml	Growth rate***	Month and year isolated
440	ELS	Polio III	9.3	Fast	9-56
468	ELP	Polio III	9.5	Fast	10-56
647	ELP	Polio III	9.5	Fast	3-57
657	ELD	Polio III	9.3	Fast	3-57
Total 35					

*East Lansing Pad (ELP); East Lansing Dip (ELD); East Lansing Sludge (ELS); Lansing Pad (LP); Lansing Dip (LD).

**Expressed as the reciprocal of the TCD50.

***Fast: marked CPE in 4 days or less; Moderate: marked CPE seen by the sixth day; Slow: CPE seen in 7 or more days after inoculation.

TABLE VIII

VIRUSES ISOLATED IN TISSUE CULTURE NOT IDENTIFIABLE AS ECHO VIRUSES 1-14, POLIOVIRUS TYPES I, II, OR III OR COXSACKIE VIRUSES

Sample number	Source*	Titer** TCD50 per ml	Growth rate***	Month and year isolated
97	ELS	8.6	Moderate	10-55
195	ELP	Not done	Moderate	1-56
365	ELP	Not done	Slow	7-56
424	ELS	8.6	Fast	9-56
451	ELP	9.3	Fast	9-56
452	LD	Not done	Slow	10-56
488	LP	5.7	Moderate	10-56
554	LP	8.6	Moderate	12-56
797	ELP	9.3	Moderate	7-57
833	LP	8.5	Moderate	8-57
870	ELP	8.3	Moderate	8-57
877	ELP	7.5	Moderate	8-57
912	ELP	7.5	Fast	9-57
1035	ELP	7.0	Moderate	9-57
1057	ELS	8.6	Fast	10-57
1066	ELP	Not done	Slow	10-57
1165	ELS	7.3	Fast	11-57

Total 17

*East Lansing Pad (ELP); East Lansing Sludge (ELS); Lansing Pad (LP); Lansing Dip (LD).

**Expressed as the log of the TCD50.

***Fast: CPE manifested in 4 days or less; Moderate: CPE visible by the sixth day; Slow: CPE not seen until the seventh day or later.

moderate or slow; a marked cytopathogenic effect was not evident for at least six days following inoculation in the case of those classified as moderate and seven days or longer for those classified as having a slow growth rate. This was in contrast to the group of identified viruses in which only eight of the 35 were classified as possessing a moderate or slow growth rate. This group of "unknowns" was negative upon passage of the original specimen and subsequent tissue culture passage material in infant mice and did not infect 21-day old mice when inoculated intercerebrally.

Of the 110 tissue culture cytopathogenic agents, 36 were also found to be positive when inoculated into suckling mice. Nineteen of these viruses were positive when the original sewage specimen was inoculated into the mice. An additional 17 specimens, which were positive in tissue culture, failed to infect infant mice when the original specimen was tested. However, when the tissue culture passage material was inoculated into one-day old mice, paralysis and death occurred. This information is in Table IX.

Twenty-two tissue culture cytopathogenic agents lost their ability to produce visible CPE in monkey kidney cells after their original isolation. In Table X are presented the data which showed that the growth rate of these agents was either slow or moderate. These agents were passed at least twice in tissue culture after original isolation and one was passed an additional seven times.

When it became evident that the latest passage material had lost its ability to produce a CPE in tissue culture, blind passages were made using the older passage materials, which had been stored at

TABLE IX

DUPLICATE ISOLATIONS OF CYTOPATHOGENIC AGENTS IN TISSUE CULTURE AND COXSACKIE VIRUSES IN SUCKLING MICE

Specimens positive in tissue culture and suckling mice upon original isolation procedures		
Specimen number	Source*	Date of collection
17	LD	9-55
63	ELS	9-55
369	ELP	7-56
393	ELD	8-56
395	ELP	8-56
405	ELP	8-56
427	ELP	9-56
724	ELD	5-57
741	ELP	6-57
807	ELP	7-57
811	ELP	7-57
825	ELP	8-57
831	ELP	8-57
840	ELP	8-57
869	ELS	8-57
876	ELS	8-57
931	ELS	9-57
1093	ELS	10-57
1121	ELS	10-57
Specimens positive in tissue culture and negative in suckling mice upon original isolations procedures. Tissue culture passage material positive in mice		
813	ELS	7-57
849	ELS	8-57
952	ELS	9-57
960	ELP	9-57
967	ELP	9-57
974	ELP	9-57
980	ELS	9-57
981	ELS	9-57
1001	ELS	9-57
1009	ELP	9-57
1022	ELS	9-57
1043	ELS	10-57
1057	ELS	10-57
1064	ELS	10-57
1073	ELP	10-57
1079	ELS	10-57
1157	ELS	11-57
Total 36		

*East Lansing Pad (ELP); East Lansing Dip (ELD); East Lansing Sludge (ELS); Lansing Dip (LD).

TABLE X

TISSUE CULTURE CYTOPATHOGENIC AGENTS RECOVERED FROM SEWAGE BUT LOST ON SUBSEQUENT PASSAGE OR STORAGE

Specimen Number	Source*	Date of collection	Growth rate**	Number of passages
41	LD	9-55	Slow	7
57	LD	9-55	Moderate	3
59	LD	9-55	Slow	5
120	LP	11-55	Moderate	6
132	ELP	11-55	Moderate	6
198	LP	11-55	Moderate	4
221	LP	2-56	Slow	3
228	ELP	2-56	Slow	5
233	ELS	2-56	Slow	4
241	LD	3-56	Slow	3
250	LP	3-56	Slow	5
278	LD	4-56	Slow	3
307	LP	6-56	Slow	3
327	ELP	6-56	Slow	4
333	ELD	6-56	Slow	3
366	ELD	7-56	Slow	2
385	ELP	8-56	Moderate	2
437	ELD	9-56	Slow	2
456	LP	10-56	Slow	2
494	ELP	11-56	Slow	2
501	LP	11-56	Moderate	2
699	LD	4-57	Slow	3
Total 22				

*LD (Lansing Dip); ELD (East Lansing Dip); LP (Lansing Pad); ELP (East Lansing Pad): ELS (East Lansing Sludge).

**Moderate: CPE manifested in 5-6 days; Slow: CPE manifested in 7 or more days.

-20 C. Blind passage techniques, although successful in restoring the CPE in some instances, did not result in success with the 22 agents listed in Table IX.

An attempt was made to demonstrate the effects of sewage treatment in removing viruses present in the influent. To demonstrate this, samples were taken from seven locations in the East Lansing treatment plant; influent, raw sludge, primary tank effluent, activated sludge tank, activated sludge return, final settling tank, and the final, unchlorinated, effluent.

Daily samples were collected from each of the seven sampling points from August 27 through November 6, 1957. However, due to lack of funds only 214 specimens were processed either in monkey kidney tissue culture and/or suckling mice. There was a progressive, significant decrease in the number of viruses isolated from stages of treatment followed through the plant (Table XI). The influent yielded 14 viruses from the 43 specimens tested (32.6 per cent). From 42 samples of raw sludge 16 viruses were isolated (38.1 per cent). Samples from the primary tank effluent were positive on 5 of 21 occasions (23.8 per cent). The number of isolations obtained from the activated sludge tank dropped to two of 18 samples (11.1 per cent). This low incidence continued when only 2 of 30 samples from the activated sludge return were positive (6.7 per cent). Samples from the final settling tank yielded virus only once in 19 attempts (5.3 per cent). The final, unchlorinated, effluent contained virus on four of 41 occasions (9.8 per cent).

During the first half of the Salmonella study, using tetra-

TABLE XI

ISOLATION OF ENTERIC VIRUSES FROM VARIOUS LOCATIONS IN A SEWAGE PLANT

Sampling location	August (27-31)	September (1-30)	October (1-31)	November (1-6)	Total	Per cent positive
Influent	4/5*	9/23	1/14	0/1	14/43	32.6
Raw sludge	2/4	8/19	4/16	2/3	16/42	38.1
Primary tank effluent	2/5	2/9	1/5	0/2	5/21	23.8
Activated sludge tank	0/5	0/8	1/4	1/1	2/18	11.1
Activated sludge return	0/4	1/19	1/6	0/1	2/30	6.7
Final settling tank	0/5	0/7	1/6	0/1	1/19	5.3
Final, unchlorinated effluent	0/5	1/20	3/14	0/2	4/41	9.8

*Number of samples positive out of the total number tested. Figures represent the combination of tissue culture and/or mouse inoculation tests. Samples positive in both instances were counted only as a single positive isolation for this table.

thionate broth as the enrichment medium, 32 of 142 gauze pad and sludge samples were positive for Salmonella (Table XII). From these 32 positive specimens, 49 salmonellae representing 14 species were isolated. It should be noted that from approximately the same number of gauze pad samples taken in the two cities, there was a marked difference in the recovery rates. In Lansing, six of the 59 specimens were positive and in East Lansing, 17 of 56 specimens were positive for Salmonella. Since sludge samples were collected only from the East Lansing plant, no comparison can be made with Lansing. Nine of 27 raw sludge samples were positive (33 per cent) and the per cent recovery was comparable to that from the gauze pad samples in East Lansing (30 per cent). However, the nine positive sludge samples yielded 16 salmonellae that included nine species whereas, the 17 positive gauze pad samples yielded 23 salmonellae representing six species (Table XIII).

The number of Salmonella isolated in the July through December period is also shown in Table XII. Fewer samples were taken during this phase of the study (92) and a greater number of isolations was made; 62 positive samples yielded 83 salmonellae representing 17 species (Table XIII). A new species of Salmonella was isolated from a sludge sample during this period. It has been named S. lansing and its antigenic characteristics will be published by A. P. Juenker and H. Bilanow of the Michigan Department of Health Laboratories. Of the 21 sludge samples tested, 19 yielded 20 salmonellae which included 12 different species. The gauze pad samples were positive on 43 of 71 occasions and included 54 salmonellae representing 13

TABLE XII

COMPARISON OF TETRATHIONATE BROTH AND SELENITE BRILLIANT GREEN MEDIA IN THE ISOLATION OF SALMONELLA FROM SEWAGE

	Tetrathionate Broth October 1956 - June 1957		Selenite Brilliant Green Medium July - December 1957	
	Gauze pads	Sludge	Gauze pads	Sludge
Lansing	6/59*(12%)			
East Lansing	17/56 (30%)	9/27 (33%)	43/71 (60%)	19/21 (90%)
Totals	32/142(23%)		62/92 (67%)	

*Number positive isolations/total specimens tested.

TABLE XIII

ISOLATION OF SALMONELLA FROM GAUZE PADS AND RAW SLUDGE ACCORDING TO LOCATION

Species	Tetrathionate Broth			Selenite Brilliant Green	
	Lansing	East Lansing		East Lansing	
	Pad	Pad	Sludge	Pad	Sludge
S. muenchen		10	5	2	4
S. montevideo	1	6	2	3	3
S. derby		2	1	5	5
S. anatum		2	1	9	5
S. tennessee	2	3			1
S. typhimurium	3		2		2
S. worthington			1		
S. infantis			2	1	2
S. thompson			1		
S. reading			1		
S. paratyphi B				8	2
S. heidelberg				3	1
S. san diego				18	2
S. lansing					1
S. typhi					1
S. kentucky				1	
S. berta	1			1	
S. bareilly				1	
S. give				1	
S. california				1	
S. cerro	1				
S. cubana	2				
S. blockley		1			
Totals (133)	10	24	16	54	29

species. Eight species were found frequently in both types of samples whereas, the pad samples were the only type of samples positive for S. berta, S. bareilly, S. kentucky, S. give, and S. california. Only the sludge samples were positive for S. typhi, S. lansing, S. tennessee, and S. typhimurium.

An attempt was made to see if sampling at different points in the treatment plant had any effect on the recovery of Salmonella. Although the number of samples was relatively small at some of the sampling points (Table XIV), salmonella was recovered at every point in the treatment system tested. Emphasis was placed on the sampling of the final, unchlorinated, effluent, since this material was discharged into the Red Cedar River which flows by the plant. A high recovery rate was obtained from this type of sample (22 of 35) indicating that although the sludge was removed at the early stage of treatment, the supernatant fluid contains considerable numbers of Salmonella as it passes through the plant and even in the effluent which was discharged into the river.

Difficulties were encountered in the first phase of the study, using tetrathionate broth, in that Proteus was frequently encountered in such large numbers that discrete salmonella-like colonies were not always observed. In addition to proteus overgrowth on the bismuth sulfite agar plates, paracolons, simulating Salmonella in the triple sugar iron agar tubes, were found to be prevalent. With the change to SBG enrichment medium, little difficulty was experienced with these groups of organisms. In addition to inhibiting undesired organisms, SBG medium seemed to enhance the growth of Salmonella,

TABLE XIV

ISOLATION OF SALMONELLA FROM GAUZE PADS LOCATED AT VARIOUS POINTS IN A TREATMENT PLANT

Sampling location	Number of samples	Species
Influent	3/8*	S. san diego (2), S. derby
Primary tank effluent	6/7	S. san diego (2), S. paratyphi B (2), S. anatum (2), S. muenchen, S. berta, S. kentucky
Activated sludge tank	1/6	S. san diego
Final settling tank	3/6	S. san diego (2), S. derby, S. muenchen
Activated sludge return	8/9	S. san diego (2), S. paratyphi B (2), S. anatum (3), S. montevideo, S. give, S. california
Final, unchlorinated effluent	22/35	S. san diego (9), S. paratyphi B (4), S. anatum (4), S. heidelberg (3), S. montevideo (2), S. derby (3), S. bareilly, S. infantis
Totals	43/71	54 Salmonella isolated

*Number of samples positive/total number tested.

since in a large number of samples a relatively pure culture of salmonella-like colonies were observed on the plates, which were subsequently confirmed as Salmonella. With the majority of samples, typical colonies were observed on the bismuth sulfite agar plates after only 24 hours of incubation.

DISCUSSION

The presence of enteric viruses in sewage over a long sampling period has been confirmed by the results of this study. Since sewage samples represent, in part, large numbers of stool specimens, the findings of Honig et al., (1956) and Gelfand et al., (1957) should be considered. In Table XV is presented a summary of the results obtained by the two previously mentioned groups and the data obtained in this study. The work of Gelfand represented the number of polio and non-polio virus isolations in tissue culture, utilizing stool samples from a group of normal healthy children over a 24 month period. Honig and his associates studied a group of healthy pre-school children over a 29 month period. The stool samples, collected at monthly intervals from each child, were tested in tissue culture and suckling mice to detect enteric viruses. Table XV combines the results for each month of the years that specimens were collected in each of the three studies. This type of presentation was chosen to help compensate for the yearly changes that occurred on a month to month basis in all three studies. The primary difference between the three studies was essentially the number of isolations. However, the basic concept that the incidence of enteric virus infections is at a maximum during the summer and fall months is firmly established. In the present study this peak was reached in August, which was directly comparable to Honig's results and appeared one month later than the peak incidence as reported by Gelfand. The decline from this summer peak was gradual and the months of lowest recoveries in the three studies were December through April. A comparison of the

TABLE XV

VIRUS ISOLATIONS FROM SEWAGE SPECIMENS AS COMPARED TO ISOLATIONS OBTAINED FROM STOOL SPECIMENS

Month of specimen collection	MSU 1955-1957		Honig (1956) 1951-1953		Gelfand (1957) 1954-1955	
	Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive
January	66	9.1*	142	0	295	5.4
February	62	4.8	127	0.8	292	5.1
March	51	7.8	135	0.7	303	5.9
April	58	6.9	111	0.9	301	4.3
May	43	2.3	103	0	297	11.8
June	66	9.1	146	0.7	312	16.7
July	82	14.6	152	15.8	312	27.2
August	100	29.0	129	18.9	306	22.8
September	209	19.6	153	6.5	306	22.6
October	144	22.9	157	7.6	319	20.1
November	75	13.3	96	2.1	311	9.9
December	62	4.8	107	0.9	313	7.6
Total	1018	14.9	1558	4.9	3667	13.4

*If the specimen was positive both in tissue culture and upon mouse inoculation tests it was counted only once in computing the results.

results of this study with the other two revealed a more direct correlation to the work of Gelfand in Louisiana than to the work performed in West Virginia by the Honig group. It should be kept in mind that the Louisiana workers did not inoculate infant mice with any of their specimens and the total number of isolations would have undoubtedly been increased if this procedure would have been included in the experimental protocol.

The similarity of the results between this study, and the two other studies, are quite interesting. It would appear that samples from a point in the sewage system, receiving wastes from the whole or any particular section of a community, would serve as an excellent source of material to determine the enteric viral flora of that area. Of course, the work of Gelfand and Honig served other important purposes such as obtaining information on multiple infections and the persistence of a particular virus or viruses in any member of the study.

From relatively few samples, 30, taken in the December through May period, Coxsackie viruses were found on five occasions by Kelly, Clark and Coleman (1955). In the present study, only four Coxsackie isolations were obtained from 225 samples collected in the December through May period, with isolations made in each of four different months. Only one Coxsackie virus isolation was reported out of the 725 specimens that were processed during the winter months by Honig et al., (1956). During the period when enteric virus isolation is at a maximum, the number of Coxsackie virus isolations was dramatically increased. Kelly et al., (1955) reported positive isolations from

66 of 88 samples (75.5 per cent) of sewage during the months of June through November. In the present study, Coxsackie viruses were found in 72 of the 634 (11.4 per cent) samples tested during the June through November period. These results in sewage again compared favorably with the findings of Honig's group who recorded 28 positive Coxsackie virus isolations from 833 stool specimens that were tested (3.4 per cent).

During the 27 months that sewage samples were collected polioviruses were only detected in five of 951 tissue culture isolation trials. Type I poliovirus was identified on one occasion from a gauze pad sample collected in Lansing during the month of July 1957. Type III poliovirus was isolated from four samples collected in the East Lansing sewage plant. One positive isolation was obtained in September 1956 and one in October 1956. Two additional type III polioviruses were isolated during the month of March 1957. The overall recovery rate of polioviruses, 0.5 per cent, was considerably lower than those reported by Gelfand (4.6 per cent of 3667 specimens) or by Kelly, Winsser and Winkelstein (1957), who reported that 21 per cent of 308 samples from various upper New York State communities contained polioviruses. It should be noted that Kelly's group sampled sewage coming from areas having reported cases of paralytic poliomyelitis. Although Gelfand's study was based on the findings in a group of normal children, the fact that these subjects, for the most part, came from urban New Orleans and urban Baton Rouge, Louisiana, may have accounted for the higher incidence of poliovirus isolations than reported by Honig or from the results of the present

survey of sewage. No information regarding the incidence of clinical cases of poliomyelitis during the course of the study was reported by the Louisiana researchers or by the West Virginia group. A higher incidence of poliovirus recoveries was reported by both groups, from stools of children coming from the lower socio-economic areas. In the study by Honig, 10 of the 15 poliovirus isolations came from children living in a section of the city where the environmental sanitation and the economic status was generally low. Gelfand et al., (1957) make the statement that "there is a definite consistency in effect of race and socio-economic status in each year and for both polio and non-polioviruses. The Negro group bears the greatest burden of intestinal virus parasitism, followed in order by the white-lower economic and white-upper economic groups. This order is in inverse relation to the levels of personal and environmental sanitation in these three populations."

The lack of more poliovirus isolations in the present study may stem from the absence of any reported clinical cases of poliomyelitis in the city of East Lansing during the period of the study and also that the East Lansing community represents an upper middle class socio-economic status with high standards of environmental sanitation. The area of Lansing that was sampled also represented an area where good sanitation facilities existed. Another point to consider is that a low level of poliomyelitis incidence might affect the sensitivity of the sampling methods.

Table VII contains information regarding the distribution of Echo types 1-14 viruses identified during the study. If the

assumption is made that all 10 of the viruses grouped under the heading of Echo 1 or 13 represent a single type, then it may be said that this strain, when combined with the six Echo 7 viruses accounted for 53.3 per cent of the total number of Echo viruses identified during the study.

The high incidence of the Echo 7 virus type (20 per cent) was similar to the findings of Ramos-Alvarez and Sabin (1956). However, Echo 2 virus which was identified in 44 per cent of the viruses isolated in Ohio was not found in the present work. A comparison of the present findings with those of Ormsbee and Melnick (1957), who identified the viruses isolated by Honig et al., (1956), revealed that there was a similarity between the two studies in that Echo 7, 8 and 14 accounted for 12 of the 20 Echo viruses isolated in West Virginia. Again, no correlation between the incidence of Echo 1 or 13 virus existed between the two studies. In West Virginia, these strains were reported only twice in comparison to the 10 identifications obtained in the present study. Echo 3 and Echo 9 viruses were also identified in both studies. Echo 11 virus was identified from five of the samples in West Virginia but was not seen in samples from the Lansing area.

Logically, an explanation for these geographical differences in the Echo virus strains found in Ohio, West Virginia and Michigan should be brought forth. Unfortunately, this is not currently possible. Perhaps, the climatic conditions, environmental factors and geographical locations of the three sampling areas account for the differences? All that may be said is that carriers of a particular

Echo strain were present in some of the areas and were absent in the others. How many infected individuals need to be present to ensure the spread of a particular strain in a large proportion of the susceptible population is another unknown factor.

The finding of 17 tissue culture cytopathogenic agents, reported in Table VIII, that were not identifiable as Echo viruses 1-14, polioviruses or Coxsackie viruses presents an area for further speculation. Perhaps these viruses are members of the Echo 15-19 group, the prototype strains not being available until late in the study precluded their inclusion in the identification procedures. Another possibility was that these isolates represented mixed cultures which might have evaded the serum neutralization screening tests. Still another explanation is that they may represent new enteric viruses not previously classified. Cross-neutralization tests would reveal how many of these unknown viruses were similar to one another. Ramos-Alvarez and Sabin (1956) reported finding 25 unknown viruses in their study and they were later established as being the prototype strains for Echo 7, 8, 10, 11, 18 and 19 viruses. Ormsbee and Melnick (1957) reported that 10 of their isolates could not be identified. Three of these were found to be similar and have been accepted as the prototype strains for Echo 15.

Another point that must be considered in dealing with the unknown viruses reported in the present study is that their origin can not be determined with certainty. Perhaps they represent enteric viruses from animals other than man? The orphan viruses present in animals have been reviewed by Hsiung and Melnick (1958). Enteric

cytopathogenic bovine orphan viruses (Ecbo) and enteric cytopathogenic swine orphan viruses (Ecso) are found in apparently normal animals and their relationship to disease is also being investigated. Needless to say, it would be interesting to label the unknown viruses found in this study. One must not overlook the possibility that these viral agents may be members of a group not normally considered as belonging to the enteric virus classification, such as the adenoviruses and herpes virus which have been found in feces.

During the late summer and early fall of 1957, a group of 17 viruses was isolated from the East Lansing plant that did not exhibit the usual characteristics of the Echo or Coxsackie viruses. No visible evidence was seen that indicated pathogenicity for suckling mice when the original specimens were inoculated. However, when the infected tissue culture fluids were inoculated into suckling mice there was a very marked pathogenic effect manifested by paralysis and death of almost 100 per cent of the inoculated mice. The control mice in each litter remained normal throughout the test. Serum neutralization tests in tissue culture revealed that these viruses were not members of the Echo 1-14 group, or poliovirus types I or III.

If these isolates belong to the Coxsackie B virus group, which can be determined by neutralization tests in mice or tissue culture, then some explanation should be offered as to why these 17 strains exhibited such bizarre signs. Hsiung and Melnick (1958) have claimed that they have isolated a number of Coxsackie A9 and Coxsackie B strains that failed to infect infant mice unless these agents were first passed in tissue culture. These authors offered no explanation

for this behavior and none is apparent in the present study. However, one interesting epidemiological point can be brought forth. One of these viruses was isolated in July 1957 and one in August. In September, nine of these agents were isolated and in October the number fell to five. During the first week in November one was isolated. From this type of data, contained in Table IX, it appeared that a small scale"epidemic" occurred in East Lansing. The lack of a hospital in East Lansing precluded obtaining information on aseptic meningitis admissions for these months.

In Table XI are presented the results obtained in an effort to determine the effects of sewage treatment, in an activated sludge type treatment plant, on the removal of enteric viruses before the final effluent was discharged into the Red Cedar River. It was evident that there was a considerable reduction in the number of viruses isolated from progressive stages of treatment. However, the finding of virus in the final effluent on 4 of 41 occasions indicated that complete removal was not being accomplished by the treatment process. Further discussion of the problems that these findings may lead to is included in the section that follows on the effect of treatment on Salmonella.

Another difference that this study showed was the number of Echo viruses found in this study as compared to the results of Kelly et al., (1957) which showed that Echo viruses were only isolated on nine occasions from 308 samples tested in tissue culture. Mack and Fields (in press) have reported that the ultracentrifugation method, employed in this study, was more sensitive in detecting Coxsackie viruses from

sewage than the ion-exchange method used by Kelly and her co-workers. Perhaps the ultracentrifugation method is also more sensitive in detecting Echo viruses from sewage than the ion-exchange method?

Of the 22 tissue culture cytopathogenic agents listed in Table X that were lost on subsequent passage or storage, all that may be said is that there were definite indications that these agents produced a CPE in monkey kidney cell cultures that was similar in many respects to the enteric viruses that were identified. There was no evidence that these agents belonged to the indigenous monkey viruses reported by Rustigan, Johnson and Reihart (1955).

One of the major problems that exists in enteric bacteriology is the lack of a completely suitable enrichment medium that would serve to enhance the growth of Salmonella and inhibit the growth of other non-pathogenic organisms. In an attempt to determine if a new medium, SBG, was capable of providing the results desired in an enrichment medium, a comparison was made with tetrathionate broth.

A marked increase in the recovery of Salmonella from sewage was observed when the enrichment medium was changed from the tetrathionate broth to the SBG medium of Stokes and Osborne (1955).

A comparison between the two time periods showed that the number of recoveries of Salmonella from gauze pad samples increased in East Lansing from 30 per cent (17 of 56) to 60 per cent (43 of 71) when SBG medium was used. Even greater differences in results were obtained when the number of recoveries from sludge were compared; 33 per cent (9 of 27) when tetrathionate broth was used to 90 per cent (19 of 21) when SBG medium was employed. The seasonal distribution

of enteric infections must be considered in the comparisons made in this study. It is generally accepted (Maxcy, 1956) that cases of salmonellosis occur throughout the year but are at a minimum during the winter and spring months, rising to a peak in the late summer and early fall. One cannot say whether this seasonal variation influenced the findings reported in this work. The qualitative nature of the experimental procedure did not allow such comparisons to be made, since the number of positive samples indicated only that Salmonella were present in the sewage, and did not reflect on their overall concentration. It was not practical to run parallel studies using the two enrichment media. By using a variety of differential agar media the number of isolations might have been increased since there is no one perfect selective medium and the type of medium employed depends on what particular organisms are being looked for (Edwards and Ewing, 1955).

The results obtained indicated that the raw sludge specimens were an excellent source of material for the isolation of Salmonella, and higher recovery rates were obtained from this source than from the gauze pad samples. The high recovery rate from the final effluent showed that the gauze pad method of sampling serves its purpose well in monitoring supernatant fluid.

In comparing the recovery rates from the pad samples taken in East Lansing and Lansing during the first part of the study, the fact that Lansing is a highly industrialized community while East Lansing is largely a residential area should be taken into consideration. Large amounts of industrial wastes, deposited in the system, may have

contributed to the lower recovery of Salmonella by the gauze pad method of sampling.

Whether the Salmonella isolated originated from human or animal sources cannot be stated. However, the results of the study serve to strengthen the conclusion of Greenberg et al., (1957), that "typhoid, and presumably other enteric infections, should be recognized as a definite occupational hazard for treatment of plant operators." In addition to the direct threat to the plant operators, there exists a potential health hazard to the community. Even if the water is not directly used for drinking or swimming purposes, the return of unchlorinated final effluent, containing viral and bacterial pathogens, to a river flowing through a populated area should not be condoned. It is reasonable to suspect a contaminated water source as one of the many links in the transmission of disease. Proper chlorination of final effluents would serve as good preventive medicine.

SUMMARY

1. A total of 1018 sewage samples were tested for the presence of enteric viruses in monkey kidney tissue culture and/or suckling mice.

2. Thirty-five of the isolates were identified as Echo viruses or polioviruses. Seventy-six were identified as Coxsackie viruses, 36 samples were positive both in tissue culture and in suckling mice, an additional 17 viruses were not identified and 22 tissue culture cytopathogenic agents were lost in passage or storage.

3. Selenite brilliant green enrichment medium (62 of 92 samples positive) was superior to tetrathionate broth (32 of 142 samples positive) in the isolation of Salmonella from sewage.

4. A new Salmonella was identified (S. lansing), among the 133 salmonellae isolated, along with 22 other species.

5. Raw sludge as well as gauze pad samples provided the best source of material for the isolation of enteric viruses and Salmonella. However, large amounts of industrial wastes present in a sewage system seem to have a detrimental effect on enteric virus recovery from gauze pad samples.

6. Enteric viruses and Salmonella were found in all stages of sewage plant treatment tested, including final effluent.

7. Sewage samples provide a source of material from which the enteric viral flora of a population may be determined.

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