ABSTRACT

IDENTIFICATION OF SOME HUMAN ENTERIC VIRUSES ISOLATED FROM RIVER WATER AFTER WASTE WATER TREATMENT

By

Pornsawan Nimnoi

A total of 77 enteric viruses isolated from the Red Cedar River were identified. By screening all isolates for their ability to grow on HEp-2 cell cultures much time and effort was saved in identifying the viruses. After screening to separate the polioviruses, serological identification was then simplified.

An unexpected ratio of 72 out of 77 isolates (93.5%) were found to be polioviruses. The serological typing of polioviruses resulted in finding equal number of type II and type III strains of the virus but only a few type I.

Other enteric viruses recovered from the river were one strain of Coxsackie and four strains of ECHO viruses.

The high recovery rate of poliovirus is discussed both from the cultural aspect and the incidence of this virus in the population.

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Pornsawan Nimnoi

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INTRODUCTION

For many years the enteric viruses have been isolated from the intestinal tracts of man. These viruses have been found in patients suffering from disease as well as from individuals that appear normal.

Since these viruses are present in the stool of man, they are also present in large numbers and in great concentrations in the wastewater. It is important to remove these pathogens in the wastewater treatment for the recycling of water.

It has been known that the inactivated sludge treatment of wastewaters will remove large numbers of pathogenic bacteria and viruses. The chlorination of the final effluent is designed to destroy those pathogens that have escaped removal and prevent the dissemination of disease organisms.

The purposes of the present account are as follows:

a. To identify enteric viruses isolated from the effluent of East Lansing Wastewater Treatment Plant and Red Cedar River.

b. To determine the distribution of viral types.

c. To evaluate techniques used in this identification.

REVIEW OF LITERATURE

History of Enteric Viruses

The enteric virus group was established in 1957 (20). The viruses of the group are polio, Coxsackie groups A and B, echoviruses, and possibly others. All are inhabitants of the human alimentary tract and as a group they are associated with a variety of clinical syndromes, but the most severe expression is in diseases involving the central nervous system of man (54).

The first enteric virus, poliovirus, was isolated from urban sewage by Paul, Trask, and Gard in 1940 (32). Enteric viruses were regularly found in sewage, even after waste treatment (63,64,106). Recent concern for the possible low level transmission of viruses by water has been expressed by Berg (7). Infection with enteric viruses may result in clinical disease in only a small percentage of people who were infected with them. However, it might be possible for a low transmission of viruses to occur from contact with contaminated water and not be recognized against the background disease cases caused by personal contact. The potential effect of even a small degree of water and food pollution by virulent viruses might be greater than has been generally realized. Contamination with a small quantity of a potentially pathogenic virus might be of great consequence for some individuals, even if the proportion of affected individuals in the community remained quite low (42).

Enteric viruses have been isolated from rectal swabs from healthy American children who had no known contact with clinically recognized cases of enteric diseases (80).

The use of the Sabin vaccine as an immunizing agent has resulted in interesting recoveries of viruses from water. A number of observations already suggested that vaccination has little influence upon the susceptibility of the lower gastrointestinal tract of man to poliovirus infection (53). In 1967 Theios *et al.* (100) used the technique described by MacCallum *et al.* (65) and compared the effect of two different sewage treatment methods on the recovery of poliovirus during a mass immunization program. This program was held in Lake County, Illinois. The vaccine used in this program was trivalent oral poliovirus vaccine. The results indicated that no viruses were recovered from sewage prior to the initial mass immunization. After immunization, however, varying combinations of the three types of poliovirus were isolated from the influents of treatment plants for at least as long as 56 days.

Mack *et al.* (60) found that there were fewer virus isolations in the winter and spring months, but in the warmer months viral content of sewage was high. This is also true for the seasonal distribution of *Salmonella*. The density of enteric viruses in the normal wastewater was established to be about 7000 plaque forming units (PFU) per liter. The concentration of viruses increased during periods of virus outbreaks or virus immunization campaigns (16).

The inactivation of virus by any sewage treatment process has been found to be inefficient. This implies a maximum removal by each step in the process of treatment is not sufficient to remove or inactivate large numbers of virus particles. Since the wastewater

effluents are deposited into rivers or onto soil surfaces, the presence of enteric viruses could produce a hazardous condition. Kleinman (49) calculated that as few as ten infectious tissue culture doses of poliovirus might be able to produce disease in man.

Much work has been done to obtain information on removal of virus in wastewater treatment. The available evidence indicated that only a minor amount of the total viruses was removed by the primary sedimentation (8,11,15,43,64,66).

Of the two main types of sewage treatment, trickling filtration was considered to be inferior to activated sludge treatment in its capability for removal of viruses. Trickling filtration appears to remove only minor amounts of viruses from a primary settled waste (8,11,15,43,64,66). Kelly and Sanderson (43) found that plaque counts were reduced by amount 40% in a trickling filter effluent. The activated sludge process was capable of removing 90% of viruses (8,11,15,43,45,64,100). In another study the activated sludge process removed about 1.5 log units of viruses from the wastewater, but the efficiency decreased during the winter or cold months (63).

By improving the method for isolating of viruses, the presence of enteric viruses had been demonstrated at any time of year in raw wastewater (63). These results indicated that it is important to consider the design of sewage treatment systems in terms of their ability to remove viruses, bacteria, and other agents of pollution.

Since our present system is very inefficient in removing enteric viruses, the only positive way of providing a virus free effluent would be as recommended by the following (10,29,44,59).

a. increasing chlorine concentration;

b. adjusting pH;

c. increasing the contact time with chlorine,

all of which will depend upon the amount of viruses and strains of viruses present (16,44,108). As indicated by Burns and Sproul in laboratory experiments (10), the time of contact between virus particle and chlorine dosage for virus inactivation beyond 90% was important. High combined chlorine residuals could produce a high destruction rate of the viruses. Lothrop and Sproul (59) found when working with T_2 and type 1 poliovirus in the laboratory experiment, they could eliminate 99.99% of the virus and therefore theorize somewhere between 28 and 44 ppm of chlorine residuals would be required to destroy 99.99%. These large amounts of chlorine would complicate any direct reuse of the wastewater.

Due to the ease with which the enteric viruses could be isolated from feces and sewage materials, several investigators undertook studies involving the carrier state and viruses present in the normal individuals. The refinement of tissue culture methods and the expansion of their use into the field of virology in the 1950's led to the isolation of large numbers of hitherto unknown viruses that were not pathogenic for laboratory animals. It early became apparent that these agents could be isolated from healthy children (70,80) as well as from patients with the aseptic meningitis syndrome, and that multiple types existed (80). As more investigators entered the field, more and more of these new agents became recognized. Because their relationship to human diseases was unknown, and because they failed to produce illness in laboratory animals, including infant mice, they were called "orphan"

viruses or human enteric viruses. Later the name was changed to enteric cytopathogenic human orphan viruses or ECHO viruses. This prompted the National Foundation of Infantile Paralysis to establish the Enteric Cytopathogenic Human Orphan Virus Committee in 1955 (19). By 1957, the Committee on ECHO viruses changed its name to the Committee on Enteric Viruses because the enteric viruses group consisted of more than the ECHO type (20). As the number of enteric viruses increased, some new strains did not fit the criteria of the subgroup. Furthermore, as new strains of established types were studied, some were determined to have properties different from those of prototypes. Some strains, when first isolated, grew best in cell cultures, under conditions different from those previously found optimal for the enteric viruses. Some did not readily produce the same lesions when injected into mice. Some highly variable characteristics frequently represented strain differences.

Because of the different characteristics found between strains and subgroups of the enteric viruses, the Committee in 1962 (21) established that known enteric viruses should be classified on an antigenic basis. New antigenic prototypes were then assigned enteric virus type numbers. This classification was recommended until adequate knowledge became available to permit establishment of properly defined subgroups.

A definition of enteric viruses was given by the Committee in 1962 (21). They classified these viruses as transient inhabitants of the alimentary tract (although they may also be found in the nasopharynx), which exist in multiple antigenic types, and have the following properties: (1) particle size about 28 mμ in diameter, (2) ribonucleic acid core, (3) ether resistance, and (4) cationic stabilization

to thermal inactivation. The new classification was desired to avoid future assigning of antigenically identical variants of the same virus to more than one subgroup.

In 1963 enteric viruses were established as a subgroup of picornaviruses (40). Finally the enteric viruses of human origin included the three types of polioviruses, the 23 Group A and 6 Group B Coxsackieviruses and 31 echoviruses.

Characteristics of the Polioviruses

This virus produces poliomyelitis, sometimes known as infantile paralysis. Most frequently produced inappearent and non-paralytic infection, but occasionally the well known paralytic manifestation occurred. The establishment of its viral etiology was first done by Lansteiner and Popper in 1908-09. Poliovirus was a spherical particle approximately 30 mμ in diameter. X-ray diffraction and electron microscopic studies suggested that the capsid was made up of a number of capsomeres arranged in icosahedral symmetry. The actual number of capsomeres was not yet established, but Cohen (17) suggested the number of capsomeres as 32, 42, and 60. Because of their particle symmetry, poliovirus preparations were readily crystallized; it was the first animal virus to be crystallized (89,95). Crystalline arrays of poliovirus were sometimes seen in the cytoplasm of infected cells (17).

A property which indicated an absence of lipid in poliovirus was its resistance to diethyl-ether, chloroform, bile, and detergents (55). Poliovirus was found to be stable at low pH (4,58). This resistance would protect an enteric virus from inactivation by gastric juices during its passage through the stomach (79). Also, poliovirus

was inactivated by heat, desiccation, formalin, chlorine, and ultraviolet irradiation (61,112). Wallis and Melnick (101) reported that molar divalent cations, particularly Mg, stabilized the titers of poliovirus suspensions against the effects of heating to 56 C. Growth of polioviruses was also inhibited by actinomycin-D and 2-thiouracil (2TU) as reported by Cooper (25) and Steele and Black (96).

In 1949 polioviruses were classified into 3 serotypes (9,46). The original prototype strains (Brunhilde, Lansing, and Leon) were designated as type I, II, and III. All strains thus far recovered in the field have been shown to belong to one of these three serotypes (18). Evidence that the type specificity of polioviruses resided in the RNA moiety has been reported. Infections of tissue culture host cells with viral RNA yielded progeny particles which were type-specific for the inflecting RNA (1). Each type was stable and showed no tendency for antigenic variation (17). Very minor antigenic differences among strains within a single type had been demonstrated by refined neutralization techniques (27,109). By means of a variety of properties, two distinct antigens have been identified for each poliovirus serotype (39,52,68). The D antigen, which sediments at 160 S, was associated with "complete" virus particles whether infectious or non-infectious. The C antigen, which sediments at 80 S, was associated with particles lacking RNA. The C antigen might be produced from D antigen by heating to 56° C, by ultraviolet irradiation, by alkaline pH, and by phenol (51,52,86).

Characteristics of the Coxsackieviruses

These viruses were first isolated from fecal specimens from two boys suffering from what appeared to be paralytic poliomyelitis by

Dalldorf and Sickles in 1948 in Coxsackie, New York. These two Coxsackie A viruses were the first evidence of the existence of the large and growing number of hitherto unsuspected enteric agents currently subdivided into Coxsackie viruses and echoviruses. The Coxsackieviruses had two unusual characteristics. First, among the small laboratory animals for which they were tested only the immature mice, Swiss strain, were fully susceptible; once the animals were weaned, they were resistant to infection. The second characteristic was that they induced, in susceptible animals, lesions of the striated muscles (77). Two groups of Coxsackieviruses, A and B, have been recognized by the lesion which they produced in suckling mice. Twenty-three of Group A and six of Group B have been classified by type-specific neutralization tests. With improvement in tissue culture techniques, Coxsackieviruses propagated in tissue cultures were reported (67). Coxsackievirus particles, which were about 28 mμ in diameter, resemble poliovirus particles in size and density. The crystallization of Coxsackieviruses was evidence of symmetrical capsid structure (17). Like polioviruses, these agents withstand exposure to a wide range of pH, i.e., pH 2.3 to 9.4 for one day and pH 4.0 to 8.0 for 7 days (84). Aqueous suspensions of certain strains were not inactivated at 55° C for 30 min. but were inactivated at 60° C for the same period. Temperatures 10° to 20° C higher were required for inactivation when the virus was suspended in milk, cream or ice cream (41). In the presence of high concentrations of Mg and other divalent cations, above 0.5 molar, the Coxsackieviruses were stable when exposed to temperatures as high as 50° C for 1 to 3 hours (102). Certain commonly used antiseptics, including ethanol (70%), Lysol (5%), Roccal (1%) and ether, failed to inactivate

the agents. However, treatment with 0.1 N HCl or 0.3 formaldehyde effects rapid inactivation (71). Coxsackieviruses were neither inactivated nor inhibited *in vitro* or *in vivo* by a variety of chemotherapeutic agents, including penicillin, streptomycin, chloramphenicol and tetracycline compounds.

Twenty-three different antigenic types have been identified among the Coxsackieviruses of Group A and six among those of Group B (21). Two antigenic components have been demonstrated. One is a group complement fixing antigen which reacts with human immune serum only, and is found in the light non-infective fraction separated by density gradient centrifugation. The other is a type-specific antigen which reacts with human immune serum in neutralization and precipitation tests and with monkey and mouse immune sera in complement-fixation tests. This anitigen may be converted to the group specific antigen by heat to 56° C for 30 minutes. So far, Coxsackieviruses A1 to 23 and B1 to 6 have been distinguished by type-specific neutralization and complement-fixation tests. One virus, previously classified as Coxsackievirus A23, has not been reclassified as echovirus type 9. Antigenic cross reactions between Coxsackieviruses A3 and 8, A11 and 15, and A13 and 18, and between various B strains, have been noted. A virus, which is called "Thai C-18" by Behbehani, previously believed to be a new enteric type, has been shown to belong to the Coxsackievirus A20 subgroup. The indicator strain of choice within the subgroup is A 20A. It is neutralized by antisera against all 4 variants (5). The most often encountered epidemic strain is reported to be Coxsackie A23 virus (77). To reduce complications of typing these viruses by neutralization tests, a colorimetric method for typing has been suggested (91).

Characteristics of the ECHO Viruses

The virus particle is approximately 28 mμ in diameter. By negative staining in electron micrographs it was observed that they possessed cubical symmetry with 32 capsomeres. Like other enteric viruses, echovirus contains RNA, and the virus is resistant to diethyl ether, stable at pH 3, and survives at 50° C in the presence of 1 M magnesium chloride ($MgCl_2$) (102). Echoviruses readily were inactivated by formalin, but their antigenicity may have been preserved if the inactivation was allowed to proceed in the presence of 1 M $MgCl_2$. Upon harvesting of infected tissue cultures, echoviruses do not become photosensitive, as other viruses may, by the addition of acridine dyes (proflavin, toluidine blue or neutral red) to the suspension of mature virus. However, if the virus is purified before the dye is added, the virus can be made photosensitive (103,104). If an echovirus is grown in cells containing the dye, the harvested virus is light-sensitive, regardless of the medium. Ordinary visible light destroyed the infective unit of the virus but not its immunizing antigen. This suggested a unique method for preparation of viral vaccines. Laboratory disinfectants such as alcohol (70%), Lysol (5%), and Roccal (1%) were ineffective against echoviruses (47). Also, they were resistant to action of all known antibiotics and chemotherapeutic agents (55).

Thirty-one antigenic types of echoviruses have been demonstrated with some types agglutinating human group O red cells. The hemagglutinin was the infective virus particles. The optimal conditions for hemagglutination were different for various echovirus types. This agglutination could be inhibited by specific immune sera. Neutralization and complement-fixation tests can be used for routine

typing of echoviruses. Neutralization and hemagglutination-inhibition tests were fairly specific, while a significantly greater number of heterotypic reactions were obtained with the complement-fixation test (12). Antigenic relationship between echovirus type 29 and 32 has been demonstrated (90). Since there are 31 antigenic types of echoviruses, in order to reduce expense and time consumed in typing of these viruses, neutralization of immune serum pools was suggested by Schmidt (93). Furthermore, different morphological types of plaques produced by the various types of echovirus on different types of cell have been used as a criterion for identification of echoviruses (17).

Characteristics of the Reoviruses (Respiratory Enteric Orphan Virus)

The Reovirus is listed in this thesis for the reader's information, although not all authorities agree that the reovirus is a true enteric virus. This is a group of RNA viruses which have been isolated from the feces and respiratory secretions of healthy persons, as well as from patients with a variety of clinical illnesses, particularly minor upper respiratory and gastrointestinal diseases. The reovirus virion contained double-stranded RNA with a molecular weight equivalent to about 10^7 daltons. The average diameter of reovirus, as measured by electron microscopy, was 60-75 mμ (17). The virion is probably icosahedral and possesses a capsid of 92 prismatic, hollow capsomeres. Reovirus lacks lipid, and therefore infectivity is unaffected by diethyl ether or chloroform. Thermolability varies from type to type (e.g., at 37° C the half life of infectious type 1 reovirus is 19 hours, whereas that of type 3 is less than 3 hours [26]).

Three immunological types of reoviruses can be distinguished by neutralization and hemagglutination-inhibition titrations. The three types are antigenically related by a cross-reacting soluble antigen that can be measured by complement-fixation tests. All three immunological types agglutinated human group O erythrocytes; in addition, type 3 agglutinated ox red blood cells (26).

Characteristics of the Hepatitis Agents

Although infective hepatitis appears as an enteric disease epidemiologically, it is still questionable whether it is caused by hepatitis viruses. The hepatitis agent thought to produce hepatitis has neither been transmitted to laboratory animals nor cultivated consistently in tissue cultures. There are two ways of transmission of hepatitis agents. One is transmitted by fecal-route and is called infectious-hepatitis agent. The other way of transmission is by injection of infected blood or its products and is called serum-hepatitis agent. Since infectious-hepatitis is transmitted by fecal-route, we will classify it as an enteric virus.

The infectious-hepatitis agent has been extremely difficult to prove because no experimental animal, with the possible exception of chimpanzees and marmosets, are susceptible to infection. Rightsel and his colleagues isolated an agent which they thought was the virus from the serum and feces of patients with infective-hepatitis, using Detroit 6 cells (17). These cells, which were originally derived from human bone marrow, were malignant epithelial-like cells. The agent was thought to be a very small particle about 15 mμ in diameter. It was shown to be very stable, resisting heat at 60° C for 30 minutes, and surviving at -20° C for more than 2 years. It was also resistant to

ether and to chlorine in concentration of 1 part per million (76). Infectivity could be destroyed by heating at 100° C for 30 minutes or by treatment with tricresol (0.2%), β-propiolactone (4 mg/ml), or ethylene oxide (liquid or gas) (27). It should be mentioned at this time that all these properties of this agent have not been confirmed.

Application of Tissue Culture Methods

Tissue culture methods have greatly facilitated the isolation and identification of enteric viruses. Enders (30) first utilized tissue culture methods to cultivate the 3 strains of poliovirus in human embryonic tissues. As a result of these techniques the viruses could be studied on a larger scale and with less expense than was formerly possible. In addition, the presence of a viral agent in inoculated cell cultures might be detected by observing the cytopathogenic effect, i.e., cellular degeneration, for enteric viruses. Robbins (83), using tissue culture methods, isolated poliovirus directly from the patient's feces. Riodan (82) reported isolating and serologically typing strains of poliovirus from human stools and spinal cords in roller tube cultures of monkey testes. Scherer (90) demonstrated propagation of all 3 types of poliovirus in human uterine carcinoma cell cultures (HeLa) established by Gey (34). Syverton and Scherer (98) found that monkey kidney epithelial cells were more susceptible to infection by poliovirus than was the HeLa cell line.

In 1954 Dulbecco and Vogt (28) developed a procedure for the preparation of cell suspensions of monkey kidney by trypsin treatment, which was an important step in tissue culture methods. Younger (113) improved this procedure to prepare large numbers of replicate cultures for use in isolation and identification of enteric viruses.

Utilization of tissue culture system for virologic studies is now within the realm of smaller laboratories as there are several reliable commercial sources of tissue culture media, sterile serums, and nontoxic detergents for glassware preparation. It is even possible to purchase cell cultures which are ready for inoculation, thus eliminating the need for maintaining lines of cells or animals necessary for specific tissues or cells. Most important, smaller laboratories now have access, through commercial channels, to high quality immune serums for use in identification of the viral agents which they isolate in cell cultures.

Many of the enteric viruses cause disease in man, ranging from severe paralysis, myocarditis, and skin rashes to common colds, depending upon the organ attacked. Infection is generally at the subclinical level. Clinical manifestations are being more common for some types than for others. Different enteric viruses, as well as other viruses not in the enteric virus group, might produce the same syndrome. On the other hand, the same enteric virus might cause more than a single syndrome. For these reasons clinical disease has not been considered satisfactory as a basis for classification.

Within the enteric virus subgroup of Picornaviruses, 63 serotypes have been recognized (55). Attempts to simplify the task of enteric virus identification have led to a number of new techniques and modifications or adaptations of older methods which might help to reduce time and expense. Various antiserum pools were proposed as a means of decreasing the number of neutralization tests that had to be performed.

A number of enteric viruses agglutinated human erythrocyte, and consequently could be typed by hemagglutination-inhibition tests

(31). The complement-fixation test also has been adapted to the identification of enteric virus. Screening by the use of HEp-2 cell cultures was suggested by Marchetti and Gelfand in 1963 (67). This method has enabled the laboratory to reduce significantly the number of neutralization tests necessary for preliminary identification of enteric viruses. Enteric viruses were segregated into two major groups: (a) polioviruses and Coxsackie B viruses, and (b) echo types and Coxsackie A9. Neutralization tests must be performed for the final identification of all viruses.

MATERIALS AND METHODS

Composition and Preparation of Tissue Culture Media and Reagents

I. Antibiotic Solution--Penicillin-streptomycin solution

Prepared by dissolving 1,000,000 units of penicillin and 1,000,000 mμ of streptomycin in 100 ml of Hanks' balanced salt solution 1 X. The solution was stored in a frozen state.

II. Balanced Salt and Buffered Saline Solution--Hanks' balanced salt solution 10 X concentration

A 1-liter amount of the solution contained the following: (a) NaCl, 80.0 g; KCl, 4.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; Na_2HPO_4, 0.48 g; KH_2PO_4, 0.6 g; glucose, 10 g; (b) $CaCl_2$, 1.4 g; (c) phenol red, 0.2 g. The reagents of (a) were dissolved in 800 ml of double distilled water. The reagents of (b) and (c) were dissolved separately in 100 ml of double distilled water. After dissolving, solution (c) was slowly added to solution (a). Then solution (b) was added to make the final volume of 1,000 ml. Three to four milliliters of chloroform were then added as a preservative. For use the 10 X solution was diluted to 1 X with double distilled water and sterilized by autoclaving at 10 lb pressure for 10 minutes.

III. Sodium Bicarbonate Solution

Prepared by dissolving 3.5 g of $NaHCO_3$ in 100 ml of double distilled water. The solution was sterilized by autoclaving at 10 lb pressure for 10 minutes.

IV. Cell-Dispersing Agent--Trypsin solution 0.25%

Dissolved 0.25 g of trypsin in 100 ml calcium free Hanks' solution. Sterilization was done by filtration through Seitz sterilizing filter. Calcium free Hanks' solution contained (per liter): NaCl, 8.0 g; KCl 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; Na_2HPO_4 anhydrous 0.05 g; KH_2PO_4, 0.06 g; sucrose, 1.0 g; $NaHCO_3$, 0.35 g; phenol red, 0.02 g. The reagents were dissolved in 1000 ml of double distilled water.

V. Calf Serum

Calf serum was purchased from commercial laboratories. The serum was inactivated by heating in the waterbath at 56° C for 30 minutes. Sterility test was done by inoculating 0.1 ml of inactivated serum to 5 ml of Brain Heart Infusion Broth. The cultures were observed for bacterial growth at intervals up through 72 hours.

VI. Cell Culture Media--Lactalbumin Hydrolysate Medium

A 100-ml amount of medium contained the following: lactalbumin hydrolysate, 0.5 g; double distilled water, 89 ml; Hanks' balanced salt solution (10 X), 10 ml. The solution was sterilized by autoclaving at 10 lbs pressure for 10 minutes. The $NaHCO_3$, antibiotics, and serum were added at the time of use.

The nutrient media employed for cell culture in virology may be divided into two broad categories; i.e., growth medium and maintenance medium.

Growth medium was employed for initiation of the cell cultures. They contained 10 to 20% inactivated calf serum for promoting rapid cellular proliferation. Maintenance medium was intended to maintain the cultures in a slow, steady state of metabolism during the time of viral proliferation. They contained 1 to 2% inactivated calf serum.

Tissue Cultures

I. HEp-2 Cell Line

Human carcinoma of the larynx was the origin of this cell line. The cells were epithelium-like. The strain of HEp-2 cells maintained in the laboratory were originally obtained from the Michigan Department of Public Health Laboratories, Lansing, Michigan. The cultures have been propagated in this laboratory on lactalbumin hydrolysate medium. The cell stock was grown in a milk dilution bottle using medium containing 20% calf serum, with $NaHCO_3$, and antibiotics containing 100 units of penicillin and 100 mμ of streptomycin. The growth fluid in stock cultures was changed when the medium reached pH 6.8, as indicated by phenol red in the medium.

II. Primary Monkey Kidney Cells (African-Green *Cercopithecus aethiops*)

Primary monkey kidney cells were purchased from Grand Island Biological Company, New York. The monolayer was ready to use when received.

Handling of Cell Cultures

I. Trypsin Treatment of Monolayer Cell Cultures for Subcultures

a. Growth medium was removed from the cell culture; the monolayer was then washed with medium without calf serum.

b. A 0.25% trypsin solution was added to the monolayer; the culture was held at room temperature for 30 to 60 seconds with the trypsin covering the cell sheet.

c. The culture was then inverted to remove the trypsin from the cell sheet; care must be taken not to disrupt the cell sheet.

d. The cells were suspended in 20 ml of growth media.

e. Suspended cells were transferred to 2 milk dilution bottles in the amount of 10 ml each. Each culture received 2 ml inactivated calf serum.

f. Incubated subcultures at 37° C.

II. Preparation of Cell Cultures in Tubes

The method was the same as for the subcultures, but suspended cells were transferred to tube in the amount of 1 ml for each tube. Then calf serum was added, and they were incubated at 37° C.

Titration of Virus in Tube Monolayer Cell Culture, Tissue Culture Dose 50% End Point (TCD 50%)

Tissue culture dose 50% end point is the highest dilution of virus suspension which produced degeneration in 50% of the cell culture inoculated. It is used for determining the amount of infectious virus present in a given culture fluid; TCD 50% is calculated by the method of Reed and Muench (81). The end point was determined by interpolation from the cumulative frequencies of positive and negative responses to occur in a dilution in which there would be 50% positive responses and 50% negative responses. One viral unit was considered to be contained in this dilution. The reciprocal of the dilution yielded an estimate of the number of viral units per inoculum volume of the undiluted sample of virus and was expressed in multiples of the 50% end point.

a. Logarithmic dilutions of viral suspension were prepared in medium without calf serum, pH 7.5.

b. Tube cultures were washed with medium without calf serum for approximately 30 minutes.

c. Each of three tube cultures was inoculated by 0.1 ml of each dilution.

d. The inoculated cultures were incubated at 37° C for 30 minutes to allow adsorption of viruses to the cells.

e. The maintenance medium was added to each tube in the amount of 0.9 ml.

f. All tubes were incubated at 37° C and observed microscopically every day for the cytopathogenic effects (CPE).

g. The TCD 50% was calculated by the method of Reed and Muench (81).

Neutralization Tests

a. Virus was diluted to contain 200 TCD 50% in a volume of 0.1 ml (as determined by previous titration of the virus). Viral dilution was prepared in medium without calf serum.

b. Specific rabbit antiserum against a known virus was diluted to the recommended titer with medium without calf serum in a volume of 0.1 ml dilution.

c. To each 0.2 ml of viral dilution was added 0.2 ml of antiserum and mixed.

d. These mixtures were incubated at room temperature for 30 minutes.

e. Tube cultures were washed with medium without calf serum.

f. To each of three tubes was added 0.1 ml of a virus-antiserum mixture.

g. For virus control 0.1 ml of 200 TCD 50% of virus was added to each of three tubes. All tubes were incubated at 37° C for 30 minutes.

h. To each tube of all tests was then added 0.9 ml of maintenance medium.

i. All tubes were incubated at 37° C.

j. Results were recorded daily.

Cytopathogenic Agents

All cytopathogenic agents identified in the study were isolated from water samples collected in September and October, 1969, by Mr. Robert Lu. The cytopathogenic agents were kept in a deep freezer all the time.

RESULTS

The viruses identified in this work were isolated from sewage samples collected at the East Lansing Wastewater Treatment Plant or from the Red Cedar River. The river receives the effluent from the wastewater plant. All of the viruses were isolated by this laboratory during September and October of 1969. A total of 77 cytopathogenic agents were isolated and the possibility of any one of the 63 serotypes of the Picornaviruses seemed possible.

Using the finding of Marchetti and Gelfand (67) that HEp-2 cells supported the growth of the three serotypes of poliovirus but did not support growth of many of the other enteric viruses, all isolates were first tested on this cell line.

All 77 strains of viruses were seeded upon HEp-2 cells. The results of this experiment were illustrated in Table 1. As can be

Table 1. Total numbers of cytopathogenic agents found to propagate on HEp-2 cell cultures

Month (1969)	Number positive / Number agents tested	Percent Positive
September	6/6	100
October	67/71	94.4
Total	73/77	94.8

seen, 73 out of 77 strains, or 94.8% of these viruses, produced cytopathogenic effect, indicating that the virus strains were either polio or members of the Coxsackie B group. The results of these infectivity tests also made it possible to determine the potency or TCD 50% of the majority of these viruses on this secondary cell line. In Table 1 the number of isolations for September and October are given. It was arbitrary that the six isolates in September were all propagated on HEp-2 and that less than the total isolated in October grew on this cell line.

The potency on infectivity titer was determined prior to the neutralization test to identify the particular virus. In Table 2, the infectivity tissue culture dose 50% (TCD 50%) is given for twenty of the virus strains, all of which were isolated from the final effluent (see Figure 1). The final effluent from here is discharged into the river resulting in dilution of the viruses. The TCD 50% range for these viruses was from $10^{-4.5}$ through $10^{-6.5}$ with an average of $10^{-5.82}$. All of the infectivity titers are within a range found with most poliovirus strains and indicate that these agents could all be poliovirus.

In Table 3 the results of the neutralization tests are given for these 20 virus strains isolated from the final effluent. The most frequent isolate was type II where 11 strains were recovered. This was followed by 7 from type III and 2 from type I. The average infectivity titer also indicated that the type II strains gave a higher infectivity titer than did the types I and III.

These cytopathogenic agents isolated directly where the effluent enters the Red Cedar River (see Figure 1) were also identified in a

Table 2. The infectivity titer (TCD 50%) of viruses isolated from the final effluent before discharge into the Red Cedar River

Date isolated	TCD 50% end point
9-8-69	$10^{-5.5}$
9-10-69	$10^{-5.5}$
9-22-69	$10^{-6.25}$
9-24-69	$10^{-6.25}$
9-26-69	$10^{-5.5}$
9-28-69	$10^{-5.5}$
10-1-69	$10^{-6.5}$
10-3-69	$10^{-4.5}$
10-6-69	$10^{-5.5}$
10-7-69	$10^{-6.5}$
10-8-69	$10^{-6.25}$
10-9-69	$10^{-5.25}$
10-10-69	$10^{-6.25}$
10-12-69	$10^{-6.5}$
10-13-69	$10^{-6.25}$
10-14-69	$10^{-5.75}$
10-17-69	$10^{-5.5}$
10-22-69	$10^{-6.25}$
10-27-69	$10^{-5.5}$
10-29-69	$10^{-5.5}$
Average	$10^{-5.82}$

Figure 1. Points of collection of water samples.

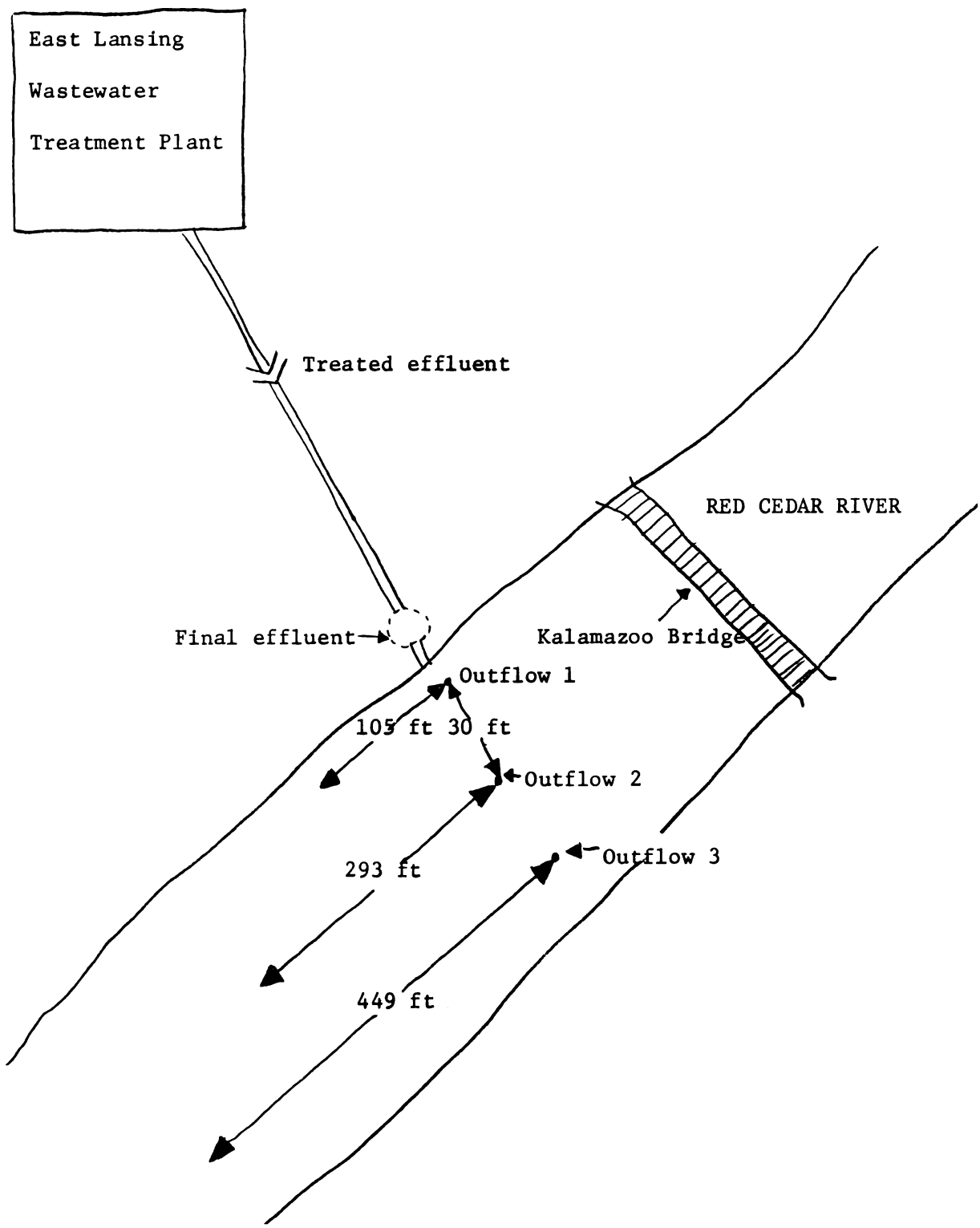

Table 3. Identification of 20 virus strains isolated from the final effluent

Neutralized by type antiserum	Number each type	Average TCD 50%	Percent of each type
I	2	$10^{-5.5}$	10
II	11	$10^{-6.02}$	55
III	7	$10^{-5.6}$	35
Total	20		

similar manner. They were separated from the agents isolated above because they were found at a point where the wastewater plant effluent is first diluted with the river water and where they might possibly be affected by the chemical discharges, if present, upstream. The infectivity titer of the 18 viruses isolated in the three outflow areas is given in Table 4. The average titer of outflow 1 was $10^{-5.39}$; that of outflow 2 and 3 were $10^{-6.38}$ and $10^{-6.45}$, respectively. There was some difference in infectivity titers of the three areas. In area 1 (see Figure 1) the area closest to the effluent proper was lowest in titer and had possible less dilution with river water than the other two outflow areas.

In Table 5, the results of the neutralization test on 18 strains of virus are given. More than half (55%) of the viruses isolated from the outflow area were type III and the average infectivity titer of this group was $10^{-6.25}$ and higher than either of the type I and type II strains.

The data in Table 6 give the infectivity titers of 35 of the strains of viruses isolated as far as 449 feet downstream from the

Table 4. The infectivity titer (TCD 50%) of viruses isolated from the outflow area (see Figure 1)

Collection point	Date isolated	TCD 50% end point
Outflow 1	10-6-69	10^{-6}
	10-7-69	$10^{-5.5}$
	10-8-69	$10^{-5.75}$
	10-9-69	$10^{-6.25}$
	10-10-69	$10^{-6.25}$
	10-12-69	$10^{-3.5}$
	10-13-69	$10^{-4.5}$
	Average	$10^{-5.39}$
Outflow 2	10-6-69	$10^{-6.5}$
	10-7-69	$10^{-6.5}$
	10-8-69	$10^{-6.25}$
	10-9-69	$10^{-6.25}$
	10-10-69	$10^{-6.25}$
	10-14-69	$10^{-6.5}$
	Average	$10^{-6.38}$
Outflow 1-3	10-3-69	$10^{-6.5}$
	10-17-69	$10^{-6.5}$
	10-22-69	$10^{-6.5}$
	10-27-69	$10^{-6.5}$
	10-29-69	$10^{-6.25}$
	Average	$10^{-6.45}$

$F_s = 4.72^*$

$^* = P<0.05$

There is a significant ($P<0.05$) added variance component among collection points for TCD 50% end point of cytopathogenic agents isolated.

Table 5. Identification of 18 virus strains isolated from the outflow area

Neutralization type antiserum	Number each type	Average TCD 50%	Percent of each type
I	3	$10^{-5.67}$	16.66
II	5	$10^{-5.75}$	27.78
III	10	$10^{-6.25}$	55.56
Total	18		

outflow area. These viruses were subjected to great dilution and mixed with other components that were present in the river water. Of the 12 viruses isolated 105 feet below the outflow, the average titer was $10^{-5.6}$. Viruses isolated 293 and 449 feet below the outflow averaged $10^{-6.37}$ and $10^{-6.06}$, respectively. Again, the longer exposure to the river water did not necessarily decrease the infectivity titer to these strains of viruses as might be expected.

When these 35 strains of virus from above were serologically typed, 34 were found to be poliovirus (Table 7). Separating the types it was found that half of 34 strains were approximately equally divided between types II and III. Only 8.57% of the poliovirus strains were type I.

The only other virus recovered and propagated on the HEp-2 cell line was a single Coxsackie B-3 virus and this was recovered 105 feet below the outflow in the river.

In Table 8, the total number of poliovirus strains isolated during September and October is given. Since only six strains were collected in September and all 6 were positive, no comparison can be made with

Table 6. The infectivity titer (TCD 50%) of virus isolated from the Red Cedar River downstream to the effluent

Collection points	Date isolated	TCD 50% end point
Red Cedar River (105 ft downstream)	10-3-69	$10^{-5.5}$
	10-6-69	$10^{-6.5}$
	10-7-69	$10^{-5.75}$
	10-8-69	$10^{-6.25}$
	10-9-69	$10^{-4.75}$
	10-10-69	$10^{-5.75}$
	10-12-69	$10^{-5.75}$
	10-14-69	$10^{-4.75}$
	10-17-69	$10^{-5.5}$
	10-22-69	$10^{-5.75}$
	10-27-69	$10^{-5.5}$
	10-29-69	$10^{-5.5}$
	Average	$10^{-5.6}$
Red Cedar River (293 ft downstream)	10-3-69	$10^{-6.5}$
	10-6-69	$10^{-6.5}$
	10-7-69	$10^{-6.5}$
	10-8-69	$10^{-6.5}$
	10-9-69	$10^{-6.25}$
	10-10-69	$10^{-6.5}$
	10-12-69	$10^{-6.25}$
	10-13-69	$10^{-6.5}$
	10-14-69	$10^{-5.75}$
	10-17-69	$10^{-6.5}$
	10-27-69	$10^{-6.5}$
	10-29-69	$10^{-6.25}$
	Average	$10^{-6.38}$
Red Cedar River (449 ft downstream)	10-3-69	$10^{-5.5}$
	10-6-69	$10^{-6.5}$
	10-7-69	$10^{-6.5}$
	10-8-69	$10^{-5.5}$
	10-9-69	$10^{-6.25}$
	10-10-69	$10^{-6.5}$
	10-12-69	$10^{-6.5}$
	10-14-69	$10^{-6.25}$
	10-17-69	$10^{-6.25}$
	10-22-69	$10^{-4.5}$
	10-27-69	$10^{-6.5}$
	Average	$10^{-6.07}$

$F_s = 8.23^{**}$; ** = $P<0.01$.

There is a significant ($P<0.01$) added variance component among collection points for TCD 50% end point for cytopathogenic agents isolated.

Table 7. Identification of 35 virus strains isolated from the Red Cedar River downstream to the effluent

Neutralized by type antiserum	Number each type	Average TCD 50%	Percent of each type
I	3	$10^{-6.41}$	8.57
II	16	$10^{-5.83}$	45.71
III	15	$10^{-6.15}$	42.86
Coxsackie B-3	1	$10^{-5.75}$	2.86
Total	35		

Table 8. Total number of polioviruses identified during September and October 1969

Month	Number positive / Number of viruses tested	Percent positive
September	6/6	100
October	66/67	98.5
Total	72/73	98.6

October findings. It is of interest to note that nearly all samples (98.6%) collected and tested during this two-month period were found to contain poliovirus.

Table 9 summarizes the types of poliovirus found in September and October and the location of isolation. Of the 72 total virus strains

Table 9. The types of poliovirus recovered from the various locations

Month	Collection points	Poliovirus type I	II	III	Total
September	Treated final effluent	1	4	1	6
October	Treated final effluent	1	7	6	14
	Outflow 1	1	2	4	7
	Outflow 2	1	1	4	6
	Outflow 3	1	2	2	5
	Red Cedar River 105	0	6	5	11*
	Red Cedar River 293	0	7	5	12
	Red Cedar River 449	3	3	5	11
	Total	8	32	32	72

*The discrepancy between Table 9 and Table 6 is that a virus was found to be Coxsackie B-3 virus and was not included in Table 9.

there were 8 type I, 32 type II and 32 type III. The fact that by far the majority of isolation, regardless of where isolated, were either type II and III is significant. In each case there were four times the frequency of isolating type II and III as type I.

Those strains of virus that had been isolated on monkey kidney cells and could not be grown on HEp-2 cells were identified in neutralization tests using monkey kidney cells. The results of these

tests are given in Table 10. Four strains of echovirus were recovered, three strains of echo-3 and a single strain of echo-9.

Table 10. Neutralization tests for echovirus type 3, 6 and 9 for virus strains which were not propagated on HEp-2 cells

Collection points	Date isolated	Echovirus type identified
Red Cedar River 105	10-13-69	9
Outflow 1	10-14-69	3
Outflow 2	10-12-69	3
Outflow 2	10-13-69	3

DISCUSSION

Of a total of 77 isolations of virus from this study, 72, or 93.5%, of the strains were identified as one of the three serotypes of poliovirus. The predominance of poliovirus in the wastewaters during this test period can only be explained by the current use of the Sabin vaccine in the community. The area being a university community with many small children and young people, there would be considerable vaccination undertaken in the area. There is also the question of interference of the growth of the other enteric viruses because of the large amount of the poliovirus present. It would be of interest to determine the number of vaccine and wild type strains present in the 72 isolates.

It would appear from these results that the type I poliovirus is less resistant to the wastewater treatment than type II and III. The predominance of the type II and III serotypes isolated, certainly if these are vaccine strains, indicate that they are more resistant. There was no clinical evidence of poliomyelitis in the community as indicated by the Michigan Department of Public Health weekly surveillance reports. It is believed, as indicated by Melnick (75) that before mass immunization with the poliovirus vaccines, the most frequent cause of paralytic poliomyelitis was the type I strain. Gelfand and his colleagues (33) have shown that both pharyngeal and fecal excretion of type III poliovirus persisted for a longer period than the excretion of type I or II. Benyesh-Melnick (6) reported that

type II poliovirus infected the alimentary tract more extensively than types I and III and gave the highest excretion rates.

The screening of large numbers of virus strains isolated from wastewater with the HEp-2 cells certainly has great advantages, as suggested by Marchetti and Gelfand (67), to the rapid identification of this numerous enteric virus group.

When the average infectivity titers of 20 virus strains isolated from the final effluent (Table 3) and the 18 strains isolated from the outflow area (Table 5) were compared, the highest titer found in the final effluent was $10^{-6.02}$ with type II virus. From the outflow area the greatest average titer was $10^{-6.25}$ with type III virus. This difference probably is a biological difference and probably has no significance as to where the virus was isolated.

Theios and his colleagues (100) studied the effect of different sewage treatment methods on the recovery of poliovirus from sewage effluent following mass oral immunization. Activated sludge treatment was found to be a good method for removal of virus. A considerable variation in recovery of poliovirus type I and II was also observed. However, an unusual aspect of that study was the virtual absence of type I poliovirus from effluent of the activated sludge treatment three days after vaccine administration. This study is in disagreement with them. Type I polioviruses were isolated in approximately 10% of the total virus isolates. This might be due to the discrepancy in isolation methods of viruses from sewage effluent.

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