ISOLATION OF COXSACKIE VIRUS FROM SEWAGE

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ISOLATION OF COXSACKIE VIRUS FROM SEWAGE

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ABSTRACT

Coxsackie virus, as well as poliomyelitis and infectious hepatitis viruses are found in sewage. The incidence of Coxsackie virus recovery from sewage is dependent upon the season of the year and the excretion of the virus from persons with infections and convalescent carriers. Adequate methods of isolating this virus give a better means of studying its distribution and prevelance.

Three methods for the isolation of Coxsackie viruses from predigested sludge were compared:

(1) an Ultracentrifugation method developed in this laboratory.

(2) Melnick's method of precipitation of the virus by ammonium sulfate followed by ultracentrifugation.

(3) Kelly's Ion Exchange method which consists of adsorption of the virus to resins and subsequent elution.

The Ultracentrifugation method gave the best results, was the easiest procedure to follow and was less time consuming than the other methods. This method produced the highest per cent of infection in suckling mice using undiluted samples and gave a relatively higher LD_{50}.

Melnick's method gave similar results but the procedure proved too cumbersome and too time consuming for routine processing of samples.

The Ion Exchange method gave poor results.

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INTRODUCTION

INTRODUCTION

In the summer of 1947, Dalldorf (1948) first isolated the Coxsackie virus from the fecal specimens of two children in Coxsackie, New York, during a mild poliomyelitis epidemic.

The agent isolated induced paralysis in suckling mice and suckling hamsters with destructive lesions of the skeletal muscle and involvement of the central nervous system. This agent was given the name "Coxsackie Virus" (Dalldorf, 1949) after the village in which the virus was isolated.

The poliomyelitis group of viruses and the Coxsackie group are similar in several characteristics. Both are about 20 millimicrons in size, are very stable, cause similar clinical syndromes and both groups are considered enteric viruses.

Coxsackie viruses are not readily inactivated by heat and are stable over a rather wide pH range (Robinson, 1950). They may be inactivated at 53 C to 55 C after 30 minutes. Some Group A and B strains survive for one day at room temperature at a pH of 2.3 to 9.4 and for several days at 4.0 to 8.0.

The Coxsackie viruses are divided into two major groups, Group A and B, according to the histopathology produced in suckling mice (Dalldorf, 1950).

Group A viruses cause degeneration of striated muscles with weakness and paralysis ending in death within a few hours, with no central

nervous system lesions present. Group B viruses cause focal lesions in the brain or fat pads or both, with infected mice showing spasms or paralysis or both. Certain Group B strains attack the pancreas (Pappenheimer, 1951) while other strains may be isolated from other organs (Somlo, 1956).

The susceptibility of infant mice to infection by Coxsackie viruses as compared to the non-fatal effects of most strains on adult mice has been of particular concern. Infant mice are most susceptible from birth with a decline in susceptibility up to 3 weeks and after which, the animal is resistant to infection. In Group A infections, as the age of the mouse increases, the development of severe muscle weakness and paralysis takes a correspondingly longer time. In other words, younger suckling mice show symptoms earlier than older suckling mice. In Group B infections, the decline in susceptibility may vary with different strains of virus (Howes, 1954).

Contrary to earlier belief that only suckling mice may be infected, it has been demonstrated that adult mice may be infected (Pappenheimer, 1951). Connecticut-5 strain produces a pancreatic disease in adult mice which is selective for the pancreas irrespective of the route of inoculation. Pancreatic suspensions from infected mice are used in passages of the virus through other mice.

Dalldorf (1954) found that gravid mice are progressively more susceptible to severe infections with pancreatic strains of Group B as gestation advances. This susceptibility disappears after delivery and the young suckling mice born of these mice showed no defects.

The resistance of adult mice can be altered by treatment with cortisone prior to inoculation (Sulkin, 1952). The effects of cortisone become apparent as natural susceptibility decreases. It has been pointed out that cortisone does not increase the severity of the disease or the titer of the virus but increases the activity of the adrenal cortex of the adults which has declined with age. It is believed that newborn mice are subject to greater amounts of adrenocortical hormones but as the cortex involutes with age, extrinsic hormones increase susceptibility (Dalldorf, 1955).

Cheever (1953) demonstrated the effects of roentgen radiation on resistance by subjecting young adult mice to roentgen radiation. The virus remained in the organs for a longer period of time as compared to the controls but there was only a moderate and irregular increase in mortality.

The role of Coxsackie viruses in human diseases has caused much concern. Both Group A and Group B viruses are involved in human Coxsackie virus infections.

Herpangina, an ulcerative condition of the pharynx, hard and soft palates or the tongue, is caused by Group A viruses. Children are affected most often, usually by person to person contact (Cole, 1951), which might explain multiple infections within families and small groups.

Group B viruses are the infective agents of pleurodynia or Bornholm Disease, which is a disease causing headaches, coughing, fever and vomiting, with most pronounced effects in the chest, abdomen and muscles (Lazarus, 1952). Group B viruses have also been isolated from the

cerebrospinal fluid of patients with diagnosed aseptic meningitis (Duncan, 1955); and concurrently with poliomyelitis (Armstrong et al., 1950).

Coxsackie viruses can be isolated from throat washings at the onset of infection and later from feces and occasionally from blood of patients infected with the virus.

Presently, there are 24 immunologically distinct types known, 19 in Group A, which cause myositis in suckling mice and 5 in Group B, which cause inflammatory changes in the dorsal fat pads and brain and are easily isolated by tissue culture technics (Rhodes, 1956).

Serological diagnosis of Coxsackie infection is carried out by virus neutralization or complement fixation tests. Beeman (1952) found that complement fixation tests detect heterotypic antibody while virus neutralization tests are type specific, which suggests a common serological type.

Because of similarities such as size, stability, clinical syndromes, sources of virus isolations, time of year infections are most prevalent and isolation of both agents, Coxsackie and poliomyelitis viruses have caused much attention to be focused on the possibility of some influence of one on the other.

Melnick (1950) isolated both agents from the acute phase stools of several paralytic patients during an epidemic of poliomyelitis. Two of the patients simultaneously developed antibodies against poliomyelitis and Coxsackie viruses.

Experiments with mice have shown that mixed infections of poliomyelitis and Coxsackie viruses have a sparing effect on the course and outcome of the poliomyelitis (Dalldorf, 1951). Sulkin (1953) has shown that if mice are inoculated with Coxsackie prior to inoculation with Lansing strain of polio virus, the normal progress of the Lansing virus is limited but there is no effect by the Lansing virus on the Coxsackie Group A strain used. However, using a Group B, Ohio-R strain for prior inoculations, the Lansing strain enhanced the infections in 4 week-old mice.

Coxsackie virus, along with other disease causing viruses, such as poliomyelitis and infectious hepatitis, has become a particular concern to sanitarians since some investigators have associated such infections with water contamination. These infections are usually transmitted by direct contact and personal hygiene is most important in their control.

Sewage, a long recognized disease vector, is a rich source of enteric viruses. Coxsackie viruses are excreted for months by convalescent carriers and asymptomatic carriers (Cole, 1951).

Recent investigations show a very high percentage of Coxsackie present in sewage (Kelly, 1957). Of 306 samples of sewage, 42 per cent were Coxsackie positive; 21 per cent were poliomyelitis positive and 13 per cent contained both poliomyelitis and Coxsackie viruses. Three per cent of the samples contained unidentified viruses. Group A strains were present throughout the summer and fall months and Group B was found

less frequently. Group A and B could be readily isolated in monkey kidney monolayer tissue culture cells.

Adequate methods of isolating enteric viruses from sewage give a better means of studying distribution of various types of Coxsackie viruses, their presence and prevalence in sewage, as well as, their survival after sewage treatment.

The Ion Exchange method for isolating enteric viruses from sewage introduced by Kelly (1953) consists of virus adsorption to ion exchange resins and elution.

A second method introduced by Melnick (1954) consists of addition of protein, saturation with ether; precipitation of the protein plus virus by half saturation with ammonium sulfate; dialysis to remove salts and redissolve the precipitate; and concentration of the virus by ultracentrifugation.

The following study was undertaken to compare the above two methods with a method consisting of concentration by ultracentrifugation developed in this laboratory.

MATERIALS AND METHODS

A pool of pre-digested sludge was made of samples collected over a period of weeks from the East Lansing sewage plant. The pooled sludge was placed in a 3 liter flask and mixed thoroughly with a magnetic stirrer for one hour. Three 100 ml. aliquants were removed and used as controls for each method to be tested.

Two ml. of a known Coxsackie brain suspension were added to the remainder of the sludge and mixed for one hour with a magnetic stirrer. The sludge was then poured out in 100 ml. aliquants into screw-cap jars and frozen at -20 C for storage until ready to use.

Preparation of Inoculum

Ultracentrifugation Method

One hundred ml. aliquants were thawed by placing them in a 22 C water bath. Immediately after thawing, samples were placed in large test tubes and centrifuged at 2400 r.p.m. for 5 minutes to remove the larger particles. The supernatant fluid of each aliquant was placed in two 30 ml. ultracentrifuge tubes, and placed in a Rotor B for ultracentrifugation. The samples were ultracentrifuged at 42,040 r.p.m. (114,610 x gravity) for one hour in a Spinco Model E Ultracentrifuge. The sediment was resuspended in 3 ml. of supernatant fluid and the remainder discarded. To the 3 ml., 1500 units of penicillin/ml and 4 mg. of streptomycin/ml were added to eliminate bacteria.

Melnick's Method

One hundred ml. aliquants were thawed in a 22 C water bath and 1.5 ml. of inactivated (56 C for 30 minutes) horse serum was added since a minimum amount of protein is needed before precipitation by ammonium sulfate occurs. Eight ml. of ether were also added and mixed for one minute with a magnetic stirrer and without interruption 40 grams of ammonium sulfate were added and stirred another minute to dissolve the salt in the emulsion.

The samples were centrifuged at 18,000 r.p.m. for 15 minutes to pack the precipitate which floated because of its ether content. The floating precipitate was removed with a sterile spatula and placed into 15 inch-lengths of cellophane tubing previously prepared by clamping at one end. After transfer of the precipitate, the open end was closed by clamping and the formed bags were then hung in a large glass cylinder and dialyzed against cold tap water for 1 to 1.5 hours to remove the ammonium sulfate.

The dialysate was placed in uncapped tubes and left in the refrigerator overnight to allow the ether to evaporate. It was then centrifuged at 3,500 r.p.m. for 15 minutes and the supernatant fluid transferred to ultracentrifuge tubes. The samples were then ultracentrifuged at 42,040 r.p.m. (114,610 x gravity) for one hour and the sediment resuspended in 1.5 ml. of sterile distilled water by using a rubber-capped glass rod to break up and disperse the pellet. The suspension was centrifuged at 16,000 r.p.m. for 20 minutes in an

International Multispeed Refrigerated Model PR-1 and the supernatant fluid was used as the inoculum.

Kelly's Ion-Exchange Method

To 100 ml. aliquants, a 30 per cent solution of egg albumin was added to give a final concentration of 0.5 per cent. Ten grams of Dowex 1 resin, 200-400 mesh, 10 per cent cross-linkage, was added to the sludge and mixed for 3 to 4 minutes. The suspension was centrifuged at 2,500 r.p.m. for 10 minutes and the supernatant fluid discarded. To the sedimented resin, 2 ml. of 10 per cent disodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$) were added and mixed for 5 to 10 minutes. The mixture was centrifuged at 3,000 r.p.m. for 15 minutes. The eluate was drawn off, saved and treated with 500 units of penicillin/ml.

Sterility tests were made on all specimens from the three methods by inoculating one loopful of the specimen into a tube of Brain-Heart Infusion broth, to which 1 gram of nutrient agar/liter had been added. The tubes were incubated for 72 hours at 37 C and observed for growth.

Those found contaminated were centrifuges at 9,000 r.p.m. for 15 minutes and 3,000 units of penicillin/ml. and 12 mg. of streptomycin/ml. were added to the specimen for titration in mice to compare the infectivity of the virus isolated using each of the three methods. All specimens were frozen and held until mice became available for inoculation.

Controls for each method were processed in the same manner as the samples containing virus.

The LD_{50}'s were calculated according to Reed and Muench (1938).

Inoculations

One to three-day old Swiss mice were used. Six hundredths ml. of the specimen was inoculated intraperitoneally into each of the suckling mice of a litter. Two mice from the same family were held as uninoculated controls and were marked by cutting off the tip of their tails. The infant mice were kept with their mothers and observed twice daily for any signs of infection. The Coxsackie was manifested by paralysis of hind-limbs and occasional deformity occurring in fore-limbs with death occurring within a few hours after onset of symptoms. Mice dying within a 24 hour period after inoculation were not considered in the results.

All mice dead or eaten by cannibalistic mothers during the critical period, from 3 to 15 days, were considered as deaths due to virus infection. All mice were observed for 21 days and nother, controls and uninfected mice were destroyed at the end of this period.

RESULTS

The results of three different titrations in mice after preparing the samples by ultracentrifugation are shown in Table I. As can be seen, the average LD_{50} was $10^{-1.6}$. The greatest dilution showing infection was 10^{-2} and none of the 10^{-3} dilutions showed infection. The data indicated that this method produces fairly consistent results. For initial Coxsackie virus isolation the Ultracentrifugation method is good since all mice injected with undiluted samples showed symptoms of disease.

The results of the titrations of the samples prepared by the method of Melnick are illustrated in Table II. In experiment 4, the LD_{50} was less than $10^{-1.0}$. However, it appeared that it should be included in the series because one could approximate the LD_{50} as being $10^{-0.7}$. This would be within the experimental error of the titration. In animal titrations, a difference of $\pm$ 0.5 log is usually considered to be insignificant. In experiments 5 and 6, the results are comparable to the results obtained with the Ultracentrifugation method. The average LD_{50} for this experiment was $10^{-1.25}$ and 100 per cent mortality was noted in the undiluted samples, indicating that this method could be used for initial isolation of Coxsackie viruses from sewage.

The Ion Exchange method resulted in low recovery rates when compared to the other two methods. The average LD_{50} (Table III) for the three experiments in this series was $10^{-0.4}$. For initial Coxsackie

virus isolation, the Ion Exchange method does not appear to be as good as the other two methods, since the undiluted samples produced 100 per cent mortality in only one of the three experiments.

The Ultracentrifugation method was the only method which gave 100 per cent infection of the mice in the undiluted samples. This appeared to be the most sensitive method for initial isolation of Coxsackie Viruses from sewage. Graph I gives a summary of the per cent of infected mice in each dilution for each experiment to further indicate the superiority of the Ultracentrifugation method.

TABLE I

RESULTS OF THREE TITRATIONS IN MICE USING THE ULTRACENTRIFUGATION METHOD

Experiments	Dilutions				LD_{50}
	Undiluted	10^{-1}	10^{-2}	10^{-3}	
1	6/6*	3/3	2/5	0/4	$10^{-2.2}$
2	8/8	3/5	0/5	0/4	$10^{-1.2}$
3	6/6	4/5	2/6	0/5	$10^{-1.6}$
Control	0/5	-	-	-	-
Average	-	-	-	-	$10^{-1.6}$

*Number of mice infected/number of mice inoculated.

TABLE II

RESULTS OF THREE TITRATIONS IN MICE USING MELNICK'S METHOD

Experiments	Dilutions				LD_{50}
	Undiluted	10^{-1}	10^{-2}	10^{-3}	
4	3/9*	1/6	0/7	0/7	**
5	5/5	2/4	1/6	0/4	$10^{-1.2}$
6	7/7	4/4	4/6	0/6	$10^{-1.3}$
Control	0/4	-	-	-	-
Average	-	-	-	-	$10^{-1.25}$

*Number of mice infected/number of mice inoculated.

**Less than 10^{-1}.

TABLE III

RESULTS OF THREE TITRATIONS IN MICE USING THE ION EXCHANGE METHOD

Experiments	Dilutions				LD_{50}
	Undiluted	10^{-1}	10^{-2}	10^{-3}	
7	3/3*	1/4	0/11	0/6	$10^{-0.7}$
8	2/4	1/5	1/6	0/8	$10^{-0.4}$
9	2/4	1/4	0/6	**	$10^{-0.2}$
Control	0/4	-	-	-	-
Average	-	-	-	-	$10^{-0.4}$

*Number of mice infected/number of mice inoculated.

**All mice died with diarrhea.

TABLE IV

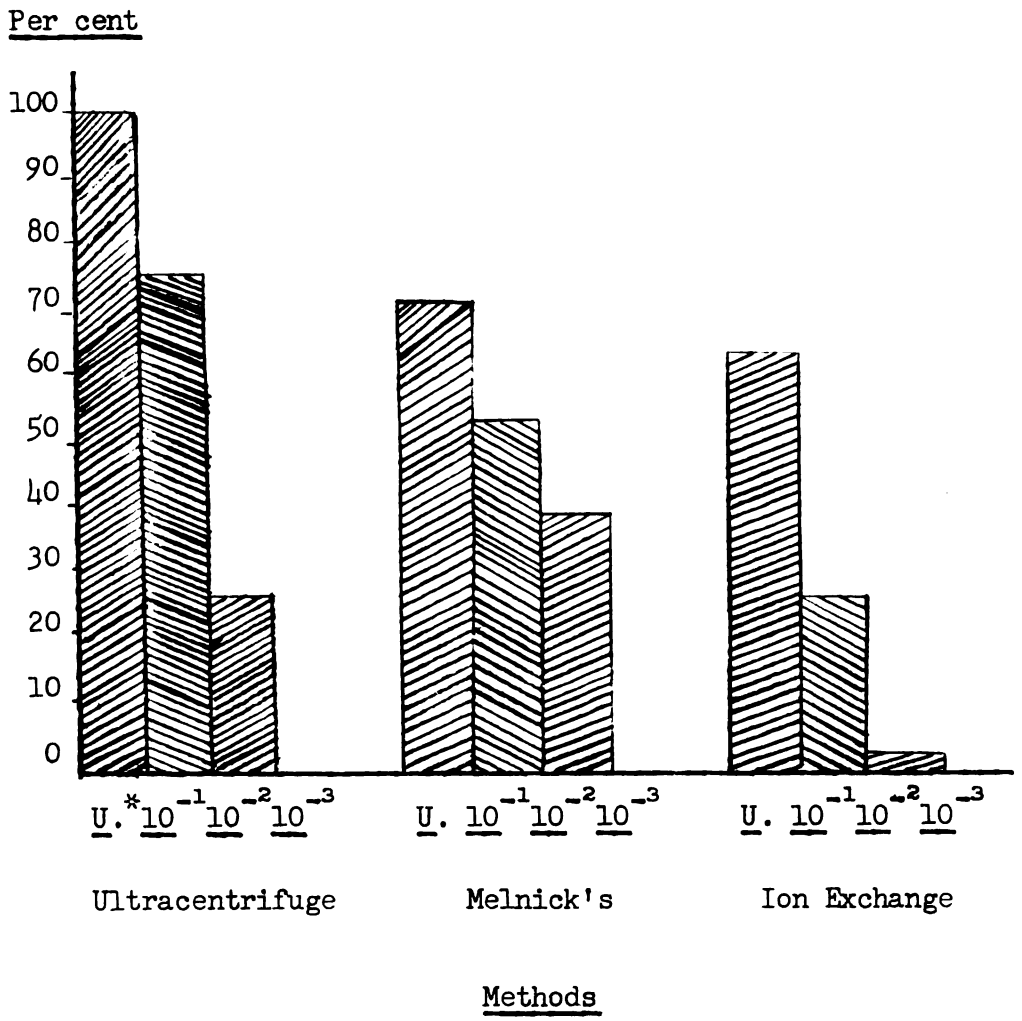
COMPARISON OF THE THREE METHODS FOR ISOLATION OF COXSACKIE VIRUS

Methods	Dilutions				LD_{50}
	Undiluted	10^{-1}	10^{-2}	10^{-3}	
Ultra-centrifugation	20/20*	10/13	4/16	0/13	$10^{-1.5}$
Melnick's	15/21	7/14	9/21	0/17	$10^{-1.1}$
Ion Exchange	7/11	3/13	1/23	0/17	$10^{-0.4}$
Controls	0/13	-	-	-	-

*Number of mice infected/number of mice inoculated.

GRAPH I

A COMPARISON OF PER CENT OF MICE INFECTED FOR THE THREE METHODS OF ISOLATION OF COXSACKIE VIRUS



DISCUSSION

The work described here was done to determine the most satisfactory method for the isolation of Coxsackie virus from raw sludge. Although the methods of Melnick and Kelly were developed to recover Coxsackie virus from dilute sewage specimens, work in this laboratory has shown that by using raw sludge samples a higher recovery of Coxsackie viruses could be obtained and for this reason, this type of specimen were selected for comparison of the three methods. The Ultracentrifugation method works equally as well for dilute and concentrated specimens. Even though Melnick's method gave similar recovery rates, the difficulty in handling raw sludge material in using this procedure makes the Ultracentrifugation method more practical because of the simplicity and speed afforded by this method.

The Ultracentrifugation method required approximately three hours for the entire preparation of four samples processed simultaneously. While Melnick includes a step in his procedure which required evaporation of ether from samples overnight before ultracentrifugation which requires two hours at minimum. The Ion Exchange method required approximately four and a half hours to process four samples of sludge because of its concentration. However, with dilute samples of sewage it can be speeded up.

The only possible advantage of using the Ion Exchange method is the fact that an ultracentrifuge is not needed. This method does not appear to be applicable for the processing of sludge samples.

The use of suckling mice for the initial isolation of Coxsackie virus is unsatisfactory at best. Having a daily supply of suckling mice for virus isolation and titration is expensive as well as requiring additional care of the mouse colony. Cannibalism often destroys the evidence of mouse infection which requires repeating many of the experiments.

No "blind" serial passages were made with tissue suspensions of suspected material. Had passages been made with suspected material, the virus isolations, especially in the undiluted samples, would have been greater. Such methods are usually employed for primary isolations of virus.

SUMMARY

Three methods of isolating Coxsackie viruses from pre-digested sludge were compared for determining the highest recovery of a Coxsackie virus suspension added to a sludge sample and the easiest and least time consuming procedure. The three methods used were the Ultracentrifugation method consisting of concentration of the virus by ultracentrifugation; Melnick's method of precipitation of the virus by ammonium sulfate followed by ultracentrifugation; and the Ion Exchange method introduced by Kelly as a method of isolating Coxsackie viruses from sewage, consisting of adsorption of the virus to resins and subsequent elution.

The Ultracentrifugation method yielded the best results, with the highest percentage of infection in suckling mice using undiluted samples and it was the easiest and fastest procedure.

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