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IX. ISOLATION OF ENTEROVIRUSES FROM SEWAGE BEFORE AND AFTER VACCINE ADMINISTRATION**

In conjunction with the city-wide program of oral vaccination conducted during the early months of 1961 in Middletown, Connecticut, tests were carried out on serial samples of sewage from various strategic points in the city as a means of virological surveillance. Objectives were: a) to determine the relative prevalence of polioviruses and other enteroviruses in the city sewage prior to, during, and subsequent to the vaccination campaign; b) to continue attempts to isolate enteroviruses from sewage for at least one year in an effort to determine how long the attenuated poliovirus vaccine strains might persist in sewers servicing communities of different sizes.

METHODS

Collection of sewage. Six sewage sampling points were selected, five throughout the city, and one in the adjacent town of Portland which served somewhat as a control. The areas from which the sewage came are illustrated in the map in Figure 1. The sampling points which were either in branch sewers or at the influent of sewage treatment plants, were designated as follows: area no. 1 (Green Street pumping station) which carried sewage from northern and northwestern parts of the city, servicing approximately 8,000 people; area no. 2 (Middletown sewage treatment plant) represented sewage not only from the center of the city but, with one exception, from all of the other sampling points of the city, servicing approximately 20,000 people; area no. 3 (the south interceptor line) carried sewage from the southern part of the city, servicing approximately 7,000 people; area no. 4 sewage came from a small group of houses representing approximately 300 people (the effluent of this line was independent of the city sewage-treatment plant); area no. 5 carried the sewage from a correctional institution with a population of 160 teenagers; and area no. 6

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(the Portland sewage treatment plant), located across the Connecticut River from Middletown, serviced approximately 4,000 people. In the collecting program, no attempts were made to avoid industrial wastes.

The collection of samples was started on January 3, 1961 and continued for a full year. It is our hope that collections can be continued well into 1962.

Sampling technique. In obtaining each specimen the swab sampling method originally devised by Moore[1] and modified by others[2-6] was employed. Details of this technique are as follows:

Three 16-ply 4" x 4" gauze sponges fastened to a nylon line were immersed in the sewage flow at the designated sites and allowed to remain for one week, or, in the pre-vaccinal period, 3 to 7 days (Table 1). When collected, each gauze pad sample was immediately put in a half-pint ice cream carton, and new pads were set. The specimens were stored at 4° C. in Middletown and were usually taken on the following day to the laboratory of the Yale Polomyelitis Study Unit in New Haven, a distance of 26 miles. However, if a delay in transportation was anticipated, specimens were frozen at -20° C. and delivered when convenient. Because of heavy rains and snow storms during the winter and spring, several collecting pads were lost in the early period when the lines to which they were attached wore through. This difficulty was overcome subsequently by placing a loop of wire through the pads to which was attached a metal snap with a swivel connected to a metal chain line.*

Preparation of specimens. The gauze pads of each collection were placed in a polyethylene bag (6x3x12") and 5 to 10 ml. of Hanks' balanced salt solution (BSS) was added. After kneading the pad within the bag for a minute or two by hand, the pH of the fluid expressed from the gauze was checked with pHydrion paper (range 6.8 to 8.4). Sufficient N/1 NaOH was added to bring the pH to 8.0 — 8.4. The sample was again kneaded for a few minutes with further adjustment of the pH if necessary, and the fluid expressed from the pad was poured into a wide-mouth beaker. The yield from each sample was usually about 45 to 60 ml. Fifteen ml. of the crude sewage suspension was centrifuged in the cold at 2,500 RPM for 20 minutes; the supernate was pipetted off, stored in a 20 ml. lusteroid tube and frozen at -20°C. After a day or two the extract was thawed again and centrifuged in similar fashion. Ten ml. of the resulting supernate was pipetted into a 40 ml. pyrex narrow-mouth centrifuge tube

* We are indebted to Mr. Sebastian Crescimanno, sanitarian of the Middletown Health Department for devising this method, and both to him and members of the Middletown Public Works Department for the collection of these specimens.

and 10 ml. of analytical ether was added. The ether and sewage extract were well mixed by sucking the material up and down in a 10 ml. pipette with a rubber bulb attached. The mixture was then stoppered with a rubber stopper and held at 4°C overnight. The following day, with the stopper removed, the opening of the tube was covered with sterile gauze held on by rubber bands. The mixture was then recentrifuged as described above. This usually brought a layer of fatty substances to the top of the fluid level. The clear subnatant fluid was then carefully taken up with a capillary pipette, placed in a sterile Petri dish and gently rocked back and forth several times. This fluid, designated as Fraction A, was then allowed to stand in the Petri dish with the cover slightly raised for two hours at room temperature to allow evaporation of the ether.

Virus isolation techniques. Primary trypsinized monkey-kidney (MK) cells, grown in three-ounce stoppered medicine bottles in a medium consisting of Hanks' BSS with 2 per cent calf serum and 0.5 per cent lactalbumin hydrolyzate, were used for viral isolations.[7] Two ml. of ether-treated sewage (Fraction A) were inoculated into each of two MK monolayer bottles. However, with sewage specimens collected after the administration of the vaccine, satisfactory results were obtained by using only one ml. of inoculum in one MK bottle. Inoculated tissue cultures were incubated at 35-36° and were observed microscopically daily for eight days. Tissue-culture fluids were harvested when the cytopathogenic effect (CPE) was well marked. Questionably positive cultures and those showing no CPE were passed once in MK culture tubes. On rare occasions, a highly toxic sewage sample, usually one which contained a black substance in solution, would destroy all the MK cells before viral CPE could be detected. This problem was overcome in most instances by treating the specimens with protamine sulfate;[8] they were then retested using one-half the usual inoculum in each of two MK bottles. All isolates were passed once in MK tubes to substantiate the specificity of the CPE. Only agents detected in MK tissue culture are described in this study for no animal inoculations were done.

Identification of isolates. In most instances the first MK passage was used for identification of agents. Second-passage material was used occasionally when the virus titer was low in the original passage. Isolates were identified by means of tube neutralization tests, as described elsewhere.[9] Combinations of antisera, types I + II, I + III, II + III, and I + II + III, were used to detect mixtures of polioviruses. With some specimens, greater precision in isolating more than one type or kind of enterovirus was achieved by retesting the original specimen in the presence

TABLE 1. DISTRIBUTION AND TYPES OF ENTEROVIRUSES ISOLATED FROM SEWAGE, LISTED BY AREA AND DATE OF COLLECTION OF SAMPLE, MIDDLETOWN AND PORTLAND, CONNECTICUT (3 JANUARY - 31 MAY 1961)

	*Pre-vaccinal period***					*Post-vaccinal (poliovirus 1)†*						*Post-vaccinal (poliovirus 2 & 3 combined)‡*											
*Area, vaccinees in population**	*Jan.*					*Jan.*	*Feb.*				*Mar.*	*Mar.*			*Apr.*				*May*				
	3	*10*	*13*	*17*	*24*	*31*	*7*	*14*	*21*	*28*	*7*	*15*	*21*	*28*	*4*	*11*	*18*	*25*	*2*	*9*	*16*	*23*	*31*
1																							
2,211 / 8,000	E 4	CB5	CB4	CB4	CB3	1	1	1	1	1	X	X	X	*2* / *3*	*2* / *3*	2 / 3	X	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3
2																							CB5 / 1 / 2 / 3
4,625 / 20,000	X	CB4	CB4	CB4	0	1	1	1	1	E 4 / 1	1	X	X	*1* / *2* / *3*	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3
3													*1* / *2*	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3	E 7 / 2	E 7 / 3	3	2 / 3	2 / 3
1,196 / 7,000	X	0	0	0	0	1	1	1	1	1	1	X	*3*										
4													1 / *2*	*1* / *2*	2 / 3	2 / 3	2 / 3	2 / 3	2 / 3				2 / 3
20 / 300	X	CB4	CB2	0	0	1	1	1	1	1	1	1	3	3	3	3	3	3	3	3	3	3	
5												2	2		2	2			2				
160 / 160	X	0	0	X	0	1	1	1	1	1	1	3	3	3	3	3	3	3	3	3	3	3	0
6													1										
19 / 4,000	3	3	0	3	0	3 / 1	1	1	1	1	1	1	*2* / *3*	*2* / *3*	2 / 3	2 / 3	2 / 3	X	3	2	2 / 3	2 / 3	2 / 3

* Numerator = no. of vaccinees living within area. Denominator = approximate total population.

** X = No sample; 0 = negative; E 4 = ECHO virus 4, etc.; CB4 = Coxsackie virus B4, etc.; 1 = poliovirus type 1; 2 = type 2; and 3 = type 3.

† Arrows indicate approximate times of vaccine administration. Type 1 (monovalent) poliovirus vaccine given from 24-31 Jan.; types 2 and 3 (bivalent) from 14-24 Mar.

‡ Virus types underlined and shown in italics were isolated after ultracentrifugation of the original sewage sample to yield a tenfold concentration.

of antiserum of the first type isolated. Thus, for example, small amounts of type II poliovirus were uncovered in specimens for which only type III —apparently present in large amounts—had been obtained on the first test.

RESULTS

Middletown sewage proved to be an excellent source of enteroviruses before, during, and after initiation of the oral vaccination program. Results

TABLE 2. FREQUENCY OF POST-VACCINAL ENTEROVIRUS ISOLATIONS FROM SEWAGE DURING LATE SPRING, SUMMER, AND AUTUMN, MIDDLETOWN AND PORTLAND, CONNECTICUT

SAMPLES COLLECTED WEEKLY, 6 JUNE - 7 NOVEMBER 1961

		Sewage samples			
Area	*Approx. population*	*No. tested*	*Per cent positive*	*No. positive for polioviruses*	*No. positive for other enteroviruses*
1	8,000	22	100	7	15
2	20,000	23	100	6	17
3	7,000	23	87	3	17
4	300	23	61	4	10
5	160	18*	0	..	..
6	4,000	22	100	6	16
Total	20,460	131	77	26**	75

* Sampling discontinued after 3 October 1961.
** Of the samples positive for polioviruses, 15 were collected in June, 7 in July, 3 in August, and 2 in early September. No polioviruses were recovered between 5 September and 7 November.

are now complete on the pre-vaccinal period (3 January to 24 January) and post-vaccinally through 31 May (Table 1). Preliminary information for the subsequent five-month period (6 June to 7 Nov.) is also available (Table 2). In most instances typing of agents isolated during this later period is incomplete, and the samples are designated simply as positive for poliovirus or for some other enterovirus.

Table I shows the types of enteroviruses isolated according to the sites and dates of collection in both pre- and post-vaccinal periods. The approximate size of the population which contributed to the sewage and the number of vaccinees living in each area are also indicated. Even though

the pre-vaccinal samples were obtained in the middle of the winter season, there seemed to be no dearth of enteroviruses, for of 25 sewage collections between 3 and 24 January, 52 per cent were positive. One strain each of ECHO 4, Coxsackie B2, Coxsackie B3, Coxsackie B5 were isolated. Coxsackie B4 was recovered six times, and poliovirus type III three times. The poliovirus strains were all obtained from the influent of the sewage treatment plant of the town of Portland, located immediately across the river from Middletown. A single suspected case of nonparalytic poliomyelitis had been reported from Portland in October 1960, at least three months prior to the collection of these specimens.

Following the administration of monovalent type I vaccine in late January, the sewage was literally flooded with this organism. All of the weekly samples obtained during the subsequent six weeks (3 January to 7 March) were positive for type I poliovirus (Table 1). Tests on all positive specimens collected during this period were repeated in the presence of specific type I antisera in order to allow lesser amounts of other viruses to multiply. In spite of this, only one isolation of a non-poliovirus enterovirus was made, namely ECHO 4, obtained from area no. 2. One strain (obviously wild) of type III poliovirus was isolated from area no. 6 where the same strain had been present in the pre-vaccinal period. Thus fewer than six per cent of specimens yielded an enterovirus other than the type I poliovirus vaccine strain which had been administered.

After the second dose of oral vaccine, consisting of types II and III, type I was quickly replaced, and virtually all sewage samples collected between 15 March and 31 May contained polioviruses II and III. During this period few specimens (less than 5%) were positive for other agents: ECHO 7 was isolated twice from area no. 3, and Coxsackie B5 once from area no. 2. Late in May, however, poliovirus type I, which had not been recovered since 28 March, appeared once again in the sewage from area no. 2. Recent tests have also shown type I poliovirus to be present in the sample of 6 June 1961 from area no. 4 and preliminary results indicate that it was found occasionally during August and September. Whether the later type I poliovirus isolates represent the vaccine strain or wild strains remains to be determined.

The frequency of enterovirus isolations from sewage collected weekly during the summer and fall (6 June through 7 November 1961) is listed in Table 2. Sampling was discontinued from area no. 5 on 3 October 1961 after the examination of 18 consecutive weekly samples had yielded entirely negative results. However, most of the samples from the other collecting points continued to remain positive for various enteroviruses.

Preliminary results show that at least poliovirus types I, II or III were still present in 15 samples during the month of June 1961; in six during July; three in August and two in September. No polioviruses were isolated between 5 September and 7 November.

DISCUSSION

The results above show how readily polioviruses may be demonstrated in the sewage of a city after oral vaccine administration. In the period immediately following the administration of type I, this type of virus was present in every sample of sewage tested over a six-week period; it was immediately supplanted by types II and III following the administration of the bivalent vaccine. These two types persisted among the larger populations for about three months. This pattern suggests that one type of poliovirus can be quickly replaced by others (presumably through interference) when the strains are administered sequentially. The results are not yet complete enough to indicate the length of time during which polioviruses may be excreted by populations of varying size such as those described in this study when no artificial interference is induced. Preliminary results indicate that little poliovirus was present after three or four months even though this carries over into summer and fall, months when polioviruses are normally at peak prevalence. There is relatively little information available to show the usual pattern of seasonal presence in the sewage of enteroviruses other than poliovirus in New England industrial cities the size of Middletown. However, data are available from the state of Michigan where Bloom, *et al.*[5] have reported virus isolations from sewage over many months in the cities of East Lansing and Lansing. In accordance with these results and some experience in other areas, the general impression is that both non-polio-enteroviruses and wild polioviruses are more frequently present in the sewage in the summer than in the winter or spring months.

It was a little surprising, therefore, to find so many agents present in Middletown sewage during the pre-vaccinal weeks in the month of January 1961. Of 25 specimens tested during this period, 13 (52%) yielded enteroviruses. This includes wild strains of type III poliovirus which were isolated from the sewage of the neighboring town of Portland, Connecticut (area no. 6, Fig. 1) on four occasions over a six-week period, even though no overt clinical cases of poliomyelitis were apparent in Portland at the time; in fact, only one suspected case had been observed during the preceding year.

Once the vaccination program had been started, polioviruses (presumably the attenuated vaccine strains) completely flooded the sewage and few other enteroviruses were recovered either during or shortly after the vaccination campaign (February through May). Of 93 specimens tested only four were positive for non-polio-enteroviruses. The question is: Did the feeding of virtually the entire juvenile population of the city with attenuated poliovirus strains force other enteroviruses out of existence,

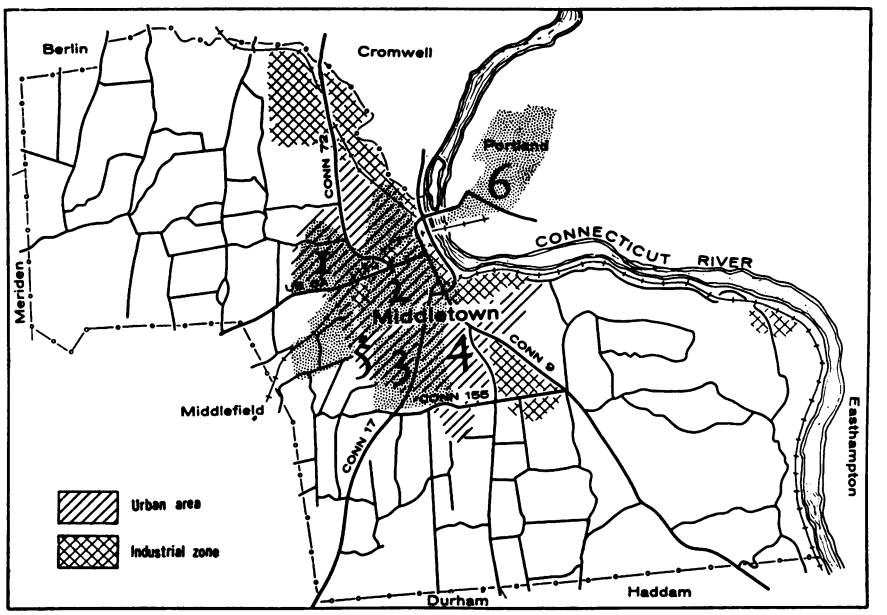

FIG. 1. Map of Middletown and Portland, Conn., 1961. The stippled portions indicate the approximate areas from which sewage drains into the selected sampling points.

i.e. interfere with their capacity to survive in the intestinal tract, or was failure to recover them due to the inadequacy of our technique of virus isolation? In other words, would the vigorous use of the more sensitive methods, including the concentration of sewage samples by ultracentrifugation, have resulted in more isolations of Coxsackie and ECHO viruses? This question remains unanswered for our techniques for the isolation of the non-polio-enteroviruses leave something to be desired. Indeed, some Coxsackie A viruses probably would have been detected if sewage samples had been tested in suckling mice as well as in tissue cultures. Bloom, *et al.*[5] reported that by using this additional method, some enteroviruses were

detected in sewage from Lansing and East Lansing, Michigan nearly every month of the 28 covered by their survey.

The relationships between the size of the population and the number of vaccinees represented in the various sewage samples and the duration of time over which polioviruses persisted in the sewage in the post-vaccinal period are difficult to analyze. One might note, however, that in area no. 5 which represented a population of 160 people, poliovirus strains were not detected beyond the end of May, and subsequently no enteroviruses were isolated from this site in 18 consecutive specimens. However, the age composition of this institutionalized group was limited to 14 years and older, and most of the individuals were 15-18; furthermore, tests indicated that more than 95 per cent had antibodies to all three types of poliovirus. These points make it unlikely that extensive or prolonged circulation of polioviruses—or other enteroviruses—would occur among this group.

Another important question to be resolved is whether the poliovirus strains isolated from sewage samples in the post-vaccinal period represent the vaccine strains or whether any of those recovered after 31 January can be identified as wild, naturally occurring types of poliovirus. Various genetic marker tests are now in progress in an attempt to settle this question.

SUMMARY

Prior to and following the administration of oral poliovirus vaccine to the juvenile population of the city of Middletown, Connecticut weekly samples of sewage were tested as part of a virological surveillance planned to cover at least one year. Most of the samples collected during the first 10 months of this period contained enteroviruses of one or more types.

Before oral vaccine administration, 13 of 25 (52%) of the sewage samples from five selected areas in Middletown and from one "control" area in Portland, Connecticut were positive for enteroviruses. Ten of the 13 strains isolated were non-polio-enteroviruses and three were type III poliovirus.

In the post-vaccinal period, results indicate that after the first dose of monovalent type I vaccine the predominating agent, found in 100 per cent of sewage samples for six consecutive weeks, was type I poliovirus. A wild strain of poliovirus type III was isolated on one occasion, and ECHO 4 virus was recovered once (in conjunction with type I poliovirus).

After the second dose of vaccine in which the combined types II and III poliovirus was administered, type I was quickly supplanted in the sewage. Types II and III then predominated for a period of 11 weeks with only

a few non-polio-enteroviruses being recovered. Subsequently, poliovirus type I was again isolated on one occasion in the 11th week of this period.

Preliminary tests of 131 additional sewage samples collected at weekly intervals later in the summer and autumn (6 June to 7 November 1961) showed that 101 (77%) were positive for enteroviruses; among these 26 contained polioviruses, and 75 yielded other enteroviruses.

ADDENDUM

Subsequent tests have revealed the presence of polioviruses in sewage collected between 28 November and 19 December, approximately four months after the last previous isolation. Type I was found in a 19 December specimen from the Middletown plant; type III was recovered from Green Street sewage collected 28 November and 5 December, and from the Portland specimen of 12 December, 1961.

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