STUDIES ON POLIOMYELITIS, 1960-1961, IN NAGASAKI
PREFECTURE, JAPAN*

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Epidemic studies on poliomyelitis in Japan up to 1947 have been reviewed by Paul¹⁾. Since 1947 there have been many other epidemiologic studies of poliomyelitis in Japan²⁾³⁾⁴⁾.

During the period from May to August of 1960, poliomyelitis was prevalent among children in Nagasaki City. It was found that this was an epidemic of poliomyelitis due to polio types I, II and III.

The present paper reports the results of the authors' study on that epidemic. The study is divided into the following parts: (1) Laboratory data from paralytic cases. (2) Distribution of cases according to age, sex, geography and season. (3) Relationship between the scale of polio epidemic and the *in vitro* markers of polioviruses. (4) Relationship between the epidemic in Nagasaki Prefecture and the epidemic in the northern part of Fukuoka Prefecture in the winter of 1960.

MATERIALS AND METHODS

Viruses

Poliovirus strains were plaque purified lines of type I (Mahoney), type II (MEF₁) and type III (Saukett); and they were used after HeLa cell passage in this laboratory. They were grown in HeLa cells maintained in cysteine medium. Virus harvests were centrifuged to remove cell debris.

Immune sera for typing

Type-specific immune sera against 3 types of poliovirus were obtained from the National Foundation for Infantile Paralysis. They were sera from monkeys that had been immunized with type I (Brunhilde), type II (YSK) and type III (Leon) viruses. These sera, which were in lyophilized form, were reconstituted with saline solution with due precaution to maintain sterility. All sera, after dilution to 1:10 in saline solution were inactivated at 62°C for 20 minutes.

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HeLa cells

HeLa cells were used in this study. The growth medium consisted of : 90 % Melnick-Hanks medium, 10 % bovine serum inactivated at 56°C 30 minutes, 100 units per ml penicillin, 100 microgram per ml dihydro-streptomycin. Abundant outgrowth was usually obtained on the fourth or fifth day after inoculation. On the day of virus inoculation, the culture was washed with Puck's solution 3 times, the nutrient fluid was replaced with 1.0 ml of maintenance medium and then 0.1 ml of virus material was inoculated. As maintenance medium the authors used a medium of the following composition :

Melnick-Earle's solution	80 per cent
skim milk solution	10 per cent
penicillin	100 units per ml
streptomycin	0.1 milligram per ml

Serum specimens

During June and August, 1961, 4 blood samples were collected from paralytic poliomyelitis patients in Nagasaki Prefecture. Serum was separated from the clot and kept frozen at -20°C until used.

Materials for virus isolation

In 1960 23 stool specimens were obtained from paralytic poliomyelitis patients in Nagasaki Prefecture. Virus isolation was attempted on these specimens. The authors collected stool specimens from paralytic poliomyelitis patients hospitalized in the clinics located in various districts of Nagasaki Prefecture. The specimens were put into small test tubes which were then sealed tightly with rubber stoppers and sent to this laboratory by messenger. The specimens were kept frozen at -20°C immediately after their arrival at the laboratory until use.

Method of isolation of cytopathogenic agent

The stool specimen was weighed and emulsified with Puck's solution which contained 500 units of penicillin per ml and 500 micrograms of streptomycin per ml to made a 10 per cent emulsion. It was then centrifuged at 3,000 rpm for 20 minutes, and 0.1 ml of the supernate was inoculated into tissue culture tubes. Three or more tissue culture tubes were used for one specimen, and the cultures were observed every day for a week. If any cytopathogenicity was noted, at the height of cellular damage the fluid and cells were scraped from the tube and harvested for second passage and were stored. If no cytopathogenicity was observed, a second passage made with culture fluid and cells were harvested at the end of the first week of cultivation. Tubes that proved negative after 2 weeks in the second passage were discarded. Cytopathogenic agents that had undergone 2 or 3 passages were preserved in a frozen state until typed.

Neutralization tests and typing of the isolated agents

Sera were inactivated at 56°C for 30 minutes before use. Neutralization tests were carried out as follows : for antibody titration of sera, samples were diluted in 2-fold steps and the neutralizing effect was tested by 100 TCD₅₀ of the viruses.

Titration of the viruses used

Serial 10-fold dilutions of the infected nutrient fluid to be tested were made,

and 0.1 ml of each dilution was introduced into 4 tubes which contained 1.0 ml of the medium. Four days after inoculation the tubes were examined microscopically for evidence of specific degeneration, and the end point was expressed in terms of TCD_{50} which was calculated by the Behrens-Kärber formula.

Neutralization test

Four-tenths ml of the serum was mixed with the same amount of virus which was diluted to contain approximately 10^2 TCD_{50} per 0.1 ml. These virus-serum mixtures were incubated at 37°C for 1 hour. Two-tenths ml of the mixtures was inoculated into a duplicate set of 5-day-old cultures. Four days after inoculation the tubes were examined microscopically, and absence of degeneration was interpreted as a positive neutralization test. For titration of the pooled serum, antibody titer was expressed in terms of the initial dilution which showed positive neutralization in all of the tubes examined.

Typing of polioviruses

The procedure of typing was almost identical with the neutralization test on the unknown serum. Type-specific monkey immune serum which was diluted 1 to 50 was mixed with an equal amount of 100 TCD_{50} virus contained tissue culture fluid which showed cytopathogenicity. The type was identified by a positive neutralization test against one of the type-specific immune sera. Cytopathogenic agents that were not neutralized by this procedure were classified as agents other than poliovirus.

Test for d character⁵⁾

This character was determined by measuring the reduced efficiency of plating under a low bicarbonate agar overlay on HeLa cells⁹⁾. In all tests, PFU per ml were compared in bottle cultures with 0.085 per cent and 0.4 per cent $NaHCO_3$ in the overlay. The nutrient medium for agar overlay consisted of: 10 times Hanks solution 18 ml, sterile distilled water 61.3 ml (or 52.6 ml), neutral red (1:1000) 3.0 ml, calf serum 3.6 ml, 7 % $NaHCO_3$ 2.9 ml (or 11.6 ml), 40,000 μ /ml penicillin 1.0 ml, and 200 mg/ml dihydrostreptomycin 0.2 ml. From the titer at the bicarbonate concentrations, the EOP (efficiency of plating) values were obtained for the test and control viruses, and the character of each strain was determined by the ratio of EOP to LSc. The strains were separated arbitrarily into three groups: d⁺ strain, having an EOP ratio to LSc greater than 0.75; intermediate, or d[±] strains, having an EOP ratio to LSc of 0.30 to 0.74; and d⁻ strains, having an EOP ratio to LSc of 0 to 0.29.

Test for T character⁷⁾

Parallel virus titrations were performed under two conditions: using 37°C and 40°C (for type III, 40.3°C) for the incubation of the inoculated HeLa cell cultures⁹⁾. The temperature variation of the 40°C incubation was $\pm 0.1^\circ C$. CPE titers were estimated on days 5, 7, 9 and 11 of the test. The maintenance medium for established cells consisted of 90 % Melnick-Earles medium (0.5 % lactalbumin hydrolysate in Earles salt solution), 10 % Carnation Brand skim milk solution, 100 μ /ml penicillin, 100 μ g/ml dihydrostreptomycin. Parallel virus titrations were performed under two conditions: using 37°C and 40°C for the incubation of the inoculated cultures. The difference in titers obtained at the two temperatures was expressed as $\log_{10}$ EOG (efficiency of growth) values ($\log_{10}$ EOG = $\log TCD_{50}$ per ml at 37°C

minus log TCD₅₀ per ml at 40°C). The authors arbitrarily determined the T value of a strain by the ratio of EOG to LSc. Here again the strains tested were separated arbitrarily into three groups: T⁺ strains — strains which had an EOG ratio to LSc greater than 0.75; intermediate or T[±] strains — those having an EOG ratio to LSc of 0.30 to 0.74; and T⁻ strains — with an EOG ratio to LSc of 0 to 0.29.

Test for MS Character³⁾

This character was determined by measuring the plaque on MS cells after 6 days of inoculation. Virus strains that produced tiny plaques smaller than 3 mm in diameter, or no plaques at all, were classified as MS⁻. On the other hand, strains that produced plaques larger than 5 mm on MS cells were considered to possess the MS⁺ character of wild strains³⁾. The nutrient medium for agar overlay consisted of: 10 times Hanks solution 18.0 ml, sterile distilled water 58.4 ml, neutral red (1:1000) 3.0 ml, calf serum 3.6 ml, NaHCO₃ (7.0 %) 5.8 ml, 40,000 μ /ml penicillin 1.0 ml, and 200 mg/ml dihydrostreptomycin 0.2 ml. Agar solution consisted of 90.0 ml of 2.7 g distilled water. After sterilization, it was cooled to 45°C before use. Just before making the overlay, equal portions of the nutrient medium (at 37°C) and the agar solution (at 45°C) were mixed.

Test for CFt character³⁾

This character was determined by the behavior of the heating antigen at various temperatures in the complement fixation test. When serial two-fold dilutions of antigen were tested against serial two-fold dilutions of homotypic sera, virus strains that indicated complement fixing ability of heating antigen at various temperatures the same or lower than nonheated antigen were classified as CFt⁻. On the other hand, the complement fixing ability of heating antigens at various temperatures higher than nonheated antigen were classified as CFt⁺. The virus strain whose highest complement fixing ability was indicated at 85°C (75°C, 65°C, 56°C) heating antigen was classified as CFt⁺₈₅ (CFt⁺₇₅, CFt⁺₆₅, CFt⁺₅₆).

EXPERIMENTAL RESULTS

Paralytic poliomyelitis in Nagasaki Prefecture, during May and August, 1960. Distribution of poliomyelitis infection.

The reported cases of paralytic poliomyelitis and their morbidity, mortality and fatality in Nagasaki Prefecture, 1959-1961, are given in Table 1.

TABLE 1
Number of Patients and Deaths from Poliomyelitis, 1959-1961, in Nagasaki Prefecture

Year	No. of cases	Morbidity*	No. of Deaths	Mortality*	Fatality‡
1959	35	1.99	2	0.18	5.7
1960	97	5.51	7	0.40	7.2
1961	46	2.62	8	0.45	17.4

* Per 100,000 population

‡ Percentage

TABLE 2
Reported Cases of Paralytic Poliomyelitis by Months, January 1960 through December 1961, in Nagasaki Prefecture

Year	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
1960	1	1	4	3	13	12	32	16	5	2	5	3
1961	1	1	0	0	1	12	14	16	0	1	0	0

Reported cases each month

TABLE 3
Reported Cases of Paralytic Poliomyelitis by Months, January through December 1960, in Nagasaki city

Year	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
1960	3	1	2	3	11	9	6	2	1	0	0	0

Fig. 1. Geographic Area and Number of Poliomyelitis Cases in Nagasaki Prefecture, 1960.

The reported cases of paralytic poliomyelitis by months, January 1960 through December 1961, in Nagasaki Prefecture and Nagasaki City are given in Table 2 and Table 3.

The geographic areas and number of poliomyelitis cases in Nagasaki Prefecture, 1960, are given in Fig. 1. Fig. 2 gives the geographic distribution of poliomyelitis in Nagasaki city, Nagasaki Prefecture, 1960.

Fig. 2. Geographic Area and Number of Poliomyelitis Cases in Nagasaki city, 1960.

Isolation and typing of poliovirus in Nagasaki Prefecture, 1960.

The authors attempted to isolate poliovirus from stool specimens of paralytic patients and their contacts by inoculation of tissue cultures of HeLa cells. The results are summarized in Table 4. Nineteen agents were isolated during March

and October in Nagasaki City and neighboring districts, Nagasaki Prefecture; 7 were type I, 7 were type II, and 5 were type III polioviruses.

TABLE 4
Isolation and Typing of Poliovirus in Nagasaki city and Neighboring Districts, 1960

DATE	CORD No.	SEX	AGE	DAYS AFTER	TYPE
Mar. 4	N- 1	M	2.6	64	
14	N- 2	M	1.1	54	
22	N- 3	F	6.7	46	
May 3	N- 5	M	5.3	35	
5	N-12	M	0.4	33	I
10	N- 8	M	2.3	28	
14	N- 9	F	1.0	24	
15	N- 4	M	2.0	23	I
16	N-10	M	3.8	22	
17	N- 6	F	0.8	21	I
18	N- 7	M	1.0	20	I
20	N-11	F	1.9	18	
26	N-12	M	1.8	12	
29	N-18	M	1.0	21	I
Jun. 1	N-15	F	7.1	14	II
3	N-14	M	1.11	11	II
7	N-17	M	8.0	7	I
8	N-19	M	8.0	12	II
17	N-20	M	1.4	3	II
28	N-29	M	1.9	10	I
30	N-26	M	1.8	13	II
Jul. 17	N-35	F	3.3	4	III
17	N-27	M	5.0	8	III
Aug. 15	N-42	M	5.0	16	II
18	N-30	M	1.0	6	III
24	N-28	M	1.6	5	II
Oct. 20	N-33	F	2.0	21	III
Feb. 7	N-36	F	1.4	11	III

d, T, MS and CFt marker test of poliovirus strains excreted by clinical poliomyelitis in Nagasaki Prefecture, 1960.

Within the past several years, studies have been made of at least seven markers of poliovirus which can be determined by tissue culture, physical property and the serological method, and which tend to discriminate between neurovirulent and attenuated strains. The d, T, MS and CFt markers were used in this study. Nineteen strains excreted by clinical poliomyelitis and two strains excreted by normal contacts in Nagasaki Prefecture were used. The results are summarized in Table 5, 6 and 7. Eight of 19 strains excreted by clinical poliomyelitis posse-

TABLE 5

d Marker test of Poliovirus Strains Excreted by Clinical Poliomyelitis in Nagasaki city, 1960

CORD No.	type	Log ₁₀ PFU/0.2ml		Log ₁₀ EOP	EOP ratio to LSc	character
		0.4%	0.1%			
N-12	I	3.5	4.0	-0.5		d ⁺
N- 4	I	6.4	5.5	0.9	0.45	d [±]
N- 6	I	6.5	6.0	0.5	0.25	d ⁺
N- 7	I	6.8	5.8	1.0	0.5	d [±]
N-18	I	7.0	6.0	1.0	0.5	d [±]
N-15	II	6.8	5.0	1.8	0.9	d ⁻
N-14	II	4.9	4.9	0		d ⁺
N-17	I	6.5	0.5	6.0	3.0	d ⁻
N-19	II	3.5	3.3	0.2	0.1	d ⁺
N-20	II	4.6	5.0	-0.4		d ⁺
Mahoney		5.5	5.5	0		d ⁺
LSc		5.5	3.5	2.0	1.0	d ⁻

TABLE 6

T Marker Test of Poliovirus Strains Excreted by Clinical Poliomyelitis in Nagasaki City, 1960

CORD No.	Type	Log ₁₀ TCD/0.1ml		Log ₁₀ EOG	EOG ratio to Lsc	T
		37°C	40°C			
N-12	I	4.5	0.5	4.0	0.8	T ⁻
N- 4	I	5.5	3.8	1.7	0.34	T [±]
N- 6	I	6.2	2.8	3.4	0.68	T [±]
N- 7	I	5.5	2.2	3.3	0.66	T [±]
N-18	I	6.5	5.2	1.3	0.26	T ⁺
N-15	II	6.5	5.5	1.0	0.2	T ⁺
N-14	II	6.5	1.8	4.7	0.94	T ⁻
N-17	I	6.2	1.5	4.7	0.94	T ⁻
N-19	II	4.8	4.2	0.6	0.12	T ⁺
N-20	II	6.5	5.5	1.0	0.2	T ⁺
Mahoney		6.5	6.5	0	0	T ⁺
LSc		6.5	1.5	5.0	1.0	T ⁻

ssed the d⁺ character, 5 were intermediate and 6 were d⁻ character. Five of 19 strains excreted by clinical poliomyelitis possessed the T⁺ character, 4 were intermediate and 10 were T⁻ character. Seventeen of 19 strains excreted by clinical poliomyelitis possessed the MS⁺ character and two were MS⁻. Nine of 10 strains excreted by clinical poliomyelitis possessed the CFT⁺₆₅ character and one was CFT⁻.

Paralytic poliomyelitis in Nagasaki Prefecture during June and August, 1961.
Distribution of poliomyelitis infection.

TABLE 7

d, T, MS and CFt Marker Tests of Poliovirus Strains Excreted by Clinical Poliomyelitis Patients and their Contacts in Nagasaki city, 1960

CORD No.	Type	d	T	MS	CFt
N-12	I	d ⁺	T ⁻	MS ⁺	CFt ⁺ ₆₅
N- 4	I	d [±]	T [±]	MS ⁺	CFt ⁻
N- 6	I	d ⁺	T [±]	MS ⁺	CFt ⁺ ₆₅
N- 7	I	d [±]	T [±]	MS ⁺	CFt ⁺ ₆₅
N-18	I	d [±]	T ⁺	MS ⁺	CFt ⁺ ₆₅
N-15**	II	d ⁻	T ⁺	MS ⁺	CFt ⁺ ₆₅
N-14*	II	d ⁺	T ⁻	MS ⁺	CFt ⁺ ₆₅
N-17	I	d ⁻	T ⁻	MS ⁺	CFt ⁺ ₆₅
N-19	II	d ⁺	T ⁺	MS ⁺	CFt ⁺ ₆₅
N-20	II	d ⁺	T ⁺	MS ⁺	CFt ⁺ ₆₅
N-23***	II	d ⁺	T ⁻	MS ⁺	CFt ⁺ ₅₀
N-22* *	II	d [±]	T ⁻	MS ⁻	CFt ⁻
N-29	I	d ⁻	T ⁻	MS ⁻	
N-26	II	d ⁻	T ⁻	MS ⁺	
N-35	III	d ⁺	T ⁻	MS ⁺	
N-27	III	d ⁻	T ⁻	MS ⁺	
N-42	II	d [±]	T ⁺	MS ⁺	
N-30	III	d ⁻	T ⁻	MS ⁻	
N-28	II	d [±]	T [±]	MS ⁺	
N-33	III	d ⁺	T ⁻	MS ⁺	
N-36	III	d ⁺	T ⁻	MS ⁺	

* contacts (*, ** same family)

The number of poliomyelitis cases in Nagasaki Prefecture, 1961, is given in Table 2.

Distribution of infections as measured by neutralizing polio antibody in Nagasaki Prefecture, 1961.

Four serum specimens from paralytic poliomyelitis patients living in Nagasaki

TABLE 8

Distribution of Infections as Measured by Neutralizing Polio Antibody in Nagasaki City, 1961

Cord No.	Sex	Age	Antibody			Result
			I	II	III	
N-1-61	F	5j 9m	1024	< 4	< 4	I
N-2-61	F	4j10m	< 4	64	< 4	II
N-3-61	M	3m	1024	< 4	< 4	I
N-4-61	M	6j 5m	< 4	1024	16	II

Prefecture were examined. The results are summarized in Table 8.

As may be seen, antibody titer against type I and type II were detected in the total of paralytic poliomyelitis patients.

DISCUSSION

Prior to 1947, only a few cases of poliomyelitis were reported and these were confined mostly to the Nagasaki Prefectural Government. Since 1947, when poliomyelitis became a reportable disease, a more accurate estimate of the number of paralytic cases per annum has been available. From 1948 to date, the number of reported cases has increased considerably. Table 1 shows the number of cases reported from 1959 to 1961.

The seasonal distribution of poliomyelitis in Nagasaki Prefecture during 1960 and 1961 is shown in Table 2.

An epidemic occurred in Nagasaki Prefecture during the early summer of 1960. Nineteen agents were isolated during March and October in Nagasaki City and neighboring districts, Nagasaki Prefecture; 7 were type I (31%), 9 were type II (40%), and 5 were type III polioviruses (29%).

On the other hand, from the winter of 1960 through the early summer of 1961, the isolation of virus and neutralization tests were studied in 121 clinical poliomyelitis cases occurring in Fukuoka Prefecture by Shingu et al.⁴⁾ One hundred twenty-one polio cases were identified; 105 of 121 cases were found to be type I (87%), 6 cases were type II (5%), and 10 cases were type III (8%).

In spite of the immense amount of literature accumulated on the serological changes caused in the living body by poliovirus infection, little information is available on the virulence of excreted virus. Within the past several years, studies have been made of at least seven *in vitro* markers of poliovirus which can be determined by tissue culture, physical property and serological method, and which tend to discriminate between neurovirulent and attenuated strains.

Nineteen strains from paralytic poliomyelitis in Nagasaki Prefecture in 1960 were examined from the view of at least four *in vitro* marker tests. The results

TABLE 9

Summary of in vitro Marker Test of Poliovirus Strains Excreted by Clinical Poliomyelitis, 1960-1961

	Type*			d			T			MS			Cft			
	I	II	III	+	±	-	+	±	-	+	±	-	[†] ₇₅	[†] ₆₅	[†] ₅₆	-
Nagasaki 60, V-VII	7 31%	9 40%	3 29%	8 42%	5 26%	6 32%	5 26%	4 21%	10 53%	17 90%	0 0	2 10%	0 0	12 80%	1 7%	2 13%
Moji & Kokura 60, IX-XI	65 83%	3 5%	10 12%	14 82%	0 0	3 18%	12 70%	4 24%	1 6%	15 88%	1 6%	1 6%	8 47%	2 12%	2 12%	5 29%
Fukuoka, Saga Yamaguchi 61, VI-VIII	40 93%	3 7%	0 0	19 83%	2 8%	2 8%	17 74%	3 13%	3 13%	16 70%	1 4%	6 26%	4 17%	9 39%	4 17%	6 26%

* Typing with virological and serological examinations

are shown in Table 9. As may be seen, there is no semblance between the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in Nagasaki Prefecture, 1960, and the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in Fukuoka Prefecture, 1960 and 1961. But there is some semblance between the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in the northern part of Fukuoka Prefecture, 1960, and the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in Fukuoka, Saga and Yamaguchi Prefectures, 1961. It is logical to believe that the epidemic of poliomyelitis in Fukuoka Prefecture, 1961, may be attributed to an epidemic of poliomyelitis in the northern part of Fukuoka Prefecture in the winter of 1960.

The number of patients and morbidity of poliomyelitis in 1960 in Nagasaki and the Kokura-Moji area are shown in Table 10. As may be seen, the poliomyelitis epidemic of the Kokura-Moji area was larger than the epidemic of the Nagasaki area. Then the virulence of poliovirus, which can be expressed as *in vitro* marker, manifested itself to a lower degree in the Nagasaki area than in the Kokura-Moji area, but the type of virus in the two epidemics was not identical.

TABLE 10
Number of Patients and Morbidity of Poliomyelitis, 1960, in Nagasaki and Kokura Moji Area

City	Population	No. of cases	Morbidity
Nagasaki	344,153	38	11.1
Kokura, Moji	286,474	70	24.4
	152,081	49	32.2
	438,555	119	27.8

SUMMARY

Poliomyelitis became epidemic in Nagasaki Prefecture in the summer of 1960. Nineteen agents were isolated during March and October in the epidemic of Nagasaki Prefecture; seven were type I, nine were type II, and three were type III polioviruses. Then 4 serum specimens from paralytic poliomyelitis patients in the epidemic of Nagasaki Prefecture, 1961, respectively, were examined. Antibody titer against type I and type II was detected at the highest percentages of this epidemic.

All polioviruses isolated in Nagasaki Prefecture, 1960, have been studied from the viewpoint of at least four *in vitro* marker tests.

There is no semblance between the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in Nagasaki Prefecture, 1960, and the *in vitro* markers of the poliovirus strains excreted by an epidemic of poliomyelitis in Fukuoka Prefecture, 1960 and 1961.

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