VIROLOGICAL STUDIES OF SMALLPOX IN AN ENDEMIC AREA

II. VIRUS CONTENT OF CLINICAL SPECIMENS AND TYPING OF VIRUS ISOLATES

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SUMMARY: From 78 patients under the surveillance of Smallpox Eradication Program in Bihar State, India, 113 virological specimens were collected by the authors in February and March, 1975. Specimens were sent through W.H.O. to the authors' laboratory in Tokyo, Japan, for the assay of virus contents under rigid freezing in liquid nitrogen.

1) Specimens from 55 cases gave positive virus isolation on chick chorioallantoic membranes (CAM); all cases were smallpox-positive in immunofluorescence (IF) diagnosis in the field study.

2) Isolated viruses were identified with variola virus by the hyperplastic focus formation on HeLa cell monolayers.

3) Average virus titers of positive specimens expressed in terms of $\log_{10}$ PFU (pock-forming units)/specimen were 2.87, 4.61, and 4.81 in throat swab (TS, 7 cases), vesicular-pustular fluid (VP, 20 cases) and scab (SB, 41 cases), respectively. Highest titers were 4.01 (TS), 6.06 (VP), and 6.78 (SB). There was significant correlation between the virus contents of TS and VP specimens and the days of illness at the collection, whereas no significant decline in virus contents of SB specimens was observed up to the 30th day of illness.

4) Scabs from one case were kept at room temperature under dry conditions. No significant loss in virus contents was shown during the first 4 weeks and a loss of only 0.7 $\log_{10}$ at the end of 16 weeks' storage, suggesting an important role of scabs in the genesis of remote outbreaks.

5) Typing of isolated virus strains by temperature-sensitivity (Ts) of hyperplastic focus formation on HeLa cell monolayers were attempted by a semi-micro culture system. Of 55 isolates, 51 were variola major type, while the remaining four strains demonstrated Ts between V. major and minor, suggesting the presence of an intermediate type in Indian smallpox. Epidemiological situations among cases of intermediate isolates are discussed.

INTRODUCTION

Proper knowledge of the infectious variola virus contents of various speci-

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mens from smallpox patients in relation to the clinical and epidemiological pictures and stages of the disease should be indispensable in establishing virological basis for the effective containment of smallpox in nonendemic areas; i.e. excretion of the infectious virus should be estimated and properly treated to prevent the transmission through the excretes, and localization of virus and viral antigen(s) should be known for the collection of diagnostic specimens for the effective and sensitive detection of early or atypical patients. Sarkar *et al.* (1973a) have traced the virus excretion in throat, urine and conjunctiva with various types of smallpox and demonstrated relatively high virus titers in the excretes. They also demonstrated infectious virus in throat swabs from 34 of 328 family contacts several days after the contact and 30 of these virus-positive cases did not develop into clinical smallpox, giving warnings about the possibility of asymptomatic cases excreting significant amounts of infectious virus (Sarkar *et al.*, 1973b).

Differentiation of variola virus types in relation to the epidemiological mechanism has been attempted in various ways. The ceiling temperature of pock formation on chick chorioallantoic membranes (CAM) has been successful to differentiate into three types, i.e. variola major, minor and intermediate (Nizamuddin and Dumbell, 1961; Bedson *et al.*, 1963), in accordance with the geographical distribution of the original outbreaks. Kitamura and Tanaka (1973) developed a tissue culture microassay system using the temperature sensitivity of hyperplastic focus formation by variola virus. Dumbell and Huq (1975) reported a typing system using tissue culture hemadsorption and suggested an intermediate group possibly coinciding with the intermediate pock typing.

The present authors had an occasion to visit smallpox patients under the surveillance of Smallpox Eradication Program in Bihar State, India, in February and March, 1975, and collected diagnostic specimens for rapid diagnosis by immunofluorescence (IF) staining in the field in combination with the virus isolation with the specimens sent to the home laboratory (Kitamura *et al.*, 1977). The IF diagnosis was demonstrated to have no possibility of false-negative results and helped the epidemiological activities there. Fifty-five out of 58 IF-positive and epidemiologically confirmed cases were positive in variola virus isolation. The present communication reports the results of virus contents of the positive specimens, analysis for the relationship between the virus titer and the days of illness and the attempts at typing the virus isolates by the temperature sensitivity of tissue culture focus formation.

MATERIALS AND METHODS

Smallpox cases and specimens collected: The 78 cases visited and specimens collected were described in the preceding paper (Kitamura *et al.*, 1977). The specimens were sealed in polypropylene tubes (Nunc, Denmark) and stored in a liquid nitrogen container until titration.

Virus titration: Vesicular or pustular fluid blotted on one cotton stab per lesion as well as a throat swab was extracted in 2.0 ml of PBS (0.01 M phosphate-buffered saline, pH 7.4, with 50 μ g/ml of gentamicin). A scab was homogenized in 2.0 ml of PBS with a mortar and a pestle. Serial 10-fold dilutions of the specimen were inoculated onto CAM's of 12-day eggs and pocks developed on at least 4 CAM's per dilution were counted after 72-hr incubation at 35 C. Variola virus nature was confirmed by parallel inoculation onto HeLa cell monolayers to allow formation of hyperplastic foci (Kitamura, 1968).

Virus typing: The semi-micro temperature-sensitivity test of hyperplastic focus formation was carried out by the modified microassay system described earlier (Kitamura and Tanaka, 1973). A semi-micro plastic culture plate having 24 flat-bottomed wells of 16 mm in diameter (Linbro No. FB16-24TC) was seeded with 0.5 ml/well of 2.0×10^5 cells/ml HeLa cell suspension as described elsewhere (Kitamura, 1968). Duplicate plates were inoculated with appropriate dilutions of the virus in Hanks' BSS, 0.1 ml/well, 2 wells/dil./plate, to be added with 0.5 ml/well of the maintenance solution after an adsorption period of 2 hr at 35 C. Plates were sealed with non-toxic sealing film (Linbro No. 64-PSM) and put in water baths, one adjusted to 35.0 C and the other to 38.3 C in accuracy within 0.05 C. After incubation for 3 days, the cell sheets were washed with PBS and stained with Wright's solution as described earlier (Kitamura and Tanaka, 1973). Focal lesions were counted as semi-micro foci of ca. 0.2 mm in diameter on the screen of a microplate projector (Medical Viewer, Model GR-3, Hoken Shizai, Tokyo) at magnification of $\times 8$. The numbers of foci formed at 35.0 and 38.3 C were compared as described in the text.

RESULTS

Virus Contents of the specimens

One hundred and thirteen specimens (54 scabs, 39 vesicular-pustular fluids, 19 throat swabs and one conjunctiva exudate) including those from non-smallpox cases were assayed and 69 (41 scabs, 20 vesicular-pustular fluids, 7 throat swabs and one conjunctiva exudate) gave positive virus contents. All but one virus isolate which was vaccinia virus from the scab of vaccination site (case No. 30) as reported earlier (Kitamura et al., 1977) were identified with variola virus. Virus titers of the specimens are plotted in Fig. 1 in terms of $\log_{10}$ PFU/specimen against days after the rash onset at the collection of the specimen. The average and highest log values are shown in Table I. As shown in Fig. 1, virus contents of the throat swabs and vesicular-pustular fluids had correlation with the lengths of time after the rash onset, the latter dropping sharply after the 15th day of illness and the former being restricted within 9 days of illness. Though we could not collect throat swabs from contacts before the rash onset, the present finding should suggest the diagnostic importance of throat swabs at the earliest stage of smallpox. This point may be confirmed by the scrutiny

Fig. 1. Variola virus titer in specimens in correlation with days of illness at the collection.

TABLE I

Virus isolation from patients' specimens.

Specimen	Number of positive case	Virus titers (log ₁₀ PFU/specimen)			Notes
		Highest	Lowest	Average	
Throat swab	7	4.01*	2.10	2.87	* 2 days after rash onset (p.e.)
Vesicular or pustular fluid	20	6.06**	2.00	4.61	** 2 cases, 3 and 4 days p.e.
Scab	41	6.78***	2.51	4.81	*** 10 days p.e.
Conjunctiva exudate	1	3.57	3.57	3.57	Case No. 39, 4 days p.e.

of cases which gave positive virus content in throat swabs (Table II). In cases Nos. 6 and 7, which were both ordinary-discrete type, 3-day specimens had significant virus but there was no detectable virus in 5 days specimens. The highest titer was found in case No. 75 2 days after the start of the papular stage. There was no significant decline in virus titer in scabs during the period between 7 to 30 days after the rash onset. The single case giving positive virus content in conjunctiva exudate was case No. 39. As described in Table II, this case was of the flat-confluent type and died 4 days after the collection. Conjunctivitis was not so severe at the collection (4 days after rash onset), but the oedema all over the eye lids was high in degree with thick mucous deposits.

TABLE II

Cases of positive virus isolation from throat swabs.

Case No.	Clinical data	Swab collection (days p.e. ^a)	Virus titer (PFU/specimen)
6	30y., m.	3	3.70×10^2 (2.5) ^b
	Ordinary, discrete	5	(—)
7	40y., f.	3	1.92×10^2 (2.28)
	Ordinary, discrete	5	(—)
39	30y., f. Flat, confluent	4 ^c	1.90×10^3 (3.28)
65	8y., f. Ordinary, discrete	6	3.34×10^3 (3.52)
69	4y., f. Ordinary, discrete	6	1.26×10^2 (2.10)
75	10y., m. Papular stage	2	1.03×10^4 (4.01)
76	8mth., m. Ordinary, discrete	8	4.04×10^2 (2.31)

a. days p.e.=days after the rash onset.

b. $\log_{10}$ PFU/specimen.

c. Died 4 days after the collection of this specimen.

Persistence of infectious Virus in Scabs at Room Temperature

About 50 scabs of case No. 11 (ordinary-discrete type; scabs collected from bedding and clothes) were put in glass tubes in a metal container with desiccant and kept at room temperature (18–25°C) for 4 months. Three scabs were taken out and homogenized as described in Materials and Methods to assay for the virus titer at 2 or 4-week intervals. The results are summarized in Table III. The virus titer of the scabs was highly stable, showing a drop during 4 months at room temperature by only 0.7 $\log_{10}$ (resulting in one-fifth of the original titer)

TABLE III

Varola virus titers in scabs kept at room temperature

Weeks at room temperature	Virus titer ($\log_{10}$ PFU/scab) ^a
0	4.99
2	5.05
4	5.00
6	4.68
8	4.75
12	4.52
16	4.26

a. Homogenates of 3 scabs were assayed.

and no significant loss during the first one month. These results demonstrated that the smallpox scabs under dry conditions could maintain their infectivity intact for several months at room temperature and may sound a big alarm about the disposal of scabs as a possible source of distant outbreaks.

Typing Variola Virus Isolates by Semi-micro Focus formation

As a preliminary test of typing by the semi-micro system, representative strains of variola major (Harvey, at 3rd CAM passage), minor (Butler, at 5th CAM passage), both designated as the International Reference Strains of two types (Fenner and Burnet, 1957), and intermediate (EA25-62 and E68-258, each at 5th CAM passage, obtained through the courtesy of Dr .K. R. Dumbell) viruses as well as one isolate in Indonesian outbreak which showed a relatively low case fatality rate (Indonesia-3, at 1st and 2nd CAM passages, obtained through Dr. S. Thaib, P. N. BioFarma, Bandung) were subjected to the test. Result are summarized in Table IV. Foci formed at 38.3 C in terms of percent

TABLE IV

Variola virus typing by the focus temperature-sensitivity test with reference and standard strains

Variola type	Strain	Average foci/well at:		Temperature-sensitivity (B/A in %)
		35.0 C(A)	38.3 C(B)	
Major	Harvey	52.0	34.0	65
Minor	Butler	124	10.0	8.0
Intermediate	EA25/62	25.0	10.5	42
	E68-258	39.5	12.5	32
(Unknown)	Indonesia-3 (E-1) ^a .	42.0	9.5	23
	" (E-2) ^b .	95.0	28.5	30

a. First CAM passage by direct inoculation of the scab homogenate.

b. Second CAM passage.

of those at 35.0 C were higher than 60, less than 10, and between 10 and 60 with variola major, minor and intermediate, respectively. A tentative category of the intermediate type was set as having a focus temperature-sensitivity (38.3 C/35.0 C in %) between 10 and 60%. By this criterion, Indonesia virus was identified as the intermediate group, irrespective of the generation of CAM passage, suggesting the efficacy of the present system in typing variola virus isolates.

Fifty-five isolates in the present study within two passages on CAM were subjected to typing. In every test, there were parallel control plates inoculated with standard strains of the three types (Harvey, Butler and EA25/62) to confirm the reproducibility of the results in Table IV. When the results were not

TABLE V

*Summary of Typing by Focus Temperature-Sensitivity (Ts)
Test on Hela Cell Monolayers*

Case No.	Ts ratio in % ^a .			Type designation
	Assay 1.	Assay 2.	Assay 3.	
1	17	46	—	Intermediate
2	60	—	—	Major
3	97	—	—	Major
4	98	—	—	Major
5	55	78	—	Major
6	76	—	—	Major
7	60	—	—	Major
9	72	—	—	Major
11	66	—	—	Major
12	63	—	—	Major
13	74	—	—	Major
14	67	—	—	Major
15	89	—	—	Major
16	96	—	—	Major
17	85	—	—	Major
18	15	49	47	Intermediate
19	9.4	34	27	Intermediate
24	66	—	—	Major
25	78	—	—	Major
26	78	—	—	Major
27	62	—	—	Major
28	62	—	—	Major
30	74	—	—	Major
32	70	—	—	Major
34	84	—	—	Major
35	67	—	—	Major
36	72	—	—	Major
37	92	—	—	Major
38	88	—	—	Major
39	80	—	—	Major
40	66	—	—	Major
41	76	—	—	Major
42	76	—	—	Major
43	40	54	—	Major
44	69	—	—	Major
46	64	—	—	Major
47	69	—	—	Major
48	88	—	—	Major
49	76	—	—	Major
50	72	—	—	Major
51	78	—	—	Major
52	76	—	—	Major

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(Table V, continued)

54	67	—	—	Major
55	73	—	—	Major
56	63	—	—	Major
57	63	—	—	Major
64	67	—	—	Major
65	84	—	—	Major
66	91	—	—	Major
69	66	—	—	Major
72	78	—	—	Major
74	83	—	—	Major
75	67	—	—	Major
76	65	—	—	Major
78	68	—	—	Major
Standard Viruses:				
Major (Harvey)	75	68	78	
Minor (Butler)	5.0	7.0	8.0	
Intermediate (EA25/62)	35	41	38	
(Summary) 55 isolates; Major 51; Minor 0; Intermediate 4.				

a. Number of foci formed at 38.3 C/that at 35.0 C expressed in per cent.

those of typical major, second or third tests were repeated as shown in Table V. Fifty-one isolates were identified as the major and the other four (cases Nos. 1, 18, 19, and 43) were the intermediate, being confirmed by the repeated tests. There was no case identified as the minor type. In cases Nos. 1, 19 and 43, individual isolates from different scabs were compared and gave identical typing patterns. In case No. 18, two isolates from vesicular fluids of two different lesions gave the same results. This should be the first report of the isolation of the intermediate type from Indian outbreaks. Epidemiological situations shall be discussed later.

DISCUSSION

Virus excretion in clinical smallpox has extensively been studied by Sarkar *et al.* (1973a, b). They demonstrated high virus excretions in throat swabs, urine and conjunctival exudate, ranging from 10^1 to 10^5 PFU/specimen. Sarkar *et al.* (1973a) reported that the severer the type of illness, the higher the virus titer in throat swabs at an early stage of the illness; thus hemorrhagic and confluent types excreted nearly 10^5 PFU/swab of the virus within 5 days of the illness, whereas the titer in cases of discrete type was not higher than 10^4 . Persistence of infectious virus in throat exudate was also longer in the cases of confluent

type, up to the 14th day of illness, while the longest persistence was 10 days in the discrete type. The results of the present studies showed an essential coincidence. Though cases studied were mostly the ordinary-discrete type, virus content showed a level of 10^3 – 10^4 PFU/swab during the first 4–5 days of illness followed by a sharp decline after the 6th day. The highest titer was $10^{4.01}$ PFU/swab in case No. 75, from which the specimen was taken at the papular stage, 2 days after the rash onset. As pointed out by Sarkar et al. (1973b) throat exudates should be infectious long before the rash onset, rather having possibly the highest infectivity during this stage causing severe air-borne infections (Wehrle et al., 1970). Thus it might be indicated that the diagnostic specimen of smallpox contacts or suspects during their incubation period should be the throat swabs for both virus isolation and morphological tests (electron microscopy and immunofluorescence). Though our preceding study (Kitamura et al., 1977) could not successfully apply the immunofluorescence test on throat swabs, it should eventually be made effective by improvements in the specificity of the labeled antibody.

Virus contents of smallpox scabs have been studied by Mitra, Sarkar and Mukherjee (1975). They reported that the virus content of 0.1 g of scab was higher than 10^5 PFU and there was no significant difference from one scab to another scab collected on different days of illness up to the 24th day. The highest titer was $10^{6.54}$ PFU/0.1 g of scab collected on the 15th day from a confluent case and the average was $10^{5.42}$. Our preliminary assay gave an average weight of a scab of about 30 mg. The highest value of $10^{6.78}$ and the average $10^{4.81}$ PFU/scab (Table I) may be converted to nearly $10^{7.28}$ and $10^{5.31}$ PFU/0.1 g of scab, respectively. Findings are in good coincidence with those of Mitra et al. (1975). Such a high titer as $10^{6.78}$ PFU/scab together with the long persistence of virus during storage at room temperature (Table II) should be taken seriously as these suggest the possible role in the etiology of remote, in time and distance, smallpox outbreaks.

Virus typing by temperature-sensitivity of focus formation on HeLa cells revealed that four out of 55 isolates in India were of the intermediate type and the other 51 were identified as the major type as expected. The detection of the intermediate type has been restricted to East African isolates (Bedson, Dumbell and Thomas, 1963; Dumbell and Huq, 1975; Baxby, 1975). Though we could not correlate our results in tissue culture focus formation with other systems such as ceiling temperature of pock formation and tissue culture hemadsorption, our isolation of four intermediates as well as demonstration of one intermediate among Indonesian isolates may suggest a rather high frequency of this type among Asian outbreaks. Epidemiological situations of four cases giving intermediate isolates are summarized in Table VI. Case No. 1 was in a village which had a big outbreak for nearly 3 months with more than 50 patients and belonged to the last several cases in that outbreak. Four other cases in the neighboring houses gave virus of the major type. Cases No. 18 and 19 occurred in a village about 50 km from the village of No. 1. There was no

TABLE VI

Epidemiological situations of the cases giving the intermediate isolates.

Case No.	Patient	Village, district	Rash onset	Clinical data	Other isolates in the same outbreak
1	██████ 3mth, m,	Pawapuri, Nalanda	1/13/75 ^b .	ordinary, discrete scab, 25 d.p.e. ^a .	4 majors (Cases No. 2, 3, 4, & 5)
18	██████ 10y., f,	Ichahose, Islampur	2/5/75	ordinary, discrete vesicular fluid, 4 d.p.e.	1 intermediate Case No. 19)
19	██████ 17y., f.	Ichahose, Islampur	1/17/75	ordinary, discrete scab, 23 d.p.e. neighboring upon case No. 18.	1 intermediate (Case No. 18)
43	██████ 7y., f.	Sarara, Madhubani	2/2/75	ordinary, discrete scab, 16 d.p.e.	5 major (Cases No. 42, 44, 46, 47, & 48)

a. d.p.e.=days after rash onset, the collection of specimen.

b. 1/13/75=13 January, 1975.

other case in its more closely surrounding villages within 10 km radius. They lived in neighboring houses and No. 18 started her illness 19 days after that of No. 19, being infected possibly in an early stage of the latter. No. 43 was the last case of an outbreak in an northern-most area of the state about 300 km apart from the villages of No. 1, 18 and 19. There was five other isolates in the same outbreak; they have been typed as major. There was a record of a smallpox outbreak of 20 cases without prior vaccination with no fatality in Sunsari District of South-eastern Nepal neighboring the northern part of Bihar state in 1974 (J. Friedman, personal communication) and etiology of some mild variant of variola virus has been suspected. It should be impossible to make further speculations about the genesis and the epidemiological significance of the isolation of intermediates among Asian outbreaks simply upon the present data. We should attempt to: (1) correlate the focus typing results with those of other typing systems, including the pathogenicity on chick embryos, to confirm that the intermediates isolated here are identical to those described by other workers (Bedson *et al.*, 1963; Dumbell and Huq, 1975); (2) repeat the typing with other isolates collected in some other areas of Asia such as Pakistan and Bangladesh.

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