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Studies On Viability Of Variola Virus*—Part I—Viability Of Variola Virus In Smallpox Material On Glass Slides, In Capillary Tubes On Filter Paper And Cotton Threads

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The results of investigation made on the viability of the variola virus in smears of vesicular fluid taken on glass slides, vesicular fluid in capillary tubes, and cotton threads soaked with the infective materials show that among the various methods of collection and despatch of specimens of vesicular fluid of smallpox cases, for laboratory diagnosis, sending a piece of cotton thread (taken from lint) soaked in the vesicular fluid and air-dried, is about the best method, since the virus is viable on the thread even for one week at room temperature. Because of the lack of enough clinical smallpox cases, 20% suspension of egg passaged virus had to be used in place of vesicular fluid in a few experiments.

Introduction

This study was designed to find out as to how long the virus can be isolated from the infective material preserved under different conditions of storage.

Material and Methods

Virus : Fluid from and scrapings of bases of papulo-vesicular lesions of smallpox cases were used for making smears on glass slides.

Fluid from vesicles of smallpox cases and 20% suspension of virus collected from chorio-allantoic membrane (CAM) of 1st egg passage of vesicular fluid of smallpox used for viability studies with capillary tubes, filter papers and cotton threads.

Collection of Materials

Smears on glass slides : Skin over the papulo-vesicular lesions of smallpox cases (on or about 6th day of disease) was cleaned gently and sterilized with absolute alcohol.

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After evaporation of alcohol, the lesions were split with a sterile scalpel or needle, and the base of the lesion was scraped with the same lancet or needle and smears made on a series of clean glass slides and allowed to be air-dried.

Capillary tubes : Capillary tubes were filled with enough quantity of 20% suspension of virus collected from CAM of 1st egg passage of vesicular fluid of smallpox case and sealed at both ends.

Filter papers : Two square cm pieces were cut from sheets of whatman No. 1 filter paper. 0·1 ml of 20% suspension of virus collected from CAM of 1st egg passage of vesicular fluid of smallpox cases or 0·1 ml of pooled vesicular fluid collected from several vesicles of smallpox case, was used to completely soak each of such bits of filter paper. After soaking, each paper was air-dried and kept in small empty sterile bottles and then corked and sealed.

Cotton threads : Cotton threads were removed from a piece of lint and cut into pieces of 2" length. 0·1 ml of 20% suspension of virus collected from CAM of 1st egg passage of vesicular fluid of smallpox cases or 0·1 ml of pooled vesicular fluid collected from several lesions of a smallpox case, was used to completely soak each of such threads. After soaking, each thread was air-dried separately and kept in small empty sterile bottles and then corked and sealed.

Methods of preservation : One set of each specimen was cultured immediately after drying, to serve as control—another set, exposed to sunlight (open Petri dish exposed for varying periods of time between 10 A.M. and 2 P.M.), a third set kept in a Petri dish in a non-airconditioned room, at room temperature for periods varying from 24 h to 216 h depending on the experiment, and a fourth set kept in a card board box, parcelled in the same manner as is done for despatching parcels by post (such parcels are kept at room temperature in a non-airconditioned room for 24 h to 216 h depending upon the experiment).

Methods of culture for variola virus

Smears on glass slides : 0·1 ml of normal saline with Penicillin and Streptomycin (P and S) was added to the smear. It was thoroughly emulsified and pipetted out and mixed with 0·9 ml of normal saline with P and S to give a dilution of 10^{-1}.

Capillary tubes : 0·1 ml of the material from the capillary tube was taken and added to 0·9 ml of normal saline with P and S and thoroughly mixed to give a dilution of 10^{-1}.

Filter paper and cotton threads : 0·9 ml of normal saline with P and S was added to each vial containing either filter paper or thread. The vials were kept in deep freeze and allowed to freeze. They were taken out of thawed rapidly at room temperature. After proper agitation and squeezing, the adsorbants were discarded. This gave a dilution of 10^{-1}. In certain instances, a dilution of 10^{-1} was used and in certain others 10^{-2} and 10^{-3} were also used. But whatever be the dilution, 0·1 ml of the antigen was used for inoculation on to CAM. For each dilution normally 2 eggs were employed. The egg inoculation was done by the standard techniques (Kempe and Vincent 1964).

Results

Table I shows the results of culture of smears taken on glass slides preserved under different conditions. Immediate inoculation of the smear produced confluent lesions on CAM. One hour's exposure to direct sunlight destroyed the virus in the smear. At room temperature there was no virus at 72 h even at 10^{-1} dilution, though inoculation of smears stored in parcels produced about 3-4 lesions on CAM at 96 h.

Table I. Results of culture of variola virus from smears of vesicular fluid of smallpox cases on glass slides

Methods of preservation	Dilution of antigen	Immediate	Results of cultures at the end of				
			1 h	24 h	48 h	72 h	96 h
	10^{-2}	c.c.c. c.c.c.	..	..	..	...	..
Exposure to sunlight	10^{-2}	.. ..	0 0·0 0·0·0	..	..	—	—
Room temperature about 35° — 37°	10^{-2}			sc. sc. sc. a. sc. 100	0·0·0 d.0.0	..	...
	10^{-1}	..	..	..	...	0·0·0 0·0·0	0·0·0 0·0·0
In parcels	10^{-2}	...	..	c. c. d. c. c. sc	0·0·0 0·0·0		
	10^{-1}	..	..	...	—	sc. 100·100 42·30·18	3·2·9 3·4·d

.. : Not done c : Confluent sc : Semiconfluent
a : Adherent membrane d : Dead embryo 0 : No lesions on CAM
Numbers indicate the number of lesions on CAM

Table II shows the results of culture of variola virus suspension kept in capillary tubes and preserved under different conditions. Immediate inoculation produced semiconfluent lesions on the CAM. Two hours exposure of capillary tubes to direct sunlight destroyed the virus. When preserved at room temperature, even at 96 h, inoculation of CAM produced discrete lesions ranging from 18-75 in number, whereas by storage in parcels the virus appeared to be less viable since only about 4-5 lesions were produced on CAM. Even though after 144 h of storage, virus could be isolated on CAM, the number of lesions were so few ranging between 3 and 6 under both the conditions of storage that specific diagnosis may not be able to be made on such findings.

Table III shows the results of culture of filter paper and cotton threads soaked in vesicular fluid of smallpox cases and preserved under different conditions. Both at room temperature and in parcels, filter paper as well as cotton threads produced semiconfluent to confluent lesions on CAM.

Table II. Results of culture from variola virus suspension (egg passage) kept in capillary tubes

Methods of preservation	Dilution of antigen	Immediate	Results of culture at the end of									
			2 h	4 h	48 h	72 h	96 h	120 h	144 h	160 h	196 h	216 h
Exposure to direct sunlight	10^{-1}	sc. sc. sc.	.. 0 0 0	.. 0 0 0	..	..	..	..	..	..	..	..
At room temperature	10^{-1}	..	..	..	61 31 57	44 31 37	52 75 8	13 0 d	0 3 0	3 4 1	0 1 d	
In parcels	10^{-1}	..	..	..	22 27 d	33 35 39	5 4 d	1 5 3	4 2 6	0 0 0	0 0 0	0 0 0

.. : Not done sc : Semiconfluent d : Dead embryo 0 : No lesions on CAM

Numbers indicate the number of lesions on CAM

Table III. Results of culture of variola virus from cotton threads and filter paper soaked in vesicular fluid of smallpox cases

Method of preservation	Dilution of antigen	Nature of Material	Immediate	Results of culture at the end of				
				48 h	72 h	96 h	120 h	144 h
	10^{-1}	CT FP	c. c. c. c. c. d.	.. ..	.. ..	.. ..	.. ..	.. ..
At room temperature	10^{-1}	CT FP		d. c. c. c. c. c.	c. c. c. c. c. c.	c. a. c. c.	a. c. d. 200·2 00 d.	c. sc. sc. c. c. c.
In parcels	10^{-1}	CT FP	.. ..	c. c. c. c. c. c.	c. c. c. c. c. c.	c. c. c. sc. 50·50	c. c. 9 sc. d. sc.	c. sc. sc. c. sc. d

c : Confluent lesions on CAM sc : Semiconfluent lesions on CAM d : Dead embryo a : Adherent

0 : No lesion .. : Not done CT : Cotton thread FP : Filter paper

Numbers indicate the number of lesions on CAM

Table IV shows the results of culture of filter paper and cotton threads soaked in 20% suspension of virus collected from CAM of 1st egg passage of vesicular fluid of smallpox cases. In this experiment the specimens were packed in parcels only. To find out the amount of virus yield, the specimens were titrated up to 3 dilutions. These results indicate that the virus was found to be viable more frequently on cotton threads than on filter paper and fair amount of virus could be released from the threads even at 144 h producing 40 to 50 lesions on CAM with 10^{-1} dilution, though beyond 144 h the number of lesions was very few. Further, the virus appeared to be more viable on threads soaked with vesicular fluid of smallpox cases, than those soaked with egg passaged material (Table III).

Table IV. Results of culture of variola virus from cotton threads and filter paper soaked in variola virus suspension (preserved in parcels only)

Results of culture at the end of	Antigen dilution	Filter paper			Cotton thread		
Immediate	10^{-1}	sc.	sc.	sc.	sc.	sc.	sc.
	10^{-2}	200	200	200	27	26	25
	10^{-3}	62	55	49	34	21	22
48 h	10^{-1}	38	41	29	200	200	200
	10^{-2}	d	0	0	62	38	25
	10^{-3}	0	1	0	5	10	9
72 h	10^{-1}	5	8	20	150	6	
	10^{-2}	0	0	0	d	55	
	10^{-3}	0	0	0	8	3	
96 h	10^{-1}	3	7	5	200	100	90
	10^{-2}	0	0	0	10	27	3
	10^{-3}	0	0	0	9	2	1
120 h	10^{-1}	0	0	0	29	35	a
	10^{-2}	0	0	0	2	0	0
	10^{-3}	0	0	0	0	0	0
144 h	10^{-1}	3	2	2	55	42	a
	10^{-2}	0	0	0	0	0	d
	10^{-3}	0	0	0	0	d	2
168 h			nd			nd	
192 h	10^{-1}	0	0	0	7	6	17
	10^{-2}	0	0	0	1	0	3
	10^{-3}	0	0	0	0	0	0
216 h	10^{-1}	0	0	0	6	7	25
	10^{-2}	0	0	0	0	2	1
	10^{-3}	0	0	0	0	0	0

sc : Semiconfluent
d : Dead embryo
0 : No lesions on CAM
a : Adherent membrane
Numbers indicate number of lesions on CAM

Discussion

The common method advocated for despatch of specimens to virus laboratories for confirmation of diagnosis of a smallpox case has been **smears of vesicular fluid on**

glass slides. It was even believed that **from vesicle or pustule fluid dried on glass slide, virus can be isolated after several months. Refrigeration is unnecessary when such specimens are transmitted by post to the diagnostic laboratory** (Downie 1959). However, it is understood that in some parts of Africa from where specimens were sent, nearly 50% of specimens yielded no virus on culture in clinically positive cases of smallpox (Henderson 1969).

In tropical and developing countries where the laboratories are situated far away from the field, and where transport facilities are not easily available, collection of materials, methods of preservation, mode of despatch and the time taken between collection and culture, play very important role in determining the results. There is every likelihood of misinterpretation of findings of laboratory in even clinically positive cases of smallpox. From the results of this study, it is evident that the virus may not be quite stable in smears on glass slides and errors of diagnosis are likely to occur if much importance is given to laboratory findings on such smears.

Though capillary tubes may be somewhat better for despatch of specimens yet it is clear that the best method is to soak 2" cotton thread (taken from a piece of lint) in vesicular fluid, air dry and despatch them in small sterile bottles. It looks as though the virus can be detected in such specimens even after 7 to 8 days. Perhaps, this is the easiest, simplest, and best method of collecting and despatching the specimen of vesicular fluid to the laboratory when cases of smallpox are detected in the early stage of disease.

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