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Studies on Freeze Dried Smallpox Vaccine as a Source of Antigen in the Precipitation-in-Gel Test

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Three hundred and sixteen batches of freeze dried smallpox vaccine manufactured at Vaccine Institute, Belgaum; Institute of Preventive Medicine, Hyderabad; King Institute, Guindy, Madras; State Vaccine Institute, Patwadangar (India); Institute of Viral Preparations, Moscow (USSR); Lister Institute, Elstree (UK) and Rijks Institute Voor De Volksgezondheid, Utrecht (Netherlands) were tested for precipitation-in-gel (PIG) test using neat rehydrated vaccine as an antigen against antivaccinia rabbit serum 90·3 per cent of the vaccine batches from Belgaum, 100 per cent (14 out of 14) from Moscow, 100 per cent batches (2 out of 2) from Lister and 60 per cent batches (3 out of 5) from Utrecht gave precipitin lines in about 2 h. which linked with adjoining lines of precipitate in about 4 h. thus establishing their specificity and became prominent in about 20 h. Better results were obtained with the neat reconstituted vaccines in comparison to their two fold-serial dillutions viz. 1: 2 and 1 : 4. The results obtained with the neat vaccines after exposure to 32°C. for 12 h. and 16 h. did not in any way differ from those observed with their original lots kept in the refrigerator. Single line of precipitate appeared in the agar-gel with the neat vaccines boiled for one h. as compared to the double precipitin lines which developed against the original unboiled lots. Fifty per cent suspension prepared from the chorioallantoic membrane (CAM) of chick embryo (egg passage 1) inoculated with some of the indigenous vaccine batches also gave better results on the PIG test. These studies indicate that freeze dried smallpox vaccine manufactured at different production centres may give variable results on PIG test.

Introduction

Gispen (1955) was the first to use the technique of double diffusion in tubes with an agar gel with various pox-virus antigens including the vaccinia virus. He observed 3 to 6 zones of specific precipitation against the standard vaccinia serum. Two fractions were distinctly precipitated while the remaining ones were faintly precipitated and not always present.

Precipitation-in-gel (PIG) on microscopic glass slides was first applied by Dumbell and Nizamuddin (1959) for the laboratory diagnosis of smallpox. The precipitin lines appeared in two h. and joined with the precipitin lines given by the known positive control antigens in 4 to 5 h. thus establishing their specificity. Subsequently this test has been widely adopted by a number of workers (Marennikova and Maltseva,

1961; WHO, 1964 and 1969; Nicoli et al., 1964; Sehgal and Murty, 1972; Nakano, 1972) for routine diagnosis of smallpox.

In the present study different batches of freeze dried smallpox vaccine manufactured at the four vaccine production centres in India namely Belgaum, Hyderabad, Madras and Patwadangar and some batches procurred from Moscow (U.S.S.R.), Lister Institute (U.K.) and Utrecht (Netherlands) were tested to study their antigenicity in the PIG test.

Material and Methods

Antigens : The following antigens were prepared.

(*i*) **Neat vaccine antigens** : The freeze dried smallpox vaccine produced at different production centres was rehydrated in an appropriate volume of McLlvaine's buffer (pH 7·2) so as to have a neat vaccine.

(*ii*) **Diluted vaccine antigens** : Some of the freeze dried vaccine batches after reconstitution were further diluted in McLlvaine's buffer in order to have two-fold serial dilutions **viz.** 1 : 2 and 1 : 4.

(*iii*) **Vaccine antigens exposed to different temperatures** : Some of the vaccine batches from Belgaum and Hyderabad after reconstitution were exposed to a temperature of 32°C. for 12 h. and those from Patwadangar and Madras for 16 h. at 32°C. Twelve vaccine batches after reconstitution were also boiled for one h. The reconstituted lots were kept in the refrigerator as well to serve as controls at the time of testing.

(*iv*) **The CAM antigens** : Four freeze dried vaccine batches, having potency titre above 1×10^8 PFU/ml. on the CAM of chick embryo, from each of the four vaccine production centres in India were passaged on the chorioallantoic membrane of 12-day old chick embryos by the usual technique. CAMs were collected after 48 h. incubation of the inoculated eggs at 35-36°C. The egg passaged vaccinia virus also exhibited potency titre above 1×10^8 PFU/ml. The CAM exhibiting confluent pock lesions were pooled and shaken vigorously in sterile MacCartney bottles containing sterile glass beads and one ml. of McLlvaine's buffer per membrane. The suspension was centrifuged, supernatant collected under sterile precautions and treated with antibiotics. Each vaccine batch supernatant was then distributed in equal volumes in three vials. These vials were kept at 4°C. 32°C. and 38°C. for about 24 h. prior to conducting the PIG test.

Antivaccinia rabbit serum : It was prepared by hyperimmunizing healthy adult rabbits with Patwadangar freeze dried smallpox vaccine by the method adopted by Kempe and Vincent (1964). The hyperimmune serum was treated with thiomersal to a concentration of 1 : 10,000 and stored at —20°C.

Normal rabbit serum : It was obtained from normal healthy rabbits prior to immunization to serve as a control in the PIG test. It was preserved in the same way as mentioned earlier.

Preparation of gel slides and PIG test : Method recommended by WHO (1969) was adopted by preparing one per cent of special Agar-Noble in 0·85 per cent saline instead of dissolving capsules containing purified agar. The hyperimmune serum was poured in the central well and the vaccine antigen in six peripheral wells. Suitable controls were also kept each time using normal rabbit serum. After charging the well with the antiserum and the antigens, the gel slides were kept on wet filter paper in a petri dish at room temperature.

Results

The results of PIG test were read with an electric table lamp as a source of bright light against a black background. Hand lens was used to detect the presence of very faint or faint lines of precipitate.

The results of 316 batches of freeze dried smallpox vaccine tested for the PIG test are shown in Table I.

Table I. Results of PIG test conducted on vaccine batches.

Origin of vaccine	No. of batches tested	Results of batches showing			
		Clear precipitin line in 2 h. which became prominent in 20 h.	Faint precipitin line in about 4 h. which become prominent in 20 h.	Faint precipitin line in 4-20 h.	No. precipitin line in 20 h.
Belgaum	104	95	..	9	..
Hyderabad	28	..	16	12	..
Madras	93	..	47	44	7
Patwadangar	70	..	..	63	2
USSR	14	14	..	..	..
Lister	2	2	..	..	..
Utrecht	5	3	..	1	1
Total	316	114	63	129	10

Ninety-five batches (90·3 per cent) of freeze dried smallpox vaccine produced at Belgaum gave clear precipitin line in about 2 h. which became prominent in about 20 h. None of the batches from Hyderabad, Madras and Patwadangar gave such clear cut lines as Belgaum batches. Sixteen batches (57·7 per cent) from Hyderabad and 47 batches (50·5 per cent) from Madras gave faint precipitin line in about 4 h. which became prominent in about 20 h. All the 14 batches (100 per cent) from USSR, 2 out of 2 batches from Lister and 3 (60 per cent) out of 5 batches from Utrecht also gave clear precipitin line in about 2 h. which became prominent in about 20 h. They were comparable with majority of the batches manufactured at Belgaum. The remaining

vaccine batches **viz.** 8·6 per cent from Belgaum, 42·8 per cent from Hyderabad, 47·3 per cent from Madras, 90 per cent batches from Patwadangar and 20 per cent from Utrecht gave faint precipitin line in 4-20 h. Two per cent batches from Madras, 10 per cent from Patwadangar and 20 per cent batches from Utrecht did not exhibit precipitin line even up to 48 h.

The results of PIG test conducted with neat vaccine antigens and their two-fold dilutions are presented in Table II.

Table II. PIG test results with neat and diluted vaccine antigens.

Source of vaccine	No. of batches tested	Results of PIG test using antigen dilutions		
		Neat	1 : 2	1 : 4
Belgaum	8	All batches gave clear precipitin lines in about 2 h.	7 batches gave clear precipitin lines in about 4 h. One batch gave faint precipitin line in 4 h which became clear in 20 h.	All batches gave faint precipitin lines in about 4 h which became clear in 20 h.
Patwadangar	8	All batches gave faint precipitin lines in 4 to 20 h.	No precipitin line in 20 h.	No precipitin line in 20 h.
Madras	6	4 batches gave faint precipitin lines in 4 to 20 h.	3 batches gave faint precipitin line in 4 to 20 h.	No precipitin line in 20 h.
		2 batches gave no precipitin line.	3 batches did not produce precipitin lines in 20 h.	
Hyderabad	6	4 batches gave faint precipitin lines in about 4 h which became clear in 20 h.	3 batches produced faint precipitin lines in 4 h which became clear in 20 h.	No precipitin line in 20 h.
		2 batches produced faint precipitin lines in about 20 h.	1 batch produced faint precipitin line in 20h. 2 batches did not produce precipitin line.	
Utrecht	5	3 batches gave clear precipitin lines in about 2 h.	2 batches produced clear precipitin lines in 2 to 4 h.	4 batches gave no precipitin line in 20 h.
		1 batch gave faint precipitin line in 20 h.	1 batch produced faint precipitin line in 2 to 4 h which became prominent in 20 h.	1 batch produced faint precipitin line in 20 h.
		1 batch gave no precipitin line.	2 batches did not produce precipitin line.	
Lister	2	Both the batches produced clear precipitin lines in about 2 h.	Both the batches produced clear precipitin lines in about 4 h.	Both the batches produced faint precipitin lines in 4 to 20 h.
USSR	4	All batches gave clear precipitin lines in about 2 h.	All the batches gave clear precipitin lines in about 4 h.	No precipitin line in 20 h.

Double precipitin lines appeared with neat, 1 : 2 and 1 : 4 dilutions of all the eight Belgaum vaccine batches and with neat and 1 : 2 dilutions of two Madras and three Utrecht batches within 20 h. of charging the wells.

The results of precipitation-in-gel test with neat vaccines after exposure to 32°C. for 12 h. and 16 h. are set out in Table III.

Table III. The results of PIG test after exposure of neat vaccine to 32°C. for 12 and 16 h.

Source of vaccine	No. of batches tested	Results of PIG test		
		4°C (Control).	32°C for 12 h.	32°C. for 16 h.
Belgaum	6	Clear precipitin lines appeared in about 2 h.	Clear precipitin lines appeared in about 2 h.	Not done
Hyderabad	6	Faint precipitin lines appeared in about 4 h. which become prominent in 20 h.	Faint precipitin lines appeared in about 4 h which became prominent in 20 h.	Not done
Patwadangar	6	No precipitin line appeared in 4 h. but faint lines observed in 20 h.	Not done	No precipitin lines appeared in 4 h but faint lines observed in 20 h.
Madras	6	Faint precipitin lines appeared in 4 to 20 h.	Not done	Faint precipitin lines appeared in 4 to 20 h.

The pattern of precipitin lines formed by the reconstituted vaccine antigens after exposure to 32°C. for 12 h. or 16 h. was almost identical as compared to their corresponding controls at 4°C.

The results of PIG test carried out with the rehydrated neat vaccine kept in boiling water for one h. and their corresponding controls kept at 4°C. are shown in Table IV.

The above mentioned freeze dried smallpox vaccine batches manufactured as Belgaum, Madras and Hyderabad after reconstitution gave double precipitin lines in agar gel in 4 to 20 h. but after boiling for one h. these vaccine batches produced single precipitin line in about 20 h.

The results of PIG test conducted with the suspension made from chorioallantoic membranes of developing chick embryos inoculated separately with different batches of freeze dried smallpox vaccine are presented in Table V. The CAM suspensions were kept at 4°C., 32°C. and 38°C. for 24 h. prior to conducting the agar gel-precipitation test.

The pattern of precipitin lines produced after exposure of the CAM suspension to 4°C., 32°C. and 38°C. was almost identical. Single clear precipitin line appeared against the individual batch in about 2 h. which joined with the adjoining line in about 4 h. and became prominent in about 20 h.

Table IV. Results of PIG test with neat vaccine after boiling for one h. and without boiling.

Source of vaccine	No. of batches tested	Results of PIG test without boiling and after boiling	
		Without boiling (control)	Boiled for 1 h.
Belgaum	6	Clear double precipitin lines appeared in about 4 h which became prominent in 20 h.	No precipitin line developed in about 4 h. Single precipitin line appeared near the margin of the well containing antigen in about 20 h.
Madras	2	Clear double precipitin lines appeared in about 4 h which became prominent in 20 h.	No precipitin line developed in about 4 h. Single faint precipitin line appeared near the margin of the well containing antigen in about 20 h.
Hyderabad	4	Clear double precipitin lines appeared in about 4 h which became prominent in 20 h.	2 batches produced single clear precipitin line in 4 h which became prominent in 20 h. 2 batches did not produce any precipitin line in 4 h but single faint precipitin line appeared in 20 h.

Table V. The results PIG test with CAM suspensions exposed to different temperatures for 24 h.

Source of vaccine	No. of batches tested	Results of PIG test after exposure of CAM suspensions for 24 h.		
		4°C.	32°C.	38°C.
Belgaum	4	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which bcame prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.
Madras	4	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.
Patwadangar	4	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.
Hyderabad	4	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.	All batches produced clear precipitin line in 2 h which became prominent in 20 h.

Discussion

Majority of the freeze dried smallpox vaccine batches manufactured at Vaccine Institute, Belgaum, after reconstitution into neat vaccine gave clear cut precipitin lines in about 2 h. in agar-gel against hyperimmune vaccinia serum which became prominent

in about 20 h. The lines of precipitate from the adjacent wells linked up in about 4 h. after the test was set up, thus establishing their specificity. Most of the batches produced double precipitin lines. No precipitin line was observed against the vaccine batches manufactured at different production centres against the normal rabbit serum which means that no nonspecific reaction was manifested. The precipitin lines which appeared against the freeze dried vaccine batches producded at Hyderabad, Madras and Patwadangar were not so prominent as compared to the Belgaum batches. The development of the faint precipitin lines especially against all the Patwadangar vaccine batches and some batches manufactured at Madras and Hyderabad indicate that they are antigenically weak for the PIG test. It is difficult to explain the reasons for the discrepancy in the results obtained against the vaccine batches produced at the four different Indian production centres. The parent seed strain in all the Institutes being identical, it was considered that this non-hemogenicity may be due to the altered production technique, time, temperature and the vacuum schedule to which the liquid lymph is exposed during primary and secondary drying at these centres. In order to ascertain the cause of this disparity some of the freeze dried vaccine batches from Belgaum and Hyderabad after rehydration were exposed to 32°C. for 12 h. and from Patwadangar and Madras for 16 h. at 32°C. Their duplicates were kept in the refrigerator to serve as controls. The results obtained on the PIG test were identical and no difference was observed which clearly invalidates the initial assumption.

Twelve batches of freeze dried vaccine which previously gave double lines of precipitate on PIG test were boiled for one h. after reconstitution. PIG tests conducted with the neat unboiled (controls) and boiled vaccine lots showed that after boiling single line of precipitate appeared instead of double lines of precipitate which developed against the unboiled lots revealing thereby that on boiling one component of the vaccinia virus antigen is destroyed. The production of the precipitin line by the second intact component of the antigen was delayed. The line of precipitate did not appear in about 4 h. but were observed in about 20 h. except in two batches from Hyderabad.

The CAM passaged vaccinia virus suspensions yielded better results on PIG test as compared to the freeze dried product itself. It indicates that the concentration of vaccinia virus on first passage in the embryonated eggs is enhanced and behaves antigenically better to produce clear cut lines of precipitate.

Two-fold serial dilutions of the freeze dried vaccine viz. 1 : 2 and 1 : 4 did not give good results on PIG test as compared to the neat (concentrated) vaccine. For conducting PIG test a concentrated virus is required for getting better results than the diluted one.

The results of this study suggest that it is not possible to get good results on PIG test with every type of freeze dried smallpox vaccine. The vaccine batches produced by some production centres may give good results while from others may not. In order to keep a known positive vaccinia antigen as a control in the PIG test for conducting

laboratory diagnosis of smallpox with a suspected smallpox specimen, the particular vaccine batch should be thoroughly checked first for the development of precipitin lines in the agar gel-diffusion test.

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