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STUDIES ON LABORATORY TESTING OF SMALLPOX VACCINES USED IN INDIA UNDER THE NATIONAL SMALLPOX ERADICATION PROGRAMME. POTENCY AND BACTERIAL STERILITY STUDIES.

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INTRODUCTION.

INITIATED in the last quarter of 1962, the National Smallpox Eradication Programme, India, is the largest single Public Health Programme of its kind in the world. Since then over 100 million people are being vaccinated annually. Liquid lymph vaccine, from fourteen production centres in India was meeting the demands of the earlier phases of the programme. Of these production centres, four (Belgaum in Mysore State, Guindy in Madras State, Hyderabad in Andhra Pradesh and Patwadangar in Uttar Pradesh) are now being developed for production of freeze dried smallpox vaccine for use in the National Smallpox Eradication Programme, India (Report 1967-68 Govt. of India, Ministry of Health, Family Planning and Urban Development, New Delhi, P14-15). However, as yet the country is not self sufficient in her needs of freeze dried smallpox vaccine which, at present, is largely imported particularly through the munificent gifts from the U.S.S.R.

The need for quality control of the vaccine as per WHO standards made the studies on testing imperative. The large-scale use of vaccine for mass prophylaxis of the Indian population, the poor 'takes' reported of some batches in primary/revaccinations stressed the need for the establishment of a Smallpox Vaccine Testing Unit at the National level. The small-scale studies initiated since August 1965 at the National Institute of Communicable Diseases, Delhi, which recently has expanded its scope and functions to a Smallpox Vaccine Testing Centre are reported here. It would be relevant to point out that the WHO standards have been made more critical since July, 1966 (WHO Biological Standards Committee Report, 1966). The results are reported as per the relevant WHO standards, i.e. prior to July, 1966 and subsequently. The WHO had earlier prescribed a potency of $5{\cdot}0 \times 10^7$/ml. and bacterial count of 1000 org./ml., while since July, 1966, the standard set is a potency of $1{\cdot}0 \times 10^8$/ml. and bacterial count of 500 org./ml.

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The studies reported in this paper, however, are (1) Potency by enumeration of pock lesions on the chorioallantoic membrane of developing chick embryos, and (2) bacterial sterility. The results reported refer to the period August, 1965 to December, 1968 inclusive.

Review of literature :

Potency studies by enumeration of pock lesions.—Studies have been carried out in the embryonated hen's eggs and the rabbit. In addition, tissue culture titration method has also been utilized in such studies. The basic principle for such potency tests was the fact that the vaccinia virus when introduced, produced 'discrete pock lesions' in the tissue. This facilitated titration of the virus and thus of potency determination of the vaccine tested.

Experiments in developing chick embryos.—Goodpasture *et al.* (1932) were the first to show that vaccinia virus readily multiplied after inoculation on the chorio-allantoic membranes (CAM) of embryonated hen's eggs. Keogh (1936) established that the viral content could be titrated by this method. He also showed that dermal and neurotropic strains could be differentiated by this method of study. This was confirmed by other workers (Overman and Tamm, 1956 ; Kaplan and Belyavin, 1957 ; Westwood *et al.*, 1957). The superiority of the CAM technique over other routes of inoculation in the developing chicken embryo (allantoic cavity and yolksac), in that the same batch of vaccine gave a 2-log titre increase in the CAM, was shown by Cabasso and Moore (1957). The intravenous injection of chick embryos was found to give comparable results as with the CAM route (Kaplan, 1960). However, the latter method is more easy than the former.

Experiments in rabbits.—The rabbit skin scarification technique for noting the pock lesions and hence the potency was first highlighted by Calmette and Guerin (1901). This technique has since been used for evaluating the potency and for thestandar dization of smallpox vaccines in some countries (Force and Leake, 1927 ; WHO Study Group on Requirements of Smallpox Vaccine, 1959 ; Kolb, Cox and Aylor, 1961). Apart from economic considerations, difficulty in procuring or rearing the very large number of rabbits of the required standards (weight and colour) precluded the use of this animal for the large-scale studies reported here.

Tissue culture.—Titration method for the assay of the potency of the smallpox vaccine has been introduced (Cutchins and Warren, 1958 ; Kolb, *et al.*, 1961 ; Subramanyam *et al.*, 1961). This technique has not been reported to be superior to CAM method of inoculation in chick embryo. The initial difficulties are for setting up tissue culture, involving considerable technical skill and its use in such large-scale studies. The WHO Expert Group (1966) recommended the use of CAM of chick embryos for 'Potency Testing.' This technique was used for the potency studies of the smallpox vaccine used in India under the National Smallpox Eradication Programme. In these studies, the indigenously prepared National Reference Vaccine was compared with the International Reference Vaccine (Copenhagen), set out under 'Results' in this paper.

MATERIALS AND METHODS.

Vaccines.—Freeze-dried vaccines imported from U.S.S.R., Lister Institute U.K., Netherlands, Utrecht, Copenhagen (Denmark), and obtained from the four freeze-dried vaccine Production Centres in India referred to earlier and lymph received from a number of vaccine producing centres in India were tested for potency and bacterial sterility.

The vaccines, freeze-dried or lymph, immediately on their receipt, were stored in the deep freeze cabinet at —20°C. prior to subjecting them to laboratory studies.

Initially, two ampoules and later on 4 amopules of Indian and 5 ampoules of U.S.S.R. freeze-dried vaccine were used. Their contents were rehydrated in one ml. of citrate-buffer (McLivaine's citric acid buffer composition—Appendix I) solution pH 7·2 and pooled so as to have concentrated vaccine. From this, 10-fold serial dilutions ranging from 10^{-1} to 10^{-6} were then made in the said diluent. Contents of four vials of liquid smallpox vaccine were pooled (it was possible with those batches where a number of vials were received) for making 10-fold serial dilutions. These dilutions were stored in the refrigerator from half an hour to three hours prior to inoculation on the chorio-allantoic membranes of developing chick embryo.

Eggs.—Fresh fertile hen's eggs from disease-free flock were purchased from the Govt. Poultry Farm, Delhi. The eggs were cleaned with water to remove the faecal material and dust, wiped with spirit or 2 per cent Savlon and kept in a commercial egg incubator at 38°C. for 12 days. During this period the eggs were rotated thrice a day. Eggs having 12-day old active embryos and normal air sacs were used.

Potency testing by chorioallantoic membrane inoculation.—The technique of inoculation on the chorioallantoic membrane of 12-day old chick embryos as described by Westwood *et al.* (*loc. cit.*) was followed.

The egg was first candled and the boundary of the air sac marked. The tapering end of the egg was then kept on the candling lamp and the albumin sac was located. It was then reversed and marked for drilling on the side opposite to the albumin sac where an appropriate area of the chorio-allantoic membrane was uppermost and free from blood vessels. A slit was then made with an electric dental drill on the air sac and two slits on the side of the egg which were previously marked, without damaging the shell membrane.

For dropping the chorioallantoic membrane, a drop of citrate buffer solution, previously warmed to 50°C., was placed on the cut made on the side of the shell. The shell membrane at the air sac was then punctured with the sharp tip of the needle. It was also punctured on the side of the egg through the buffer drop without injuring the chorioallantoic membrane. A drop of citrate buffer solution was then dropped on the CAM by sucking air through the aperture in the air sac with the help of a rubber bulb teat. After dropping the CAM, the eggs were again candled and those showing haemorrhage or defective artificial air sac were discarded to avoid development of non-specific lesions.

Inoculation was usually carried out after one and a half to three hours after dropping the membranes with 1 ml. tuberculin syringe using 26 G × ½" needle. 0·1 ml. of the inoculum (10^{-5} and 10^{-6} dilutions of the virus) was introduced through the second slit. The eggs were then rocked gently to distribute the inoculum on the CAM. The shell holes were sealed with cellophane tape. After inoculation the eggs were kept in the bacteriological incubator at 36°C. for about 48 hours, in a horizontal position with the cellophane seal uppermost. Five chick embryos were used for each dilution. Suitable controls with vaccinia virus of known potency and later on in the studies the National Reference Vaccine were also put up.

Harvesting.—After 48 hours incubation at 36°C. the eggs were removed one by one, the shell of each egg was then cut longitudinally with scissors. The embryos, yolk sac and the albumin sac were removed and discarded. The CAM around the site of inoculation was removed with forceps, washed in water and spread in a Petri dish on a black background. The CAM from the dead embryos was discarded. Pock lesions were then counted and the average pock count determined. The titre of the vaccinia virus was then calculated for one ml. of undiluted vaccine. The chorioallantoic membranes showing pock lesions of 10 or more were taken into consideration (*W.H.O. Tech. Rep. Ser.*, 180, *loc. cit.*).

Bacterial sterility studies :

Review of literature.—Prior to 1959, the total number of living bacterial or other extraneous organisms permissible were up to 20,000 orgs/ml. of the liquid smallpox vaccine (Ghosh, 1965). Later on, the W.H.O. Expert Committee (*W.H.O. Tech. Rep. Ser.* 180, *loc. cit.*) recommended that the total bacterial count in the freeze-dried smallpox vaccine should not exceed 1000 orgs/ml. In 1966, however, the W.H.O. Expert Group (*W.H.O. Tech. Rep. Ser.* 323, *loc. cit.*) further reduced the total permissible bacterial content to 500 orgs/ml. of the freeze-dried smallpox vaccine.

Total bacterial count.—Earlier 0·1 ml. of 1 : 10 dilution of smallpox vaccine was streaked on the surface of blood agar plate which were then incubated at 37°C. for 48 hours. Later on, pour plate method on nutrient agar (*W.H.O. Tech. Rep. Ser.* 323, *loc. cit.*) was adapted using 3 plates for each of 1 : 10 and 1 : 100 dilution. The nutrient agar culture plates were first kept at 15 to 20°C. for 3 days followed by 37°C. for 2 days. The number of colonies appearing on each plate was counted and total number of living bacteria were then calculated for one ml. of the undiluted vaccine.

Results.

The results of potency and bacterial sterility tests of the indigenous and imported batches (Table I) are set out in Tables II through VIII. Each Table indicates the results of the tests conducted on six monthly basis and results analysed as per recommendations of the WHO Expert Committee for the period before July, 1966 (Tables II and III only) and from July, 1966 to December 1968 (Tables IV, V, VI, VII and VIII.)

TABLE I.

Source of freeze-dried (F) as well as lymph (L) vaccine and number of batches thereof including total (T) tested by 6 months period.

Source of vaccine.	PERIOD OF TESTING/NUMBER OF BATCHES AND THEIR STATE (FREEZE-DRIED OR LIQUID LYMPH):																				
	August 1965 to December 1965			January 1966 to June 1966			July 1966 to December 1966			January 1967 to June 1967			July 1967 to December 1967			January 1968 to June 1968			July 1968 to December 1968		
	F	L	T	F	L	T	F	L	T	F	L	T	F	L	T	F	L	T	F	L	T
Indigenous	17	7	24	16	36	52	24	38	62	17	12	29	164	8	172	436	36	472	551	32	583
Imported	57	..	57	70	..	70	41	..	41	29	1	30	110	..	110	70	..	70	95	..	95
Total	74	7	81	86	36	122	65	38	103	46	13	59	274	8	282	506	36	542	646	32	678

TABLE II.

The results of laboratory tests for smallpox vaccine carried out at the Smallpox Vaccine Control and Testing Unit, N. I. C. D., Delhi, from August 7, 1965 to December 31, 1965 (standard potency titre $5{\cdot}0 \times 10^7$/ml. and above, and total bacterial count below 1000 orgs./ml.)

Origin of vaccine.	TITRE BY ENUMERATION OF POCK LESIONS ON THE CAM OF CHICK EMBRYOS :															TOTAL BACTERIAL COUNT :								
	Below $1{\cdot}0 \times 10^7$/ml.			$1{\cdot}0 \times 10^7$ to $5{\cdot}0 \times 10^7$/ml.			$5{\cdot}0 \times 10^7$/ml. to $1{\cdot}0 \times 10^8$/ml.			$1{\cdot}0 \times 10^8$/ml. and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	24	27	51	5	7	12	12	9	21	3	8	11	4	3	7	10	10	20	2	1	3	..	..	..
U.S.S.R. batch from Nepal	..	2	2	..	..	..	..	2	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Lister Instt., England	3	1	4	1	..	1	..	..	..	..	..	..	2	1	3	2	..	2	..	..	..	..	..	..
Patwadangar	15	..	15	..	..	..	..	..	..	5	..	5	10	..	10	9	..	9	1	..	1	..	..	..
Guindy, Madras	2	..	2	..	..	..	..	..	..	..	..	..	2	..	2	..	..	..	2	..	2	..	..	..
Total	44	30	74	6	7	13	12	11	23	8	8	16	18	4	22	21	10	31	5	1	6	..	..	..
Liquid vaccine																								
Trivandrum	2	..	2	..	..	..	2	..	2	..	..	..	..	..	..	..	..	..	..	..	..	2	..	2
Amritsar	..	1	1	..	1	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1	1
Patwadangar	1	..	1	..	..	..	..	..	..	..	..	..	1	..	1	..	..	..	..	..	..	1	..	1
Calcutta	1	..	1	..	..	..	..	..	..	1	..	1	..	..	..	..	..	..	..	..	..	1	..	1
Nagpur	2	..	2	2	..	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	2	..	2
Total	6	1	7	2	1	3	2	..	2	1	..	1	1	..	1	..	..	..	..	..	..	6	1	7
Grand Total	50	31	81	8	8	16	14	11	25	9	8	17	19	4	23	21	10	31	5	1	6	6	1	7

P=Production Centre; F=Field specimen; T=Total. Thirty-seven batches of freeze-dried vaccine could not be tested for total bacterial count.

TALBE III.

The results of laboratory tests for smallpox vaccines carried out at the Smallpox Vaccine Control and Testing Centre, N. I. C. D., Delhi, from 1st January, 1966 to 30th June, 1966 (standard potency titre $5{\cdot}0 \times 10^7$/ml. and above and total bacterial count below 1000 orgs/ml.)

Origin of vaccine.	TITRE BY ENUMERATION OF POCK LESIONS ON THE CAM OF CHICK EMBRYOS :															TOTAL BACTERIAL COUNT :								
	Below $1{\cdot}0 \times 10^7$/ml.			$1{\cdot}0 \times 10^7$ to $5{\cdot}0 \times 10^7$/ml.			$5{\cdot}0 \times 10^7$/ml. to $1{\cdot}0 \times 10^8$/ml.			$1{\cdot}0 \times 10^8$/ml. and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	58	11	69	19	3	22	21	7	28	12	1	13	6	..	6	54	11	65	4	..	4	..	..	..
Patwardangar	10	..	10	..	..	..	..	..	..	4	..	4	6	..	6	10	..	10	..	..	..	..	..	..
Netherlands	1	..	1	..	..	..	..	..	..	..	..	..	1	..	1	1	..	1	..	..	..	..	..	..
Belgaum	6	..	6	..	..	..	..	..	..	..	..	..	6	..	6	6	..	6	..	..	..	..	..	..
Total	75	11	86	19	3	22	21	7	28	16	1	17	19	..	19	71	11	82	4	..	4	..	..	..
Liquid																								
Nagpur	6	..	6	6	..	6	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	6	..	6
Namkum	24	..	24	..	..	..	2	..	2	2	..	2	20	..	20	24	..	24	..	..	..	..	..	..
Belgaum	3	..	3	1	..	1	2	..	2	..	..	..	..	..	..	3	..	3	..	..	..	..	..	..
Amritsar	3	..	3	3	..	3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	3	..	3
Total	36	..	36	10	..	10	4	..	4	2	..	2	20	..	20	27	..	27	..	..	..	9	..	9
Grand Total	111	11	122	29	3	32	25	7	32	18	1	19	39	..	39	98	11	109	4	..	4	9	..	9

P = Production Centre ; F = Field specimens ; T = Total.

TABLE IV.

The results of laboratory tests for smallpox vaccine carried out at the Smallpox Vaccine Control and Testing Centre, N. I. C. D., Delhi, from 1st July, 1966 to 31st December, 1966 (standard potency titre $1{\cdot}0 \times 10^8$/ml. and above and total bacterial count below 500 orgs/ml.)

Origin of vaccine.	Titre by enumeration of pock lesions on the CAM of chick embryos :																Total bacterial count :								
	Below $1{\cdot}0 \times 10^7$/ml.			$1{\cdot}0 \times 10^7$/ml. to $5{\cdot}0 \times 10^7$/ml.			$5{\cdot}0 \times 10^7$/ml. to $1{\cdot}0 \times 10^8$ ml.			$1{\cdot}0 \times 10^8$/ml. and above.						Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																									
U.S.S.R.	37	3	40	9	3	12	12	..	12	6	..	6	10	..	10	31	2	33	1	..	1	5	1	6
Copenhagen	1	..	1	..	..	..	..	..	..	..	..	..	1	..	1	1	..	1	..	..	..	..	..	..
Belgaum	18	..	18	..	..	..	..	..	..	3	..	3	15	..	15	18	..	18	..	..	..	..	..	..
Patwadangar	3	..	3	..	..	..	..	..	..	..	..	..	3	..	3	3	..	3	..	..	..	..	..	..
Hyderabad	3	..	3	..	..	..	1	..	1	2	..	2	..	..	..	3	..	3	..	..	..	..	..	..
Total	62	3	65	9	3	12	13	..	13	11	..	11	29	..	29	56	2	58	1	..	1	5	1	6
Liquid																									
Namkum	8	..	8	..	..	..	1	..	1	1	..	1	6	..	6	8	..	8	..	..	..	..	..	..
Nagpur	3	..	3	1	..	1	2	..	2	..	..	..	..	..	..	..	..	..	..	..	..	3	..	3
Indore	27	..	27	27	..	27	..	..	..	..	..	..	..	..	..	24	..	24	..	..	..	3	..	3
Total	38	..	38	28	..	28	3	..	3	1	..	1	6	..	6	32	..	32	..	..	..	6	..	6
Grand Total	100	3	103	37	3	40	16	..	16	12	..	12	35	..	35	88	2	90	1	..	1	11	1	12

P = Production Centre ; F = Field specimens ; T = Total.

TABLE V.

The results of laboratory tests for smallpox vaccines carried out at the Smallpox Vaccine Control and Testing Centre N. I. C. D., Delhi, from January 1, 1967 to June 30, 1967 (standard potency titre—$1{\cdot}0\times10^8$/ml. and above, and tolal bacterial count be low 500 orgs/ml.)

Origin of vaccine.	TITRE BY ENUMERATION OF POCK LESIONS ON THE CAM OF CHICK EMBRYOS :															TOTAL BACTERIAL COUNT :								
	Below $1{\cdot}0\times10^7$/ml.			$1{\cdot}0\times10^7$/ml. to $5{\cdot}0\times10^7$/ml.			$5{\cdot}0\times10^7$/ml. to $1{\cdot}0\times10^8$/ml.			$1{\cdot}0\times10^8$/ml. and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	22	2	24	..	..	..	5	2	7	9	..	9	8	..	8	22	2	24	..	..	..	..	..	..
U.S.A.	3	..	3	..	..	..	..	..	..	..	..	..	3	..	3	3	..	3	..	..	..	..	..	..
W. Germany	2	..	2	..	..	..	..	..	..	1	..	1	1	..	1	2	..	2	..	..	..	..	..	..
Patwadangar	12	..	12	2	..	2	4	..	4	1	..	1	5	..	5	12	..	12	..	..	..	..	..	..
Hyderabad	5	..	5	..	..	..	2	..	2	1	..	1	2	..	2	5	..	5	..	..	..	..	..	..
Total	44	2	46	2	..	2	11	2	13	12	..	12	19	..	19	44	2	46	..	..	..	..	..	..
Lymph																								
Namkum	1	..	1	..	..	..	1	..	1	..	..	..	..	..	..	..	..	..	..	..	..	1	..	1
Trivandrum	2	..	2	1	..	1	1	..	1	..	..	..	..	..	..	..	..	..	2	..	2	..	..	..
Bangalore	4	..	4	3	..	3	..	..	..	..	..	..	1	..	1	1	..	1	3	..	3	..	..	..
Guindy, Madras	4	..	4	4	..	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	4	..	4
Nagpur	1	..	1	..	..	..	1	..	1	..	..	..	..	..	..	..	..	..	..	..	..	1	..	1
U.S.A.	1	..	1	..	..	..	1	..	1	..	..	..	..	..	..	1	..	1	..	..	..	..	..	..
Total	13	..	13	8	..	8	4	..	4	..	..	..	1	..	1	2	..	2	5	..	5	6	..	6
Grand Total	57	2	59	10	..	10	15	2	17	17	..	17	20	..	20	46	2	48	5	..	5	6	..	6

P=Production Centres ; F=Field specimens ; T=Total.

TABLE VI.

The results of laboratory tests for smallpox vaccines carried out at the Smallpox Vaccine Control and Testing Centre, N. I. C. D., Delhi, from July 1, 1967 to December 31, 1967 (standard potency titre $1{\cdot}0 \times 10^8$/ml. and above, and total bacterial count between 500 orgs/ml.)

Origin of vaccine.	TITRE BY ENUMERATION OF POCK LESIONS ON THE CAM OF CHICK EMBRYOS :															TOTAL BACTERIAL COUNT :								
	Below $1{\cdot}0 \times 10^7$/ml.			$1{\cdot}0 \times 1{\cdot}0^7$/ml. to $5{\cdot}9 \times 10^7$/ml.			$5{\cdot}0 \times 10^7$/ml. to $1{\cdot}0 \times 10^8$/ml.			$1{\cdot}0 \times 10^8$/ml. and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	83	14	97	..	9	9	5	4	9	16	..	16	62	1	63	83	14	97	..	..	..	..	..	..
U.S.S.R. batch from Nepal	..	1	1	..	..	..	..	..	..	..	..	..	..	1	1	..	1	1	..	..	..	..	..	..
Patwadangar	91	..	91	6	..	6	3	..	3	2	..	2	80	..	80	90	..	90	..	..	..	1	..	1
Hyderabad	13	..	13	..	..	..	..	..	..	..	..	..	13	..	13	13	..	13	..	..	..	..	..	..
W. Germany	4	1	5	..	1	1	..	..	..	..	..	..	4	..	4	4	1	5	..	..	..	..	..	..
U.S.A. (Reconstituted Vaccine Jet Gun)	..	5	5	..	5	5	..	..	..	..	..	..	..	..	..	..	5	5	..	..	..	..	..	..
Netherland	2	..	2	..	..	..	..	..	..	..	..	..	2	..	2	2	..	2	..	..	..	..	..	..
Coonoor T.C.	7	..	7	7	..	7	..	..	..	..	..	..	..	..	..	7	..	7	..	..	..	..	..	..
Belgaum	7	..	7	..	..	..	3	..	3	..	..	..	4	..	4	7	..	7	..	..	..	..	..	..
Guindy, Madras	46	..	46	..	..	..	..	..	..	..	..	..	46	..	46	46	..	46	..	..	..	..	..	..
Total	253	21	274	13	15	28	11	4	15	18	..	18	211	2	213	252	21	273	..	..	..	1	..	1
Liquid																								
Patwadangar	1	..	1	..	..	..	..	..	..	..	..	..	1	..	1	..	..	..	..	..	..	1	..	1
Shillong	3	..	3	..	..	..	..	..	..	..	..	..	3	..	3	..	..	..	..	..	..	3	..	3
Belgaum	4	..	4	..	..	..	4	..	4	..	..	..	..	..	..	4	..	4	..	..	..	..	..	..
Total	8	..	8	..	..	..	4	..	4	..	..	..	4	..	4	4	..	4	..	..	..	4	..	4
Grand Total	261	21	282	13	15	28	15	4	19	18	..	18	215	2	17	256	21	277	..	..	..	5	..	5

P=Production Centre ; F=Field specimens ; T=Total.

TABLE VII.

The results of laboratory test of smallpox vaccines carried out at the Smallpox Vaccine Control and Testing Unit N. I. C. D., Delhi from January 1, 1968 to June 30, 1968 (standard potency titre $1{\cdot}0 \times 10^8$/ml. and above, and total bacterial count below 500 orgs/ml.)

Origin of vaccine.	TITRE BY ENUMERATION OF POCK LESIONS ON THE CAM OF CHICK EMBRYO :															TOTAL BACTERIAL COUNT :								
	Below $1{\cdot}0\times10^7$/ml.			$1{\cdot}0\times10^7$/ml. to $5{\cdot}0\times10^7$/ml.			$5{\cdot}0\times10^7$/ml. to $1{\cdot}0\times10^8$/ml.			$1{\cdot}0\times10^8$ and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	57	9	66	..	1	1	4	2	6	14	3	17	39	3	42	57	9	66	..	..	..	..	..	..
U.S.A.	3	..	3	..	..	..	..	..	..	..	..	..	3	..	3	3	..	3	..	..	..	..	..	..
W. Germany	..	1	1	..	..	..	..	..	..	..	..	..	..	1	1	..	1	1	..	..	..	..	..	..
Patwadangar	207	..	207	..	..	..	2	..	2	7	..	7	198	..	198	207	..	207	..	..	..	..	..	..
Belgaum	141	..	141	..	..	..	..	..	..	..	..	..	41	..	141	141	..	141	..	..	..	..	..	..
Guindy, Madras	52	..	52	..	..	..	..	..	..	..	..	..	52	..	52	52	..	52	..	..	..	..	..	..
Hyderabad	22	..	22	..	..	..	..	..	..	..	..	..	22	..	22	22	..	22	..	..	..	..	..	..
Coonoor Tissue Culture vaccine	14	..	14	7	..	7	6	..	6	1	..	1	..	..	..	14	..	14	..	..	..	..	..	..
Total	496	10	506	7	1	8	12	2	14	22	3	25	455	4	459	496	10	506	..	..	..	..	..	..
Liquid vaccine																								
Belgaum	4	..	4	4	..	4	..	..	..	..	..	..	..	..	..	4	..	4	..	..	..	..	..	..
Bangalore	3	..	3	..	..	..	1	..	1	2	..	2	..	..	..	3	..	3	..	..	..	..	..	..
Amritsar	24	..	24	13	..	13	11	..	11	..	..	..	..	..	..	..	..	..	..	..	..	24	..	24
Namkum	5	..	5	5	..	5	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	5	..	5
Total	36	..	36	22	..	22	12	..	12	2	..	2	..	..	..	7	..	7	..	..	..	29	..	29
Grand Total	532	10	542	29	1	30	24	2	26	24	3	27	455	4	459	503	10	513	..	..	..	29	..	29

P = Production Centre ; F = Field specimens ; T = Total.

TABLE VIII.

The results of laboratory tests for smallpox vaccines carried out at the Smallpox Vaccine Control and Testing Unit, N. I. C. D., Delhi from 1st July, 1968 to 31st December, 1968 (standard potency titre 10×10^8/ml. and above, and total bacterial count below 500 orgs/ml.)

Origin of vaccine.	Titre by enumeration of pock lesions on the CAM of chick embryo :															Total bacterial count :								
	Below 1.0×10^7/ml.			1.0×10^7/ml. to 5.0×10^7/ml.			5.0×10^7/ml. to 1.0×10^8/ml.			1.0×10^8/ml. and above.			Below 500 orgs/ml.			500 to 1000 orgs/ml.			Above 1000 orgs/ml.		
	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T	P	F	T
Freeze-dried																								
U.S.S.R.	54	38	92	..	13	13	1	13	14	3	7	10	50	5	55	54	38	92	..	..	..	..	..	..
W. Germany	3	..	3	..	..	..	..	..	..	..	..	..	3	..	3	3	..	3	..	..	..	..	..	..
Patwadangar	205	1	206	..	..	..	..	..	..	..	..	..	205	1	206	205	1	206	..	..	..	..	..	..
Belgaum	144	4	148	..	..	..	..	..	..	..	..	..	144	4	148	144	4	148	..	..	..	..	..	..
Hyderabad	49	..	49	..	..	..	..	..	..	..	..	..	49	..	49	49	..	49	..	..	..	..	..	..
Madras	138	..	138	..	..	..	..	..	..	..	..	..	138	..	138	138	..	138	..	..	..	..	..	..
*Coonoor T.C.	10	..	10	..	..	..	4	..	4	1	..	1	5	..	5	10	..	10	..	..	..	..	..	..
Total	603	43	646	..	13	13	5	13	18	4	7	11	594	10	604	603	43	646	..	..	..	..	..	..
Liquid																								
Amritsar	27	..	27	27	..	27	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	27	..	27
Namkum	5	..	5	5	..	5	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	5	..	5
Total	32	..	32	32	..	32	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	32	..	32
Grand Total	635	43	678	32	13	45	5	13	18	4	7	11	594	10	604	603	43	646	..	..	..	32	..	32

P = Production centre ; F = Field specimen ; T = Total ; *T.C. = Tissue culture vaccine.

TABLE IX.

Results of potency tests carried out on selected batches of freeze-dried smallpox vaccine at N.I.C.D. Delhi, and WHO International Reference Laboratory, Utrecht.

Batch	Results of USSR Protocols	Results of N.I.C.D		Results of WHO Reference Laboratory
	Potency titre PFU/ml.	National Reference Vaccine potency titre PFU/ml.	U.S.S.R. batches. Potency titre PFU/ml.	U.S.S.R. batches. Potency titre PFU/ml.
U.S.S.R. 0183	$2{\cdot}2 \times 10^8$	$2{\cdot}66 \times 10^8$	$3{\cdot}84 \times 10^7$	$2{\cdot}4 \times 10^7$
U.S.S.R. 0184	$1{\cdot}8 \times 10^8$	$2{\cdot}66 \times 10^8$	$4{\cdot}24 \times 10^7$	$2{\cdot}5 \times 10^7$
U.S.S.R. 0185	$1{\cdot}8 \times 10^8$	$2{\cdot}95 \times 10^8$	$4{\cdot}96 \times 10^7$	$1{\cdot}6 \times 10^8$
U.S.S.R. 0187	$1{\cdot}8 \times 10^8$	$2{\cdot}68 \times 10^8$	$5{\cdot}11 \times 10^7$	$5{\cdot}1 \times 10^7$
U.S.S.R. 0182	Protocol not received	$2{\cdot}58 \times 10^8$	$3{\cdot}87 \times 10^7$	$5{\cdot}4 \times 10^7$
U.S.S.R. 0193	$2{\cdot}4 \times 10^8$	$3{\cdot}01 \times 10^8$	$4{\cdot}5 \times 10^7$	$1{\cdot}9 \times 10^8$

Utrecht Reference Vaccine Batch No. R.I.V. 6664 Potency titre $1{\cdot}5 \times 10^9$/ml.

An analysis of the results of the potency and bacterial studies carried out as set out in Tables II to VIII is set below :

Table II (Period 7-8-1965 to 31-12-1965).

Freeze-dried vaccines.—Seventy-four batches were studied (from production centres in U.S.S.R. and India as well as from field). 35·2 per cent of batches from U.S.S.R. and 100 per cent of batches from State Vaccine Institute, Patwadangar, U.P., had a potency titre of $5{\cdot}0 \times 10^7$/ml. and above. Thus, 51·5 per cent of the total batches were up to the WHO standard. Bacteriologically, all the 37 batches studied had a total bacterial count below 1,000 orgs/ml. Thirty-seven other batches could not be tested for bacterial sterility.

Liquid vaccine.—Seven batches from various production centres in India were tested. Only 28·5 per cent of them were up to the WHO standard for potency of $5{\cdot}0 \times 10^7$/ml. and above. Bacteriologically, all the batches were beyond the WHO upper limit prescribed in that they had a total bacterial count above 1,000 org/ml.

Table III (Period 1-1-1966 to 30-6-1966)

Freeze-dried vaccine of 86 batches.—The potency studies showed that only 41·7 per cent of all the batches met the WHO standard. Of the imported vaccines only 27·5 per cent of 69 batches from U.S.S.R. had a potency titre of $5{\cdot}0 \times 10^7$/ml. and above. Of the indigenous 16 batches, 6 from Belgaum and 10 from Patwadangar, all were noted to have a titre of $5{\cdot}0 \times 10^7$/ml. and above, thus making a 100 per cent grade.

Liquid vaccine.—Of the 36 batches tested, 61·0 per cent of all (91·6 per cent batches from Namkum) had a titre of $5{\cdot}0 \times 10^7$/ml. and above. Bacteriologically, 75 per cent batches had a total bacterial count below 1,000 orgs/ml.

Table IV (Period 1-7-1966 to 31-12-1966. WHO standard raised to $1{\cdot}0\times10^8$/ml. and below 500 org/ml.).

Freeze-dried vaccine.—A total of 103 batches was tested consisting of 65 batches of freeze-dried vaccine, 24 from India and 41 from other sources. 46·1 per cent of all the batches (25·0 per cent of batches from U.S.S.R., 83·3 per cent of batches from Belgaum and 100 per cent batches from Patwadangar) had a potency of $1{\cdot}0\times10^8$/ml. and above. 89·2 per cent of the total batches (82·5 per cent U.S.S.R. batches, 100 per cent of Belgaum and 100 per cent Patwadangar batches) had a total bacterial count of less than 500 org/ml.

Liquid vaccine.—Of 38 batches tested, 15·7 per cent had a potency titre of $1{\cdot}0\times10^8$/ml. and above (75 per cent of batches of vaccine from Namkum, Bihar State included). Bacteriologically, 83·3 per cent of the total batches (100 per cent from Namkum, Bihar and 88·8 per cent batches from Indore, Madhya Pradesh) had a total bacterial count below 500 org/ml.

Table V (Period 1-1-1967 to 30-6-1967)

Freeze-dried vaccine.—A total of 59 batches comprising of 46 batches of freeze-dried vaccine (17 indigenous and 29 imported) was tested. 41·3 per cent of all the batches (33·3 per cent from U.S.S.R., 100 per cent from U.S.A., 41·6 per cent from Patwadangar and 40 per cent from Hyderabad) had a potency titre of $1{\cdot}0\times10^8$/ml. and above. 100 per cent of the total batches, imported and indigenous, had a total bacterial count of less than 500 orgs/ml.

Liquid vaccine.—Of 13 batches (12 indigenous and 1 from U.S.A.) tested, 7·7 per cent had a potency titre of $1{\cdot}0\times10^8$/ml. and above. Only 15·4 per cent batches had a total bacterial count below 500 org/ml.

Table VI (Period 1-7-1967 to 31-12-1967).

Freeze-dried vaccine.—A total of 282 batches was tested consisting of 274 batches of freeze-dried vaccine (110 imported and 164 indigenous). 77·7 per cent of the total batches (64·9 per cent from U.S.S.R., 87·9 per cent from Patwadangar, 100 per cent from Hyderabad, 80 per cent from West Germany, 57·1 per cent from Belgaum and 100 per cent from Madras) had a potency titre of $1{\cdot}0\times10^8$/ml. and above. 99·6 per cent of the batches (100 per cent U.S.S.R., 98·9 per cent of Patwadangar, 100 per cent from Hyderabad 100 per cent from West Germany ; 100 per cent from Belgaum and 100 per cent from Madras) had a bacterial count below 500 org/ml.

Liquid vaccine.—Of 8 batches tested, only 50 per cent had a titre of $1{\cdot}0\times10^8$/ml. and above. Bacteriologically, 50 per cent of the batches had a total bacterial count below 500 org/ml.

Table VII (Period 1-1-1968 to 30-6-1968)

A total of 542 batches comprising of 506 batches of freeze-dried vaccine (436

batches from indigenous sources and 70 imported), and 36 batches of indigenously produced lymph was tested. 90·7 per cent of the total batches (63·6 per cent from U.S.S.R., 95·6 per cent from Patwadangar, 100 per cent from Belgaum, 100 per cent from Madras and 100 per cent from Hyderabad) had a potency titre of $1{\cdot}0\times10^{8}$/ml. and above. Bacteriologically, 100 per cent of the total batches (imported and indigenous) had a bacterial count below 500 org/ml.

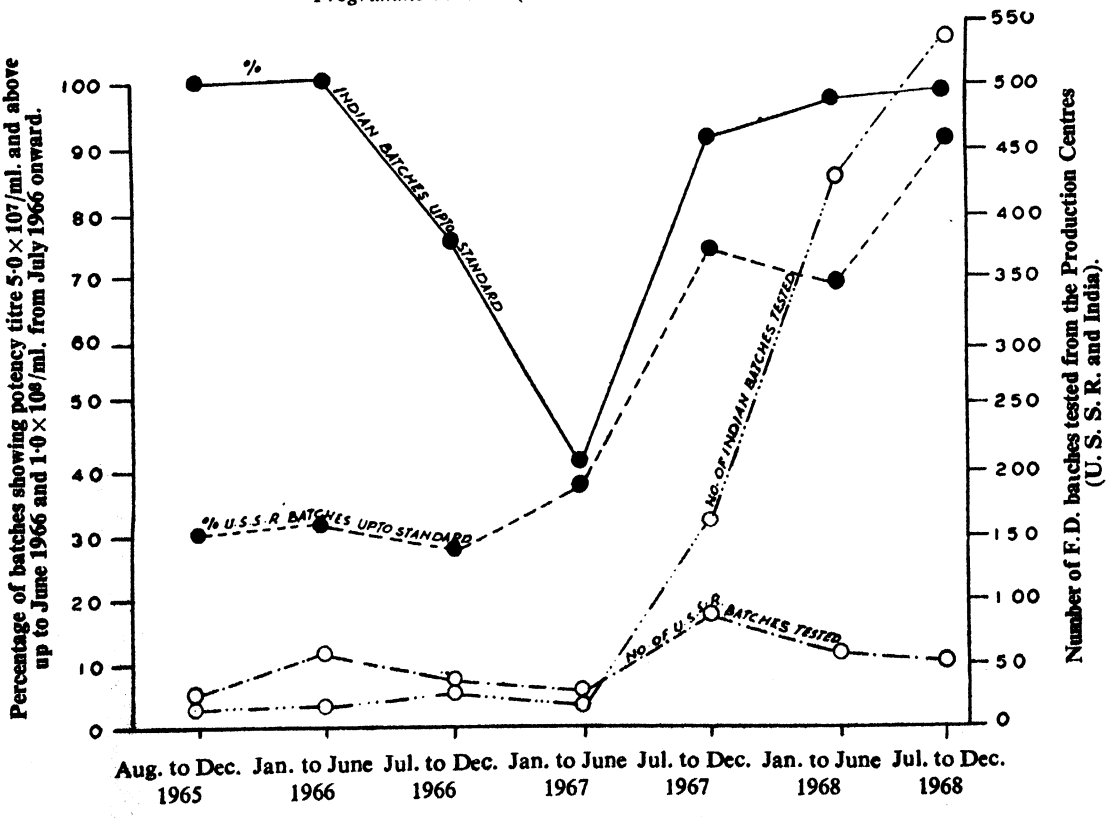
Liquid vaccine.—Of 36 batches tested, not a single batch had a potency titre of $1{\cdot}0\times10^{8}$/ml. Only 19·4 per cent of the batches had a bacterial count below 500 org/ml.

Table VIII (Period 1-7-1968 to 31-12-1968)

A total of 678 batches consisting of 646 batches of freeze-dried vaccine (551 indigenous and 95 imported) and 32 batches of indigenously produced lymph were tested. 93·5 per cent of the total batches (59·7 per cent from U.S.S.R., 100 per cent from West Germany ; 100 per cent from Patwadangar, 100 per cent from Belgaum and 100 per cent from Hyderabad ; 100 per cent from Madras and 50 per cent from Coonoor) had a potency titre of $1{\cdot}0\times10^{8}$/ml. and above on the chorioallantoic membrane of chick

GRAPH.

Results of testing freeze-dried smallpox vaccine batches used under National Smallpox Eradication Programme in India (U. S. S. R. and indigenous).



embryo. Bacteriologically, 100 per cent of total batches (indigenous and imported) had a bacterial count below 500 org/ml.

Liquid vaccine.—Not a single batch out of 32 batches of liquid vaccine showed a potency titre of $1{\cdot}0 \times 10^8$/ml. when tested in embryonated eggs. Bacteriologically, 100 per cent of the batches had bacterial count more than 1,000 org/ml.

From the foregoing, it would be noted that the freeze-dried vaccine (imported and indigenous batches) have since July, 1967 shown an upward trend of meeting the revised WHO standards (1966). These facts are brought out in the Graph.

In this context, it may be pointed out that all the batches are tested as compared with the National Reference Vaccine made in India, details of which are set below.

Comparison of National Reference Vaccine with the International Reference Vaccine (Copenhagen)

The need for a National Smallpox Reference Vaccine was accepted which was to be compared with the International Reference Vaccine as has been laid down by WHO (1966). Accordingly, a large batch of freeze-dried smallpox vaccine was prepared at State Vaccine Institute, Patwadangar, and this reference vaccine was compared for potency and bacterial sterility with the International Reference Vaccine. It was observed that the National Reference Vaccine as well as the International Reference Vaccine and Vaccine from other countries had more or less the same potency titre, viz. $1{\cdot}0 \times 10^8$/ml. and above and bacteriologically all of them were found to conform to the WHO standard, i.e. less than 500 org/ml. In some of the batches of imported vaccine differences were noted in the test at N.I.C.D. and protocols. A few batches of imported vaccine tested at the N.I.C.D. and found substandard as compared to the protocols were also tested at an International Reference Laboratory. The results are set out in Table IX. It would be noted from therein that the results obtained at the recently established N.I.C.D. Smallpox Vaccine Testing Unit are definitely comparable to those obtained at the well established WHO International Reference Centre.

SUMMARY.

The large-scale use of smallpox vaccine under the National Smallpox Eradication Programme in India and the poor 'takes' reported of some batches in primary and the revaccinations stressed the need for testing the quality of the vaccine batches produced indigenously (freeze-dried and liquid lymph) and imported as well. Results of potency and bacterial sterility studies carried out at the National Institute of Communicable Diseases, Delhi, as per the WHO standard, are described. Results of potency studies showed that 1,398 (82·3 per cent) out of 1,697 batches of freeze-dried vaccine and 35 (20·6 per cent) out of 170 batches tested of liquid lymph were up to the WHO standards. In the same period total bacterial count of 1,652 (99·5 per cent) out of 1,660 batches of freeze-dried vaccine and 72 (42·3 per cent) out of 170 batches of liquid lymph was found up to the WHO standard. A national Reference Vaccine produced indigenously served as a control. An improvement in the quality of the indigenously produced as well as imported freeze-dried vaccines has been noted since July, 1967. Some of the imported vaccine batches found sub-standard by the N.I.C.D.

were sent to an International Reference Laboratory by WHO for a second opinion. The results of the International Reference Laboratory by and large corroborated the results of the National Institute of Communicable Diseases, Delhi.

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APPENDIX—1.

(*McLivaine's citric acid buffer*)

The buffer solution was prepared as follows :

1.	Citric acid	0·84 g.
	distilled water	1 litre
2.	$Na_2 HPo_4 . 2H_2o$	0·712 g.
	distilled water	1 litre

Mixed 90·0 ml. of solution 1 with 910·0 ml. of solution 2 to get pH 7·2.

The buffer was then filtered through Whatman filter paper No. 1, checked its pH on Beckman pH meter and sterilized by autoclaving at 15-lb. pressure for 15 minutes. The pH was checked again on Beckman pH meter.