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Evaluation of Egg Inoculation and Precipitation in Gel Test for Laboratory Diagnosis of Smallpox

C.L. Sehgal and S.C. Pal

National Institute of Communicable Diseases, Delhi.

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Two hundred and eighty-four samples in the form of vesicular/pustular fluid or crusts collected from suspected cases of smallpox in different parts of the country were subjected to laboratory diagnosis by using a combination of two tests viz: chick embryo chorioallantoic membrane (CAM) culture and agar gel-precipitation. 73·9 per cent of the specimens were positive for variola on either or both the tests. The efficiency percentage of the CAM method was 97·6 and that of the precipitation-in-gel test 93·3. The difference in the efficiency of the two techniques has not been found statistically significant.

Introduction

A combination of at least two laboratory methods have been utilised by various workers in different laboratories in the world to confirm the presumptive clinical diagnosis, particularly the atypical cases of smallpox. Kempe and Vincent (1964) have mentioned six methods which can be used for laboratory diagnosis of smallpox. They consist of light microscopy, agar gel-precipitation, complement-fixation, CAM culture, tissue culture and serology. Nakano (1972) has listed four diagnostic methods comprising of electron microscopy, gel-precipitation test, CAM culture and tissue culture which are currently in use at the Centre for Disease Control (CDC) Atlanta, Georgia (USA). However WHO (1969) have recommended three simple tests viz. examination of stained smears under light microscope, precipitation-in-gel and the culture of specimens on the CAM of chick embryos.

In the present investigation two laboratory tests consisting of culture on the CAM of chick embryos to study the pock morphology and precipitation-in-agar gel were employed to evaluate the efficiency of these two methods for the diagnosis of smallpox.

Material and Methods

Antigen : Vesicular/pustular fluid aspirated from a number of vesicles or pustules in capillary tubes or drawn by sterile syringe or 2 to 12 crusts collected from a suspected case of smallpox were sent to the laboratory in sterile MacCartney bottles. The specimens were received either through a special messenger or by post. The time taken from the collection of material to the arrival in the laboratory varied from a few hours to a few days.

Vesicular/pustular fluid : It was diluted to 50 per cent in 0·004 M McLlvaine's buffer (pH 7·2) containing penicillin and streptomycin, in case the fluid was not found in sufficient volume to conduct the tests. In this concentration it was employed as an antigen in agar gel-precipitation test but for egg inoculation 10-fold serial dilutions ranging from 10^{-1} to 10^{-3} were made in the said buffer.

Crusts : The crusts were ground in a sterile pestle and mortar and worked up into a thick suspension by adding two drops of 0·004 M McLlvaine's buffer containing antibiotics per medium sized scab. This thick suspension served as an antigen in the precipitation-in-gel (PIG) test. Ten-fold serial dilutions 10^{-1} through 10^{-3}, were then made in the same buffer for inoculation on the CAM of chick embryos.

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

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

Known positive antigen : In the early stages of this study a known positive variola material in the form of scabs suspension was used in the PIG test to serve as a known positive control. Later on it was replaced by the freezedried smallpox vaccine manufactured at Vaccine Institute, Belgaum. The freezedried vaccine was rehydrated in McLlvaine's buffer in such a way so as to have a neat vaccine.

Preparation of agar medium, agar gel slides and procedure for the precipitation-in-gel test : The method adopted was the same as recommended by WHO (1969) except that one per cent agar solution was prepared by using Difco powdered agar/Special Agar-Noble in 0·85 per cent saline instead of capsules containing purified agar.

Dropping of CAM of chick embryo : The CAM of 12-day-old chick embryos were dropped according to the technique described by Westwood **et al.** (1957) with slight modification. 0·1 ml. of 10-fold viral dilution was dropped on to the CAM of each egg. Usually two eggs were used for each dilution. The inoculated eggs were then kept at 35-36°C for three days in BOD incubator.

Results

Between December, 1971 to July, 1973, the National Smallpox Reference Laboratory at N.I.C.D. tested 284 specimens by both the tests viz. PIG and CAM inoculation. The details of the specimens, their source and the results obtained are presented in Table I.

Fifty specimens showing confluent growth of variola virus on the chorioallantoic membranes at the first egg passage were further confirmed by PIG test.

The results of a combination of two tests have been summarised in Table II.

Table I. The results of smallpox specimens tested by both the methods.

Source	No. of specimens tested			No. of specimens found positive for variola on both tests					
				CAM inoculation			PIG test		
	V/P* fluid	Crusts	Total	V/P fluid	Crusts	Total	V/P fluid	Crusts	Total
Andhra Pradesh	—	8	8	—	3	3	—	3	3
Delhi (I.D. Hospital)	115	82	197	107	61	168	100	55	155
Gujarat	—	17	17	—	—	—	—	—	—
Haryana	—	5	5	—	3	3	—	2	2
Jammu & Kashmir	—	5	5	—	2	2	—	2	2
Madhya Pradesh	—	15	15	—	9	9	—	9	9
Mysore	—	1	1	—	—	—	—	—	—
Punjab	—	2	2	—	—	—	—	—	—
Rajasthan	—	1	1	—	—	—	—	—	—
Uttar Pradesh	—	5	5	—	3	3	—	3	3
West Bengal	—	7	7	—	6	6	—	6	6
W.H.O., S.E.A.R.O., New Delhi									
Bihar	—	5	5	—	2	2	—	5	5
Maharashtra	—	1	1	—	1	1	—	1	1
Mysore	—	7	7	—	3	3	—	4	4
Pakistan	—	8	8	—	5	5	—	6	6
Total	115	169	284	107	98	205	100	96	196

*V/P fluid = Vesicular/Pustular fluid.

Table II. Results of CAM inoculation and PIG tests.

CAM inoculation	Precipitation-in-gel test		Total
	Specimens positive for variola	Specimens negative for variola	
Specimens positive for variola	191	14	205
Specimens negative for variola	5	74	79
Total	196	88	284

It would be observed from Table II that 191 (67·2 per cent) out of 284 specimens were found positive for variola on both the tests whereas 74 (26·0 per cent) specimens were negative. Individually 205 (72·2 per cent) specimens out of a total of 284 samples were found positive for variola on CAM inoculation and 196 (69·0 per cent) specimens

positive for variola on PIG test. The difference in the results of the two tests is not statistically significant.

If a specimen was found positive by either of the two or by both the tests, it was declared as a case of smallpox. The efficiency of the two tests for the laboratory diagnosis of smallpox has been shown in Table III.

Table III. Relative efficiency of CAM inoculation and PIG tests.

Test	No. of specimens examined	No. positive for variola	Positive percentage	Efficiency percentage*
PIG	284	196	69·0	93·3
CAM culture	284	205	72·2	97·6
Either or both the tests	284	210	73·9	

*Efficiency percentage = $\frac{\text{No. of positives by a given test}}{\text{No. of positives by both the tests}}$

Results obtained with the vesicular/pustular fluid and crusts on the CAM culture and precipitation-in-gel test are shown in Table IV.

Table IV. Percentage positives with vesicular/pustular fluid and crusts.

Test	Vesicular/pustular fluid	Crusts	Total
CAM culture	93·0	58·0	72·2
PIG	87·0	56·8	69·0

It would be seen that the positive results obtained from the crust specimens were much less in comparison to those of vesicular/pustular fluid.

Discussion

In the present study 210 out of 284 specimens were found positive by either of the tests or by both.

Fourteen (4·9 per cent) specimens which were positive for variola on egg inoculation were declared negative for variola on PIG test. It could be due to the fact that for agar gel-precipitation test higher concentration of virus is required than that for virus isolation on the CAM of chick embryos. These specimens were received in small quantities and it is probable that sometime owing to excessive dilutions no precipita-

tion lines are seen in the agar gel test. Only 1·7 per cent of the specimens (5 out of 284) which gave negative results for variola on the CAM inoculation, were positive by agar-gel test. This might have been due to inactivation of the virus on exposure to adverse field conditions and en-route to the laboratory. In order to determine the cause of this discrepancy, a preliminary experiment was conducted in which thick crusts suspensions from six different specimens which were previously found positive for smallpox on both the tests, were inactivated by heating in a water bath at 60°C for 30 min. These inactivated materials gave positive results for variola on PIG test but were found negative on egg inoculation.

Nakano (1972) observed that the percentage of positives (30) obtained with the agar gel-precipitation method was significantly (statistically) less than that obtained with the electron microscopy (EM) or CAM method. This is contrary to our findings. In the present study we observed that 69 per cent of the specimens were positive for variola on PIG test and 72·2 per cent with the chick embryo CAM technique. The data was analysed statistically. No difference of any statistical significance was observed.

It was further observed (Table IV) that the percentage of positive results obtained was higher in case of vesicular/pustular fluid on both the tests as compared to the crusts. This observation is in conformity with the findings of Nicoli **et al.** (1964) who also found positive results in 71 of 86 (83 per cent) pustular fluids and 22 of 32 (69 per cent) crusts. The level of positive results rose up to 90 per cent where close control over the collection of specimens was possible.

It has been found that agar gel-precipitation test is very sensitive, accurate, reliable and efficacious. It is very simple to perform and gives results in 2 to 20 h. It is inexpensive and can be conducted with great ease even in the laboratories which do not possess adequate facilities to conduct more elaborate test of chick embryo chorioallantoic membrane cultures. The CAM technique in the embryonated hen's eggs, though, accurate and gives slightly higher percentage of positive results as compared to the gel test, is expensive, cumbersome, time consuming and requires skilled workers.

Well equipped laboratories in advanced countries may perform more sophisticated tests such as electron microscopy, tissue culture, fluorescent antibody technique besides the traditional tests **viz.** culture on the chorioallantoic membrane (CAM) of developing chick embryos, agar gel-precipitation and light microscopy for examination of stained smears.

In endemic and developing countries where sufficient laboratory facilities are not available agar gel-precipitation test may be conducted with highly potent antivaccinia serum and specimens found positive for smallpox on this test need not be tested in eggs. They should be declared positive for smallpox after comparing the results with the clinical findings. Specimens which give negative results on gel test must be tested on the CAM of chick embryo before declaring the specimen negative for variola.

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