Indian J Med Res 58, 3 March 1970

Precipitation in Gel Test in Diagnosis of Smallpox*

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Received for publication June 7 1969

Precipitation in gel test is being used for diagnosis of smallpox by testing the vesicular fluid of suspected case against a known antibody (smallpox convalescent serum or hyper-immune anti-vaccinia serum). Since the antibody response to infection with variola and vaccinia are almost identical and since contacts of a smallpox case are invariably vaccinated after exposure, diagnosis of subclinical infection with variola by antibody studies poses a problem. Precipitation in gel test in the detection of subclinical infection with variola showed that nearly 22 % of sera of recently successfully vaccinated persons gave positive result with vaccinia antigen, but none was positive against variola antigen. 100% of the sera taken between 10th and 30th day of the disease of all the surviving cases of smallpox proved positive for this test against both variola and vaccinia antigen. This suggests that the precipitating antibodies produced by the vaccinia antigen (vaccinees' sera) precipitate only vaccinia antigen and not variola, whereas precipitating antibodies produced by the variola antigen, precipitate both variola and vaccinia antigen as demonstrated by this test. Subject to confirmation therefore, precipitation in gel test appears to be a useful tool in the diagnosis of clinical cases of smallpox in acute stage, as well as after recovery within a period of 3 months (retrospective diagnosis) and also in diagnosis of sub-clinical infection with variola virus.

Introduction

During epidemics, occurrence of subclinical cases amongst close familial contacts is not infrequent. In the case of smallpox, however, there are no authentic reports of such occurrence. The fact that nearly 60% of the unvaccinated and 98% of the vaccinated familial contacts escape clinical disease, despite close exposure (Rao et al 1968) tends to show that in smallpox, like in other diseases, subclinical cases do occur in members of the infected families or even of localized communities.

The difficulty with smallpox, is that in the absence of rash, it is not possible to state whether a person was infected or not. Of course, an appreciable rise in antibody titre, may indicate infection with variola. However, since the antibody response is

*This project was sponsored and financed by the WHO and Indian Council of Medical Research

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almost identical with both the viruses, vaccinia and variola, and since contacts of smallpox patients are invariably vaccinated immediately after notification of the case, it is rather difficult to say whether the rise in titre, observed in them was due to vaccination or variola infection.

Downie (1958) says that "the presence of demonstrable CF antibodies, therefore, in a patient who has not been vaccinated within the previous one year, is a good evidence of recent variola infection". Accordingly he found in six variolar contacts, who suffered febrile attack without rash, variolar nature of illness. Applying the same principle Rodrigue de Silva **et al** (1963) have reported inapparent (asymptomatic) infection in 6 out of 37 contacts. Some of these were vaccinated successfully some years before, and some unsuccessfully even recently. Since there are several factors which may determine the level of antibody titres after vaccination, like the vaccination takes, time lag after the previous vaccination, the basic immunity of the population, endemicity of smallpox in a locality, vaccination coverage of population etc., it is doubtful that mere "presence of demonstrable CF antibodies in a person who has not been vaccinated within the previous one year" is a good evidence of variola infection.

As long as it is a fact that CF antibodies can be demonstrated in sera of both vaccinees as well as smallpox cases, the titre alone, perhaps may not justifiably be used as an index to differentiate the type of infection, variolar or vaccinial, especially amongst familial contacts exposed to smallpox and vaccinated after exposure.

It has been observed (WHO TRS 393) that "precipitating antibodies are rarely found in post vaccination sera by means of precipitation in gel test" (PIG). It is stated that even after successful vaccination (pirmary or revaccination) this particular antibody cannot easily be demonstrated by this technique in the vaccinees' sera. If so this could be utilized to differentiate vaccinia infection from that of variola.

This study was therefore undertaken to find out :

Whether or not precipitating antibodies can be demonstrated by the PIG test in post vaccination sera,

whether these antibodies can be demonstrated invariably in sera of smallpox patients by this test ? If so, from what day of the disease and how long after recovery can they be demonstrated,

whether the results of PIG test will be the same or they vary depending upon the antigen used for the test, viz. the variola and vaccinia, and

whether it would be possible to use the PIG test

(*i*) to diagnose a case of smallpox easily even in recently vaccinated person

(*ii*) to detect subclinical cases of smallpox amongst the exposed contacts, if they exist

(*iii*) to undertake a retrospective diagnosis of smallpox in persons who present themselves with pock marks with a history of an attack of eruptive fever a few weeks before.

Material and Methods

New recruits for temporary appointment at Infectious Diseases Hospital, Madras for cholera epidemic work, were the subjects for study of antibody level in the sera before and after vaccination.

A few cases of virus (other than smallpox) and bacterial diseases admitted to Infectious Diseases Hospital and a few persons who were attending the public health laboratory of the Corporation of Madras for examination (biochemical, haematological) of their bloods for various other conditions, were the subjects for study of basic levels of antibodies in sera against variola-vaccinia group of viruses.

Smallpox cases admitted to Infectious Diseases Hospital, during 1966-69 and also those detected by the WHO/ICMR epidemiological unit in the state of Tamil Nadu (Madras) were the subjects of study of antibody levels in post variolar sera.

Some of the available and willing familial contacts of the above cases of smallpox were the subjects of study for the occurrence of subclinical disease amongst familial contacts of smallpox.

Blood was drawn from the new staff recruits of Infectious Diseases Hospital, just before and again on 14th day of vaccination. Vaccination was performed by multiple puncture method with bifurcated needle. The vaccine used was freeze dried vaccine manufactured at King Institute for Preventive Medicine, Guindy. Reactions to vaccinations were read on 7th day of vaccination and classified as described by WHO Scientific group on smallpox eradication (WHO TRS 393). A part of the blood drawn for various investigations in public health laboratory was utilized for our study.

From patients suffering from various diseases like chickenpox, measles, mumps, cholera, typhoid etc admitted to I.D. Hospital, blood was drawn during the course of the disease usually a day or two after admission but invariably before vaccination. No second samples were collected from this group after vaccination. From all cases of smallpox, blood was drawn during the course of the disease on different days. Samples were also collected in a few instances long after recovery.

Samples of blood were collected from available and willing familial contacts of smallpox cases, usually on the day of notification of the primary case. But in a few instances, second samples were also taken later.

All the specimens of blood were kept in the refrigerator overnight and the sera were separated the next day, by light centrifugalization, and were preserved at —20°C till they were studied.

Precipitation in Gel (PIG) test : The presence of precipitating antibodies was detected by the PIG test using the technique described by Kempe and Vincent (1964). The only deviation was that in the preparation of agar solution, plain double distilled water was used instead of buffer. As variolar antigen, a pooled vesicular fluid of smallpox patient was used for study of all sera, not only of smallpox patients but of others too. The test has been repeated with some of the sera using vaccina virus (first egg passage) as antigen also for comparative study.

Haemagglutination inhibition antibody (HIA) estimation : All the sera were studied for HIA titres also, so as to find out whether there is correlation between HIA response and precipitating antibody response. The technique employed is the same as described by Kempe and Vincent (1964) except for the fact that instead of using 2 haemmagglutinating units of antigen, 4 units were used in the present study.

Results

Serological studies of persons not suffering from smallpox : 286 persons, not suffering from smallpox were studied. These included 85 patients suffering from various diseases other than smallpox, 110 healthy persons, and 91 persons who were attending the public health laboratory for various haematological and biochemical investigations of their blood. Most of them were adults and were vaccinated in infancy, and some were re-vaccinated too. Sera of these persons were tested for precipitating antibody by PIG test using vaccinia (first egg passage) as well as variola (pooled vesicular fluid of smallpox case) as antigens. HIA titres were also estimated for comparison.

One hundred and nine of these persons were vaccinated. Their sera were collected before, and again on the 14th day of vaccination and were examined for precipitation antibody by PIG test. HIA titres also were estimated in some. None of the sera, irrespective of the basic vaccinial status, gave positive PIG test either with vaccinia or variola antigens before vaccination, though about 17% of them had a titre of 1 in 40 and more of HIA. Of the sera collected on 14th day after vaccination nearly 44% had a four fold and greater rise in HIA titre. 16·1% of these sera gave positive PIG test with vaccinia antigen. But none gave a positive PIG test with variola antigen (Table I).

Of the 110 persons vaccinated, vaccination takes could be read only in 95. Of these, 4 had primary take, 71 had major take, and the remaining 20 equivocal take. All persons with primary take, 45·7% of persons with major take, and 35% of those with equivocal takes had four-fold and greater rise in HIA titre. Irrespective of vaccination takes, none of the 95 sera tested gave positive PIG with variola antigen. On the other hand with vaccinia antigen, 66·7% sera of those with primary takes and 21·7% of those with major takes gave positive PIG. None of those with equivocal takes were positive (Table II).

Serological studies on smallpox patients : Altogether 89 sera were collected from a total of 48 cases of smallpox on different days of disease. They were tested for presence of precipitating antibodies by PIG test and HIA titres also were estimated to see whether there is any correlation between the two antibody responses. Vesicular fluid of smallpox case only was used as variola antigen in PIG test on the sera. Results are shown in Table III. Seventy-six of the 89 sera were from survivals and the remaining 13 were from fatal cases. 48·7% of the sera from survivals had HIA titre of more than 1 in 160 and 65·8% (50/89) gave a positive PIG with variola. Amongst these positive sera, nearly 68% had HIA titre of more than 1 in 160 and 96% more than 1 in 40.

Amongst the specimens taken from survivals, none of the 7 taken before 7th day the disease gave positive result with PIG test. 41·7% of sera collected between 7th and

Table I. Results of PIG test before and after vaccination with reference to basic vaccination status

Basic vaccinial status of persons studied before current vaccination	Number of persons studied	Before current vaccination				On 14th day of vaccination				
		Results of PIG test				Results of PIG test				HIA titre
		With variola		With vaccinia		With variola		With vaccinia		
		Number examined	Percentage positive	Number examined	Percentage positive	Number examined	Percentage positive	Number examined	Percentage positive	Percentage who had four-fold and more rise
No vaccination scars seen	34	34	Nil	5	Nil	5	Nil	5	40·0	40·0
Only primary vaccination scars seen	180	177	Nil	58	Nil	68	Nil	56	10·7	48·5
Primary vaccination scars seen with history of recent revaccination	72	69	Nil	35	Nil	36	Nil	32	21·9	12·5
Total ..	286	280	Nil	98	Nil	109	Nil	93	16·1	44·0

Table II. Results of PIG test before and after vaccination with reference to the reactions of vaccination

Before vaccination					Reactions of vaccination observed	After vaccination					
	Results of PIG test						Results of PIG test				HIA titre
Number of persons studied	With variola		With vaccinia			Number studied	With variola		With vaccinia		
	Number examined	Percentage positive	Number examined	Percentage positive			Number examined	Percentage positive	Number examined	Percentage positive	Percentage of persons whose sera showed four-fold and more rise
					Primary take	4	4	Nil	3	66·7	100·0
95	92	Nil	83	Nil	Major take	71	71	Nil	60	21·7	45·7
					Equivocal take	20	20	Nil	41	Nil	35·0
					Total	95	95	Nil	77	19·5	44·7

9th day of disease, 100% of these collected between 10th and 29th day, and 88·9% of these collected between 29th and 72nd day were positive for PIG test with variola. After 72nd day of disease, these antibodies tend to disappear slowly. Beyond 210th day of disease none of the 7 sera taken were positive. In 12 survivals, whose blood was negative for PIG before 9th day of disease, it was invariably positive after 10th day. In none of the survivals, one who was negative before 9th day continued to be negative after 10th day. There was a good correlation between PIG test and HIA titre. In fatal cases, of the 13 sera tested, only 2 were positive for PIG test and these were taken between 10th and 29th day of fever. These two sera had a high rise in HIA titre also. All others which were taken from cases who died on or before 10th day were negative.

Table III. Results of PIG test on sera of smallpox patients with reference to the day of disease and clinical outcome of the case

Day of disease when blood was drawn	Total number of sera studied	In survivals			In fatal cases		
		Number Tested	Percentage positive for PIG test	Percentage which had HIA titre of more than 1 in 160	Number tested	Percentage positive for PIG test	Percentage which had more than 1 in 160 HIA titre
Within 7 days	10	7	Nil	12·3	3	Nil	Nil
7th to 9th day	17	12	41·7	33·3	5	Nil	Nil
10th to 29th day	32	27	100·0	63·0	5	40·0	60·0
30th to 72nd day	18	18	88·9	66·7			
90th to 170th day	5	5	40·0	40·0			
Beyond 210th day	7	7	Nil	12·3			
Total	89	76	65·8	48·7	13	15·4	23·1

With reference to the clinical variety, irrespective of the day of disease when the sera were drawn, they were all negative for PIG in cases belonging to haemorrhagic and flat varieties. Between 10th and 26th day 100% (29/29) of specimens tested both of ordinary and modified varieties were positive.

The vaccinial status of the patient does not seem to have any influence on the result of PIG test. Whether vaccinated or unvaccinated, it is the day of the disease and clinical outcome that determined results of PIG test. Fatal cases were rarely positive for PIG and majority of sera taken amongst the survivals between 10th day of disease and 170th day were positive. Sixty of the total 89 sera were tested for PIG using vaccinia as antigen also. The results in 52 specimens were identical with those obtained with variola.

Serological studies on contacts of smallpox patients : There were, in all, 157 contacts for 33 smallpox cases studied. Of these, 14 developed clinical smallpox. Amongst the remaining 143 apparently healthy (i.e. who had not developed clinical

smallpox) contacts, only from 94, sera could be collected for PIG studies. In all, 114 samples of sera were collected on different days of exposure to the primary case, from these 94 contacts. These sera were studied for the presence of precipitating antibodies as evidenced by the PIG test using variola antigen and of haemagglutination inhibition antibodies using vaccinia antigen.

Since the purpose of this study was to detect subclinical disease amongst the contacts, and since it was found that these precipitating antibodies can invariably be detected after 10th day of disease by PIG test in smallpox cases, it was presumed that a contact, if infected with variola virus, would give a positive PIG test with variola antigen invariably after 24th day of exposure (14 days of incubation period plus 10 days of subclinical disease). Hence a comparison has also been made between the results of PIG test on sera collected before 24th day of exposure and on those collected after 24th day. Table IV shows the results of PIG test on sera of these contacts taken on different days of exposure. 8·1% (5/62) of sera collected before 24th day of exposure were positive for PIG as against 37·8 (17/45) of these collected between 24th and 45th day of exposure. 8·3% (1/12) of sera collected on or after 90th day were positive. Unfortunately no sera were collected between 45th and 90th day of exposure. Of the 12 specimens collected on or after 90th day, 2 were first specimens and both were negative, though one of them had a very high HIA titre of 1 in 2560. Sera of 7 contacts which were positive earlier, became negative after 90th day. Only in one isolated instance, it continued to be positive even on 344th day of exposure. The contacts whose sera were positive even within 24 days of exposure to primary cases might have got infected from the primary case itself or from an extra familial source which was common to both for the primary case as well as the contact.

Table IV. Results of PIG test on sera of familial contacts of smallpox patients with reference to the day of exposure to primary case

Day of exposure to primary case	Number of sera tested	Number positive for PIG test	Percentage positive for PIG test
Within 24 days	62	5	8·1
Between 24th and 45th day	45	17	37·8
90 days and beyond	12	1	8·3

Table V shows the actual number of familial contacts, these 32 cases of smallpox had, with reference to their age group and vaccinial status, how many of them developed clinical smallpox, and also how many of the apparently healthy contacts were studied for subclinical disease, as evidenced by positive PIG test with their results.

Of the 143 apparently healthy familial contacts, sera of 49 could not be studied. Irrespective of vaccinial status, age, and day of exposure, 23·4% (22/94) of the remaining contacts studied gave a positive PIG with variola antigen indicating possibility that they were suffering from subclinical disease. In 48 contacts, sera were examined only

Table V. Results of PIG test on sera of apparently healthy familial contacts of smallpox cases

Vaccinial status	Vaccinated				Unvaccinated				Total vaccinated and unvaccinated				No data regarding vaccinial status and age available	Grand total
Age group	0—4	5—14	15+	Total	0—4	5—14	15+	Total	0—4	5—14	15+	Total		
Total number of contacts	13	26	79	118	10	4	10	24	23	30	89	142	15	157
Number who developed clinical smallpox	0	1	3	4	5	2	3	10	5	3	6	14	0	14
Number of apparently healthy contacts	13	25	76	114	5	2	7	14	18	27	83	128	15	143
Number actually studied	5 (4)	16 (2)	55 (12)	76 (18)	2 (2)	1 (0)	5 (1)	6 (3)	7 (6)	17 (2)	60 (13)	84 (21)	10 (1)	94 (22)
Number studied														
Within 24 days	2 (1)	11 (0)	20 (1)	33 (2)	2 (2)	1 (0)	3 (1)	4 (3)	4 (3)	12 (0)	23 (2)	39 (5)	9 (0)	48 (5)
Between 25th and 45th day	3 (3)	5 (2)	34 (11)	42 (16)	0	0	1 (0)	1 (0)	3 (3)	5 (2)	35 (11)	43 (16)	1 (1)	44 (17)
90 days and after	0	0	1 (0)	1 (0)	0	0	1 (0)	1 (0)	0	0	2 (0)	2 (0)	0	2 (0)

( ) Positive for PIG test using variola antigen

within 24 days of exposure and of these only 5 (10·4%) were positve for PIG, whereas 17 out of 44 (38·5%) who were examined between 24th and 45th day of exposure were positive. Two contacts who were examined for the first time on 90th and 93rd day of exposure were both negative.

There were 118 vaccinated and 24 unvaccinated contacts in this series. 3·4% (4/118) of the vaccinated and 41·7% (10/24) of the unvaccinated developed clinical smallpox. These are almost similar to our previous findings on clinical smallpox amongst familial contacts (Rao **et al** 1968). 23·7% (18/76) of the vaccinated and 37·5% (3/8) of the unvaccinated healthy contacts were positive for PIG test using variola antigen irrespective of the day of exposure.

With reference to age 85·7% (6/7) of the children belonging to the age group 0-4 were positive for PIG as against 11·8% (2/17) of the age group 5-14 and 20% (12/60) of those above the age of 14. It has to be admitted however that less number of contacts in the age group 5-14 were examined after 24th day of exposure. The same trend is seen even with reference to age and vaccinial status also.

Discussion

At the outset, it must be admitted, that the number of samples of sera tested in this study is rather small. However, from the data available, this study seems to suggest that this simple "precipitation in gel" (PIG) test may be a useful tool not only in diagnosis of variola infection but also in differentiating it from vaccinia infection. The statement made in WHO TRS 393 that "precipitating antibodies are rarely found in post-vaccination sera by means of PIG test" does not seem to be wholly correct. This study has shown that 16·1% of vaccinees' sera had demonstrable precipitating antibodies which can be detected by PIG test using vaccinia as antigen on the 14th day of vaccination.

The results of PIG test seem to have a definite correlation with reaction of current vaccination. In this study, it has been found that as many as two thirds of sera of persons with **primary** take, and about 1/5 of those of persons with **major** take, gave positive PIG whereas none of the sera of persons with "equivocal" take were positive. But of these sera none were positive when variolar virus (i.e. vesicular fluid of smallpox patient) was used as the antigen which is of great interest.

This study has also shown that these precipitating antibodies cannot be usually detected in sera of smallpox patients before 7th day of disease. But 41·7% of sera collected between 7th and 9th day of disease, 100% of sera collected between 10th and 26th day, and nearly 90% of these collected between 26th and 72nd day, do have demonstrable precipitating antibodies as can be detected by PIG test. After that day, the positive rate falls down and none were positive after 7th month after the attack. This of course is applicable to survivals only. In fatal cases, especially those belonging to the haemorrhagic and flat varieties, the sera are invariably negative. But if they survive beyond 15th day of disease, these antibodies can be demonstrated even in fatal cases too. In testing of all the sera of smallpox patients, variola virus (vesicular fluid of smallpox case) was used as antigen. Fifty-two out of 60 sera tested with vaccinia virus as antigen also gave identical results.

From the above studies, it appears that in variola infections, precipitating antibodies may be formed which give positive precipitation both against variola as well a vaccinia. But in vaccinia infections the antibodies formed do not seem to precipitate variola antigen though they do precipitate vaccinia antigen in a few. If this hypothesis is correct, this PIG test using variola (vesicular fluid of smallpox patient) as the antigen, can be used as a tool in diagnosis of smallpox both clinical, as well as subclinical disease. Since in clinical disease, the PIG test is invariably positive only after 10th day, one should expect the sera of exposed contacts to be invariably positive after 24th day of expsoure to the primary case, if they have been infected.

In this study sera taken on different days of exposure from 94 contacts of smallpox cases were tested against variola antigen. 23·4% of these gave positive PIG when their sera were tested against variola antigen suggesting occurrence of subclinical disease in them. 38·5% of contacts who were tested between 24th day and 45th day of exposure gave positive PIG test. Most of the sera examined after 90th day were negative though a few of the sera were positive even before 24th day of exposure.

When compared to clinical smallpox there is comparatively greater incidence of subclinical infection amongst the vaccinated contacts, than in the unvaccinated. This is understandable since nearly 40% of the unvaccinated contacts developed clinical smallpox as against only 3·4% of the vaccinated contacts.

As in the case of clinical smallpox there is a suggestion that even subclinical disease is relatively more common in the young children of the age group 0-4.

If our presumption that a positive PIG test using variola antigen is an indication of infection with variola virus is correct then this study suggests that nearly 40% of the apparently healthy familial contacts of a smallpox case do develop subclinical disease.

Though further work has to be done on these lines, yet there is an indication from this study, that PIG test using vesicular fluid of smallpox patient as antigen, can usefully be employed in diagnosis of variola infection both clinical as well as subclinical, and that subclinical infections with smallpox do occur in a community in epidemics. Perhaps this may be one of the reasons why not all susceptibles do develop clinical disease and why smallpox does not produce explosive epidemics but spreads slowly and dies out even automatically (Rao 1968).

Acknowledgment

The authors thank Thiru. K.J.M. Shetty, IAS, Commissioner, Corporation of Madras for having permitted to conduct the work in Infectious Diseases Hospital, Madras and also permitted to publish this paper. Our thanks are due to Director General and other staff members of Indian Council of Medical Research for their financial and secretarial assistance.

The authors wish to thank Dr. D.A. Henderson, Chief, Smallpox Eradication, Geneva, who has given valuable suggestions and advice.

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World Health Organization—Technical Report Series No 393.