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POTENCY TESTING OF SMALLPOX VACCINE

by

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In recent years, in the course of our testing service for freeze-dried smallpox vaccine at the WHO Reference Laboratory for Smallpox Vaccine, we have noticed that there have been some discrepancies between the titres determined by our laboratory and those by the vaccine producers who submitted the samples. Although it is difficult to pinpoint the causes for differences in test results, we feel that detailed information on the procedures employed in our laboratory would be of some assistance to ensure more uniformity between the results of potency titration in our laboratory and those of various producers.

The procedures described here are based on our experiences in testing more than 1000 production batches submitted to our laboratory from different laboratories and are recommended methods with good reproducibility. This note may be considered as supplementary to the "Methodology of freeze-dried smallpox vaccine production" issued in 1968 (WHO document SE/68.3 Rev. 1).

1. Instruments and reagents for testing

The glassware (bottles, tubes and pipettes) and other tools needed for the titration of vaccinia virus should be clean and sterile. The cleansing methods and sterilization procedures are not different from the methods routinely used in virus laboratories. All receptacles should be clearly labelled and pipettes should be properly calibrated. The chemicals and antibiotics used for the preparation of diluents should be as pure as possible and always from the same supplier. Diluents should be prepared by one technician, strictly following the same method of preparation each time. Diluents should be stored at refrigerator temperature.

2. Preparation of the virus dilutions

2.1 Use of 0.004 Molar McIlvaine buffer solution as diluent

In Table 1 the results of a number of virus titrations are given in which different diluents were used: (a) physiological saline (NaCl 0.85%); (b) 0.2 Molar McIlvaine buffer, and (c) 0.004 Molar McIlvaine buffer (the standard solution).

As is apparent from these results, use of the correct diluent in the prescribed concentration is absolutely essential when titrating vaccine. Directions for preparing 0.004 Molar McIlvaine buffer pH 7.2-7.4 are given in Table 2. If desired 400 IU per ml of penicillin and 0.100 mg per ml of streptomycin may be added.

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2.2 Method of pipetting

In our laboratory, the sterile glass tubes in which dilutions are made are 15 cm long with an inner diameter of 15 mm. They are sealed with a plug of cotton (Figure 1). For accurate work, the quantities to be pipetted should be large. Quantities of 1.8 ml of diluent are pipetted into the above-mentioned tubes with a sterile 10 ml pipette (Figure 1). The pipette is kept in a vertical position and the tip of the pipette is held against the inner wall of the tube so that the diluent can flow to the bottom via the inner wall of the tube. The advantage of working with this small quantity of diluent in a relatively large tube is that only a small part of the tube is filled, so that when 1.0 ml pipettes for diluent and vaccine (as described in 2.3) are dipped into this diluent, only the lowest part of the exterior of these pipettes gets wet.

If automatic or semi-automatic pipettes or pipetting machines are used, one should make certain that the correct amount of diluent to be pipetted remains the same throughout the procedure. In our laboratory we have experienced difficulty with automatic pipettes in adjusting them so as to ensure that the amount of diluent pipetted stays the same throughout the whole procedure. Another disadvantage is that for sterilization most automatic pipettes must be taken apart. Re-assembling means a chance for error. Every new cylinder must be calibrated.

In our laboratory, pipetting is done by mouth. In each pipette there is a cotton plug in the upper part to prevent fluid being inadvertently taken into the mouth. As a further precaution, however, all the workers are vaccinated against smallpox every two to three years.

2.3 Dilution of vaccine for inoculation

Two different dilutions of the vaccine sample and two different dilutions of a control vaccine of known satisfactory potency (about 1 x 10^8 pfu/ml) should be prepared for inoculation of eggs. Suitable dilutions should be determined so that the number of pocks on the chorio-allantoic membrane will be about 15 to 20 with one of the dilutions, taking into consideration the expected titre of the vaccine sample. Depending on the expected titre, simple ten-fold dilutions may be made although in some instances it may be necessary to prepare dilutions which are intermediate between ten-fold dilutions.

2.3.1 Ten-fold dilution

For the preparation of the 10^{-1} (1/10) virus suspension, 0.2 ml of reconstituted vaccine is needed. Sufficient vaccine should be reconstituted and collected in one ampoule. In our laboratory it is routine to reconstitute at least 0.4 ml of vaccine and to withdraw from this the 0.2 ml of vaccine required. A 1.0 ml pipette is used (Figure 1). As already described above, the pipette is kept in a vertical position just above the surface of the diluent and the 0.2 ml of vaccine flows downwards via the inner wall of the tube from the marking points "0.7" to "0.9" on the pipette. To avoid vaccine accumulating on the exterior of the pipette and subsequently flowing into the tube, the pipette should not be inserted too deeply into the reconstituted vaccine. This general principle should be followed each time vaccine or vaccine dilutions are transferred from one tube to another.

Before transferring 0.2 ml of the first virus suspension to the next tube to make the next dilution, the virus suspension should be thoroughly mixed. In our laboratory this is routinely done with a 1.0 ml pipette. The pipette is kept in the suspension and the suspension is moved up and down in the pipette five times. For proper mixing, care should be taken that the virus suspension is sucked up into the pipette to the same height each time. Each time that the virus suspension is mixed and transferred, a fresh, sterile pipette is used. Therefore to make a virus dilution of 10^{-6}, six 1.0 ml pipettes are needed. All dilutions steps should be sealed and labelled properly (Figure 2).

Thorough mixing of each dilution of the virus suspension can also be done with the aid of a machine where the contents of the tube are stirred rapidly by vibration of the tube. In our laboratory we have found that different mixing procedures can result in slightly different titration results, therefore the method of mixing of the suspensions should be standardized.

2.3.2 Vaccine dilutions which are intermediate between ten-fold dilutions

Table 3 has been prepared to show the dilution to be made, the amount of this suspension and the amount of diluent to be mixed together, and the amount to be inoculated following mixing. The last column shows the average number of pocks expected.

3. Inoculation of the eggs

The virus suspensions should be inoculated not later than two hours after they are made. Twelve-day-old chick embryos are used. The eggs to be inoculated are candled and dead embryos and non-fertile eggs are discarded. Sufficient eggs are prepared to permit counting the pocks on at least five membranes at each dilution tested.

On all eggs selected for potency assay the site of inoculation is pencil-marked on the shell, carefully defining an area which is not in proximity to blood vessels. A hole is made through the shell and the shell membrane into the air sac of each egg. An area of shell approximately 4 x 4 mm is then carefully removed from the previously marked inoculation site without penetrating the shell membrane (Figure 3). Two drops of 0.004 Molar McIlvaine buffer pH 7.2 are placed on the exposed shell membrane and the membrane is penetrated with a needle. Care must be taken not to damage the chorioallantoic membrane (CAM). Suction produced at the air sac hole with a rubber mouth piece or teat, drops the CAM completely. (Dropping of the CAM can also be achieved by leaving the eggs in the incubator for one to four hours). The shell membrane covering the inoculation site is cut away with a pair of scissors (Figure 3) and the CAM in the eggs inspected with the naked eye. Eggs in which whole blood or other abnormalities are found on the CAM are discarded. Each egg is inoculated with 0.1 ml of virus dilution using a 1.0 ml pipette. The eggs are sealed with scotch tape and incubated at 35-37°C for 42-46 hours.

4. Collection of the membranes

Pock counting has to be done on membranes removed from the eggs. In order to take the membranes out of the eggs a handle is made by fixing a piece of scotch tape on the inoculation site. Eggs are opened by making a small hole in the shell and cutting the egg lengthwise into two halves (Figure 4). The embryo is dropped out of one of the halves and the CAM carefully taken out with the aid of a cover-glass pincet. In order to remove blood and other material the membranes are washed in two or three petri dishes (14 cm) with distilled water. Finally the membranes are placed in a petri dish in distilled water containing 1% formalin. All membranes inoculated with one dilution can be placed in a single petri dish.

5. Pock counting and calculation of titre

The pocks are counted on a black background. The membranes in the petri dishes are spread with the aid of two egg pincets so that the pocks can be easily counted (Figure 4). The number of pocks should be recorded immediately after counting. Dead embryos should be discarded. Zero pocks should be taken into account. Only the dilution giving an average number of 15-20 pocks per membrane is used for calculation. The potency of the control and the test vaccine are calculated as pock forming units per ml (pfu/ml). The results of 10 potency tests of reference vaccine 6713-18 and the calculation of the $\log_{10}$ titre are given in Table 4.

Conversion of original pock count number to $\log_{10}$ titre is illustrated in Table 5.

Potencies equal to or greater than the potency of the control vaccine (1×10^8 pfu/ml) following incubation of the vaccine for four weeks at 37°C indicate satisfactory vaccine. In Table 6 a number of typical examples are given of the results of titration of different vaccines showing the calculations made or, in some instances, additional steps to be taken.

TABLE 1. POTENCY OF SMALLPOX VACCINE DILUTED WITH DIFFERENT BUFFER SOLUTIONS

Batch no. of vaccine	Test no.	Log_{10} titre (pfu/ml) of Vaccinia Virus after making the virus dilution in:		
		Physiological saline	0.2 mol McIlvaine buffer	0.004 mol McIlvaine buffer
6713-18	1	7.6	-	8.1
"	2	7.8	-	8.0
"	3	7.7	-	7.9
"	4	7.7	-	8.2
"	5	7.7	-	8.1
"	6	7.0	-	8.2
"	7	-	7.5	8.2
"	8	-	7.0	8.1
"	9	-	7.2	8.2
"	10	-	7.3	8.1
"	11	-	7.1	8.3
7134	1	7.6	-	8.7
KO 72/2C	1	6.8	-	7.7
6547-G	1	8.1	-	9.0
6547-V	1	8.2	-	9.0
6664	1	-	8.5	9.3
6922-7112 II	1	-	7.0	8.9
6840-W-R	1	-	6.5	8.8
6840-X-R	1	-	6.0	8.3
6840-Y-R	1	-	5.4	7.6
6840-Z-R	1	-	4.8	7.1

- Not done

TABLE 2. PREPARATION OF 0.004 MOLAR McILVAINE BUFFER

1. Make up Stock solution A.

   Dissolve 2.1 grams of analytical grade citric acid ($C_6H_8O_7.H_2O$) in 100 ml of distilled water.

2. Make up Stock solution B.

   Dissolve 35.6 grams of analytical grade disodium phosphate ($Na_2HPO_4.2H_2O$) in 1000 ml of distilled water.

3. Mix 10 ml of Stock solution A with 90 ml of Stock solution B.

4. Add distilled water to mixture of solutions A and B to make 5000 ml.

5. Determine pH. Normally, this will be between pH 7.2 and 7.4. If it is above pH 7.4, a very small amount of Stock solution A will lower the pH to the proper range. If pH is below 7.2, discard.

Note: Stock solutions as well as buffer should be kept refrigerated. At refrigerator temperatures, the phosphate in solution B will crystallize out of solution. Before using, the solution must be warmed until crystals redissolve.

TABLE 3. PREPARATION OF VIRUS DILUTIONS WHICH ARE INTERMEDIATE BETWEEN TEN-FOLD (LOG) DILUTIONS

Expected titre (pfu/ml)	Amount of diluent to be mixed with amount of virus dilution			Final dilution of virus suspension to be inoculated originating from foregoing mixing	Average number of pocks expected
	diluent (ml)	virus (ml)	from dilution		
5×10^7	1.4	0.6	10^{-5}	3.0×10^{-6}	15
6×10^7	1.5	0.5	10^{-5}	2.5×10^{-6}	15
7×10^7	1.6	0.4	10^{-5}	2.0×10^{-6}	14
8×10^7	1.6	0.4	10^{-5}	2.0×10^{-6}	16
9×10^7	1.7	0.3	10^{-5}	1.5×10^{-6}	13-14
1×10^8	1.7	0.3	10^{-5}	1.5×10^{-6}	15
2×10^8	0.5	1.5	10^{-6}	7.5×10^{-7}	15
3×10^8	1.0	1.0	10^{-6}	5.0×10^{-7}	15
4×10^8	1.2	0.8	10^{-6}	4.0×10^{-7}	16
5×10^8	1.4	0.6	10^{-6}	3.0×10^{-7}	15
6×10^8	1.5	0.5	10^{-6}	2.5×10^{-7}	15
7×10^8	1.6	0.4	10^{-6}	2.0×10^{-7}	14
8×10^8	1.6	0.4	10^{-6}	2.0×10^{-7}	16
9×10^8	1.7	0.3	10^{-6}	1.5×10^{-7}	13-14
1×10^9	1.7	0.3	10^{-6}	1.5×10^{-7}	15
2×10^9	0.5	1.5	10^{-7}	7.5×10^{-8}	15
3×10^9	1.0	1.0	10^{-7}	5.0×10^{-8}	15
4×10^9	1.2	0.8	10^{-7}	4.0×10^{-8}	16

TABLE 4. POTENCY TESTING OF REFERENCE VACCINE 6713-18

Date 1971	Number of eggs inoculated	Virus dilution inoculated	Number of membranes counted	Dead embryos	Number of pocks per membrane	Average	Calculation of the titre (pfu/ml)	Log_{10} titre
26/10	9	1.5×10^{-6}	9	0	16,12,21,23,21, 10,8,9,10	$\frac{130}{9} = 14.44$	$\frac{14.44}{1.5} = 9.626 \times 10^{6}$ per 0.1 ml or 9.626×10^{7} per 1.0 ml	8.0
27/10	9	1.5×10^{-6}	9	0	23,13,20,19,14, 15,18,11,26	$\frac{159}{9} = 17.66$	$\frac{17.66}{1.5} = 1.177 \times 10^{7}$ per 0.1 ml or 1.177×10^{8} per 1.0 ml	8.1
29/10	9	1.5×10^{-6}	9	0	19,25,25,25,31, 25,18,16,20	$\frac{204}{9} = 22.66$	$\frac{22.66}{1.5} = 1.510 \times 10^{7}$ per 0.1 ml or 1.510×10^{8} per 1.0 ml	8.2
10/11	9	1.5×10^{-6}	9	0	33,31,12,35,28, 36,41,34,38	$\frac{288}{9} = 32.00$	$\frac{32.00}{1.5} = 2.133 \times 10^{7}$ per 0.1 ml or 2.133×10^{8} per 1.0 ml	8.3
12/11	9	1.5×10^{-6}	9	0	15,16,16,10,17, 6,18,12,12	$\frac{122}{9} = 13.55$	$\frac{13.55}{1.5} = 9.033 \times 10^{6}$ per 0.1 ml or 9.033×10^{7} per 1.0 ml	8.0
15/11	9	1.5×10^{-6}	9	0	17,11,18,13,16, 9,20,23,19	$\frac{146}{9} = 16.22$	$\frac{16.22}{1.5} = 1.081 \times 10^{7}$ per 0.1 ml or 1.081×10^{8} per 1.0 ml	8.0
16/11	9	1.5×10^{-6}	8	1	38,28,40,16,15, 22,16,39	$\frac{214}{8} = 26.75$	$\frac{26.75}{1.5} = 1.783 \times 10^{7}$ per 0.1 ml or 1.783×10^{8} per 1.0 ml	8.3
17/11	9	1.5×10^{-6}	8	1	33,8,16,24,16, 14,11,19	$\frac{141}{8} = 17.625$	$\frac{17.625}{1.5} = 1.175 \times 10^{7}$ per 0.1 ml or 1.175×10^{8} per 1.0 ml	8.1
19/11	9	1.5×10^{-6}	9	0	27,20,15,28,22, 9,26,15,11	$\frac{173}{9} = 19.22$	$\frac{19.22}{1.5} = 1.281 \times 10^{7}$ per 0.1 ml or 1.281×10^{8} per 1.0 ml	8.1
22/11	9	1.5×10^{-6}	9	0	23,8,19,35,13, 28,14,32,16	$\frac{188}{9} = 20.88$	$\frac{20.88}{1.5} = 1.392 \times 10^{7}$ per 0.1 ml or 1.392×10^{8} per 1.0 ml	8.1

TABLE 5. CONVERSION OF ORIGINAL POCK COUNT TO LOG_{10} TITRE

Pock count per ml		Log_{10} titre*
Original count	Number in power of 10	
1×10^7	$10^{7.00}$	7.00
2×10^7	$10^{7.30}$	7.30
3×10^7	$10^{7.47}$	7.47
4×10^7	$10^{7.60}$	7.60
5×10^7	$10^{7.69}$	7.69
6×10^7	$10^{7.77}$	7.77
7×10^7	$10^{7.84}$	7.84
8×10^7	$10^{7.90}$	7.90
9×10^7	$10^{7.95}$	7.95
1×10^8	$10^{8.00}$	8.00
2×10^8	$10^{8.30}$	8.30
3×10^8	$10^{8.47}$	8.47
4×10^8	$10^{8.60}$	8.60
5×10^8	$10^{8.69}$	8.69
6×10^8	$10^{8.77}$	8.77
7×10^8	$10^{8.84}$	8.84
8×10^8	$10^{8.90}$	8.90
9×10^8	$10^{8.95}$	8.95
1×10^9	$10^{9.00}$	9.00
2×10^9	$10^{9.30}$	9.30
3×10^9	$10^{9.47}$	9.47
4×10^9	$10^{9.60}$	9.60
5×10^9	$10^{9.69}$	9.69

* Titre may be expressed as round number - say 7.47 is 7.5.

TABLE 6. CALCULATION OF VIRUS TITRE

Vaccine	Number of eggs inoculated	Virus dilution inoculated	Number of membranes counted	Dead embyros	Number of pocks per membrane	Average	Calculation of the titre (pfu/ml)	Log_{10} (pfu/ml)
a	10	10^{-6}	10	0	12,16,18,21,9, 17,11,15,16,12	$\frac{147}{10} = 14.7$	$14.7 = 1.47 \times 10^{7}$ per 0.1 ml or 1.47×10^{8} per 1.0 ml	8.2
b	10	10^{-6}	10	0	18,14,21,25,13, 0,10,12,0,17	[a]		
c	10	10^{-6}	9	1	37,38,45,50,45, 48,43,39,34	[b]		
c	10	4×10^{-7}	10	0	18,14,17,21,12, 11,16,9,18,21	$\frac{157}{10} = 15.7$	$15.7 = 3.92 \times 10^{7}$ per 0.1 ml or 3.9×10^{8} per 1.0 ml	8.6
d	10	10^{-6}	9	1	15,8,18,6,17,3, 21,12,5	[a]		
e	10	10^{-7}	10	0	0,0,4,6,2,0,1, 5,4,3	[c]		
e	10	5×10^{-7}	9	1	15,10,8,17,21, 14,12,19,16	$\frac{132}{9} = 14.66$	$14.66 = 2.93 \times 10^{7}$ per 0.1 ml or 2.9×10^{8} per 1.0 ml	8.5
f	10	10^{-6}	10	0	0,0,10,12,9,11, 16,0,8,15	[a]		
g)	10	10^{-5}	10	0	50,45,41,53,48, 56,31,47,49,59	[d]		
g)	10	10^{-6}	10	0	17,18,12,21,14, 19,15,9,17,16	[d]		
h	10	1.5×10^{-7}	9	1	14,22,17,16,19, 12,15,18,19	$\frac{152}{9} = 16.88$	$16.88 = 11.25 \times 10^{7}$ per 0.1 ml or 1.13×10^{9} per 1.0 ml	9.1

[a] No further calculation. The variation in the number of pocks between membranes is too large. Test should be repeated.

[b] No further calculation. Too many pocks per membrane. Test should be repeated with a more diluted suspension.

[c] No further calculation. Number of pocks per membrane is too low. Test should be repeated with a less diluted suspension.

[d] No further calculation. The difference in the number of pocks at the two different dilutions is much less than should be observed as there is a 10-fold difference in the concentration of the two suspensions and only a two to three-fold difference in the number of pocks. Test should be repeated.

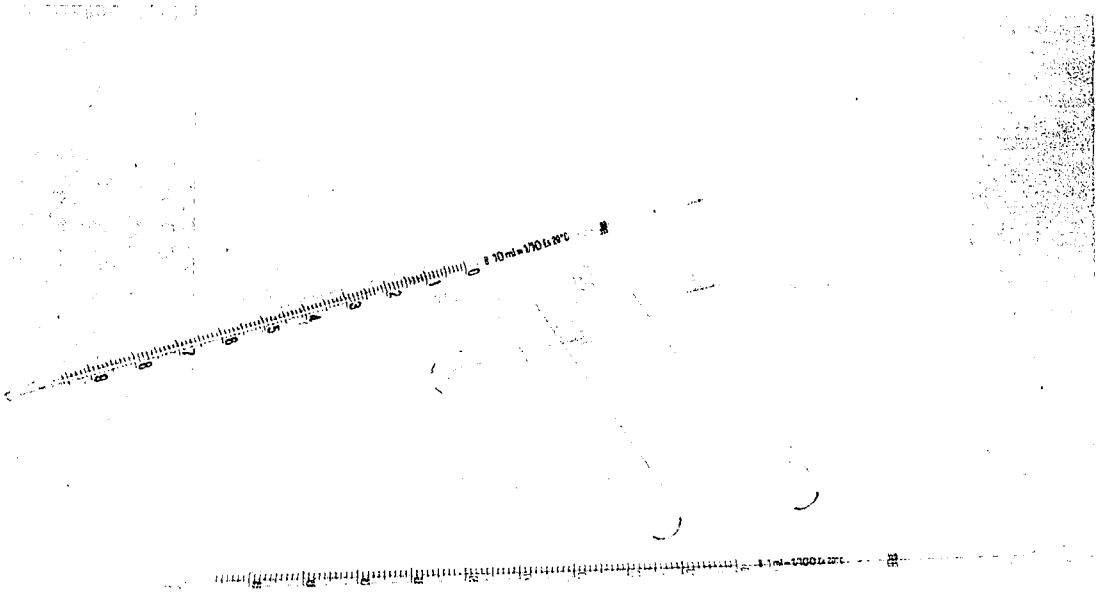

FIGURE 1. A 10 ml pipette is used to transfer diluent to sterile glass tubes which are sealed with a cotton plug. A 1.0 ml pipette is then used to add reconstituted vaccine to the diluent.

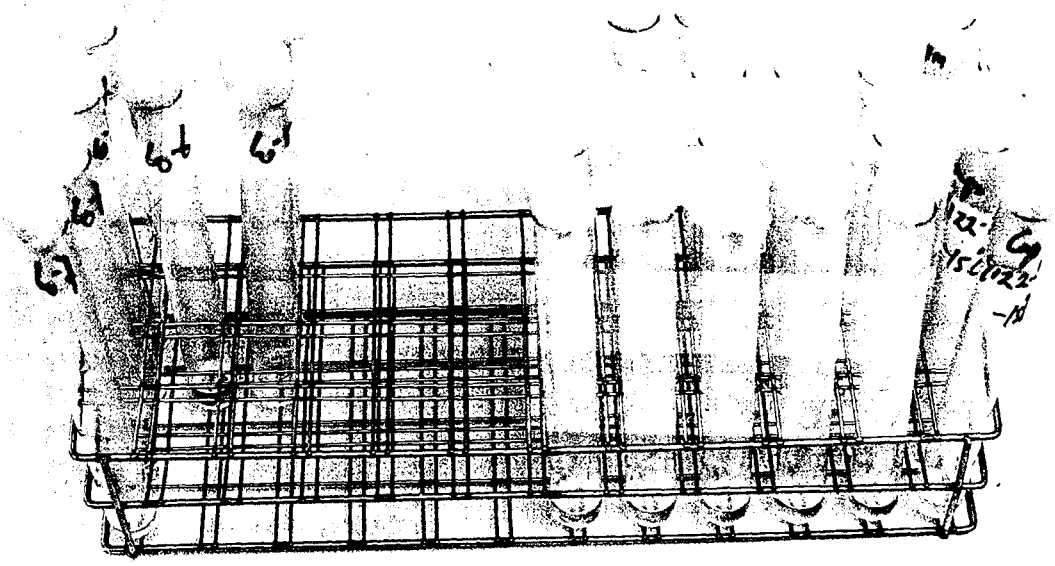

FIGURE 2. Dilution steps should be sealed and labelled properly.

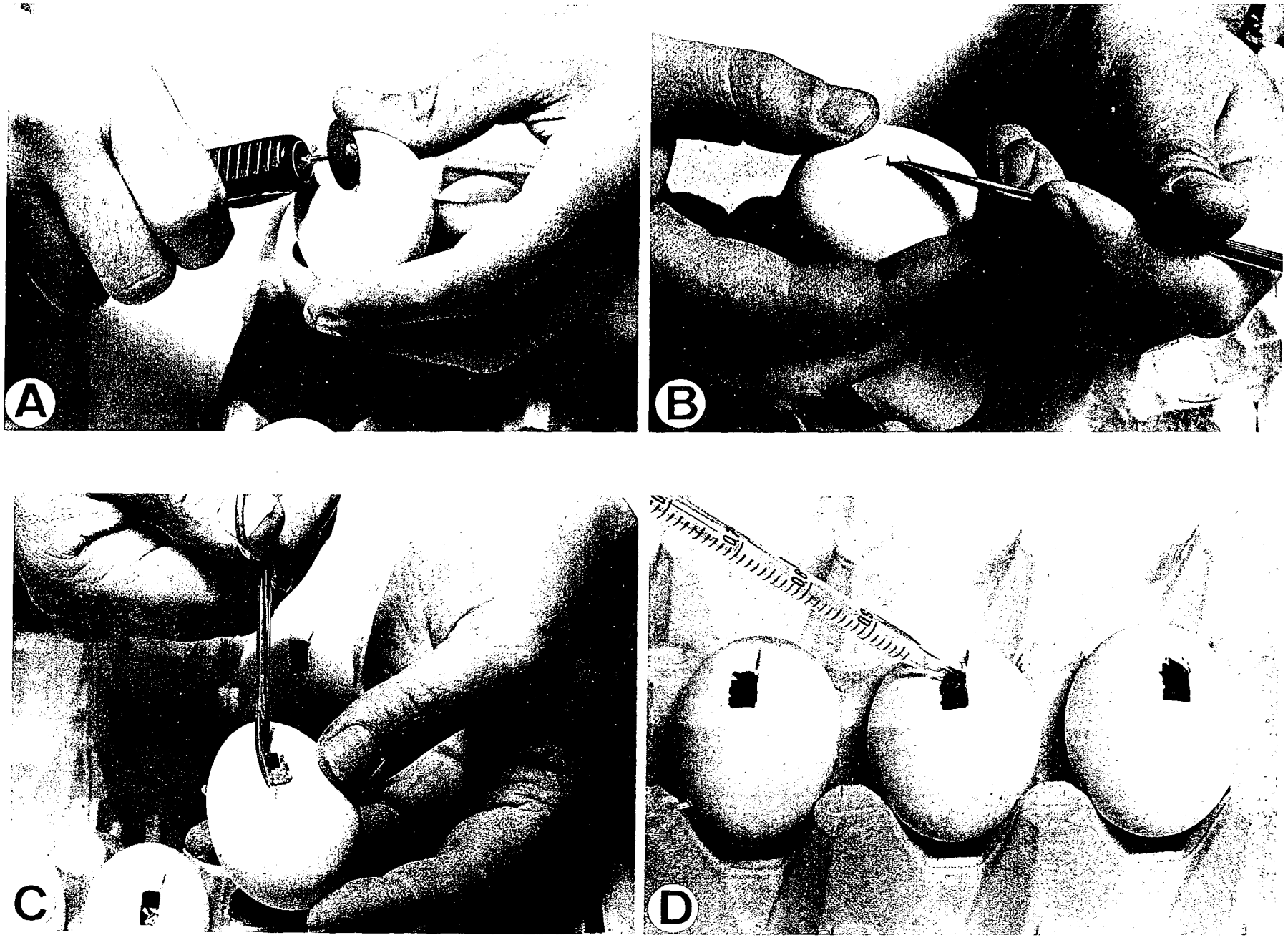

FIGURE 3. Inoculation of eggs. After marking the inoculation site with a pencil the shell is cut (A) and a 4 X 4 mm area of shell is removed (B). The shell membrane is cut away (C). Each egg is inoculated with 0.1 ml of virus dilution (D) and sealed with scotch tape.

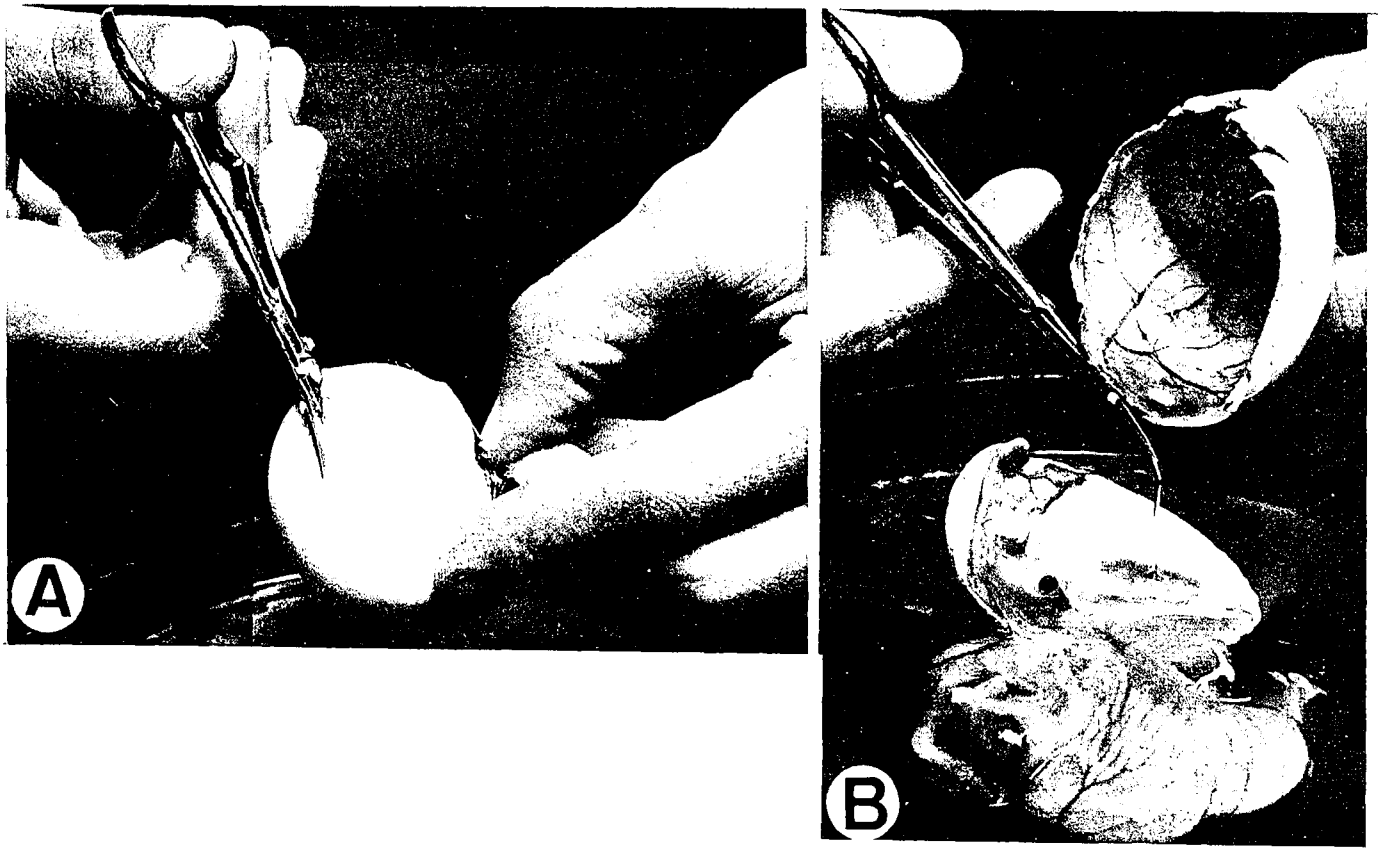

FIGURE 4. Collection of the membranes. A handle of scotch tape is fixed over the inoculation site and the shell is cut along the oval axis (A). The embryo is dropped out (B) and the CAM removed (C). Only the virus dilution giving 15-20 pocks per membrane are used to calculate potency (D).