WORLD HEALTH ORGANIZATION

The World Health Organization (WHO) is one of the specialized agencies in relationship with the United Nations. Through this organization, which came into being in 1948, the public health and medical professions of more than 120 countries exchange their knowledge and experience, and collaborate in an effort to achieve the highest possible level of health throughout the world. WHO is not concerned with problems that individual countries or territories can solve with their own resources. It deals, rather, with problems that can be satisfactorily solved only through the co-operation of all or several countries—for example, the eradication or control of malaria, schistosomiasis, smallpox, and other communicable diseases, as well as some cardiovascular diseases and cancer. Progress towards better health throughout the world also demands international co-operation in many other activities: for example, setting up international standards for biological substances, for pesticides and for pesticide spraying equipment; compiling an international pharmacopoeia; drawing up and administering the International Sanitary Regulations; revising the international lists of diseases and causes of death; assembling and disseminating epidemiological information; recommending non-proprietary names for drugs; and promoting the exchange of scientific knowledge. In many parts of the world there is need for improvement in maternal and child health, nutrition, nursing, mental health, dental health, social and occupational health, environmental health, public health administration, professional education and training, and health education of the public. Thus a large share of the Organization's resources is devoted to giving assistance and advice in these fields and to making available—often through publications—the latest information on these subjects. Since 1958 an extensive international programme of collaborative research and research co-ordination has added substantially to knowledge in many fields of medicine and public health. This programme is constantly developing and its many facets are reflected in WHO publications.

GUIDE TO THE LABORATORY DIAGNOSIS OF SMALLPOX

FOR

SMALLPOX ERADICATION PROGRAMMES

WORLD HEALTH ORGANIZATION
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PREFACE

This guide is intended to provide information on procedures that can be performed by laboratories participating in the surveillance activities of smallpox eradication programmes in endemic areas. It is hoped that it will assist the setting up of such diagnostic laboratories, an aim in accordance with the recommendations of the WHO Scientific Group on Smallpox Eradication.[1]

A first draft of the guide was prepared in April 1968 by Dr A. S. Benenson,[2] *Dr A. W. Downie,*[3] *and Dr J. Noble,*[4] *in consultation with the WHO Secretariat.*[5] *This draft was circulated to a number of reviewers* [6] *and later revised to take into account their comments and suggestions. Dr G. Meiklejohn* [7] *assisted the WHO Secretariat in the revision of the text.*

With the exception of Plate 3, the photographs used to illustrate the procedures described were taken by the Training Branch of the National Communicable Disease Centre, Atlanta, Ga., under the guidance of Dr A. S. Benenson and Dr J. Noble. Plate 3 is reproduced, with the permission of the publisher, from a book by Dr K. R. Dumbell (see page 41) and the photographs were taken by Dr P. N. Cardew. The World Health Organization is most grateful to all those who have participated in the preparation of this guide.

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1. INTRODUCTION

Smallpox can be diagnosed readily on clinical examination in 80–90% of cases. Thus, in endemic areas, laboratory confirmation of the diagnosis is not usually necessary. However, as the incidence of smallpox is reduced in the course of eradication programmes, the laboratory study of suspected cases becomes increasingly important. In fact, in smallpox-free areas and in areas where only a few cases are occurring, every suspected case should be subjected to laboratory investigation. Diagnostic tests should be performed as quickly and as reliably as possible.

Whenever specimens from suspected cases are submitted to the laboratory, such cases should be reported immediately to appropriate health authorities and necessary containment measures initiated. Laboratory tests may confirm the presumptive clinical diagnosis, or they may reveal that the illness is not caused by variola virus. In the latter case, containment measures can be suspended.

Although laboratory services should be readily available, the creation of large numbers of laboratories is not practicable, since the staff of a laboratory lose their skill if they are not regularly processing specimens. In smaller countries that are at present smallpox-free, a single national laboratory should be satisfactory, while in larger countries, such as India or Indonesia, which may become smallpox-free in different areas over a period of 2-3 years, as many as five or six laboratories may be required. When these countries become smallpox-free, the number might be reduced to one, or perhaps, because of geographic considerations, to two or three laboratories per country.

As part of the smallpox eradication programme, WHO is assisting in the establishment of a network of laboratory services, which consists of international and regional reference centres and national diagnostic laboratories.

Laboratories that are specially equipped and have skilled personnel may elect to perform a variety of more complex diagnostic procedures, such as electron microscopy, tissue culture isolation of virus, serological tests, etc. However, such laboratories are exceptional in endemic countries. The present guide deals, therefore, only with basic techniques, specifically with the staining and examination of smears, the precipitation-in-gel test, and the culture of specimens on the chorioallantoic membrane (CAM) of chick embryos. Necessarily, laboratories carrying out these procedures must have a regular supply of fertilized eggs.

The basic tests described above are relatively easy, provide reliable results quickly and do not require elaborate equipment. A presumptive diagnosis may be provided by detection of elementary bodies in a stained smear, which can be prepared and examined in one hour, or by a precipitation-in-gel test, which can be interpreted within 2 to 24 hours. A definitive diagnosis requires culture of the virus on the CAM of chick embryos and a final diagnosis is made only when

variola virus has been recovered by this method. This will require at least two to three days after the eggs have been inoculated.

2. COLLECTION OF SPECIMENS

2.1 General considerations

The efficacy of laboratory diagnosis is governed partly by the quality and quantity of material submitted for examination. The laboratory functions most effectively when a member of the laboratory staff actually joins the field team investigating outbreaks and collecting specimens. When suitable material is submitted, a presumptive report can usually be made within hours after specimens are received and a definitive report in three to seven days.

The techniques used for the collection and shipment of specimens for the diagnosis of smallpox must be such that there is no possibility of spreading infection through the community as a result of breakage of specimen containers in shipment, etc. Those who collect the specimens must, of course, have been properly vaccinated within the preceding twelve months, and must wash their hands thoroughly with soap and water or weak disinfectant after contact with a smallpox suspect.

2.2 Collection kit

Specially designed kits, as provided by WHO, will facilitate the collection of specimens. These should contain:

1. A sterile Hagedorn needle (or vaccinostyle), used for scraping maculopapular lesions, for opening and scraping the base of vesicles and pustules, or for removing scabs.
2. Clean glass slides for smears.
3. Sterile capillary tubes or a sterile Pasteur pipette with a rubber bulb for collection of vesicular and pustular fluid.
4. A labelled screw-capped sterile container for submission of crusts or vesicular or pustular fluids.
5. A double container, made of metal or wood, for forwarding the material to the laboratory.
6. Instructions for specimen collection.
7. A form for information on the patient, to accompany specimens (Annex 1).

2.3 Method of collection

2.3.1 *Maculopapular stage*

Maculopapular lesions are scraped with the Hagedorn needle until the surface becomes moist; gross admixture of blood is undesirable. The material on the needle is rubbed on the slide with a circular motion covering an area about

1 cm in diameter. At least six lesions should be sampled; material should be placed on six slides, several smears (from different lesions if possible) being made on each slide. The smears are allowed to dry in the air and should not be heated or exposed to a fixative or a disinfectant. After collection of the specimens, the slides should be separated from each other by means of string, rubber bands, or small pieces of cardboard.

Normally two slides will be used for staining of the smears. The remainder will be useful for preparation of suspensions for precipitation-in-gel tests and inoculation of eggs.

2.3.2 *Vesicular and pustular stages*

Capillary tubes or a Pasteur pipette are used to aspirate the contents of six to twelve vesicles or pustules. After this procedure has been completed, the fluid may be drawn up from the tip so that an air space remains and the tip is sealed in the flame of a match. Care must be taken that the fluid that has been collected is not heated during this procedure. The capillary tube or pipette is replaced in the sterile tube which is then tightly closed. In addition, material should also be obtained from the base of the lesions by scraping with the Hagedorn needle and smeared on glass slides (as in 2.3.1).

2.3.3 *Crusting stage*

Crusts should be pried loose with the Hagedorn needle. At least twelve or more crusts should be collected in the container provided. In late stages of the disease there may be few crusts remaining on the patient, but search of the patient's bed may yield sufficient crusts for study.

2.4 Labelling and packing

When smallpox specimens are collected, each tube or slide should be labelled with the patient's name and the date of collection. A form (Annex 1) should accompany the specimens giving the following minimum information: name, age, sex, and address of the patient; date of collection of specimen, date of onset of illness, date of onset of rash; history of vaccination, vaccination scar, and exposure to smallpox; history of previous chickenpox (varicella) or exposure to chickenpox; the name of the person who collected the specimens and the name of the person to whom the report should be sent. Annex 1 should be included with the specimens inside the container.

Specimens from only one patient should be enclosed in the container. There should be enough padding in the container to prevent any free movement of the tubes and to absorb any fluid if breakage should occur in transit. The outside of the container should be wiped with disinfectant. Refrigeration is not essential during transportation, but should be used if available.

2.5 Shipment

The most rapid form of shipment available should be used. Within the country, this may be done by messenger, bus, boat, train or plane, depending on

the local situation. All containers should be clearly addressed to the smallpox laboratory and the pakcage should be clearly marked on the outside as containing highly infectious and perishable material, to be opened only in a smallpox laboratory.

When shipment is across national borders, the requirements of the international postal convention must be followed.[1] Specimen kits provided by WHO, if properly used, satisfy these requirements.

3. THE SMALLPOX LABORATORY

The work of a smallpox diagnostic laboratory must be so conducted that spread of virus will not occur. It is therefore necessary to select the best qualified personnel and to ensure proper management of the laboratory.

3.1 Vaccination requirements

Nobody should be admitted to the laboratory who has not been vaccinated within the preceding twelve months. This applies not only to the laboratory staff, but also to visitors, janitors, maintenance staff, window cleaners, and others who may have business there. It is advisable also that families of the laboratory staff be revaccinated annually.

3.2 The laboratory

The laboratory should have a washable floor and walls that can be mopped down with disinfectant as required. A single room of a general microbiology laboratory will suffice; it should be well lit. The diagram in Annex 2 presents a convenient arrangement and the minimum equipment necessary. The door to the room should be clearly marked with a sign stating " SMALLPOX—KEEP OUT ". If feasible, as an additional safety measure, the door should open into a vestibule or anteroom, separated by a second door from the working laboratory.

When the smallpox laboratory is on the same site as a laboratory producing smallpox vaccine, the diagnostic laboratory must be housed in a completely separate building.

3.3 Disinfection

Variola virus is easily destroyed by boiling. It is also destroyed by certain disinfectants, including coal tar derivatives (phenolic compounds) such as Lysol (2.5%), quaternary ammonium compounds (1% or greater), oxidants such as chloramine solution (5%), and formalin solution (2.5–5%). For disinfection, all articles should be left in disinfectant overnight since killing by chemicals requires a period of contact. Contamination of the room is reduced by covering the work surface with towelling kept damp with disinfectant (Fig. 1).

[1] These are to be found in Article 120 of the requirements established by the International Postal Convention (Vienna, 1964). See also *Wld Hlth Org. techn. Rep. Ser.*, 1968, No. 393, p. 49.

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Laboratory personnel should wear gowns that fasten at the back. These should be disinfected in the laboratory by boiling at the end of each day's work. The soles of shoes should be disinfected by walking on a cloth mat soaked with disinfectant inside the door of the laboratory; it is preferable that shoes or sandals be available for use in the laboratory only.

All articles, except records and reports, should be sterilized by disinfection or boiling before they are taken out of the laboratory. Records and reports can be disinfected by exposure to sunlight for at least two hours in a place where they are protected against theft or dispersion by wind. Both surfaces should be exposed.

3.4 Handling of packages received in the laboratory

Packages containing specimens for examination must be opened only within the smallpox laboratory. The wrapping should be removed and dropped directly into a bucket of disinfectant. The outside of the container should be wiped with disinfectant and the contents then removed over disinfectant-soaked towelling on the work bench. The packing should be dropped into the disinfectant bucket (Fig. 2).

2

The receipt of the package and the nature of the contents should be recorded in the laboratory records (Annexes 1 and 3).

4. MICROSCOPIC EXAMINATION OF STAINED SMEARS: GISPEN'S METHOD

The elementary bodies of vaccinia and variola virus can be stained on a slide by relatively simple techniques. If positive, a *presumptive* diagnosis is available within an hour. With experience, this technique gives reasonably reliable results in early cases. Stained smears are useful in the maculopapular stage when there is very rarely sufficient antigen for a positive precipitation-in-gel test. Consequently, it would otherwise be necessary to wait for three or more days until inoculated eggs could be harvested in order to establish the diagnosis. *A negative smear does not exclude smallpox.*

Gispen's method for staining smears has the advantages that it can be used on relatively thick smears as well as on thin ones and on specimens taken up to the pustular stage of the disease. It is a modification of Morosow's silver impregnation technique.

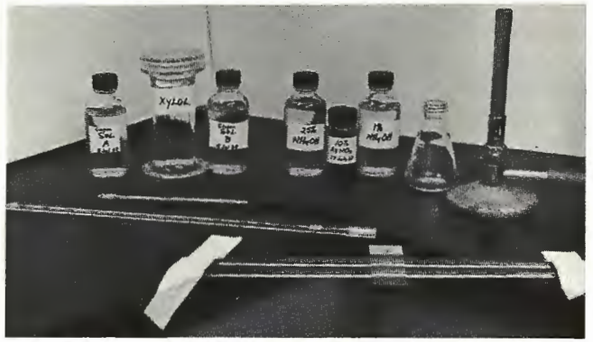
4.1 Reagents and equipment (Fig. 3)

Reagents

Solution A (fixative) (glacial acetic acid, 1 ml; formalin, 2 ml; distilled water, 100 ml)
Xylol
Solution B (mordant) (carbolic acid, 1 ml; tannin 5 g; distilled water, 100 ml)
Ammonium hydroxide, 25% stock solution; 1% solution in distilled (or demineralized) water, freshly prepared daily. (The stock solution must be tightly stoppered to ensure full potency; the 1% solution should have a strong smell of ammonia)
Silver nitrate, 10% solution in distilled (or demineralized) water
Immersion oil

Equipment

Conical flask, 100 ml
Bacteriological pipette, 1 ml
Graduated cylinder, 50 ml
Staining jar with cover
Microscope with oil-immersion lens
Bacteriological loop
Alcohol lamp or other heat source
Glass rods for supporting slides



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4.2 Preparation of ammoniacal silver solution

1. Clean and rinse a 100-ml conical flask with distilled water.
2. Introduce a loopful of 25% ammonia solution into the drop of water remaining in the flask (Fig. 4).

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3. Add with a pipette 0.5 ml of 10% silver nitrate (Fig. 5).

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4. Add 20 ml of distilled water (Fig. 6).

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5. Add 1% ammonia solution drop by drop (about 1.4–1.8 ml) until the ammoniacal silver precipitate redissolves (Fig. 7).

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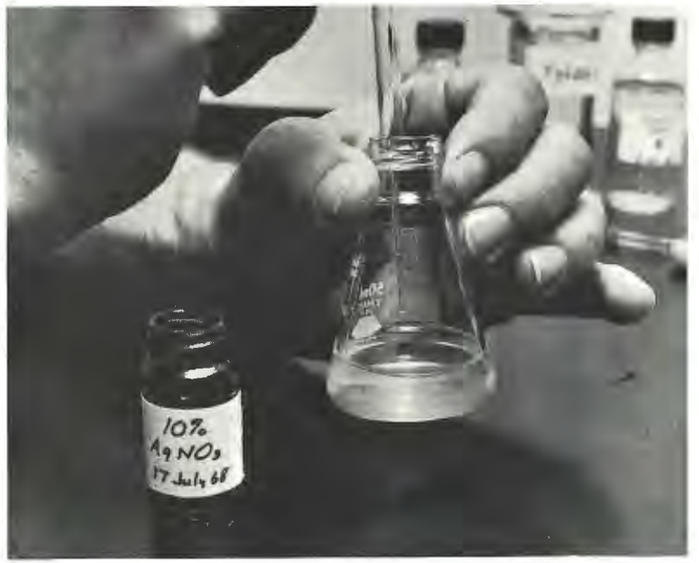
6. Add one drop of 10% silver nitrate solution producing a faint bluish opalescence (Fig. 8). This ammoniacal silver solution must be freshly made before use.



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4.3 Staining procedure

1. Treat an air-dried thin smear (see section 2.3.1) with solution A for one minute (Fig. 9).

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2. Rinse in tap water and dry in air.
3. Immerse slide in xylol in a staining jar for five minutes and dry in air (Fig. 10).

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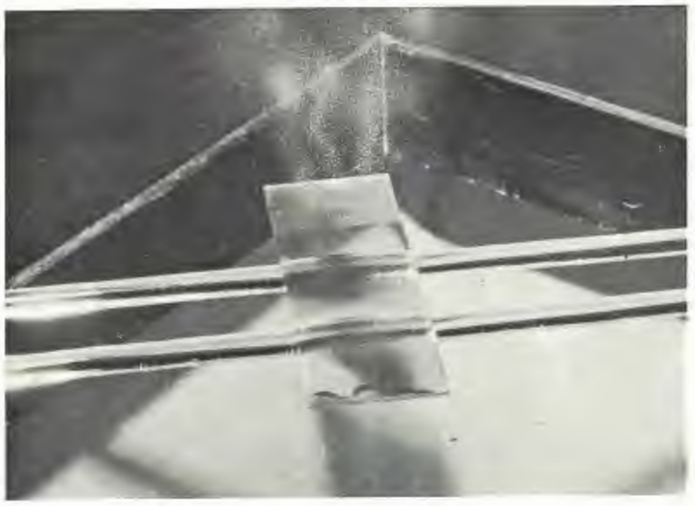
4. Support the slide in a suitable manner and treat with solution B. Heat until steam rises and continue heating for one minute (Fig. 11).



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5. Rinse thoroughly with tap water (Fig. 12) and wash with distilled (or demineralized) water (Fig. 13).

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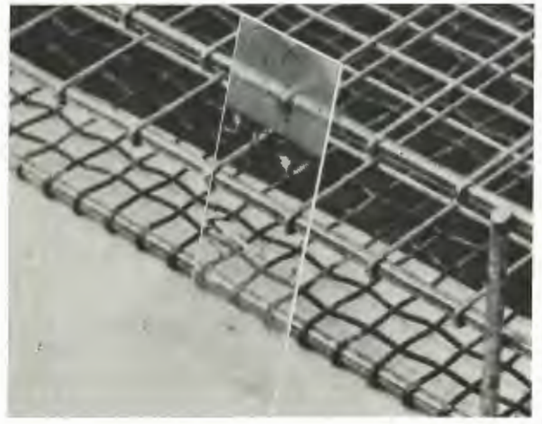
6. Cover the slide with the freshly prepared ammoniacal silver solution and heat until steam rises and continue heating for two minutes.
7. Rinse in tap water, support in an inclined position and dry in air (Fig. 14).



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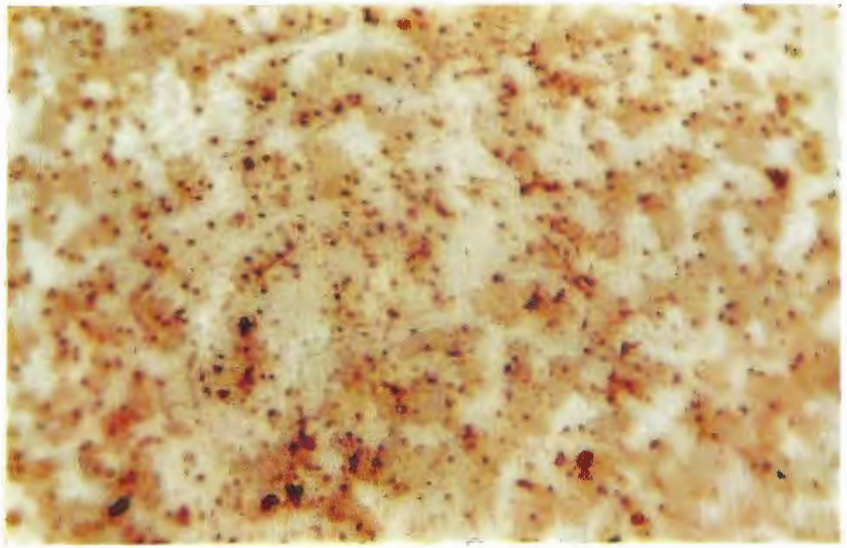
8. Examine under oil-immersion lens.

4.4 Reading

Virus particles are uniform in size, round, and about one-third the diameter of a staphylococcus; they are stained brownish-black against a pale-yellow background (Plate 1). The virus particles are confined to the area of the smear, and are not obviously related to cellular structures. A positive reading is presumptive only, and does not differentiate vaccinia from variola virus.

PLATE 1. POSITIVE SMEAR STAINED BY GISPEN'S METHOD



The success of the technique depends on the care with which the original smears were prepared. It is desirable to select for staining those slides with thin smears. The chances of success are greater when smears of several lesions are placed on one slide.

4.5 Interpretation of stained smears

The results of the staining technique are, at best, presumptive. A positive test only suggests the presence of a member of the vaccinia-variola group of pox viruses. Smears from varicella or herpes simplex do not show the numerous, deeply stained elementary bodies characteristic of the vaccinia-variola viruses. A negative result does not exclude the diagnosis of smallpox; material that is negative on direct examination may often yield virus on the chorioallantoic membrane (CAM). *Thus, whether the smear is positive or negative, the precipitation-in-gel test and culturing on the CAM are always indicated.* Positive smear

results are reported to the individual who submitted the specimen and to the appropriate smallpox eradication officer as " presumptively positive ". A negative smear is not reported until the results of other tests become available.

5. PRECIPITATION-IN-GEL METHOD

Pox virus antigens will react with specific antibody to form a line of precipitation in agar gel. This precipitation reaction provides a good test for the diagnosis of smallpox. Specimens from vesicular, pustular and crusting stages of the disease are suitable for testing if sufficient material is provided, and a diagnostic result may be obtained in two to twenty-four hours.

5.1 Reagents and equipment

Reagents

Antivaccinia serum, Positive antigen, Normal serum (Prepared according to the instructions and stored at 4°C)
Phosphate-buffered saline (PBS) (one tablet added to 100 ml of distilled or demineralized water)
Antibiotic tablets (one tablet added to 100 ml of PBS provides 500 IU of penicillin and 500 μg of streptomycin per ml)
Purified agar in capsules

Equipment

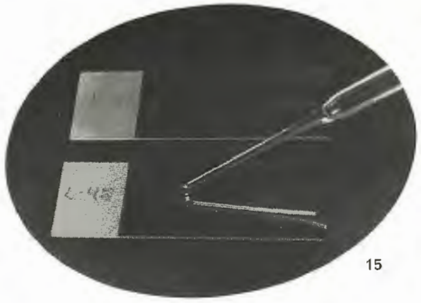
Tissue grinder
Template
Agar-cutting tube
Slides
Oblong pieces of slides
Bifurcated needles or suction tube
Petri dishes
Pasteur pipettes
Rubber bulbs
Rubber bands
Graduated cylinder, 100 ml
Hand lens
Black tile or paper
Beaker, 25 ml

5.2 Preparation of specimen

5.2.1 *Smears*

Smears on 3 or 4 glass slides may provide enough material to give a positive result if the material on the slides is very thick and is suspended in only 0.2 ml of PBS containing antibiotics. The same suspension is used for virus isolation (see section 6.5.1). There is often insufficient antigen present for a positive precipitation test; egg inoculation is required for virus isolation (see section 6).

1. Place 0.2 ml of PBS on the smear. Scrape the material into the fluid with the tip of a pipette (Fig. 15) or the edge of a clean slide.
2. Transfer the fluid with a pipette to each of the other 3 or 4 slides in turn and repeat the scraping, thus making a concentrated suspension. This suspension is also used for virus isolation (see section 6.5.1).
3. Alternatively, scrape the material with the tip of a pipette or the edge of a clean slide into a beaker (Fig. 16). Repeat this with each of the other slides. Care must be taken to avoid loss of the suspension due to its adherence to the large surface of the beaker.



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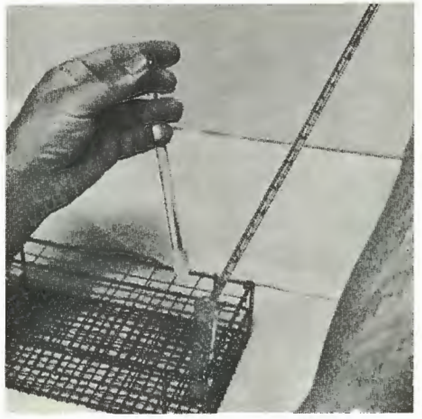
5.2.2 *Vesicular or pustular fluid*

Samples of vesicular or pustular fluid should be used undiluted if possible. If only a very small amount of material is available, it is diluted in approximately 0.1 ml of PBS to provide enough material to fill the well of the agar slide. The remainder is retained for culture on the CAM (see section 6.5.2).

5.2.3 *Crusts*

1. As many crusts as possible (up to 12) are placed in a tissue grinder, together with two capillary tubes (ground glass will assist the grinding). The thicker the suspension the better the results.
2. Add PBS with pipette (Fig. 17), the volume depending on the number of crusts: for 2–5 crusts, 0.2 ml; for 6–10 crusts, 0.4 ml.



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3. Insert the grinding rod and grind up the crusts (Fig. 18). The material after grinding should be homogeneous and liquid enough to be drawn up in a Pasteur pipette (Fig. 19). If too thick, a small volume of PBS may be added.

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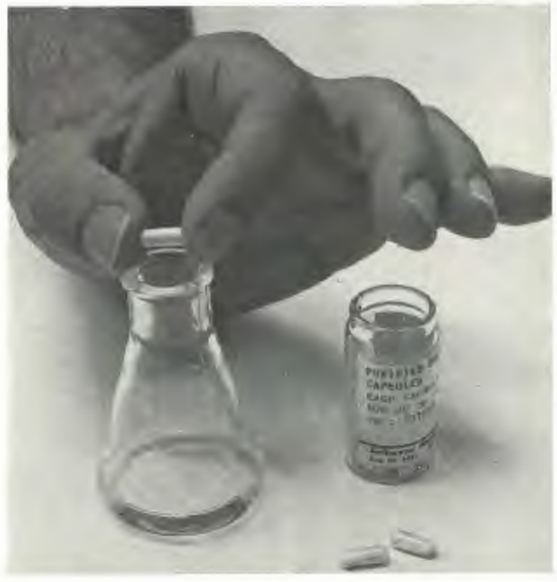
5.3 Preparation of agar slide

1. A 1% solution of purified agar is prepared by dropping one capsule into 10 ml of distilled or demineralized water (Fig. 20), which is then brought to a boil over a flame or on a hot plate and kept above 50°C until needed.



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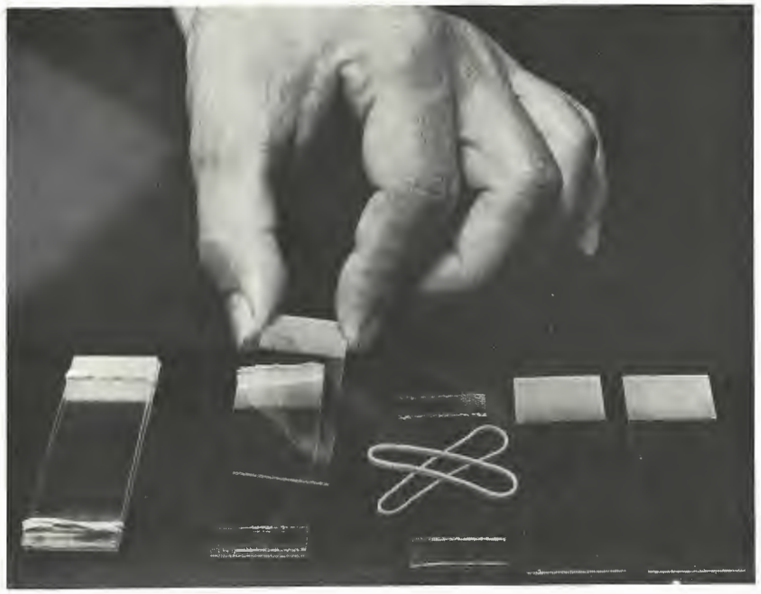
2. Two glass slides are kept apart by oblong pieces of glass slide about 1 mm thick, the inner edges of which should be about 12 mm from ends of the slides. The latter are then held together with rubber bands (Fig. 21).



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3. The melted agar is pipetted between the slides (Fig. 22, 23).

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4. When the agar has solidified (10–15 minutes at room temperature), one of the glass slides is slid off with care so as not to dislodge the agar layer from the other slide (Fig. 24, 25).

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5. The plastic template used for the test has two sets of four holes and a central group of seven holes; one set of four holes is used to test each specimen. The central group is used only for specialized procedures. The holes are numbered 1, 2, 3 and 4 as shown in Fig. 26.

26

6. The template is placed above the agar layer (Fig. 27) and the hollow metal agar-cutting tube placed in each of the four holes in turn and pushed through the underlying agar (Fig. 28). To facilitate holding the template and agar layer in position, rubber bands may be used.

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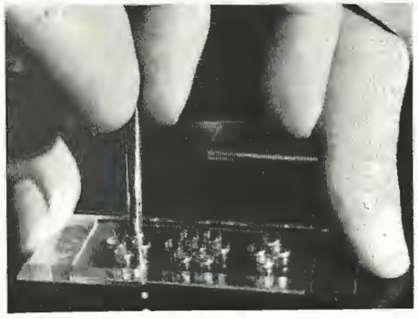

7. The template is removed and the agar discs are aspirated with a Pasteur pipette by mouth suction (Fig. 29) or lifted out with the point of a bifurcated needle.

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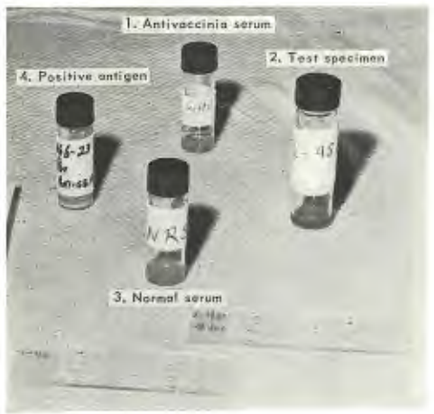
8. Slides should be kept at room temperature in a closed Petri dish or plastic box containing moist filter paper or cotton wool to prevent drying of the agar and should be used within a few hours after preparation.

5.4 Procedure

For each test specimen, one agar slide is normally used. When sufficient material is available, it is advisable to do the test in duplicate, using both sets of 4 wells in the agar slide. The reagents are added to the wells using Pasteur pipettes. A separate pipette is used for each reagent. Drawing the tip of the pipette to a fine point provides optimum control. This is important because, although the wells should be filled to near the top, care must be taken that fluid does not flow over the surface of the agar.



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The same arrangement of reagents is always used, with the antivaccinia serum in well 1, the test specimen in well 2, the normal serum in well 3, and the positive antigen in well 4 (Fig. 30).

The wells should be filled in the following sequence:

1. The normal serum is placed in well 3.
2. The antivaccinia serum is placed in well 1.
3. The positive antigen is placed in well 4 (Fig. 31).

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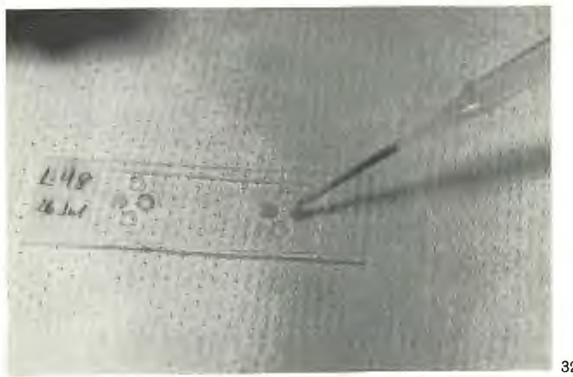
4. The vesicular fluid, pustular fluid, crust suspension or smear suspension is placed in well 2 (Fig. 32).



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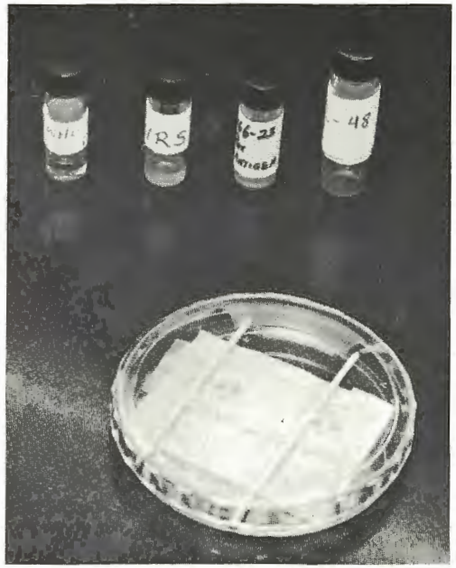
When there is more than one specimen to be tested it is convenient to add normal serum, antivaccinia serum and positive antigen to the appropriate wells of all the slides at one time.

5. The slide is placed in a Petri dish containing water-soaked filter paper, towelling or newspaper. The dish is covered and kept at room temperature (Fig. 33). If the humidity is very low, the paper should be remoistened, or the lid of the Petri dish should be sealed to the bottom dish with petroleum jelly.



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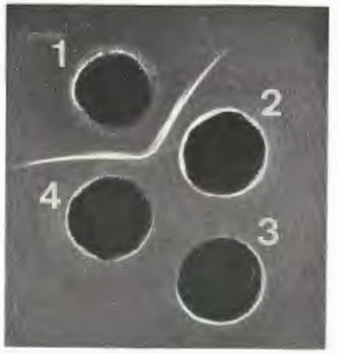
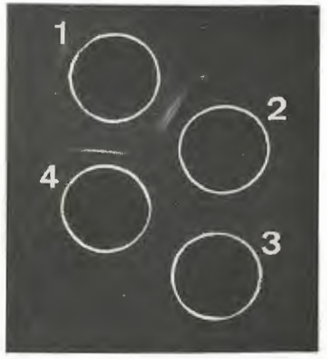
5.5 Reading and interpretation

The slides should be read with a bright light against a black background, using a hand lens to bring out the presence of faint lines and to increase the accuracy of reading. The egg candler provides an excellent light source. A line should always form in the agar between well 1 (antivaccinia serum) and well 4 (positive antigen).

If the vesicular or pustular fluid or crust suspension contains smallpox or vaccinia antigen, lines of precipitate will appear (usually in 2–3 hours) in the agar between well 1 and well 2 (test specimen). When the line between wells 1 and 2 joins that between wells 1 and 4 (usually after 4 or more hours) the test is considered positive (Fig. 34). Lines that cross or do not join may represent antigens other than variola or vaccinia. When antigen is present in the specimen only in small amount, the line between wells 1 and 2 may not extend far enough to join the line between wells 1 and 4 or may not become continuous with it (Fig. 35). In these cases, the result can only be considered presumptively positive. If a line also appears between well 2 and well 3 (normal serum), the result must be considered doubtful and further tests must be done to establish the nature of the infection.



Positive: line between wells 1 and 2 joins that between wells 1 and 4. 34



Presumptively positive: line between wells 1 and 2 does not join that between wells 1 and 4. 35

Negative. 36

If the only precipitation line is between well 1 and well 4 the result is negative (Fig. 36), but the test is not considered negative until the preparation has been allowed to stand for twenty-four hours. In most cases of smallpox the test gives a positive result, i.e., the lines join, after the lesions have become vesicular. However, the test may be negative if inadequate material is provided. The diagnosis of variola cannot be excluded until culture on CAM has also given negative results.

Some tests may present a turbid halo around the wells, making interpretation of the test difficult. In such cases, the test must be repeated. Rarely, a line of precipitate will appear between the test specimen (well 2) and the positive control antigen (well 4), joining a line between wells 1 and 4. This indicates that the specimen contained antibody, either from a patient in a late stage of smallpox or from a patient with generalized vaccinia.

6. ISOLATION OF VIRUS ON CHORIOALLANTOIC MEMBRANE OF CHICK EMBRYO

Inoculation of the CAM of chick embryo is a sensitive and reliable method for the laboratory confirmation of the diagnosis of smallpox. Fertile hens' eggs incubated at 37°C to 39°C for 11–13 days are required. In an emergency, 10-day or 14-day embryos may be used.

6.1 Materials and equipment

Materials

Eggs
Petroleum jelly
PBS tablets (see section 5.1)
Antibiotic tablets (see section 5.1)

Equipment

Incubator, bacteriological
Candler
Egg punch
Pen nibs and holders or blood lancet
Rubber bulb
Disposable tuberculin syringe and needle
Scissors
Forceps
Screw-capped vials
Petri dishes

6.2 Supply of eggs

Eggs should be obtained from an egg producer or hatchery that has a healthy flock containing roosters as well as hens. The eggs must be gathered from the hen house at least every day. Ideally, more than 80% of the eggs procured should be fertile. The following factors should be considered if the fertility rate is low. Fertility is greatest if the chickens are less than two years old, are well nourished and free from disease. Exposure of the eggs to sunlight or heat reduces fertility rates (fertility rates tend to be lower during the summer months). Washing the eggs removes a protective substance from the shell and increases embryo mortality; eggs may be brushed to clean the shell, but not washed.

Enough eggs should be ordered to provide a minimum of twelve fertile eggs for use during each week. The number of eggs ordered may be increased, depending upon the number of specimens that are anticipated. Four to six eggs should be used per specimen.

6.3 Preliminary incubation of eggs

If the eggs have to be kept for a day or two before incubation they should be held at room temperature in a cool place to prevent premature development.

The eggs should be incubated at 37° to 39°C. The incubator should preferably be outside the smallpox laboratory so that if the eggs are not required for smallpox diagnosis they may be made available for other purposes. A pan of water to maintain a high humidity in the incubator is desirable. If the eggs are

PLATE 2. SUCCESSFUL AND UNSUCCESSFUL DROPPING OF THE CAM

A. Normal egg

B. CAM dropped, creating artificial air sac

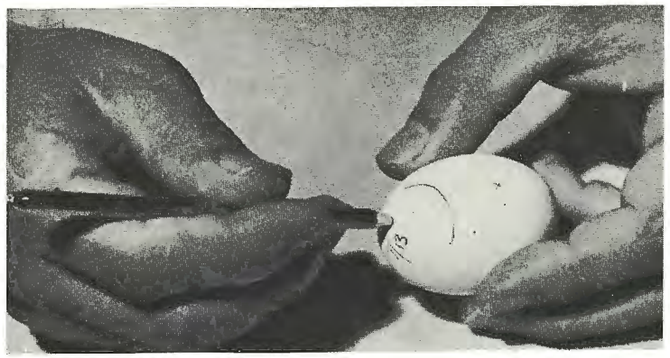
C. Failure of CAM to drop owing to puncture of CAM with formation of air space under CAM; not suitable for use.

set in the incubator, either on Thursday and Sunday or on Wednesday and Saturday, a supply of embryos 10–13 days old can be available on every day of the week. The eggs should be marked with the date of setting. Eggs should be turned once a day.

Eggs not used for smallpox diagnosis may serve two purposes: (1) they may be inoculated with vaccinia virus (from smallpox vaccine) so that the staff retains familiarity with the technique and the appearance of specific virus-induced lesions; (2) the membranes may be " dropped " (see section 6.4, paragraphs 8–10) and inoculated with PBS or broth to provide experience with negative membranes and non-specific opacities (see section 6.10).

6.4 Preparing eggs for inoculation

1. The inoculated eggs are candled in a dark area to check viability. It should be possible to see movements of the embryos and clearly defined red blood vessels in the CAM (Plate 2A).
2. The edge of the air space is marked on the shell with a pencil and a mark is made over the centre of the CAM in an area free of large blood vessels.
3. Without sterilizing the surface, the shell over the air space is punctured with the pointed end of the hand punch using a rotary motion (Fig. 37). The shell membrane must also be punctured.
4. An inoculation hole is made on the marked spot over the CAM, using a rotary motion (Fig. 38). This time, *the shell membrane must remain intact.*
5. The shell dust is blown away.
6. It is helpful to spread a little petroleum jelly over and around the hole.
7. A drop of sterile PBS at room temperature is placed over the inoculation hole (Fig. 39).
8. The tip of a bent blood lancet or curved pen nib is inserted through the drop of saline at an angle of about 20° to the horizontal (Fig. 40).
9. The tip is passed directly under the shell as far as it will go.
10. The handle is then raised towards the vertical (Fig. 41); this tears the shell membrane and the drop of fluid is drawn inside as the CAM falls away, creating a new air space (Plate 2B).
11. It is advisable to place the eggs in the incubator for thirty minutes to permit maximum enlargement of the new air space.
12. The eggs are then candled to determine that the CAM has dropped.
13. If the CAM of any eggs has not dropped, slight suction is applied with a rubber bulb over the hole at the blunt end of the egg (Fig. 42).
    If the vessels adhere to the shell over the new air space (Plate 2C), the CAM has been punctured and the egg should be discarded.
14. The eggs must be inoculated within the next 2 hours.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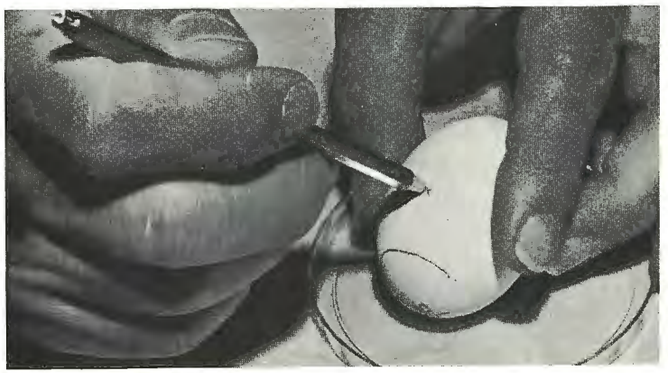
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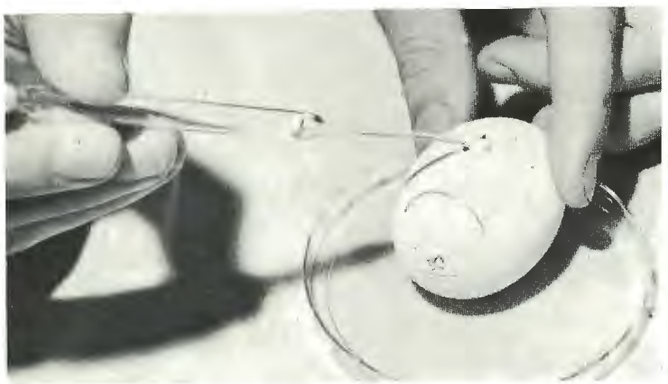
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6.5 Preparation of inoculum

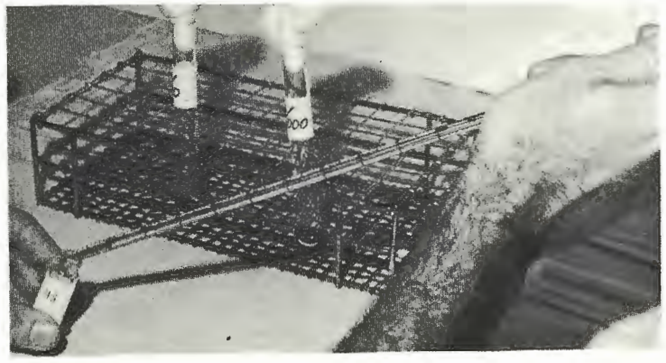
When more than one type of specimen has been submitted, the CAM should be inoculated with vesicular fluid, pustular fluid or crust suspension, rather than with a suspension prepared from smears on slides. Original specimens should be conserved and not discarded.

6.5.1. *Smears on slides*

A suspension of smears in phosphate-buffered saline containing 500 IU of penicillin and 500 μg of streptomycin per ml as described for the precipitation-in-gel test, is used for the inoculation of the CAM for virus isolation (see section 5.2.1). The remaining suspension may have to be diluted by addition of PBS to provide the volume of fluid necessary for egg inoculation.

6.5.2 *Vesicular or pustular fluid*

1. 0.1 ml of the fluid is taken up in a pipette (Fig. 43) and added to 0.9 ml of PBS with antibiotics to make a 1 : 10 dilution (Fig. 44).



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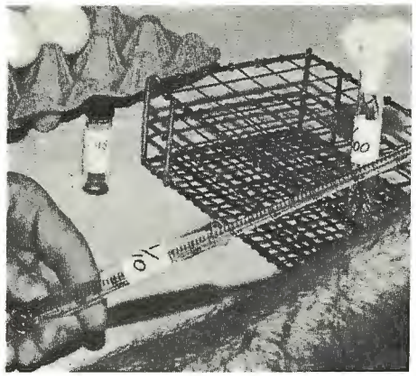
2. 0.1 ml of the 1:10 dilution is taken up in a pipette (Fig. 45) and added to 9.9 ml of PBS with antibiotics to make a 1:1000 dilution (Fig. 46).



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If only a very small amount of material is available, it is diluted in approximately 0.1 ml of PBS for the precipitation-in-gel test (see section 5.2.2) and the remainder is taken up in 0.5 ml of PBS with antibiotics. This is considered to be a 1:10 dilution. 0.1 ml of this diluted fluid is further diluted in 9.9 ml of PBS with antibiotics, giving an approximately 1 : 1000 dilution.

6.5.3 *Crusts*

1. The crust suspension remaining from the precipitation-in-gel test is suspended in 0.5 ml of PBS with antibiotics (approximately 1 : 10 dilution).
2. 0.1 ml of this suspension is added to 9.9 ml of PBS with antibiotics (approximately 1 : 1000 dilution).
3. The suspensions are permitted to settle for 5–10 minutes and the clear supernate is used.

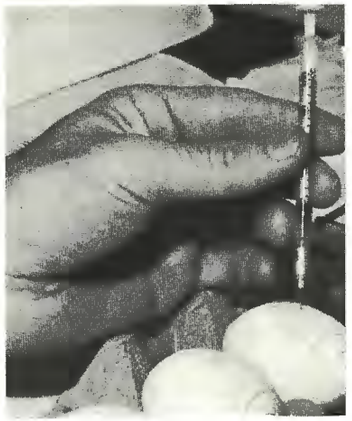
47

6.6 Inoculation of eggs

A different disposable syringe with firmly fixed needle should be used for each specimen. Of two dilutions from one specimen:

1. The more dilute suspension (1:1000) is inoculated first into 2 or 3 eggs (Fig. 47).

2. The tip of the needle is introduced through the inoculation hole approximately 2–3 mm beyond the shell membrane (Fig. 48).



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3. 0.1 ml is dropped on the CAM.
4. The same syringe is used to inoculate 2 or 3 more eggs with the 1 : 10 dilution (Fig. 49).

49

5. The eggs are then tilted in each direction to ensure that the fluid is spread over the entire surface of the CAM (Fig. 50).

50

6. Petroleum jelly or a mixture of petroleum jelly and paraffin is used to seal the inoculation hole (Fig. 51). Scotch tape and quick drying cement can also be used.

51

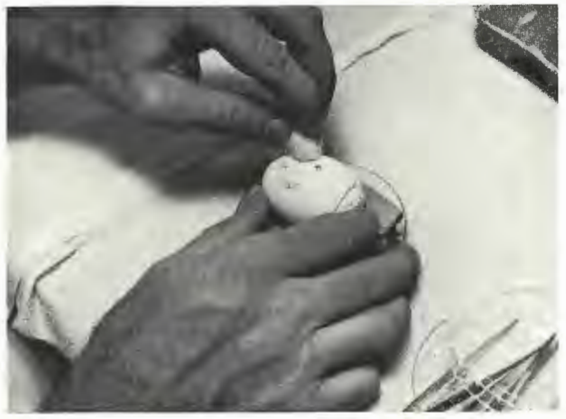
7. All syringes and other equipment in the working area should be immediately dropped into boiling water or disinfectant, even if they have not been used (Fig. 52).

52

8. The eggs are placed in an incubator at 35°C to 37°C with the inoculation hole uppermost. The incubator temperature *must remain below 37°C*, since variola virus may not grow above this temperature.
9. After 48 hours, one egg inoculated with each dilution is examined; the other eggs are examined after 72 hours.

6.7 Examination of the chorioallantoic membrane

1. The work area should be covered with towelling soaked in disinfectant. A roll of paper towelling is soaked in disinfectant and placed in a Petri dish to support the egg. A set of sterile instruments including a coarse and a fine pair of forceps and scissors should be available. Separate sterile instruments and glassware should be used for specimens from each patient.
2. The petroleum jelly or other material is wiped from the shell with a swab soaked in alcohol (Fig. 53).



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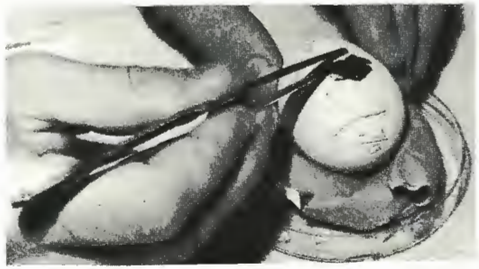
3. With the sterile coarse forceps the shell and the shell membrane are broken away over the CAM (Fig. 54) and placed directly in the disinfectant.



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4. The CAM is then picked up with the fine sterile forceps and gently pulled up and away from the shell (Fig. 55).

5. With the scissors the membrane is cut free, commencing cutting a few millimetres beyond the point of attachment to the shell (Fig. 56).

56

6. The membrane is rinsed in a beaker or Petri dish containing PBS or distilled or demineralized water (Fig. 57).

57

7. The membrane is transferred to a Petri dish containing 2 ml of PBS and is spread out (Fig. 58). The Petri dish is placed on a well-lit black surface. If the space between the dish and the black surface is filled with water, lesions on the membrane are more easily visible.

58

8. After the membranes have been removed, the eggs are discarded and dropped into disinfectant, where they remain in the laboratory until the next day to ensure that the virus has been killed (Fig. 59).

59

6.8 Storage of chorioallantoic membrane and specimen

Membranes should be stored in the refrigerator or in the freezing compartment in a screw-capped vial (Fig. 60). All specimen containers should be clearly labelled with the laboratory number and the date of isolation. All specimens and the retained membranes should be kept in the laboratory for at least 3 months or sent to another laboratory according to instructions.

60

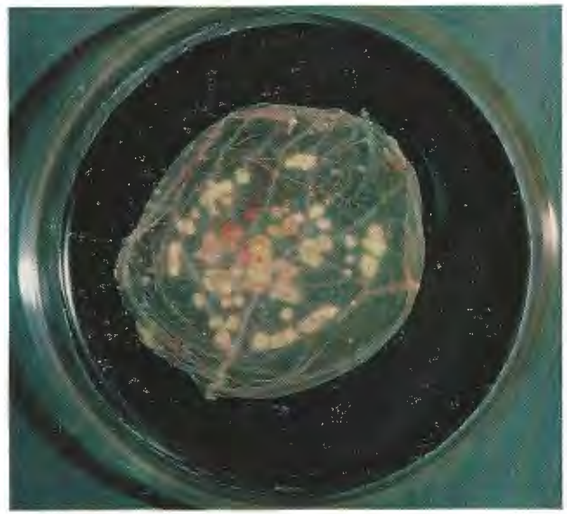
6.9 Interpretation

With high virus concentration, confluent lesions may produce a general thickening of the CAM. For interpretation, a dilution that gives isolated lesions is required: the 1 : 1000 dilution will usually serve.

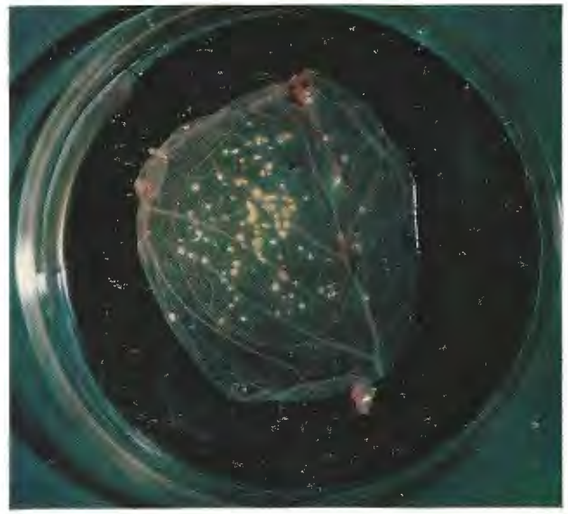
The lesions produced by variola virus, even after 48 hours, are typical in appearance; they are round, grayish-white, less than 1 mm in diameter, smooth on the surface, and raised above the CAM. After 3 days' incubation, the pocks are a little larger, being about 1 mm in diameter (Plate 3A). Pocks formed by vaccinia virus are *much larger*, being approximately 2 mm in diameter at 48 hours and 3–4 mm in diameter after 72 hours of incubation (Plate 3B). Vaccinia virus lesions are flatter than those of variola virus, are often ulcerated on the surface, and may show haemorrhages into the pocks or into the CAM. Thus, the gross appearance of the pocks will serve to distinguish variola from vaccinia virus.

PLATE 3. APPEARANCE OF VARIOLA, VACCINIA AND HERPES SIMPLEX LESIONS ON THE CAM *

A. Variola lesions after 3 days at 35°C.



B. Vaccinia lesions after 3 days at 35°C.



C. Herpes simplex lesions (genital strain) after 3 days at 35°C.

* Reproduced, by permission, from Dumbell, K. R. (1968) *Laboratory aids to the control of smallpox in countries where the disease is not endemic (Progress in medical virology, No. 11)*, Basle, Karger.

Varicella virus does not produce pocks on the CAM. The virus of herpes simplex produces lesions on the CAM similar to variola but they are usually much smaller and not as raised or opaque. However, genital strains of herpes simplex may produce large pocks of varying size (Plate 3C) which might be confused with variola.

6.10 Non-specific lesions on chorioallantoic membrane

Non-specific lesions may arise on the CAM from a variety of causes, complicating the interpretation of the test. Most frequently they are a response to egg-shell dust or trauma from the drill or punch while dropping the CAM. Haemorrhage often causes thickening of the membrane. The use of iodine or other disinfectant to sterilize the outer surface may produce thickened areas. Inflammatory reactions to foreign material, such as fragments of crust and fungal or bacterial infection, will produce suggestive local opacities. The use of control eggs in conjunction with the test frequently offers little help in differentiating non-specific lesions.

If non-specific lesions occur frequently the egg technique is at fault. The technique is best evaluated by using leftover eggs for control inoculations with vaccinia virus, saline, and other non-infectious material. In using vaccinia virus, careful sterile technique must be practised to avoid contamination of the laboratory and erroneous isolation of this virus.

A diagnosis of variola from a *single lesion* on the CAM is rarely, if ever, justified. When a single lesion is noted, or if local opacities are present, a suspension of the membrane should be inoculated into other eggs.

6.11 Confirmation of diagnosis

All membranes considered positive should be confirmed as pox virus by the precipitation-in-gel test. This test is positive only if the membranes have numerous lesions. For this purpose:

1. One or more membranes are placed in a bottle containing beads.
2. The bottle is shaken vigorously for 5 minutes.
3. A drop of the fluid that separates is tested for antigen by the precipitation-in-gel test (see sections 5.3, 5.4 and 5.5). This will confirm the isolate as variola or vaccinia virus. The remainder of the fluid is preserved.

Membranes showing atypical or doubtful lesions should be tested further:

1. The membranes are shaken up with sterile glass beads in 2 ml of PBS containing antibiotics.
2. 1 : 10 and 1 : 1000 dilutions are made (see section 6.5.2).
3. Three eggs are inoculated with a 1: 1000 dilution and 3 eggs with the supernate from the initial dilution and incubated (see sections 6.6 and 6.7).

All specimens on which atypical or unusual results are obtained should be forwarded to the appropriate reference laboratory, together with all particulars, for further testing (see section 7).

7. RECORDS AND REPORTS

Details of specimens and results should be entered in a permanent laboratory record book (see Annex 3). Specimens should be assigned consecutive numbers, and the following information recorded: the date the sample was taken, the date the sample was received, the name, address, age, and sex of the patient, the name of the person submitting the specimen, the results of the individual laboratory, whether the material was forwarded to any other laboratories, and the date of the final report.

The results must be immediately reported to responsible public health officials (see Annex 1).

If the specimen is to be forwarded to another laboratory for confirmatory examination, copies of the form containing the information on the patient for smallpox laboratory tests (Annex 1), together with necessary comments on results that require further testing, should be submitted with the specimen.

Annex 1

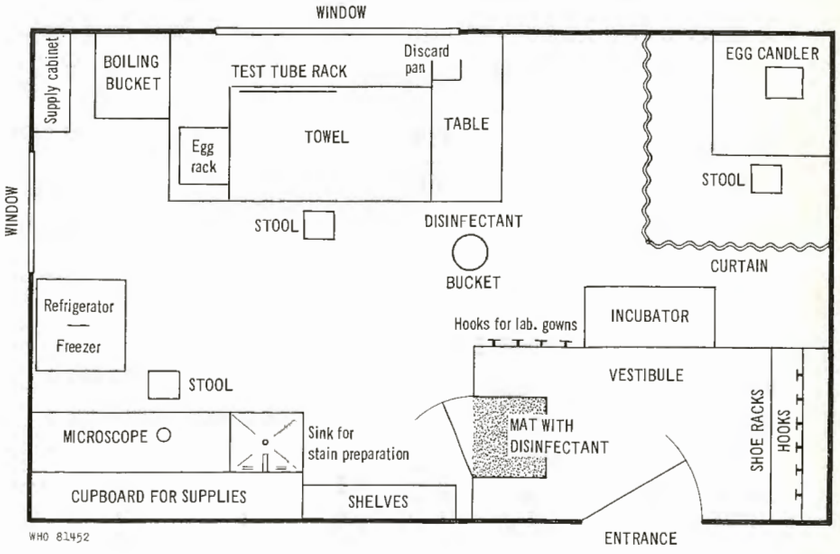
INFORMATION ON PATIENT FOR SMALLPOX LABORATORY TESTS

(*Sample form*)

PERSONAL DATA	COUNTRY				PATIENT No.....		
	DATE CASE NOTIFIED						
	NAME		AGE		SEX		
	ADDRESS		VILLAGE OR CITY		COUNTY, DISTRICT		
PRESENT ILLNESS	DATE OF ONSET OF FEVER _____ DATE OF ONSET OF RASH _____		HOSPITALIZED: ☐ NO ☐ YES		NAME & LOCATION OF HOSPITAL		
	PREDOMINANT SKIN LESIONS AT HEIGHT OF ERUPTION:		OUTCOME: RECOVERED ☐ DIED ☐ _____ DATE OF DEATH				
	VESICULAR ☐ PUSTULAR ☐ HAEMORRHAGIC ☐						
HISTORY	EARLIEST VACCINATION DATE _____ MOST RECENT VACCINATION DATE _____ VACCINATION SCAR: POSITIVE ☐ NEGATIVE ☐						
	HAS PATIENT EVER HAD: SMALLPOX: YES/NO DATE _____ CHICKENPOX: YES/NO DATE _____						
CONTACT HISTORY	WAS PATIENT RECENTLY EXPOSED TO: A CASE OF SMALLPOX ☐ _____ DAYS AGO A CASE OF CHICKENPOX ☐ _____ DAYS AGO ☐ NONE						
DIAGNOSTIC TESTS	SPECIMEN	DATE				RESULTS (INDICATE + OR —)	
		COLLECTED	RECEIVED	REPORTED	STAINING	PRECIPITATION-IN-GEL	VIRUS ISOLATION
	SMEAR FROM MACULOPAPULAR LESION						
	VESICULAR FLUID OR PUS						
	CRUST						
	OTHER (SPECIFY)						
	NAME AND LOCATION OF TESTING LABORATORY						
	PERSON SUBMITTING SPECIMEN NAME _____ TITLE _____ ADDRESS _____						

Annex 2

SUGGESTED ARRANGEMENT FOR A SMALLPOX DIAGNOSTIC LABORATORY



LIST OF MAJOR ITEMS OF EQUIPMENT

Large table (1)
Small tables or laboratory benches (3)
Stools or simple laboratory chairs (3)
Cupboard or wall cabinets for supplies (2)
Buckets with boiling water; stove or other heat source (2)
Discard buckets with disinfectant and cover (3)
Discard pans (4)
Microscope and microscope light (1)
Staining rack (1)
Test tube rack (1)
Egg rack (1)
Egg candler (1)
Incubator for egg culture (35°-37°C) (1)
Incubator for preliminary incubation of eggs (37°-39°C) (kept outside the laboratory) (1)
Refrigerator with freezer compartment (1)
Demineralizer (if distilled water is not available) (1)

Annex 3

SMALLPOX LABORATORY RECORD BOOK *(sample form)*

LABORATORY No.	DATA ON PATIENT					DATA ON SPECIMEN			RESULTS						SPECIMEN FORWARDED FOR CONFIRMATORY TESTING		REMARKS
	CODE No.	NAME	AGE	SEX	ADDRESS	SUBMITTED BY	DATE COLLECTED	DATE RECEIVED	STAINING SMEAR	PRECIPITATION IN GEL	VIRUS ISOLATION	DIAGNOSIS	DATE REPORTED		DATE	NAME OF LABORATORY	