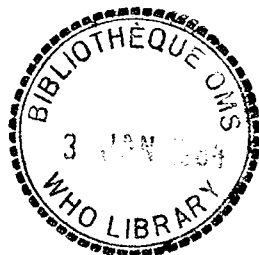


WORLD HEALTH
ORGANIZATION

EXPERT COMMITTEE ON SMALLPOX

Geneva, 14-20 January 1964



ORGANISATION MONDIALE
DE LA SANTÉ

Smallpox/WP/2
3 December 1963

ENGLISH ONLY

LABORATORY ASPECTS

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SMALLPOX AND ALASTRIM

The diagnostic procedures to be used for the minor and major forms of smallpox are identical, and the sections "Diagnostic procedures" and "Collection and dispatch of specimens" are applicable to both diseases. Until recently it was impossible to distinguish the two viruses by laboratory methods. This can, however, now be simply effected by isolation of virus from the patient's tissue and determining the growth characteristics of the virus on the chick chorioallantoic membrane (CAM). At 35° and 37°C the viruses produce identical lesions on the CAM. However, strains of alastrim virus will not produce visible lesions on the CAM of eggs incubated at 38°C or higher, whereas the virus of variola major will produce such lesions on the CAM up to a temperature of 38.5°C. To identify the virus as that of variola major or variola minor it is therefore necessary to inoculate the CAM of two sets of 12-day eggs. One set of eggs is incubated at 36°C and the other at 38.25°C. The eggs are examined after three days. Controls of known strains of variola major and variola minor should be inoculated at the same time. If the virus under examination produces lesions at 36°C but none at 38.25°C, then it is variola minor. If lesions are produced at both temperatures, the virus is variola major. The only exceptions to this so far encountered are certain African strains of alastrim which behave irregularly (Bedson, Dumbell & Thomas, 1963).

DIAGNOSTIC PROCEDURES

Histological methods

This technique is not often required as other simpler methods are more reliable. Histological examination will be most useful in distinguishing between lesions of varicella and those of smallpox. In varicella, vesicles tend to be more superficial and intranuclear inclusions are present in the epithelial cells at the sides and base of the vesicles. In addition, multinucleated cells are usually to be found. On the other hand, in smallpox lesions, intranuclear inclusions are not common, multinucleated cells are not usually present and cytoplasmic inclusions (Guarnieri bodies) may be seen.

Microscopic demonstration of virus particles

Although not specifically a histological method, smears from papular or vesicular lesions made on clean slides may be fixed and stained for virus particles by Gutstein's, Hertzberg's or Gispén's techniques. If such smears are properly prepared from early cases of smallpox, innumerable virus particles will often be found. The only other lesions which may present a similar picture are those of vaccinia or cowpox. In smears from varicella or herpes simplex, elementary bodies are scanty, stain poorly and appear smaller. They present quite a different appearance from that seen in cases of smallpox. With experience, this simple staining of smears will often provide a presumptive positive diagnosis in the majority of smallpox cases examined in the early eruptive phase of the disease. It should, however, be emphasized that once the lesions have become pustular, the results are not satisfactory. Moreover, to obtain good results, smears must be made of material obtained by scraping macules, papules or the base of vesicles with a small knife or needle and not from vesicle fluid (see also Immunofluorescence).

Electronmicroscopy

This method will, in the hands of those experienced in this technique, as shown by Peters, give satisfactory results and enable a presumptive diagnosis to be made within a few hours. This has the advantage over the simple staining of smears

mentioned above, in that it may serve to detect typical pox virus particles from pustular fluid and even from crusts in the late stage of the disease. It should, however, be emphasized that this method requires experience in the recognition of smallpox virus particles in electron microscope pictures.

Virus isolation

This is the most reliable and sensitive of all laboratory techniques to be used in the diagnosis of smallpox. It should always be used as a confirmatory test to supplement any other diagnostic method employed. Isolation of virus may be carried out in tissue cultures or on the CAM of 12-day chick embryos. It is advisable to add antibiotics to the material obtained from the patient's lesions before inoculation (penicillin 500 μ /ml and streptomycin 500 μ /ml). This technique should give positive results at all stages of the disease, from the appearance of the first macule to the disappearance of all crusts from the patient's skin. In severe cases, and particularly cases which are to be haemorrhagic, virus may be isolated from the blood in the first day or two of illness. The buffy coat from the blood is more likely to give a positive result than the whole blood.

Tissue cultures (human or monkey cells) may be used instead of the CAM for isolation of virus. The growth of virus in tissue culture may be detected after 48 hours by the specific adsorption of fowl red cells added at this time. When a virus has produced cytopathic effects in tissue culture, however, it is essential that the virus be identified by neutralization or other serological test. While this is also advisable for any virus isolated on the CAM, the typical appearances produced on this tissue within three days may enable a tentative diagnosis to be reached without serological confirmation.

When confirmation of the identity of a virus isolated in tissue culture or on the CAM is required, this may be done by demonstration of specific variola-vaccinial antigen by haemagglutination, complement-fixation or gel-diffusion techniques, using a specific antivaccinial serum prepared in the rabbit; or specific neutralization of the virus by immune serum may be determined in tissue culture or on the CAM. These tests, however, will not serve to distinguish variola from vaccinia virus.

For differential identification, the character of the lesions produced in tissue culture or on the CAM or failure of variola virus to produce transmissible infection in the rabbit skin, may be useful. On the CAM vaccinia virus will produce demonstrable lesions at an incubator temperature of 40°C while neither kind of variola virus will grow at this temperature.

While virus may be isolated and identified from the skin lesions or by culture of blood, it may also be sought in garglings from the patient during the first ten days of disease. Although isolation of virus in tissue culture of the CAM has been reported from garglings in the pre-eruptive phase of the disease, this has not been the universal experience.

Serological methods in the early stages of the disease

Precipitation technique Gel-diffusion technique

The precipitation technique is a reliable diagnostic procedure if sufficient material is obtained from the skin lesions in a suspected case. Vesicle or pustule fluid or extracts in saline of one or two crusts should suffice. When preparing an extract of crusts, the volume of fluid must be kept to a minimum, i.e. not more than two or three drops of saline for each crust. A hyperimmune antivaccinial serum prepared in the rabbit should be used for precipitation tests, although smallpox convalescent serum will sometimes give satisfactory results.

The older technique of carrying out precipitation tests in tubes, using a clarified crust extract against hyperimmune serum, has now given place to the gel-diffusion method. The test is best carried out on microscope slides, as described by Dumbell & Nizamuddin (1959). The immune serum should be placed in one cup and extracts placed in surrounding cups. It is always advisable to include in this test a known positive extract of smallpox or vaccinial material, and extracts should also be tested against a normal rabbit serum as an additional control. If a suitable hyperimmune serum is used, a positive result from this precipitation technique should be available within three or four hours. If a weaker serum is used, results may be easier to read after 24 hours. A good convalescent zoster serum may be included, as this will give a positive result in 24 hours with vesicle fluid, if the case should be one of vericella.

Complement fixation

This technique is a very sensitive method of detecting smallpox antigen in vesicle or pustule fluid or extract of crusts. Such material should be tested with a hyperimmune antivaccinial rabbit serum and known positive and negative preparations should be included with all diagnostic tests. The results should be available within 24 hours. Overnight fixation in the cold gives more satisfactory results than a shorter period of fixation at room temperature or at 37°C. This technique for detecting antigen is more sensitive than the agar gel-diffusion method, but the results are not so quickly available and the technique is rather more complicated.

Immunofluorescence

This method for detecting virus particles is relatively simple if the necessary reagents are available. The indirect technique, using an immune rabbit serum to treat smears, followed by an anti-rabbit serum coupled with a fluorescent dye, gives more reliable results than the direct immunofluorescent technique. Murray's results, published in the Lancet 1963, do not suggest that it has any great advantage over microscope examination of stained smears from suitable cases.

Serological methods in the later stages of the disease

While the tests under the heading "Serological methods in the early stages of the disease" were designed to detect virus antigen in the patient's lesions, this section is concerned with the detection of antibody in the patient's serum after the first week of illness. In these tests, therefore, one requires a specific antigen for the detection of antibody in the patient's serum. It will usually be advisable to obtain two samples of serum, one as early as possible in the illness for comparison with the second, collected after the first week.

Complement fixation

This is probably the best of the three techniques for detecting an increase in antibody in smallpox cases. The antigen to be used may be either crust extract from known cases of smallpox, or vaccinial antigen prepared in the rabbit. If a smallpox crust extract is to be used, this should be treated with 0.2 per cent. formalin to inactivate any virus present. The test usually becomes positive about

the seventh or eighth day of illness. A positive result may also be given by the serum of a recently vaccinated person but this becomes negative within six to twelve months. The serum from a smallpox case taken after the first week of illness may give a positive titre up to a dilution of 1/80 or 1/640, depending on the technique used. A positive result in a serum dilution over 1/10 would usually be significant if the patient has not been vaccinated within the previous six months.

Haemagglutination inhibition

This technique for measuring antibody will often give high titres (over 1/1000) but it has the disadvantage that this antibody may persist for some years after vaccination against smallpox. Vaccinial haemagglutinin prepared in eggs is the antigen used.

Neutralization

The serum of suspected cases may be tested for its power to neutralize vaccinia or variola virus in tissue culture or on the CAM. It therefore requires that an active suspension of one or other of these viruses should be at hand. This test is rather more time-consuming than either complement-fixation or haemagglutination inhibition. The results are not always easy to interpret if the patient has been previously vaccinated against smallpox. Neutralizing antibodies may persist in the serum for many years after smallpox vaccination. It is, therefore, essential that a fourfold or greater rise in titre should be demonstrated during the course of illness by this technique.

CHOICE OF TECHNIQUES AT DIFFERENT STAGES OF THE DISEASE

The laboratory methods to be used in the diagnosis of smallpox cases will depend to some extent on the facilities available and the experience of the virologist. The choice of methods suggested below is based on the experience of British workers.

The pre-eruptive illness

In febrile contacts in the absence of a rash, an attempt may be made to isolate virus by culture of blood (buffy coat) on the CAM or in tissue culture. Similarly, garglings may be used in the same way. However, in many cases of smallpox these examinations will give negative results.

Macular and papular stage

The most useful test will be the demonstration of virus particles in scrapings of the skin lesions by: (a) stained smears; (b) immunofluorescence; or (c) by the electron microscope. The same material should give positive results by inoculation on the CAM or in tissue culture.

Vesicular stage

Virus should be demonstrable microscopically in smears made from the base of vesicles. Virus antigen should be demonstrable in vesicle fluid by complement-fixation or gel-diffusion techniques, and the virus should be isolated in culture.

Pustular stage

Virus may be demonstrable by the electron microscope. Complement-fixation and agar gel-diffusion tests will give a positive result with the contents of pustules and culture will always be positive.

Crusting stage

Virus may be demonstrable by the electron microscope. Extracts will give a positive test for specific antigen by complement-fixation or gel-diffusion and virus will be demonstrable in culture.

Tests for antibody

Test for a rise in antibody in the patient's serum is never as satisfactory as the methods outlined above, but may have to be used: (a) in mild cases who never develop an eruption; or (b) in retrospective diagnosis of cases from whom no crusts or other material are available for the detection of virus or virus antigen.

In the interpretation of results of antibody determinations in these instances, the history of vaccination must be taken into account.

COLLECTION AND DISPATCH OF SPECIMENS

Collection

Blood

If blood is to be used for examination for virus in the first few days of illness, a few cubic centimetres should be collected into citrate in a small screw-capped bottle or other suitable container. If required for antibody studies, the blood should be allowed to clot.

Garglings

Saline or broth should be used, and garglings collected in screw-capped bottles.

Material from skin lesions

For microscopic demonstration of virus, material obtained by scraping with a Hagedorn needle, macules, papules or the base of vesicles, should be smeared on clean glass slides. Five or six lesions should be sampled. The slides should be allowed to dry in air and should not be placed in a fixative or in a disinfectant. The slides should be separated from each other by means of rubber bands or small pieces of cardboard, and wrapped in grease-proof paper and placed in a container for dispatch to the laboratory.

Vesicle fluid and pustular fluid

These are best collected in small glass capillary tubes which should not be sealed but put in a small screw-capped bottle for dispatch to the laboratory. If capillary tubes are not available, the material may be spread thickly on glass slides and allowed to dry in air. In the laboratory, the material on the slides may be washed off in a small volume of saline, to be used for the detection of antigen and/or for culture.

Crusts

At least half-a-dozen crusts should be collected from suitable cases and placed in a screw-capped bottle for dispatch to the laboratory.

All the specimens mentioned above and the instruments used for their collection, must be regarded as highly infective. The virus is known to withstand drying for many weeks. It is therefore essential that all specimens and containers should be securely packed in metal, wooden or strong cardboard containers for dispatch to the laboratory.

Dispatch

Specimens should reach the laboratory with the least possible delay. If they cannot be delivered by hand or by ambulance, they should be sent by the most rapid transport available and the receiving laboratory should be informed by telephone of the time of arrival of the specimens.

With all specimens sent to the laboratory, details of the patient's age, name, address, history of contact, history of vaccination, date of onset of illness, date of appearance of rash (if any), should be given.