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The Role of the Diagnostic Laboratory

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When the global smallpox eradication programme began, it was not immediately apparent what the requirements might be for facilities for the laboratory diagnosis of smallpox. Originally, it was felt that a network of laboratories would need to be developed, including international and regional reference centres and one or more national laboratories in each of the endemic countries. Partly, this assumption was based on experience with regard to other diseases. For the control of cholera and diphtheria, for example, definitive diagnosis is often in doubt and, commonly, epidemiological investigation provides very few clues.

As the programme progressed, it became increasingly apparent, however, that smallpox presented a uniquely different situation. In endemic areas, the clinical and epidemiological information almost invariably provided more than ample information for confirmation of diagnosis. The laboratory examination of specimens from endemic areas was clearly little more than an academic exercise. The rash and the evolution of disease were found to be sufficiently characteristic in at least 85% of the cases so as to leave even the modestly experienced worker in little doubt as to the diagnosis. For questionable cases, the vast majority could be readily identified on epidemiological grounds - the clinical characteristics of the index case from whom the person acquired infection and subsequent cases to whom the disease was transmitted providing the necessary clues to diagnosis. For the rare cases in which neither the clinical nor the epidemiological appraisal was definitive, it was most practical to consider the individual

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to have smallpox and to undertake requisite containment measures. Undoubtedly some modest "over reporting" of cases occurred because of errors in diagnosis but I'm sure this was of neglible importance and in no way affected the direction of the programme.

Thus, to date, in countries and states (of large countries), where transmission has been known to be occurring, no efforts have been made to obtain specimens for laboratory diagnosis. The satisfactory results so far observed in the global programme indicate this to have been a sound policy. I believe this is still the most practicable scheme to follow irrespective of the availability of laboratory testing facilities.

However, when transmission is believed to have been interrupted in a country or state (of a large country) each case becomes of signal importance. If the case is really smallpox, it implies either an introduction from elsewhere which must be documented or the existence of a persistent focus of smallpox which must be identified. Thus, in an area in which transmission is believed to have been interrupted, specimens from every suspect case should be examined in the laboratory and the results correlated with clinical and epidemiological observations.

Several steps should thus be taken when a laboratory diagnostic service is established or when the possibility of routinely dispatching specimens to a WHO Reference Laboratory is considered:

1. Areas wherein it is believed that transmission has been interrupted should be specifically and clearly delineated by the national smallpox programme authority. Efforts should be made to obtain specimens from at least one case in every outbreak in all such areas. The smallest practicable area which should be so delineated would be, for example, for India, a state, and for Bangladesh, a division. Except under special circumstances, no specimens should be accepted from other areas.

2. With this plan, all specimens are necessarily obtained from cases of considerable importance to the programme. Since it is most important that adequate material be obtained and since detailed clinical and epidemiological studies must immediately follow should the case prove to be smallpox, specific individuals with epidemiological competence should be designated as the ones to collect specimens. Experience has shown that it is impossible to establish and maintain a large staff with the requisite competence. Accordingly, in most countries, specimens are collected only

by state or national surveillance teams who, at the same time, evaluate the patients both clinically and epidemiologically. Wide distribution of collection kits to hospitals and health centres so as to permit anyone to collect and submit specimens, has invariably led to confusion and delays in investigation.

3. Of necessity, an answer from the laboratory in the shortest possible time is required. A provisional response (based on agar-gel precipitation) should always be available within 24 hours after receipt of the specimen and usually a definitive answer (virus isolation on CAM) within three or at the most six days. This implies the need for laboratory staff to be available 7 days a week and to have available, at all times, eggs for inoculation which have already been properly incubated.

Eventually the need for examination of specimens by electron microscopy will become evident. This is well-illustrated in Dr Nakano's paper (WHO/SE 72.44). Most frequently, cases of adult chickenpox are mistaken for smallpox. As the chickenpox virus dies quickly and, in any event, does not grow on CAM, a specimen from a chickenpox case yields no "line of identity" in the agar gel test and nothing is isolated on CAM. The laboratory can only report "no virus isolated or identified". The result is exactly the same if a specimen is taken from a smallpox case in which the quantity of material is too little or the virus is inactivated by continued exposure to light. The epidemiologist who receives a response of "no virus isolated or identified" is left with the uncomfortable decision as to whether the illness was indeed caused by some virus other than variola or whether there was something faulty in specimen collection. With the electron microscope, a skilled operator may identify chickenpox virus particles and variola-vaccinia type particles even when the specimen has been mishandled or quantity of the specimen is too small to permit diagnosis by conventional means. The identification of chickenpox particles with electron microscopy has been most reassuring in regard to many worrisome clinical puzzles in which the epidemiological investigation has been of little or no help.

4. The competence of the laboratory must, at all times, be assured. Inevitably in all laboratories there are changes in staff, inadvertant modifications in reagents, changes in the supply source of eggs etc. False positive as well as false negative results have been obtained. Three measures should be routinely taken to assure, to the extent possible, the quality of the laboratory work.

a. Test specimens should be sent on a regular basis (at least quarterly) from a reference center to provide assurance that the laboratory is able to identify specimens correctly.

b. Each month, the designated programme officer should arrange to send crust or pustular material from a primary vaccinee (without indication to the laboratory as to the source of material) to assure that the laboratory can identify properly the presence of vaccinia virus.

c. If there is a discrepancy between the laboratory result and the clinical and epidemiological observations, specimens should immediately be forwarded to a reference laboratory and higher level consultation sought. While there is a tendency in some to regard the laboratory examination as "final" and "definitive", the ultimate diagnosis must take into account the clinical and epidemiological observations. Even the best of laboratories may err. On one occasion, for example, the clinical and epidemiological findings in a particular case indicated the diagnosis to be most probably chickenpox but a laboratory of recognized competence reported the isolation of variola virus. More detailed clinical and epidemiological studies were conducted which confirmed the original impression. Finally, blood specimens were obtained from the patient. Antibody studies showed that the correct diagnosis was chickenpox. The laboratory had erred. Many other such illustrations could be given.

Specimen collection

Proper specimen collection is most important and here the Expert Committee Report on Smallpox may be appropriately quoted.

"The surveillance team responsible for investigating the epidemiological aspects of each suspected case should also be responsible for and specifically trained in the collection of specimens. WHO has available for supply to the central surveillance authorities a simplified specimen-collecting kit consisting of a plastic, screw-capped tube containing a cotton swab mounted on a stick.

The tube is inside a screw capped container which itself is in another screw-capped container. Swabs are used to collect the contents of 5 or 6 pustules for each laboratory. When the patient's lesions have formed crusts, not less than 6 scabs should be obtained.

"The specimens must be regarded as highly infective and must therefore be enclosed in suitable containers. These are supplied as part of the WHO kit. Specimens should reach the laboratory promptly and, when possible, should be refrigerated. If they cannot be delivered by hand they should be sent by the most rapid means of transport available, and the receiving laboratory should be informed by telephone or telegraph of the estimated time of their arrival. Packages sent through the post should conform to national and international postal requirements.

"With all specimens sent to the laboratory, details should be given of the patient's age, name, address, history of vaccination, date of appearance of rash, and date of collection of specimen. This requires the completion of the form supplied with each kit".

Interpretation of results

Most laboratories are able to perform two tests, the precipitation-in-gel test and isolation of the virus on the chorioallantois of embryonated eggs. These tests are described briefly below.

Precipitation-in-gel

This is an immunodiffusion test for the recognition of poxvirus antigens. It is a reliable diagnostic test when sufficient material is used. False negative results are seen when too little material is employed. In this test, a suspension containing pustular material or an extract of 2 or 3 crusts and a highly potent antivaccinial serum are used. The test is done on microscope slides on which a layer of agar is prepared and reservoirs or cups are cut. The antiserum is placed in one cup and pustular fluid, crust extracts, and control materials are placed in surrounding cups. If the material is from a smallpox patient, precipitation lines should appear in the agar between the antigen and antiserum cups within 2 hours, linking with those in the positive control within 4-5 hours. If a weak serum or little antigen is present in the pustular fluid or crusts, a positive result may be delayed for 24 hours.

A positive result is strong presumptive evidence that the specimen contains virus material of the variola-vaccinia group, but vaccinia and variola viruses cannot be differentiated by this test. A negative result indicates that the patient may have had some other illness or that insufficient antigen may have been present in the specimen. Virus isolation must be done in confirmation and to identify the virus.

Virus isolation

Poxviruses may be isolated by inoculating material from the lesions of the patient on the chorioallantoic membranes of chick embryos. Normally, 72 hours are required for growth. Isolation of virus should always be attempted as a confirmatory test and to supplement other diagnostic methods. Variola, vaccinia, cowpox, monkeypox, and herpes simplex viruses produce different types of lesion and thus may be distinguished by this technique. Varicella virus does not grow on the chorioallantoic membrane. Failure of pock formation on the membrane may indicate that the suspected case is not smallpox or that the sample was inadequate or improperly handled before it reached the laboratory. The use of appropriate tissue culture in the diagnostic procedure may provide an additional indication of the specific virus infection.

When there are strong clinical grounds for diagnosing smallpox, the physician should not allow his judgment to be unduly influenced by failure to identify the virus. However, when very few cases of smallpox are occurring, isolation of the virus from a suspected case is welcome confirmation of the clinical diagnosis.

It is noted particularly that a negative precipitation-in-gel test or the failure to isolate variola virus does not mean that the patient did not have smallpox. Diagnosis of each case must always take into consideration the clinical, epidemiological and laboratory findings.