Diagnostic Methods in Clinical Virology

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Preface

This book has been written as a guide to clinical pathologists. It is intended for experienced laboratory workers who wish to make use of the recently developed but now well-established techniques of diagnostic virology. The methods described are based on practical procedures found satisfactory in the diagnostic work of the Regional Virus Laboratory, Glasgow. No attempt has been made to deal with every one of the numerous viruses causing human disease, nor to describe the very latest advances and untried innovations of technique. Most of the methods included in this book are standard procedures or modifications of techniques devised by others, and only in a few cases is originality claimed.

During the development of our methods we have benefited from the advice and help of many colleagues. We are particularly indebted to Dr C.M.P.Bradstreet and Dr A.D.Macrae and other members of staff of the Standards Laboratory for Serological Reagents and the Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London, to Dr A.J.Tyrrell and other members of staff of the Common Cold Research Unit, Harvard Hospital, Salisbury, and to our own long-suffering technicians.

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List of abbreviations

[A] = see appendix
BSS = balanced salt solution
CAM = chorioallantoic membrane
CF = complement fixation
CPE = cytopathic effect
HA = haemagglutination
HAI = haemagglutination inhibition
HD = haemadsorption
HDI = haemadsorption inhibition
MEM = minimum essential medium
PBS = phosphate buffered saline
PBSA = phosphate buffered saline with penicillin (100 units/ml) and streptomycin (100 μg/ml)[A]
PS = stock solution of penicillin (10,000 units/ml) and streptomycin (10,000 μg/ml)[A]
RBC = red blood cells
RMK = rhesus monkey kidney
TCD_{50} = 50 per cent tissue culture infectious doses

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Chapter 1

Introduction to clinical virology

Clinical virology comprises the examination of specimens from persons suspected of infectious disease for evidence of virus infection, followed by the attempt to judge whether or not any virus infection detected is the cause of the patient's disease. The demonstration of virus or of specific changes (generally immunological) due to viral activity can prove the presence of infection. Whether this infection is or is not causally related to the patient's disease must then be judged taking into consideration not only the full clinical and other evidence in the particular case but also the background of established knowledge of the virus infection demonstrated.

Much epidemiological and experimental work may be required to demonstrate that a particular virus causes a particular disease. The problem is increased by the limited range of host response: many types of disease (e.g. pneumonia; nonbacterial meningitis) may be caused by any of a large number of viruses. Conversely, many viruses (e.g. poliovirus; mumps) may cause any of a variety of disease patterns ranging from fatal or permanently crippling to minor or completely inapparent. For these reasons, and because many viruses are very prevalent, coincidental and even multiple virus infections are often encountered. Fulfilment of Koch's postulates may be impossible, particularly if no experimental animal is susceptible to infection by the virus in question or if the experimental disease is totally unlike the illness in man.

In many cases, e.g. isolation of variola virus from the rash of a case of smallpox, there is no doubt of the significance of a positive test. Although there is little doubt of the significance of isolating poliovirus from the faeces of a case of paralytic poliomyelitis, one must consider the possibility that the poliovirus may have originated from oral vaccine ingested by the patient some days or weeks previously or even from a silent infection with a 'wild' strain of virus. Paralysis in such a case may be due to infection with Coxsackie A7 or some other virus. The

source from which virus is isolated may be helpful in evaluating the test. Thus in the investigation of nonbacterial meningitis, mumps virus is more likely to be the cause if isolated from cerebrospinal fluid (CSF) than from saliva; echovirus type 9 is more probably the cause if isolated from CSF than from faeces. Yet the undoubtedly neurotropic poliovirus is rarely demonstrable in the CSF.

VIRUS ISOLATION

Since viruses unlike bacteria are incapable of growth in non-living culture media, their isolation necessitates inoculation of susceptible living cells, e.g. tissue culture, embryonated hen eggs or animals. Fortunately, the majority of important viruses can be isolated in tissue cultures in which they usually produce more or less characteristic morphological changes or death of infected cells (CPE). They may give rise to specific CF antigens or haemagglutinins, or may be demonstrable by haemadsorption after the addition of RBC which adhere to infected cells at whose surface viral haemagglutinin has been produced. Some viruses can be cultivated in fertile eggs, the presence of virus being indicated by the appearance of haemagglutinin in the extra-embryonic fluids (e.g. influenza), antigen in tissue extracts (e.g. influenza 'S' antigen in embryo lungs; psittacosis group antigen in yolk sac), or characteristic focal lesions (e.g. chorioallantoic membrane 'pocks' caused by herpes, smallpox or vaccinia virus). After inoculation into animals, certain viruses may cause characteristic sickness or death (e.g. flaccid paralysis of suckling mice by Coxsackie A virus), characteristic pathological changes (e.g. psittacosis virus visible in stained impression smears of brains of intracerebrally inoculated mice), or development of specific antibodies (e.g. to *Rickettsia burneti* in guinea-pigs inoculated intraperitoneally with material from suspected Q fever). Virus isolation recognized by such 'indicator effects' in inoculated hosts must be confirmed by specific tests (usually immunological) and sometimes by additional tests of pathogenicity, host range or other properties.

SEROLOGICAL DIAGNOSIS

The standard methods of serological diagnosis are: (*a*) complement fixation, the most convenient test for routine purposes; (*b*) haemagglutination inhibition, which is based on the ability of antisera to prevent agglutination of red blood cells by haemagglutinating viruses; and (*c*) neutralization, i.e. measurement of the ability of antisera to inhibit infection of susceptible living cells in cultures or

organisms inoculated with serum-virus mixtures. Immunofluorescent, precipitin and other serological reactions may also be used in some cases.

A fourfold or greater rising titre of specific antibodies to a particular virus observed in tests of sera collected during the course of illness establishes a close temporal relationship between the illness and the infection. To this extent the observation increases the probability that the relationship is causal rather than casual, but is not alone sufficient to prove this. For demonstration of a rising antibody titre two blood samples are necessary, one taken as early as possible in the course of illness (preferably within the first week), the other taken not less than a week later and usually during the third week after onset of illness; in children under 3 years old, blood collected as late as the fourth or fifth week after onset of illness may be necessary to show the antibody response. If antibody titres are high in both sera without a significant (fourfold) increase, this may indicate that the interval between bleedings was too short, that the first specimen was collected too late, or that the infection had been established for longer than the history of illness suggested. In some cases, particularly with CF tests, high antibody levels may persist for only a few weeks or months after infection. In such cases, a high titre may constitute evidence of 'recent infection', which may have caused the patient's illness. In other cases, the unchanging antibody level may merely be similar to that of many sera from the general population and cannot be interpreted as evidence of recent infection. Evaluation of what constitutes a 'high' antibody level must be judged from the laboratory's cumulative experience of routine tests with the particular antigen and technique. In many cases antibodies (especially those measured by neutralization tests) may persist for years, and their presence cannot be interpreted diagnostically in the absence of a rising titre; such antibodies may nevertheless be useful for measuring the past prevalence of a virus and the present immune status of the population.

ANTIGEN TESTS

Direct demonstration of virus antigen is sometimes useful. The best established method of this type is the test for pox group antigen in material from the eruption of suspected smallpox: CF or agar gel microprecipitin procedures have superseded the original flocculation method. Staining with fluorescent antibody holds great promise for rapid diagnosis of infections which give rise to specific antigen in clinical specimens.

MICROSCOPY

Apart from low powered scrutiny of tissue cultures, microscopy has had very limited usefulness in diagnostic virology. With the exception of the pox group, true viruses are too small to visualize with the optical microscope. It is nevertheless useful to seek elementary bodies in smears from suspected smallpox, and low magnification is sufficient for examining smears for the characteristic giant cells found in the vesicles of varicella, zoster and herpes simplex. The development of immunofluorescent techniques is increasing the importance of microscopy for diagnostic work. Electron microscopy is not generally available, but can be useful for demonstrating virus particles of characteristic morphology in material from skin lesions deposited directly on grids and 'stained' for negative contrast by potassium phosphotungstate.

PRACTICAL AND THEORETICAL IMPORTANCE OF CLINICAL VIROLOGY

Diagnostic virology will remain of limited value in the clinical management of individual patients until effective methods of specific treatment of virus infections become available. Recent work on thiosemicarbazones (e.g. N-methylisatin β-thiosemicarbazone) in smallpox and halogenated nucleosides (e.g. 5-iodo-2′-deoxyuridine) in herpetic keratitis give promise of useful antiviral compounds, while the discovery of interferons suggests that biological substances with 'broad spectrum' activity may be obtainable. In the meantime, virological investigations can be of help in precise clinical diagnosis, but their most obvious practical value is less to the individual patient than to the community. For example they make possible the rapid specific diagnosis of smallpox or poliovirus infection, or the detection of new variants of influenza or even previously unknown viruses threatening the health of the population. Laboratory support is also required for field trials and subsequent surveillance of vaccines. Selection of which virus infections are sufficiently important to justify attempts at specific control depends on accurate epidemiological information which can also suggest the best approach, e.g. whether a vaccine might be effective or whether the antigenic variability of the virus or its pattern of pathogenesis make such an approach unlikely to succeed. Knowledge of the ecology of a virus may suggest other methods of control, e.g. breaking the chain of transmission.

At the present stage of knowledge, therefore, diagnostic virology can make a useful contribution in the clinical field, but has a wider potential importance for viral epidemiology and public health. It may

be of limited interest to a clinician to know that his patient was infected, not just with an agent with the properties of an enterovirus but precisely

TABLE 1.1 Viruses pathogenic for man

PICORNAVIRUSES:	Poliovirus, types 1–3
	Coxsackievirus, types A1–24, B1–6
	Echovirus, types 1–9, 11–32+
	Rhinovirus, 50+ types
REOVIRUSES:	Reovirus, types 1 (formerly ECHO 10), 2, 3.
ARBOVIRUSES:*	Group A (15 types: Western equine encephalitis, etc.)
	Group B (33 types: louping ill, yellow fever, etc.)
	Group C and numerous other types
MYXOVIRUSES:	Influenza, types A-A^1-A^2, B, C
(and related viruses)	Newcastle Disease virus
	Parainfluenza, types 1 (Sendai; HA2), 2(CA), 3(HA1), 4
	Mumps
	Measles
	Respiratory syncytial virus
	Rabies*
ADENOVIRUSES:	Adenovirus, types 1–31+
PAPOVAVIRUSES:	Infectious warts*
HERPESVIRUSES:	Herpes simplex
	Varicella-zoster
	Cytomegalovirus*
POXVIRUSES:*	Variola (variola major & alastrim)
	Vaccinia
	Cowpox
	Contagious pustular dermatitis (orf)
	Molluscum contagiosum
UNCLASSIFIED VIRUSES:	Rubella*
	Hepatitis*
PSITTACOSIS-LGV GROUP:*	Psittacosis-ornithosis
(not true viruses)	Lymphogranuloma venereum
	Trachoma
	Inclusion conjunctivitis
	Cat scratch disease (?)
RICKETTSIAE:*	*Rickettsia burneti* (Q fever)
(not viruses)	*R. prowazeki*, etc. (typhus group)
	R. rickettsi, etc. (spotted fever group)
	R. quintana (trench fever)

* Because of technical difficulties or unimportance for human disease in Britain, no detailed treatment is given in this book to arboviruses (other than louping ill), rabies, infectious warts, cytomegalovirus, poxviruses (other than variola-vaccinia group), rubella, hepatitis, psittacosis-LGV group (other than psittacosis and LGV), or rickettsiae (other than *R. burneti*).

with one particular type—information which may have taken weeks or months to obtain, possibly with the help of local, national or international reference centres. Nevertheless, definitive identification of the virus is essential for working out both the changing prevalence of enteroviruses in the community and also the ecology and pathogenicity of individual viruses. This fragment of evidence becomes useful when related to established and future knowledge of the same and other viruses in the local community and in the wider world (Table 1.1).

This, then, is the context within which clinical virology makes its full contribution. The practical implication is that it is best practised on a scale sufficient not only to keep the range of techniques regularly employed (and therefore efficient) but also to furnish significant epidemiological information.

Chapter 2

General organization

A. SAFETY

Safety is of particular importance in the virus laboratory because effective antiviral therapy is not yet available. Safe procedures must be followed for the protection of staff and their contacts, and to avoid cross-infection in the laboratories. Viruses such as smallpox, poliovirus and infective hepatitis are relatively thermostable and resistant to many chemical disinfectants. Some, e.g. poxvirus, influenza virus, and *Rickettsia burneti*, may remain infectious in dried particles of dust in the laboratory.

Awareness of risk and observance of scrupulous technique are even more important in the diagnostic than in the research laboratory because the hazards of diagnostic specimens are unknown in advance and because numerous agents, known and unknown, are handled in the laboratory during the same period. Not only viruses but also pathogenic bacteria and parasites may reach the laboratory in diagnostic specimens. It should be remembered that all human blood, serum, tissue specimens and tissue cultures, eggs or animals may be infectious. Smoking, eating or drinking in the laboratory are forbidden. Since slight contamination of the immediate working area is inevitable, this should be kept small; paper towels on a metal tray provide an absorbent working surface. Inoculation hoods with exhaust ventilation and ultraviolet irradiation are useful and even essential for handling highly dangerous agents. Ideally, only one virus or isolation specimen should be worked with at a time. All apparatus not actually in use should be closed or covered; equipment should be safely disposed of immediately after use, the working area cleaned up and the hands washed before changing to work on a different agent or specimen.

Risks arise from manipulating tissues (e.g. animal dissection, egg harvesting), pouring and pipetting fluids (avoid frothing, squirting and manipulate highly infectious fluids by 'Propipette'[A] in inoculation

hood). Grinding in mortars and homogenization in blenders cause splashing or infective aerosols and should be done under hoods, allowing half an hour for spray to settle before opening. Dangers of centrifugation arise from leakages and breakages, and from overfilling bottles in angle centrifuges. Ampoules of freeze-dried virus should be opened under a hood by scratching across, cracking the glass with a hot rod, and allowing pressure to equilibrate before opening.

Accidents should be avoided by firm closures of blenders and bottle caps (especially in centrifuge and deep freeze); caps should be kept dry to avoid wetting screw threads and leakage by capillarity. Glassware with chipped rims or cracks should not be used. Risk of spillage by knocking over bottles is reduced by supporting them in metal racks. Bottles of fluid to be frozen should not be more than two-thirds full and should be slanted without wetting the cap liner while freezing. The tips of pipettes and sharp instruments should be protected by sterile tubes.

Used apparatus should be disposed of safely after use—infected instruments into closed receivers. Bottles and tubes should be capped or bunged for temporary safety until autoclaved. 'Chloros'[A] is satisfactory for receiving potentially infected pipettes, and is an excellent general purpose disinfectant but is caustic for skin and attacks some metals. Strong acids, alkalis and halogen solutions are virucidal but all chemical disinfectants are weakened in their action by the organic matter which usually accompanies virus and the final disposal of infected material should usually be by autoclaving or incineration.

Staff may be protected by immunization against various viruses. Routine poliovaccination and annual smallpox vaccination are advisable. Passive immunization may be possible for many accidental infections. Several antiviral sera are available, and gamma globulin (1·5 g) may be injected intramuscularly as a precaution after exposure to accidental infection by human pathogenic viruses.

B. SPECIAL FACILITIES

Refrigeration

Long term storage of the most highly labile materials is best achieved by sealing in glass ampoules and freezing in liquid nitrogen (−196°C) or solid CO_2 (−78°C). The technical problems and expense of using these refrigerants make electrically powered mechanical freezers preferable for most purposes. Temperatures around −70°C, attainable by two stage mechanical freezers, are satisfactory for labile viruses,