Some advances in the diagnosis of respiratory virus infections

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Respiratory infections are among the commonest causes of a paediatric patient being brought for attention and for each such patient there are several acute respiratory infections in the home. They are especially frequent in the early years of life, the exact peak depending on the age at which frequent contacts with other children begin. Five illnesses a year are not unusual in the first five years of life, and it is probable that most of these are virus infections. Minor infections such as colds continue to occur at the rate of two or more a year for the rest of life. Of course, bacterial infections also occur, and may indeed cause disease, such as otitis media or mycoplasmal pneumonia: even so a viral infection may predispose to bacterial invasion, eg influenza and pneumococcal or staphylococcal pneumonia. Thus, even when a positive diagnosis of a bacterial infection is made by clinical examination and laboratory testing the possibility of a virus infection is not excluded. However, on clinical grounds, many illnesses such as the common cold or bronchiolitis almost certainly imply a virus infection; some, such as pharyngitis may be due either to viruses or bacteria, though viruses are more common. Most respiratory virus infections recover without anti-bacterial treatment even if bacteria are present and it seems that viruses are responsible for most of the symptoms and signs.

Although infection with some viruses, such as measles or varicella, can be diagnosed confidently on clinical grounds, respiratory viruses each cause a range of syndromes which overlap with each other, and laboratory tests are essential for an exact diagnosis. Nevertheless, there may be clues to the identity of the virus that is causing the respiratory illness in the signs and symptoms observed, eg in a case of pharyngitis, the lymphadenopathy and blood picture of glandular fever indicate Epstein-Barr virus (EBV) infection, the skin lesions of hand, foot and mouth disease point to coxsackie virus A16, while a catarrhal conjunctivitis suggests an adenovirus infection. In addition, although it seems that almost any respiratory virus can cause almost any respiratory syndrome, there is a tendency for certain viruses to be particularly associated with particular clinical pictures (Table 1).

Respiratory infections may be associated with a range of more serious illnesses either representing extension of the infection, eg wheezy bronchitis, probably febrile convulsions and encephalopathy or exacerbations of underlying disease, eg renal disease or juvenile chronic arthritis: in each of these may be found infection with any one of a variety of respiratory viruses.

However, in order to make a specific diagnosis of a virus infection the use of appropriate laboratory methods is essential and the rest of this article will outline how this may be done, with particular emphasis on some recent advances. Before describing the methods, some general principles must be set out. During an infection several events take place. First, the virus replicates in the cells of the respiratory epithelium, and is then shed into the respiratory secretions, sometimes by way of the fragments of disintegrated cells—thus a virus infection may be detected by collecting secretions and showing the presence in them of infectious virus, viral peptides or viral nucleic acid. Following infection, the host responds by producing a range of cell products. Some, such as interleukins and acute phase proteins are not specific for virus infections, but others, such as interferon (IFNα) are, and so to detect IFNα in secretions or serum may give an early indication that a virus infection is taking place. Specific antibodies are produced; in the serum these typically comprise IgM followed by IgG and IgA, although the early IgM response is less striking in respiratory infections than in some others: thus emphasis is placed on demonstrating a rising titre between sera collected in the acute phase and about two weeks later.

Table 1. Common associations between respiratory virus groups and clinical syndromes in children.

| Common cold, rhinitis | Rhinoviruses (100)*
|-----------------------|------------------------
|                       | Coronavirus (2)
| Laryngotracheo bronchitis, croup | Parainfluenza types 1 and 2
| Bronchiolitis | Influenza
| Pneumonia, pneumonitis | Respiratory syncytial virus (RSV)
| Pharyngitis, sore throat | RSV, parainfluenza 1 and 3
|                       | Enterovirus — echo, coxsackie (70+)
|                       | Adenovirus types
|                       | 1, 2, 3, 4, 5, 7, 14, 21
|                       | Epstein-Barr virus
|                       | Herpes simplex

*Total number of serotypes associated with disease.

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Methods available

Virus detection

The concentration of virus may be quite low so it is important to collect a substantial sample of respiratory secretions in the first few days of the illness. Aspirated nasopharyngeal secretion (NPA) is probably the best sample in infants or young children, whatever the clinical diagnosis, but a well-taken nose or throat swab can also be used for virus isolation. The original methods depend on inoculating secretions into tissue cultures of several different virus susceptible cells, incubating at 33°C for several days, and examining the tubes for the presence of virus effects, usually a cytopathic effect, but sometimes haemadsorption, for example, for influenza and parainfluenza viruses. If positive, the virus is characterised by testing for various properties, such as stability and morphology by electron microscopy (EM). Eventually, the virus is identified and typed, usually by immunological methods. As some respiratory viruses are very labile it is desirable to inoculate cultures as soon as possible after they are collected, ideally at the bedside, although this is only practical as a research project. They are therefore usually mixed with a transport medium and may be stored at −70°C or lower for subsequent testing. Non-infectious virus cannot be detected in this way and it is therefore an attractive idea to look for viruses by EM. However, this is only successful if large amounts of virus of a characteristic size and shape are present; otherwise they cannot be recognised in the presence of cellular and other debris. The method is often successful in faeces in cases of diarrhoea but is not satisfactory for respiratory infections.

Antigen detection

For certain of the more common viruses with a limited range of antigenic types it has now been shown that virus antigen (which represents the peptides or glycopeptides of the virus particle) can be detected by antibody binding techniques. The first methods were based on immuno-fluorescence (IF) and cells obtained from NPA were prepared as films and stained with fluorescein conjugated sera. There were doubts at first that they were sufficiently sensitive and specific, but it was shown that respiratory syncytial virus (RSV) antigen detection correlated well with the results of virus isolation tests and standard serology. Under the name of ‘rapid virus diagnosis’ they have been introduced into certain laboratories and can detect influenza, para-influenza and adenovirus antigens as well as RSV[1]. These methods are rapid in that they yield results in a matter of hours, rather than days. However, they still take up quite a lot of bench time and require the staff to spend hours at the microscope—and even then the results are to some extent subjective. But, if the reagents and methods are rigorously standardised (and this was a major reason for founding the European Rapid Virus Diagnosis Group) reliable and reproducible results can be obtained in diverse laboratories. Monoclonal antibodies offer some advantages because they show affinity only for viruses and not for cell components. However, they may also have disadvantages; they will stain only one epitope of one antigen and they may not bind strongly. Nevertheless, several suitable tests based on monoclonals have now been successfully developed and evaluated[2].

Antibody binding can, of course, be measured by other methods such as radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA) and these have also been developed[3]. A number of laboratories have methods for the detection of RSV. Several in Europe do such rapid assays for a range of viruses on a routine basis[4,5]. The tests are almost as sensitive as IF but care is still needed in collecting and processing the specimens and the reagents and methods have to be carefully selected to avoid non-specific binding and other forms of ‘background’. Even so, it takes more time to get a result by ELISA than by IF. It is often advantageous to use a ‘capture’ method in which the specimen is placed on a plastic surface such as the well of a microtitre plate that has been previously coated with an antiviral antibody which holds the antigen while other components are washed away. The antigen is then revealed by a detection system consisting of a secondary antibody and an enzymic or other label. With these methods the results can be read automatically and the positives and negatives determined by objective criteria, for example, greater than 2SD above background.

The development of these methods continues and W. Al-Nakib and his colleagues at our Unit now have methods that are sensitive and specific for the diagnosis of certain rhinovirus infections[6].

Nucleic acid detection

The nucleic acids of virus are just as characteristic as their peptides and it is now possible to test for these by using the techniques of ‘genetic engineering’ which enable one to clone DNA into bacteria where it can be replicated, harvested and hybridised specifically with either DNA or RNA of viral origin. It has been known for some time that in principle the technique can be used to detect virus nucleic acids in clinical material, but, again, the methods are only just becoming sufficiently refined and convenient to be applied more generally. In a typical hybrid dot assay nasal secretions or washings are treated to extract the nucleic acid and applied to a nitrocellulose filter. Then the DNA probe, previously labelled with radioactive phosphate or biotin, is applied to the area and allowed to hybridise with viral DNA or RNA and then the whole is washed. If the nucleic acid sequences in the specimen and the probe correspond then the probe is retained and can be detected by autoradiography or a suitable enzymic detection system. The method has been used for in vitro studies and examination of tissue or urine, for instance for cytomegaloviruses[7], but it has not been successfully developed for use in respiratory infections in general, although an ingenious modification of it has been used to detect adenoviruses[8] and another version detects these viruses in faeces[9]. Al-Nakib has found that cDNA from the 5’ end of several rhinoviruses detects RNA from a range of viruses, and can be used to detect homologous
virus in nasal washings [10]. The exact methodology used is critically important. We can expect such methods to be used more widely in research in the near future and they may eventually be applied in practice.

**Detection of IFNα**

There are genes for a number of IFNα molecules in human DNA and in response to infection with viruses these are particularly likely to be activated—as compared with those for IFNβ or γ. Furthermore, although only small amounts may be produced after mild infections such as colds, these can be detected by sensitive assays applied to good samples of secretions such as nasopharyngeal aspirates [11]. In more severe illnesses, such as influenza, enough IFNα may be formed to be detected conveniently in the serum. Thus, for the more severe respiratory infections, a serum assay may give useful supplementary diagnostic information [12]. Sensitive bioassays are available, and by combining these with neutralisation with specific antiserum the IFN can be positively identified and typed. Immunoradiometric assays are now available as an alternative. The reagents are expensive and unstable, but the method can be more rapid than bioassay. Certainly for research purposes IFNα assay can be a useful way of detecting infection with a wide range of viruses and in the future it may become available for clinical practice.

**Antibody responses**

**CF tests.** The standard methods for detecting a rising titre of antibody to a virus are well known. They consist of complement fixation (CF) tests for antibody against influenza A and B, adenoviruses, parainfluenza virus and respiratory syncytial virus (RSV). They are often called a ‘respiratory virus screen’ because of the nature of the antigens and the test. The results show broad specificity—for example, a positive response can be expected whatever serotype of influenza A or adenovirus has infected the patient. Certain bacteria may be included in the screen to test for Q fever and oromithosis. Similar batteries are also used for cases of meningitis or meningoencephalitis. CF tests may be supplemented with haemagglutination inhibition and other more specific tests; for rhinoviruses neutralisation tests are sensitive, but are worth doing only when one knows what virus is likely to have infected the patient, since they are highly specific and over 100 serotypes of rhinovirus are known. For coronaviruses, complement fixation tests have been used but are insensitive.

**ELISA tests.** Experience shows that for many purposes CF tests may soon be replaced by ELISAs. These are highly sensitive assays and use very small amounts of serum and antigens and have already been used in clinical research, eg for RSV and influenza. Further, the reagents are stable and non-radioactive. We have found that an ELISA is ideal for serodiagnosis of coronavirus infections [13] and Al-Nakib and his colleagues are now evaluating an ELISA for rhinovirus antibodies [14]. The situation is complicated by the number of virus serotypes, the presence of C antigens in damaged particles, and different responses by IgA and IgG class antibodies. Nevertheless, in due course it seems likely that assays will emerge that are more sensitive and convenient than the present neutralisation tests and will increase the possibilities for the serodiagnosis of infections with rhinoviruses and other organisms.

It is well known that additional information can be obtained by determining the class of antiviral antibody present: in particular, the first antibody to be found during an infection is likely to be IgM. In the past, methods of detecting IgM were laborious and slow. However, ELISA tests lend themselves to rapid and simple measurement of antiviral IgM—for instance, the IgM may be ‘pulled out’ of serum by placing the specimen on a plastic surface coated with anti-class serum: the antibody thus ‘captured’ can be shown in later stages of the test to bind a viral antigen. Alternatively, virus may be coated on to plastic and all the serum antibodies bound—the amount of, say, IgM may be estimated by using an antia IgM detector system. The tests may not be easy to set up, and in many respiratory virus infections specific IgM assay is not particularly informative, but they can be used to solve difficult problems like the detection of chronic enterovirus infections in myocarditis [15], although this has been supplemented recently by detecting virus RNA by hybridisation tests on cardiac muscle biopsy material. Similarly, the presence of IgM antibody suggests an active or recent infection [16] with rubella, hepatitis A or cytomegalovirus and may also be useful in certain respiratory virus infections [17].

Various tests of antibody to HIV have been developed for the diagnosis of AIDS and related conditions. Mostly these are straightforward ELISA binding tests using viral antigens derived from cultures of infected human cells, but the Wellcome test incorporates a further refinement in that the antibody is tested by competition rather than simple binding and the test is thus rather more specific and also requires fewer steps. To make quite sure that such a serious diagnosis is correct it is normal to confirm the result by using an independent test such as ‘Western blotting’ (ie binding the antibody to virus antigens separated out on an electrophoretic strip) or by immuno-fluorescent staining of infected cells.

**Some further developments**

With the advent of biotechnological products, further advances are being made. For instance, virus peptides can be produced by cloning viral nucleic acid or cDNA into expression vectors and harvesting viral antigen from bacteria, yeast or tissue cultures of mammalian cells. Thus purer, cheaper antigens may become available. There is an additional advantage in the case of dangerous viruses, like Lassa fever virus or HIV in that the virus itself is not propagated and thus special containment facilities are not needed.

It may not be necessary to synthesise a whole virus peptide, since the antigenic activity may all be limited to
groups of a few amino acids. Thus, oligopeptide antigens are now being made which detect antiviral antibodies. Such peptides and monoclonal antibodies can now be used in competition tests to determine the exact chemical site or epitope against which an antibody is directed. Studies of influenza and rhinoviruses have already shown that a single amino acid substitution can prevent a monoclonal antibody from binding to or neutralising a virus [18] and therefore a new generation of tests will become available. It should soon become possible to answer questions such as 'Does antibody against a particular chemical site on a virus protect against infection?'

Some abiding principles

Although we know that there are respiratory viruses that we still cannot identify [19], most of those that cause serious respiratory disease of children are now known. Methods of detecting infection with them are available and are being improved.

In spite of all the recent and forthcoming laboratory advances, our old principles must still be obeyed and the first of these is that there is no substitute for an initial full clinical evaluation of the patient. I still remember my helplessness when, as a laboratory worker, I received a request form stating only 'Virus agglutinins please'. It is quite impossible to do all possible tests for all possible viruses. The laboratory has to be guided by being told the type of case, time of onset and all the clues the clinician has picked up and also any suspicions there may be about the source of infection, e.g. possible drug abuse or recent travel in a specific developing country.

A second principle is that laboratory results have to be interpreted at the bedside. I well remember making a tentative diagnosis of an influenzal encephalitis and becoming fixed in this opinion when the laboratory showed that the patient was infected with an influenza virus, only to discover soon after that the patient had positive tests for neurosyphilis.

This example leads to the final principle that, as a matter of scientific logic, it is often possible to say that in groups of patients disease is associated with a particular virus infection. In the individual case we can only conclude that there is a higher or lower probability that the disease was due to the virus infection detected and therefore decide to manage the patient accordingly. That is what clinical medicine is all about.

This article is based on a paper given at the Paediatric Conference held at the College in October 1986.

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