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Respiratory Syncytial Virus and Other Pediatric Respiratory Virus Infections

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1. Introduction

1.1. Respiratory Virus Infection of Infants

Infants and young children undergoing their primary infection with common human respiratory viruses are at risk of serious, even life-threatening, lower respiratory tract infection. A multiplicity of viruses infect the human respiratory tract but a relatively small number are responsible for the majority of significant illness. Of these the most commonly diagnosed in the pediatric population is respiratory syncytial virus (RSV), which infects essentially all children in their first or second year of life, bringing approx 1% into the hospital with bronchiolitis or pneumonia *(1)*. Children with underlying cardiac or pulmonary disease, or born prematurely are particularly at risk. The virus also causes problems in the immunosuppressed and mortality rates are alarmingly high for RSV pneumonia after bone marrow transplantation *(2)*. The availability of therapy with ribavirin *(3)* or high titer anti-RSV γ-globulin *(4)*, which may be of benefit in these at risk groups, places a premium on rapid and accurate, but cost effective, diagnosis.

Significant lower respiratory tract disease may also result from infection with the parainfluenza viruses, the influenza viruses, and adenoviruses. Measles virus, currently relatively rare in countries operating a successful vaccination regime, may also be regarded as an important respiratory pathogen. Influenza C virus, the coronaviruses, the rhinoviruses, the reoviruses, and the enteroviruses, also commonly demonstrable in respiratory secretions, are generally considered less significant respiratory pathogens, mainly restricted to

the upper respiratory tract. Nonetheless all may be associated, at least occasionally, with serious lower respiratory tract disease.

1.2. Virus Diagnosis

There are a number of routes to the diagnosis of a respiratory virus infection, not all of which are suitable for routine pediatric use. Serological diagnosis has proved unreliable in infants, the younger of whom still possess transplacentally acquired maternal antibodies that can interfere with or mask serological responses *(1)*. The detection of viral nucleic acid in nasopharyngeal secretions by polymerase chain reaction (PCR) offers great sensitivity, but the precautions required to prevent false-positive results, and the greater expense and time taken to achieve a diagnosis make it currently unattractive for routine use *(5)*.

Demonstration of infectious virus by inoculation of secretions into cell cultures of high virus susceptibility, although necessarily laborious and slow, was for a long time the gold standard against which other techniques were measured. Although this approach can be speeded up by the immunofluorescence staining of early cultures with virus-specific monoclonal antibodies (MAbs), culture can never match the speed of direct antigen detection in specimens using either immunofluorescence or enzyme immunoassay. Although in most studies virus culture shows marginally greater sensitivity than the rapid techniques, occasionally antigen detection may succeed where virus culture fails. Whether antigen detection methods alone, without the backup of virus culture, are adequate to provide a reliable diagnostic service remains a controversial issue.

Enzyme-linked immunosorbent assay (ELISA) techniques are amenable to automation and attractive to laboratories with a large throughput. Although ELISA techniques are often relatively slow and of poor sensitivity, some rapid and sensitive commercial kits are now available that should be considered where the cost can be justified *(6)*.

Immunofluorescence staining of virus antigens in exfoliated cells collected from nasopharyngeal secretions is the most widely used technique for the demonstration of respiratory viruses in pediatric populations. Where suitable antibodies, evaluated directly on clinical material, are used, sensitivity and specificity are comparable with any other technique, with the possible exception of PCR *(5,7,8)*. In addition, the direct observation of the specimen provides useful visual feedback on specimen quality allowing negative results to be reported with greater confidence Using direct immunofluorescence with conjugated MAbs, results can be available within an hour with only minor sacrifice of sensitivity. Furthermore, suitable antibodies are available for all of the major and many of the minor viral respiratory pathogens (**Table 1**) which can thus be tested for on a single multiwell slide offering great flexibility at reasonable cost. It is this technique, therefore, that will be detailed here.

Table 1
Commercially Available Monoclonal Antibodies
to Respiratory Viruses[a]

Virus	Biosoft	Dako	Novo
Respiratory syncytial virus[b]	I, D	D	I, D
Influenza A virus	I, D	D	I, D
Influenza B virus	I, D	D	I, D
Influenza C virus			
Adenovirus group	I, D	D	I, D
Parainfluenza viruses[c]	I, D	D	
Coronavirus group	I, D		
Rhinovirus group[d]			
Enterovirus group		I	I, D
Reovirus group			
Measles virus	I, D		

[a]I, unconjugated antibodies for use in indirect immunofluorescence, D, antibodies conjugated to fluorescein for direct immunofluorescence, Biosoft, Biosoft/Argene antibodies, Parc Technoloqique (Varines, France) Dako, Dako Ltd , Glostrup, Denmark, Novo, Novocastra Laboratories, Newcastle upon Tyne, UK.

[b]Two antigenically distinct subgroups of RSV isolates, A and B can be differentiated with monoclonal antibodies. The clinical relevance of sub-grouping has yet to be established but a subgroup A-specific MAb is available from Biosoft

[c]MAbs to parainfluenza virus types 1, 2, and 3 are generally available but not to type 4

[d]A group-specific rhinovirus antigen has been described (9) but no monoclonal antibodies to this antigen are currently available.

1.3. Immunofluorescence Diagnosis
of Respiratory Virus Infections in Infants and Children

Diagnosis is made by demonstrating the presence of virus-infected respiratory epithelial cells desquamated into the nasopharyngeal mucus of the infected child. Infected cells are sedimented from samples of mucus, fixed onto a glass slide, and stained either with virus specific antibodies conjugated to fluorescein (the direct technique) or unconjugated antibodies subsequently labeled with a fluorescein-conjugated secondary anti-immunoglobulin (the indirect technique). Fluorescing infected cells are then visualized under a UV microscope.

Immunofluorescence staining is also valuable in identifying viruses isolated in cell culture in tubes or shell vials, and may be carried out 1–2 d postinoculation, which is often some time before cytopathic effect is evident (10).

The detailed descriptions of these techniques below are essentially those of Gardner and McQuillin *(11)*, with minor modifications to take account of more recent, commercially available reagents.

2. Materials

1   Sterile plastic mucus extractor (Henley's Medical Supplies, London, UK)
2. Sterile polythene feeding tube, size 8 (Sherwood Medical, Crawley, UK)
3. Vacuum pump. Hospital wards commonly have a suitable vacuum supply. The Laerdal Suction Unit (Laerdal Medical, Orpington, Kent, UK) is a convenient, portable, battery-operated pump with a maximum negative pressure of 600 mmHg (12psi)
4   Virus transport medium consisting of Hank's balanced salt solution (Oxoid/Unipath, Basingstoke, UK) containing 0 2% bovine albumin (Sigma Chemical, Poole, Dorset, UK), 100 IU/mL of penicillin and 100 μg/mL streptomycin (GibcoBRL, Paisley, Scotland), adjusted to pH 7.2 with 4.4% NaHCO$_3$
5   Sterile phosphate-buffered saline (Dulbecco 'A') (Oxoid/Unipath Ltd , Basingstoke, UK).
6. Teflon-coated multiwell glass slides with the required number of wells for cell preparations (Hendley Essex Ltd. Loughton, Essex, UK).
7   Moist box. Slides may be stained on two parallel swab sticks 2 5 cm apart in a plastic Petri dish containing a wet pledget of cotton wool or moistened sponge rubber to provide a humid atmosphere
8. Evan's blue counter stain: 0 5% in PBS (Sigma Chemical), diluted 1/1000 in PBS for use.
9. Fluorescein isothiocyanate (FITC)-conjugated antimurine IgG (Dako, Glostrup, Denmark)
10. FITC-conjugated antirabbit IgG (Dako, Glostrup, Denmark)
11 Antiviral antibodies Indirect immunofllorescence with carefully prepared polyclonal antisera, appropriately absorbed to remove nonspecific staining and extensively evaluated on positive and negative clinical material gives optimal sensitivity and specificity of viral antigen detection Such high quality reagents, however, are not generally available, and where they are interpretation of fluorescence staining requires an experienced eye. For the preparation of such antisera the reader is referred to Gardner and McQuillin *(11)*. MAbs, carefully selected for sensitivity and specificity on clinical material, are commercially available (**Table 1**) and offer an acceptable alternative in many situations (*see* **Note 1**) Direct immunofluorescence with fluorescein-labeled MAbs provides a simpler, more rapid, and easily interpretable test, but MAbs rarely offer the sensitivity achievable with polyclonal antisera. The lack of sensitivity may be improved by using a pool of MAbs to different virus epitopes, preferably on different proteins, which may both increase the number of cells stained and improve the level and pattern of staining within individual cells.
12. Fluorescence microscope. The fluorescence microscope must be equipped with a lamp and an excitation and barrier filter system capable of illuminating the speci-

men with light at close to 490 nm, the peak of absorption by fluorescein, and of transmitting only apple green fluorescent light of wavelengths around 517 nm to the eyepiece (*see* **Note 2**). The most suitable modern fluorescence microscopes are equipped for incidence light fluorescence with high numerical aperture low-power and high-power (×50 or ×63) oil immersion objectives (*see* **Note 3**).

3. Methods

3.1. Collection of Nasopharyageal Secretions

Secretions collected from the nasopharynx of children with respiratory infections are the material of choice for the identification of virus as they contain a larger number of infected cells than nose and throat swabs and are more easily available than secretions from the lower respiratory tract. The latter, however, are preferable if available. Here, the simple aspiration of nasopharyogeal secretions is described although some laboratories instill a small volume of buffered saline into the nose prior to aspiration *(12)*. Cough and nasal swabs are collected simultaneously as suction for nasopharyngeal secretions is not always productive.

Nasopharyngeal secretions, cough, and nasal swabs will generally be collected by ward staff who may require some training to produce material optimal for virus diagnosis (*see* **Note 4**).

1. Swab one of the child's nostrils and break the swab into 4 mL of transport medium held on melting ice
2. Swab the back of the throat until the patient gags and coughs onto the swab Break this swab into the same bottle of transport medium which is held in melting ice
3. Attach a sterile polythene feeding tube to the inlet of a sterile plastic mucus extractor. Attach the mucus extractor to a portable vacuum pump which has a maximum suction pressure of –26psi (239 kPa)
4. Switch on the pump and insert the feeding tube into the child's nasopharynx via each nostril in turn. Some children may show signs of distress, particularly if the nose is dry and secretions sparse and viscous. Secretions collect in the mucus extractor, although if few are present in the child's nose, they may lodge in the feeding tube and will require washing through with a little sterile PBS
5. Place the mucus extractor and the cough and nasal swabs in a vacuum flask on melting ice and transport to the laboratory with minimum delay.

3.2. Preparation of Infected Cell Suspensions (see Note 5)

3.2.1. Swabs and Secretions

1. Repeatedly pipet the medium over the swabs to remove adherent secretions. Remove the fluid containing the secretions to a centrifuge tube and centrifuge at 380*g* for 10 min at 4°C to pellet the cells. Remove the supernatant, which may be used for virus isolation.
2. Resuspend the cells in 3 mL of PBS by gentle pipeting and recentrifuge as above to obtain a cell pellet.

3 Centrifuge the mucus extractor containing nasopharyngeal secretions at 380*g* for 10 min at 4°C to collect all the secretion at the base of the vessel A small aliquot may be removed for virus isolation Add 2–3 mL of PBS to the remainder and disperse the mucus by gentle pipeting with a wide-bore pipet. Add further aliquots of PBS to about 10 mL, pipeting after each aliquot until the majority of the mucus is broken up Transfer the suspension, leaving any remaining mucus lumps, to a centrifuge tube and pellet the cells at 380*g* for 10 min at room temperature

4 Resuspend cell pellets (derived from swabs or from nasopharyngeal secretions) in a minimum volume of PBS to obtain a free running solution. The volume of PBS added will depend on the amount of mucus left on the cells The aim is to produce the most concentrated cell suspension achievable while diluting any remaining mucus beyond the point where it renders the suspension sticky. Mucus stains nonspecifically and also interferes with access of the staining reagents to the cells

3.2.2. Infected Cell Cultures (see **Note 5**)

1 Remove and discard medium from infected cultures of adherent cells.

2. Wash the cells gently with PBS and discard (*see* **Note 6**) the washings. Add a volume of PBS approximately equivalent to the original volume of the culture and scrape the cells into this using a Pasteur pipet for tubes or a plastic cell scraper for larger vessels. Pellet the cells by centrifugation at 380*g* for 5 min at room temperature

3 Discard the supernatant and resuspend the cells in PBS at approx 10^6 cells/mL

3.3. Preparation of Fixed Cells on Glass Slides (see Note 5)

1. Rinse Teflon-coated glass multiwell slides in absolute alcohol and wipe dry with a lint-free cloth. Engrave identification details on the free end and wipe again with a watermoistened cloth.

2. Place sufficient cell suspension on each well to cover the glass surface (15–35 μL depending on the size of the well). Larger wells, allowing more cells to be examined, will give an increase in sensitivity.

3 Allow to air dry (a hairdryer on a cold setting or a fan may be used to speed drying).

4 Immerse slides in cold acetone (4°C) for 10 min (*see* **Note 7**)

5 Dry at room temperature and stain immediately or store at or below –40°C indefinitely. (Samples stored next to the door can be repeatedly freeze/thawed on opening and closing)

3.4. Indirect Staining of Mixed Cell Preparations for Immunofluorescence

1 Place freshly prepared fixed cell preparations or those removed from –40°C storage in a moist box and allow to equilibrate to room temperature

2 Carefully spread one drop (10–15 μL) of relevant primary antibody or control reagent (*see* **Note 8**), diluted to the recommended working dilution in PBS, over each cell preparation (*see* **Note 9**). A bacteriological loop may be used to ensure that the whole well is covered.

3. Incubate for 30 min at 37°C in a moist box.
4 Gently rinse antibody from each slide with PBS applied via a Pasteur pipet
5. Immerse the slide in PBS in a Coplin jar or staining trough and soak for 10 min (*see* **Note 10**).
6 Discard and replenish the PBS and soak for a further 10 min Repeat, for three 10-min soaks total
7. Drain the slides and air dry.
8. Staining of the immunoglobulin-coated cells with the appropriate FITC-conjugated antiglobulin is carried out immediately as described below **(Subheading 3.5.)** for direct staining of cells.

3.5. Direct Staining of Fixed Cells for Immunofluorescence

This procedure may be applied to fixed cells already reacted with unconjugated antibodies by the indirect technique described in **Subheading 3.4.** using the appropriate species-specific FITC-conjugated anti-γ-globulin. Alternatively it may be used with FITC-conjugated antiviral monoclonal or polyclonal antibodies for single-step visualization of virus-infected cells. Suitable control reagents must be included in each test to ensure the specificity of the fluorescent antibody staining observed (*see* **Note 11**).

1. Dilute the relevant FITC-conjugated antibody to the recommended working dilution in Evan's blue counterstain (*see* **Note 9**) The diluted conjugate may be kept at 4°C but only so long as it remains sterile. Long-term storage of diluted reagent is not recommended
2. Carefully spread one drop of FITC-conjugated antibody over each cell preparation.
3 Incubate for 30 min (*see* **Note 12**) at 37°C in a moist box
4 Rinse as in **steps 4–7** of **Subheading 3.4.**
5 Immerse slides in distilled water and soak for 1–2 min to remove PBS, which will crystallize if allowed to dry on the slide
6 Allow to dry in air and either immediately examine microscopically immediately or store at 4°C in the dark in a closed container for examination the next day. Quality of staining deteriorates on storing of unmounted preparations, particularly if immersion oil has been applied Mounting in Fluokeep (Biosoft; TCS, Buckingham, UK) under a coverslip not only increases the intensity of fluorescence but protects the cells from the deleterious effects of immersion oil

4. Notes

1. A vast range of MAbs to viral antigens are available commercially and through other channels, but very few of these are likely to be suitable for diagnostic purposes. Some currently available MAbs or MAb pools designed for antigen detection in clinical material are listed in **Table 1**. Also noteworthy are the polyvalent pools of antibodies to RSV, the influenzaviruses, the parainfluenza viruses, and adenoviruses produced by some manufacturers. These allow one-test screening

for significant respiratory virus pathogens, reducing the costly screening of negative samples against multiple type specific reagents.

2. For more information prior to purchasing consult E O. Caul *(13)*

3. Not all objectives are suitable for fluorescence microscopy owing to autofluorescence of some components or excessive internal light scattering. Manufacturers should be able to give advice on those most suitable.

4 Adequate specimens must contain a reasonable number of respiratory epithelial cells derived from the nasopharyngeal epithelium Squamous epithelial cells, derived from the anterior air passages, are not generally infected

5. It is advisable to carry out the handling of material likely to generate airborne pathogens in a class 2 containment cabinet.

6 Cell cultures exhibiting extensive cytopathic effect may detach on washing In this case, the cells should be scraped and pipeted directly into the culture medium. Cells are pelleted at 380*g* for 5 min at room temperature, resuspended to original volume in PBS to wash, and recentrifuged to obtained a washed cell pellet. A similar procedure is adopted for virus-infected cultures of nonadherent cells

7 Acetone should not be stored in conventional refrigerators as it poses an explosion hazard A Coplin jar of acetone in an ice bath is convenient for fixing a small number of slides. For cell culture preparations, which are free from mucus, fixation may be reduced to 5 min.

8. Specificity of reagents cannot be taken for granted, particularly when clinical material is being investigated and adequate controls must be included to reveal non-specific reactions. The following should be included for the indirect test·

   a A cell preparation similar to the test specimen but known to be negative for the virus in question, stained in parallel with the test specimen Once sure of all the reagents and methods and familiar with the patterns of staining they produce, experienced workers may choose to omit this control.

   b. A duplicate preparation of the test specimen stained with a negative antibody as similar as possible to the virus-specific antibody in use, followed by the FITC-conjugated antiglobulin. Where a polyclonal antiserum from an animal is employed, the ideal control would be the preimmune serum from the same animal. This is rarely available for commercial products and here a serum from an animal immunized by a similar protocol with an antigen likely to be absent from the specimen (e g., another virus) must suffice. Where MAbs are employed a MAb of the same immunoglobulin class but specific for an irrelevant antigen known to be absent from the specimen should be used

   c. A duplicate preparation of the test specimen stained with PBS, instead of the virus specific antibody, followed by the FITC-conjugated antiglobulin.

9 Although the maker's instructions or advice from other laboratories may be taken as a guide, optimal working dilutions vary widely from laboratory to laboratory *(14)*. It is always advisable to titrate new antibody preparations in your laboratory. Normally, testing preparations fivefold either side of the recommended dilution on known positive control material will suffice.

10. For cell culture preparations, in which nonspecific staining is less troublesome, 5-min soaks will suffice.
11. In the direct test the controls should include:
    a. A cell preparation similar to the test specimen but known to be negative for the virus in question and stained in parallel with the test specimen.
    b. A duplicate preparation of the test specimen stained with a negative control conjugated antibody as similar as possible to the virus-specific conjugated antibody to be employed. Where a polyclonal antibody from an animal has been purified and conjugated, the most suitable control would be the preimmune serum from the same animal similarly purified and conjugated. Where such is not available, a similar conjugated antiserum from the same species but specific for an antigen known to be absent from the test specimen will suffice. Where conjugated MAbs are employed, a conjugated MAb of the same immunoglobulin class but specific for an antigen known to be absent from the test specimen should be used.

    The specificity of fluorescent staining can be confirmed in a subsequent blocking test. Here, preparations of the test specimen are preincubated with either unconjugated virus-specific antibody or unconjugated negative-control antibody. On subsequent staining with conjugated antibody, specific fluorescent staining will be blocked by the latter but not the former
12. In the direct immunofluorescence test this incubation period may be reduced to 15 min for many conjugated MAbs in line with manufacturer's instructions.

Acknowledgments

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