Recent advances in the rapid diagnosis of respiratory tract infection

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Molecular techniques have enabled major advances in the speed and sensitivity of the laboratory diagnosis of respiratory infections. Although the polymerase chain reaction is the most commonly used, there are several other methods available, which have applicability across the range of microbial pathogens.

This review does not intend to be a comprehensive treatise on the rapid diagnosis of respiratory infections. There are several excellent textbooks that cover the field in detail. It will, instead, focus on recent and future trends.

General considerations

Laboratory diagnosis is essential in the overwhelming majority of clinical conditions that require specific therapy. It is a misconception, for example, that streptococcal pharyngitis can be reliably differentiated on clinical grounds from viral pharyngitis in an individual patient. Probability estimates can be made (for instance common cold symptoms have an 80% negative predictive value for the diagnosis of bacterial pharyngitis), but confirmation is only after laboratory examination.

Most diagnostic advances in the last decade have focused on detection technologies. It should not be forgotten, however, that sampling inadequacies are the prime reason for failure to identify aetiology. It is well recognised that, for most respiratory viruses, the delay in transport of the specimen to the laboratory is usually the major factor in both slowness of the process and a failure to make a diagnosis. There has been some success in applying near-patient testing. This has, in general, made the diagnosis of respiratory tract virus infection more reliable and speedier. It has had particular use in differentiating respiratory syncytial virus cases who might need admission from other causes of viral respiratory tract infection.

Another important consideration is the type of specimen used. For lower respiratory tract bacterial infections, sputum is the gold standard specimen, but the quality of the specimen is key because oral and gastrointestinal secretions may contaminate it. Ideally, there should be fewer than 10 squamous cells and greater than 25 leukocytes in a low power (x100...
magnification) field. A report on the Gram stain provides the initial ‘diagnosis’ for empirical therapy. In practice, patients with COPD are able to provide satisfactory samples, but this is less often the case with patients who have pneumonia. In one study, only a third of 250 patients with clinically-diagnosed acute community-acquired pneumonia (CAP) admitted to hospital had Gram-stained smears which were useful for diagnostic purposes. With *Pneumocystis carinii* pneumonia (PCP) in which unproductive cough is common, induction of sputum with nebulised 3% salt solution in 10% N-acetylcysteine has been tried with moderate success. In difficult cases, transtracheal aspiration, transthoracic needle biopsy and broncho-alveolar lavage (BAL) have also been used. Of these more invasive techniques, there has been the best validation of BAL, but even this sample has the potential to be contaminated from the upper respiratory tract and strict diagnostic criteria should be used with the same organism being found either in another bodily fluid and/or by another specimen.

**Molecular probes**

Molecular probes have the advantages over conventional culture methods of greater speed, specificity and sensitivity. In addition, they may be the only applicable techniques as many micro-organisms are fastidious and cannot be easily grown for phenotypic analysis in the laboratory. Apart from detection, molecular methods are now becoming standard technologies for establishing quantitative estimates, particularly with regard to monitoring treatment, and resistance to therapy. Table 1 shows a comparison of molecular probes with more conventional methods.

Cheaper cultural methods will, no doubt, be used as a standard but there are four areas where nucleic acid based diagnosis will be the method of choice: (i) the identification and characterisation of fastidious organisms; (ii) rapid typing of isolates for epidemiological and other purposes; (iii) rapid determination of antimicrobial resistance; and (iv) identification of organisms in a non-cultivable state.

**Viruses**

Viruses possess either RNA or DNA unlike all other infectious agents, which possess both. From the viewpoint of probe technologies it is useful to know the type of target nucleic acid (Table 2). In theory, it should be possible to diagnose virus infections rapidly by electron microscopy and culture. In practice, the former is insensitive, requiring about $10^6$ virions to be present in a sample, and the latter slow and not always possible. Molecular probes, and gene amplification, overcome these problems and have become routine procedures in the diagnostic setting.
Detection of virus

Almost all human viruses have had probe methods described for their detection. The whole gamut of hybridisation test formats has been used but, generally, amplification methods are now employed.

Polymerase chain reaction (PCR) has had widespread applicability to the detection of viruses in a range of specimen types. The principle of this method is illustrated in Figure 1. For DNA viruses this is straightforward, for RNA viruses an additional reverse transcription step is required either by a separate reverse transcriptase (RT) enzyme or a DNA polymerase, such as *Thermus thermophilus* DNA polymerase, which has RT activity. Under appropriate conditions, the use of the single enzyme is not only more convenient but can be shown to be more efficient.

Some viruses can be routinely grown in cell culture but so slowly as to be clinically unhelpful. An example of this is cytomegalovirus. An innocuous virus mostly, it can cause life-threatening pneumonia in the immunocompromised. Standard cell culture techniques can take up to 3 weeks for positive isolation, although this can be speeded up by immunofluorescent or enzymatic detection of early antigens in 24–48 h. PCR can effect a diagnosis in less than 24 h. The extreme sensitivity of PCR is useful if the clinical features are suggestive, but if these are ambiguous may cause confusion. Positive results may occur because of latent virus, which is common in adults. The likeliest benefit will come from examining material from neonates and clinical specimens that would not normally harbour the virus. In one study in which primers were used that hybridised to regions of the MIE gene, all 44 culture-positive neonates were PCR positive in their urine but none of the 27 culture-negative neonates\(^4\).

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**Table 1** A comparison of molecular probes with conventional methods for the detection of micro-organisms

|                      | Culture | Immuno-fluorescence | ELISA | Non-amplification probes | Gene amplification methods |
|----------------------|---------|---------------------|-------|--------------------------|---------------------------|
| Speed to produce result | +       | +++                 | +++   | ++                       | ++/++/++                  |
| Sensitivity          | +++     | ++                  | ++    | ++                       | +++                       |
| Specificity          | +++     | ++                  | ++    | +++                      | +++                       |
| Quantifiability      | ++      | ++                  | +     | +                        | ++                        |
| Ease of use          | +       | +                   | +++   | +                        | ++/++/++                  |

**Table 2** Types of genome possessed by specific respiratory viruses

| DNA                  | Adenoviruses | Herpesviruses, e.g. cytomegalovirus |
|----------------------|--------------|-----------------------------------|
| RNA                  | Picornaviruses, e.g. rhinovirus | Coronavirus, e.g. human coronavirus 229E |
|                      |              | Orthomyxoviruses, e.g. influenza   |
|                      |              | Paramyxoviruses, e.g. respiratory syncytial virus |

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When viruses are expected to be present in low concentrations, nested PCR is often used in which initial amplification is followed by another round of amplification using primers internal to the first set. It adds to specificity; however, the increased sensitivity also makes the risk of false positives due to contamination greater. With respiratory specimens, which may have a number of different viruses or even mixed infections,
Multiplex PCR methods have been developed. This involves using probes to several different viruses in a single amplification reaction. Another use of multiplex PCR is to type viruses, such as parainfluenza viruses.

Although PCR methods have been the most commonly used, other nucleic acid amplification technologies have also been applied to viruses. Target amplification systems, which produce multiple copies of the target nucleic acid rather than the probe, such as self-sustained sequence replication (3SR) have been used to amplify viral genes. Probe amplification techniques such as ligase chain reaction (LCR) and Qβ replicase have also been used and are being developed commercially. LCR is useful for the detection of mutations. LCR depends on the ability of a thermostable enzyme, DNA ligase, to seal nicked double stranded DNA. If two primer probes bind contiguously to the target DNA, they can then be amplified by repeated hybridisation/disassociation cycles.

Qβ replicase is an enzyme derived from the Qβ RNA bacteriophage. This enzyme is capable of replicating a 221 bp RNA named MDV-1. If MDV-1 containing a probe is bound to target nucleic acid, this results in multiple copies of the recombinant. Qβ replicase amplification appears to be less sensitive than PCR because of high background amplification of unhybridised probe recombinant. Its advantages are that it can detect both RNA and DNA targets and is more easily quantifiable than PCR.

**Molecular epidemiology**

Viruses evolve through mutation and recombination events at a rate much faster than living organisms; this is greater in those with an RNA genome. It is helpful to be cognisant of this variation for a number of reasons: (i) to identify the spread of outbreaks; (ii) to understand the natural epidemiology of viruses including animal reservoirs; and (iii) to develop more effective vaccines. As genetic variation is a precursor of antigenic variation, subtler differences can best be detected by genome analysis. Multisegmented viruses exhibit gross changes by variation in size of those segments: these can be visualised by agarose or polyacrylamide gel electrophoresis. The genomes of large DNA viruses can be analysed by restriction fragment length polymorphism (RFLP) mapping (see below) if sufficient virus is available. Oligonucleotide fingerprinting is a useful method for typing RNA genomes. Digestion of the genome with an enzyme, such as ribonuclease T1, for defined periods results in fragments which can be separated by two-dimensional electrophoresis. If radiolabeled, the fragments can be visualised by autoradiography to form a characteristic pattern.

DNA probes are useful for determining defined sequence variants but, as with diagnostics, PCR has offered a more rapid and sensitive approach. Two methods have been commonly employed, PCR-restriction endonuclease (PCR-RE) and PCR-single-strand-conformation polymorphism (PCR-SSCP).
analysis. The former is also termed PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and involves restriction enzyme digestion of PCR amplicons. PCR-SSCP employs a non-denaturing polyacrylamide gel to compare melted single strands of amplicons. In theory, a strand with a single base mutation will migrate differently from the non-mutant type. In practice, this degree of resolution requires meticulous techniques.

Methods such as RAPD used for bacterial typing (see below) could also be applied to larger viral genomes and gene sequencing applied for the ultimate discrimination.

**Quantitative viral estimation**

Quantification of viral load is becoming increasingly important as a means of monitoring antiviral therapy. PCR, nucleic acid sequence-based amplification (NASBA) and branched chain DNA (bDNA) amplification methods have all been applied to quantification of viral load in the clinical setting: LCR and QB could also be applied.

Quantitative competitive PCR includes a target sequence mimic (imitator) which contains a template (control sequence) which is amplified as efficiently as the actual target. Viral load is then compared to the control load. ‘Real-time’ quantitative PCR, using Taqman technology that allows earliest detection of a positive signal rather than after a fixed number of cycles with subsequent post-PCR detection, has been shown to be more sensitive than tissue culture for the detection and differentiation of influenza A and B8.

NASBA is an isothermal RNA amplification system, which has a similar lower limit of detection than PCR. Like 3SR, it a transcription-based amplification system (TAS) which utilises 3 enzyme activities: RT, RNAse H and T7 RNA polymerase. An oligonucleotide probe primer is bound to target RNA and the RT makes a DNA copy. RNAase H removes the RNA portion of the RNA-DNA hybrid and allows a second probe primer to anneal downstream. RT then acts as a DNA-dependent DNA polymerase to extend from one probe binding site to the other. One probe primer has a T7 promoter site incorporated so that this enzyme can then produce a further RNA copy to allow the process to start again. Typically, a $10^9$ amplification can be achieved. Quantification is usually by competitive amplification with a control as with PCR. NASBA has an inherent advantage over PCR of not being as sensitive to differing amplification conditions and can be used with accurate quantification on a wide range of specimen types.

bDNA amplification does not require an internal control template to be quantifiable. It is a signal amplification method that uses branched chain DNA probes that can then act as substrates for further hybridisation reactions if the template is present initially. Consistency of test format is important as the viral load value is not equivalent for each
format. The more sensitive assays will have an advantage if complete reduction of viral load ever becomes a possibility on therapy.

**Measurement of antiviral resistance**

Even conventional phenotypic assays based on cell culture have not until recently been used routinely. This has partly been because of the paucity of effective antivirals, but also because antiviral resistance has not been frequently documented. This picture is slowly changing with more antivirals and increased use, so that antiviral susceptibility testing is now available in reference centres. Genotypic determination of viral (as opposed to clinical) resistance relies on identification of mutations that confer this state. Thus, for example, it is possible to detect mutations in the UL97 phosphotransferase gene and the UL54 DNA polymerase genes of cytomegalovirus that confer resistance to ganciclovir and/or foscarnet.

**Detection of novel agents of disease**

Novel respiratory viruses continue to be identified. The identification of such viral pathogens has been achieved by subtractive technologies, allied to amplification methods. The principle of subtraction involves the removal of common sequences in infected and non-infected material with complex sequences. When two samples of broadly similar sequences are compared, the control source is termed the ‘driver’ and the experimental (infected) source termed the ‘tester’. In general, a large excess of driver dsDNA is allowed to hybridise to single-stranded tester nucleic acid. Any non-hybridised single-stranded tester nucleic acid can be separated as presumed unique sequences. Subsequent gene amplification can be achieved by adding unique linkers to all nucleic acids. One of these methods, representational differential analysis (RDA) has been used to identify a novel herpes virus from a vascular tumour common in AIDS patients, Kaposi’s sarcoma. RDA simultaneously combines amplification and subtraction. After restriction enzyme digestion to reduce genome complexity, linkers are added to the products to enable subsequent PCR amplification. Smaller fragments are amplified most efficiently. Thus the resulting amplicons may represent only a proportion (typically 10%) of the DNA restriction fragments. By the judicious use of single restriction enzymes for digestion of the genome, it is possible to produce amplicons that represent the entire genome. This is done for both tester and driver DNA. Subsequent steps in the RDA process are shown in Figure 2. RDA is being applied to a number of diseases of potential viral aetiology.

The applicability of RDA is not, however, universal as the two nucleic acid sources required for the tester and driver need to be highly matched for practicability. Other methods, such as genome mismatch scanning might offer an alternative approach. This identifies regions of identity between two sources of nucleic acid. Suppression subtraction
Childhood respiratory diseases

Tester amplicon

Clone and analyze

Target-enriched difference product

Digest with restriction endonuclease

Digest ss-DNA with mung bean nuclease; PCR amplify

Ligate to dephosphorylated adaptors

Mix, melt and anneal

Hybrids

Fill in the ends

Add primer; PCR amplify

Linear amplification

No amplification

Exponential amplification

No amplification

Driver amplicon (in excess)

Fig. 2 Principles of RDA.
hybridisation (SSH) is an alternative to RDA, and is designed to selectively amplify differentially expressed cDNA and simultaneously suppress non-targeted DNA amplification.

The identification of a virus, or any other micro-organism, from patients with a disease is, of course, not sufficient grounds to establish cause and effect. Although not conclusive, the finding of nucleic acid within a diseased cell, but not a normal cell, provides convincing evidence of aetiology. *In situ* hybridisation enables this. As with other probe methods, *in situ* PCR is becoming the advancement of choice. *In situ* PCR can be direct or indirect. Indirect methods involve adding standard PCR reagents to the fixed cells and then post-amplification the products are detected by standard *in situ* hybridisation. Direct *in situ* PCR involves the use of a labelled dNTP in the PCR reaction so that it can be detected: a biotin- or digoxigenin-dUTP detected by labelled avidin or anti-digoxigenin are common examples.

*Bacteria*

Bacteria are responsible for the majority of currently treatable infectious disease and because of this an accurate rapid diagnosis and determination of susceptibility to antibiotics is important. In addition, typing is important for epidemiology and, ultimately, control of these organisms.

**Identification**

Bacteria have a circular DNA genome that represents its chromosome. In addition, there may be extrachromosomal elements on plasmids, which may encode virulence determinants. Methods have been developed which facilitate the detection of chromosomal, plasmid or total DNA in a wide range of bacteria. In addition, the advent of methods such as PCR has enabled the detection of previously undiscovered pathogens. Indeed, molecular methods have been important in the understanding of pathogenesis and have led to a revision of Koch’s postulates. The use of probes to 16S ribosomal RNA sequences is peculiar to bacterial diagnostics enabling both detection and phylogenetic analysis.

Slow-growing or fastidious bacteria are particularly appropriate targets for molecular diagnostics. The prime example of the former is *Mycobacterium tuberculosis*. Traditional methods of diagnosis, as with other bacterial pathogens, have relied on microscopy and culture. Microscopic identification with Ziehl-Neelsen (ZN) stain allows a rapid and inexpensive means of identification, but is not specific for *M. tuberculosis*. It is also well-recognised to be commonly false-negative. Culture on specialised media, such as Lowenstein-Jensen, takes several
weeks. Earlier identification of a positive culture can be achieved by radiometric detection of $^{14}$CO$_2$-labelled palmitic acid released from liquid media, or by activation of fluorescent dyes by CO$_2$ produced. Neither method is, however, as rapid as PCR-based methods.

In the main, PCR-based methods for $M$. tuberculosis use probes to either rRNA$^{10}$ or to a repetitive DNA sequence, IS6110$^{11}$, although methods based on the detection of other genes, such as those that code for 65 kDa$^{12}$ and 38 kDa$^{13}$ proteins have also been described. Utilisation of the IS6110 target has an inherent advantage in that, although both $M$. tuberculosis and the related $M$. bovis Bacille-Calmette-Guerin strain are detected, the differences in copy number of the target sequences within the two organisms results in differing patterns of amplification products. Mycobacteria can be found in many bodily fluids but most methods have been developed for use on sputum. Eisenach et al$^{12}$ examined 162 sputa for the presence of IS6110 genes. All those that were ZN smear positive were also PCR positive. One of two smear-negative, culture-positive samples were PCR-positive. Two of the 43 smear-negative, culture-negative specimens were also PCR-positive. 26 non-mycobacterial specimens were PCR-negative and one of 42 non-tuberculous mycobacterial specimens. Although the sensitivity was poor with smear-negative specimens, the sample was small. Other studies have supported the finding, however, that PCR-based methods are more sensitive than Ziehl-Neelsen stains$^{14}$. Although culture still appears to be more sensitive than single-round PCR, advances such as multiple primer and nested methods improve sensitivity.

Apart from PCR, the literature describes other amplification methods for the detection of bacteria. The diagnosis of $Chlamydia$ trachomatis by the probe amplification method, LCR (ligase chain reaction) has been shown to be both sensitive and specific. Nucleic acid sequence based amplification (NASBA) has been applied to the identification of mycobacteria using universal primers, with subsequent species-typing with specific probes$^{15}$. This method is isothermal and more reliably quantitative than PCR. Strand displacement amplification (SDA) has been described for the detection of $M$. tuberculosis. SDA relies on the ability of certain restriction endonucleases to nick double-stranded DNA. This nick is then extended at the 3’ end by an enzyme, Klenow polymerase, such that the downstream strand is displaced. This is an isothermal amplification method that is capable of generating a 10$^7$-fold amplification in 2 h at 37°C$^{16}$.

Many bacteria can exist in both a pathogenic and non-pathogenic state. Merely finding the organism does not imply that it is causing the disease. In this scenario, genotypic methods can be used to detect virulence determinants. Not all virulence determinants will be chromosomally mediated, but probes are equally suited to detect toxin genes on plasmids.
Genotypic methods of identification have the advantage over phenotypic methods of identification, such as zymotyping, of being less variable and dependent on assay conditions. The fact that the genes exist for, for example, a virulence determinant implies that the organism has the capacity to cause damage. Indeed it is now recognised that specific virulence genes are only expressed in the host but not under standard laboratory conditions. Gene detection is more reproducible than detection of the expressed products or their effects even under differing assay conditions.

The main drawback of most probe methods that are described in the literature is that they do not differentiate between ‘live’ and ‘dead’ bacteria. The most common approach to resolve this is to detect messenger RNA (mRNA) by RT-PCR. This has been successfully applied to the differentiation of, for example, viable from non-viable Legionella pneumophila. False positives due to contamination with minute quantities of DNA can be minimised by DNAse digestion of template or by RS-PCR in which the cDNA is tagged with a unique sequence so that only cDNA, produced by the RT step, is subsequently amplified.

Transcription-mediated amplification (TMA) systems have several advantages compared to PCR and LCR: they can use both RNA and ssDNA targets, increasing sensitivity, and the resulting RNA amplicon is labile so reducing the risk of contamination of subsequent testing. One system employs TMA of rRNA followed by a hybridisation protection assay (HPA). HPA uses acridinium ester-labelled probes to bind to the target amplicon. The addition of a selection reagent results in the hydrolysis of the acridinium ester which releases light detectable by luminometry. Methods are available for the detection of M. tuberculosis complex in respiratory secretions and C. trachomatis in urethral swab specimens and urine. Developments should enable these kits to be used with automated immunoanalysers, such as the VIDAS system, which are commonly used for serological assays.

**Typing of isolates**

Epidemiological typing of isolates is useful for tracking an outbreak of infection prospectively, and for finding a source retrospectively. The latter is its more common use, in practice, as the aim is to abort the spread of infectious disease. Although it is recognised that within one bacterial species, there may be many genetic clones, in clinical practice the source of an outbreak is most frequently a monoclonal. Genotypic typing is more discriminatory between related isolates than phenotypic methods: for example, it has been shown that Staphylococcus aureus isolates that were identical by zymotype and serotype were distinguishable by genotype, although isolates of an identical genotype were also of the same phenotype. As with their use in identification,
there are a number of targets for probes: (i) chromosomal DNA; (ii) plasmid DNA; and (iii) repetitive DNA.

Chromosomal DNA was the target for an early genotypic typing scheme for bacteria called BRENDA (for bacterial restriction endonuclease analysis). Chromosomal DNA is extracted from, ideally, a pure bacterial culture and digested with frequent-cutting restriction enzymes. Subsequent electrophoresis of the fragments results in an RFLP pattern. This technique is not as easily applied to bacteria as it is for some viruses because the much larger genome produces a large number of restriction fragments, some of which may be difficult to resolve on a gel. In an outbreak of 32 cases of *L. pneumophila*, this technique was used to incriminate a water system as the source. Although genotypic matching was found, phenotyping by monoclonal antibody showed that the patient and water isolates belonged to Pontiac and Bellingham types, respectively. These techniques are dependent on the organism being cultivable.

Gene amplification techniques are now more commonly employed for the typing of microbial isolates. The first described was arbitrarily-primed PCR (AP-PCR) in which an arbitrary primer is to detect amplification length polymorphisms. This has now been adapted as the RAPD (random amplified polymorphic DNA) method. Both methods have been generically described as DNA amplification fingerprinting (DAF). A short single primer oligonucleotide, five nucleotides or longer, is used so that binding is of low specificity to a large number of loci in the target DNA. These primers are typically of random sequence. After PCR amplification, complex banding patterns can be visualised in agarose or polyacrylamide gels. In theory it is possible to obtain a specific fingerprint. The original application of AP-PCR was to identify 24 strains of five staphylococcal species, 11 strains of *Streptococcus pyogenes* and three varieties of the rice plant, *Oryza sativa*.

Gene sequence analysis is the ultimate discriminatory tool but until recently has been too labour intensive for routine use. Automated methods using fluorescent chemical markers has made this a more practical proposition.

Plasmid DNA has been recognised, almost since antibiotics were first introduced, as a means of transferring genetic information, and particularly antibiotic resistance, between bacteria. This is regardless of the species of bacterium but is clinically most important amongst Gram-negative bacteria. Analysis of the plasmids that are carried, a plasmid profile, has been used commonly to investigate and characterise outbreaks of infection epidemiologically. The plasmids usually characterised are those that confer antimicrobial resistance, termed R (for resistance)-factors. These have been shown to be useful epidemiological markers of the spread of nosocomial infection within hospital settings.
pitfall with using plasmid profiles for epidemiology is that not all bacteria will carry them, or they have them in low copy number. Repetitive DNA is common in bacterial genomes. The identification of the differing frequency of repetitive sequences at differing positions within the chromosomal DNA allow a ‘fingerprint’ to be established. Chromosomal DNA is digested then hybridised to radioactive, or chemiluminescent, probes. The IS6110 sequence of Mycobacterium tuberculosis is present in low copy number and has been used to describe the epidemiology of tuberculosis as well as to identify the organism.

Rep-PCR involves the PCR amplification of regions of the genome between repetitive elements. Differing sizes of product occur as a result of variations between the repeat sequences. The repetitive sequences that code for tRNA are conserved and may have variable intervening genome lengths. With judicious use of primer probes to tRNA genes, it is then possible to produce DNA fingerprints with multiple bands or single bands of differing size which can be separated by migration rate on agarose gel electrophoresis. It has been possible to type streptococcal and staphylococcal species and other bacteria by this method.

Ribotyping (vide infra) can be used for epidemiological purposes and is particularly useful for investigating outbreaks due to those bacteria which are not easily typed by phenotypic methods. These include mucoid Pseudomonas aeruginosa strains, Legionella and Rhodococcus spp. Problems still occur, however, because these methods may be too discriminatory.

**Antimicrobial resistance**

Conventional determination of antimicrobial resistance consists of culture and identification of the organism and determination of growth in the presence of antibiotic as separate procedures. Gene probes can be used to determine the presence of genetic sequences coding for resistance or chromosome; if the latter, it may be possible to combine detection of the organism and determination of antibiotic resistance simultaneously. A number of genes responsible for antibiotic resistance have now been identified (Table 3). One of the best characterised is the mecA gene of Staph. aureus. This gene encodes a penicillin-binding protein and is present in the majority of methicillin-resistant isolates of Staph. aureus (MRSA), but absent in those that are susceptible. Detection of the mecA gene correlates strongly with phenotypic determination of resistance. In one study, in which the mecA gene was detected using PCR, 42 of 46 isolates of Staph. aureus and Staphylococcus epidermidis that were mecA-positive were also methicillin resistant. Bacterial cells were lysed then genetic material directly probed and amplified within one single day. This enables appropriate infection control measures and antibiotic therapy, if required, to be instituted more rapidly than by conventional
methods. The cost of the methodology is more than outweighed by the potential savings in health resource.

**Fungi**

The majority of the common fungal causes of human diseases can be rapidly diagnosed by Gram stain or potassium hydroxide microscopy. This is cheap and sensitive and would remain the method of choice for candidoses and dermatophyte infections. Even with chronic infections, such as zygomycoses, microscopy of infected material enables a diagnosis within minutes, compared to the hours that a molecular probe method would take.

Molecular probes will, however, have a limited role in diagnostic mycology. Life-threatening infections due to fungi are becoming more prevalent because of acquired immunosuppression due to AIDS and transplantation procedures. Rapid diagnosis of disseminated candidiasis and invasive aspergillosis could be improved by molecular methods. Currently, diagnosis depends on culture and/or serology. Both of these are insensitive, slow, or both. The use of antigen detection tests speeds up the process, but has less than 100% sensitivity or specificity, neither exceeding 70% in practice.

Although direct probe methods have been described, as with the rest of diagnostic microbiology, amplification methods are more likely to be used in a routine setting. Highly conserved ribosomal DNA (rDNA) targets have been used as non-genus specific targets for PCR amplification\(^{27}\). The use of PCR to detect genus-specific targets, such as the chitin synthase gene, have been shown to detect as few as 10 organisms in blood and other samples within 6\(^{28}\) h. PCR detection can also be used to monitor therapy. The use of 5S rDNA probes in a PCR amplification assay to detect \textit{P. carinii} in sputum samples from AIDS patients was used to monitor treatment with pentamidine. The shedding patterns correlated consistently with clinical

| Antibiotic resistance genes | Examples of organisms | Antibiotic(s) affected |
|-----------------------------|-----------------------|----------------------|
| mecA                        | \textit{Staphylococcus aureus, Staph. epidermidis} | Penicillins          |
| rpoB                        | \textit{Mycobacterium tuberculosis, M. leprae}   | Rifampicin           |
| katG                        | \textit{Mycobacterium tuberculosis} | Isoniazid            |
| TetK                        | \textit{Staph. aureus, Streptococcus spp.}       | Tetracycline         |
| TetM                        | \textit{Streptococcus, Staphylococcus, Neisseria, Haemophilus, Mycoplasma, Bacteroides, Bacillus spp.} | Tetracycline         |
| ErmA                        | \textit{Staph. aureus}                               | Erythromycin         |
| TEM-1                       | \textit{Neisseria, Haemophilus spp.}                | \(\beta\)-lactams    |
| AacC3                       | \textit{Pseudomonas spp.}                            | Aminoglycosides      |
outcome even though the organisms were not reliably detected by cytology\textsuperscript{29}. The specificity of PCR for the detection of \textit{P. carinii} is high, but its sensitivity is also high, higher than immunofluorescence, and in one study the organisms was detectable in the absence of clinical symptoms\textsuperscript{30}.

### Future prospects

Nucleic acid probes are becoming a standard diagnostic methodology in microbiology. Evaluation studies are already showing that for many microorganisms they are cost-effective when compared to standard cultural and serological methods. Automation is the key to the high through-put and rapid turnover of specimens that will be required. This will not only lead to a more specific and sensitive detection of micro-organisms, but drive down the financial costs of molecular diagnostics. The next major advance will be the incorporation of DNA chip technology into routine diagnostics. Probes are bound to a solid phase for detection of unknown, ‘interrogated’, DNA. The use of a solid phase to bind probes is not novel, being used in commercial PCR systems, but DNA chips involve a very large array of probes of known sequence on a solid surface, ‘the chip’. This technology has already been used, with hexamer probes, for DNA sequencing. It has also been applied, empirically, to detect mutations\textsuperscript{31}. With the increasing recognition that most bacteria are yet to be recognised, DNA chip technology, allied to sequencing, may speed up the process of establishing true bacterial biodiversity.

Biosensor technology to detect binding of bound probe also offers the prospect of rapid through-put and automation. One possibility is the use of membranes that respond chromatically to target-probe binding, \textit{e.g.} the diacetylenic lipids. Their use has already been described for the detection of bacterial toxins\textsuperscript{32}.

### Other specific technologies

The standard technique for rapid diagnosis by using immunofluorescence should not be forgotten. Although less sensitive than the molecular techniques described, they are well-established and are useful rapid screening tools for respiratory viruses.

Direct antigen detection methods are available and used for a number of bacterial pathogens, including \textit{Strep. pneumoniae} and other streptococci. These are reported to have a sensitivity of 60–95%, relative to culture, with adequate training in their use a key to minimising both false negatives and positives. In many laboratories, a dual swab/specimen system is used, one for direct antigen detection and another for routine culture.
ELISPOT (enzyme-linked immunospot) assays which detect antigen-specific T-cells are also coming out of research laboratories. One assay to detect *M. tuberculosis* is reported to allow earlier detection than the tuberculin skin test. These assays are quantifiable and have the potential to be automated for epidemiological screening.

Flow cytometry is a method whereby a specimen is passed through a ‘capture’ system that results in a detectable signal. Antigen, antibody or nucleic acids can be captured and depending on the signal used, common examples being fluorescence and change in electrical impedance, can be quantified. It has also been adapted for antimicrobial susceptibility testing. It has been applied to the detection of several viral and bacterial respiratory pathogens but primarily in a research setting. Although it is possible to obtain results within 4 h, the method is still hampered by specificity issues and the costs of setting up the technology.

### Non-invasive markers

Non-specific markers of airways inflammation are being evaluated to allow early detection of inflammation of the lower respiratory tract, particularly in those at-risk patients with chronic lung disease, which may signal infection. The most studied of these is the measurement of exhaled gases such as nitric oxide (NO), carbon monoxide (CO), ethane, pentane and non-volatile substances in breath condensate such as hydrogen peroxide.

An example would be high levels of exhaled NO which seem to occur in exacerbations of chronic obstructive pulmonary disease (COPD). This rise in NO levels occurs in both viral and bacterial infections: indeed there is often a rise in viral upper respiratory tract infections in patients without COPD. Nasal NO rises have also been used as markers of nasal inflammation in allergic rhinitis and could also be used as a beacon of upper respiratory tract viral infection although this has not yet been reported.

The analysis of breath condensate where a patient breathes through a mouth piece into Teflon tubing that is connected with a dry ice container also offers utility as a non-specific marker. The 1 ml of breath condensate that can usually be collected after 5 min of breathing can be assayed for inflammatory mediators such as tumour necrosis factor and interleukin-1. This has not yet been applied to the early diagnosis of infection *per se*, but there have been short reports of studies in detecting early inflammation in asthma and COPD.

### Conclusions

The pace of laboratory diagnosis is possibly greater than in other areas of clinical practice. The future is likely to be focused on rapid, automated tests.
Key points for clinical practice

- Adequate specimen collection and transport are an essential part of rapid microbiological diagnosis
- Consider more than one specimen sample to enable several techniques to be applied
- Laboratory methods are automated and should enable faster results
- It is essential to understand the basis of the new technologies if the correct interpretation is to be made of the result

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