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and every procedure involving blood (phlebotomy, surgery, dialysis, endoscopy, transfusion) carries a risk of spread of infection to hospital staff or other patients. The risk is greatest when contaminated hollow-bore needles cause a deep injury, or when blood contaminates open wounds or mucosal surfaces (including the eye).

Cross-infection with blood-borne viruses is rare because of control measures, including sterilization of surgical instruments before use, and single use or sterilization of needles and other pieces of equipment that come into contact with sterile tissues and blood. Correct handling of blood samples and disposal of used needles and other contaminated sharp equipment is also important. In the past, attempts were made to identify likely virus carriers and take greater precautions with them, but it was realized that carriers were often unrecognized. More recently, the concept of ‘universal precautions’ has evolved, based on the fact that any patient or sample might contain blood-borne viruses and should be handled with this risk in mind. Contaminated sharps waste should be disposed of in specific yellow, rigid (needle-proof) containers, which are then incinerated. Re-sheathing of needles after use was a major cause of sharps injuries and is now strongly discouraged.

Controlling and preventing nosocomial infections

The methods of controlling and preventing hospital infections are as varied and complex as the infections themselves, and range from hospital design to vaccination policies to personal hygiene. Some are discussed above, and summarized in Figure 4.

Molecular diagnostic techniques

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The impetus for the development of new diagnostic techniques comes primarily from three sources:

• the need for more rapid diagnosis of infections caused by micro-organisms that are slow or difficult to grow (e.g. mycobacteria) or cause acute fulminating disease
• a search for highly sensitive methods that can detect low pathogen numbers (e.g. as found in early meningitis)
• the increasing importance of old, or discovery of new, pathogenic micro-organisms (e.g. hepatitis C virus – HCV).

Despite initial optimism, many new diagnostic techniques are never accepted into routine practice, usually because they are too labour intensive and/or expensive. Also, though new tests may be theoretically more sensitive than traditional culture-based methods, problems with specificity and reproducibility between centres may be significant. For example, quality-control surveys of laboratories that have introduced polymerase chain reaction (PCR)-based methods for the detection of mycobacterial or HCV DNA/RNA found that false-positive results were widespread. In response

What’s new?

• Real-time PCR enables detection and quantification of pathogen DNA from clinical specimens in a single test
• Using DNA microarrays, many chemiluminescent or fluorescent probe-based assays are performed simultaneously on a single slide or ‘chip’
• Highly discriminatory classification systems have been developed for several infectious agents, based on the comparison of DNA sequence variation

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to similar findings in the USA, an independent quality-assurance programme has been established by the Virology Committee of the AIDS Clinical Trials Group; for continuing certification, each participating laboratory must correctly test eight whole blood samples, and must include HIV-1 DNA copy standards with each PCR assay performed. As increasing efforts are made to standardize methodology and to identify and eliminate factors responsible for reduced sensitivity and specificity, new techniques are gaining widespread acceptance.

Another problem has been how to validate the accuracy of nucleic acid tests that may be more sensitive than any previous technology (i.e. better than the ‘gold-standard’ test). In practice, this situation is resolved by comparison of the new test with results from multiple different alternative testing methods (discrepant analysis).

DNA probes (Figure 1)

A wide variety of established DNA probe-based techniques (e.g. Southern blot, dot blot, in situ hybridization) are commercially available as identification kits. In direct hybridization, target nucleic acid amplification is not performed first; larger specimens are therefore required and sensitivity tends to be lower. Generally, use of DNA probe technology is economically viable only for identifying relatively common pathogens in the patient population under investigation, and/or for micro-organisms for which conventional methods are unsatisfactory (e.g. Mycobacterium tuberculosis, M. avium–intracellulare complex). In immunosuppressed patients, rapid identification of fungi in clinical specimens can be of great importance in clarifying diagnosis and treatment.

AccuProbe has been successful in the identification of Cryptococcus neoformans, Histoplasma capsulatum and Blastomyces dermatitidis, and can identify mycobacterial isolates with high specificity (> 99%) using DNA probes complementary to target rRNA sequences (which are highly conserved and present in multiple copies). The test takes only 1 hour and the system performs well on cultured bacteria, but is not sufficiently sensitive for identification of isolates directly from clinical specimens. This problem has been partially overcome with the introduction of Hybrid Capture systems.

Hybrid Capture involves signal amplification rather than target amplification to increase sensitivity. Resultant DNA/RNA
hybrids are detected using a chemiluminescent enzyme-linked immunoassay in which the signal can be amplified more than 3000-fold. DNA probe Hybrid Capture systems are now available for the detection of human papillomavirus (HPV), cytomegalovirus (CMV), Chlamydia trachomatis, Neisseria gonorrhoeae and hepatitis B virus (HBV).

DNA microarrays (Figure 2) have considerable potential in diagnostic microbiology. To overcome the need to undertake multiple assays to cover different possible pathogens, large numbers of oligonucleotide probes are immobilized on glass slides, where they can hybridize specifically with target nucleic acid sequences present in material extracted from clinical samples. A single assay (or ‘chip’) can therefore detect and identify large numbers of infectious agents in parallel at the genus or species level. Target material is detected using chemiluminescent or fluorescent reporter systems, the sensitivity of which may be increased when used in conjunction with PCR analysis. This sequence-specific extension and dye-labelling of captured nucleic acid target sequences has been successfully applied to the detection of rifampicin-resistant strains of M. tuberculosis.

It has been estimated that, in clinical applications, chip technology can reliably detect infectious agents within 1 working day. However, the highly complex task of optimizing specific systems coupled with the high cost and relatively low flexibility currently limits its application in routine clinical microbiology diagnosis; only technical studies involving Listeria, Cryptosporidium and rotavirus have been undertaken.

Nucleic acid amplification techniques (Figure 1)

PCR analysis: many different PCR-based techniques have been developed to detect and amplify nucleic acid sequences of infecting micro-organisms (Figure 3a). These techniques can detect DNA or RNA specific to certain micro-organisms and do not rely on recovery of the micro-organism or the ability of the individual to recognize the pathogen (i.e. to mount an immune response). Amplified products were conventionally detected by comparing the position of bands obtained following electrophoresis with the profile of known standards (Figure 4a) and after transfer of DNA from gel to nitrocellulose paper (Southern blotting). Hybridization of bands with complementary standards (probes) has also been used to confirm the identity of amplified nucleic acid (Figure 4b).

Although amplification-based specific detection initially appears an appealing approach, several disadvantages have limited its application in routine practice.

- False-positive results may be caused by nucleic acid contamination from other clinical samples and PCR products from previous amplifications.
- False-negative results may originate from the low sensitivity of the PCR system, or from the presence of DNA or RNA polymerase

DINOSIS OF INFECTION

Polymerase chain reaction

| Template DNA | Original target sequence | Cycle 1 | Primers, deoxyribonucleoside triphosphate and DNA polymerase |
|--------------|-------------------------|---------|---------------------------------------------------------------|
| Two copies   | 5'                      | 3'      | -                                                             |
| Four copies  | 5'                      | 3'      | -                                                             |
| Eight copies | 5'                      | 3'      | -                                                             |

Target sequence: Primer: New strand

Each cycle of a typical PCR reaction comprises three stages performed at different temperatures – DNA denaturation, primer annealing and amplicon extension. Each cycle results in a net doubling of template molecules to be amplified in the next cycle of the reaction.

TaqMan reporter probe

| DNA denaturation followed by annealing of primer and reporter probe |
| 5' | 3' |
| Amplicon extension causing, displacement of reporter probe |
| 5' | 3' |
| Amplification cycle complete, accompanied by fluorescent signal |
| 5' | 3' |

b As a consequence of primer-directed synthesis, a fluorescent signal is emitted during each amplification cycle. A reporter probe is designed to anneal within the target region. The probe is labelled with both a reporter dye and a quencher dye. When the probe is intact, the dyes are in close proximity and the reporter dye is quenched. During amplicon extension, the probe is displaced and cleaved by exonuclease activity of the DNA polymerase. The reporter and quencher dyes are separated and a fluorescent signal is detected. In this manner, a positive signal is produced only from amplification processes specified by the PCR primers and probe.

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inhibitors (particularly several factors in blood) in the clinical sample.

Commercial PCR kits now generally include built-in controls at each stage to detect potential contamination and/or inhibition of the test.

**Real-time PCR** is a more recent innovation enabling detection and quantification of amplified DNA as the PCR reaction proceeds, thereby removing the need for time-consuming post-amplification processes (e.g. gel electrophoresis). A fluorescent signal is produced during each amplification cycle as a consequence of primer-directed synthesis; the intensity of the signal increases in proportion to the amount of amplified product generated. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of target DNA is first detected rather than by the amount of target accumulated after a fixed number of cycles (Figure 5). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The number of target copies in the original sample can be established by processing alongside internal control reactions with nucleic acid of known concentration.

The first detection systems using this technique relied on fluorescent dyes that bound nonspecifically to DNA molecules in the PCR reaction (SYBR Green I). A more recent innovation uses specific probes designed to anneal within the target region. If successful amplification occurs across the occupied site, the probe is displaced and a fluorescent signal is emitted (TaqMan technology, Figure 3b).

**Typical real-time polymerase chain reaction results**

Reactions are characterized by the point in time during cycling when amplification of target DNA is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. For example, undiluted DNA is detected at cycle 16, whereas 1:1000 diluted DNA is not detected until cycle 26.

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**Figure 5**

- **4a** Electrophoresis of clinical samples following two polymerase chain reactions using different primers, one specific for the genus *Mycobacterium* (top), one for *M. tuberculosis*. Inhibition controls were added to the top reaction to check that DNA replication was not prevented; these are represented by the highest molecular weight bands (lanes 12 and 14 are negative – hence, these samples contain inhibitors of DNA polymerase). Lane 11 contains a positive control and lane 10 is positive in both reactions for mycobacterial DNA. Lanes 1 and 16 contain molecular size standards. 
- **b** The products of the bottom reaction were transferred by Southern blotting and then probed for a sequence within the amplified DNA. Lanes 10 and 11 are again positive, confirming the results of the polymerase chain reaction analysis.
**Other amplification methods:** probably as a result of the comprehensive patent protection of PCR technology and commercial interest in diagnostic procedures, several alternative nucleic acid amplification methods have been devised.

**Ligase chain reaction** (LCR) has many similarities with PCR technology. LCR amplifies very short DNA fragments comprising a pair of oligonucleotide primers that have become joined through the presence of thermostable DNA ligase in the amplification mixture. Only primers at a precise position on the DNA template are joined, and only ligated primers are amplified successfully. Thus, LCR is more stringent than standard PCR, and has been applied commercially to the detection of *N. gonorrhoeae*, *C. trachomatis* and *M. tuberculosis* with excellent sensitivity and specificity.

**Nucleic acid sequence-based amplification** (NASBA, also termed ‘self-sustained sequence replication’ – 3SR) allows amplification of specific RNA fragments. The technology combines three different enzyme activities at the same temperature, thereby eliminating the need for temperature cycling equipment, and can amplify an RNA signal by more than 10⁶-fold in only 30 minutes. Use of NASBA/3SR has concentrated on detection of HIV-1 and *M. tuberculosis*.

**Strand displacement amplification** (SDA) is similar to 3SR isothermal technology except that DNA is amplified and the system operates at higher temperatures. Clinical experience with this technology concentrates mainly on the detection of mycobacteria and, most recently, *C. trachomatis* and *N. gonorrhoeae*.

**Branched DNA signal amplification** (bDNA) is similar to Hybrid Capture systems in that a colorimetric/chemiluminescent signal is amplified rather than target DNA/RNA. Because no nucleic acid amplification occurs, the risk of contamination is reduced. This system has recently been applied to the detection and quantification of HIV-1, HBV, HCV and CMV.

**Previously uncultured microorganisms:** PCR has been successfully used to identify previously non-culturable micro-organisms such as *Bartonella* (*Rochalimaea*) *henselae* (cat-scratch fever and bacillary angiomatosis), *Ehrlichia chaffeensis* (ehrlichiosis) and *Tropheryma whippelei* (Whipple’s disease). A similar approach showed that a coronavirus was the infectious agent responsible for severe acute respiratory syndrome. In such cases, primers have been used to amplify regions of evolutionarily conserved microbial ribosome genes; these are then sequenced and the results compared with genetic databases of known micro-organisms to confirm the identity of these ‘new’ infectious agents. This approach (broad-ranged PCR) has also been used in the detection and sequence-based identification of bacteria from otherwise sterile clinical specimens (e.g. whole blood, CSF), supplementing diagnosis of infective endocarditis and sepsis in the acute critical-care setting.

**Difficult-to-grow organisms:** because DNA amplification-based detection systems were initially too complex and expensive for use in diagnostic laboratories, they have generally been used in the more difficult areas of pathogen detection (viruses, slow-growing, fastidious or non-culturable bacterial pathogens, certain fungi and protozoa). For these organisms, the current alternatives (culture or serology) are slower than nucleic acid amplification methods, are equally labour intensive, or lack sensitivity; in some cases, there are no alternatives available. Many laboratories originally developed in-house PCR diagnostic methods, but these were often poorly reproducible. Commercial amplification-based kits include tests for HCV, herpes simplex virus (HSV), HIV-1, *M. tuberculosis*, *C. trachomatis* and *Legionella* spp. Results are available the same day and are claimed to be highly sensitive and specific.

**Established molecular diagnostic tests in infectious disease**

Several nucleic acid detection tests are now widely available and are becoming accepted as the new gold-standard diagnostic methods.

**Clinical management of HIV-positive patients:** HIV RNA load-testing is routinely performed by quantitative PCR analysis, which has become a cornerstone of management in HIV disease. Viral load testing (Figure 6), in conjunction with clinical features and other laboratory markers (e.g. CD4 count), is used to assess prognosis and for therapeutic monitoring of antiretroviral therapy in HIV-1 infected patients. Periodic monitoring of viral load is important for prompt identification of treatment failure, possibly through the emergence of resistance.

**Clinical management of HBV and HCV:** detection of HBV DNA and HCV RNA is now integral to the management of hepatitis B and C.

- A rapid and sustained decrease in HBV DNA in patients receiving therapy can be used to predict a favourable outcome in acute HBV infection.
- Early detection of HCV RNA allows early clinical intervention, which may improve the effectiveness of antiviral therapy. Detection of HCV RNA demonstrates viral replication, indicating active HCV infection.

**Plasma viral load in HIV infection**

Measurements of plasma viral load are now used routinely in clinical practice. Serial measurements are a powerful predictor of risk of disease progression. Such measurements can also help to determine when to initiate antiretroviral therapy, to evaluate the effectiveness of an antiretroviral regimen, and to identify when such a regimen is failing.

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infection. Response to treatment is determined using HCV RNA testing at the conclusion of therapy and on follow-up 6 months later.

Several amplification systems are available for the quantification of HBV and HCV nucleic acid in clinical specimens. The COBAS AMPLICOR HBV and HCV MONITOR tests (PCR) and the VERSANT HCV 3.0 RNA Assay (bDNA) are commonly used viral load monitoring systems. A significant proportion of patients with no detectable HCV RNA after the usual treatment subsequently relapse and exhibit detectable levels 6 months later. In this situation, qualitative HCV assays (e.g. COBAS AMPLICOR HCV v2.0, VERSANT HCV RNA), which are more sensitive than the real-time PCR monitoring assays, identify a greater number of patients in whom HCV persists in the blood.

**Diagnosis of M. tuberculosis** infection: detection of **M. tuberculosis** and other mycobacteria has been revolutionized by nucleic acid testing; in particular, the time required to achieve a result has been reduced from weeks to 1 or 2 days in some cases. Most commercially available systems have been evaluated only against smear-positive respiratory specimens, and investigations of other, equally important specimens still rely on ‘in-house’ nucleic acid detection assays. Available kits include BD ProbeTec ET (real-time SDA) and COBAS AMPLICOR MTB for the detection of **M. tuberculosis** complex (**M. tuberculosis**, **M. bovis** BCG, **M. africanum**, **M. microti** and **M. canetti**) direct from clinical respiratory samples. Collectively, these and other systems (including AccuProbe) offer tests for the identification of **M. avium**, **M. intracellulare**, **M. kansasii** and **M. gordonii**, optimizing detection of opportunistic infections in immunosuppressed patients. Systems are also available for simultaneous detection of **M. tuberculosis** complex and the genes responsible for antibiotic resistance (e.g. rifampicin – INNO-LIPA Rif. TB), enabling early detection of multi-drug-resistant **M. tuberculosis** strains.

**Diagnosis of C. trachomatis** infection: a population-based screening programme for the detection of active genital chlamydia was recently initiated in England, as outlined in the Department of Health’s National Strategy for Sexual Health and HIV. This initiative has increased demand on diagnostic laboratories providing **C. trachomatis** testing and has created a need for more automated, high-throughput assays. Nucleic acid amplification tests can be performed successfully on cellular material from non-invasive (urine) specimens, in addition to conventional urethral and endocervical samples. Specimens can be processed much faster than in conventional tests (tissue culture, enzyme-linked immunosorbent assay – ELISA). Furthermore, this amplification-based technique has much greater sensitivity, improving detection of asymptomatic infections. These techniques are the methods of choice in the Department of Health screening strategy.

**Diagnosis of viral and bacterial meningitis/encephalitis**: **HSV** is the most commonly detected virus in laboratories. Because of the availability of potentially life-saving therapy against **HSV** in patients with meningococcal encephalitis, rapid detection of this virus is important. Conventional methods using clinical specimens (including cell culture, ELISA, latex agglutination, nucleic acid probes and fluorescent antibodies) are either time-consuming (24–48 hours for tissue culture) or of low sensitivity when low titres of **HSV** are present in the sample. Nucleic acid amplification techniques, particularly PCR analysis, are more sensitive. Recent evaluations involving the LightCycler system (real-time PCR) have reported that results can be available within 2 hours of receipt of the specimen in the laboratory, and that **HSV** detection is better than with the gold-standard method, virus culture. In addition, this system clearly determines **HSV** type (1 or 2), aiding effective management of the infection.

Nucleic acid amplification tests have improved diagnosis of meningococcal sepsis. This approach can detect meningococcal-specific DNA in blood (or other specimens), and can also identify the **N. meningitidis** group and thereby provide early information on cluster recognition (Figure 7).

**Typing techniques (Figure 1)**

**DNA fingerprinting and typing**

Techniques based on micro-organism genotype rather than phenotype (e.g. whole cell protein and lipopolysaccharide profiles, antibiotic susceptibility profile, biochemical tests) are now used preferentially to determine the relatedness of clinical isolates. These techniques are useful in the investigation of outbreaks of infection, and for determining routes and sources of infection; for example, HCV RNA analysis provided evidence that an infected surgeon may have transmitted HCV to five of his patients during open-heart surgery. Molecular typing techniques are also powerful epidemiological tools, and have been used to identify women who are more likely to progress to high-grade squamous intra-epithelial lesion status or to develop cervical cancer following infection with high-risk HPV types such as 16 and 18.

It is important to note that the discriminatory power of these genomic techniques may vary significantly when applied to different species. It should not be assumed that a single fingerprinting method will be superior for any given collection of isolates.
Plasmid profiles are useful only when the strains contain plasmids (which may also be unstable), and are of limited discriminatory power. Chromosomal or plasmid DNA can be examined or specific sequences targeted after amplification using PCR (e.g., restriction fragment length polymorphism and single-strand conformation polymorphism analyses).

Restriction endonuclease analysis (REA) – DNA may be ‘fingerprinted’ by cutting with restriction endonucleases (which recognize specific nucleotide sequences) and then separating the resulting fragments by electrophoresis. DNA probes may also be used to identify bands containing the complementary target DNA sequences.

Pulsed-field gel electrophoresis (PFGE) – some restriction endonucleases cut bacterial chromosomes into DNA fragments that cannot be separated using conventional electrophoresis, and PFGE must be used instead. This technique is more difficult to perform, but the DNA fingerprints obtained are generally less complex than those obtained by standard restriction endonuclease digests (because fewer but larger DNA fragments are produced). Although PFGE is currently recognized as the gold-standard molecular typing technique, some bacterial isolates have become non-typeable because of unstable DNA (e.g., certain strains of Clostridium difficile). Recently, systems have emerged to facilitate exchange of DNA fingerprints electronically via the Internet. ENEMTI is a network of the European Laboratories Scientific Research Foundation that aims to standardize methods and data exchange protocols for Internet-based comparison of microbial fingerprinting data. CDC-PulseNet is a network of public health laboratories in the USA and Canada that performs and exchanges PFGE typing on food-borne pathogens including Campylobacter, Escherichia coli O157, Listeria monocytogenes, Salmonella and Shigella.

Ribotyping is a powerful typing technique that uses rRNA probes to detect complementary sequences in restriction endonuclease digests of chromosomal DNA, with resultant relatively simple banding patterns.

PCR-based typing

Random amplified polymorphic DNA (RAPD)/arbitrary primed PCR (AP-PCR) – there are subtle differences between RAPD and AP-PCR techniques, but the terms are often used interchangeably. These methods enable detection of genetic polymorphisms of an individual bacterial strain without prior knowledge of the target nucleotide sequence. At low-stringency temperatures, primers whose sequences are not directed at any specific sequence in the genome hybridize at random sites along the bacterial DNA. In turn, fragments of amplified material form ‘fingerprints’ that differ according to the degree of relatedness of the strains under investigation. The specificity of the amplification process depends on primer–target interactions, and variations in these components can affect the reproducibility of the system.

Rep-PCR is similar to RAPD/AP-PCR, but is not randomized because the primers are based on specific sequences that appear repeatedly throughout the genome of the test organism.

Ribospacer PCR exploits strain-specific differences in the spacer regions of 16S–23S ribosomal RNA in bacteria. The system is based on specific primer-directed amplification and is therefore more stable and reproducible than RAPD/AP-PCR. Generally, fewer DNA fragments per profile are produced, and the results are therefore slightly easier to interpret than RAPD/AP-PCR and certain rep-PCR profiles.

Amplified fragment length polymorphism (AFLP) combines REA and PCR techniques. Bacterial DNA is initially cut into fragments using restriction endonucleases. Adaptor oligonucleotides are ligated onto these digestion fragments to facilitate their amplification by PCR. DNA fragments are resolved using agarose gel electrophoresis, producing a fingerprint based on the organization of restriction sites along the bacterial chromosome. AFLP exploits the discriminatory power of REA, while producing clearer and more easily interpretable DNA fingerprints. Using DNA digests from more than one enzyme increases the discriminatory power of this technique.

Variable-number tandem repeats (VNTR)/mycobacterial interspersed repetitive units (MIRU) – the genome of M. tuberculosis contains 41 genetic loci with repeat DNA sequences. The number of repeats per locus varies between strains, and this variability is the basis of M. tuberculosis typing. VNTR is associated with tandem repeat sequences in six genetic loci found to have high variability and therefore achieves the best strain discrimination. More recently, the MIRU technique has exploited this variability in twelve such loci, and a combination of VNTR and MIRU is used for strain classification in M. tuberculosis epidemiology.

Sequence-based typing

Single-nucleotide polymorphism (SNP) analysis and multi-loci sequence typing analyse bacterial genetic information directly by nucleotide sequencing rather than indirectly using the electrophoretic mobility of DNA fragments. SNPs are DNA sequence variations that occur when a single nucleotide in the pathogen genome sequence is changed. A highly discriminatory bacterial classification system is produced by the sequencing and comparison of one genetic locus (in SNP analysis) or several different loci (in MLST). These techniques produce typing data that are both unambiguous and portable and therefore can be exchanged via the Internet. Such web-based databases are appearing for pathogens including Staphylococcus aureus, N. meningitidis, Haemophilus influenzae, Campylobacter jejuni, E. coli, Candida albicans and C. glabrata. It is hoped that this ‘multi-user’ approach will increase collaboration and revolutionize the typing and global epidemiology of important infectious pathogens.

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