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new pathogens, or the concern about bioterrorism, has brought an added urgency to the development of more efficient and rapid methods to detect pathogens and predict their potential virulence.

**Genotypic-based Tests**

Many of the traditional diagnostic tests are now being complemented or replaced by genotypic analysis, i.e., detection of DNA or RNA fragment sizes or specific sequences through nucleic acid hybridisation techniques, or increasingly today DNA amplification by the polymerase chain reaction (PCR). An expression frequently found in microbiologic DNA testing is NAT (nucleic acid amplification technique). NAT refers to a number of techniques apart from PCR that can be used to amplify DNA, e.g., ligase chain reaction. For convenience, the description “PCR” will be used interchangeably with “NAT” to indicate broadly based amplification of DNA. To synthesise or design a DNA probe or primer, it is necessary to sequence an organism’s DNA to identify species-specific regions (see Table A.2, which provides more information about probes and primers). Today, whole genomes from many pathogens have been sequenced, and this information is available on databases. From this sequence (1) DNA primers can be
synthesised for PCR. (2) DNA probes can be isolated or synthesised (oligonucleotide probes) for nucleic acid hybridisation techniques.

Ribotyping: A useful target for a probe is repetitive DNA, an example of which is ribosomal RNA (rRNA). rRNA and the rRNA genes in chromosomal DNA provide naturally derived amplified products that enhance their hybridisation potential. In these circumstances, radio-labelled DNA probes can be avoided since the signal-to-noise ratio is increased because of the amplified target sequence (Figure 8.1). This method of differentiating between bacteria is called ribotyping. For ribotyping, DNA is broken into fragments using restriction endonucleases. DNA probes that are specific for rRNA genes, then allow various fragments to be identified and compared. This approach is very similar to DNA mapping or Southern blotting in eukaryotic DNA (see the Appendix), and the bacterial hybridisation patterns are comparable to eukaryotic DNA polymorphisms.

Pulsed field gel electrophoresis (PFGE): This type of DNA electrophoresis is capable of separating very large DNA fragments. These fragments result from digesting the organism's genome with a restriction endonuclease enzyme such as NotI that cuts DNA very infrequently (see Table A.1). The pattern of the fragments generated in this way is sufficiently discriminatory to allow bacterial strains to be distinguished. Read more about PFGE under Legionella.

DNA hybridisation: 16SrRNA and 12SrRNA contain highly conserved regions common to all bacteria as well as highly variable regions unique to particular species. Therefore, multifunctional DNA or RNA probes can be prepared since they can be generic for all bacteria, or specific for different species or strains.

**PCR**

It is reasonable to say that whatever can be done by the various DNA probes or hybridisation techniques just described can also be accomplished, and probably better, with PCR. Thus, there are genus-specific and species-specific PCR-based assays and many other strategies appropriate for a range of activities involving microorganisms in the environment, food industry, research and clinical practice. Ultimately, the most information that can be obtained about a microorganism's DNA is through sequencing of its genome. This is now increasingly possible as a consequence of the Human Genome Project and the sequencing of many model
organisms (see Table 8.2). The spread of epidemics or hospital-acquired (nosocomial) infections will be followed and characterised with more accuracy by the identification of unique DNA fingerprints for individual pathogens. The value of the DNA approach to emergent infections was recently illustrated by SARS (severe acute respiratory syndrome—discussed in more detail below).

Real-time PCR is starting to make a significant impact on molecular microbiology. This technique is particularly valuable because the risk for contamination is reduced (real-time PCR is conducted in a closed system—see Chapter 2, Appendix). Results from real-time PCR are quickly available. Semi-automation is possible, which is essential when dealing with common infections for which the number of referred specimens would be high. Related to real-time PCR is quantitative PCR (Q-PCR). This technique is essential for accurate viral quantitation (HIV, HCV).

### Test Utility

A number of parameters are used when assessing the value of a diagnostic test. **Sensitivity** refers to the percentage of true positives that will be detected as being positive by the test—or the probability that the test will be positive when the disease is present. **Specificity** refers to the percentage of true negatives that will be detected as being negative by the test—or the probability that the test will be negative when the disease is not present (Table 8.3). The above parameters are largely a product of the test and do not alter according to the population tested. However, they do not always directly demonstrate a test’s usefulness. This is better assessed by the predictive value of positive and the predictive value of negative results coming from the test.

**Positive predictive value (PPV)** refers to the percentage of all positive test results that are truly positive (or the probability that the disease is present when the test is positive). PPV is influenced by the specificity but also by the prevalence of what is being tested for in that population. The higher the prevalence, the higher will be the PPV since there will be less chance of false positive results. The **negative predictive value (NPV)** refers to the percentage of all negative test results that are truly negative (or the probability that the disease is absent when the test is negative). NPV is influenced by test sensitivity as well as prevalence. A high NPV will be easier to obtain if the infection has a low prevalence in a population. **Tests with high PPVs** are required for conditions in which a false diagnosis will have significant consequences; e.g., treatment regimens are potentially toxic; there are medical or psychological stigmas associated with a pos-

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### Table 8.2 Some pathogens that have had their genomes sequenced, with the focus here on those likely to have major pathogenic effects

| Grouping | Examples |
|----------|----------|
| CDC category A<sup>a</sup> | *Yersinia pestis* (plague), *Bacillus anthracis* (anthrax) |
| CDC category B<sup>b</sup> | *Brucella melitensis* (brucellosis), *Coxiella burnetii* (Q fever), *Salmonella enterica* (typhoid fever), *Vibrio cholerae* (cholera), *Clostridium perfringens* (gas gangrene) |
| Others | *Borrelia burgdorferi* (Lyme disease), *Mycobacterium leprae* and *tuberculosis* (leprosy and TB), *Neisseria meningitidis* (meningitis), *Helicobacter pylori* (ulcers), *Treponema pallidum* (syphilis), various strains of *Chlamydia*, *Mycoplasma*, *Staphylococcus*, *Streptococcus* |

<sup>a</sup>By December 2004, about 188 bacterial genomes had been sequenced. See this list as well as all other published genome projects (239 in total) on the Gold<sup>TM</sup>, Genomes OnLine Database (http://www.genomesonline.org/).<sup>b</sup>CDC—Centers for Disease Control category A are organisms posing a major threat to national security particularly in relationship to high mortality, infectiveness and public health impact. CDC category B is the next level because these organisms are easy to disseminate and cause morbidity, but not to the same degree.

### Table 8.3 Sensitivity and specificity in an infectious disease scenario (180 individuals in the population—50 are infected and 130 are not infected, Strohl et al 2001)

(See Table 2.10 for more information on this topic.)

| Description of test | Number infected (from total 50) who test positive | Number not infected (from total 130) who test positive | Comments |
|---------------------|-----------------------------------------------|------------------------------------------------|---------|
| High sensitivity    | 50                                            | 0                                              | All infected people detected and no false positives recorded. |
| Low specificity     | 50                                            | 60                                             | All infected people detected, but also false positives are recorded. |
| High sensitivity    | 30                                            | 0                                              | No false positives, but missing some infected people (i.e., getting some false negatives). |
| Low sensitivity     | 20                                            | 70                                             | Not all infected people are detected (i.e., some false negatives), and some detected are falsely positive. |
itive test as might occur in the case of sexually transmitted diseases. Tests with high NPVs are required when it is essential that positives are not missed, e.g., blood screening tests, treatable infections with fatal outcomes if missed.

**INFECTIOUS DISEASE DNA LABORATORY**

To date, DNA testing in microbiology has been directed predominantly to the detection of organisms that are difficult to culture *in vitro*, or for various reasons (delayed transport, low titre pathogens, prior treatment with antibiotics) growth is unlikely. Infections in which there is a mix of pathogens might also be usefully approached through DNA analysis. Apart from the straightforward diagnostic applications, DNA microbiological testing has been used to detect antimicrobial resistance (methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci* spp) or toxigenic forms of *E. coli*. More recently, the availability of DNA technology to quantitate HCV and HIV has been useful in planning and monitoring treatment. Table 8.4 provides some scenarios involving DNA testing.

Unlike molecular genetic (DNA) testing, commercially produced kits have made an early entry into molecular microbiology, and they allow a wider range of laboratories to participate in this technology. A very important boost to this area has been the Human Genome Project (Chapter 1). The successful completion of genomic sequencing for a number of microorganisms was both a catalyst for the human sequencing endeavour as well as the characterisation of genomes from additional model organisms. The next developments with a major impact on molecular microbiology will be microarrays (see Chapter 5, Appendix) and nanotechnology (Chapter 5). The latter will enhance the potential for point of care testing.

In contrast to the genetics DNA laboratory, the microbiology DNA laboratory faces greater technical challenges because infected samples invariably have little of the pathogen’s DNA to function as a template for PCR, and the limited amount of DNA that is available will be mixed with DNA from many other organisms that are present. Because of the small target for PCR, it is not surprising that contamination from other PCR products (other samples or previous amplifications) becomes a major source of error. Another inherent problem in the microbiology setting is the potential for inhibitors in the various samples tested to interfere with DNA amplification. Finally, interpretation of some microbiological DNA results is difficult because the test shows only the presence of pathogen DNA. Whether this indicates active

| Organism | Traditional test | DNA test | Comments |
|----------|------------------|----------|----------|
| *C. trachomatis* | Fastidious grower; antigen detection not sensitive or specific enough. | PCR very accurate with sensitivities and specificities in the 90–100% range. | Can be detected in a number of samples and can be multiplexed as a PCR test to detect other organisms in STD, e.g., *N. gonorrhoeae*. |
| *M. tuberculosis* | Fastidious grower, but culture still plays an important role in overall management and remains the gold standard. Only way in which to measure drug sensitivity. | PCR demonstrates high sensitivity and specificity (if specimen has acid fast-staining bacteria detectable). | Particularly useful when rapid diagnosis is essential, e.g., TB meningitis. |
| Herpes simplex virus | Difficult to detect with conventional assays in scenarios such as encephalitis. | PCR can detect virus in CSF. | Avoids need for brain biopsy. |
| *Bordetella pertussis* | Detection through swab culture possible. | PCR-based method more rapid and easier. | Early detection possible. |
| HIV, HCV | Cannot quantitate with conventional assays. | PCR quantitation now available in commercial kits. | Drug treatment in AIDS can be monitored by CD4+ cell counts and viral loads. |
| *S. aureus* (methicillin), *Enterococci* spp. (vancomycin), *M. tuberculosis* (various drugs), HSV (acyclovir), CMV (ganciclovir) | Culture for sensitivity testing (when possible) takes a few days. | PCR-based assays targeting various antibiotic or drug-resistance genes can be completed in a few hours. | As more information becomes available about the genomes of pathogens, it will be possible to test for various DNA markers to predict the pathogen's effect. |
infection or residual DNA after the pathogen has died cannot be determined. On the other hand, the finding of mRNA (i.e., cDNA by PCR) is more likely to indicate an active infection. If one then adds that DNA tests are relatively expensive compared to the traditional approaches, it is not surprising that many of the optimistic predictions about molecular microbiology have been slow to materialise. However, there is a steadily growing acceptance of DNA diagnostics. The threat of bioterror or emerging global infections such as was feared with SARS will best be resolved if there is a DNA or RNA component as part of the overall containment strategy.

**HUMAN IMMUNODEFICIENCY VIRUS (HIV)**

In December 2003, an estimated 34–46 million people were infected with HIV, and more than 20 million had died from AIDS. Included in this number are 2.1 million children under 15 years of age, and more than 2 million HIV-infected women give birth each year (http://www.who.int/whr). All regions of the world have AIDS, including Africa, Asia, Latin America, North America, Europe, the Caribbean, and Oceania. Three quarters of cases are found in sub-Saharan Africa. A very worrying trend is occurring in India, with estimates of the HIV infected somewhere between 2.2 to 7.6 million people.

There are two viral types (HIV-1, HIV-2). HIV-1 was first isolated in 1984 (Figure 8.2). A year later, a similar virus (HIV-2) was found in Paris from West African patients with AIDS but seronegative for HIV-1. HIV-2 is predominantly found in a number of West African countries, including Senegal, Ivory Coast, Gambia, Ghana and Nigeria. It has spread to other countries that have historical or other links to the African sources; i.e., France, Portugal, Angola and Mozambique. However, with the ease of air travel, sporadic cases can appear in any country. Overall, HIV-2 spreads less rapidly and takes longer to

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**Fig. 8.2 Structure of HIV-1.**

*(Top)* A schematic representation of the HIV genome (family Retroviridae, genus Lentivirus) shows that it comprises two identical strands of RNA within a core of viral proteins. Key viral enzymes are reverse transcriptase (viral DNA synthesis), integrase (integration into host cell chromosome) and protease (viral assembly). The core is surrounded by a protective envelope (env) derived from viral glycoprotein and membrane from the previous host cell. Components of the envelope are a transmembrane protein, gp41, and a surface protein, gp120. Abbreviations: gp41—glycoprotein molecular weight of 41,000; p24—protein molecular weight 24,000. *(Bottom)* A number of genes make up HIV. Gag (group specific antigens) codes for capsid, matrix and nucleocapsid proteins. Pol codes for reverse transcriptase, protease, integrase and ribonuclease. Env (envelope) codes for a glycoprotein precursor, gp160, that is cleaved to give envelope structural proteins gp120 and gp41. Although an RNA virus, HIV is able to produce DNA through its reverse transcriptase enzyme once it infects a cell. The viral DNA then integrates (via integrase) into double-stranded host DNA to produce the proviral form. Not shown are six regulatory genes (rev, tat, nef, vpr, vpu and vif) that give HIV a more complex structure compared to the animal retroviruses. LTR—long terminal repeats of the retrovirus.
develop than HIV-1. The lower viral load associated with HIV-2 may also explain why it is less likely to be transmitted from mothers to babies.

HIV-1 and HIV-2 are retroviruses (so called because they transfer the order of information from RNA to DNA) belonging to the lentivirus genus, which predominantly affects the nervous and immune systems. Transmission occurs by one of three routes: (1) sexually—both male to male and male to female, (2) blood or blood products, including HIV-contaminated needles and (3) perinatally—during birth or from breast feeding. There is presently no effective vaccine against AIDS. Perinatal transmission can be reduced by treating the mother with an antiretroviral agent, avoiding breast feeding and early weaning. Prevention is also possible through screening blood (discussed below) and the practice of safe sex. Treatment options have dramatically improved the outlook for AIDS. However, less than 10% of those requiring treatment in developing countries have access to antiretroviral drugs (Box 8.1).

The urgency and potential implications of the AIDS pandemic have resulted in many research programs directed to better diagnostic tests, more effective therapeutic regimens and a greater understanding of the viral biology. The identification of HIV viruses has required extensive changes to be made in blood screening protocols (see below). In all these areas, rDNA technology is playing a major role. In the context of the present theme of laboratory detection, molecular medicine has an important function to play since opportunistic infections are the chief cause of death in AIDS. All who have AIDS will develop infections at some time in their illness. The pathogens involved include those that normally do not produce overt disease such as Candida albicans, and the more exotic organisms that will not usually be seen in clinical practice, e.g., JC virus, which gives rise to a demyelinating neurological disorder called progressive multifocal leukoencephalopathy. In these circumstances, standard microbiological detection methods often prove ineffective.

### Screening and DNA Testing for HIV

Many commercial ELISA (enzyme linked immunosorbent assay) kits detect antibodies to HIV-1 and HIV-2 (Figure 8.3). They are used to screen those who are at risk and blood donors. The assays are well standardised with good sensitivities and specificities. In HIV infection, screening tests must have the highest sensitivity so that positives will not be missed. False positives are then excluded by using a second assay with a high specificity, e.g., a western blot.

HIV-1 testing by DNA analysis can be directed towards the gag, env or pol genes of the virus (see Figure 8.2). Laboratory techniques and detection strategies (algorithms) utilising these sequences have been constructed

### Box 8.1 Treatment of HIV/AIDS.

AIDS represents the clinical phase of HIV infection that by now is well established. Therefore, effective treatment must be started early and take into consideration a feature of this infection—the rapid development of mutant viruses. This occurs because (1) HIV infection is associated with significant viraemia. (2) The viral population is very heterogeneous due to an error-prone reverse transcriptase (unlike DNA polymerase, which makes few mistakes in the replication of DNA). Therefore, mutations are constantly being produced in the HIV genes, and mutant viruses will form. This, combined with the high viral titres present, facilitates the development of resistance. The key to success with AIDS treatment has been HAART (highly active antiretroviral therapy), which became available in 1996 and involves the use of multiple drugs directed to different viral pathways.

| Class of antiretroviral drug | Mechanism of action | Examples |
|-----------------------------|---------------------|---------|
| Nucleoside and nucleotide analogues | Act as DNA chain terminators, thereby inhibiting the reverse transcription of viral RNA into DNA | Zidovudine (AZT), lamivudine |
| Non-nucleoside reverse transcriptase inhibitors | Bind and inhibit viral reverse transcriptase | Nevirapine |
| Protease inhibitors | Block viral protease required for making the inner core of viral particles | Nelfinavir |
| Entry inhibitors | Block virions from penetrating target cells | Enfuvirtide |

Combination drug therapies involve the use of three drugs having a synergistic rather than additive effect on reducing the viral load, and so they delay the development of drug resistance. Various combinations are used, for example, two nucleoside and nucleotide analogues with the third drug a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor. More recently, a fourth class of drug with a completely new mechanism of action has been developed. This involves inhibiting the entry of HIV-1 into CD4+ lymphocytes. The major questions now requiring resolution include when to start HAART and what are the optimal drug combinations (more than 20 drugs are approved by the FDA for treating HIV infection). Despite the use of HAART, HIV re-emerges once therapy is stopped, indicating a virostatic rather than virocidal effect. Better drugs are still needed, but more importantly, a vaccine that will allow the prevention of HIV-AIDS.
to optimise sensitivity and specificity and minimise false positive or false negative results. It has been estimated that one HIV-1 proviral copy can be detected against a background of $10^5$ mononuclear cells in peripheral blood. Virus isolation is the only other method with comparable sensitivity although various problems with this test exclude it as a routine assay.

Variations of the PCR technique including nested PCR (see Chapter 2, Appendix) can be used to reduce the false positive rate, which is due, in most cases, to contamination of samples or reagents. On the other hand, false negative results can reflect the presence of inhibitors in the specimen being tested or chemical substances such as anticoagulants. Another source of a false negative result with HIV occurs because of the considerable diversity found in the viral DNA sequence so that a mismatch between the usual primer pairs inhibits the subsequent PCR step. This difficulty can be avoided by use of primer pair combinations derived from conserved segments of the viral genome. For the present, serological testing for HIV-1 and HIV-2 remains the preferred screening method for at-risk individuals, including blood donors, because it is fast, relatively cheap and able to be automated (see also blood screening below).

**TRANSFUSION-RELATED INFECTIONS**

A number of the important recent viruses have reached notoriety through their association with blood transfusions. Previously, blood transfusion services based their donor and blood screening programs on detecting antibodies or antigens in the donor or blood supply. However, this approach has been shown to be inadequate, and an important new addition to the screening protocols is the use of PCR to identify viral DNA or RNA. The advantages of a NAT assay include (1) higher sensitivity and (2) greater reliability during the window period, which is the time between a blood donor becoming infectious and donor screening tests becoming positive; i.e., seroconversion has occurred.

PCR-based assays for screening blood donations are used to test (1) pools of donations, for example, 16–24 donations simultaneously, and (2) individual donations. The former is more rapid and cheaper, but the very rare case of HIV and hepatitis C virus (HCV) being missed means the testing of individual donations is the method of choice. Screening by NAT has a number of potential drawbacks, including (1) It involves more sophisticated technology (therefore, trained staff are needed, and tests

**Fig. 8.3 Diagnosis of HIV/AIDS infection.**
The ELISA test is the mainstay for routine HIV detection. It is rapid and able to test many samples. It has the disadvantage of both false positives and false negatives, and so confirmation by a western blot (or second ELISA or an immunofluorescence test) is essential. Apart from technical and clerical mistakes leading to a false negative result, the most common reason for this type of error is the window period since it takes between 4–12 or more weeks after infection for antibodies to form. A second independent sample is then tested to reduce the potential for technical or clerical problems before a definitive diagnosis is made. Quantitative PCR (Q-PCR) is used to assess viral load, which helps to decide when to start therapy and can be used to monitor response to therapy.
are invariably more expensive). (2) Assays are technically demanding, including the strict requirement to prevent contamination. (3) Results from NATs take longer than those from serologic ELISAs (enzyme linked immunosorbent assays). However, in response to a number of cases of contaminated blood, many blood transfusion services have opted for NAT testing.

Blood transfusion services screen blood and donors for a range of infectious agents, e.g., hepatitis B virus (HBV), HCV, HIV-1, HIV-2 (and in some regions human T-cell lymphotropic virus types I and II [HTLV-I, HTLV-II]), syphilis, cytomegalovirus (CMV) and, more recently, West Nile Virus in the USA. Some screening tests are undertaken only in selected circumstances; e.g., CMV-free blood is required for an immunosuppressed patient, fetus or neonate.

The risks of blood-borne viral infections have dramatically decreased with the implementation of donor questionnaires that allow self-exclusion (particularly important for infections that are not routinely tested for), donor and blood testing with ELISA and PCR-based techniques. The latter’s sensitivity is several magnitudes higher than culture or ELISA methods. To exclude the potential for slow virus transmission (i.e., CJD and vCJD) through blood, transfusion services have deferred the use of donors who have lived in the UK over certain time periods (Table 8.5). Other reasons for deferral include fever with headaches the week before donation (possibility of West Nile Virus and others) or travel to risk regions (for example, the possibility of malaria).

Apart from HIV, another success with blood transfusion screening has been HCV. Since the single-stranded ~10 kb RNA sequence of the HCV was reported in 1989, it has been possible to use an enzyme immunoassay to detect antibodies against a number of viral antigens in patient testing or blood donor screening. However, the first-generation immunoassays gave rise to many false positives. This problem with serological testing was overcome by the availability of more sophisticated second- and third-generation enzyme immunoassays. Similar to what happens with HIV, the resolution of false positive results is undertaken by an additional assay such as an immunoblot that incorporates multiple viral-specific antigens. Viral antigens in many of the modern tests have been prepared by using rDNA expression systems such as E. coli or yeast (see Chapter 6). Indeterminate or equivocal results can be further evaluated with PCR.

Table 8.5 Some success stories with screening in blood transfusion services (Goodnough 2003)

| Virus       | Screening                                                                 | Outcomes                                                                 |
|-------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| HIV         | Donor and blood with full-range tests including NAT. First blood-related infections in 1982. | In the USA, there were 714 HIV cases in the year before testing was implemented. That number fell to about five cases per year in the next five years after NAT testing started in 1999. Risk of blood transfusion–acquired HIV in the post-NAT era is 1 : 1 900 000. |
| HCV         | Donor and blood with full-range tests including NAT.                      | There are serious consequences of blood-borne HCV infection. However, following testing protocols, HCV is a rare complication. Risk of blood transfusion–acquired HCV in the post-NAT era is 1 : 1 600 000. |
| HBV         | Donor and blood with third-generation serologic assays for hepatitis B surface antigen. | Risk of blood transfusion–acquired HBV is still relatively high (1 in 220 000) although only about 10% of post-transfusion hepatitis is due to HBV. Unlike HCV, risk of chronic liver disease is less with HBV. |
| CJD, vCJD   | No useful test available.                                                 | In the USA, deferral protocols have been in place since 2000; i.e., individuals living in certain European countries during defined times are excluded. No direct evidence that blood can transmit CJD or vCJD, although the infection is transmitted with tissues such as cornea and pituitary extracts. In the UK, plasma for fractionation is obtained from the USA. |
| West Nile   | Most useful is NAT for viral RNA. Serologic testing of donors is not sensitive. | In 2003, 23 cases were reported—resulting in the development in a very short time (2003) of a PCR-based test used to screen all donors. Also included in the donor questionnaire is history of fever with headaches in the week before donation (yes answer leads to deferral). |

*It is worthwhile comparing infectious risks of blood transfusions with incompatible blood transfusions with their associated 10% mortality. The FDA reports over twice the frequency for incompatible blood transfusion compared to all infections, and in the UK, adverse events related to incompatible blood transfusions were 10 times higher than for infectious diseases.*
PATHOGENESIS

The pathogenesis of many infections, particularly viral ones, has been deduced from experimental strategies based on light and electron microscopy, cell culture and immunoassay. To these research tools can now be added nucleic acid (DNA, RNA) probes for in situ hybridisation or NAT. Advantages provided by DNA techniques include the ability to detect latent (non-replicating) viruses and to localise their genomes to nuclear or cytoplasmic regions within cells. Tissue integrity remains preserved during in situ hybridisation, and so histological evaluation can also be undertaken. Nucleic acid probe techniques or NAT can be manipulated to enable a broad spectrum of serotypes to be detectable. This is particularly valuable in those emerging infections where the underlying serotypes are unknown. More recently, the most powerful application of DNA techniques is the ability to sequence whole genomes, and so identify the pathogen, and from its genomic sequence (1) predict its role in disease pathogenesis and (2) identify regions of the genome suitable for NAT-based detection methods.

HOST RESISTANCE

The host’s response to an infection involves a complex mix of genetic and environmental factors. In humans, evidence for a genetic component contributing to the outcome of an infectious disease comes from the observation that some ethnic groups are more resistant to infections, whereas others appear to have an increased susceptibility; e.g., resistance to malaria in black Africans and susceptibility to chronic carrier state for HBV in Chinese. Not all exposed to HIV-1 get infected, and those who do progress to AIDS show different responses. For example, there can be a rapid progression within 1 to 5 years, or individuals can demonstrate a more benign progression extending up to 20 years. Because host resistance is likely to represent multiple genetic effects—i.e., QTLs (quantitative trait loci), which interact with the environment—they are difficult to detect and so have been sought by the usual molecular genetic approaches, particularly association (case control) studies and the investigation of candidate genes (see Chapter 4 for further discussion of this strategy and QTLs).

HIV-1 Infection

HIV-1 targets the macrophages, monocytes and T lymphocytes that carry the CD4 cell surface protein. HIV-1 first attaches to CD4 and then the chemokine receptor CCR5. Once this occurs, the HIV gp41 protein can penetrate the cell membrane, enabling HIV to infect the cell. In some circumstances, particularly as the disease progresses, a mutation in the env gene enables a move away from the CCR5 receptor to the CXCR4 receptor. Therefore, two host-related genetic factors can influence the infectivity of HIV-1 in humans. They are (1) chemokine receptor genes and (2) major histocompatibility complex (MHC).

CCR5 is a chemokine and functions as a co-receptor required for HIV to enter cells. CCR5 is highly polymorphic, with variants found in different ethnic groups. One important variant involves a 32 bp deletion in the coding region leading to a truncated protein. It is now known that homozygotes for CCR5 Δ32 (approximately 1% of Caucasians) have significant resistance to HIV infection because the virus has lost one of its entry points into the cell. Heterozygotes do not appear to have enhanced resistance to HIV infection although progression to clinical AIDS is delayed, and viral loads are lower. Other genetic variants related to CCR5 have been reported to influence HIV infection and progression (Table 8.6).

The second and probably the more important of the genetic factors influencing HIV infections came from an observation of apparently HIV-resistant female sex partners. Unlike the protective effect of the CCR5 Δ32 defect, there is some controversy about the role played by the other genetic variations because they have all been identified through genetic association studies. Two other important genes that influence progression from HIV to AIDS are IL10 and IFNγ. These genes produce cytokines that inhibit HIV replication. Hence, genetic variants that lead to overexpression of these genes will favour a milder disease, and the converse occurs with genetic variants that are associated with reduced expression.

Table 8.6 Genetic variants in chemokine receptors influencing HIV infectivity (O’Brien and Nelson 2004)**

| Variant | Mechanism |
|---------|-----------|
| CCR5    | Δ32 defect decreasing the amount of this receptor. Homozygotes for this have a strong resistance to HIV infection (although this is not absolute). Progression to AIDS is slowed in heterozygotes. |
| CCR5    | Apart from the 32bp defect, mutations in the promoter have also been associated with increased gene function, thereby accelerating the development of AIDS. |
| RANTES  | Mutations in the promoter can affect both susceptibility as well as HIV progression, depending on how RANTES expression is affected. |
| CCR2    | The variant V64I appears to delay progression of HIV perhaps by impairing the transition from the CCR5 receptor to an alternative one (CXCR4). |
| CXCL12  | Variant in the 3’ end appears to impair HIV entry into the cell perhaps through the CCR5 to CXCR4 transition. |

*Unlike the protective effect of the CCR5 Δ32 defect, there is some controversy about the role played by the other genetic variations because they have all been identified through genetic association studies. **Two other important genes that influence progression from HIV to AIDS are IL10 and IFNγ. These genes produce cytokines that inhibit HIV replication. Hence, genetic variants that lead to overexpression of these genes will favour a milder disease, and the converse occurs with genetic variants that are associated with reduced expression.
workers in Nairobi. These women were shown to have a particular HLA type (HLA A2, HLA A28), and it has been proposed that this enhanced their resistance through a better presentation of HIV antigens to T lymphocytes. Therefore, the virus was more rapidly cleared by the immune system. Subsequently, there have been many associations described between HLA types and increased resistance or greater susceptibility to HIV infection and its progression to AIDS (Table 8.7).

Another observation involving HLA is that mothers and children who are mismatched at the MHC are less likely to have perinatal transmission because immune surveillance is enhanced. However, the HLA protective effect is not observed if HIV is acquired through breast feeding. Other HLA associations in AIDS (as well as other infections) have been reported, including polymorphisms of interleukins IL4 and IL10 (Table 8.8). Like association studies in genetic disorders, the reports have produced many correlations between genetic markers and response to infections although they often remain controversial. The confusion that can be found with association studies is well demonstrated by the HCV example in Table 8.8.

**Malaria**

Each year there are more than 500 million cases of falciparum malaria in Africa, and around 1–2 million deaths will result. A resurgence in malaria reflects socio-economic factors (poverty, overcrowding), emergence of mosquitoes that are resistant to insecticides and development of drug resistance. The two common forms of malaria (*P. falciparum* and *P. vivax*) produce severe anaemia, and in addition, *P. falciparum* is associated with cerebral malaria, respiratory and metabolic complications. This differing spectrum is partly explained by *P. falciparum* being able to invade a large proportion of red
blood cells compared to \( P.\) \textit{vivax}, which invades only the reticulocytes. Another explanation is the mode of entry of these parasites into red blood cells, with \( P.\) \textit{falciparum} having a number of different invasion pathways compared to \( P.\) \textit{vivax}, which is able to enter only red blood cells that carry the Duffy blood group.

Host factors providing some protection from malaria have been identified. They include single gene effects seen in haemoglobinopathies such as sickle cell anaemia or thalassaemia, and the Duffy negative blood group. The former are explained by suboptimal red blood cell environments that do not provide a good milieu for the parasite. The Duffy negative blood group has a more powerful protective effect since it is the receptor by which \( P.\) \textit{vivax} enters the red blood cell. Thus, \( P.\) \textit{vivax} is not seen in West Africa because the populations are Duffy negative.

**Hepatitis B**

Although vaccination for HBV has made a very significant reduction in new cases, worldwide there still remain about 350–500 million infected individuals. Long-term complications with this DNA virus occur in about 15% of those infected and include chronic hepatitis, cirrhosis and hepatocellular carcinoma. Why some go on to develop a chronic carrier state is not known, with the course of the illness after infection very variable. This has been explained on the basis that it involves the viral genotype and the host's immune responses; i.e., following acute infection, a vigorous T cell response against the various viral-specific components (core, envelope and polymerase) limits the infection. In contrast, those with a poor cellular immune response are more likely to develop a chronic carrier state. In this scenario, the MHC will play a key role since class II molecules from the HLA complex are critical for T cells to function. Evidence for an MHC-related effect in hepatitis B infection is presented in Table 8.8.

**Animal Models**

Polygenic traits contributing to pathogen susceptibility or resistance in humans would be difficult to separate into their individual components because of the genetic variability in the human genome. However, this can be overcome by using different strains of inbred animals, particularly mice. Since chromosomal segments in the mouse can be traced to their homologous or syntenic regions in humans, it becomes possible to identify a genetic locus or gene in a mouse and then go back to the corresponding region in the human genome to find the equivalent gene.

Forward genetic screens (phenotype to genotype) result from the observation that a particular mouse strain (or one created by, for example, a chemical mutagen such as ENU—Chapter 5) has a phenotype that alters the animal's expected response to an infection. An example would be a mouse strain shown to have resistance to \( Leishmania donovani\) infection. From this observation, a gene was isolated by conventional positional cloning. The gene was called \( N r a m p 1\) (now renamed \( S l c 11 a 1\)). Mice with mutations in this gene (particularly G169D) are resistant to three intracellular pathogens (\( M y c o b a c t e r i u m b o v i s, S a l m o n e l l a t y p h i m u r i u m\) and \( L e i s h m a n i a d o n o v a n i\)). This gene is likely to play a key role in infection since it is exclusively expressed in macrophages, key cells in the control of the three intracellular pathogens just mentioned.

A reverse genetic screen (genotype to phenotype) is exemplified by the transgenic knockout mouse when the particular phenotype associated with a gene can be confirmed by inactivating that gene. One interesting knockout involves the removal of the TNF\(\alpha\) (tumour necrosis factor) gene, which leads to a mouse that is very susceptible to the pathogens \( M.\) \textit{tuberculosis\) and \( L.\) \textit{monocytogenes}. A similar susceptibility is seen if the mouse's interferon-\(\gamma\) gene is knocked out. Both these genes play key roles in the immune response.

Another strategy allowing a mouse model to be used for an infection that is found exclusively in humans is to “humanise” the mouse for the particular host-tropism that explains the species specificity. An example of this is the polio virus that does not normally infect mice. However, transgenic mice that express the human poliovirus receptor CD155 develop polio if infected intracerebrally, but not if they are infected orally. In addition to creating a suitable animal model, this experiment also indicates that the oral phase of infection with poliovirus is likely to involve another receptor.

**DRUG RESISTANCE**

**Antimicrobial Drugs**

Resistance to antibiotics has always been a concern. However, new antibiotics in the 1950s–60s temporarily addressed the problem, and this false sense of security was reinforced in the next two decades with newer and more powerful antibiotics. Today, the increasing trend in antibiotic resistance is alarming, and is not being matched by new drug development. This led to the World Health Organization in 2001 developing a plan to address what has become a global problem.

Resistance to antibiotics occurs by three major mechanisms: (1) reduced antibiotic uptake by a cell or increased efflux from the cell, (2) modification or inactivation of the antibiotic and (3) altering the target for the antibiotic. The development of resistance may be an intrinsic feature of an organism, or it may result from mutations or the acquisition of resistance genes. Transfer of resistance genes can occur via plasmids or trans-
The latter represent mobile DNA elements that can move between plasmids or between plasmids and chromosomes, thus having the potential to disseminate widely (and stably) drug resistance genes.

The genes involved in antibiotic resistance are being characterised, enabling a better understanding of how this develops and how to detect it quickly by DNA-based approaches, and finally, identifying strategies by which the resistance pathways can be bypassed. Apart from the inappropriate use of antibiotics in medicine, agriculture and animal feeds (about 50% of antibiotics are used in the beef and poultry industry!) contributing to the increasing resistance, other factors involved include travel and tourism.

Pneumococci are important pathogens in the community leading to central nervous system infections, pneumonia and otitis media in children. Resistance to macrolide antibiotics (e.g., erythromycin) in some countries is reaching alarming proportions; for example, 65% of *Streptococcus pneumoniae* are resistant to erythromycin in parts of Asia. Two major mechanisms explain resistance in this organism: (1) Bacteria carrying the *ermB* gene can methylate a specific amino acid on the ribosome (the site of action for macrolides) and so interfere with binding of the antibiotic to this region. (2) Bacteria carrying the *mefA* gene (an efflux pump gene) can export out the antibiotic and so protect the ribosome.

Multidrug resistance (MDR) occurs with both antibiotics and anti-cancer agents, and refers to the development of resistance to a range of drugs that may be unrelated or differ widely in their structure or target. Common pathways are seen with MDR. One of these involves the P-glycoprotein (*MDR1*) gene, which is a plasma membrane–spanning multidrug transporter protein. One of the most important mechanisms for MDR is the ability to extrude out the antibiotic (or anti-cancer drug) from the cell (Figure 8.4). Antibiotic resistance to many gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter* spp, and the *Enterobacteriaceae*) occurs through an efflux-mediated mechanism.

**Mycobacterial Infection**

Rifampicin occupies a pivotal place in the World Health Organization’s multidrug resistance program for tuberculosis (TB) and leprosy. Despite this, about 3.2% of new TB cases are caused by multidrug-resistant strains. The development of resistance has major therapeutic and public health implications. Since *M. leprae* grows slowly, it is essential to confirm the development of drug resistance by alternative means to the traditional culture approach. Similarly, to avoid the use of what will be ineffective drugs (a cost consideration as well as a way to optimise antimicrobial therapy), the Centers for Disease Control recommends that resistance patterns for TB should be reported within 28 days of the specimen being received. However, this is a challenge because of the slow-growing *M. tuberculosis*. Therefore, an alternative approach is to use molecular diagnostic techniques to identify resistance genes in the mycobacteria.

Until recently, the use of various drug combinations selected from isoniazid, rifampicin, ethambutol, streptomycin and pyrazinamide as well as improved living standards was instrumental in producing a declining incidence for tuberculosis. However, this is now changing. The number of new cases is increasing. The traditional dogma that active cases usually arise from infections acquired years earlier is no longer applicable since DNA studies have shown that approximately one third of active cases in some cities are the result of person-to-person transmission. Contributing factors to
the increase in tuberculosis and the finding of multidrug-resistant strains are HIV infection, intravenous drug use and the decline in living standards resulting from political changes or war. At the molecular level, the genes associated with drug resistance are being defined (Table 8.9). To deal with these challenges, modern global strategies directed to tuberculosis (and leprosy) will require laboratory facilities that utilise state-of-the-art technology to provide health professionals with accurate information in an efficient and timely fashion.

**Malaria**

During the latter half of the twentieth century, chloroquine was the drug of choice for treating falciparum malaria. However, the spread of drug-resistant *Plasmodium falciparum*, and to a lesser extent *Plasmodium vivax*, has made the prophylaxis and treatment of malaria a global public health issue. Chloroquine-resistant *P. falciparum* is present in every major region where malaria is endemic. Air travel means that drug-resistant strains can appear in any city. Resistance is now spreading since the 1950s from a limited number of foci in South East Asia or South America and then spreading slowly worldwide. In contrast, resistance to pyrimethamine and proguanil has arisen independently in many different regions where these drugs have been used, consistent with a single gene being involved.

The molecular basis for resistance in malaria is slowly being unravelled, and many genes are now implicated (Table 8.10). As the molecular defects become more comprehensively characterised, it will be possible to predict the drug resistance pattern for various geographic regions and so optimise drug selection. It might also be feasible to use other drugs or genetic-based therapies to bypass the resistance defect in the parasites, allowing the continuing use of what are cost-effective and relatively safe drugs such as chloroquine. Another positive development in the therapy of malaria is the availability of the DNA sequence from the genomes of the important human and mouse malaria parasites. This development will enable new targets to be found, i.e., genes for metabolic pathways that differ between the parasite and humans.

**HIV**

Monitoring response to AIDS drug treatment can be undertaken by measuring the CD4+ cell count, as well as the assessment of viral load by Q-PCR. In the USA, about half the patients are infected with viruses that have some resistance to drugs. The mutations causing resistance, as well as their likely modes of action, are well understood for HIV. They include many single base changes and the occasional insertions of a few amino acids. Cross resistance, once it occurs, is restricted to drugs within the same class although all classes of drugs can develop resistance, which tends to occur slowly over a period of time (Box 8.1 describes the various drugs). Resistance is the result of many mutations accumulating within HIV, although one exception is the M184V mutation in reverse transcriptase, which alone produces complete resistance to lamivudine. When plasma viral loads rise despite therapy, a change in regimen is needed. Because of the
cross-resistance within classes, what drug to substitute is not an easy decision. Assays to detect HIV resistance include genotypic (detecting RNA mutations) as well as phenotypic (*in vitro* viral cultures).

**VIRULENCE FACTORS**

**Streptococcal Virulence Factors**

Group A streptococcus (GAS) is responsible for a wide range of human infections. Virulence factors in this organism produce a broad spectrum of toxic effects from antiphagocytic, cellular adherence, internalisation, invasion and systemic toxicity. An important toxin associated with GAS is now better understood through analysis of its gene. This is the \( \beta \) haemolysin effect, which was difficult to study without DNA technology. The \( \beta \) haemolysin effect in GAS is due to a powerful cytotoxin called streptolysin S, which has a very broad toxic effect on cells and is non-immunogenic. The gene for streptolysin S has now been isolated and is called \( \text{sag} \). It comprises 9 different contiguous genes \( \text{sagA} \) through to \( \text{sagI} \). The corresponding gene for \( \beta \) haemolysin in group B streptococci is \( \text{cyl} \) and involves 11 contiguous genes.

**Helicobacter Pylori Virulence Factors**

*H. pylori* is an important gram negative slow-growing bacterium that colonises the gastric epithelium and can lead to chronic gastritis, ulceration and cancer. Infection is strongly associated with socioeconomic status, with prevalences >80% in developing countries and much lower ones (20–50%) in affluent regions. Disease outcome depends on a number of factors including bacterial genotype. Infection with *H. pylori* can be treated with antibiotics. However, because it is so common in some communities, a vaccine approach for prevention would be more economical and effective. To identify potential targets for vaccines in this organism, the various virulence factors have been identified. They include urease, vacuolating toxin, a cytotoxin and an adherence factor. The genome of this bacterium was sequenced in 1997, and from this it was possible to understand better the bacterium’s acid tolerance and genes involved in pathogenesis. A number of the virulence-producing genes have been characterised. They include \( \text{vacA} \)—exotoxin-producing vacuoles in epithelial cells associated with greater risk for ulcers; \( \text{cag} \) PAI region associated with the induction of a more intense host inflammatory response leading to more severe disease in Western populations. The significance of \( \text{cag} \) PAI in the developing world is less obvious because almost all strains are \( \text{cag} \) positive.

**CANCER**

DNA and RNA viruses cause tumours in humans. Oncogenic viruses include hepatitis B (DNA), hepatitis C (RNA)—hepatocellular carcinoma, and papillomavirus (DNA)—cervical cancer. The Epstein-Barr virus (EBV), a DNA virus, is associated with the development of both lymphoid and epithelial tumours.

**Hepatitis B Virus (HBV)**

The hepatitis B virus is a hepatotropic DNA virus. Its replication cycle within the liver nucleus leads to the formation of mature virions via reverse transcriptase, and they are exported from the cell. Alternatively, the newly formed viral DNA can be recycled back into the nucleus for conversion to a plasmid form that generates further genomic transcripts and so maintains a constant intranuclear pool of templates for transcription. The HBV replication cycle within the hepatocyte is not directly responsible for liver damage, consistent with the observation that many HBV carriers are asymptomatic with minimal liver damage. It has also been observed that those with immune defects tend to be chronic carriers of HBV but usually in association with mild liver damage. Therefore, it has been proposed that liver damage following chronic HBV infection is predominantly the result of the host’s immune response, in particular: (1) cytoxic T cells, natural killer T cells and (2) inflammatory products, including tumour necrosis factor (TNF), free radicals and proteases.

Most primary HBV infections in adults are self-limited (compare this with hepatitis C virus discussed under Future). About 5% of primary infections in adults continue and lead to persistent infection. It is estimated that about 350 million people worldwide are HBV carriers. About 20% of chronic carriers go on to develop the serious complication of cirrhosis. Liver transplantation in HBV will be associated with a recurrence of the infection in more than 80% of patients unless preventative measures are undertaken to reduce the risk for reinfection. They include the use of hepatitis B immune globulin and an antiviral agent such as lamivudine that is also used for treating AIDS (see Box 8.1). Another serious consequence of chronic HBV infection is hepatocellular carcinoma, with carriers being 100 times more likely to develop this than non-carriers. Those who are HBeAg positive carriers are particularly at risk, which is not surprising since this serologic marker is associated with high HBV titres. Despite the HBV genome being sequenced and characterised, it is disappointing that even today there is incomplete understanding how this DNA virus leads to the development of hepatocellular carcinoma.
Papillomavirus

Papillomaviruses are DNA viruses that exhibit species specificity and induce hyperplastic epithelial lesions as a result of infection. Cervical cancer is the second most common cancer in women worldwide, and there is now unequivocal evidence implicating certain types of human papillomavirus (HPV) as the primary cause of cervical cancer and its precursor, cervical intraepithelial neoplasia. HPV is acquired mainly through sexual activity. More than 80 types of HPV have been identified, and about 40 can infect the genital tract.

Cervical cancer begins as a preinvasive neoplastic change in cells (histologically described as CIN—cervical intraepithelial neoplasia). This may regress, remain unchanged or progress to an invasive malignancy. Human papillomavirus has been implicated in the various stages of cervical cancer with different types correlating with the histological findings. Human papillomavirus has more recently been implicated in anal cancer (particularly in those who are HIV positive), penile, laryngeal and oral cancers. Human papillomavirus–related skin cancers can be found in the immunosuppressed.

Human papillomaviruses induce cellular transformation and may also interact with other viruses, oncogenes or carcinogens to bring about neoplastic changes. The virus is not the sole factor in the progression to invasive cervical cancer (Figure 8.5). The E6 and E7 oncoproteins code for proteins essential for viral replication in HPV types 16 and 18. E6 binds to and inactivates TP53, thereby disrupting a key cell cycle checkpoint (see Figure 4.13). The E7 oncoprotein binds to and inactivates retinoblastoma gene products, leading to further uncontrolled cell cycle progression.

The various types of human papillomaviruses can be distinguished on the basis of their DNA sequences. Comparisons between histological findings and viral types have enabled classification into three groups—low, intermediate and high risk (Table 8.11). Commercial kits based on PCR assays are now available to identify most of the high-risk HPVs. This test is considered to be very sensitive. However, as mentioned previously, a DNA test indicates only the presence of viral DNA. It cannot predict whether the infection is active or transient. Hence, the suggestion that HPV DNA testing would replace the traditional Papanicolaou smear is incorrect, particularly in young women who are more likely to have a transient HPV infection. However, used in conjunction with the Papanicolaou smear, the papillomavirus DNA screen will be helpful if there is a suspicious result, or this combination might become the preferred method for screening women over 30 years of age. In this way the combined tests will increase the overall sensitivity of screening, and so increase the interval between testing.

Epstein-Barr Virus (EBV)

EBV is a ubiquitous human herpes virus that infects most adults. After being infected, the individual remains an EBV carrier for life. In developing countries, primary EBV infection is asymptomatic and occurs early in life. In developed countries, the primary infection is delayed until adolescence or adult life, when it manifests as infectious mononucleosis. An in vitro characteristic of EBV is its ability to immortalise lymphocytes, an application that

Table 8.11 Classification of HPV types and their risk for cervical cancer (Munoz et al 2003)

| High-risk HPV (probable high risk) | Indeterminate-risk HPV | Low-risk HPV |
|-----------------------------------|------------------------|--------------|
| Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, (26, 53, 66) | Types 34, 57, 83 | Types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108 |

![Fig. 8.5 Human papillomavirus (HPV) in cervical infection.](image)

Similar to what has been described for colon cancer, the development of cervical cancer is a staged process predominantly in response to infection with HPV. Infection can be transient (and so reversal of the HPV effects on cervical epithelium is possible), but once HPV infection is persistent, it can lead to pre-cancerous lesions that can progress to cervical cancer. Other factors associated with the development of cervical cancer include early sexual activity (<16 years of age), >4 sexual partners, and a history of genital warts. Other independent risk factors that might interact with the HPV include HIV infection, immunosuppression and cigarette smoking.
is very useful in the research laboratory to provide a permanent supply of a particular cell line (or an unlimited source of DNA). In the immortalised lymphocyte, EBV does not replicate but remains as multiple extrachromosomal copies of the circular viral DNA genome in the cell. Latent infection with EBV is accompanied by the expression of a set of viral latent genes. These genes and their respective proteins are well characterised and include (1) six nuclear antigens: EBNAs 1, 2, 3A, 3B, 3C and LP; (2) three latent membrane proteins: LMPs 1, 2A and 2B and (3) EBERs 1, 2—small, non-polyadenylated non-coding RNAs.

Inappropriate expression of EBV latent genes leads to tumour development. Tumours in which EBV is found include Burkitt’s lymphoma, post-transplant B cell lymphoma, Hodgkin’s disease and nasopharyngeal carcinoma. In each, various latent genes and proteins will be detected. Two important ones (these are always present in the laboratory-transformed lymphoblastoid cell lines) are EBNA2 and LMP1. The former is a transcriptional regulator; hence, inappropriate expression of this gene will have far-reaching effects on cell proliferation. LMP1 in experimental studies behaves as an oncogene. Nasopharyngeal carcinoma is a tumour that is predominantly found in China and South East Asia. The latency genes EBNAl, EBERs and LMP1 are frequently found in this tumour. A recent study used Q-PCR to quantitate EBV-specific DNA (in the form of EBNA3 and LMP1) in the plasma and tumours from nearly 100 patients with nasopharyngeal carcinoma. This study showed that the amount of EBV DNA in the plasma correlated closely with the stage of the cancer with the most DNA found in tumours that had metastasised. The authors (Lin et al 2004) suggested that monitoring for EBV DNA in the plasma was an effective way to predict outcome to treatment.

**EPIDEMIOLOGY**

Conventional typing of pathogens based on their phenotypes (phage susceptibility; biochemistry, antigen profiles; antibiotic resistance; immune response; fimbriation; etc.) is not always successful in epidemiological studies. A changing spectrum of infectious agents, particularly in the immunocompromised host and in hospital outbreaks, has meant that newer epidemiological approaches are required to complement or replace the more traditional methods. Five strategies based on characterisation of pathogen-derived DNA or RNA are useful in these circumstances: (1) nucleic acid hybridisation, (2) plasmid identification, (3) chromosomal DNA banding patterns, (4) PCR and (5) sequencing.

**ANTIGENIC VARIATION**

**Influenza A**

The three RNA influenza viruses (A, B, C) are distinguished by their internal group-specific ribonucleoprotein. Only influenza A and B are medically significant since epidemics or pandemics have not occurred with influenza C. Influenza A has the potential to produce pandemics because it infects other species apart from humans (for example, birds, pigs and horses). In contrast is influenza B, which infects only humans, and so its antigenic structure does not become sufficiently different to cause pandemics. Influenza A viral envelope has two important antigenic glycoproteins—haemagglutinin (HA—composed of 15 different types) and neuraminidase (NA—9 different types) (Figure 8.6). Although the envelope antigens are capable of producing many different combinations (as seen in water birds), a smaller number are found in humans (Table 8.12). To date, only a limited number have been implicated in human-to-human spread (H1N1, H2N2, H3N2, H1N2, H5N1, H9N2 and H7N7).

As the influenza A virus passes through its hosts, the most important of which in terms of global spread are the water birds, it undergoes genetic changes that are of two types: (1) **Genetic (antigenic) drift**—new viral strains frequently result from the accumulation of point mutations in the surface glycoproteins (HA and NA). These mutations are occurring continuously over a period of time. The new strains produced are antigenic variants that nevertheless remain related to viruses from preceding epidemics. Thus, they can avoid immune surveillance in those who have developed immunity, and so outbreaks occur. Because some immunity is still present, only epidemics result. (2) **Genetic (antigenic) shift**—this results from an abrupt major change in antigenicity of the HA protein or the HA and NA protein combination. Either this is the result of a novel HA alone, or in combination with a novel NA. This virus is antigenically distinct from previous ones and has not arisen from them by mutation. It is likely that the sources of the new viral gene are water birds that have a large reservoir of different HA and NA genes. They then get into humans via other animals such as pigs or directly from chickens such as occurred with the H5N1 virus discussed below. The new viral subtype is the precursor to a pandemic because populations have never been exposed to it before (Figure 8.7).
Avian Influenza (Avian Flu, Bird Flu)

A contemporary worldwide threat to health is the large avian flu epidemic (influenza A—H5N1) presently infecting chickens across many South East Asian countries and mainland China. In 1997, the first cases of human infection from exposure to sick birds or their droppings were reported in Hong Kong. Eighteen patients were admitted to hospital and six died. Fortunately, the culling of more than a million chickens controlled this particular outbreak. Since then, there have been other human cases, with the most recent reported in Vietnam and Thailand.
By late 2004, 44 humans had contracted the avian flu, and 32 of them had died (73% mortality if the infected person was ill enough to be admitted). The “Spanish” (H1N1) flu epidemic in 1918 killed over 20 million people. However, mortality associated with this infection was estimated to be 2.5%, and so considerably less than what is being seen with H5N1 avian flu. It is possible that there have been milder cases of the H5N1 flu that have gone unrecognised, but this worrying comparison as well as the broadening host range for the H5N1 virus (particularly the involvement of the pig) remain important global concerns.

In contrast to the common human influenza virus (H3N2), which is highly contagious but rarely lethal, the avian flu in chickens is a particularly virulent type that can kill rapidly and that causes widespread organ damage. On the other hand, it is not easily transmitted from birds to humans or human to humans. So far only two probable cases of human to human transmission have occurred. However, swapping of genetic material should an individual be co-infected with both might produce a hybrid H5 (avian flu) N2 (human flu) virus with devastating effects. Another way for this mixing to occur (called reassortment) would involve an animal such as a pig being simultaneously infected with both the human influenza virus and the bird flu virus. This mixing might produce a new virulent virus if it had human genes (allowing human-to-human spread) and lethal genes from the bird virus. DNA sequencing of the viral genome from various outbreaks has shown: (1) Viruses from 1997 and 2003 Hong Kong infections have mutated; i.e., their DNA sequences are different. (2) Viruses from the most recent Vietnam and Thailand outbreak show that the virus is resistant to two of the four antiviral agents available. (3) Fortunately, the genes remain of bird origin; i.e., the virus has not acquired genes from human influenza virus, the precursor to a pandemic.

Other outbreaks of bird flu recently reported show sub-types H7N3, H5N2, H7N2, H2N2. These outbreaks have involved poultry in various parts of Canada and the USA. The H7N3 infection resulted in two humans getting a mild flu-like illness following exposure to sick poultry. One person died in New York in late 2003 from an H7N2-type influenza A infection although details of how this occurred are not known. The culling of many birds has controlled the risk of avian flu to humans, but the virus continues to re-emerge in poultry in several Asian countries, with a suggestion that H5N1 might be increasing in pathogenicity and becoming more widespread. Close surveillance by the WHO, the CDC and other bodies continues. Another concern is that clinical features and travel history of individuals who might have SARS (discussed below) and flu virus H5N1 are similar. Hence, vigilance by health professionals and airport monitoring continues, particularly in those with a travel history and respiratory symptoms suggestive of these infections. In this unpredictable environment, the value of rapid DNA-based diagnostics is obvious.

**Vaccines**

Characterisation of the different viral types and their mode of development depends on the analysis of the HA and NA amino acids, and more recently, the sequencing of the single-stranded RNA genome of this virus. For this purpose, the WHO has a number of collaborating centres that sequence (by RT-PCR) various viral strains, particularly the HA and NA genes, to monitor the changes as they occur. The ability to identify the functional components of the viral ribonucleoprotein complex and then produce these by rDNA means will provide a potential source of antigens for immunisation programs.

Another contribution from molecular medicine has come in developing a vaccine to the potentially serious avian flu virus. Normally, influenza vaccines are prepared in chicken embryos, but since H5N1 is very lethal to chickens, it cannot be grown in this way. An additional step is necessary using recombinant DNA means to take out the H5N1 genes required for the vaccine and place them in a less toxic laboratory virus that will grow in the chicken embryos.

**TAXONOMY**

**Echinococcus**

Worldwide, the tapeworm *Echinococcus granulosus* is an important public health problem leading to the development of cysts in many body organs (hydatid cyst disease). An interesting feature of this parasite is the phenotypic heterogeneity observed in the various isolates, including those obtained from different intermediate hosts such as sheep, cattle, goats and camels. The parasite’s variability has made it more difficult to understand its life cycle, to distinguish pathogenic from non-pathogenic types and to determine the best approaches to treating animals that are carriers. However, these issues are now less problematic, with DNA studies providing a more objective way to distinguish the different types, including the potential to distinguish the parasites in their intermediate hosts, making it possible to determine which parasitic strains are more likely to be infective for humans.

The DNA-based taxonomic classification of *Echinococcus* has identified nine different genotypes (G1–G9). Some of these genotypes are found in particular animal intermediate hosts; for example, G1 is the sheep form; G2, Tasmanian sheep; G6, camel; G5, cattle; and so on. The horse strain (G4) is not infective to humans. Another strain (G8) is associated with *E. granulosus* infections in Alaska and was not considered to be highly pathogenic until DNA analysis of a severely affected
Anopheles

Anopheles are insects that are medically important because of their association with malaria, filariasis and arbovirus infections. There are nearly 500 recognised species of the Anopheles mosquito. However, only a small number are vectors for human diseases. The importance of being able to identify them is illustrated by a mosquito eradication program in Vietnam that failed because the wrong mosquito was targeted. This error was discovered by DNA typing, and it occurred because the female forms of different strains in that particular geographical region were difficult to distinguish, and so a non-vector form of mosquito was mistaken for one involved in the spread of malaria.

Taxonomic classification of these mosquitoes relies on the traditional morphology, to which has been added chromosome analysis, and DNA markers. Recently, the publication of the complete genome sequence for Anopheles gambiae has opened up a new source of information that will allow even better characterisation of the Anopheles mosquito. There is also the potential for microarray studies of gene expression to understand better the mosquito's metabolic pathways and so expand the options for therapeutic targets.

Fungi

Fungi are eukaryotes that belong to a separate kingdom from plants and animals. They are ubiquitous in the environment and, in addition, have many commercial applications. In humans, infections caused by fungi (mycoses) can be superficial (e.g., skin, hair, nails) or deep (e.g., pneumonia, systemic infection, septicaemia). At particular risk are the immunocompromised for whom mycoses can be life-threatening. Treatment options in fungal diseases are limited. Until recently, there were few medically important fungi. Today, many new infectious opportunistic fungi are emerging.

The large diversity in fungal morphology, their ecological habitats and the wide spectrum of clinical consequences have complicated diagnostic approaches and our understanding of infections caused by these organisms. An important objective in studying fungi is to deduce evolutionary comparisons and, from them, determine relatedness. Fungal phylogenetics relies on parameters described earlier such as morphology, biochemistry and staining characteristics. In addition, life cycles (e.g., morphological appearances, particularly of the sexual reproductive structures) provide additional sources of information for comparison. However, traditional typing methods are not always helpful or sensitive enough and even life cycles can be uninformative; e.g., the presence of asexual forms of Coccidioides immitis has made classification particularly difficult.

An early breakthrough in fungal taxonomy based on DNA typing was the finding that Pneumocystis carinii, a very common and serious infection in AIDS as well as in other immunocompromised patients, is a fungus and not a protozoan as considered initially. Culture of human-derived P. carinii was always difficult. Its life cycle and metabolic processes were poorly understood. However, based on rRNA and DNA sequence comparisons, this organism is now considered a fungus. Today, there are new opportunities in fungal research as a result of the number of organisms that have had their genomes sequenced. This started with the S. cerevisiae yeast genome project that was completed in 1996, and there are many others in progress.

Genomics approaches are now very much part of basic research into fungi, and so microarrays (both genomic and protein) are being applied to study important pathogens. By cataloguing the gene profile of these organisms, it is expected that virulence pathways can be identified and then targeted by specific therapies. In an
epidemiologic sense, an understanding of virulence pathways facilitates the investigation of disease outbreaks, particularly nosocomial ones, since it allows a mix of organisms that are likely to be present to be accurately typed and, from this, determine which strains or subtypes are responsible for the infection.

**NOSOCOMIAL INFECTIONS**

Nosocomial (hospital acquired) infections include bloodstream infections, pneumonia, ventilator-associated pneumonia, intra-abdominal sepsis, wound infections and infections in immunocompromised patients. Pathogens causing these infections are often resistant to many antimicrobial agents. Suboptimal treatment within the hospital environment only exacerbates the problem of drug resistance. In one study of intensive care patients, the effect of bloodstream (bacteremia and septicemia) nosocomial infections increased the stay in intensive care units as well as the associated financial costs.

It is important in nosocomial infections to have methods to type pathogens to enable their source(s) and mode of spread to be identified. A composite of DNA polymorphisms will provide a unique DNA pattern for an individual person. DNA fingerprinting, as it is popularly known, is now well established in forensic practice (see Chapter 9). The DNA/RNA profile of infectious agents is similarly being exploited for diagnostic or epidemiological purposes. DNA markers can be selected from a conserved region of the microbial genome if less discrimination is required. On the other hand, choosing a DNA probe from a highly variable (i.e., polymorphic) region of the genome will allow discrimination between closely related organisms. The ultimate in DNA fingerprinting is now available in infectious diseases, and this is the use of DNA sequencing including the option to sequence the pathogen’s entire genome.

### Table 8.13 Water supply and nosocomial infections (Merlani and Francioli 2003)

| Organism | Traditional sources of infection | New source of infection from DNA analysis |
|----------|----------------------------------|------------------------------------------|
| Pseudomonas aeruginosa | In intensive care units, *P. aeruginosa* infections can originate from the endogenous gut flora of the patients. | Genotyping of the organisms showed in some studies that the taps in the patients’ rooms were the source of infection in more than a third of cases. |
| Mycobacteria spp. | Non-TB forms of mycobacteria can cause a wide range of infections including abscesses from the use of catheters or endoscopes, the surgical sites or sites related to dialysis. | Non-sterile ice and the inappropriate uses of tap water (rinsing of instruments) have been shown by DNA typing to be another source of these infections. |
| Fungi | Aspergillus is a life-threatening infection in immunosuppressed patients and is thought to be caused by fungal particles in the air. | In a three-year prospective study, aspergillus was detected in a hospital’s water system. Higher titres were found in bathrooms. Genotyping one isolate of *Aspergillus fumigatus* from an infected patient showed that this fungus was the same as isolates recovered from the shower wall in the patient’s room. |

**Legionella**

*Legionella* spp. are ubiquitous bacteria present in domestic and industrial water systems, tanks and other sources of pooled or collected water. About 45 different species of the genus *Legionella* are described. It is now considered to be a relatively common pathogen involving both community acquired as well as nosocomial legionellosis. Infection with this organism is easily overlooked because traditional diagnostic tests are not sensitive enough, particularly with species other than the common *L. pneumophila*. Although air conditioners and cooling towers are recognised sources of this organism, more recently the finding of *Legionella* spp. in drinking water provides another explanation for sporadic or larger outbreaks.

*Legionella* spp. are found in the water supply of many hospitals. In one study, 17 of 20 hospitals tested were positive, and genotyping showed that each hospital had its own different *Legionella* spp. It is considered that water-borne *Legionella* causes infection when contaminated aerosols are inhaled or there is aspiration of contaminated water. The link between hospital water supplies and various other types of nosocomial infections is slowly growing as DNA typing provides the evidence for similarity between the infecting organisms and those found in the water supply (Table 8.13).

A proportion of cases with Legionnaire’s disease is considered to be nosocomial in origin. Both sporadic cases and outbreaks of this respiratory disorder have been reported. The organism (e.g., *L. pneumophila*) is difficult to grow, and so it is frequently necessary to obtain bronchial aspirates or lung biopsies for direct detection. Serological testing is the mainstay of diagnosis although a number of DNA-derived tests are now being developed. These tests can utilise DNA hybridisation with probes that are often rRNA-specific. Another useful rDNA approach to fingerprinting bacterial genomes such as *Legionella* spp. is pulsed field gel electrophoresis (PFGE).
The technique allows large segments of DNA to be separated by using restriction enzymes such as NotI and SfiI that digest DNA very infrequently. The upper limit for resolution in conventional DNA gel electrophoresis is approximately 30 kb, whereas for pulsed field gel electrophoresis it is approaching 10 Mb ($10 \times 10^3$ kb). Pulsed field gel electrophoresis has been particularly attractive in microbiological work because the genomes of infectious agents are relatively small. For example, total DNA from Legionella spp. can be cleaved into a limited number of fragments (5–10 with NotI, depending on the strain), and these fragments or fingerprints can be directly visualised from an ethidium bromide stained gel, thereby avoiding the requirement for a DNA probe and a hybridisation step. However, the technique is very demanding. Not surprisingly, PCR-based approaches are increasingly becoming the preferred method for DNA fingerprinting. Typing of Legionella spp. for epidemiological purposes follows similar strategies.

**Methicillin Resistant Staphylococcus Aureus (MRSA)**

The first isolate of methicillin resistant *S. aureus* was reported in 1961, one year after methicillin became available. This was followed by the emergence of vancomycin-resistant MRSA in 1996. MRSA developed when *S. aureus* acquired a large segment of DNA (called SCCmec) that integrated into the *S. aureus* chromosome. This segment of DNA looks like an antibiotic “resistance cassette” because it contains genes for $\beta$-lactam resistance and genes for resistance against non-$\beta$-lactam antibiotics. Vancomycin resistance is thought to have occurred with the emergence of a strain of *S. aureus* that had a thickened cell wall, thereby preventing the vancomycin from getting into the pathogen.

Antibiotic-resistant *S. aureus* is an important and frequent cause of nosocomial infections in hospitals. When this occurs, infection control protocols need to identify the source(s) of the methicillin-resistant *S. aureus* and then determine whether there has been a breakdown in infection control practices so that more effective preventative measures can be implemented in future. For the above to occur, the different bacterial subtypes need to be distinguished. Phage typing has been the traditional tool in epidemiological studies involving *S. aureus*. However, this method has poor reproducibility, it is not able to type all isolates and it is impractical for most laboratories since it requires a large number of phage stocks to be maintained. Phage typing is now being replaced by DNA-based tests. Like the example of *Legionella*, MRSA typing can involve a wide range of techniques from PFGE to PCR. Although these techniques are often more discriminatory than the traditional approaches, they now need to be standardised to allow better inter-laboratory comparisons to be made.

**EMERGING INFECTIONS**

Emerging infections describe infections that are newly identified or whose incidence in humans has significantly increased in the past 20 years. Many factors contribute to the emerging infections, including (1) changes in human behaviour, (2) globalisation (i.e., increased travel, tourism), (3) technologic advances, economic development and changes in the environment and (4) lapses in public health measures including those resulting from poverty and war. Very few of the emerging infections represent novel pathogens. Most are the result of a change in the epidemiology or virulence of a pathogen, or secondary to microbial adaptation. Important sources of emerging infections are animals.

**ZOOONESSES**

In the past, the emergence of infectious agents reflected changed patterns of human movements that disrupted traditional geographical boundaries. For example, yellow fever is thought to have emerged in the New World as a result of the African slave trade, which brought the mosquito *Aedes aegypti* in ships’ water containers. More recently, *Aedes albopictus*, a potential vector for dengue virus, has become established in the USA following its conveyance from South East Asia in old car tyres. With this, the threat of dengue in the North American continent has become real. Most emergent viruses are zoonotic; i.e., they are acquired from animals, which are reservoirs for infection. Thus, completely new strains are less likely than the appearance of a virus following a change in animal reservoirs. This is particularly relevant to the modern world where the consequences of easy migration, deforestation, agricultural practices, dam building and urbanisation are making, and will continue to have, a major impact on the ecology of animals. At the same time, humans have increasingly populated rural areas and are pursuing more outdoor recreational activities. There is also a growing trend for keeping exotic animals as household pets. Changes in global climate may also contribute directly, through their effects on vegetation, insect and rodent populations.

Table 8.14 lists a number of zoonoses that have become established as new infectious diseases or are emerging as problems for the future. Some of them are newly acquired in the West, whereas others remain endemic to specific countries. In many cases, molecular
| Pathogen                  | Clinical problems                                                                 | Epidemiology                                                                 | Emergence                                                                                       | Role of DNA technology                                                                 |
|--------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| West Nile Virus          | Febrile illness complicated by meningoencephalitis, weakness and paralysis.        | Transmitted by mosquitoes; blood or organ donation; pregnancy, lactation; infected needles or laboratory specimens. The virus is maintained by a bird-mosquito-bird cycle. | Isolated in 1937 from Uganda and found in many parts of the world. Appeared in the USA in 1999 and has rapidly spread across North America. | NAT is used to screen blood donors and is potentially useful for immunosuppressed individuals who cannot mount an antibody response for serologic testing. |
| Monkeypox virus          | Self-limited febrile illness with vesiculo-pustular eruptions. Can be confused with more serious illnesses. | Primary animal reservoir is the rat.                                         | Recognised in 1958 and remained localised to Africa. In 2003, outbreak in Midwest USA shown to be monkeypox. Traced back to rats imported from Africa to which native prairie dogs were exposed and became infected and then infected humans. Appears to be contained. | DNA characterisation helped in identifying this virus as monkeypox.                      |
| Ebola                    | Haemorrhagic fever in humans (mortality 20–100%)                                  | Example of increased human-to-animal contact in tropical forest. Animal host remains unknown (bats are suspected). | First isolated in 1976 from Sudan and Zaire. Since then, sporadic outbreaks have occurred. Remains isolated to Africa. | PCR-based assays for rapid and sensitive diagnostic tests described.                      |
| Lassa fever              | Haemorrhagic fever with 20% having severe multisystem disease.                    | Rats infect humans (by contact or if eaten). Virus excreted in human urine/ semen for months after infection. | Endemic in West Africa since 1950s.                                                          | Nil                                                                                      |
| Hantavirus               | Haemorrhagic fever with renal syndrome and pulmonary syndrome (severe disorders). | Infection occurs through exposure to rodent excreta.                         | Isolated in 1979 in Korea. Now established within the Eurasian continent. Outbreaks in the USA in 1993 and 1998 thought to be due to climatic changes, increasing vegetation and so an increase in the rodent population. Now established throughout North Central and South America. | The types of Hantaviruses in different locations show a close relationship to their rat host. But now shown by DNA analysis that hantaviruses can jump from one rat species to another. This will make it more difficult to know from studying the rat populations if a particular type is likely to be virulent. |
| Lyme disease             | Early non-specific malaise can be complicated by arthritis, neurologic and cardiac problems. | Tick (*Ixodes* spp.) transmitted disease. Mice, rodents and birds are the intermediate hosts. | First recognised in the USA in 1957; since then reported in many countries.                   | DNA characterisation useful for epidemiologic purposes and to explain different clinical features in various countries. |
| HIV                      | Serious acquired immunodeficiency disorder.                                      | Cross-species transmission from non-human primates followed by human-to-human spread. | Evidence for the link between non-human primate and human disease includes (1) similar viral genomes, (2) prevalence in the natural host, (3) geographic co-location. | DNA technology has been helpful in all phases of this particular disease from diagnosis to prognosis (in terms of viral load determination and detection of viral resistance). |
medicine offers little to current management including
diagnosis, although much knowledge about these infec-
tions has come from analysis of the organisms’ DNA or
RNA. However, the potential for spread of any disease is
high because of international travel or the mass disloca-
tion of large populations following civil unrest. There is
also an increasing awareness that a number of pathogens
could be used for bioterrorism. Some of the zoonoses
associated with a viral haemorrhagic clinical picture can
be confused with other clinical infections including
malaria, leptospirosis and Neisseria meningitidis, and in
these potentially fatal conditions, a rapid screening test
is essential. In terms of bioterrorism and the differential
diagnosis of haemorrhagic fevers, PCR (NAT) based
assays are presently the only options with the potential
to allow rapid and sensitive diagnostic tests to be
developed.

### SARS

SARS (severe acute respiratory syndrome) attracted a lot
of publicity and provoked fear in 2003. It has been
described by some as the first pandemic of the twenty-
fifth century since it involved over 8000 patients and
caused more than 800 deaths in 30 countries on five con-
tinents (Peiris et al 2003). The economic impact of this
infection in Hong Kong was estimated to be nearly US$6
billion. SARS also represents a contemporary paradigm
that illustrates the value of molecular-based DNA
approaches in dealing with a serious emerging infection.
The journal Science acknowledged the impressive work
undertaken to control SARS, citing it as the outstanding
achievement of the year: “SARS: a pandemic prevented”
(see Table 8.11). A chronology of events in the SARS story
is given in Table 8.15.

Traditional approaches such as viral culture, electron
microscopy and serology played a key role in character-
ising the SARS virus now shown to be a coronavirus
(CoV). Nevertheless, the molecular approach enabled the
following key information to be obtained in a very short
time frame:

- Molecular typing of this virus from an outbreak in
  Taiwan confirmed that the virus was identical in its
  DNA sequence to a virus isolated from a Hong Kong
  outbreak; i.e., human-to-human spread had occurred
  through an individual who had been in Hong Kong.
  Similar comparisons were possible, linking various
  outbreaks through molecular epidemiology.

- Rapid whole genome sequencing of the viral RNA
  provided a firm base for the development of PCR-
  based diagnostic assays, a critical requirement for
  acute infections such as SARS-CoV.

- In searching for animal reservoirs, RT-PCR–based
  techniques were used. They enabled the SARS-CoV to
  be detected, as well as identified genetic differences
  between the human and animal virus.

- Future understanding of the virus, how it evolved,
  what strains were pathogenic and its mode of spread
to populations could be addressed through DNA
  approaches. For this, the US National Institute of
  Allergy and Infectious Diseases released a genome
  chip containing the full 29700 base pairs of the SARS
  viral genome.

The SARS outbreak ended just as quickly as it started.
Only the occasional cases were reported in early 2004,
and none was reported after the end of April that year.
However, there remain many unanswered questions,
including the inconsistent human-to-human transmission
with documented cases of one infected passenger trans-
mitting to a number of others on a particular flight, while
on another flight no transmission occurred despite a
number of passengers being ill with this virus. These
inconsistencies were explained by proposing that some

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**Table 8.14 Continued**

| Pathogen | Clinical problems | Epidemiology | Emergence | Role of DNA technology |
|----------|------------------|--------------|-----------|------------------------|
| BSE, CJD, vCJD | Fatal spongiform encephalitis with a long incubation period. | BSE detected in mid-1980s in cattle, resulting from transmission of prions in meat and bone meat products used as animal feed in the UK. | vCJD, the human equivalent of BSE, identified from 1996 due to the ingestion of meat from infected cattle. Theoretical risk that it can be transmitted through blood, although not proven. | Infectious agent does not have nucleic acid and so not detectable by conventional means. DNA analysis has shown mutations in the PRNP gene (see also Figure 8.8 and Chapter 4). |
| PERV | Xenotransplants (especially from pigs) considered as a source of organs or cells because there are insufficient human donors. | Concern that endogenous pig retroviruses (PERV) might jump the species barrier particularly if immunosuppression is needed for the xenotransplant. | Theoretical risk based on in vivo evidence that PERV can infect human cell lines. Nevertheless, a concern holding back xenotransplantation. | DNA testing can detect PERV but does not necessarily indicate infectivity. |
of those infected were “super-spreaders.” Another intriguing observation with SARS is the relatively large numbers of health workers that have been infected; i.e., SARS transmission seems to be more likely within a hospital setting.

Although a number of emerging viruses have been described recently, SARS-CoV is particularly worrying because it is one of the few that has shown human-to-human transmission, although fortunately this seems to be relatively inefficient. For the future, a key issue will be early and prompt recognition of new cases to enable traditional public health measures (the reason for effective containment on this occasion, although the seasonal changes may also have contributed) to be implemented even faster. The WHO has also flagged the importance of laboratory containment when dealing with this virus. This became an issue when two of the nine persons infected in China in 2004 worked in a reference laboratory conducting research into SARS-CoV. A similar scenario was reported earlier from Singapore. The latter case was clearly documented on DNA sequencing of the virus to be due to a contaminated laboratory culture that the scientist had been working with three days before showing signs of the infection.

An issue that is still outstanding with SARS-CoV is how this virus is normally spread. Communicable respiratory infections traditionally infect others because of droplet spread or contact with contaminated surfaces. Some preliminary data are now being reported that SARS-CoV involves airborne transmission. If proven, this will be an interesting public health challenge requiring a better understanding of this mode of transmission, including its relationship to modern housing, travel and working environments. Detecting viral particles involved in airborne spread will not be easy, and for this PCR will become an indispensable tool for the public health professional.

### BIOTERROR

After the 2001 anthrax bioterror cases in the USA, the world has been placed on alert. A new form of modern terrorism had been launched. In Chapter 9 reference is made to the US government’s move to develop “microbial forensics” as a way in which to deal with bioterrorism or biocrimes involving the use of human or animal pathogens. DNA technology will be a particularly valuable asset in microbial forensics, allowing the rapid identification of various pathogens, and as part of the detective work, to find the source of the infection. As discussed further in Chapters 1 and 9, it is fortuitous that a component of the Human Genome Project was the sequencing of genomes from a number of pathogens. This information will be needed in combating bioterrorism. Biological warfare or bioterrorism is possible through many ways, including the contamination of food or water.

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**Table 8.15 Chronology of events in the SARS story**

| Date          | Event                                                                 |
|---------------|----------------------------------------------------------------------|
| November 2002 | Atypical pneumonia cases in Guangdong Province, China.               |
| January 2003  | Outbreaks of pneumonia in same province.                             |
| February 11, 2003 | WHO notified about outbreaks of pneumonia in Guangdong Province. At this stage concern that this represents influenza A (H5N1) cases identified in Hong Kong. |
| February 21, 2003 | Resident from Guangdong Province checks into Hong Kong hotel. He becomes ill and is hospitalised. Subsequently shown that he has infected at least 17 other visitors/guests at the hotel (they will later travel to Vietnam, Singapore and Toronto to spread SARS locally). |
| February–March 2003 | Contacts at Hong Kong hotel become ill, and some infect health care workers. Hotel contact dies in Toronto. |
| March 12–14, 2003 | WHO issues global alert; clusters of atypical pneumonia are reported in Singapore and Toronto (later, it will become evident that these clusters are linked to the Hong Kong hotel). |
| March 15, 2003  | The atypical pneumonia is called SARS; more than 150 new cases are reported; WHO issues a travel warning. |
| Mid-late March 2003 | Novel coronavirus isolated from SARS patients. |
| May 12, 2003   | The complete sequence of the coronavirus is announced; virus now called SARS-CoV. |
| June 2003      | Virus related to SARS-CoV isolated from animals.                     |
| July 2003      | Official end of the SARS outbreak in humans.                        |
| August 2003    | The identification of SARS-CoV as the cause of an outbreak of mild respiratory infection in a Canadian nursing home is recanted. Error based on laboratory misdiagnosis. |
| September 2003 | Laboratory-acquired SARS-CoV infection (mild) reported in Singapore. |
| October 2003   | Concern expressed that the definitive animal reservoir for SARS-CoV remains unknown and so future outbreaks are possible. Virus detected in civet cats and a raccoon dog in a Chinese market. These are a likely source for the human infections, but the source for these animals remains unknown. |
| December 19, 2003 | Breakthrough of the Year by Science magazine: SARS: A pandemic prevented. |
| January–April 2004 | In January, two suspected cases of SARS-CoV confirmed in Guangdong. The last cluster of nine cases (one fatal) reported in China in April. No further cases of SARS described after April 29, 2004. |
Supplies, or via infected animals including insects. A more serious form would involve the use of aerosols containing the pathogens because now there is the risk for infecting a very large number of people. The length of the incubation period is also a consideration since it would, to some degree, influence the number infected before containment or treatment was initiated. Biosensors are being developed to assist early detection. In some infections, DNA-based chip technology is being tried as a way in which microbes can be detected directly through their DNA sequence. This will ensure rapid detection and typing of pathogens.

Two pathogens of particular relevance to bioterror are anthrax and smallpox. DNA-based genotyping has already proven its value in the case of the 2001 US anthrax cases (Box 8.3). Promising reports now suggest that a pathogen such as anthrax could be detected within an hour by real-time PCR. Smallpox is another serious infection since routine vaccination was stopped in the USA in the early 1970s, leaving many unvaccinated and so vulnerable targets in this and other countries. In Asia, case fatality rates of around 30% were observed during epidemics, and there are no known treatments for smallpox. As part of its biologic warfare program, the former Soviet Union produced smallpox, anthrax and other pathogens, and it remains a concern that some could fall into the hands of potential terrorists.

Box 8.3 Bioterrorism using anthrax (Griffith et al 2003).

Anthrax is caused by B. anthracis, a gram positive spore-forming organism. It is usually acquired by humans through exposure to infected animal products or contaminated dust. The major forms of anthrax are cutaneous (95% of cases, with a mortality of about 20%) and pulmonary (100% mortality if not treated before symptoms develop). In October and November following the September 11, 2001, World Trade Centre disaster, 7 cutaneous and 11 inhalational cases of anthrax occurred in Florida, New York City, New Jersey, Washington DC and Connecticut. They were examples of bioterrorism since all bacteria came from the one source based on DNA typing, which showed an identical genotype. The likely route of infection was intentionally contaminated mail. Apart from two cases, all others were mail or government workers, or employees at news media outlets. The two exceptions were a 61-year-old woman in New York City and a 94-year-old woman in Connecticut. Both died after developing inhalational anthrax. It was possible to detect a likely source of infection in the case of the Connecticut victim. The postal processing and distribution centre (PDCs) for the letter-related cases had been identified through the finding of contaminating bacteria. However, for this victim, no samples from her home or personal items tested positive for B. anthracis. It was known that she had received mail (including junk or bulk mail) from one of these PDCs. It was also subsequently shown that her local PDC (Southern Connecticut) had a heavily contaminated sorting machine. From this, it was assumed that B. anthracis identical to those involved in the other outbreaks had infected her through a letter directly contaminated in a PDC implicated in the earlier infections, or secondarily contaminated in the Southern Connecticut PDC.
Over the past century, there has been a steady decline in infectious diseases in affluent countries, and these diseases have become relatively minor problems when compared to the increasing role played by chronic and neoplastic diseases. During the 1980s this situation started to change with the onset of AIDS and its related infections such as tuberculosis. Other emerging infections have appeared and are important concerns for the future.

The hepatitis C virus is an example of a pathogen that is starting to impact on the health and well-being of many communities. Although it does not have the same emotive force as HIV, it is an important modern scourge that will need to have its spread controlled more effectively, and those infected will need access to antiviral agents (Box 8.4).

A key to controlling the spread of modern pathogens will be effective vaccines that are developed on the basis of the organism’s genome sequence (some call this reverse vaccinology). This is illustrated by the group B meningococcus for which a vaccine could not be developed by traditional means. However, soon after the genome was sequenced, it was possible to identify many potential vaccine candidates that are now being tested in clinical trials. The genomic era has opened up new opportunities for the control of infectious diseases.

The SARS example also illustrates the value of genomics in public health medicine. In particular, the very rapid sequencing of the genome of what appeared to be a coronavirus-like pathogen enabled a definitive diagnosis to be made and potential vaccine targets to be identified, some of which are already in the animal phase of testing.

Developments in technology are addressing many of the potential problems related to DNA testing in microbiology. A challenge for the future will be a better understanding of what the finding of a DNA (or RNA) sequence means in terms of infectivity and pathogenesis. The traditional approach has many years of experience to help in interpreting a result such as a positive culture. The same experience is not available with DNA testing, but it will be necessary to do the hard work and determine the significance of various DNA tests. An example would be persistence of mycobacterial growth in culture despite treatment. This would suggest a compliance issue or the development of drug resistance. The finding of a persistently positive DNA test in this circumstance would be more difficult to interpret. The validation of positive DNA test results will be necessary for a wide range of infections; for example, the finding of CMV DNA by PCR in a patient’s serum could mean active disease or latent infection. How these issues are resolved will be important for the integration of molecular diagnostics into routine microbiological laboratory practice.

No doubt the potential for genomics will allow novel ways to detect infection with pathogens. One early model described involves the use of microarrays to detect gene profiles in the patient rather than attempts at detecting pathogen-specific nucleic acids. Using this approach, it was claimed that peripheral blood mononuclear cells exposed to M. tuberculosis, a protozoan (Leishmania donovani) and a worm (Brugia malayi) demonstrated discrete expression profiles particular to the infectious agent.

**Box 8.4 Hepatitis C virus (HCV).**

Thirteen years after the term “non-A, non-B” hepatitis was first used, the HCV virus was found. A milestone in molecular virology came when HCV was cloned in 1988, years before it was visualised, cultured or even characterised. Based on its amino acid sequence and genomic organisation, HCV was classed in a separate genus in the viral family Flaviviridae. HCV is an RNA virus and has six genotypes (1–6). The main source of infection today is intravenous drug use. Transfusion-related HCV is rarely seen because of the effective screening methods in place. Unlike HBV, mother-to-infant and sexual transmission is rare. Nearly 170 million people worldwide are infected with HCV, which is now the most common reason for liver transplantation in the USA. Unlike HBV, which infrequently leads to chronic hepatitis, about 70% of those infected with HCV develop this complication. Like HBV, the hepatic damage caused by HCV is thought to be secondary to an immune response rather than a direct viral effect. In addition, age, male sex, alcohol consumption, coexistent HIV infection, low CD4 count and metabolic disorders such as diabetes predispose to the development of liver cirrhosis and subsequently hepatocellular cancer. Like HIV, excellent serologic or DNA-based assays are available for accurate diagnosis. Quantitative RNA assays and genotyping enable predictions to be made of how an individual will respond to antiviral therapy. Treatments using interferon alpha and ribavirin are effective, particularly with HCV genotypes 2 and 3 (88% response rates compared to 48% for the other four genotypes). However, because of inadequate detection (or the money to pay for the above drugs), there is considerable concern that what is being seen at present is only the tip of the iceberg since the virus has a long incubation period, and many who are carriers remain asymptomatic until complications develop.
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