

## CHAPTER 3.2.

# BIOTECHNOLOGY IN THE DIAGNOSIS OF INFECTIOUS DISEASES

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## INTRODUCTION

*Modern concepts of livestock breeding and increased global trade demand the development of early warning diagnostic platforms, global capacity and diagnostic harmonisation to enhance livestock production and health and contribute towards uniting pathogen detection technologies as a basis of the global one-world, one-health initiative.*

*The global development of improved transport infrastructure has contributed to increased international movement of people and goods. Consequently, the risk of pathogen transfer between countries or even between continents is increased. In response to global climate changes, diseases endemic in certain areas, such as bluetongue and African swine fever, are showing a tendency to move or extend to other geographical areas outside their normal range. Finally, the constant intensification of animal production requires different approaches related to the preventive measures, diagnosis and control of animal diseases.*

*In order to be widely accepted, modern diagnostic methods have to be validated, sensitive, specific, rapid, user friendly, cost effective, and eventually automated to facilitate the evaluation of large numbers of samples.*

*Eradication of rinderpest is a unique example of the application of new technologies to the control and eradication of a disease, where the virus neutralisation test was replaced with a well validated enzyme linked immunosorbent assay that was used in the eradication programme worldwide.*

*The purpose of this chapter is to provide general background information for the non-specialist. Two issues of the OIE Scientific and Technical Review are concerned with biotechnology and the diagnosis of animal diseases and are available on the OIE website. The following is an outline of the topics briefly reviewed in this chapter.*

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## A. DETECTION OF NUCLEIC ACIDS

Efficient management of infectious diseases, in particular those of a transboundary nature, require rapid, sensitive, specific and confirmatory identification of the pathogen. Conventional diagnostic techniques such as the isolation of the pathogen (the gold standard for disease diagnosis), are not only laborious for fastidious pathogens that are difficult to cultivate *in vitro*, but could also pose a risk to diagnostic technicians if they are zoonotic in nature. As these limitations often require high level security laboratories (e.g. BSL3 level), the amplification of nucleic acids has been shown to have advantages. The polymerase chain reaction (PCR)-based platform technologies have, in particular, quickly revolutionised infectious disease diagnoses. The DNA amplification platforms are sensitive, specific, rapid and robust as they use several non-infectious nucleic acid templates as starting material. The platforms are also cost effective and prone to automatisisation and therefore ideal for high throughput.

### 1. Nucleic acid extraction

An important step in the molecular diagnostic procedure is extraction of 'clean' nucleic acid mixtures to act as a template for the reactions. While it is relatively easy to extract DNA from bacterial cultures or blood, it is technically more challenging to prepare suitable material from field samples, such as faeces, internal organs or abortion material. If the target material has not been purified of contaminants in the clinical sample, the assay is compromised and may yield false results. On the other hand, having to process large numbers of samples increases the risk of contamination, leading to false-positive results. Manipulation steps during the extraction of nucleic acids are critical cause of cross contamination of samples, leading to false positive results. From this reason, nucleic acid extraction should be performed using strict working procedures, in an isolated room, separated from other phases of the PCR procedure (such as preparation of the master mix, addition of extracted samples into the master mix or amplification). Multiple negative controls (such as DEPC [diethylpyrocarbonate] water) are usually inserted between individual samples of the tested sample sets in order to detect eventual cross contamination.

Several types of samples are known to inhibit the PCR reaction, leading to false-negative results. Including an internal control in the assay is important for the quality control of the nucleic acid extraction, to prove the absence of PCR inhibitors. These internal controls can be either a house-keeping gene, an endogenous gene, a constant basal cell-expressed gene, such as the gluceraldehyde-3-phosphatase (GPDH3) or the  $\beta$ actin, or an exogenous nucleic acid that is not present naturally in the preparation but added at the extraction step (Belák & Thorén, 2001). A number of specialised methods for particular types of samples and tissues, most of which are now commercially available either as manual or automated systems for robotic workstations. The development and accessibility of the robotic extraction platforms not only minimises the risk of contamination, but also enables processing of large numbers of samples under constant reaction conditions and minimal operator manipulation. Consequently, these platforms have contributed to the establishment of high-throughput, robust diagnostic assays, shortening the processing time required per sample from hours to minutes (Belák *et al.*, 2009). These are destined to improve the reliability of nucleic acid extraction from different samples, but it still remains a challenging area.

As an alternative to nucleic acid extraction, biotechnologists are increasingly focusing on polymerases that are resistant to PCR inhibitors and several are now available on the market for direct amplification of nucleic acids from pathological specimens without any extraction step. Assays increasingly use an internal control to demonstrate that PCR inhibitors are not present.

## 2. Polymerase chain reaction (PCR), real-time PCR

The PCR exploits natural DNA replication mechanisms and results in the *in-vitro* production of large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences (Saiki *et al.*, 1988). PCR can amplify a selected region of from fifty to several thousand base pairs into billions of copies. The specificity of the amplified region can be targeted by specific primers (short synthetic molecules of DNA complementary to both strands and flanking the target sequences), which are annealed to the single-stranded template and extended with the DNA polymerase.

The amplification of DNA in PCR protocols is accomplished using a succession of cyclic incubation steps at different temperatures. The target DNA is first heat-denatured to separate the two complementary strands to provide a single-stranded template. Specific primers are then annealed to the single-stranded template at a low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesised a new strand of DNA, the product is separated from the template by heating to a higher temperature. These steps, referred to as cycles, are repeated 20–40 times, resulting in amplification of target DNA sequences. The key to the geometric amplification of target DNA sequences by the PCR is the selection of paired primers that, when extended, will create additional reciprocal primer-annealing sites for primer extension in subsequent cycles. To detect RNA (e.g. RNA viruses), a cDNA copy of the RNA must first be made using reverse transcriptase (RT). The cDNA then acts as the template for amplification by the PCR. This technique is referred to as reverse transcription PCR (RT-PCR).

The identity of the PCR product is defined by its characteristic size, and/or confirmed using DNA probes (see below), or restriction digests, which can be used to provide restriction fragment length polymorphisms (RFLP) (see Section A.5). More commonly, since the advent of automated cycle sequencing techniques, identification can be made unequivocally via direct sequencing of the PCR product. For example, sequencing is used in the virulence typing of avian influenza A virus, in which virulence-associated structural motifs at the haemagglutinin gene cleavage site are reliable indicators of high pathogenicity in chickens. The sensitivity of a PCR may be enhanced by the use of a second set of primers to amplify a sub-fragment from the PCR product of the first reaction. This technique is commonly referred to as 'nested PCR' and has been used to detect low levels of pathogens in the sample. However, the use of nested PCR can increase the rate of environmental contamination during the second (subfragment) amplification and consequently increase the risk of false-positive results.

PCR is a highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when only a small number of host cells are infected. PCR can target and amplify a gene sequence that has become integrated into the DNA of infected host cells. It can also target and amplify unintegrated viral gene sequences. It is clear that PCR has a role in the testing of vaccines to detect contamination. However, it does not differentiate between viable and nonviable organisms or incomplete pieces of genomic DNA, and this may complicate interpretation of results and affect the applicability of PCR in this role.

PCR may prove to be very useful in the diagnosis of chronic, persistent infections, such as bovine viral diarrhoea (BVD), enzootic bovine leukosis or caprine arthritis/encephalitis virus. These diseases present serious problems in terms of diagnosis and prevention as infected animals are a constant potential source of transmission.

To expand its utility in veterinary diagnostics and pathogen identification, PCR has been extensively modified over the years. PCR using broadly conserved primers is designed for identification of classes of pathogens. Using PCR primers that are complementary to these conserved sequence regions, the presence in the sample of any bacteria of a desired class can be determined. It must be noted that a positive PCR result needs to be further characterised by hybridisation with species-specific probes, analysis by RFLP, or by sequencing. Similarly, consensus PCR can be designed to use degenerate primers targeted at conserved sequence regions or motifs of a group of related pathogens. The targeting of degenerate primers (i.e. a mixture of similar primers with different bases in some positions) has led to the identification of many previously unrecognised viruses in various animal species. On the other hand, multiplex PCR has been designed to use two or more primer pairs directed at pathogen-specific unique sequences within a single reaction for simultaneous detection of multiple pathogens. Multiplex PCR has the advantage of a high degree of sensitivity and specificity. However, there have been reports that multiplexing can reduce sensitivity compared with single reactions, because of competition. If it is important to have a very sensitive assay, this should be considered during the validation procedure (assay development and optimisation).

Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now widely replaced with real-time PCR assays. In real-time PCR assays, intercalating dyes or a target-specific probe or primer (labelled with fluorescent dye) are used. The measured fluorescent signal is proportional to the number of specific DNA fragments produced. Thus, during the real-time PCR, the accumulation of PCR products can be monitored in each consecutive cycle as a change in the degree of fluorescence. In other words, the assay can be used for absolute or relative quantification of the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid, has a closed-tube format (decreased risk of cross-contamination), is highly sensitive and specific thus retaining qualitative efficiency, and provides quantitative

information. In many cases, real-time PCR assays have proved to be more sensitive than existing reference methods. The development of portable real-time PCR machines and assays raises the prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

When PCR is used for diagnosis, a great deal of care is required to avoid contamination of the samples because the exquisite sensitivity of the technique can easily lead to false-positive results. Multicentre studies have shown that positive samples are detected consistently between laboratories, but that false positives are frequently obtained with known negative samples, indicating the continuing presence of contamination problems (Schweiger *et al.*, 1997). A new generation of robotic workstations is now available where PCR reactions may be set up with only a single tube open at any one time. This greatly reduces the risk of contamination. It is also important to control for potential 'negative' results caused by the presence of PCR inhibitors in the reaction mixture. A template, independent of the target DNA, known to produce a PCR product (mimics) with specific primers can be used as a control for the PCR inhibitors, thus indicating false-negative results (Belák *et al.*, 2009). Use of these precautions allows the PCR to become a realistic option for the diagnostician.

The generation of the signal in a real-time PCR assay has been limited, until recently, to certain chemistries, such as intercalating dyes (SYBR Green and EvaGreen), hydrolysis probes, molecular beacons, primer probe energy transfer (PriProET), scorpion primers, dual hybridisation probes and dye-labelled oligonucleotide ligation (Belák *et al.*, 2009). Alternative labelling has been developed using tags that enable high multiplexing of the assay. The mass tag PCR assay is an improvement of the real-time PCR platform, in which the primers are tagged with tags of known, but different, molecular weights. After amplification of the targeted DNA fragments, the tags are released using UV light and subsequently measured using mass spectrometry. This approach enables multiplexing of much larger panels of target DNA fragments (and hence multiple diseases), as the assay is not limited to the number of dyes available. Application of the mass tag PCR assay has been already proven in differential diagnosis of syndromic diseases (respiratory, haemorrhagic, enteric pathogens, meningitis/encephalitis syndrome) and detection of new clades of pathogens (Lipkin, 2010). A modification of this method uses matrix-assisted laser desorption-ionisation (MALDI), which directly measures the molecular weights of the PCR products and compares them with known databases (Lipkin, 2010).

Additional improvement in the sequencing technology has been achieved by shifting from photometric to chemical detection of the PCR reaction in real time (Neidringhaus *et al.*, 2011). The technology is called ion torrent sequencing (pH-mediated sequencing, silicon sequencing or semiconductor sequencing) and is based on detection of the release of hydrogen ions, when a nucleotide is incorporated into a strand of DNA by the polymerase. Hydrogen ions will change the pH of the solution, which can be detected by an ion sensor (micro pH meter). The whole setup uses high-density array of micro-machined wells to perform the biochemical process in a massively parallel way. Fourth generation sequencing sequencing platforms, such as nanopore sequencing technologies, long read extension methods and methods based on direct video recording of nucleic bases, have already been developed as a proof of principle (Neidringhaus *et al.*, 2011). Their impact within the scientific community is still to be evaluated.

### **3. Isothermal amplification**

Isothermal amplification technologies offer the advantage of omitting thermocycling, enabling DNA amplification at constant temperature. These technologies include nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), signal-mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), isothermal multiple displacement amplification (IMDA), Helicase-dependent amplification (HDA), circular helicase-dependent amplification (cHDA), single primer isothermal amplification (SPIA) and strand invasion-based amplification (SIBA) (Gill & Ghaemi, 2008). The most widely used method is the LAMP, which deploys four primers forming a stem-loop DNA by self-primed DNA synthesis and a DNA polymerase with strand displacement activity. The result of the amplification process is the production of loops at the ends of the complementary strands, which are continually extended. The amplification process is indicated by either the occurrence of turbidity in the reaction mixture or the generation of a fluorescent signal using fluorescent dyes (Gill & Ghaemi, 2008).

### **4. Detection of nucleic acids without DNA amplification**

A new approach to detection of target DNA has been developed using surface-enhanced Raman scattering (SERS) (Harpster *et al.*, 2009). The method is based on capturing a thiol-conjugated probe and a methylene blue-labelled reporter probe, which are attached to the complementary (target) DNA. A Raman label positions the methylene blue on a gold nanoparticle, to an optimal distance for SERS enhancement. The production of a measurable, capture-specific signal of the Raman spectrum is produced by elicitation of surface-enhanced plasmon resonance by laser excitation. The analytical sensitivity of the method is in the nanomolar range. The signal can also be measured by quartz crystal microbalance-dissipation (a method based on the induction of a piezoelectric effect), which enables quantitative interpretation of the results. A modification of this method uses paramagnetic nanoparticles as a carrier for the capture probe. Once the target DNA is attached to the capture

and reporter probes, the complexes are attracted magnetically to a defined position, where the “concentrated” signal is measured. Quantitative evaluation of the results is also possible using this method.

## 5. Diagnosis by restriction fragment length polymorphisms (RFLP) and related DNA-based approaches

Serological tests that are commonly used to identify microorganisms may not distinguish between isolates of closely related pathogens, whether they are viruses, bacteria, fungi or parasites. DNA-based procedures can offer a higher degree of discrimination that is often required. An appropriate starting point may be analyses for restriction fragment length polymorphisms (RFLP).

The RFLP approach is based on the fact that the genomes of even closely related pathogens are defined by variation in sequences. For example, the linear order of adjacent nucleotides comprising the recognition sequence of a specific restriction enzyme in one genome may be absent in the genome of a closely related strain or isolate.

In practice the RFLP procedure consists of isolating the target pathogen, extracting DNA or RNA (with subsequent reverse transcription to cDNA), and then digesting the nucleic acid with one or a panel of restriction enzymes. These restriction enzymes are endonucleases that recognise and cleave double-stranded DNA at specific nucleotide sequences called restriction sites. The individual fragments within the digested DNA are then separated by gel electrophoresis and visualised by staining with ethidium bromide. Ideally each strain will reveal a unique pattern, or fingerprint. Many different restriction enzymes may be considered at the outset, so that analyses of many molecular fingerprints from digestions with several individual restriction enzymes may be undertaken; a combination of the best set of results will allow a comprehensive differentiation between strains or isolates (Loza-Rubio *et al.*, 1999).

Of greater utility to the study of pathogens is a modification to the basic RFLP technique whereby the PCR is incorporated as a preliminary step. The PCR method is used to amplify a specific region of the genome (known already by the investigator to be variable in sequence between different pathogens), which then serves as the template DNA for the RFLP technique. This combination (PCR-RFLP) offers a much greater sensitivity and comparability for the identification of pathogens and is especially useful when the pathogen is present in the infected animal in low numbers or is difficult to isolate in culture. The involvement of specific strains or types in a disease outbreak can be thus determined and the epidemiological tracing of isolates within a country or between countries should be possible. The incorporation of pulsed-field gel electrophoresis (PFGE) facilitates the separation of large (up to megabase size) fragments of DNA and can be a useful adjunct to the basic RFLP analysis. These technologies are extensively used in the official programme for detection and discrimination of food-borne pathogens (*Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Listeria*, or *Campylobacter*) by the Center for Disease Control, US Department of Health and Human Services (<http://www.cdc.gov/pulsenet/>).

RFLPs have a clear value for use in epidemiological studies but more critical interpretation of RFLP data involves the construction of databases to determine whether the RFLP profiles are clinically significant and linked to factors such as virulence or host range. In practice, it is usual not to rely on one restriction site but to use sites from several locations within the genome to classify an isolate. A continuing dilemma for veterinary diagnosticians is the correct assessment of any molecular differences that might be found between different isolates of a pathogen, as the loss or acquisition of restriction endonuclease site(s) may not be associated with differences in the ability of the pathogen to cause disease, i.e. an RFLP difference may not be functionally significant, except as a distinguishing feature.

Random amplified polymorphic DNA (RAPD) is performed using 8–12-mer random primers that will or will not amplify a segment of DNA depending on complementarity to the primers' sequence. If a mutation occurs in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. RAPD markers that define individual strains, may be sequenced and then used as a sequence-confirmed amplified region (SCAR). Thus conversion of an anonymous polymorphic marker to a SCAR means that a single PCR may be done to more simply identify a specific genome (Lewin *et al.*, 2002).

Although the RFLP and PCR-RFLP are much less powerful compared with the modern sequencing technologies, they are inexpensive, easy to perform and sufficiently descriptive for epidemiological investigations of outbreaks and identification of individual strains of pathogens. The RFLP/PCR-RFLP motif investigation is a valuable tool to differentiate the origin of an infection (especially where animal-to-human transfer has occurred) and/or to differentiate between field and vaccinal strains of individual pathogens.

## 6. Genome sequencing

The techniques by which DNA from a pathogen may be detected and characterised continue to improve and evolve. Presently, the ultimate discriminatory procedure is that of genome sequencing. Since 1977, the Sanger method (Sanger *et al.*, 1977) has been the dominant approach and gold standard for DNA sequencing.

Conventional DNA sequencing is based on cycle sequencing of targeted DNA fragments with labelled di-deoxy nucleotides, which have a property to stop the elongation at their place of binding. Each di-deoxy nucleotide is labelled with different dye, enabling distinction between individual di-deoxy nucleotides. As each di-deoxy nucleotide competes with the 'normal' nucleotides for their complementary binding sites, the result of such PCR amplification will be a mixture of DNA fragments of different length, each ending with a defined di-deoxy nucleotide (identifiable by its colour). The PCR mixture is then analysed using capillary electrophoresis, which separates the fragments by length and reads the colour of each fragment. Analytical software is then used to convert the colour signals to a layout of nucleotides.

Development of microarray technologies, as well as the improvements in DNA manipulation, have contributed significantly to the development of direct sequencing protocols capable of detecting unknown pathogens. This technology enables sequencing of large DNA fragments, allowing sequence comparison with sequence databases available locally or in the public domain.

Sequencing of a well characterised portion of the genome is playing an important role in pathogen characterisation and epidemiological studies. Sequencing the products amplified by PCR using degenerate primers targeting a gene common to the viruses in the same family has become an important diagnostic tool, especially for identification of previously unrecognised members of the family. Sequencing the products in a defined region of the genome is used in epidemiological studies to evaluate the genetic similarity to other pathogens of the same species (subtype), to determine the phylogenetic properties or to determine the origin of an outbreak/infection. Additionally, by analysing and comparison of different sequence motifs, this technology offers the possibility of predicting the tendency of pathogens to mutate into more pathogenic strains, allowing, to a certain level, tracing forward the spread of an outbreak or infection (Horimoto & Kawaoka, 1995).

The state of the art technologies, such as high mass tag-PCR and high-throughput sequencing (Belák *et al.*, 2009; Lipkin, 2010) have opened much greater diagnostic opportunities, compared with conventional targeted sequencing. The mass tag-PCR has been successfully used for simultaneous detection and differentiation of syndromic diseases, such as respiratory diseases, diarrhoeas, encephalitides/meningitides and haemorrhagic fevers (Lipkin, 2010). Moreover, high-throughput sequencing applied in multiplex platforms is capable of generating random whole genome sequencing, giving the opportunity for simultaneous pathogen detection and comparison in different regions of the genome.

The commercial launch of the first parallel pyrosequencing, 454 DNA sequencer platform in 2005, introduced the new era of high-throughput genomic analysis now referred to as next-generation sequencing (NGS). This allows the sequencing of a large genome in a short time, facilitating the study of genetic material recovered directly from environmental samples, or metagenomics. These new technologies have made it possible quickly to identify an unknown pathogen (emerging pathogens) or one difficult to cultivate *in vitro*, or to identify a variant that is present in small quantities within a mixture (Kunin *et al.*, 2008).

High-throughput sequencing is a significant challenge for the bioinformatics solutions needed to analyse the vast quantities of data generated, in order to answer specific biological questions including possible amplifications of high numbers of unexpected pathogens and their interactions with the host cell genome (Kunin *et al.*, 2008). The process of differentiation of these pathogens, when performed using conventional blasting, is still time-consuming and inappropriate for routine use. Several approaches are currently under development to solve this problem, such as sample preparation (removal of the host cell eukaryotic DNA during the extraction phase), reducing the entry datasets for evaluation (submitting parts of the obtained amplicons instead of the whole genome), targeting towards a limited (reduced) panel of pathogens (e.g. animal pathogens, only viruses/bacteria, only a group of viruses/bacteria, etc.) and optimisation of bioinformatics (production of specialised software platforms capable of analysing large amounts of data using built in algorithms). Each of these approaches has advantages and disadvantages, however it seems that careful sample preparation and optimisation of bioinformatics can offer the best solutions.

## **7. Diagnosis by DNA probes and DNA microarray technology**

Conventional DNA probing and microarray analysis are different but closely related processes. Fundamental to both processes is the binding (hybridisation) of DNA, derived from a sample suspected of containing a pathogen (the 'unknown'), with highly characterised DNA derived in advance from a pathogen of interest (the 'known' DNA).

In conventional DNA probing, the unknown DNA (or RNA) – the target – is immobilised on a solid surface e.g. a membrane. The known DNA, made into a probe by labelling or tagging it in some way, is in the liquid phase and is applied to the target.

Additionally, in conventional DNA probing, the target can be nucleic acids extracted from clinical material or cultured cells and either (a) added to membranes (a dot or slot blot) or (b), less conveniently in a diagnostic context, transferred to a membrane after gel electrophoresis. The amount of pathogen in a clinical sample might

be too low for detection. Consequently the nucleic acid might be amplified by PCR or RT-PCR, the PCR product being applied to a membrane. To visualise a probe bound to its target, the probe can be labelled with a radioactive nuclide or, more commonly and safely, 'tagged' non-radioactively. For example, biotin or psoralen–biotin may be incorporated into the probe, the bound probe then being detected by addition of streptavidin linked to an enzyme for subsequent generation of colour or light (chemiluminescence).

In microarray diagnosis, it is the known DNA (large oligonucleotides or complementary DNA) that is the target, immobilised on a glass slide, and the unknown DNA, in the liquid phase that is labelled to make a probe.

In microarray probing the probe is made from the nucleic acid of the test sample. The nucleic acid is extracted from a sample and a PCR or RT-PCR performed using random oligonucleotide primers. In this way, part of all the nucleic acids in the sample – both of host and pathogen origin – are amplified. These PCR products, representative of every nucleic acid in the sample, are labelled with a fluorescent dye and applied to the microarray. Under optimised conditions only the DNA derived from the pathogen will bind to the DNA on the glass slide. If detection of a particular pathogen or group of related pathogens is the object then pathogen-specific oligonucleotides can be used to amplify these within the sample for probe production.

A microarray is so-called because it can comprise several thousand different known DNAs, each DNA being spotted onto glass slides to form the array. Each spot is only around 10 µm in diameter. DNAs complementary to parts of selected genes of pathogens can be used to make the arrays (Belák *et al.*, 2009). However, if large numbers of pathogens are to be investigated then it would be easier logistically to use large oligonucleotides.

Microarrays for detecting pathogens can be designed for several levels of differentiation. In the case of oligonucleotide target DNAs oligonucleotides may initially be designed to be able to detect and differentiate pathogens at the genus level. A number may be chosen, perhaps 10 or so, of oligonucleotides with a high degree of sequence conservation (consensus oligonucleotides) within a given genus, such that a probe made from a field sample containing a member of that genus would be likely to hybridise to at least some of the oligonucleotides, while not hybridising (or hybridising to a lesser degree) to those corresponding to related genera, e.g. to differentiate Aphthovirus (foot and mouth disease virus [FMDV]) isolates from Enterovirus strains in the Picornaviridae family. Other sets of oligonucleotides, placed on the same array slide, able to characterise a pathogen more specifically, e.g. to differentiate the seven types of FMDV, and potentially for even further refinement at the subtype level, could then be selected.

In conventional DNA probing the detection of a pathogen is limited by the number of probes used, whereas microarray analysis is limited only by the number of target DNAs on the array. If a microarray has 1000 different oligonucleotides, then to achieve the same resolving power by conventional probing would require 1000 probes and 1000 separate probing reactions. The great advantage of microarray analysis in searching for pathogens is that hundreds of pathogens can be looked for simultaneously when probing a single microarray slide. Clearly, microarray analysis has great potential when investigating diseases of unknown aetiology or diseases where more than one pathogen might be present and when subtyping is required. To enhance sensitivity in pathogen detection, microarrays can be coupled with PCR amplifications. These PCRs are usually designed to amplify one or more conserved genes, or multiple sequences, such as PCR using broadly conserved primers, consensus PCR and multiplex PCR. When a particular pathogen needs to be identified, then the use of a microarray would be less justifiable, as the production and hybridisation of slides is relatively expensive. Instead, for these more simple cases, it would be more appropriate to use pathogen/subtype specific PCRs, followed by sequencing or RFLP for confirmation.

Currently, most of the novel technologies, such as mass tag PCR, SERS, high-throughput sequencing, etc. are compatible with certain microarray formats. This enables high multiplexing of the tests in a single run, enabling generation of a large amount of data from a single sample. Appropriate software are used for internal quality control/quality assurance purposes in the microarrays, as well as for data recording related to the status of an individual sample.

If past developments in biotechnology are indicative of the future, then microarray equipment and reagents would be expected to become less expensive, leading to greater application of this technology in animal disease diagnosis. It will assist in the search for hitherto undiscovered viruses or the characterisation of bacterial strains in terms of virulence, anti-microbial sensitivity or other important markers. One of the main challenges faced when using array-based approaches is the handling and analysis of the very large data sets that are generated.

## 8. Metagenomics

Metagenomics is defined as a culture-independent study of the collective set of microbial populations (microbiome) in a sample by analysing the sample's nucleotide sequence content. (Bexfield & Kellam, 2011). The technology is based on amplification and sequencing of the whole DNA and/or RNA content of a given sample followed by extensive filtering of obtained data using specific software solutions. Metagenomics is a powerful

tool for random detection of existing or new pathogens. There are several considerations related to this technology, such as the ratio between the number of target and the number of total amplified sequences (0.00135% reads in case of detection of arena virus (Palacios *et al.*, 2008), sample selection (amount of pathogens in the targeted sample), time consuming data acquisition and data analysis time. Two limitations are currently of major concern: i) as the method relies on finding similarities with known pathogens, there is still no solution for definition of unmatched sequences, and ii) software solutions that may facilitate the interpretation of the results. To minimise these disadvantages, three solutions are currently considered, the increased pathogen load (target samples with high probability of pathogen multiplication), reducing the resulting data sets by limiting the number of targeted pathogens and/or excluding host reference sequences from data analysis and optimising bioinformatics for “profession related” application.

## **B. DETECTION OF PROTEINS**

The boundaries between what might be termed “classical biological tests” and “biotechnology-based methods” are not clear cut, and both are used to detect proteins specific to disease agents, or antibody responses to those proteins. Most of the methods rely on immune interactions between antigens and antibodies. It is also difficult to clearly differentiate what is an antibody and what is an antigen detection test, as most of these assays can be used for both, antigen or antibody detection, depending on the respective unknown antibody or antigen component. Therefore, the classification in this section on antigen and antibody detection methods is conditional, and a qualification is given for each method. For completeness, a brief description of conventional immunoassays such as agglutination and complement fixation, as well as more recent developments in biotechnology, such as the different ELISA platforms, are given in this section.

### **B1. DETECTION OF ANTIGENS**

#### **1. Antigen-capture enzyme-linked immunosorbent assay (AgELISA)**

The antigen-capture enzyme-linked immunosorbent assay (AgELISA) facilitates detection of antigen from pathogens present in an animal prior to or during clinical disease. The AgELISA commonly follows a sandwich assay format using capture and detecting antibodies (either specific MABs or polyclonal antibodies). Antigen from the test sample is first captured by a specific MAB or polyclonal antibody bound to a solid-phase support and its presence is detected through use of a second MAB or polyclonal antibody, which may either be radio- or more generally, enzyme-labelled. If the detecting antibody is not labelled then an anti-species conjugate (reactive to the detector antibody) is used. The capture antibody selects the target antigen from other competing protein in sample suspensions and ensures that it is semi-concentrated to increase the chances of its detection. The desired characteristics of the capture MAB are strong binding to the pathogen, recognition of a conserved epitope highly specific for the target agent, and the ability to attach to an ELISA plate without loss of reactivity. In addition, a second MAB recognising an epitope other than that recognised by the capture MAB that is bound to the ELISA plate is often used as part of the indicator system. However, it may be difficult to identify MABs of comprehensive intra-type reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants.

AgELISAs have been developed for detection of *Anaplasma* (Trueblood *et al.*, 1991), BVDV (Mignon *et al.*, 1991), rinderpest and PPR (Libeau *et al.*, 1994). Related antigen-capture methods using immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria*, *Salmonella* and *Escherichia coli*. The principle of this technology relies on immunomagnetic separation, i.e. using small super-paramagnetic particles or beads coated with antibodies against surface antigens of cells. Both intact bacteria and their soluble antigenic determinants can be detected after magnetic extraction from the test sample using a second antibody in a sandwich format. As solid surface-free binding, the antigen-capture assays using immunomagnetic beads can enhance the kinetics of an antigen-antibody reaction. As a result, both nonspecific binding and the incubation time are reduced.

#### **2. Quantitative immunoassays (radioimmunoassay and quantitative enzyme immunoassay)**

Quantitative ELISA (qELISA) is not used specifically for disease detection. However, it is widely used for measurement of the concentration of specific proteins (thyroxine, steroids) or other proteins or haptens in the body or body fluids. Hence, these methods are often used for diagnosis of hormonal disorders, mainly in animal reproduction. The principle is very similar to competitive ELISA (cELISA). The plates are coated with antibodies against the substance that is to be measured. The competitive substance is labelled with a marker (an isotope in a radioimmunoassay and an enzyme or biotin in an enzyme immunoassay) and is titrated to a concentration that will saturate the coating antibodies. A set of standards of known concentration is added to the wells of the ELISA plate. As the concentration of the standard increases, the labelled competitor is increasingly displaced in

appropriate wells, resulting in reduced colouration at the end of the reaction. Based on this correlation, a standard curve is drawn, showing the discoloration (percentage of displacement) as a function of the known concentration. Once the standard curve is drawn, the percentage of displacement caused by the unknown sample is measured and the concentration of the targeted substance read via the standard curve.

### 3. Fluorescence antibody test

Fluorescent antibody test (FAT) is used for detection of pathogens in animal tissues or fluids, using specific antibodies against the targeted antigen. The antibodies are labelled with fluorescent dye (most commonly used is fluorescein isothiocyanate [FITC]). Once the sample is prepared according to a predefined laboratory procedure, labelled antibodies are added onto the sample, the sample is incubated under defined conditions, washed to remove the unbound antibodies and examined under fluorescent microscope. Visible fluorescence appears at the binding sites of the specific antibodies. The method is commonly used for detection of the rabies virus in the brains of dead animals and classical swine fever virus in tissues of suspected pigs. As the method is based on direct binding of the labelled antibody to the antigen (pathogen) present in the sample, it is commonly called direct immunofluorescence.

### 4. Immunohistochemistry

As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry has become a standard tool for identification of pathogens, and also for confirmation of the results obtained using high-tech diagnostic technologies (e.g. mass tag PCR). The *in situ* detection of antigens in fixed tissues offers a number of advantages over other diagnostic techniques. These advantages are: (i) convenience of sample submission; (ii) safe handling of potential human pathogens; (iii) retrospective studies of stored specimens; (iv) rapidity; and (v) the detection of nonviable organisms (Haines & Clark, 1991). Immunohistochemistry is also used for the detection of abnormal prion protein (PrP<sup>Sc</sup>) in brain tissue to confirm scrapie, BSE and other transmissible spongiform encephalopathies, and has proved to be more sensitive than standard histopathological examination (Thorgeirsdottir *et al.*, 2002). As the number of MAbs to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmune diseases and neoplasia is increasing. As formalin fixation can denature the antigenic epitopes (i.e. the three dimensional structure recognised by MAbs) the limiting step in the application of immunohistochemistry is to identify an appropriate MAb/antigen combination that will bind in formalin-fixed tissues. This may be overcome by using frozen sections or employing antigen retrieval techniques (e.g. proteolytic enzyme digestion, microwaving) before immunostaining.

### 5. Immunochromatography (lateral flow devices)

Immunochromatography provides a convenient method for detection of antigens in several minutes without special apparatus. The sample is applied on one end of a filter in which antibody is immobilised and microbeads (such as colloidal gold) conjugated with antibody are applied. The antigen in the sample forms an immunocomplex with the antibody labelled with colloidal gold. This complex moves along with the liquid sample, and makes a contact with the antibody immobilised on the membrane, where it forms an immuno-complex with the immobilised antibody, generating a colored product that can be visualised by eye.

## B2. DETECTION OF ANTIBODIES

### 1. Agglutination

Agglutination is a method using the property of specific antibodies to bind many pathogenic organisms into single clumps thereby forming large complexes, which are easily precipitated. The precipitation can be macroscopically or microscopically visible. Different technical applications of this principle are still widely used in veterinary laboratories, especially for detection of specific antibodies against brucellosis and leptospirosis (see disease-specific chapters in this *Terrestrial Manual*). The method can be used for detection of both antibodies, when known antigen is used, or antigen, when well defined sera are used.

### 2. Haemagglutination inhibition test

The haemagglutination inhibition method relies on the property of some pathogens (mainly viruses) to nonspecifically agglutinate erythrocytes. In the presence of specific antibodies, this activity of the virus can be 'blocked' and it will be inhibited from agglutinating the erythrocytes. The test is used for qualitative and quantitative detection of both antigens and antibodies. It has been used extensively for detection of serotype-specific antibodies against avian influenza, peste des petits ruminants (PPR) and others. The test can be also

used for detection of antigen (detecting presence of an avian influenza virus from the allantoic fluid after attempted isolation) (Mulder & Brans, 1952).

### **3. Agar gel immunodiffusion test**

The agar gel immunodiffusion test is based on the property of proteins to randomly diffuse in an agar of certain concentration. Antibodies and antigen are placed in wells at a defined distance. After incubation, at the site in the agar gel where antibodies and the antigen meet and bind, precipitation lines are formed, visible under a beam of intense oblique light against a black background. The test is most commonly used for detection of antibodies against avian influenza (matrix antibodies), equine infectious anaemia (Coggins test) and enzootic bovine leukosis. Under certain circumstances the test can be used for detection of antigen (monitoring the success of avian influenza virus isolation in allantoic fluid).

### **4. Complement fixation test**

Complement is a thermolabile system of over 25 proteins present in the blood that are sequentially activated by antigen/antibody complexes to assist in eliminating pathogens. The complement fixation test (CFT) relies on the property of some antibodies to use complement as a mediator. The test is based on two independent reactions, each requiring complement as a mediator: (i) the 'main' reaction in which the test serum (heat-inactivated to destroy the complement) is incubated with an external source (usually rabbit or guinea pig) of complement and the antigen; and (ii) the 'helper' (indicator) reaction, where anti-erythrocyte antibodies ('haemolysin') are incubated with washed sheep erythrocytes in predefined concentration. If the tested serum in the main reaction is positive (specific for the targeted antigen), the complement will have been used and the whole mixture will therefore lack active complement. If the reaction is negative, the complement will not have been spent and the mixture will contain active complement. After an appropriate incubation period, a defined volume from the 'main' reaction mixture is added to the 'helper' reaction mixture. In the case of positive reaction (no active complement in the 'main' mixture), the erythrocytes in the 'helper' reaction will not be haemolysed and will form a discrete round button on the bottom of the tube (or well in tests where a microplate format is used). The supernatant in these tubes (wells) will be clear. In the case of a negative reaction (complement in the 'main' mixture is active); the erythrocyte in the 'helper' reaction will be haemolysed. In this case there will be no formation of the discrete button on the bottom of the tube (or well in tests where microplate format is used). The supernatant in these tubes (wells) will be redish and turbid. The CFT can be used for qualitative and semiquantitative detection of specific antibodies. It is still widely used for detection of antibodies against brucellosis in cattle, sheep and goats), Q fever, contagious bovine pleuropneumonia (CBPP) and several other diseases.

### **5. Indirect ELISA (iELISA)**

In an iELISA, the target antigen (whole or purified) is bound to the solid phase in an ELISA plate. Serum samples at an appropriate dilution are added. If specific antibodies against the coated antigen are present, they will bind to the antigen. The ELISA plates are washed to remove any unbound antibodies. Anti-immunoglobulin antisera conjugated with an enzyme (usually a peroxidase), specific for the immunoglobulin of the animal species examined are then added. If specific antibodies against the coated antigen have been present in the first phase, the conjugated anti-immunoglobulins will bind to them and will not be removed during the second washing step. The substrate buffer is then added. In positive cases (presence of specific antibodies) the colour of the substrate buffer will change. The colour development is measured at a defined wavelength using a spectrophotometer and is proportional to the level of antibodies present in the sample.

### **6. Competitive ELISA (cELISA)**

The cELISA is an immunoassay that can be used to detect or quantify antibody or antigen using a competitive method. The cELISA for detection of specific antibodies has largely replaced the iELISA for large-scale screening and sero-surveillance. The cELISA offers significant advantages over an iELISA, as samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test. Many antigens are extremely difficult or time consuming to purify. When used in an indirect assay, they can produce high background values because of nonspecific binding. However, relatively crude antigens may be used in the cELISA provided the 'detecting antibody' has the desired specificity. The principle of a competitive assay for the detection of antibodies is competition between the test serum and the detecting antibody. Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate. A reduction in the expected colour obtained is caused by binding of antibodies present in the test serum with the antigen, which therefore prevents binding of the specific detecting antibody.

### **7. Blocking ELISA (bELISA)**

The basic setup in the bELISA is similar to that described for the AgELISA (paragraph B2.1.). Antibodies specific to the targeted antigen are bound to the solid phase of the ELISA plate. The antigen, prior to adding to the

antibody coated plate, is incubated with the samples. If the samples contain antibodies against that antigen, they will bind (block) to the antigen during this incubation. When added to the wells, the blocked antigen will be unable to bind to the coating antibodies. Consequently, when the detecting antibody is added to the wells it will not be able to recognise any bound antigen (no colour development). In contrast, if the sample is derived from a negative case, binding will occur and there will be colour development.

## 8. Immunoblotting

Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immunochemical detection and offers a means of identifying immunodominant proteins recognised by antibodies from infected animals or monoclonal antibodies (MAbs) directed against the target agent. The immunoblotting procedure can be divided into six steps: (i) Preparation of the sample, (ii) Resolution of the antigen by gel electrophoresis, (iii) Transfer of the separated polypeptides to a membrane support (nitro-cellulose membrane, polyvinylidene difluoride [PVDF]), (iv) Blocking nonspecific binding sites on the membrane, (v) Incubation with detecting antibody, and (vi) Detection of bound antibody.

The choice of detecting antibody is critical. Polyclonal sera are composed of a range of antibodies reflecting the full repertoire of the immune response to a particular complex antigen. They will therefore detect a number of distinct polypeptides giving a characteristic 'profile' of reactivity. MAbs bind to only one epitope; therefore they are useful in identifying highly specific polypeptides. After incubation with the detecting antibody, any antibodies bound to specific protein bands are visualised using enzyme-labelled anti-species antisera and a suitable substrate/chromogen.

Immunoblotting is performed chiefly in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or using known antigens to detect a specific serological response. False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (Molina Caballero et al., 1993). As an example of antigen detection, immunoblotting has been used on a large scale as a major screening method for bovine spongiform encephalopathy (BSE) and scrapie; it has been used on millions of brain stem samples in Europe and elsewhere for the detection of prion protein (Schaller et al., 1999). It has now largely been replaced as a screening test by AgELISA or lateral flow device based methods but is still an important confirmatory test and integral to the differentiation of transmissible spongiform encephalopathies strains into typical and atypical bovine spongiform encephalopathies and scrapie. Immunoblotting is also often used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant expressed proteins) may also be transferred to nitrocellulose or PVDF membranes by immunoblotting to examine the reactivity of test sera to individual proteins. This characteristic profile of reactivity may be used to help distinguish between animals that have been vaccinated or infected, such as the enzyme-linked immunoelectrotransfer blot (EITB), a western blot for FMD that is widely used in South America (Bergmann et al., 1993). The major factor affecting the success of an immunoblotting technique is the nature of the epitopes recognised by the antibodies. Most high resolution gel techniques involve some form of denaturation of the antigen. This destroys conformational determinants and allows only the detection of linear or non-conformational epitopes. Most polyclonal antisera contain antibodies to both linear and conformational epitopes, but MAbs are directed at single epitopes; thus if they target conformational epitopes, they will not react with denatured protein.

## 9. Fluorescent antibody

Modification of this method can be used for detection of antibodies. Essentially, the modification is in the use of secondary antibody, specific for the antibodies of the species examined. For example, if a dog is examined for the presence of specific antibodies against leishmania, leishmania antigen is fixed onto a slide, incubated with the examined dog serum, washed to remove unbound antibodies and again incubated with labelled anti-dog-antibodies (antibodies raised by immunisation of different animal species with immunoglobulines of a dog). Fluorescence appears only if the examined animal has specific antibodies against the target antigen.

## B3. DEVELOPING TECHNOLOGIES FOR PROTEIN DETECTION

The following protein-detection technologies are in the developmental stage:

- i) Multiplex ELISA platforms that can be deployed to create "disease package assays" (BVDV, Infectious bovine rhinotracheitis [IBR], parainfluenza virus 3 [PI3] and respiratory syncytial virus [RSV]). The test uses the same principle as the conventional ELISA, adapted into a micro-format.
- ii) Time-resolved fluorescence resonance energy transfer (TR-FRET) used as a source of signal generation. The labelling of the conjugate and the antigen is performed using two fluorophore dyes. The principle has been tested for competitive and sandwich format.

- iii) “Potentiometric ELISA” based on production of an electrical, instead of a colour signal, requiring minimal sample preparation and producing results within 2–15 minutes.
- iv) A SERS assay (using colloidal gold as a ligand) for detection of DNA/RNA and proteins in immunological reactions using piezoelectric effect, as well as amplification of the signal using magnetic capture.
- v) Multiplex protein assay based on signal generation using piezoelectric effect and the oscillation modulation caused by specific analyte binding. The assay has been developed for rapid result generation (60 seconds), multiplex capacity, qualitative and quantitative measurements, does not need reagents and/or incubations. The device is the size of a mobile phone, and integrated wireless transmission of the results can be used to expedite data analysis. The principle of the work has been scientifically proven and preliminary investigations on its applicability to avian influenza diagnosis are in progress.
- vi) Proximity ligation assays (PLA) for detection of antigens  
PLA are a modification of the conventional immunohistochemistry. It is based on the application of two antibodies targeted towards different epitopes of a single protein, detected by secondary antibodies labelled with short strands of known DNA. Upon binding of the secondary antibodies, their DNA strands are amplified and complementary detector probes (labelled with fluorescent dyes) in turn, then bind to their complementary sequence on the amplified stands. This results in multiple amplification of the signal and enables detection of minimal amounts of target proteins present in the sample. The technique is used as a tool for rapid scanning of multiple proteins or proteomes
- vii) MALDI-TOF analysis for determinative bacteriology  
MALDI-TOF mass spectrometry uses an ultraviolet absorbing matrix mixed with a polymer in an appropriate solvent. The sample/matrix mixture is placed onto a sample probe tip. Under vacuum conditions the solvent is removed, leaving co-crystallised polymer molecules homogeneously dispersed within the matrix molecules. Molar masses are determined using a pulsed laser beam with appropriate frequency. The technique is increasingly being used in microbiological laboratories for detection and differentiation of viral, bacterial and fungal pathogens.

## B4. PROTEOMICS

The proteome is the total complement of proteins expressed within a cell, a tissue or an organism and proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins, on a large scale. As not all proteins are expressed at all times, but are dependent on physiological and environmental factors, proteomics can provide an excellent overall view of disease processes at the protein level. Because the application of proteomics to novel drug discovery promises huge economic returns, companies all over the world have rapidly poured resources into this new research field.

Many methods used in proteomics, including two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) were established many years ago. However recent advances in MS techniques, together with whole genome sequencing and the development of powerful bioinformatics and robotics platforms, have revolutionised protein identification. The general principle of proteomics is that proteins are separated, usually by 2DGE on polyacrylamide gels, then protein spots are excised, digested with trypsin, and the resultant peptides analysed by MS. The masses of these peptides are then compared with the predicted masses of peptides derived by computational analyses of genome databases, resulting in gene identification. MS can also be used to deduce the amino acid sequence of peptides and to characterise post-translational modifications such as glycosylation or phosphorylation. 2DGE has some drawbacks, particularly for the separation of hydrophobic proteins, and other separation techniques based on liquid chromatography are now finding favour for certain applications. Nevertheless, 2DGE is the method of choice for creating quantitative maps of protein expression and many thousands of proteins can be analysed in a short space of time.

Alterations in the proteome of body tissues or of fluids such as serum, urine or cerebro-spinal fluid can be measured directly so changes that occur in a disease state can be accurately pinpointed. As well as identifying molecules that may be targets for novel therapies, this approach is a very powerful tool for early-stage diagnosis of disease. The best-established clinical applications of proteomics so far are in the identification of markers for the early diagnosis of cancers, such as bladder cancers, in urine. However, considerable research efforts are also ongoing in other areas such as heart disease, Alzheimer's disease and insulin-dependent diabetes.

The use of proteomics for the diagnosis of infectious disease is in its infancy but may prove to be of considerable importance. For example, definitive diagnosis of chronic hepatitis B virus (HBV) infection still relies on liver biopsy, but proteomic analysis of serum samples shows that the expression of at least seven serum proteins is changed significantly in chronic HBV patients. Similarly, the ante-mortem differential diagnosis of Creutzfeldt-Jakob disease (CJD) may be aided by proteomics as preliminary data show that seven proteins in cerebro-spinal fluid (CSF) are differentially expressed between patients with variant or sporadic CJD (Choe *et al.*, 2002).

An extremely useful application of proteomics to the diagnosis of infectious disease is in the identification of novel diagnostic antigens by screening serum from infected and uninfected individuals against immunoblotted, 2DGE mapped proteomes of infectious agents.

Within the veterinary field, proteomics-based research projects are now underway and these will undoubtedly yield novel diagnostic tools for the future. Proteome maps are being derived for a range of veterinary pathogens including bacteria (Mujer *et al.*, 2002), protozoa (Rout & Field, 2001) and nematodes (Yatsuda *et al.*, 2003).

## **B5. PRODUCTION OF ANTIGENS BY RECOMBINANT DNA TECHNOLOGY**

Advances in molecular biology and genetics in the 1970s initiated the development of recombinant DNA technology. Since then the impact of this technology is such that it plays a vital role in scientific research as well as in the diagnosis and treatment of disease. Recombinant DNA technology simply refers to the transfer of genes from one organism into another: literally the recombination of DNA from different sources. The objectives of recombinant DNA technology include identifying genes, isolating genes, modifying genes, and re-expressing genes in other hosts or organisms. These steps permit scientists and clinicians to identify new genes and the proteins they encode, to correct endogenous genetic defects, and to manufacture large quantities of specific gene products such as hormones, antigens for use in vaccines, and other proteins produced by biological agents of interest. Of particular value is the degree of specificity attainable in diagnostic tests by the use of recombinant protein.

Native proteins are perhaps the ideal antigens, providing sequence-specific and surface structural epitopes. Many current diagnostic tests require test antigens that need to be continuously produced from cell culture or harvested from an infected animal. Such antigen preparations are expensive and often have a short shelf-life, with each new batch of antigen requiring standardisation. Natural proteins are rarely available in a completely pure form, and antibodies often develop against contaminating polypeptides that can lead to false-positive results. Recombinant DNA technology produces antigens that offer many advantages over antigens isolated from other biological sources. These advantages include a high purity, high specific activity and as the protein is synthesised in genetically modified laboratory-grown cells, each preparation of the protein product is identical to the previous preparation, ensuring batch-to-batch consistency. When recombinant antigens are used in combination with the cELISA format, purification of the recombinant antigen from the lysate may not be necessary as the specificity of the cELISA resides mainly in the MAb used.

An outline of the procedure for the production of an antigen by recombinant DNA technology is as follows. The identification of an antigen of potential diagnostic or scientific significance is achieved through the study of the antibody response of the host to the proteins of the organism in question. Immunodominant antigens, defined proteins of the organism against which the host responds with the highest potential antibody titre, are of particular interest as they are likely to be major stimuli of cellular and humoral immunity against the pathogen of interest. Antigen recognition studies are widely used to identify biologically relevant, immunodominant antigens for use in generating MAbs as well as in vaccine development. Once a protein of interest has been identified, the gene encoding the protein is generated using messenger RNA (mRNA) from the organism as a template for making cDNA. This method of cloning the gene encoding the protein of interest requires a prior knowledge about the gene sequence, either directly from the organism of interest or through the use of gene sequences from closely related species. An alternative method, when gene sequence data are not available, is the generation of recombinant libraries from the genomic DNA of the organism or from cDNA synthesised from mRNA. Fragments of the recombinant libraries can be cloned into a protein expression system, which may be prokaryotic or eukaryotic, and the gene library screened for expression of the protein.

There is a wide choice of expression systems. Protein may be expressed in bacteria, usually *E. coli*, yeast, insect cells using baculovirus, or in eukaryotic cells by infection with appropriate viral vectors or by permanent transfection. Differences in glycosylation when prepared in bacterial, insect or mammalian cell cultures can modify protein structure and its reactivity with antibody. Antigen may need to be extracted from the cell or it may be secreted. Purification is often, but not always, necessary. An upcoming trend in the production of antigens for use in assays is in the development of synthetic peptide antigens. This allows antigens to be tested as diagnostic reagents based on the gene sequence, expression of the whole protein being unnecessary, thus shortening the process.

Genome sequences of numerous pathogens have already been determined. A gene responsible for an antigen or immunogen can easily be cloned with PCR technologies and characterised by nucleotide sequence (Rappuoli & Covacci, 2003). In short, the amplified DNA can be cloned in a bacterial plasmid vector and expressed as a recombinant antigen. The cloned gene can be designed in a way that the recombinant protein is fused at its N or C-terminal with a detection tag that can be used for its purification by affinity chromatography. There are many tags but the most popular are polyhistidine (6-8 residues), glutathione S-transferase (GST) or the Flag peptide Flag composed of 8 amino acid residues. The antigenicity of the gene products can then be determined. Systematic

screening of the antigen gene in silico, from genome sequence data, accelerates the development of diagnostic kits and vaccine (Tortorella *et al.*, 2000).

## B6. IMPLICATIONS OF THE NEW TECHNOLOGIES

There are a number of trends in diagnostic technologies that will have an impact on the way in which disease diagnosis will be approached in the future, affecting the laboratory environment, data analysis and disease control:

- i) The global development of chip technologies has led to a strong trend towards miniaturisation of the test format in both molecular and protein detection assays. The test formats range from several millimetres to several centimetres.
- ii) Although miniaturised, multiplex platforms can be developed that are capable of detecting from several up to thousands of pathogens in a single sample.
- iii) Miniaturisation of the test format is being accompanied by similar miniaturisation of laboratory equipment, enabling on-site testing. This trend is resulting in the use of sophisticated equipment, previously only used in the laboratory, in the field, facilitating the rapid and early diagnosis of infectious diseases, as well as the modes of reaction of appropriate Competent Authorities.
- iv) The development of alternative sources of signal generation/amplification, replacing light with mass measurement, piezoelectric effect or concentration of the ligand will lead to the development of a whole new platform of technologies.
- vi) Although development of new technologies can often mean improved capability, consideration should always be paid to the actual value and the role of the confirmatory test in diagnosis!
- viii) As the diagnostic platforms are continuously changing in the ways described above, several components of the disease control chain will be affected. Appropriate communication technologies/information systems will need to be developed in order to systematically collect, store and analyse large datasets produced by the new technologies in a relatively short time. There is likely to be an increasing trend towards real-time inputs of results via mobile telephones or the Internet that will require relevant development of IT and data-handling systems.
- ix) Appropriate infrastructure and preparedness have to be an integral component of technology update. This will include distance training in testing in the field (sample collection and preparation, testing, interpretation of the test results, sample processing for dispatch to reference laboratories and reporting to appropriate authorities).
- x) Adaptation of national contingency plans towards simultaneous management of multiple diseases, as well as management of new, unexpected diseases.

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