CHAPTER 2.1.2.

BIOTECHNOLOGY ADVANCES IN THE DIAGNOSIS OF INFECTIOUS DISEASES

INTRODUCTION

Modern methods of livestock production and increased global trade demand the development of early warning diagnostic platforms, global capacity, effective assays and diagnostic harmonisation to enhance livestock productivity and health and to facilitate trade in healthy animals as the basis of the global One Health initiative.

The risk of pathogen-transfer between countries or continents has increased markedly in recent years, due to several factors, such as increased national and international movement of people, animals, food and feed products, and various goods, and climatic changes, causing new epidemiological scenarios and threats. This new situation highlights the requirement for the development and application of a wide range of powerful novel diagnostic methods that can detect spreading pathogens very rapidly, with high diagnostic specificity and sensitivity.

The purpose of this chapter is to provide general information and updates on the most important biotechnology-based methods, currently used in our diagnostic laboratories. For further information, two issues of the WOAH Scientific and Technical Review *concerned with biotechnology and the diagnosis of animal diseases are available on the WOAH website (https://www.woah.org/en/what-we-do/publications/scientific-and-technical-review/; Potential applications of pathogen genomics: Volume 35, Issue 1, 2016; Biotechnology applications in animal health and production: Volume 24, Issue 1, 2005).*

A. TECHNIQUES FOR THE DETECTION AND ANALYSIS OF NUCLEIC ACIDS

The direct detection and amplification of specific nucleic acid sequences in the genomes of pathogens have been shown to have considerable advantages. Polymerase chain reaction (PCR)-based technologies have revolutionised the diagnosis of infectious diseases. DNA amplification platforms are highly sensitive, specific, rapid and robust. The platforms are also cost-effective and adaptable to automation and therefore ideal for high-throughput screening. The equipment required is highly adaptable to the detection of many different targets, however the quality of the specimens is key to successful use of these technologies.

1. Nucleic acid extraction

An important initial step in most molecular diagnostic procedures is the extraction of 'clean' nucleic acid mixtures from the clinical sample. While it is relatively easy to extract DNA from bacterial cultures or blood, it is technically more challenging to prepare suitable material from field specimens, such as faeces, internal organs or aborted fetuses. Care during sampling in the field and in laboratory processing during the extraction of nucleic acids is important to avoid cross contamination of specimens, leading to false positive results. For this reason, nucleic acid extraction should be performed using strict working procedures, in an isolated room separated from nucleic acid amplification procedures.

Nucleic acid isolation and purification consists of three main steps: disruption of cells, removal of proteins and contaminants, and recovery of nucleic acid suitable for analysis. There are numerous methods of nucleic acid extraction, including manual extraction using phenol-chloroform and guanidinium thiocyanate to concentrate DNA in a hydrophilic phase, centrifugal spin column-based elution where nucleic acid binds to a solid phase such as silica and proteins are removed with the aid of centrifugation and magnetic bead extraction where the nucleic acid binds to magnetic particles while the proteins are washed away. While extraction methods may vary in the relative yield

and purity of the targeted nucleic acids, effective and reproducible extraction is an essential part of nucleic acid detection and thus pathogen detection. PCR, or other methods described below, can amplify and detect the specific target, but the quality of the starting material is an inherent part of the detection process.

Several specialised extraction methods for particular types of samples and tissues 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 with 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; Oberacker *et al.,* 2019). These are destined to improve the reliability of nucleic acid extraction from different specimens, but it remains a challenging area.

As an alternative to nucleic acid extraction, biotechnologists are increasingly focusing on PCR-inhibitor resistant polymerases and novel amplification assays, which are now available to directly amplify nucleic acids from pathological specimens without the requirement for extraction steps (Pavsic *et al.,* 2016).

2. Conventional polymerase chain reaction (PCR) and real-time PCR

PCR is a widely used, highly sensitive diagnostic tool for detecting pathogen-specific nucleic acid sequences in host tissues and vectors. Over the past 30 years, PCR has revolutionised the diagnostic process, allowing rapid, highly sensitive and specific detection of a wide range of pathogens.

PCR can identify and amplify a specific, selected target region of nucleic acid from a complex mixture of heterogenous sequences (Saiki *et al.,* 1988). PCR achieves *in-vitro* amplification by exploiting natural DNA replication mechanisms, using a succession of cyclic incubation steps at different temperatures. Amplification facilitates the detection of target nucleic acid sequences within a sample, even when only a small number of targets were present originally.

Diagnostic PCR is performed on nucleic acids extracted from clinical samples. The heterogenous sample DNA is first heat-denatured to separate the two complementary strands, thereby creating a single-stranded template. Specific primers are then used to identify the target regions for amplification. Primers are short, synthetic, single-stranded DNA molecules complementary to the target sequences. If the target sequence is present in the sample, the primers will anneal to these regions as the temperature cycle cools. Following binding, the cycle enters an intermediate temperature phase in which bound primers enable extension of the target sequence by DNA polymerase. Once the polymerase has synthesised a new complementary strand of DNA, the product is separated from the template by heating to a higher temperature and the cycle repeated.

These cycles are repeated, usually between 35–50 times, resulting in the amplification of target DNA sequences. Each cycle has the potential to double the number of targets from the previous cycle leading to the exponential increase in target DNA that gives the method its high sensitivity. The key to the 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 complementary DNA (cDNA) copy of the RNA must first be made using reverse transcription (RT). The cDNA then acts as a template for amplification by PCR. This technique is referred to as reverse transcription PCR (RT-PCR).

Once amplified, the target sequence can be detected. Amplified products are detected most frequently using methods that also help to confirm the identity of the PCR products, including:

i) Methods that define their characteristic size (such as gel electrophoresis)

ii) DNA probes (see below)

iii) High resolution melting (see Section A.2.1)

iv) Fluorescence-based detection systems (real-time technology)

v) Nucleotide sequencing technologies

Increasingly, since the advent of automated cycle sequencing techniques, identification is made more and more frequently via PCR techniques and agent characterisation by direct sequencing of the PCR products.

To provide robust, reproducible results, the PCR protocols should be carefully designed, optimised and fully validated. Chapter 2.2.3 *Development and optimisation of nucleic acid detection methods* provides comprehensive information and guidance on such assays.

To expand its utility in veterinary diagnostics and pathogen identification, PCR has been modified extensively over the years. Traditional PCR requiring detection at the end-point of the reaction, typically by agar gel electrophoresis, has been largely superseded by detection while the reaction is occurring i.e. real-time PCR. The most common variations; the main advantages and the limitations of PCR technologies are summarised in Table 1.

Table 1. Summary of commonly used variations in PCR techniques, their advantages and disadvantages

PCR: amplifies a selected region of target nucleic acid in a sample			
Variations	**Details**	**Advantages**	**Disadvantages**
Nested PCR	Two rounds of PCR, with the second round further amplifying a pre-amplified genomic fragment from the first round. Generally superseded by the real-time PCR	High sensitivity	High risk of contamination as it is not a closed system and cross-contamination or carry-over may occur via handling of amplified products
Consensus PCR	Targets a conserved genomic region in a group of pathogens	Can identify new but closely related pathogens	Not specific, may require further characterisation
Real-time PCR	Results of amplification are detected in real time, through fluorescence of a region-specific probe. Quantification is possible, if standards with known copy numbers are used in the run (hence the alternative name of quantitative PCR)	Highly sensitive and highly specific; decreased risk of contamination as the system is closed and there is no post-amplification manipulation	Cost (probes; platform). Risk of false negatives with highly mutable agents: primer probe sets can be designed to be highly target specific or universal to detect sequences conserved across lineages and strains
Multiplex real-time PCR	Detects multiple targets at once	Allows for screening numerous pathogens in one assay	Sensitivity can be compromised. Limited by the number of different fluorescent dyes available

2.1. High resolution melting

For DNA organisms such as parasites and bacteria, and for DNA viruses, the use of high-resolution melting (HRM) allows the genotyping of subtypes of a pathogen based on nucleotide differences. HRM is a post-PCR analysis method for analysing the melting (dissociation) of the PCR products in the presence of improved double-stranded DNA-binding dyes. As most real-time PCR machines have integrated this feature, HRM represents a cost-effective method of choice as no labelled probe is required. It is possible to increase the specificity and genotyping accuracy of HRM methods by using unlabelled probes to interrogate a short segment of the PCR amplicon, where the targeted mutation is present. The unlabelled probes must be blocked at the 3'-end to prevent polymerase extension. HRM techniques can be used in singleplex or multiplex format for the simultaneous detection and differentiation of species within the same genus (Banowary *et al.,* 2015; Gelaye *et al*., 2013), drug-resistance screening (Loiacono *et al.,* 2017) or differential diagnosis (Gelaye *et al.,* 2017).

2.2. Mass-tag PCR

Until recently, the detection of amplified target sequences in a real-time PCR assay relied on the use of various chemistries, such as intercalating dyes, hydrolysis probes, molecular beacons, primer probe energy transfer (PriProET), scorpion primers, dual hybridisation probes and dye-labelled oligonucleotide ligation (Belák *et al.,* 2009). The availability of detection methods limited the number of different targets (and thus, different pathogens) that could be detected simultaneously in a multiplex PCR. Mass-tag PCR assays have been developed to overcome this limitation.

In a mass-tag, multiplex PCR assay, the various primers are marked 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 detection of a far larger range of amplified DNA targets than previously possible, as the assay is not limited to the number of dyes available. This allows a single assay to simultaneously test for a large panel of diseases. Application of the mass-tag PCR assay has been proven already in the differential diagnosis of syndromic diseases (respiratory, haemorrhagic, enteric pathogens, meningitis/encephalitis syndrome) and in the 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 (Angeletti, 2017; Jang & Kim, 2018).

2.3. Digital PCR (dPCR)

In recent years there has been renewed interest in the application of digital PCR (dPCR), where single copies of target DNA and RNA are isolated and individually amplified by PCR. The technique was first described in the 1990s and has since been revitalised due to progress in chemistry and equipment development. The distinctive feature of dPCR is the separation of the reaction mixture into thousands to millions of partitions, which is followed by real-time or end-point detection (conventional PCR) of the amplification. Target sequences are distributed into partitions according to the Poisson distribution model, thus allowing accurate and absolute quantification of the target from the ratio of positive partitions against all partitions at the end of the reaction. This omits the need to use reference materials with known target concentrations and increases the accuracy of quantification at low target concentrations compared with real-time PCR. dPCR has also shown higher resilience to inhibitors in many different types of specimens (Gutierrez-Aguirre *et al.,* 2015). Several research papers have been published on the application of dPCR to the detection and quantification of animal pathogens such as African swine fever virus and pseudorabies virus, demonstrating increased sensitivity and linearity (Ren *et al*, 2018; Wu *et al,* 2018).

2.4. Portable real-time PCR machines

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. However, the isothermal amplification techniques described below may be better, as these require less complex and costly equipment.

3. Isothermal amplification

Isothermal amplification technologies offer the advantage of omitting thermocycling, enabling DNA amplification at a constant temperature. Consequently, the required equipment is less complex and therefore cheaper. The basic principle of this technology has been developed, resulting in several distinct methodologies (Gill & Ghaemi, 2008).

The most widely used method is the loop-mediated isothermal amplification (LAMP) method, which deploys four or more 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, the generation of a fluorescent signal using fluorescent dyes (Gill & Ghaemi, 2008) or, if primers are biotinylated, the reaction products can be observed using lateral flow devices (see Section B.3 Immunochromatography). The process is also less prone to inhibition from contaminants compared with PCR; however, protocols based on this technology might be difficult and complex to develop. Isothermal amplification is less suitable for multiplex assays than PCR, as the potential for primer dimer formation and/or competitive reactions is higher and the assay design is more difficult in certain cases.

4. Analysis by restriction fragment length polymorphisms (RFLP) and related DNA-based approaches

The restriction fragment length polymorphisms (RFLP) method allows individual genomes to be identified based on unique patterns caused by restriction enzymes cutting in specific regions of DNA. 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. The RFLP procedure consists of digesting the pathogens' nucleic acid (DNA or PCR products) with one or a panel of restriction enzymes and then separating the DNA fragments by agarose gel electrophoresis to determine the number of fragments and their relative sizes. A restriction enzyme is an endonuclease that recognises and cleaves double-stranded DNA at specific nucleotide sequences called restriction sites (Loza-Rubio *et al.,* 1999).

Pulsed-field gel electrophoresis (PFGE) can be used for 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 programmes for detection and discrimination of food-borne pathogens (*Escherichia coli* O157:H7, *Salmonella, Shigella, Listeria*, or *Campylobacter*) worldwide (https://www.cdc.gov/pulsenet/).

RFLPs have a clear value for use in epidemiological studies, however, an RFLP difference may not be functionally significant. Although the RFLP and PCR-RFLP are much less powerful compared with the modern sequencing technologies, they are relatively inexpensive, easy to perform and sufficiently descriptive for epidemiological investigations of outbreaks and identification of individual strains of pathogens.

5. Genome sequencing

In recent times, the cost of sequencing has reduced and genome sequencing has become increasingly important in the detection and characterisation of pathogen nucleic acid in diagnostic samples. Genome sequencing involves determining the order in which nucleic acid bases occur in a gene. Sequencing of PCR products targeting an informative region of a gene is particularly useful for pathotyping various organisms. For example, the sequence analysis of influenza virus A haemagglutinin gene is commonly used to locate the cleavage site and thereby classify the virus as highly pathogenic or low pathogenic influenza. Complete genome sequencing is currently the ultimate discriminatory procedure for pathogen identification. Since 1977, the Sanger method (Sanger *et al.,* 1977) has dominated DNA sequencing, although recent advances in high-throughput, next generation sequencing are revolutionising this approach.

Genome sequencing is a complex process. The basic principle is cycle sequencing of targeted DNA fragments with labelled di-deoxy nucleotides, which halt the elongation on binding. Each of the four di-deoxy nucleotides is labelled with a different fluorescent dye, enabling distinction between individual di-deoxy nucleotides. During the PCR process, these labelled di-deoxy nucleotides bind to the DNA fragments wherever their complementary base pairs exist. Once bound, they block further elongation of the DNA fragment. This process creates a mixture of DNA fragments, each ending with a defined nucleotide. The reaction mixture is then analysed using capillary electrophoresis, which separates the fragments by length and reads the fluorescence of each fragment. Analytical software is then used to convert the specific fluorescence signals into a nucleotide sequence.

Genome sequencing allows the analysis of pathogens detected during the diagnostic process. Such analyses include:

i) Identifying pathogenic strains to facilitate the implementation of appropriate control efforts and policies and where indicated, the update of vaccines and development of specific diagnostic tests (Beerens *et al.,* 2017; Busquets *et al.,* 2019).

ii) Evaluating the genetic similarity of isolates to other pathogens of the same species (subtype), to determine their phylogenetic properties or to determine the origin of an outbreak/infection. This can be valuable in the epidemiological analysis of outbreaks.

Importantly, conventional sequencing methods can be used to detect and characterise newly emerging pathogen strains. This overcomes one of the major limitations of PCR-based methods, which require known primers for pathogen detection. For example, sequencing is commonly used to identify a new virus within a family by using degenerate primers (a mix of similar but not identical primers) to amplify a gene common to that virus family, followed by sequencing of the PCR amplicon. Similarly, universal primers that anneal to a conserved sequence can be used to amplify and sequence genes in all bacterial species; the most common application is the use of 16S rRNA gene for bacterial identification (Cai *et al.,* 2014).

Advantages and limitations of the various sequencing techniques commonly applied in diagnostics are summarised in Table 2.

Table 2. Summary of commonly used variations of nucleic acid sequencing techniques, their advantages and disadvantages

Sequencing: determines the nucleotide composition of a selected region of target nucleic acid in a sample			
Variations	**Details**	**Advantages**	**Disadvantages**
Sanger sequencing (also known as di-deoxy sequencing)	DNA fragments from a PCR product are labelled with di-deoxy nucleotide, which can be detected through fluorescence	Highly specific Cost effective for short lengths of DNA Quick and simple work flow and analysis	Sequence length is limited and has no depth. For one sequencing reaction, only one sequence read is generated
High throughput sequencing (HTS) also known as next generation sequencing (NGS) and massive parallel sequencing (MPS)	Genomes are fragmented, and universal adapters and primers are used to generate sequence. HTS is enabling the following types of studies: • Whole genome sequencing • Pathogen discovery (*de novo* sequencing) • Metagenomics • Microbiome detection • Transcriptome detection	No prior knowledge of target is required as the reaction is primer independent Can analyse multiplex samples, using barcodes, which brings the cost down	Cost Requires large data storage and deep bioinformatic analysis, as the amount of data generated is enormous Can take several days to get results however this is offset by the large amount of data generated (e.g. whole genomes) Access to bioinformatics skills and data libraries can be problematic

5.1. High throughput sequencing (HTS) and mobile technologies

High throughput sequencing (HTS) is the term used to describe highly parallel sequencing methods. These methods produce data at the genome level and provide greater diagnostic opportunities compared with conventional sequencing (Belák *et al.,* 2009; Lipkin, 2010). Although various instruments are available, each with its own sequencing methods, the so-called second-generation sequencing technologies share the same general strategy, consisting of clonal amplification of DNA templates followed by sequencing in massively parallel sequencing reactions to produce thousands or millions of reads, ranging in length from 50 to 700 base pairs. These reads are subsequently assembled into longer sequences, known as contigs, using bioinformatics tools (see Chapter 1.1.7 *Standards for high throughput sequencing, bioinformatics and computational genomics*). The availability of bench-top versions of these instruments with reduced costs has made HTS accessible to average-sized laboratories for pathogen identification and characterisation. These new technologies have not only increased the speed of sequencing but also reduced its cost radically. As a consequence, these sequencing methods are becoming ever more significant tools in pathogen detection and definition.

Another application of HTS is whole genome sequencing of infectious agents. HTS allows the assembly of the complete genome or genome segment and reveals reassortment or recombination events and virulence markers. HTS can be used to perform deep re-sequencing of viral sequences within single specimens to assess the evolutionary dynamic (Granberg *et al.,* 2016). Phylogenetic studies and tracing the source of infections can also be undertaken using HTS. However, most HTS platforms produce short reads and so have widely accepted limitations in the *de novo* assembly of new genomes. In this context, a third generation of sequencing methodology producing longer read lengths but lower throughput, provides a means to increase the resolution of the genome assembly, thus enabling more efficient *de novo* sequencing of microbial genomes (Levy & Myers, 2016). At present, portable devices in a flash-drive format are available and can produce reads of 5–50 kb (Granberg *et al.,* 2016). Although the error rate of these instruments, 5–20%, looks very high, the hybrid assembly of short reads from second generation sequencing platforms facilitates the *de novo* assembly of novel microbial genomes. Today, a common technology applied by the portable sequencing devices relies on the principles of nanopore sequencing, which is based on measuring changes in electrical properties as the DNA molecule traverses a pore. These electrical changes are then used to identify the exact DNA base going through the pore.

5.2. Multilocus sequence typing

Multilocus sequence typing (MLST) is a method of sequencing used in pathogen typing and epidemiological studies. Using this approach, fragments of a number of gene targets are amplified by PCR and sequenced. For each locus, unique alleles are assigned arbitrary numbers and, based on the allelic profile, the sequence type is determined. Most MLST methods give highly reproducible results due to the international harmonisation of the nomenclatures; allele sequences and profiles are available in large central databases with companion on-line analysis tools.

6. Metagenomics

Metagenomics refers to culture-independent applications of HTS for investigating the complete viral and microbial genetic composition (microbiome) of samples (Bexfield & Kellam, 2011). Significantly, it is an unbiased approach as organisms are not selected by favourable culture methods (or deselected by unfavourable conditions) and does not require prior knowledge. It is important to consider that without targeted amplification, the metagenomic technologies are less sensitive for pathogen-detection compared with PCR-based methods. However, they are being used more frequently in diagnostic laboratories, because, by detecting a wide range of infectious agents simultaneously, they are powerful diagnostic tools. These technologies have also made it possible to identify pathogens more rapidly, including pathogens that can be difficult to identify by cultivation *in vitro* (Hoper *et al.,* 2017).

From a diagnostic viewpoint, the capacity to detect a wide range of infectious agents simultaneously (Belák *et al*. 2013) is a significant advantage of metagenomic technologies. Some of the agents identified may be novel and metagenomic approaches, accompanied by bioinformatics, have led to the discovery of a high number of infectious agents, including novel bocaviruses, Torque Teno viruses, astroviruses, rotaviruses and kobuviruses in porcine disease syndromes and new virus variants in honey bee populations. It should be noted that the power of metagenomic technologies to identify all organisms present in a sample in an unbiased manner, can present the diagnostic laboratory with the challenge of identifying which of the many organisms detected in a sample might be the causative agent of the disease.

These results, and the recent experience gained in several diagnostic centres worldwide, indicate that the metagenomic detection of infectious agents is becoming a powerful, cultivation-independent and useful diagnostic tool, both in veterinary and human medicine (Granberg *et al.,* 2016; Wylezich *et al.,* 2018).

B. IMMUNODIAGNOSTIC TECHNIQUES

All immunoassays exploit the innate ability of antibodies to bind to an antigen (or epitope(s) within the antigen) with high specificity and affinity. Antibodies are often referred to as being monoclonal or polyclonal. Monoclonal antibodies (MAbs) are derived from a single B-lymphocyte, meaning that each antibody produced is identical to the next and will therefore bind only to a single specific epitope on an antigen. Polyclonal antibodies (PAbs) are essentially a collection of different MAbs as they are derived from a population of B-lymphocytes, so that each antibody is different to the next and may therefore bind specifically to different epitopes within the antigen.

MAbs are produced by immortalized B-lymphocyte clones called hybridomas, which are formed by the fusion of a myeloma cell with an individual B-lymphocyte taken from the spleen of an immunised animal. PAbs are produced in animals that have been exposed to a foreign antigen such as a pathogen or a vaccine and represent the full antibody response to that antigen. Thus, sera being tested will contain polyclonal antibodies whereas antibodies used as reagents in a test may be polyclonal or monoclonal.

Immunoassays are designed to detect pathogen-specific antigen or antibody in test samples, as indicators of active or prior infection. Antigens may be the pathogen itself, one or more of its subunits or proteins, a recombinant protein or a synthetic polypeptide. Antibodies will be those of host origin that the assay targets as a marker of infection and/or the reagent antibodies used to bind specific antibody or antigenic markers of disease. Reagent antibodies are commonly described according to their function. As examples, a primary antibody is used to bind directly to an antigen; a secondary antibody will bind to the primary antibody and be used to carry a label for detection; and a capture antibody will be used to immobilise an antigen.

Depending on the immunoassay format, antibody or antigen is first bound to a solid phase – typically the surface of micro-beads, a membrane, or the wells of an enzyme-linked immunosorbent assay (ELISA) plate. This provides a stationary point to which a specific target of interest binds or is captured. Thus, an immunoassay designed to detect an antibody response in a serum sample from an infected animal might use an antigen bound to the solid phase that is bound by pathogen-specific antibody in a test serum and this in turn is detected using a labelled secondary antibody. Where the target in a test sample is an antigen, an antibody on a solid phase is used to capture the antigen via a specific epitope and a second (often labelled) antibody directed towards an alternative epitope sandwiches the antigen and is used for detection. There are many variations on these assay formats but in all cases the specificity of antibodies for a given antigen forms the basis of the immunoassay. A wide range of useful reports is available in the literature, where various diagnostic systems were compared and recommendations were given for diagnostic laboratories concerning the specificity, sensitivity and usefulness of the various immunodiagnostic assays (Kittelberger *et al.*, 2011).

1. Enzyme-linked immunosorbent assay (ELISA)

ELISAs are the predominant immunodiagnostic tool used in veterinary diagnostic laboratories around the world. ELISAs exploit specific antigen-antibody interactions to detect specific antibodies to a pathogen, or a specific antigen of the pathogen. The conventional design of the ELISA has been extensively modified to meet a range of diagnostic requirements.

1.1. Principles of operation

The basic principle that underpins all ELISAs is the specific binding of antigen to antibody. Each type of ELISA exploits this interaction in a different way.

1.1.1. Indirect ELISA

Indirect ELISAs (I-ELISA) are used for the detection of antibodies in a serum sample to an antigen. In I-ELISA, the target antigen (whole or purified) is bound to the solid surface of the wells of an ELISA plate (solid phase). To perform the test, the following steps are performed:

i) Serum samples at an appropriate specified dilution are added to the wells. If specific antibodies against the coated antigen are present in the serum, they will bind to the antigen.

ii) The ELISA plates are then washed to remove any unbound antibodies.

iii) Antibodies raised against the immunoglobulins of the animal species being examined, are then added to the wells. These anti-species antibodies (or sometimes Protein A or G) are conjugated with an enzyme (usually a peroxidase).

iv) As all unbound antibodies from the serum sample are removed during the initial washing step, conjugated antibodies added at step (iii) can only bind to those antibodies already bound to the solid phase of the ELISA during step (i) (i.e. antibodies that are specific to the antigen of interest).

v) A second washing step is performed to remove any unbound conjugated antibodies.

vi) The enzyme substrate with a suitable chromogen, in substrate buffer, are then added. If any bound conjugated antibodies are present in the well (i.e. wells containing the antibodies of interest), the presence of the conjugated enzyme will cause the colour of the substrate buffer to change.

vii) This colour development is measured at a defined wavelength using a spectrophotometer. Higher quantities of antibodies in the sample result in increases in optical density.

I-ELISA is very sensitive for antibody detection, because reagents are added in excess (excess reagent is washed away) and this promotes rapid reactions and the amplification of signal due to the continual action of the enzyme that generates the colour change in the final step of the assay. The format can be made even more sensitive by adding more enzyme per molecule of conjugate. This may be done by biotinylating antibodies and then adding streptavidin-conjugated enzymes, or by using detection reagents that have scaffolds to which many more molecules of enzyme are attached, e.g. poly-HRP (horseradish peroxidase) streptavidin.

1.1.2. Antigen-capture ELISA

Antigen-capture ELISAs (Ag-ELISA) are used to detect the presence of pathogen-specific antigens in a sample and are useful for diagnosis prior to or during clinical disease.

The Ag-ELISA commonly follows a sandwich assay format. MAb or PAb specific to the antigens of interest are bound to the solid surface of the wells of an ELISA plate. The sample is then added to the well and any target antigens are captured by the bound antibodies. Captured antigens are subsequently detected through the addition of a second, enzyme-labelled MAb or PAb. The wells are then washed to remove any unbound conjugated antibodies before enzyme substrates and buffers are added. As for I-ELISAs, this will result in a detectable colour change in positive wells. Note that if the detecting antibody is not labelled, then an enzyme-conjugated antibody that targets the detecting antibody is used.

The desired characteristics of the capture antibodies 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. The detection antibody should recognise an epitope other than that recognised by the capture antibody that is bound to the ELISA plate. 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.

Ag-ELISAs have been developed to detect many infectious agents, e.g. bovine viral diarrhoea virus (BVDV, see Mignon *et al.,* 1991), rinderpest and peste des petits ruminants virus (PPRV) (Libeau *et al.,* 1994). Related antigen-capture methods using antibody-coated immunomagnetic beads are now important and well accepted methods for detecting certain bacterial infections, including *Listeria, Salmonella* and *E. coli.* The principle of this technology relies on immunomagnetic separation, i.e. using small super-paramagnetic particles or beads coated with antibodies against bacterial surface antigens. The magnetic properties of the beads allow them to be retained whilst the beads are washed and moved to different buffers in a manner analogous to the methods described above for ELISA. Antigens such as intact bacteria or their soluble antigenic determinants can be detected after magnetic extraction from the test sample using a second antibody in a sandwich format. Antigen-capture assays using immunomagnetic beads can enhance the kinetics of the antigen–antibody reaction as the beads mix within the sample reducing the incubation time.

1.1.3. Blocking ELISA

Blocking ELISA (B-ELISA), used for detection of serum antibodies, shares some similarities with Ag-ELISA. Antibodies specific to the target 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. 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 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.

1.1.4. Competitive ELISA

Competitive ELISA (C-ELISA) is a variant of ELISA used to detect or quantify antibody or antigen using a competitive method. The principle of a competitive assay for the detection of antibodies is competition between antibodies that may be present in the test serum and the detecting antibody (which in this case binds directly to the antigen). Specific binding of the detecting antibody is detected using an appropriate anti-species conjugate or the antibody itself may be labelled. A reduction in the expected colour obtained is caused by binding of antibodies present in the test serum that are specific to the antigen, which therefore competes with the specific detecting antibody for antigen-binding sites. The C-ELISA offers significant advantages over I-ELISA; for example, samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test, assays may be performed using less purified antigens, and may be faster to perform as they require less incubation time. In some cases, C-ELISAs may have lower analytical sensitivity than indirect ELISAs. Where the C-ELISA relies on competition between antibodies in the test serum and an MAb (detecting antibody) for binding to a single epitope on the antigen, false negatives may occur where the humoral response

of the host to the pathogen has targeted distinct epitopes on the antigen that differ from the MAb target epitope.

2. Immunoblotting

Immunoblotting is performed in diagnostic laboratories to identify and/or characterise infectious agents based on antigen specificity or using known antigens to detect a specific serological response. Immunoblotting combines the high resolution of gel electrophoresis with the specificity of immuno-detection and offers a means of identifying immunodominant proteins recognised by antibodies from infected animals or antibodies directed against the target agent. 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 Ag-ELISA or lateral flow device-based methods but is still an important confirmatory test and is integral to the differentiation of transmissible spongiform encephalopathies strains into typical and atypical BSE and scrapie.

False-positive and false-negative results in other diagnostic assays can often be resolved by immunoblotting (Molina Caballero *et al.*,1993). Immunoblotting can also be used to determine the specificity of individual MAbs. Individual purified polypeptides (or recombinant proteins) may also be transferred to nitrocellulose or polyvinylidene difluoride 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 foot and mouth disease (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, which 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 recognise single epitopes; thus, if they target conformational epitopes, they will not react with denatured protein.

3. Immunochromatography (lateral flow devices)

Immunochromatography provides a convenient method to detect antigens, antibodies (or any labelled molecule for which a ligand has been coated onto the chromatographic membrane, e.g. biotinylated LAMP products [see Section A.3 Isothermal amplification]) in several minutes without special apparatus (Fowler *et al.,* 2014; Hanon *et al.,* 2016; Waters *et al.,* 2014).

The sample is applied (usually with buffer) to one end of a membrane where there is a pad containing antibody- or antigen-conjugated microbeads (beads such as latex or colloidal gold). The antigen or antibody (dependent on format) in the sample forms an immunocomplex with the labelled microbeads. This complex moves along the membrane due to capillary action, until it makes contact with a corresponding antibody, antigen or ligand immobilised on the membrane, where it forms an immuno-complex and generates a coloured product that can be visualised by eye or read with a portable hand-held reader. Lateral flow devices (LFDs) show great potential for development into portable, pen-side diagnostic tests. Microbeads incorporating fluorophores can be used to produce LFDs with greater analytical sensitivity when applied with specific portable detectors (Liang *et al.,* 2017). Low cost, paper-based fluidics, may also have a greater role in the future, especially in the pen-side context (e.g. Yang *et al.,* 2018).

4. Reporter virus neutralisation tests

Reporter viruses may be derived from genetically manipulated viruses that are fully attenuated and that bear a marker gene encoding, for example, green fluorescent protein, or the *Renilla* or firefly luciferases. Alternatively, the envelope glycoproteins of the virus to be studied are expressed on a replication-deficient particle of a heterologous viral species that carries a marker gene, for example lyssaviral glycoproteins expressed on retroviral particles (viral pseudotypes). These innovative approaches, which reduce the bio-risk for the veterinary diagnostician and increase the sensitivity of virus neutralisation tests (VNT), have been developed for a range of viral pathogens, including rabies virus (Wright *et al*., 2008), influenza A virus (Carnell *et al.,* 2015), PPR, rinderpest and canine distemper viruses (Logan *et al.*, 2016a; 2016b), and Rift Valley fever virus (Schreur *et al.,* 2017). These technologies allow rapid quantification of neutralising antibodies as they obviate the need to wait for the development of plaques or other

cytopathic effects. They can potentially be multiplexed to develop VNT assays capable of detecting neutralising antibodies against distinct antigens (Molesti *et al.,* 2014).

5. Luciferase immunoprecipitation system

The luciferase immunoprecipitation system (LIPS) can also be used for pathogen-specific antibody detection. A recent application of this approach was described for the detection of PPRV-specific antibodies (Berguido *et al.,* 2016). In this assay, a reporter enzyme, *Renilla* luciferase (Ruc), is fused to an antigen of interest and expressed in mammalian cells. Subsequently, the Ruc-antigen fusion protein is recognised by antigen-specific antibodies, and antigen–antibody complexes are captured by protein A/G beads, which recognise the Fc region of the IgG antibody. The relative amount of antibody bound to the Ruc-tagged antigen is determined using a luminometer to measure the light produced when adding coelenterazine, the substrate for Ruc. This approach is species independent, provides a very specific and sensitive detection and requires very low volume of test serum.

6. Rapid homogeneous assays

Rapid homogeneous assays are those where the reagents are mixed together to form a homogeneous solution or suspension within which the antibody and antigen reactions rapidly take place. The assays require no further separations or washing and can be read without further liquid-handling steps. Examples of such assays include agglutination assays whereby a serum sample is mixed with a suspended particulate antigen and where the presence of serum antibody against the antigen causes the antigen to agglutinate (due to the multivalent nature of antibodies). More sophisticated methods include the fluorescence polarisation assay (FPA). This measures how the rate of spin of a molecule, for example an antigen, changes as it becomes larger due to the binding of antibody. An example of this approach is the *Brucella* FPA using fluorescein conjugated O-polysaccharide (Nielsen *et al.,*1996). A limitation of this approach is the need for highly purified antigen and an antigen that is small enough to spin rapidly when not bound to antibody. Two readings are required, one to measure the background fluorescence of the sample and the other to measure the fluorescence of the reaction after antigen addition. Other methods that are used in the pharmaceutical industry for high-throughput compound screening include competitive methods where complementary ligands, analogous to antigens and antibodies, are labelled with tags that measurably interact with each other on a proximity basis, with signals increasing as they get closer (indicating binding has taken place). The introduction of competing reagents, for example unlabelled serum antibody, impedes this reaction leading to the consequent measurable reduction of signal (McGiven *et al*., 2009). When compared with other diagnostic methods, FPA assays exhibited high sensitivity, e.g. for the diagnosis of bovine brucellosis (Praud *et al*., 2016).

C. BIOTECHNOLOGY APPROACHES FOR THE PRODUCTION OF DIAGNOSTIC REAGENTS

1. Recombinant DNA technology to produce diagnostic reagents

These technologies are now also being used to produce protein-conjugated polysaccharides (Cuccui & Wren, 2015) as the genetic code for the protein carrier, the proteins required for the synthesis of the glycans and the code for the proteins required to conjugate the glycans to the carrier can all be inserted into host bacterial cells (such as *E. coli*). Although directed primarily towards the generation of vaccines, such recombinant glycoproteins can also be used for serodiagnosis, for example the detection of anti-*Brucella* antibodies in cattle and pigs. (Ciocchini *et al*., 2014; Cortina *et al.,* 2016). These antigens offer the traditional advantages of recombinant proteins, such as purity of product and avoiding the need to culture the native organism (in this case a highly infectious zoonotic agent) but also enable the production of highly important non-protein diagnostic antigens such as glycans in a form (conjugated) that facilitates their use as diagnostics and vaccines.

Recombinant DNA technology enables the production of antigens for various diagnostic applications such as ELISA, agglutination, haemagglutination inhibition (HI) tests, agar gel immunodiffusion (AGID) tests, complement fixation tests (CFT), as well as microarray and bead-based technologies.

For antibody production, recombinant antibodies are used as an alternative to the traditional hybridoma-based technology in generating high quality antibodies. Phage display, yeast display and ribosomal display are some of the approaches used to produce antibodies in prokaryotic, eukaryotic and *in-vitro* systems, respectively (Sutandy *et al.,* 2013).

2. Synthetic antigen biology

By circumventing biological systems completely, the recreation of antigen structure via synthesis also offers many advantages over native antigen extraction. This includes high levels of purity and reproducibility, simple scale up capability and bespoke antigen design.

An upcoming trend in the production of antigens for use in assays is 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 is unnecessary, thus shortening the process. Glycosylated peptides can also be synthesised (Bednarska *et al.,* 2017). The cost and benefits are such that recombinant production systems still dominate for the production of large proteins. However, for non-protein antigens where the underlying genetic basis for production is unclear and thus recombinant production is not possible, synthesis is a very viable and successful option for diagnostic antigen production. This has been shown for brucellosis where the epitopes that exist in the native structure of the O-polysaccharide from *B. abortus* S99 and *B. melitensis* 16M have been synthesised and the synthetic antigens have been shown to be highly effective for serodiagnosis (Guiard *et al.,* 2013; McGiven *et al.,* 2015).

Synthetic DNA can be generated from sequence data and then used with recombinant technology to generate proteins and even complete viruses for the rapid production of vaccines, such as influenza (Dormitzer *et al.,* 2013).

D. TECHNOLOGIES APPLICABLE TO DETECTION AND ANALYSIS OF ANTIGENS, ANTIBODIES AND NUCLEIC ACIDS

1. Microarray technologies

A microarray is a two-dimensional arrangement of specific biological probes (e.g. DNA, proteins, peptides, glycans) immobilised on solid substrates such as a glass slide, polymer-coated glass, plastics, nitrocellulose, etc. (Barbulovic-Nad *et al.,* 2006). A microarray provides high multiplexing capability, i.e. hundreds or thousands of detections can be performed at a time. Microarray chips can be created to identify the cause of syndromic disease, for example, a chip that targets panels of encephalitic pathogens or to target species-related diseases, e.g. swine disease chips. Similarly, chips of very high specificity may be designed to detect multiple targets for a given pathogen. Using array-based approaches can present some challenges in handling and analysis of the very large data sets that are generated. Instrumentation and consumables may also be cost-prohibitive for some laboratories.

1.1. DNA microarrays

DNA microarrays exploit the ability of complementary strands of nucleic acids to hybridise, and involves the following steps:

i) Nucleic acids isolated from biological sources are amplified and labelled with a fluorescent dye.

ii) The single-stranded, labelled DNA products are then added to the surface of the DNA microarray. This results in hybridisation of the sample nucleic acids with the complementary biological probes in the microarray, creating a labelled double-stranded molecular structure on the surface of the array.

iii) The microarray is then rinsed to remove non-specifically bound target molecules and evaluated using a laser scanner.

DNA microarray technology is a useful tool with a variety of diagnostic applications. This technology offers the possibility of detecting and identifying pathogens of veterinary and public health importance in the target sample (Ojha & Kostrynska, 2008). It can also be used for epidemiological investigations and genotyping of pathogens. With technological advances, DNA microarrays have also progressed toward the discovery of new emerging pathogens. For instance, an array consisting of highly conserved 70-mer probes from all sequenced reference viral genomes was used to demonstrate that a coronavirus was responsible for severe acute respiratory syndrome (SARS).

DNA microarrays are particularly useful for multiplex assays and have been used to detect and determine all possible HA and NA subtypes of avian influenza virus (Belák *et al.,* 2009). The most recent generation

of microarrays enables DNA sequence analysis, often providing complete genome sequences in a single experiment.

1.2. Protein microarrays

Protein microarrays are produced similarly to DNA arrays by immobilising proteins at high density on a solid surface (Sutandy *et al.,* 2013). The arrays contain specific probing molecules such as antigens or antibodies that can be recognised via fluorescent labels or detected by mass spectrometry (MS) (SELDI-TOF [surface-enhanced laser desorption/ionization-time of flight] or MALDI-TOF [matrix-assisted laser desorption/ionisation-time of flight]) (Sutandy *et al.,* 2013; Yu *et al*., 2006). The principle of the reaction between an immobilised capture molecule and a protein target analyte present in the sample relies on antibody/antigen recognition or protein/protein interaction. Protein arrays are used for antigen or antibody detection in blood samples, the discovery of disease biomarkers and the discovery of the mechanism of pathogenesis and immune response to a pathogen by different host. For example, a protein microarray containing 1406 predicted *Brucella melitensis* proteins was used to screen sera from experimentally infected goats and naturally infected humans and to demonstrate the differences in the immune response in goat and humans following *B. melitensis* infection (Liang *et al*., 2010). In addition, protein microarrays can be used for detection of antibodies directed against specific pathogens and monitoring the changes in cellular protein expression.

Surface-enhanced laser desorption/ionisation-time of flight mass spectrometry

Technological advances have enabled the microarray platform to achieve sufficient standardisation and method validation as well as efficient probe printing, liquid handling and signal visualisation. Some major challenges from using protein microarrays in routine diagnostics are the need for highly specific antibodies to prevent false-positive results and the need to produce large numbers of antibodies in a high throughput fashion (Sutandy *et al*., 2013).

2. Bead-based arrays

Bead-based arrays and cytometric bead arrays are variants of probe-based assays that provide the opportunities for multianalyte profiling targeting nucleic acids, antigens or antibodies (Christopher-Hennings *et al.,* 2013). In the technique, pathogen-specific nucleic acid, antigen or antibody is covalently linked to microsphere beads. An advantage of the technology is its multiplex capability that results from the beads themselves having a fluorescent signature (such that each bead can carry a specific probe). Bead-based assays are increasingly being used in multiple pathogen detection tests to look for the nucleic acids of several pathogens (Boyd *et al.,* 2015) or antibodies to different pathogens in a single sample (Sánchez-Matamoros *et al.,* 2016). If there is a requirement for testing many serum samples for a panel of diseases (e.g. several different antigens), including the possibility of multiple antigens per disease, then multiplex bead-based arrays offer an efficient means for this. Recent examples exploiting the multiplex capability of this technology are the application to genotyping of African swine fever virus (LeBlanc *et al*., 2013) and DIVA (differentiation between vaccinated and infected animals) test for FMD (Chen *et al.,* 2016).

3. Biosensors

Biosensors use an immobilised biosensing element (DNA, RNA, antigen/antibodies or glycans), also known as a bioreceptor, to recognise a characteristic biomarker of the pathogen. The resulting biochemical interaction between the biomarker and the bioreceptor is converted into a measurable signal by the transductor and displayed (Vidic *et al.,* 2017). Biosensing is based on optical electrochemical and mass-based transduction methods (Alahi & Mukhopadhyay, 2017). This approach has been used for influenza A antigen detection (Hideshima *et al.,* 2013; Lee *et al.,* 2013), and the detection of antibodies to *Mycoplasma bovis* (Fu *et al.,* 2014).

4. Mass spectrometry

Mass spectrometry (MS) can detect biomarkers present in a sample, based on their mass. Infection with a microorganism can be detected by comparing the profile of biomarkers present in a sample to a database of samples known to be positive for the organism of interest. This is the premise of diagnostic modalities including MALDI-TOF MS. This approach was used for *Staphylococcus intermedius* and for the direct identification of bacteria in blood cultures (Guardabassi *et al.,* 2017). Some approaches have also been developed to identify protein biomarkers as a means of virus identification. MALDI-TOF MS can also be used for sequencing short DNA

fragments, as an alternative to conventional sequencing when high throughput automated screening for mutations is needed.

One electrospray ionisation (ESI)-based platform, combining the accuracy and the sensitivity of MLST with the speed and throughput of MS, has been developed for rapid identification of pathogens (Kailasa *et al*., 2019). Similarly, several applications using the MALDI-TOF MS are available for genotyping or single nucleotide polymorphisms (SNP) typing and resequencing.

4.1. Proteomics

MS is also used in proteomics. The proteome is the total complement of proteins expressed within a cell, a tissue or an organism. Proteomics is the study of proteins, including their expression level, post-translational modification and interaction with other proteins. 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. 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). Within the veterinary field, proteomic studies have been developed for a variety of applications for animal and zoonotic diseases (Katsafadou *et al*., 2015; Patramool *et al*., 2012; Torre-Escudero *et al*., 2017).

5. *In-situ* detection of antigens and nucleic acids

Different techniques are available for the direct detection of pathogen proteins, antigens or nucleic acids in animal tissues or body fluids. In some cases, the same technology can be applied to the detection of antibodies in serum.

5.1. Immunofluorescence

The fluorescent antibody test (FAT) is used for the detection of pathogens in animal tissues or fluids, using specific antibodies against the targeted antigens. As the method is based on direct binding of the labelled antibody to the antigens of the infectious agent present in the sample, it is commonly called direct immunofluorescence. The method is commonly used in diagnostic laboratories, e.g. for the detection of the rabies virus in the brains of dead animals and classical swine fever virus in tissues of suspected pigs.

A modification of the FAT can be used for the detection of specific antibodies, produced by the immune system against various pathogens during infections. Essentially, the modification is in the use of secondary antibody specific for the antibodies of the species examined. This procedure is commonly referred to as indirect immunofluorescence. The method is commonly used in diagnostic laboratories for the detection of antibodies raised against a wide range of pathogens, e.g., African swine fever virus (Cubillos *et al*., 2013), *Coxiella bernetii*, causative bacteria of Q fever (Roest *et al.,* 2013) and many other infectious agents in veterinary medicine.

Another modification of direct immunofluorescence uses an unlabelled primary antibody derived from a specific species to recognise pathogen-specific antigen, and this antibody is subsequently bound by a secondary anti-species immunoglobulin antibody that is conjugated to a fluorophore. This procedure is also commonly referred to as indirect immunofluorescence.

5.2. Immunohistochemistry

Immunohistochemistry involves the *in-situ* detection of antigens in fixed tissues using labelled antibodies. As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry has become a standard tool to identify pathogens in tissue, and for confirmation of the results obtained using other diagnostic technologies. Immunohistochemistry is commonly used for the detection of abnormal prion protein (PrP^{Sc}) in brain tissue to confirm scrapie, BSE and other transmissible spongiform encephalopathies, and is more sensitive than standard histopathological examination (Thorgeirsdottir *et al*., 2002). In recent years, diagnostic tests based on immunohistochemistry have been successfully

developed and applied to rabies virus detection in clinical specimens (Rahmadane *et al*., 2017). As the number of antibodies to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmune diseases and neoplasia is increasing.

Due to the use of fixatives to prepare samples, IHC offers several advantages over pathogen isolation methods, including:

i) convenience of sample submission;

ii) safe handling of zoonotic pathogens;

iii) retrospective studies of stored specimens;

iv) rapidity; and

v) the detection of nonviable organisms (Haines & Clark, 1991).

As formalin fixation can denature the antigenic epitopes (i.e. the three-dimensional structure recognised by some antibodies) the limiting step in the application of immunohistochemistry is to identify an appropriate antibody/antigen combination that will bind antigen 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.

5.3. *In-situ* hybridisation (ISH)

This technology exploits two natural phenomena: the unique genetic code or signature a pathogen contains in its genome and the ability of single-stranded nucleic acid sequences (DNA or RNA) to anneal or bind with complementary single-stranded nucleic acid sequences to form double-stranded hybrids. In its simplest form, *in-situ* hybridisation (ISH) uses single-stranded DNA or RNA probes that are produced synthetically and are designed to complement a short specific region of a pathogen's genome. Probes are coupled to labels that are readily detectable using microscopy and may include fluorescent dyes, fluorescent nanoparticles or enzymes that produce a chromogenic product when treated with substrate.

Recent commercial supply of ISH reagents has made the technique more accessible and is likely to see greater uptake by diagnostic laboratories in the future. A noteworthy application of ISH is in the rapid development of tests for detection of newly emerging pathogens. As soon as sequence data generated during identification of the novel pathogen by high throughput sequencing (see Section D.vi) becomes available, probes may be designed and assays developed. In contrast, antibody/antigen-based reagents required for the development of immunoassays take considerably longer to generate.

E. IMPLICATIONS OF THE NEW TECHNOLOGIES

There are several trends in diagnostic technology development, e.g. multiplexing of assays, evaluation of complexity in the biology of infection, that will have an impact on the way disease diagnoses 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. In parallel, a wide range of simple diagnostic tools, such as lateral flow assay devices were developed for the improved, on site (point-of care) diagnosis of many diseases. These changes enable on-site testing, facilitating the rapid and affordable diagnosis of the targeted infectious diseases. Appropriate infrastructure and preparedness must be an integral component of technology update, including training in the use and interpretation of such methods.

ii) Simplification of technologies, development of smaller, more simple and affordable devices is also seen in other fields of veterinary diagnostic microbiology, for example in rapid and high throughput sequencing technologies. The development of simple devices and improved sample preparation technologies will facilitate the direct sequence analysis of clinical samples not only in central institutes, but also on site in the field or in simply equipped field laboratories. During this work it will be very important to focus not only on the

rapid technical development of sequencing equipment, but in parallel, to strengthen the knowledge of bioinformatics and to maintain general knowledge in veterinary medicine and epidemiology.

iii) 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.

iv) Although the development of new technologies can often mean faster results and improved capability, consideration should always be paid to the actual value and the role of the confirmatory test in diagnosis and of the modes of reaction of appropriate Competent Authorities. In this context, the expertise present in laboratories remains as important as ever in explaining the significance and limitations of diagnostic results.

v) Increasingly, traditional, well understood methods that use low cost equipment are being replaced with more complex methods requiring increased investment. These newer methods often require higher diversity of expertise in laboratory personnel and are associated with increased turnover in platforms as relatively recent systems become redundant. This has implications for laboratory resourcing and the structure of laboratory networks within and between countries as laboratories become more specialised.

vi) In-depth analysis of pathogens, for example through high throughput sequencing, is increasingly becoming an expected part of reference laboratory operations. 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 to systematically collect, store and analyse the large datasets produced by the new technologies in a relatively short time. For example, there is likely to be an increasing trend towards real-time inputs of results via mobile platforms. These trends will require ongoing development of bioinformatics, information technology and data-handling systems.

vii) Methods with high levels of analytical sensitivity and specificity are increasingly available to laboratories and are enabling the rapid identification and response to infectious veterinary disease, improving the effectiveness of control and eradication methods. These developments provide new opportunities for the identification and characterisation of the infectious agents and for the improved control of infectious diseases in veterinary medicine.

viii) Novel, biotechnology-based methodologies open a wide range of new possibilities and challenges in diagnostic microbiology. Thus, it is strongly advised to consider powerful new methods and to introduce these new technologies at our diagnostic laboratories. However, it is important to determine what is the exact diagnostic capacity and value of the new technologies and to what extent can these methods replace the classical diagnostic approaches. In general, the safest way is to maintain a multidisciplinary complex of diagnostic approaches and capacities at our laboratories, providing a practical combination of powerful new and classical technologies and a properly balanced knowledge of veterinary diagnostic medicine, infection biology and epidemiology.

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NB: FIRST ADOPTED IN 1996 AS BIOTECHNOLOGY IN THE DIAGNOSIS OF INFECTIOUS DISEASES AND VACCINE DEVELOPMENT. MOST RECENT UPDATES ADOPTED IN 2021.