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Review Article

PROSPECTS OF THE USE MOLECULAR TOOLS IN THE CONTROL OF INFECTIOUS DISEASES

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SUMMARY

Molecular diagnostics continues to advance very rapidly, and its impact in the diagnosis of infectious diseases is obvious and very effective. Molecular tools have played a pivotal role in discovering and characterizing several emerging infectious agents and have now become the gold standard for the diagnosis of infectious diseases caused by fastidious or sterile agents. Multiple challenges still remain for the widespread use of cost-effective, validated, and commercially available molecular tools. Automated instruments capable of sample processing and multiplex nucleic acid amplification and post amplification analysis have already been approved by the US Food and Drug Administration (FDA) for use in the clinical setting. Nanobiotechnology is beginning to impact laboratory diagnostics in the clinical setting.

Keywords: Molecular tools, Clinical laboratories, Infectious diseases.

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INTRODUCTION

Several infectious diseases caused by microorganisms, including protozoans (malaria, leishmaniasis, trypanosomiasis, amoebiasis.), bacteria (cholera, gastritis and gastric ulcers, meningitis, tuberculosis, leprosy.), and viruses (dengue fever, yellow fever, influenza, chikungunya fever, ebola, human immunodeficiency syndrome.), are threats to public health. In order to control outbreaks, emergence, and reemergence of these infectious diseases, diagnosis, correct identification, treatment, control and notification of

pathogenic agents are necessary. Adequate clinical supervision of infectious diseases relies primarily on the accurate identification of the causal microorganism and the production of reliable information on its antimicrobial susceptibility (Foxman *et al*., 2005). Due to their clinical importance, misidentification of these pathogens in clinical samples can have serious consequences in increased morbidity and mortality. Over the past century, scientists have searched for more rapid and efficient means of microbial identification (Tang *et al*., 1997). The identification and differentiation of micro- organisms had mainly relied on microbial morphology and growth variables. These traditional methods of microbial identification rely solely on the phenotypic characteristics of the organism. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include iso-enzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids (Thurm *et al.,* 1994; Lin *et al*., 1995; Blanc *et al*., 1994;).

Application of molecular techniques in clinical and veterinary microbiology laboratories have now progressed beyond identification of antibiotic resistance and tolerance genes, and are making inroads in the rapid and direct detection of etiologic agents of disease directly from clinical samples without the need for culture. Increased use of automation and user-friendly software makes these technologies more widely available, more efficient, less laborious, cost effective and gives room for versatile application. While the role of molecular techniques has increased in the developed world and are now part of routine specimen processing in many of these countries, it has continued to be a theoretical exercise in many developing countries and the evaluations of this technology for adoption in laboratories have generally been limited by several factors. As these molecular methods are further refined and become more widely available, we need to understand their clinical applications and be aware of their potential advantages, limitations and clinical utility.

CLINICAL APPLICATIONS

The diagnosis of any infectious disease requires active communication between clinicians and clinical laboratory personnel, usually in the form of □□consultations□□ from clinicians to the laboratory director □□on call□□ or directly with the laboratory technologists. Arriving at a correct diagnosis in a timely fashion begins with the acquisition of an adequate clinical specimen (blood, cerebrospinal fluid, urine, respiratory secretions, bronchoalveolar lavage, feces) and its transportation to the laboratory in an appropriate container. Specimens for molecular diagnostic tests sometimes require different methods of transportation/ preservation from those of regular specimens to ensure that the techniques, used efficiently, will detect the suspected infectious agent. The classic diagnostic principles in clinical medicine still apply when using molecular methods of diagnosis. Patients and populations are evaluated clinically and epidemiologically, and case definitions are created so that test parameters can be defined such as sensitivity, specificity, and predictive values. Likewise, understanding the

concept of screening versus confirmatory testing is also important.

The 3 major steps in molecular assays are specimen processing, nucleic acid amplification or hybridization, and product detection (Nolte *et al.,* 2007: Persing *et al.,* 2004;). Processing of the specimen is one of the most important steps for successful detection of nucleic acids. Protocols vary, depending on the specimen received, and the specimen ??matrix?? plays an important role in nucleic acid extraction owing to the presence of extraneous material in some matrices that could potentially interfere with the molecular assays being performed. The most widely known example of such interference is the presence of Tag polymerase inhibitors in nucleic acid extracts, leading to false-negative results. Nucleic acid extraction protocols are usually cumbersome, and automation has been relatively challenging. However, several instruments have been designed for the clinical laboratory in which automated extraction protocols have been incorporated. Automation has also been incorporated for nucleic acid amplification methods, including conventional and real-time polymerase chain reaction (PCR), branched DNA, and isothermal technologies that obviate the usual thermal cycling and potentially speed up the time required for amplification. Automated platforms, however, have decreased the cycling time by changing more quickly the temperatures required for the different PCR cycles. Real-time PCR systems are usually coupled with automated detection systems that also vary in complexity. A few instruments are also capable of complete automation, including extraction, nucleic acid amplification, and detection systems. Examples of automated systems are given in Table 1.

Table 1: SELECTED AUTOMATED SYSTEMS FOR MOLECULAR DIAGNOSTICS

INSTRUMENT	MANUFACTURER	CAPABILITIES
MagNA Pure	Roche, Pleasanton, California	High throughput processing
m200 generic	Abbott, Des Plaines, Illinois	High throughput processing
QIAcube	Qiagen, Salt Lake City, Utah	Nucleic acid extraction
COBAS	Roche, Pleasanton, California	Conventional PCR amplification
System 340	Bayer, Berkeley, California	Branched-DNA amplification
ABI Prism	Celera, Alameda, California	Real-time PCR
LightCycler	Roche, Pleasanton, California	Real-time PCR
SmartCycler	Cepheid, Sunnyvale, California	Real-time PCR
COBAS TaqMan analyzer	Roche, Pleasanton, California	Real-time PCR
Abbott m2000	Abbott, Deerfield, Illinois	Processing,amplification,detection
TIGRIS	Gen-Probe Inc, San Diego, California	Processing,amplification,detection
AmpliPrep COBAS TaqMan	Roche, Pleasanton, California	Processing,amplification,detection
Ibis T5000	Ibis Biosciences, Carlsbad, California	Processing,amplification,detection
GenXpert Diagnostics	Cepheid, Sunnyvale, California	Processing,amplification,detection
Verigene System	NanosphereInc, Northbrook, Illinois	Processing,amplification,detection
LX 100/200	Luminex Corp, Austin, Texas	Processing,amplification,detection

(Source: Juan *et al.*, 2011)

Table 2: Comparison of molecular and conventional methods

Nucleic acid based method (Molecular method)	Conventional (Phenotypic) method
Advantages	Advantages
1. Saves time, results can be in minutes or hours. 2. Highly specific and highly sensitive. 3. Detects even fastidious or microorganisms that cannot be cultured at all on artificial media. 4. Can still be used when antimicrobial agents are present or administered (that is, outcome of result is not affected by presence of antimicrobial agents in the patient or source of sample). 5. Detection of certain properties such as toxin and other virulent genes or antimicrobial resistance. 6. Delay in sample collection and transportation has little or no effect on the final result.	1. Some results can be obtained in minutes or hours (eg malarial parasite test) 2. Rise in antibody titer of serological procedure can suggest involvement of pathogen in infectious process. 3. Can demonstrate viability of the target microorganism in culture. 4. Phenotypic antimicrobial testing allows laboratories to test many microorganisms and can detect newly emerging as well as established resistance patterns. No prior knowledge of resistance gene is required. 5. Comparatively less expensive than molecular methods in terms of storage and use in developing countries. 6. Interpretation of results are usually not very difficult because they are not very complex.
Disadvantages 1. Technically complex procedures. Though there are simple types, most requires highly skilled labour. 2. High cost of initially establishing the laboratory. 3. Potential for false-negative test results (e.g., because of presence of PCR inhibitors interfering with nucleic acid amplification).	Disadvantage 1. Less complex procedure 2. Less expensive, but delay in obtaining results could still lead to loses and wastages. 3. Potential for false-negative results (e.g., when antibiotic are used before sample collection).

(Source: Aniesona 2012)

FACTORS LIMITING THE AVAILABILITY AND USE OF MOLECULAR TOOLS

LACK OF SKILLFUL MANPOWER

A 2002 estimate suggested that only 13% of the world's scientist live and work in Africa, Latin America or Middle East.[10] In addition to the scarcity of experienced scientists, only very few teachers or lecturers are conversant with the techniques and practical applications of molecular diagnostic methods in developing countries. Only those with such skilled knowledge can make the subject interesting and impact effectively.

LACK OF MOTIVATION BY INDIVIDUALS, POLICY MAKERS AND GOVERNMENTS

The African Union (AU) leaders at the January 2007 Addis Ababa summit, proposed to boost funding for scientific education and the environment as well as to set in place policies towards biotechnology development and intellectual property protection (Koenig, 2007; Masood, 2007). The AU leaders stopped short of approving a science and innovation fund and an independent assessment body for science on the continent. Lack of interest by our leaders, law makers and wealthy individuals who travel abroad for diagnostic research,

medical treatments or to enjoy the products and services of other scientists abroad makes it difficult to acquire, transfer and adapt such technology to developing countries (Ashok, 2005).

ENVIRONMENTAL FACTORS

In many developing countries, the establishment of specialized research institutes was intended to make research and some of these molecular techniques available to the local community, but lack of patronage by the community who may not have confidence on the local scientist have often been unfavorable.

COST OF ESTABLISHMENT AND MAINTENANCE

Cost of most molecular techniques is fairly high; this is because they require costly material and apparatus. The sensitivity of the molecular techniques makes the risk of contamination a very important issue in the establishment of laboratories for molecular techniques. To avoid false positive results due to laboratory contamination, relatively large laboratory areas are required for physical separation of reagent preparation, specimen preparation and product detection areas together with a high level of staff training and skill. Cost of purification, preservation etc can be discouraging (Pfaller, 2001; Foxman et al., 2005).

POOR SCIENTIFIC TEAMWORK

Cooperation among scientists in developing countries is very poor. Multi-center discourse needed to build collaborations is often accessed by conferencing with other scientists addressing similar problems (Okeke, 2007). A preference for developed country conference venues by scientists, unjustifiably high airfares from developing countries to the conference venues, difficulties in obtaining foreign visas, and limits in financial support for travel still hamper the participation of scientists from developing countries in international meetings.

SCARCITY OF RELEVANT AND UP-TO-DATE LITERATURES

Significant roadblocks must also be overcome to bring the most up-to-date and relevant biomedical literature and electronic research resources to the desks of many scientists in the poorest countries. Many regional publications, which carry the bulk of papers authored by developing country scientists, have typically been neither free nor online.

POOR MOTIVATION FROM SCIENTIFIC SOCIETIES AND ACADEMIA

Many at times scientific organizations and the academic societies believed the establishment and management of such specialized laboratories should be better funded by the government, unfortunately the government may not be willing since the politicians are not clued-up and may not be properly informed on the existence and importance of such diagnostic tools.

MOLECULAR-TOOLS TECHNIQUES

Molecular biological-tools techniques have become increasingly applicable to the diagnosis of infectious diseases and vaccine development. To become widely used, the methods need to be easy, safe, sensitive, reproducible and eventually automated to facilitate the evaluation of large numbers of samples.

The following is an outline of the molecular tools techniques briefly reviewed in this work

A. Detection of nucleic acids

1. Polymerase chain reaction (PCR) and real-time PCR

2. Diagnosis by DNA probes and DNA microarray technology

B. Detection of protein

1. Immunohistochemistry

3. Antigen-capture enzyme-linked immunosorbent assay (ELISA)

C. Antibody detection

1. Competitive ELISA (C-ELISA)

2. Production of antigens by recombinant DNA technology

E.Nanotechnologies in diagnosis

1. Disease diagnosis

2. Vaccine development

A. DETECTION OF NUCLEIC ACIDS

1. Polymerase chain reaction (PCR) and 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 50 to several thousand base pairs into billions of copies. A detailed discussion on the methodology and applications of PCR is given in (Mullis *et al.,*1994). The amplification of DNA by the PCR is accomplished through a cyclic succession of 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 (short synthetic molecules of DNA complementary to both strands and flanking the target sequences) are then annealed to the single-stranded template at low temperature and extended with DNA polymerase at an intermediate temperature. Once the polymerase has synthesized 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 (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 RT-PCR.

Any PCR product generated has, by definition, a characteristic size. Its identity is generally confirmed using DNA probes or restriction digests, which can be used to provide RFLPs. More commonly, since the advent of automated cycle sequencing techniques, identification is via direct sequencing of the PCR product. For example, sequencing has been 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 *Anaplasmamarginale*in persistently infected cattle (Torioni *et al.*,1998). However, the use of nested PCR can increase the rate 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 those caused by retroviruses (bovine leukaemia virus, caprine arthritis/encephalitis virus). These diseases present serious problems in terms of diagnosis and prevention since infected animals are a constant potential source for transmission.

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). Systems have been developed to deal with this problem, for example the dUTP-UNG system (d-uracil triphosphate and uracil-N-glycosylase). This system uses an enzymatic reaction to specifically degrade PCR products from previous PCR amplification (in which dUTP has been incorporated) without degrading native nucleic acid templates (Burkhardt *et al.,* 2000). This, of course, does not exclude contamination of the sample with extraneous virus. 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 interfering substances in the PCR reaction mixture or patients sample by the inclusion of a template known to produce a PCR product.[20] Use of these precautions allows the PCR to become a realistic option for the diagnostician.

To expand its utility in veterinary diagnostics and pathogen identifications, PCR has been extensively modified in the past years. PCR using broadly conserved primers is designed for identification of classes of pathogens. The best example is the use of sequences of the 16s rRNA gene, an evolutionarily conserved gene in bacterial species (Greisen *et al.*, 1994). Using PCR primers that are complementary to these conserved sequence regions, one can determine the presence of any bacteria of a desired class from the sample. It must be noted that a positive PCR result needs to be further characterized by hybridization with species-specific probes, analysis by restriction enzyme digestion, or by sequencing. Similarly, consensus PCR is designed to use degenerate primers targeting conserved sequence regions or motifs of a group of related pathogens

(Vandevanter *et al.*, 1996). Use of degenerate primers targeting the sequence regions of the herpes viral DNA polymerase gene has led to identification of many previously unrecognized herpes viruses in various animal species (Ehlers *et al.*, 1999; Li *et al.*, 2000). On the other hand, multiplex PCR is designed to use two or more primer pairs directed at pathogen-specific unique sequences within a single reaction for simultaneous detection of multiple pathogens that are of interest (Elnifro *et al.*, 2000). 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. Classical PCR methods for diagnosis of pathogens, both bacterial and viral, are now being complemented and in some cases replaced with real-time PCR assays. Real-time PCR monitors the accumulation of PCR product during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. In other words, the assay can be used to reliably quantify the DNA or RNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid than conventional PCR techniques, has a closed-tube format therefore decreasing risk of cross-contamination, is highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. In many cases, the real-time PCR assays have proved to be more sensitive than existing reference methods (Heim *et al.,* 2003; Weidmann *et al.*, 2003). The recent development of portable real-time PCR machines and assays (Risatti *et al.*, 2003) raises the exciting prospect of these techniques being used for rapid (less than 2 hours) diagnosis of disease outbreaks in the field.

2. Diagnosis by DNA probes and DNA microarray technology

Conventional DNA probing and microarray analysis are two sides of the same coin. Fundamental to both processes is the binding (hybridization) of DNA, derived from a sample suspected of containing a pathogen, with highly characterized DNA derived in advance from a pathogen of interest (the known DNA). In conventional DNA probing the unknown DNA (or RNA), the target, is immobilized on a solid surface e.g. a filter. The known DNA, made into a probe by labeling or tagging it in some way, is in the liquid phase and is applied to the target. In microarray diagnosis it is the known DNA (large oligonucleotides or complementary DNA) that is the target, immobilized on a glass slide, and the unknown DNA, in the liquid phase that is labeled to make a probe. In conventional DNA probing the target can be nucleic acids extracted from clinical material or cultured cells and either (a) added to filters (a dot or slot blot) or (b), less conveniently in a diagnostic context, transferred to a filter after gel electrophoresis. The amount of pathogen in a clinical sample might be too low for detection. Consequently one might amplify the nucleic acid by PCR or reverse transcription PCR (RT-PCR), the PCR product being applied to a filter. In order to visualize a probe bound to its target, the probe can be labeled with a radioactive nuclide or, more commonly and safely, tagged non-radioactively. For example, biotin or psoralen biotin may be incorporated into the probe, bound probe being detected by addition of streptavidin linked to an enzyme for

subsequent generation of colour or light (chemiluminescence). A microarray is so-called because it can comprise 20,000 or more 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 (Boonham *et al.,* 2003). However, if large numbers of pathogens are to be investigated then it would be logistically easier to use large oligonucleotides. The microarray that was used to identify the SARS virus as being a coronavirus had oligonucleotides comprising 70 nucleotides (70-mer) (Wang *et al*., 2002). In microarray probing it is the sample from which a probe is made. Essentially nucleic acid is extracted from a sample and a (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 labeled with a fluorescent dye and applied to the microarray. Under optimized conditions only the DNA derived from the pathogen will bind to the DNA on the glass slide. If one is interested in detecting only a particular pathogen or group of related pathogens then pathogen-specific oligonucleotides can be used to amplify these within the sample for probe production. Microarrays for detecting pathogens can be designed for several levels of differentiation. In the case of oligonucleotide target DNAs one might initially design oligonucleotides to be able to detect and differentiate pathogens at the genus level. One would choose a number, 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, whilst not hybridising (or hybridising to a lesser degree) to those corresponding to related genera, e.g. to differentiate *Apthovirus* (foot and mouth disease, FMDV) isolates from *Enterovirus* strains in the *Picornaviridae* family. One could then select 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 to even further refinement at subtype level. In conventional DNA probing the detection of a pathogen is limited by the number of probes used, whereas in microarray analysis one 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, 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 one is investigating diseases of unknown aetiology, 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 as mentioned in the above section. When one has a particular pathogen in mind, then the use of a microarray would

be less justifiable, since the production and hybridization of slides is relatively expensive. Instead, for these more simple cases, one might use pathogen/subtype specific PCRs, followed by sequencing or restriction fragment analysis for confirmation. If previous experience of biotechnology is indicative of the future, then one would expect microarray equipment and reagents 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 vast data sets that are generated.

B. DETECTION OF PROTEIN

1. Immunohistochemistry

As an adjunct to the isolation of causative organisms from tissue, immunohistochemistry is rapidly becoming a standard tool in diagnostic laboratories for the identification of antigens associated with viral, bacterial and protozoa microorganisms and transmissible spongiform encephalopathy (Procop *et al.*, 2001). 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 *et al.*, 1991). Immunohistochemistry is also used for the detection of abnormal prion protein (PrPSc) in brain tissue to confirm scrappy, bovine spongiform encephalopathy and other transmissible spongiform encephalopathies, and has proved to be more sensitive than the standard histopathological examination for diagnosis of these diseases (Thorgeirsdottir *et al.*, 2002). Demonstration of PrPSc in lymphoid tissue biopsies, e.g. nictitating membrane, can also be used for the preclinical diagnosis of scrapie (O□rourke *et al.*, 2001). As the number of monoclonal antibodies (MAbs) to defined antigens increases, the use of immunohistochemistry for the identification of organisms and other specific markers for autoimmunity and neoplasia will increase. The limiting step in the process of immunohistochemistry is identifying a 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.

2. Antigen-capture enzyme-linked immunosorbent assay (ELISA)

The antigen-capture enzyme-linked immunosorbent assay (ELISA) facilitates detection of antigen from pathogens directly from an animal prior to or during clinical disease. The ELISA 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- labeled (conjugated). If the detecting antibody is not conjugated 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-typic reactivity and polyclonal antisera may be preferred to increase the likelihood of reaction against all antigenic variants. Examples of antigen-capture ELISAs are the system for detection of *Anaplasma marginale* in the blood of preclinical cattle (Trueblood *et al.*, 1991). the use of antigen-capture ELISA on cattle blood samples for the detection of bovine viral diarrhoea virus (Mignon *et al.*, 1991; Sandvik *et al.*, 1995). and the rapid detection of rinder pest and peste des petits ruminants virus antigens in clinical samples (Libeau *et al.*, 1994). Respiratory syncytial antigen in nasal secretions was captured using ELISA with MAbs directed against epitopes of the viral capsid (Obert *et al.*, 1988). Several capture ELISAs for the detection of prion protein have been extensively validated and are used as rapid screening methods worldwide (Europeran 2003; Efsa 2004; Moynag *et al.,* 1999). Capture ELISAs are also widely used for chronic wasting disease testing in deer (Hibler *et al.*, 2003) and scrappy testing in sheep and goats (Efsa 2003). A related antigen capture method, the Priostrip test, which is a dipstick method, is also used as a rapid BSE screening test (Efsa 2004). 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, 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 the nonspecific binding and the incubation time are reduced. Validation of tests to detect antibody is addressed in the review Principals of validation of diagnostic assays for infectious disease.

C. ANTIBODY DETECTION

1. Competitive enzyme-linked immunosorbent assay (C-ELISA)

Competitive ELISA (C-ELISA) is an immunoassay that can be used to detect or quantify antibody or antigen using a competitive method. The C-ELISA for detection of specific antibodies has largely replaced the indirect ELISA for large-scale screening and sero-surveillance. The C-ELISA offers significant advantages over the indirect assay since samples from many species may be tested without the need for species-specific enzyme-labeled conjugates for each species under test. Many antigens are extremely difficult or time consuming to purify. If used in an indirect assay, they would result in high background values due to nonspecific binding. However, relatively crude

antigens may be used in the C-ELISA 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 due to binding of antibodies in the test serum, which prevent binding of the detecting antibody. The detecting antibody may be polyclonal or monoclonal depending on the required specificity. MAbs directed against highly conserved epitopes will give broadly reactive assays whereas those directed against highly specific epitopes will result in a highly specific test. One of the early reports on the use of the C-ELISA was its use in detecting anti-bluetongue virus antibody (Anderson, 1984). This used a MAb against a highly conserved epitope on bluetongue virus (BTV) P7 and allowed detection of antibodies to all 24 serotypes of BTV. The epitope was not shared in any of the other closely related Orbivirussero groups, therefore the test was also BTV-specific. The specificity of the assay can therefore be tailored depending on the specificity of the detecting antibody. Sensitivity of C-ELISA is improved using detecting antibody directly conjugated with an enzyme. The C-ELISA format has been successfully used in the screening of large numbers of pig sera for classical swine fever antibodies, the detection of antibody to malignant catarrhal fever virus in in apparently infected sheep, deer and bison and antibodies to *Babesiaequi* and *B. caballi* in persistently infected horses . A competitive ELISA for brucellosis, based on the immunodominent *Brucella* smooth lipopolysaccaride, has been widely used as a screening test for brucellosis in bovine, caprine and ovine), porcine and sea mammals. More recently, a solid-phase C-ELISA was used for the large-scale serological surveillance during the UK FMD outbreak in 2001 (Paiba*et al.*, 2004). this facilitated the testing of some 3 million sera over a period of less than one year.

2. 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 a gene 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 importance is the degree of specificity in diagnostic tests attainable by the use of recombinant protein. One example is the use of ESAT-6/CFO-10, (immunogenic secreted antigens) present invirulent *Mycbacterium bovis* and *M. tuberculosis* but not in avirulent BCG or most environmental mycobacteria, for the diagnosis of tuberculosis in cattle and humans (Buddle *et al.*,1999; Ulrichs *et al.,*

1998). Overlapping peptides of *M. tuberculosis* antigens ESAT-6 and CFP-10 increased specificity when they were used in the ELISPOT assay for gamma interferon detection for the diagnosis of *M. tuberculosis* infection (Hill *et al.*, 2005). This has the potential for providing a degree of specificity in diagnosis not achievable with purified protein derivative (PPD), the bacterial extract currently used.

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. These antigen preparations are expensive and often have a short shelf-life, with each new batch of antigen requiring standardization. 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 since the protein is synthesized 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 C-ELISA format, purification of the recombinant antigen from the lysate may not be necessary as the specificity of the C-ELISA resides mainly in the MAb used. An example of the procedure is the cloning of the envelope genes of caprine arthritis/encephalitis lentivirus in a vaccinia expression vector (Lichtensteiger *et al.*, 1991). Synthetic peptides can also be used as valuable antigens for veterinary laboratory diagnosis. The peptide-based diagnostic tests rely on the selection of short fragments containing the most potent antigenic epitopes that are recognized by specific antibodies induced by the whole viral proteins. In recent years, synthetic peptides that mimic specific epitopes of infectious agents proteins have been used in diagnostic systems for various human and animal diseases. Both recombinant proteins and synthetic peptides as antigens are useful for the companion diagnostic tests in DIVA, differentiating infected from vaccinated animals. Marker vaccines carry at least one less antigenic protein than the corresponding wild-type virus, which allows the serological tracing of wild type strains in vaccinated individuals (Henderson 2005).

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 diagnostic titre, are of particular interest as they are major stimulants of cellular and humoral immunity against the disease of interest. Antigen discovery 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 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 is not available, is the generation of recombinant libraries from the genomic DNA of the organism or from cDNA synthesized from mRNA. Fragments of the recombinant libraries can be cloned into an 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.* (*Pines et al., 1999)* yeast (Carter *et al*., 1987), insect cells using baculovirus (Stewart *et al*., 1993), or in eukaryotic cells by infection with appropriate viral vectors (Smith *et al*., 1983) 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 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, without expression of the whole protein being necessary, thus shortening the process. An example is the production of peptide antigens from two immunodominant antigens, reported to be promising candidates as diagnostic reagents for the detection of *M. bovis* infection in cattle (Villarreal *et al*., 1998). In recent years, the use of plants for a protein expression system has shown promise. For the expression of candidate antigens in plants, plant viruses offer the advantages of speed of product development, flexibility, and high levels of gene expression among others (Gleba *et al*., 2007).

Genome sequences of hundreds of bacteria and thousands of viruses have already been determined. The antigen gene can easily be cloned with PCR technology, using primers designed from the nucleotide sequence of closely related species (Covacci *et al*., *2003*). The gene can also be expressed and its product can be purified using tag peptide. 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).

D. NANOTECHNOLOGIES IN DIAGNOSIS

Nanotechnologies involve working at the atomic, molecular and supra-molecular levels in the length scale of 1–100 nm range, in order to understand, create and use materials, devices and systems with fundamentally new properties and functions because of their small structure. The potential use of nanotechnologies in relation to animal health is currently being examined (Oie 2007).

1. Disease diagnosis

Nanotechnology is being considered of great use for medical diagnosis (Anon 2007). Nanoparticles have exhibited tremendous potential for detecting disease markers, pre-cancerous cells and fragments of viruses. Also, metal coatings and metal nanoparticles functionalized with different biomolecules have been found useful in detecting specific proteins and antibodies. For example, researchers

have synthesized a specially charged silicon nano-wire connected with an antibody receptor that can detect the presence of cancer markers in the blood even if the concentration of these antigens in blood is about a hundred-billionth of the protein content. These sensors are much more accurate than currently available technologies. Also, in disease diagnosis, devices are being reported that resemble a glass microscope slide with tiny nanoscale imbedded pores that allow the device to examine DNA molecules in the blood for signs of disease (Anon 2007). New studies (Anon, 2006) have shown that ultra sound waves sense nanoparticles and the nanoparticles can brighten the resulting image. These bright spots may indicate that a few cells in an area may be on the verge of mutating and growing out of control. There is the suggestion that by combining ultrasound and nanotechnology definitive diagnoses may be reached without using invasive procedures such as biopsies. Nanoparticles Diagnostics is running a suit of projects focusing on the development of rapid, portable diagnostic tests in animal and human health (Anon, 2007).

2. Vaccine development

The majority of nanotechnology based applications relating to vaccines are focused on enhancing vaccine delivery and effectiveness. For example, a nanoemulsion (Anon, 2006) has been developed consisting of very tiny droplets of oil suspended in water and stabilised by detergents. The droplets in the nanoemulsion are surface active and react specifically with the outer membrane of infectious organisms. The technology works differently than antibiotics or traditional antiseptics, and is safe for humans, animals and the environment. In the vaccine work, the mixture of nanoemulsion and either whole virus or protein is applied directly to the nose of animals. This presents the immune system components required to create a vaccine. It is speculated that there is great promise for vaccines based on this technology because they can be administered without the use of needles or refrigeration and this would have particular appeal to developing countries. In a specific application (Bielinska *et al.*, 2007). scientists were able to trigger a strong immune response by treating the inside of animal□s noses with a nanoemulsion and a recombinant protein of *Bacillus anthracis.* The animals developed several types of immune response after only two administrations. On the challenge, all the immunized animals survived, whereas none of the control animals did. Further projects (Anon, 2007). Are underway in regards to TransDermal delivery using patches structured on the skin side with microprotrusions which hold the drugs to be delivered. The protrusion face of the patch is applied to the skin where the protrusions cross the outer surface layer of the skin only reaching as far as the interstitial space and avoiding nerves and blood vessels. In this interstitial space, the nano-structured compounds are released from the surface of the protrusions and taken up by the cells of the immune system for vaccination applications. The patches are intended for applications in human and animal health for delivering vaccines, proteins and peptides, peptide hormones and other drugs.

CONCLUSION.

Advances in the use of molecular tools will continue to grow at a very rapid pace,

and the field of nucleic acid‐based diagnostics will undoubtedly grow in parallel. Molecular tools will also continue to have an impact on disease prognosis and response to therapeutic interventions. In the near future, the development of fully automated multiplexing assays for relevant clinical syndromes will have a great impact in clinical laboratories.

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