Viruses, diagnosis and vaccines

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According to the most recent FAO/OIE/WHO yearbook, the world animal population is estimated at 1.3 billion cattle (including 229 million dairy cows and 156 million buffaloes), 1.7 billion small ruminants, 900 million pigs, 58 million horses, 19 million camelidae and 12.2 billion poultry. The diseases that affect these animal populations vary depending on whether they have been extensively reared or intensively produced, especially in industrialised countries. Excluding diseases specifically related to intensive production systems, such as bovine spongiform encephalopathy or 'mad cow disease', (Kitching, 1997), as well as parasitic diseases, the major trends in contagious diseases (epizootics) can be identified. The most serious of these diseases, the so-called 'List A' diseases, which are the most damaging to the world economy and international trade, are shown in Table I and are all virus diseases.

A few examples include rinderpest (Crowther, 1997), which now only affects a few north-western areas of Sub-Saharan Africa, the Middle East and the Indian sub-continent and is controlled through a coordinated vaccination programme, the 'Global Rinderpest Eradication Programme' (GREP), aimed at eradicating the disease over the next ten years.

Foot and mouth disease is either absent or under control in Oceania, Japan, the North American continent and the southern cone of Latin America, as well as in all western European countries. It is being closely monitored on the fringes of central Asia and there is a special disease control programme in south-east Asia. Contamination of a region by this disease can result in substantial financial losses because of the need to slaughter affected animals and impose an export ban. This was the case in Taipei, China, at the beginning of the year, when four million pigs were slaughtered over a period of a few weeks at a reported overall economic cost of 10 billion dollars (Donaldson, 1997)

Contagious bovine pleuropneumonia is still a major concern in Africa, with the exception of North Africa and Southern Africa. In east Africa it has developed rapidly, spreading to the south of the continent. This disease, which is a major obstacle to the development of cattle rearing, can be controlled by vaccination and/or slaughtering infected animals.

Classical swine fever has recently made a spectacular comeback in Europe. The European Union's chosen strategy of slaughtering all diseased or infected animals has resulted in a very high number of animals having to be killed. The financial losses associated with this disease have topped 250 million US dollars in the Netherlands since the beginning of the year.

African swine fever has been virtually eliminated from Europe. It persists in certain sub-Saharan African countries where it sometimes causes significant losses since no preventive vaccine is available.

African horse sickness has been eradicated in Europe and North Africa and is no longer being reported in some African countries south of the Sahara.

Newcastle disease can infect practically all birds in all countries of the world, including farmed poultry, pets and migratory wild birds and is controlled through vaccination. Other diseases (the so-called 'List B' diseases) are also a source of great concern in some regions and include rabies. Although rabies is coming under control in western Europe due to the oral vaccination of foxes, it is a growing threat in developing countries and more recently in eastern European countries.

Each year, direct or indirect losses associated with the development or spread of all contagious animal diseases total billions of US dollars. A considerable effort is needed to control such diseases. Virus diseases are then predominantly trans-boundary in nature and affect both developed and developing countries. The importance of particular diseases and the control measures adopted, often depends on the perceived economic importance even where resources are available. Diseases caused by viruses represent the largest general problem to human and animal populations, as well as to agricultural production in general,

through damage to crops. Agriculture represents a large component in all economies and the results of virus diseases can be dramatically direct, through killing or severely disabling individuals; or insidiously chronic and be more difficult to recognize.

Overall management of any disease requires that complex of methodologies is pursued within a relatively well developed infra-structure. Laboratory confirmation of diagnosis and the use of vaccines are two elements in control, but these can neither be disassociated from each other nor from other factors such as epidemiological surveillance, disease reporting, veterinary staff availability and mobilization, information exchange technologies and pure and applied scientific research.

Consideration of animal viruses requires there "range" to be defined. Inherent in the definitions is the identification of the host or hosts which is affected and in turn this affects what efforts are used to control diseases. The availability of methods *per se* which are described below does not mean that there is equality of effort to control diseases. There is a constant need to be educative on the true cost of animal virus diseases and that this should be examined from long term and trans-boundary perspectives (Murphy, 1994)

Groups of animals affected should be considered since they focus on the problems associated with diagnosis and control. Diseases can be regarded according to incidence as endemic or exotic; and as to their infectivity and scale of effect. Endemic infections require eradication campaigns with the use of intervention measures e.g. vaccines and strict control measures, while exotic can be regarded as mainly animal control problems.

The major livestock groups already mentioned, i.e. cows, sheep, goats, and pigs can be supplemented by wild animals; pets (dogs, cats, rabbits); zoo animals; laboratory animals- chimps, monkeys; guinea pigs, rabbits; mice; amphibians; fish, bees/insects and birds. Considerations of the interaction of species (including man) and disease agents also has to be made in control strategies. This is particularly evident in zoonotic diseases. Thus interventions also must include vector/pest control.

Viruses are by their genetic nature obligate parasites. Consideration of developments in diagnostic methods and vaccines in general and animal viruses in particular, has to recognize the tremendous ability of viruses to rapidly produce mutations which, through selection, can exploit biological "niches". Thus there is a constant evolution of potential "strains" which can exploit both there "normal" and also more "foreign" species. Indeed such exploitation can often result from direct intervention strategies, e.g. vaccination and from more indirect changes concerning human patterns of endeavor (

The extent of animal virus disease can be appreciated through reference to The Office International des Epizooties (OIE) Manual of Standards for Diagnostic Tests (3rd Edition). This contributes to the harmonisation of methods of surveillance and control of important animal diseases and standard methods are described for laboratory diagnostic tests and the production and control of biological products (principally vaccines) for veterinary use in laboratories across the world. The availability of such standards should increase the effectiveness of measures undertaken to improve animal health worldwide. The Manual has been written and revised by experts of established international standing and is unique in that each chapter has been approved by the Veterinary Services of all the OIE Member Countries.
The Manual contains general information on sampling methods, Good Laboratory Practice (GLP), validation of diagnostic assays, sterility tests, laboratory safety, vaccine production and biotechnology, as well as chapters on specific diseases. The area of Good Laboratory Practice in the context of laboratory recognition (certification by outside authorities) is taking on a higher significance in the context of World trade.
The subject of "emerging diseases" caused by viruses has been developed for human diseases, (Morse and Schluederberg, 1990; Morse, 1993), but is no doubt also highly relevant in both the zoonotic diseases and those affecting only animals. Viruses are humanity's only real competitors, acting as both parasites and genetic elements in their hosts. They show considerable genetic plasticity, enabling them not only to evolve in new directions, but also to produce genetic and metabolic relationships with cells, uniquely positioning them to mediate subtle, cumulative evolutionary changes in their host. This poses problems in vaccine design

and strategies as well as to diagnostic methods. Virus diseases are also able to rapidly destroy large proportions of populations and the mere fact that long term natural selection favours mutualism offers only limited encouragement to our species, with millions of people and animals suffering before an equilibrium can be reached.

New patterns of human movement and practices, leading to new contacts across what had once been geographic boundaries, have been seen to give rise to a variety of emergent infections. Most emergent viruses are zoonotic, with natural animal reservoirs a more frequent source of new viruses than from spontaneous evolution. Rodents and arthropods are most commonly involved in direct transfer, and changes in agricultural practices or urban conditions that promote rodent or vector multiplication favour increased incidence of disease.

Other animals, especially primates, are important reservoirs for transfer by arthropods. Approximately 100 of the more than 520 known arthropod borne viruses (arboviruses) cause human disease. At least 20 of these might fulfill the criteria for emerging viruses, appearing in epidemic form at generally unpredictable intervals. These viruses are usually spread by the bites of arthropods, but some can also be transmitted by other means, for example through milk, excreta or aerosols. The arbovirus infections are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by blood-sucking insects; they multiply to produce viraemia in the vertebrates, multiply in the tissues of the insects and are passed on to new vertebrates by the bites of insects after a period of extrinsic incubation. The names by which these viruses are known are often place names such as West Nile or Rift Valley, or are based on clinical characteristics like yellow fever.

Most arboviruses are spherical, measuring 17-150 nm or more, a few are rod-shaped, measuring 70 x 200 nm. All are RNA viruses. Many circulate in a natural environment and do not infect man. Some infect man only occasionally or cause only a mild illness; others are of great clinical importance causing large epidemics and many deaths. Specifically, these belong to the Togaviridae, the alphaviruses, flaviviruses, the Bunyaviridae, nairoviruses, phleboviruses and other subgroups. Vertebrate hosts can be defined according to their role in maintenance or amplification of virus. Maintenance hosts are essential for the continued existence of the virus and there is usually no actual disease, but the development of antibodies. These include migrating birds which travel over long distances carrying these and other similar viruses; rodents and insectivores such as rats, hedgehogs, lemmings and chipmunks are known to carry louping ill and Colorado tick fever; primates such as monkeys which carry Dengue fever; Leporidae (rabbits and hares) which carry Californian encephalitis; Ungulates (cattle and deer) which are implicated in the transmission of European tick-borne encephalitis; bats which carry Rio Brava virus; and marsupials, reptiles and amphibia such as kangaroos and snakes which also harbour encephalitis-causing viruses.

Incidental hosts become infected, but transmission from them does not occur with sufficient regularity for stable maintenance. Man is usually an incidental host, often, but not always, being a dead end in the chain. These hosts may or may not show symptoms. Link hosts bridge a gap between maintenance hosts and man, for example, between small mammals and man by goats (via milk) in tick-borne encephalitis. Amplifier hosts increase the weight of infection, as is the case with pigs which act between wild birds and man in Japanese encephalitis.

Table I. OIE defined animal diseases

List A diseases (all virus diseases)

African horse sickness (AHS), African swine fever (ASF) Bluetongue Classical swine fever (hog cholera), Contagious bovine pleuropneumonia Foot and mouth disease (FMD) Highly pathogenic avian influenza (fowl plague) Lumpy skin disease Newcastle disease Peste des petits ruminants (PPR) Rift Valley fever (RVF), Rinderpest Sheep pox and goat pox, Swine vesicular disease, (SVD) Vesicular stomatitis (VSV)

List B diseases
Acariosis of bees, American foulbrood, Anthrax, Atrophic rhinitis of pigs, Aujeszky's disease Avian chlamydiosis, Avian infectious bronchitis, Avian infectious laryngotracheitis Avian mycoplasmosis (Mycoplasma gallisepticum), Avian tuberculosis. Border disease, Bovine anaplasmosis, Bovine babesiosis, Bovine brucellosis, Bovine genital campylobacteriosis, Bovine spongiform encephalopathy, Bovine tuberculosis, Bovine viral diarrhoea. Caprine and ovine brucellosis (excluding B. ovis infection), Caprine arthritis/encephalitis and Maedi-visna, Contagious agalactia, Contagious caprine pleuropneumonia, Contagious equine metritis, Cysticercosis. Dermatophilosis, Dourine, Duck virus enteritis, Duck virus hepatitis Echinococcosis/Hydatidosis, Enterovirus encephalomyelitis (previously Teschen/Talfan diseases), Enzootic abortion of ewes (ovine chlamydiosis), Enzootic bovine leukosis, Epizootic lymphangitis, Equine encephalomyelitis (Eastern and Western), Equine infectious anaemia, Equine influenza, Equine piroplasmosis, Equine rhinopneumonitis, Equine viral arteritis, European foulbrood. Fowl cholera (avian pasteurellosis), Fowl pox, Fowl typhoid and Pullorum disease Glanders. Haemorrhagic septicaemia, Heartwater, Infectious bovine rhinotracheitis/Infectious pustular vulvovaginitis. Infectious bursal disease (Gumboro disease) Japanese encephalitis Leishmaniosis, Leptospirosis Malignant catarrhal fever, Mange, Marek's disease, Myxomatosis. Nairobi sheep disease, Nosemosis of bees. Ovine epididymitis (Brucella ovis), Ovine pulmonary adenomatosis. Paratuberculosis (Johne's disease), Porcine brucellosis, Porcine reproductive and respiratory syndrome. Q fever. Rabies. Salmonellosis, Scrapie, Screwworm (Cochliomyia hominivorax), Surra (Trypanosoma evansi) Theileriosis. Transmissible gastroenteritis, Trichinellosis, Trichomonosis, Trypanosomosis (Tsetse-borne), Tularaemia. Varroosis, Venezuelan equine encephalomyelitis, Viral haemorrhagic disease of rabbits

Viruses are thus adapted to extremely diverse niches. Arthropod-borne viruses are spectacular examples of emergence and re-emergence resulting from innocent environmental manipulation or natural environmental change. Important aspects of ecological change and their relation to arbovirus life cycles are: 1) Population movements and the intrusion of humans and domestic animals into new arthropod habitats, particularly tropical forests; 2) Deforestation, with development of new forest-farmland margins and exposure of farmers and domestic animals to new arthropods; 3) Irrigation, especially primitive irrigation systems, which are oblivious to arthropod control; 4) Uncontrolled urbanization, with vector populations breeding in accumulations of water (tin cans, old tires etc.) and sewage; 5) Increased long distance air travel, with potential for transport of arthropod vectors; 6) Increased long-distance livestock transportations, with potential for carriage of viruses and arthropods (especially ticks); and 7) New routing of long-distance bird migration brought about by new man-made water resources.

Recent Advances

The most recent advances in molecular biological and other instrumentation technologies have been advantageous in improving diagnostic potential and for the improvement of vaccines. Relevant biotechnological advances from product development and better research to improve understanding of viruses can be highlighted. These are genetic engineering (molecular biological advances); hybridoma technology and large scale (industrial production) of tissue culture; improvements in adjuvants and delivery systems for vaccines and molecular modeling for designing active anti-viral pharmaceuticals.

These improvements are a positive exploitation of the fundamental basic research into both the pathogen and also the host (e.g. better understanding of the immunology, allowing a better understanding of the mechanisms of pathogenesis), and the needs driven application of this research. Advances also in other technologies are relevant, in particular the performance of computers in collecting, analysing and storing data and developments in instantaneous communication. Table II lists areas of exploitation with respect to diagnostic and vaccine improvements. The areas covered are extensive and these will be considered in more detail. They are also linked and pertinent to all other biological fields. The two areas of vaccines and diagnostics can now be considered in light of the statements shown in the table.

Vaccine Development

The vast majority of virus diseases cannot be treated with the equivalent of antibiotics for bacteria. Once infected, the individual's immune system has to combat disease. Prevention of disease through prophylactic measures is therefore necessary and this is achieved through administration of vaccines. The history of virus vaccines goes back to Jenner's cowpox vaccine for smallpox in 1798. Advances in tissue culture methods and reagents in the sixties and seventies allowed the successful production of vaccines for man and animals. The more recent development of biotechnology coupled with research has offered some alternative strategies and production possibilities (Brown, 1996).

Classical vaccines

Classical vaccines are prepared in one of two ways. Inactivated vaccines are produced by growing virus in large amounts and then chemically inactivating this under conditions where the immunogenicity is retained, e.g. current foot-and-mouth disease vaccines. Attenuated vaccines are produced by reducing or eliminating the pathogenicity of the virus with regard to the target host, through growth in an unnatural host or under special conditions. Such a virus multiplies in the target host but does not cause disease. An excellent example of an attenuated virus is that for rinderpest. Despite the past and present record of success for conventional vaccines , they have several disadvantages. Killed vaccines must be proven free of any live organism and attenuated strains must not demonstrate reversion to virulence. There is also the possibility of contaminating adventitious agents. Attenuated viruses also can be unstable and have to be kept cool under physiological conditions. This factor also affects the vaccination per se once a fully formulated vaccine is constituted in the field. Such stability factors (the virus must be "live"), affect the strategies of a campaign necessitating setting up of cold chains.

Newer strategies

Developments in the human sphere offer the models for approaches in animal virus vaccines. In 1981, the first vaccine against a non-propagable agent and, in fact, the first against human cancer, was derived from antigen purified from the blood of Hepatitis B carriers. The plasma derived sub unit was purified to ensure the destruction of all possible life forms. Once attached to an alum adjuvant, it proved safe and effective. However, the purification was slow and tedious and there were fears about the safety of human blood as a source of antigen.

Molecular biological methods offer ways of inserting genes into vectors so that these are expressed to produce proteins. There are only two ways in which these the many

expression systems available can be exploited for vaccine manufacture. In the first, the vector is cultivated in vitro to produce large amounts of protein for use as an inactivated vaccine. This can be regarded as being "safer" than where infectious virus is produced and then possibly not completely inactivated. In the second, the foreign DNA is inserted into a live vector capable of replicating in the host species in the same way as an attenuated strain.

The first recombinant vaccine, also against Hep B, was produced in 1986 was produced in yeast . The vaccine was constructed by excising the S antigen gene of the viral DNA complete with flanking promoter and terminator sequences, and inserting it into the ring structure of a plasrnid vector. Transvectored into yeast cells, the plasmid encoded for production of the antigen which could be readily purified, however, the vaccine offered poor protection. This is now attributed to a deficiency of helper T cell determinants in an outbred population. Moreover, even with adjuvants, multiple doses were required to generate high levels of antibodies and sometimes these levels were short-lived. Nonetheless, research continued to develop other recombinant vaccines which could be expressed in host cells.

The E. coli vectors although easy to use, produce non-glycosylated proteins and have proved to produce poorly immunogenic vaccinating products. The mammalian and yeast cell systems producing glycosylated proteins have been found to produce more immunogenic proteins, particularly against enveloped viruses, presumably since they resemble the surface antigens of lipid containing viruses more closely. Such approaches also include the use of transgenic animals with acquired genes to produce high levels of "antigen" e.g. for porcine reproductive and respiratory syndrome (PRRS), an emerging viral disease of pigs in the United States and other swine producing countries causing losses due to acute and persistent chronic infections in pigs of all ages. Current immunization strategies rely on the use of attenuated live-virus because killed-virus vaccines have been found ineffective against PRRSV infection. The "in vivo" production of antigenic and immunogenic PRRSV glycoproteins in the milk of transgenic animals and the further use of recombinant PRRSV proteins as subunit vaccines is being explored.

Table II. Outline of technologies relevant to vaccine and diagnosis of virus diseases in animals

Technology Area	Use	Example
DNA manipulation Polymerase Chain Reaction	Gene insertion into live vectors. Production of large amounts vaccinating product in vectors in heterologous systems including transgenic animals Engineering and production of cytokines. Gene deletion. Amplification of genes. Rescue and amplification from samples (RNA and DNA viruses). Direct diagnosis.	Vaccinia virus /sheep pox virus vaccine encoding e.g. rabies; rinderpest genes. E.coli, yeasts, mammalian cells, chimeric proteins. Baculovirus expression. Improved adjuvants for vaccines, linked to delivery system improvements e.g. porcine, avian, bovine Pseudorabies vaccine. Linked to diagnostic kit identification of infected and vaccinated animal differentiation. Expression specific proteins for use as diagnostic reagents. Allows rapid sequencing and comparison (molecular epidemiology) Detection/differentiation of genes with specific primers. Sequencing.
DNA Synthetic proteins	Use to make vaccine directly excluding protein. Hybridization reactions. Peptides identified and produced.	Fastest growing research area In situ hybridization in diagnosis/Restriction endonuclease mapping Not too successful as vaccines. T and B cell epitopes needed. As reagents for diagnosis. Foot and Mouth Disease
	Epitope characterisation.	Methods evaluating monoclonal antibody defined epitopes. Pepscan, phage libraries.
Hybridoma technology - Monoclonal antibodies (MAbs)	Large supply MAb from tissue culture. Production of defined product Panels of MAbs with different specificity Production of MAb escape mutants	Therapeutic- prophylactic applications Direct diagnosis in immunoassays (standardisable by relating activity to weight). Improved specificity and sensitivity over polyclonal serum based assays. Rapid differentiation between and within closely related strains. e.g. rabies, foot-and-mouth disease in binding assays. Linking of sequence of mutants to epitope identified by MAb, characterisation of epitopes at molecular level. Paratope profiling (determination of antibody spectrum).

Table II (continued)

Technology Area	Use	Example
Instrumentation	Rapid measurement of various signals in immunoassays.	Florescence polarization, Enzyme Linked Immunosorbent Assay (ELISA), bioluminometry, chemoluminescence.
Biosensors	"Instant" measurement in a single instrument.	Strong developments for diagnosis and environmental monitoring.
Computers	Data collection, analysis, storage, communication of results.	Essential in sequencing and comparative studies relating large amounts information. Essential as instrumentation "brain".
Commercial reagents/equipment	In all fields of biology.	Restriction enzymes, DNA polymerase, reverse transcriptase, labelled bases, conjugated antibodies (enzymes, gold particles, fluorescent markers), dif-labeling, cell culture, affinity purification, cytokines, MAbs, microtitre equipment, thermocyclers,
Services	Move away from local production. Sequence data banks/ host and agent	Sequencing, labelling , oligonucleotide primers, antibody production (including MAbs). Primer identification.
Kits	Commercial exploitation.	PCR. PCR/ELISA Many Enzyme Immunoassays (virus diseases swine, ovine, bovine, poultry, fish, dogs, cats).

Virus vectors have also been used for the expression of other "foreign" virus antigens, including vaccinia virus for the control of rabies in foxes. However, because of safety concerns, application of vaccinia recombinants have been very limited. The use of Sheep and goat pox instead of vaccinia is being actively pursued e.g. genes encoding the H and F proteins of rinderpest have produced successful results. (Giavedoni et al, 1991)

Engineering viruses to delete genes is illustrated well in Pseudorabies vaccine. Here, the virulence of the vaccine strain of Aujeszky's disease was modified by engineering a mutation of the thymidine kinase (TK) gene, so that the vaccine has no detectable TK activity. In addition to a TK deletion, a second deletion removed a gene coding for viral glycoprotein which prevents antibodies being produced against the glycoprotein. This second deletion allows vaccinated pigs to be identified by ELISA from pigs naturally infected and kits have been developed.

A process for producing live, non-pathogenic, vaccines for the pathogens RNA tumour virus utilizes gene-altering technology to produce an altered genome which codes for the antigenic determinants of a pathogen, but has no genes coding for pathogenicity. The vaccine is the phenotypic expression of the altered genome. Specifically, an avian RNA tumor virus env gene is cloned into the non-pathogenic RNA virus RAV-O and the resulting recombinant product is replicated in host cells to provide a recombinant vaccine for the pathogen avian RNA tumor virus.

Vaccines based on nucleic acids show promise, (Dixon, 1995) This began with the observation that in vivo inoculation of purified genetic material can elicit immune responses against the encoded antigens in mice and other animals. Immunisation with purified genetic

material allows presentation of the antigen in a native form, synthesised by the host in a similar way to that by which the antigens are synthesised during infection by that pathogen, and so it is possible to code for pathogens that escape the immune system by modifying their antigens. An additional advantage of purified nucleic acid vaccination over viral carrier systems is that only genes coding for antigens are delivered, and not the genetic material of the carrier organism. The disadvantage of this approach is the concern over possible integration of the DNA into the human genome. This could be overcome by using by using RNA, but the short lived molecules may not generate the required long lasting protective response.

Another field of vaccine research is the designed construction of synthetic vaccines, (Lerner, 1983). These have the advantage of being non-infectious and free of nucleic acids . As long as the synthetic antigen has peptide epitopes recognised by helper T cells and by antibodies, the immune system should react. However proteins separated from virus particles are generally much less immunogenic than the intact particle. Protective antibody mediated responses depends on the presentation of the antigen to the immune system and this must mimic the configuration of an intact organism. So far, successful methods include the creation of particles containing many copies of the immunogenic site or linking the protein to an immunostimulating complex (iscom) with activities equivalent to those of the virus particle.

Adjuvants

The main functions of the adjuvant in a vaccine are to keep the antigen at, or near, the injection site and to activate antigen presenting cells to achieve effective antigen processing and interleukin production. There is currently great interest in developing new adjuvants, particularly those which act as 'antigen depots' providing controlled release of antigen over a long time span. Candidates include oil-water emulsions, liposomes, iscoms and biodegradable microspheres.

Cytokines

The immune system is regulated and activated by hormone-like cytokines. Immune responses to vaccines depend on complex cytokine mediated interactions and it is known that injection of certain cytokines can augment responses to vaccines. Recently cytokine genes have been engineered into live virus vectors enabling production of cytokines in a very localised environment. The expression of certain cytokines by vaccinia virus can selectively stimulate particular responses in mice following immunisation.

Diagnosis

Viruses produce disease and particular signs in animals which are observed in the field. The confirmation of clinical disease diagnosis is usually made at laboratories. Figure 1. illustrates the relationships of various actions in the field and laboratory systems for confirmation of diagnosis. The newer technologies offer a level of sophistication to more local laboratories which potentially can improve the rapidity of results and the confidence in those results. A wider view may be taken with regard to control and monitoring of virus diseases, particularly since vaccines are being considered. Thus the methods relevant to confirmation of disease status, disease monitoring and surveillance will be examined.

Samples for test can be taken and examined for the presence of virus or virus antigens (proteins) and/or antibodies, produced against the virus (serum). The sample may be examined directly for virus and/or virus antigens or may be grown in tissue culture to amplify virus and the specific cell tropism used to help confirm diagnosis. Actions of veterinary authorities rest on laboratory confirmation so that tests have to be rapid, accurate and standardised with results transmitted readily. Most clinical observations lead to rapid restrictions of animal movement until a control strategy based on vaccination, slaughter and movement control is signaled depending on test results. With virus diseases, it is likely that observation of clinical signs is some days after infection and that a great deal of virus has

been produced to infect other animals. Knowledge of the exact nature of the disease is therefore essential for control. This includes differential diagnosis, separating different virus diseases producing similar signs; evaluation of an antigenic type to allow choice of the appropriate vaccine e.g. foot and mouth disease; or examination of smaller variations within an antigenic type (subtype). During control phases laboratories are involved with monitoring the efficacy of vaccination (sero–monitoring to detect antibody conversion) and maintaining surveillance for disease.

The most recent developments have allowed improvements in the rapidity, analytical sensitivity and specificity of tests. They have also allowed more sophisticated methods to be extended from only the relatively sophisticated laboratories The latest methodologies allow the detection of viruses or evidence for viruses directly from samples and also where no infectious virus exists e.g. Polymerase Chain Reaction (PCR). It must be emphasised that the modern technologies should not always be regarded as the most appropriate for all laboratory situations, nor that the older methods should be automatically displaced. Tests should always be complimentary, indeed the modern technologies e.g. PCR, often need the assurance of other tests e.g. ELISA, to allow them to be fully validated.

Serological methods

A major generalisation can be made in reviewing methods, by saying that the technology which has revolutionised most diagnostic applications is that of Enzyme Immuno Assay (EIA), in particular solid phase assays based on Enzyme Linked Immunosorbent Assays (ELISA) using microtitre plates and equipment. Although the test is over 25 years old, it is still the most flexible and useful of binding assays used for both detection of antibodies and/or antigens. Over 60% of all tests in the diagnostic field are based on ELISA. There are a great many specific systems employed and all have the basic advantages of high capacity for sample analysis, low cost, and quantification possibilities through reading using multichannel spectrophotometers with the consequential analysis of data using statistical parameters. Developments in the availability of conjugates, antibodies, plastics and in specific systems (e.g. use of monoclonal antibodies) are continuing and will provide the answers to problems produced through virus variation into the future. The ELISA is also suitable for use in developing countries allowing sensitive and specific assays mainly through the supply of kits from International organisations. The commercial sphere also thrives on ELISA kit manufacture and these are steadily improving in terms of quality and bringing down prices. The ELISA is pertinent to local (including veterinary practice, regional and International laboratories especially where suitability controlled kits are available.

Monoclonal Antibodies

The advent of Monoclonal antibody (MAb) technology has improved many assays in terms of specificity and sensitivity. MAbs can now be routinely produced and offer a fully standardisable reagents of defined binding characteristics. This definition can be extended to understanding the exact epitope reacting with the MAb and related to amino acid composition. MAbs form the basis of many ELISA (particularly competition systems) and other assay systems for the unequivocal identification of viruses and for differentiation of strains (through use of one MAb or panels of a number of MAbs reacting with different epitopes).

The measurement of specific antigens is also possible with MAbs as well as assays which can differentiate between vaccinated and infected animals, either by the specific detection of or confirmation of the lack of a particular antibody population The specificity of MAbs is useful in designing agglutination assays (latex) and in developing "dip-stick" technologies based on the development of lines on a solid phase matrix. One such development has been made for rinderpest and is illustrated in Figure 2. Variations on this technology are suited for the rapid examination of samples in the veterinary practice or in the field (pen-side confirmation) which has a place in developing countries where the sending of samples takes a long time and where laboratory support is limited. MAbs are also reagents

exploited in various forms of biosensor development which measures the energy changes in antigen/antibody binding through a variety of transduction technologies.

One elegant application of MAbs, results from the relatively easy generation of MAb escape mutants (Crowther, 1993). These are made by growing virus in the presence of particular virus neutralising MAb, where mutants resistant to neutralisation grow. On cloning and re growth in the presence of the MAb, stable MAb resistant mutants can be isolated. Sequencing studies then highlight differences between parental and mutants and thus the amino acid sequence changes elicited by the MAb pressure (to allow neutralisation to be avoided) are mapped. In this way distinct epitopes can be measured on viruses as well as mutants themselves being used as antigens to measure specific antibody populations. Where X ray crystallographic studies are available on parental and escape mutant viruses, the epitopes can be definitively modelled. Such approaches are leading to higher probabilities that specific virus receptor drugs may be developed. The development of new MAbs depends on continued research into animal virus diseases.

Immunoblotting

This is still often the first step in identifying immunodominant proteins recognised by sera from infected animals where the proteins are detected using various polyclonal /monoclonal antibodies after separation by SDS polyacrylamide gel electrophoresis and transfer onto nitrocellulose paper. After incubation, the protein bands (immune complexes) are visualised with peroxidase-conjugated protein and a substrate/chromogen or use of chromogenic substrate and X-ray film. The qualitative determination of antibodies with reference to standardised antigen preparations offers a method of differentiating vaccinated and non-vaccinated animals.

Molecular Methods in Diagnosis

These can be groups into direct methods for detection of virus genomes or proteins and into those applications improving reagents used in diagnostic assays. The uses of Restriction fragment length polymorphisms (RFLPS), the Polymerase chain reaction PCR) and DNA probes illustrate applications from the first group.

Restriction fragment length polymorphism analysis (nucleic acid fingerprinting) can detect differences in the genomes of closely related viruses. If it is desired to map the genome of a microorganism, the DNA is extracted and clipped into fragments of specific nucleotide sequences with restriction endonucleases. The resultant DNA fragments are then separated in agarose gel by electrophoresis and visualised with ethidium bromide. The fragments can then be hybridised with complementary DNA (CDNA) tagged with ^{32}P to determine the differences or similarities in the genomes.

The main use is in epidemiological studies where the involvement of a particular virus isolate in a disease outbreak can be documented, and the epidemiological tracing of isolates within a country or between countries should be possible. Epidemiological investigations utilising RFLP analysis of Aujeszky's virus disease isolates shed light on the direct and indirect origin of the field isolates in Hungary. Regional variants of the rabies virus in Canada were identified by RFLP analysis which showed consistent differences in the viruses from distinct geographical regions.

A question confronting veterinary diagnosticians is what level of differences between virus isolates is significant? This is a common feature relating molecular results to those of serological studies. Only through continued parallel evaluation of serological and molecular techniques can any significance be drawn. Where a “molecular marker” of a particular activity, say of pathogenicity, can be ascribed, the molecular techniques offers great advantages. For example, RFLP analysis can detect single base pair substitutions in DNA based on the loss or acquisition of a restriction endonuclease site. However, if the loss or acquisition of restriction endonuclease site(s) is not represented by differences of the ability of the viruses to cause disease, it may be concluded that the difference detected by RFLPs is not significant.

Polymerase chain reaction

The polymerase chain reaction (PCR) procedure exploits natural DNA replication, producing, *in vitro,* large quantities of a desired sequence of DNA from a complex mixture of heterogeneous sequences . The various PCR methods can amplify copies of a small region of 100-400 or more base pairs into millions of copies. The amplification of DNA by the PCR is accomplished via a succession of incubation steps at different temperatures, facilitated through use of thermo cycling machines. The target DNA is heat denatured to separate the two complementary strands. Specific primers (short strands of DNA complementary to both strands of DNA) are then annealed at low temperature and extended with DNA polymerase at an intermediate temperature utilising the target DNA as a template. These steps, referred to as cycles, are repeated 20 to 40 times, yielding amplification of target DNA sequences. The key to geometric amplification of target DNA sequences by the PCR is selection of paired primers which, when extended, will create additional reciprocal primer annealing for primer extension in subsequent cycles. With RNA viruses, a CDNA copy of the RNA must be made using reverse transcriptase, then the PCR can be used for amplification. The identity of the PCR product must be confirmed using DNA probes or RFLP. The PCR products can be sequenced to further characterise the genome. This has proved on great importance in molecular epidemiological studies e.g. in rinderpest, and FMD, where unequivocal identification of strains can be made, as well as examination of evolutionary traits and retrospective variation.

PCR is a thus highly sensitive procedure for detecting infectious agents in host tissues and vectors, even when a small number of host cells are infected. It does not differentiate between viable and non-viable organisms. PCR can also be used to amplify a gene sequence that has become integrated into the DNA of infected host cells and also to amplify non-integrated viral gene sequences. It is clear that PCR will have a role in the testing of vaccines to detect contamination.

The PCR may thus prove to be very useful in the diagnosis of chronic-persistent infections such as those caused by retroviruses (bovine leukemia virus, caprine arthritis-encephalitis virus, etc.) and latent infections caused by herpesviruses (e.g. infectious bovine rhinotracheitis and Aujeszky's disease). These diseases present serious problems in terms of diagnosis and prevention since infected animals often do not demonstrate clinical signs until there is advanced disease, and infected animals appear to be 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, contamination of the laboratory in general, because the exquisite sensitivity of PCR can easily lead to false positives. Problems with samples per se also have to be solved for each individual system and there are many anomalies to be resolved.

Diagnosis by DNA probes

The use of DNA probes used directly proved to give poor sensitivity, but DNA hybridisation procedures combined with PCR provide a powerful tool in the diagnosis of virus diseases. To make a probe, DNA is heated or treated chemically until the two strands separate. Each strand will recognise and bind to a strand of DNA that has complementary nucleotide bases. A DNA probe will 'search' the tissues of an animal or an insect for the complementary nucleotide sequence of a pathogen. To determine whether binding (hybridisation) has occurred, the single strand of the DNA probe is usually labelled with radioactive ^{32}P. Denatured DNA is freed from clinical specimens (blood, saliva, urine, exudates) and applied to a solid support system such as nylon or nitrocellulose membranes. If the DNA sequence of the probe and the target DNA of the clinical specimen are complementary they will hybridise. After washing to remove the unbound probe, the membrane is placed in contact with X-ray film. The X-ray film is then examined for the presence of the radioactive ^{32}P label of the probe. The specimen is positive for the pathogen if the label is detected. If the specimen is negative, the labelled probe will not bind to the sample and will be washed away in the procedure. Radioactive probes although offering high sensitivity have a short half life and present a bio-hazard. To facilitate the practical use of DNA probes,

radioactive tags can be replaced with sensitive, long shelf-life non-radiolabeled tags e.g. where the DNA probe is labelled with biotin and is detected by streptavidin-linked to horseradish peroxidase or alkaline phosphate which yield conspicuous colour in the presence of their substrates and can be assayed. Streptavidin can also be conjugated with a fluorescent dye.

Production of antigens by recombinant DNA technology

Diagnostic tests require test antigens must have until recently been produced from cell culture or harvested from an infected animal. These are expensive, often have a short shelf-life, need to be standardised with each new batch, and potentially contain additional antigens which may be recognised by animals immunised with vaccines prepared from cell culture systems and may yield false positive tests. Molecular cloning potentially overcomes these problems and can provide fully defined antigens in large quantities. Furthermore, recombinant antigens may not need purification when used in combination with specific MAbs e.g. in competition ELISA.

Antigen(s) of potential diagnostic significance is identified by studying the antibody response of the host to the proteins of the organism in question. For example, immunodominant antigens may be defined as those organism proteins against which the host responds with the highest antibody titre. Specific reagents such as MAbs or monospecific polyvalent sera may then be generated against these for use in screening recombinant gene libraries for the protein(s) of interest. Recombinant libraries can be produced from the genomic DNA of the organism or by CDNA synthesis using organism messenger RNA (MRNA) as a template. Fragments of the genomic DNA or CDNA are molecularly cloned into a prokaryotic or eukaryotic expression systems, and the gene library screened for expression of the desired protein. Problems of expression and antigenicity have to be considered to allow the true diagnostic use of such proteins to be evaluated and production of glycosylated proteins has advantages in this respect.

Conclusions

Virus diseases of animals pose the most important and continuously evolving threat to economics of both developed and developing countries. Control measures include the use of vaccines and the need for rapid and accurate diagnosis. Conventional methods of tissue culture growth and inactivation or attenuation of viruses are still those most used to prepare vaccines. Molecular biological methods has offered the chance to develop new strategies involving identification and manipulation of genes for expression of protein in vectors to produce large amounts of vaccinating protein or insertion into new “safe” replicating agents. The immunological basis of host/pathogen relationship has been better understood through use of the latest molecular and serological advances. This understanding will improve the chances of designing appropriate vaccines in future.

Diagnostic improvements stem from the identification of relevant “diagnostic” proteins, the availability of defined products in large amounts through gene manipulation and expression and the improvements in methods, particularly those based on MAbs and PCR technologies and developments in instrumentation. These offer potentially highly sensitive and specific methods for unequivocal results concerning disease or immune status.

The exploitation of the newer methods has not been as rapid in the veterinary sphere as in human medicine. The reasons for this come from the highly fragmented nature of the problems encountered in animal science which is not so attractive to commercial interests. There is also a misunderstanding of the damage caused by virus disease in both developed and developing countries. The newer methods do offer a new level of sophistication to all laboratories, including those in developing countries. All rely on the continued research into virus disease at all levels, molecular, serological and epidemiological as well as the applied production of defined reagents, including kits for serology and molecular biology. This is particularly important in developing countries but is essential to all involved in disease control, particularly since virus diseases have to be regarded as trans-boundary in nature.

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