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HEALTH and CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
**C2 - Management of scientific committees; scientific co-operation and networks**

# Diagnostic Techniques and Vaccines for Foot-and-Mouth Disease, Classical Swine Fever, Avian Influenza and some other important OIE List A Diseases

Report of the Scientific Committee on

Animal Health and Animal Welfare

Adopted 24-25th April 2003

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## 1. ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| ABTS | 2,2-azino-di-3-ethylbenzthiazoline sulfonate |
| Ag | Antigen |
| AGID | Agar gel immunodiffusion |
| AI | Avian influenza |
| AIV | Avian influenza virus |
| AP | Alkaline phosphatase |
| APMV1 | Avian paramyxovirus serotype 1 |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BDV | Border disease virus, member of the pestivirus genus |
| BSE | Bovine spongiform encephalopathy |
| BVDV | Bovine viral diarrhoea virus, member of the pestivirus genus |
| CCD | Low-light-sensitive charge-coupled device |
| cDNA | Complementary DNA, made by conversion of (viral) RNA into DNA by reverse transcription |
| CMI | Cell-mediated immunity |
| CPE | Cytopathic effects |
| CRL | Community Reference Laboratory |
| CSF | Classical swine fever |
| CSFV | Classical swine fever virus, member of the pestivirus genus |
| DIF | Direct immunofluorescence |
| DISC | Defective in second cycle |
| DIVA | Differentiating infected from vaccinated animals |
| DNA | Deoxyribonucleic acid |
| E2 | The most immunogenic glycoprotein of CSFV |
| EDTA | Ethylene diamine tetra-acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| $E^{rns}$ | One of the glycoproteins of CSFV |
| EU-FMD | European Commission for the control of Foot-and-Mouth Disease |
| FAO | Food and Agriculture Organization |
| FAT | Fluorescent antibody test |
| FDA | Food and Drug Administration |
| FITC | Fluoresceine isothiocyanate, a fluorescent dye |
| GIS | Geographical information system |
| GMP | Good Manufacturing Practice |

| | |
|---|---|
| H5, H7 | Examples of haemagglutinin subtypes of influenza A viruses |
| HA | Haemagglutinin antigen |
| HAV-5 | Human adenovirus 5 |
| HI | Haemagglutination inhibition |
| HPAI | Highly pathogenic avian influenza |
| HRP | Horseradish peroxidase |
| ICPI | Intracerebral pathogenicity index |
| $ID_{50}$ | Infectious dose in 50% of animals tested |
| IFN | Interferon |
| IgA | Immunoglobulins of isotype A, secreted on mucous membrane surfaces |
| IgG | Immunoglobulins of isotype G, major Ig fraction |
| IgM | Immunoglobulins of isotype M, earliest appearing Ig isotype |
| iIFA | Indirect Immunofluorescent antibody test |
| ILTV | Infectious laryngotracheitis virus |
| ISCOMS | Immuno-stimulating complexes |
| ISO | International Organization for Standardization |
| IVPI | Intravenous pathogenicity index test |
| LCR | Ligase chain reaction |
| LPAI | Low pathogenic avian influenza |
| mab | Monoclonal antibody |
| MHC | Major histocompatability complex |
| mRNA | Messenger RNA |
| N1, N7 | Examples of neuraminidase subtypes of influenza A viruses |
| NA | Neuraminidase antigen |
| NASBA | Nucleic acid sequence-based amplification |
| NCR | Non-coding region |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |
| NP | Nucleoprotein |
| NP-ELISA | Nucleoprotein ELISA |
| NRL | National Reference Laboratory |
| NS3 | Non-structural protein 3 of CSFV, BVDV, and BDV; conserved among pestiviruses |
| NSP | Non-structural protein |

| | |
|---|---|
| OIE | Office International des Epizooties |
| PAV | Porcine adenovirus |
| $PD_{50}$ | Protective dose in 50% of animals tested |
| PRV | Pseudorabies virus |
| RNA | Ribonucleic acid, viral genome substrate |
| RT-PCR | Reverse transcription- polymerase chain reaction |
| SDA | Strand displacement assay |
| SP | Structural protein |
| SPF | Specific pathogen free |
| SR | Singleton reactor |
| TCID | Tissue culture infective dose |
| TMA | Transcription mediated amplification |
| TMB | Tetramethylbenzidine |
| TNF | Tumour necrosis factor |
| TSE | Transmissible spongiform encephalopathy |
| VLP | Virus-like particle |
| VN | Virus neutralisation |
| VNT | Virus neutralisation test |
| WHO | World Health Organization |
| WRL | World Reference Laboratory |

## 2. MANDATE

The Scientific Committee is requested to provide a report including:

- updated information on the developments and the state of art of the real-time PCR and other newly developed diagnostic techniques for diseases of the List A of the OIE, with particular reference to foot-and-mouth disease, classical swine fever and avian influenza, including techniques to discriminate the vaccinated and healthy animals from the infected ones;

- recommendations on the most appropriate steps to rapidly standardise and validate these techniques, so that they can be available and successfully used in the Community in case of an emergency, as well as recognised at international level;

- recommendations on the potential use of these techniques in the event of an emergency, in the framework of the measures envisaged for disease control, with the main aims to detect apparently healthy but infected animals and to reduce the need for slaughter and destruction of non-infected animals as much as possible.

## 3. BACKGROUND

Recent epidemics of highly contagious animal diseases included in the List A of the OIE such as foot-and-mouth disease (FMD), classical swine fever (CSF) and avian influenza (AI) have led to the need to slaughter and destroy many millions of animals, which were infected, suspected to be infected or had an uncertain health status as regards the disease in question.

During the emergency of a major contagious disease, rigorous and rapid measures must be taken to interrupt the chain of infection. Such measures may include the pre-emptive cull of animals suspected of being infected or contaminated due to their direct or indirect contacts with a confirmed outbreak. In the past this situation has led to the slaughter of a high number of animals in which the infection could not be confirmed post-mortem or on the basis of conventional diagnostic tests on samples taken at the time of slaughter.

Indeed, the use of vaccines in such emergencies is also limited by the possibility that vaccinated animals may, after infection with the infectious agent, spread the virus further. At the same time they cannot be easily and rapidly identified and distinguished from vaccinated but uninfected animals.

The slaughter and destruction of a very large number of animals in relation to animal diseases, in particular for those diseases that do not pose a risk for human health, gives rise to considerable public concerns. It has also led to very high costs and losses for the Community budget, Member States, stakeholders and ultimately for consumers.

In recent years new diagnostic tests such as "real time PCR" and specific immunoassays have been developed or are being developed for the rapid detection of apparently healthy but infected animals and to discriminate the infected animals from those vaccinated and uninfected animals.

## 4. PREAMBLE

In order to consider techniques for discriminating between infected and vaccinated animals, as requested in the mandate, it was necessary to describe the corresponding vaccines that have been used or are under development. The Scientific Committee therefore decided to establish two working groups to address this mandate, one to consider diagnostic techniques and the other to deal with vaccines and their application in disease control. As outlined in the mandate to this report, particular reference is made to FMD, CSF and AI, while other OIE List A diseases that occur in the EU are also briefly considered. Previous reports of the Committee have also considered aspects relevant to these diseases and some of these reports are included in the list of references.

In the past, EU Member States have been successful in the eradication of FMD and CSF from their territories. Preventive and systematic vaccination and diagnostic systems were crucial tools in such eradication programmes. In anticipation of the common internal market, when FMD and CSF viruses were exotic in most EU Member States, the EU adopted a non-vaccination policy for these OIE List A diseases. However, due to expanding international tourism and global trade of domestic and wild animals and animal products, the risks of the re-importation of exotic diseases into EU Member States have continued to increase since that time. Recent outbreaks of CSF, FMD and AI have occurred in several Member States and resulted in the slaughter of large numbers of animals as well as severe economic consequences. As an example, with regard to the UK FMD outbreak in 2001, the cost to the public sector was estimated at over 4.5 billion euro, and the cost to the private sector at over 7.5 billion euro (NAO, 2002). The following tables indicate the scale and impact of FMD outbreaks in the EU in 2001 in terms of numbers of animals slaughtered.

**Table 1. Outbreaks of FMD in the EU in 2001 (NAO, 2002)**

| Country | No. of infected premises | No. of animals killed# | Duration of outbreak (first and last confirmed cases) |
|---|---|---|---|
| France | 2 | 58,000 | 12 to 23 March 2001 |
| Republic of Ireland | 1 | 60,000 | 22 March 2001 |
| The Netherlands | 26 | 268,000 | 21 March to 22 April 2001 |
| Northern Ireland | 4 | 50,000 | 1 March to 22 April 2001 |
| Great Britain | 2,026 | 4,200,000* | 20 February to 30 September 2001 |

# Approximate figures, rounded up or down

**Table 2. Numbers of animals killed for disease control purposes during the FMD outbreak in Great Britain 2001 (DEFRA, 2003)**

| Total animals slaughtered | Infected premises | DC° (contiguous premises) | DC° (non-contiguous premises | Slaughter on suspicion | Total |
|---|---|---|---|---|---|
| **Cattle** | 295,752 | 195,615 | 78,071 | 13,226 | 582,664 |
| **Sheep** | 986,607 | 1,013,605 | 1,381,244 | 112,226 | 3,493,682 |
| **Pigs** | 21,916 | 54,799 | 68,711 | 2,498 | 147,924 |
| **Goats** | 870 | 699 | 721 | 295 | 2,585 |
| **Deer** | 28 | 492 | 450 | 3 | 973 |
| **Other** | 553 | 370 | 6 | 3 | 932 |
| **Total** | **1,305,726** | **1,265,580** | **1,529,203** | **128,251** | **4,228,760** |

° DC: Dangerous contacts- premises where animals were subject to direct contact with infected animals or have in any way been exposed to infection

A rapid and accurate diagnosis and efficacious vaccination (including emergency vaccination) can contribute significantly to safeguarding animal welfare, by limiting the spread of an epizootic disease and thereby the pain and suffering that such disease may engender in the animals (e.g. ulcerations in the mouth and on the feet of an animal suffering from FMD may result respectively in an inability to eat and lameness). Furthermore, in cases where a 'stamping out' disease control policy is adopted (i.e. killing to eliminate a disease), these diagnostic and vaccination tools can reduce the number of animals to be killed. This can improve overall animal welfare as the obvious difficulties of killing animals on a large scale (whole herds of animals over widely dispersed areas) raises serious welfare concerns. For example, there are inherent practical difficulties with large numbers of animals being required to be killed in a short time period on farm premises in varying circumstances. There may be difficulties in confirming that each animal has indeed been killed, as well as difficulties associated with the killing of very old and very young animals. The fear engendered in the animals waiting to be killed also needs to be considered. It should also be emphasised that the use of mathematical models and the testing of various intervention strategies can make an important contribution to disease control programmes, helping to prevent disease spread and the consequent negative impact on animal welfare.

During the FMD outbreaks in a number of EU Member States in 2001, the Committee drew attention to some important issues regarding control of outbreaks of this disease and the need for urgent research in a number of areas. It should be noted that during the oubreak of FMD in Great Britain in 2001 2.3 million animals were slaughtered for animal welfare reasons as a consequence of animal movement restrictions or due to limited market outlets for these animals, e.g. the "light lambs" scheme. Furthermore, the need for disease control to take into account not only socio-economic consequences but also the effects on the welfare of animals was highlighted (SCAHAW, 2001).An additional issue concerns the welfare of animals involved in the safety and potency testing of batches of vaccines. While the experimental design and the methods of challenge are laid down in monographs by the EU in the Pharmacopoeia (Council of Europe, 1997) they sometimes may not adequately take into account current scientific knowledge in some technical aspects or in experimental design (see Cussler *et al.,* 1999a; Hendriksen and Morton, 1999). Moreover, the legal requirements under current animal research legislation to cause a minimum of suffering under European legislation (CEC, 1986) and the Council of Europe Convention ETS 123 need to be considered (Cussler *et al*., 1999b). For example, in some tests animals are allowed to die rather than being euthanised when they first show irreversible signs of death, or when the scientific objective has been achieved (e.g. clinical signs of early stages of the disease in potency and safety tests). Positive control animals could also be euthanised at an early stage of the experiment when it is clear that the challenge has been with live virus. It is noteworthy that the OECD requires early killing of animals when testing chemicals (OECD, 2001).

Although there are strict international animal health regulations in force, it cannot be totally excluded that unrecognised infected animal products or indeed infected live animals could enter the EU. The feeding of swill to farm animals can be the final trigger for outbreaks of either FMD or CSF. Poultry are at risk of contracting AI[1], especially

[1] The definition of avian influenza referred to in EU legislation differs from that used by the OIE, and revised definitions are currently under consideration. In this paper the term 'avian influenza' will be used where differentiation between viruses of low and high virulence is not necessary. The term 'highly pathogenic avian influenza' (HPAI) is used for viruses falling within the current EU and OIE

poultry kept in open holdings since migratory birds are often the source of an outbreak. Once a primary outbreak has occurred, regional risk factors may contribute to the rapid spread of the infection (e.g. high concentration of susceptible animals, lack of awareness among farmers or veterinarians and intense animal trade over long distances). The vast majority of primary outbreaks in farm animals in recent years have not been completely contained and follow-up outbreaks and epizootics have developed (Elbers *et al*., 1999; Capua *et al*., 2000; Fritzemeier *et al*., 2000; Gibbens *et al.,* 2001; Gibbens and Wilesmith, 2002). Recent epidemics of FMD, CSF and highly pathogenic AI (HPAI) have been dealt with by a stamping out policy and other zoo-sanitary measures at the cost of many millions of animals killed and severe socio-economic consequences. New emergency vaccination strategies and modern diagnostic techniques may offer alternative means to eradicate incursions of exotic diseases. However, of utmost importance is the constant vigilance required for the detection of early clinical signs of exotic diseases. There is an additional requirement to maintain the thresholds for the early submission of laboratory samples by veterinarians and farmers in cases of suspicion of these diseases as low as possible (including 'psychological' and regulatory thresholds).

**Diagnosis:**

Although for all the infectious diseases under consideration in this paper there are well-proven diagnostic tests available, recent disease outbreaks have highlighted the necessity to improve existing tests and to develop new methods, in order to more rapidly recognise new cases of exotic diseases and to shorten the time interval between introduction of the infectious agent and control measures being taken. In cases of primary outbreaks, or questionable results in the case of secondary outbreaks, it remains the task of the authorised central laboratories, using the most up to date repertoire of diagnostic techniques, to confirm results of diagnostic tests that have been performed in regional laboratories or on farms. However, the time between the clinical suspicion and laboratory confirmation of the exotic disease can be relatively long due to the logistics of sending samples to central laboratories. On the other hand, in most cases, regional laboratories do not have the expertise, equipment or facilities to diagnose exotic diseases. A further difficulty is the lack of tests to detect animals in the early stages of infection and this allows the virus always to be 'ahead' of the investigators, thus being able to spread unrecognised to other susceptible animals and herds.

The recent epidemics of FMD, CSF, and HPAI in several Member States have added further impetus to the development of so-called 'marker vaccines' for emergency vaccination. The first generation of marker vaccines is available for FMD and CSF. However, in both systems a clear limitation is obvious: the serological recognition of infection within a vaccinated population is not always possible at the level of the individual animal, as opposed to being recognisable at herd level, using currently available techniques (Mackay *et al*., 1998a; Floegel-Niesmann, 2001).

The market potential for veterinary diagnostics is also relatively small and therefore there are few incentives for the industry to invest in research and development. As a consequence many diagnostic procedures are not based on commercially available and licensed diagnostic kits but on assays developed 'in-house' in particular laboratories for use in response to a specific need. The use of tests that are not always fully validated,

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definitions, and the term 'low pathogenic avian influenza' (LPAI) for viruses not covered by those definitions.

standardised and accredited, reduces the confidence in the results of such tests for the diagnosis of these infectious diseases.

With respect to the limitation of current diagnostic techniques the following needs could be addressed:

- Development of methods for the effective and inexpensive screening of animal products for exotic pathogens.

- Development of simple and rapid tests (e.g. 'pen-side' tests) for use in the field or in regional laboratories, to support clinical suspicions of diseases.

- Development of rapid and highly sensitive tests, that will detect animals as soon as possible after they become infected. When these tests can be performed on a mass screening basis they could be used to detect follow-up outbreaks, before clinical suspicion arises.

- Development of more sensitive and specific tests to detect infection in an individual vaccinated animal without the need for screening whole herds.

- Development of rapid and sensitive methods for differential diagnosis of diseases that induce similar clinical signs.

The use of pen-side tests for on-farm screening is required to be restricted to official veterinarians. Test sensitivity needs to be high, whereas specificity is less critical, since a false-positive result will only lead to restriction measures of a non-infected herd/flock and results in any case need to be verified by the competent central laboratory. Laboratories with restricted resources with respect to equipment, personnel and specific expertise for exotic diseases (e.g. regional laboratories) would benefit from test formats designed for their circumstances. This would enable regional laboratories to play a role in the early diagnosis of exotic infectious diseases, thereby saving time in the eradication process and relieving the authorised central laboratory in times of a full disease epidemic. However, strict biosecurity, containment and statutory requirements would need to be fulfilled in all cases.

With regard to the characteristics and performance of diagnostic tests two applications should be borne in mind: either the analytical detection at laboratory level or diagnostic performance when applied to an individual animal or herd. Analytical sensitivity and specificity apply at laboratory level, while diagnostic sensitivity and specificity relate to testing of individual animals or a herd (including false positive and false negative results- see Annex, also Jacobson, 1998). In order to increase confidence in the diagnosis of infectious diseases it is essential that the methods used are fully validated and standardised. Assay performance must also be monitored through quality control and proficiency testing procedures using specially designated reference materials and a network of designated laboratories.

**Vaccination:**

The classical vaccines against FMD (inactivated) and CSF (live-attenuated) are efficacious and safe and are widely used around the world. Both types of vaccines are powerful weapons in the struggle aimed at controlling disease outbreaks and eradication of these infections. A great disadvantage of the use of classical vaccines is their inability to allow the differentiation of infected from vaccinated animals. However, in the case of

FMD, a test has been developed that, in principle, allows laboratories to make that distinction, provided the vaccines used are well-purified and thus free of non-structural viral proteins.

The use of classical vaccines against CSF still precludes the discrimination of infected from vaccinated pigs, making it more difficult to prove freedom from infection, which often causes limitations for intra-community and international trade. The advent of biotechnology into vaccinology, however, has enabled researchers to develop a first generation of marker vaccines, i.e. two subunit vaccines against CSF that allow the discrimination of infected pigs from vaccinated pigs using the accompanying differential antibody tests. Both vaccines have recently been licensed for market use and potentially more powerful recombinant vaccines are under development. Unfortunately, CSF has entered the wild boar population in a number of European countries and is thus a constant threat for new outbreaks in the domestic pig populations. Therefore an oral vaccine has been developed to attempt to reduce the prevalence in wild boar.

Following the HPAI outbreaks of 1999-2000 in Italy, H7N1 virus of low pathogenicity AI (LPAI) re-emerged and a 'DIVA' (differentiating infected from vaccinated animals) strategy was developed and applied to stop the spread of the LPAI virus. This strategy consisted of the use of an inactivated vaccine containing the same haemagglutinin subtype as the outbreak virus, but a different neuraminidase: H7N3. Infected poultry could then be distinguished from vaccinated poultry by the use of a differential test for detection of antibodies against the neuraminidase of the outbreak virus. Thus the course of the epidemic could be monitored.

The development of more efficient diagnostic methods and emergency vaccination using vaccines allowing differentiation between virus-infected and vaccinated animals will provide important tools for the improvement of current 'stamping-out' disease control strategies. However, compliance with regulatory requirements may sometimes hinder the availability of newly-developed products on the market. Another constraint for vaccine development, as for diagnosis, is the limited commercial potential of the end products. Consumer concerns regarding the possible use of vaccines and their acceptance by the general public would also need to be addressed.

This report describes existing diagnostic methods and vaccines, reviews the progress that has been made in the above fields and draws conclusions and recommendations on the potential use of such techniques.

## 5. RECENT DEVELOPMENTS IN THE DIAGNOSIS OF INFECTIOUS DISEASES

### 5.1. Introduction

Viral infections can be divided into a number of stages. Initially, virus replication is localised, often at the portal of entry. Subsequently, there may be a viraemia and a phase of more generalised virus replication. This phase may lead to the host's death, or else to the development of immunity, including the production of anti-viral antibodies, which may remain at detectable levels for prolonged periods – often years. As immunity develops, the levels of virus decline rapidly, although in some infections residual virus may persist locally, giving rise to animals known as virus carriers. Diagnostic tests for viral infections are of two main types, firstly tests for the

presence of virus and secondly tests for the presence of specific anti-viral antibodies.

So far, other specific and non-specific early indicators of infection, such as T-helper cells, chemokines and cytokines, have not been exploited for the diagnosis of either exotic or non-exotic viral infections although there is ongoing progress in this field. Strong interferon IFN α/β and IFNγ responses were detected in the blood of calves within 4-6 days of inoculation with a non-cytopathic bovine viral diarrhoea virus (Charleston *et al.,* 2002) and IFNγ was detected in the blood of pigs 4 days after inoculation with porcine reproductive respiratory syndrome virus (Greiner *et al.,* 1998). CSF virus (CSFV) specific IFNγ-secreting cells were detected in peripheral blood mononuclear cells of CSFV vaccinated and unvaccinated pigs soon after challenge (Suradhat *et al.*, 2001), while Murphy *et al.* (2002) reported fluctuating levels of mRNA for IFN α/β, IFNγ, interleukin 1α during experimental infection of pigs with FMDV.

In the early stages of an infection there may be little virus present or shed and no antibodies detectable. There will usually also be no clinical signs of disease (i.e. preclinical infection) and diagnosis at this stage is therefore very difficult. Subsequently, there is an increased chance of detecting virus, which may coincide with the period of illness and manifestation of clinical signs. During and after convalescence anti-viral antibodies are detectable, indicating previous infection (or vaccination) with or without concurrent virus carriage, whilst virus detection becomes progressively more difficult or impossible.

Tests for virus detection include the isolation of live virus in cell cultures or embryonated eggs, the detection of viral proteins (antigens) and the detection of viral genome fragments (Murphy *et al*., 1999). Virus isolation is the 'gold standard' method for many viruses that can be grown *in vitro*, including FMD virus (FMDV), CSF virus (CSFV) and AI virus (AIV) (OIE, 2000). It may be used on a wide range of sample types. Although time-consuming, the method is very sensitive, and offers the maximum opportunity for subsequent viral characterisation. It is necessary to confirm the identity of isolated viruses by other techniques such as the detection of viral proteins or genome fragments.

Immunoassays are the most commonly used techniques for detecting viral proteins and rely on the use of specific antibodies to bind and visualise the target antigens. Examples include fluorescent antibody tests used for immunolabelling virus in sections of infected organs and enzyme-linked immunosorbent assays (ELISA) carried out on blood or tissue homogenates in microplates. Another example is the use of chromatographic strip tests as pen-side assays. Used on their own, without prior virus amplification *in vitro*, these methods are less sensitive than virus isolation, but are quicker and easier to perform.

Viral genomes may be detected in a wide variety of sample types by hybridisation of labelled, complementary nucleic acid probes. The sensitivity of the method can be greatly increased if the target region of the viral genome is also amplified for example by reverse transcription-polymerase chain reaction (RT-PCR), in which case, the sensitivity of detection may

match or exceed that of virus isolation (McGoldrick *et al*., 1999; Reid *et al*., 2002; Spackman *et al*., 2002).

The gold standard for testing blood samples for the presence of antibodies to FMDV and CSFV is virus neutralisation (VN), in which known amounts of virus are mixed with serial dilutions of the test serum, prior to the mixture being incubated with susceptible cell cultures. If there are neutralising antibodies present in the serum, they will prevent virus replication in the cell cultures, which may be measured by an absence of a specific viral cytopathic effect or a lack of virus-specific immunostaining. Although very sensitive and specific, neutralisation tests are slow, cumbersome to perform and must be carried out in high containment laboratories because of the requirement to work with live viruses. They do not distinguish between antibodies elicited by vaccination and those elicited by infection. In the case of AIV, sera are classically tested for anti-viral antibodies by measuring the ability of a serum sample to inhibit the haemagglutination of red blood cells by influenza virus antigens.

Simpler and quicker tests, especially ELISA, have been developed as alternatives to VN tests (Crowther, 1998). A commonly used approach is to utilise tests such as ELISA for primary antibody detection, reserving VN tests for the confirmation of inconclusive and positive results. As a general guide to the reader, Tables 3 and 4 give qualitative estimations of properties of various methods available for antibody or antigen detection.

**Table 3. Qualitative estimations of properties of various methods to detect viruses or their components**

| Method | Applied for | Relative sensitivity | Relative specificity | Capacity for automation | Pen-side feasibility | Cost | Reproducibility/ Robustness | Speed of obtaining results | Biosafety/ Containment needed | Level of complexity for user |
|---|---|---|---|---|---|---|---|---|---|---|
| **Immunoassays** | | | | | | | | | | |
| FAT | CSF | MEDIUM | MEDIUM | NO | NO | LOW | MEDIUM | MEDIUM | YES | MEDIUM |
| ELISA | FMD, CSF (k), AI (k) | MEDIUM | MEDIUM | HIGH | N/A | LOW | HIGH | MEDIUM | YES | MEDIUM |
| STRIP TEST | FMD (k) | MEDIUM | MEDIUM | N/A | HIGH | LOW | HIGH | HIGH | N/A | LOW |
| **Virus Culture** | FMD, CSF, AI | HIGH | HIGH | NO | NO | HIGH | MEDIUM | LOW | YES | HIGH |
| **Nucleic acid detection** | | | | | | | | | | |
| Amplification – PCR | FMD, CSF, AI | HIGH | HIGH | MEDIUM | LOW | MEDIUM | LOW | MEDIUM | NO | HIGH |
| Hybridisation-Microarrays* | N/A | LOW | HIGH | MEDIUM | N/A | MEDIUM * | MEDIUM | MEDIUM | NO | HIGH |

FAT: fluorescent antibody test, ELISA: enzyme linked immunosorbent assay, PCR: polymerase chain reaction, (k) indicates the availability of commercial kits. Specific test components are commercially available in some cases, * is used to indicate expected values.

The biosafety/containment requirements of ELISA and PCR methods will depend on whether the samples are inactivated (e.g. by heating) prior to testing.

**Table 4. Qualitative estimations of properties of various methods for antibody detection**

| Method | Applied for | Relative sensitivity | Relative specificity | Capacity for automation | Pen-side feasibility | Cost | Reproducibility/ Robustness | Speed of obtaining results | Biosafety/ Containment needed | Level of complexity for user |
|---|---|---|---|---|---|---|---|---|---|---|
| IFA | CSF | MEDIUM | MEDIUM | NO | NO | MEDIUM | MEDIUM | MEDIUM | YES | MEDIUM |
| AGID | AI | MEDIUM | MEDIUM | NO | N/A | LOW | MEDIUM | LOW | NO | LOW |
| ELISA | FMD (k), CSF (k), AI | LOW TO MEDIUM | MEDIUM | HIGH | LOW | LOW | MEDIUM | HIGH | NO/LESS | MEDIUM |
| Haemagglutination inhibition | AI | MEDIUM | HIGH | LOW | N/A | LOW | MEDIUM | MEDIUM | YES[#] | MEDIUM |
| Virus neutralisation | FMD, CSF, AI | MEDIUM | LOW TO MEDIUM | NO | NO | HIGH | LOW | LOW | YES | HIGH |
| Strip test* | N/A | MEDIUM | MEDIUM | N/A | HIGH | LOW | HIGH | HIGH | N/A | LOW |

IFA: indirect fluorescent antibody test, ELISA: enzyme linked immunosorbent assay, AGID: agar gel immunodiffusion, (k) indicates the availability of commercial kits. Specific test components are commercially available in some cases.

# biosafety/containment is required for haemagglutination inhibition tests unless inactivated antigen is used, * indicates expected values

## 5.2. Screening of animal products

The risks associated with the introduction of disease via food and feed products and animal products such as semen and embryos are mitigated by measures such as the checking of imports, quarantine restrictions, testing of the donor animals etc. In general, for the diseases under consideration, there are no tests available or in prospect for large-scale screening of animal products for virus detection. Available methods lack sensitivity and/or are not suitable for use on a sufficiently large scale.

Although there are recent reports on virus or antibody detection in meat (muscle) samples (Thuer and Hofmann, 1998) or meat juice (Mortensen *et al*, 2001), even the most sensitive detection methods have proven to be unreliable, suffering from a significant lack of sensitivity. In these cases virus detection only proved to be successful if the specimens originated from clinically ill animals where the samples contained high levels of virus. Furthermore, depending on the sample type, inhibitory effects may be observed, e.g. CSFV detection from semen (Floegel *et al*., 2000). There is some potential for screening and detecting antibodies from product extracts but it seems unlikely that technical improvements (e.g. enrichment methods and immunomagnetic capture assays) could overcome the inherent difficulty of sampling very large consignments. Regarding AI, specific screening tests are not generally used but import of poultry and associated products into the Community must satisfy the conditions laid down in Community legislation (CEC, 1990).

## 5.3. Nucleic acid amplification methods

During the last decade there has been widespread application of techniques based on the amplification of specific nucleic acid sequences by polymerase chain reaction (PCR), ligase chain reaction (LCR) and nucleic acid sequence-based amplification (NASBA), and these have been applied increasingly for veterinary diagnostic uses (Pfeffer *et al*., 1995; Belak and Thoren, 2001; Leutenegger, 2001). The processes involved in nucleic acid amplification with relevance to suitability for diagnostic use are (1) extraction of nucleic acid from the sample, (2) nucleic acid amplification, (3) evaluation or detection of amplified nucleic acid and (4) qualitative confirmation.

In theory, these methods are sensitive enough to amplify a single nucleic acid molecule by a million fold, so that the level of amplified nucleic acids (amplicons) can be detected by conventional DNA/RNA detection methods, with the added benefit of speed and high specificity. Despite these obvious advantages, their reliability is dependent upon very thorough validation and quality control, which has limited their uptake into routine diagnostic work. The highly sensitive nature of these assays means that extremely high laboratory standards are required to prevent both cross-contamination between samples and contamination by amplicons from previous assays. However, increasingly reliable methods and processes are becoming available and robotic processing systems are being used to enable nucleic acid detection methods to be applied to the screening of samples on a large-scale. There is also a growing interest in the possibility of using PCR tests in the field or in mobile laboratories (Callahan *et al*., 2002). The main obstacles are the need for specialist equipment, technical difficulties in simplifying

sample preparation and nucleic acid extraction, and considerations regarding quality control.

5.3.1. Extraction of nucleic acid from the test sample

Before the nucleic acid can be extracted, solid tissue samples have to be homogenised, and cells have to be lysed. This is usually performed by solubilising the cellular components and at the same time inactivating enzymatic activities that can degrade the nucleic acids, using chaotropic salts (substances that break up hydrogen bonds and hydrophobic interactions) (Chomczynski and Sacchi, 1987).

Three approaches can be used for general nucleic acid extraction:

- Selective precipitation of protein followed by nucleic acid precipitation with alcohol (isopropanol, ethanol);

- Organic separation of proteins using phenol-chloroform followed by nucleic acid precipitation with alcohol;

- Attaching the nucleic acid to a matrix (silica, magnetic beads, glass), with washing and subsequent elution in buffer.

In order to improve the success of extracting nucleic acid targets from biological samples, new strategies are under development by which nucleic acid can be enriched: hybridisation capture, immuno-capture of the infectious agent with specific antibodies before nucleic acid extraction.

All of these processes can be labour intensive, although improved robotic systems are available that can simplify handling and substantially increase throughput. For instance, high-throughput robotic systems are routinely used for the isolation of chromosomal DNA from sheep blood cells, PCR amplication and purfication, to test sheep for susceptibility to scrapie. More than 2,000 samples per day can be handled and these systems can also be used for enrichment procedures, e.g. by the use of coated magnetic beads (Bossers, pers. comm.). Development and validation of commercial robots and devices for effective nucleic acid extraction are of crucial importance and need to be encouraged. In order to make them useful for mass screening they need to be suitable for handling high numbers of samples without increasing the probability of cross-contamination of samples. It could be feasible that technological developments comparable to those in the field of automatised genotyping, e.g. for sheep genotype resistance against scrapie, could improve the throughput of these methods (Lemieux, 2000; Grant and Phillips, 2001; Galvin, 2002).

Table 5 gives a qualitative comparison of various nucleic acid extraction methods.

**Table 5. Qualitative comparisons of different nucleic acid extraction methods useful for nucleic acid amplification**

| Procedure | Commercially available as | Format* | Yield of nucleic acid** | Ease of use | Time needed | Automation possible |
|---|---|---|---|---|---|---|
| Precipitation of proteins/ nucleic acid | Reagents | Protein-DNA Precipitation Solution, etc. | Good | Good | 1 hr | No |
| Solution-based | Reagents | Bottle containing phenol/ thiocyanate solution. | Excellent | Good | 1–2 hr | No |
| Silica or glass adsorption | Kits | Tips, columns, microtitre plates, beads. | Very good | Excellent | 10-45 min | Yes |
| Hybridisation capture | Reagents | Magnetic beads, microtitre plates | Very good | Excellent | ~30 min | Yes |
| Immunocapture | Reagents | Magnetic beads, microtitre plates | Very good | Good | ~30 min | Yes |

* All methods except hybridisation capture and immunocapture require (low speed) centrifugation steps

** Scheibner *et al*., 2000

### 5.3.2. Target amplification systems

Systems for amplifying target nucleic acids use a variety of bacterial, viral or recombinant enzymes originally involved in intracellular DNA or RNA replication.

#### 5.3.2.1. Polymerase chain reaction (PCR)

For PCR, a specific segment of target DNA is amplified using two primers, one for each strand. These direct selective replication by the enzyme DNA polymerase and as each primer is extended, it creates a copy of the original template. After thermal denaturation, four strands are available for replication in the next cycle. By employing successive cycles of high temperature denaturation and lower temperature primer annealing and chain extension, exponential amplification of the target sequence flanked by the two primers is achieved. This is performed in a programmable thermocycler, using thermostable DNA polymerases. The methodology can be applied to RNA by first preparing a DNA copy of the target RNA using reverse transcription (RT) (Win-Deen, 1996; Belak and Thoren, 2001).

The entire amplification process can be performed *in vitro* using relatively small amounts of sample in a few hours or less. The process is generally most simply done in liquid phase but solid phase applications are also used to enhance target availability through direct attachment, immunocapture or hybridisation.

The conventional approach to the detection of amplicons uses analytical examination by gel electrophoresis and staining with DNA intercalating agents (e.g. ethidium bromide). The specificity of the amplified product is confirmed by its size in relation to reference standards and/or by its sequence

composition, using restriction fragment length polymorphism, sequence analysis, or hybridisation. As this approach is unsuited to automation, it is not widely used in large-scale diagnostic applications. Alternative methods to detect specific PCR products include hybridisation with labelled probes on a solid phase or the use of ELISA, which is suitable for automation and higher throughputs.

5.3.2.2. Real-time PCR

A more recent development is the application of real-time PCR in which the concentration of the PCR product is measured as the reaction proceeds, enabling the viral load within a sample to be measured (Callens and De Clercq, 1999). This method utilises fluorescent dyes either for non-specific staining of double-stranded DNAs or in a variety of systems involving hybridisation probes. In addition, the melting/dissociation temperature profile of the product can be calculated, to confirm the specificity of the amplification process. Real-time PCR offers the advantage that it can be performed in a single tube without having to open or manipulate it, thereby reducing the risk of laboratory contamination, in addition to making it suitable for automation (Belak and Thoren, 2001; Mackay *et al*., 2002) and for use in small mobile laboratories (Callahan *et al*., 2002; Risatti *et al.*, 2003). Real-time PCR using a fluorescent hybridisation probe for the detection of CSFV (McGoldrick *et al*., 1999) was successfully applied in England as part of the routine diagnosis, during the disease outbreak in 2000 (Paton, 2002).

5.3.2.3. Multiplex PCR

In conventional (gel-based) multiplex PCR, several primer sets are used to allow amplification of multiple templates within a single reaction. Such tests are currently emerging in the literature for veterinary diagnostics (Carvalho *et al*., 2000; Kim *et al*., 2000; Sykes *et al*., 2001; Pang *et al*., 2002). Multiplex PCR enables the presence of nucleic acids from several pathogens to be checked for in one test, but care must be taken to avoid interference between primer pairs or templates. Currently, the EU is funding an animal health project on novel multiplex nucleic acid tests for some viruses of farm animals (Project reference QLK2-2000-00486).

5.3.2.4. Multiplex real-time PCR

The term multiplex real-time PCR is used to describe the use of two to four fluorogenic oligoprobes for the discrimination of multiple amplicons (Mackay *et al*., 2002). To date, there have been only a few truly multiplexed real-time PCR assays described in the literature, and few of these have been applied to the diagnosis of infectious diseases. This is due to the limitations set by the number of fluorophores that can be combined and clearly distinguished when compared to the discriminatory abilities of conventional multiplex PCR. The use of non-fluorescent quenchers and the continuous development of better light sources in the machines are helping to overcome these problems (Mackay *et al*., 2002) and first applications for virus detection are becoming available (Read *et al*., 2001). An alternative approach is to use the same fluorescent oligoprobe to detect all the different

amplicons in a multiplex screening reaction and then to perform further analysis on positive samples to find out which pathogen is present.

5.3.2.5. Ligase chain reaction (LCR)

The ligase chain reaction is a probe-based DNA amplification method based on repeated cycles of oligonucleotide hybridisation and ligation (Carrino and Lee, 1995; Win-Deen, 1996). The method employs sets of oligonucleotides specific to stretches of the target sequence that are in close proximity to each other, as well as another set of oligonucleotides that is complementary to the first set. The protocol is very similar to PCR, except that LCR uses a heat-stable ligase. Polymerase activity is not needed since the primers constitute virtually the entire length of the target sequence. Therefore, the length of the amplicon will generally be limited by the availability of longer oligonucleotides (Weidmann *et al*., 1994). Although LCR has been available for many years, it is very rarely used in routine diagnostics compared to PCR (Carrino and Lee, 1995; Pfeffer *et al*., 1995). In veterinary virology, it has been used to assess the virulence of Newcastle disease viruses (Collins *et al*., 2003), and to differentiate orthopoxviruses (Pfeffer *et al*., 1994).

5.3.3. Transcription-based amplification systems

These methods are based on RNA-directed amplification. Unlike PCR, which theoretically doubles the amount of amplimer per cycle, each cycle of transcription amplification makes 40-100 copies of RNA resulting in a million-fold amplification in 4 to 5 cycles. The methods include nucleic-acid sequence-based amplification (NASBA), transcription mediated amplification (TMA) and the strand displacement assay (SDA) (reviewed in Win-Deen, 1996).

5.3.3.1.Nucleic acid sequence based amplification (NASBA)

NASBA is an isothermal nucleic acid amplification process involving the simultaneous activity of three enzymes; reverse transcriptase, RNase H and T7 RNA polymerase (Compton, 1991; Kievits *et al*., 1991), thus mimicking the process of retroviral replication (Compton, 1991). The technique utilises two oligonucleotide primers in which the downstream antisense primer contains a highly conserved 5' promotor sequence recognized by T7 RNA polymerase. Since reverse transcriptase is incorporated into the amplification mixture, RNA can be added directly to amplification reactions without prior manipulation such as the generation of cDNA templates, as are required for RT-PCR, thus providing a single-tube amplification format. With NASBA, contaminating double stranded DNA, which is often a problem in RT-PCR assays, is not denatured at the isothermal amplification temperature (41°C) and therefore does not participate in the amplification procedure, obviating the need for rigorous RNA purification (Deiman *et al.,* 2002). In veterinary virology, NASBA has been used for the detection of several RNA viruses (Romano *et al*., 1996; Lanciotti and Kerst, 2001; Collins *et al*., 2002; Jordan *et al*., 2002). Table 6 provides a qualitative comparison of methods for nucleic acid detection.

**Table 6. Qualitative comparison of methods for nucleic acid detection**

| Method | Amplifies | Signal detection | Additional steps required | Specificity/ sensitivity | Multiple assays | Suitable for large-scale detection | Suitable for high-throughput typing |
|---|---|---|---|---|---|---|---|
| NASBA | RNA | Gel electrophoresis/ ELISA | NO | MEDIUM /HIGH | NO | NO | NO |
| LCR | DNA | Gel electrophoresis/ ELISA | NO | HIGH / HIGH | NO | NO | NO |
| PCR | DNA/ RNA | Gel electrophoresis/ ELISA | NO | MEDIUM /MEDIUM | YES | YES | NO |
| Real-time assays | DNA/ RNA | Automatic read-out | NO | HIGH / HIGH | YES (max. 4) | YES | YES |
| DNA microarrays | N.A. | Automatic read-out | YES (e.g. PCR) | HIGH / MEDIUM | YES | NO | YES |

## 5.4. DNA Microarrays

Microarrays consist of ordered sets of DNA molecules of known sequence (the probes) fixed in spots to small solid surfaces (microscope slides: 1 x 25 x 76 mm; or cassettes: 12.7 x 12.7 mm). These DNA chips are prepared on glass or on nylon substrates by hand or more generally by making use of high-speed robotics, and will contain thousands of spots (probes). When the array is exposed to a sample containing target DNA or RNA previously labelled (target = free nucleic acid sample whose identity/abundance is being detected), the probes on the array surface will bind (hybridise) their complementary sequences. Hybridisation results will be imaged and analysed, allowing the detection of thousands of genes or DNA/RNA sequences included in the labelled target simultaneously and their expression level to be analysed.

Despite the potential of microarrays to detect a large number of pathogens at one time, there is as yet little information concerning the use of DNA arrays for diagnosis of infectious diseases. A method has been described for typing and subtyping human influenza virus using glass DNA microarrays, containing DNA fragments of the hemagglutinin, neuraminidase and matrix protein genes, in conjunction with multiplex RT-PCR (Li *et al*., 2001). In addition, an assay for typing of Hepatitis C virus is commercially available (Anthony *et al*., 2001). Presently, the major applications for DNA microarrays are drug discovery (Debouck and Goodfellow, 1999), the analysis of DNA variation on a genome-wide scale (sequence discrimination, gene/gene mutation) and the determination of expression levels (abundance) of genes (Lander, 1999; Lockhart and Winzeler, 2000; Shoemaker and Linsley, 2002).

According to the properties of the probe DNA, there are two basic variants of the microarray technology:

a) Oligonucleotide microarrays. Oligonucleotides (usually 20-60 nucleotides in length) can be prefabricated and printed onto the chip by micropipetting, or synthesised directly on the solid support. This can be achieved by the accurate dispensing of the nucleotides in each round of the synthesis reaction using piezoelectric printing (similar to colour inkjet printing) or by photolithography (Anthony *et al*., 2001),

b) DNA fragment microarrays. DNA fragments (hundreds of nucleotides long), usually made by PCR, are accurately stamped onto the solid surface of the pre-designed microarray (Afshari, 2002).

For sample preparation, DNA or RNA need to be isolated and fluorescently labelled. As large amounts of nucleic acid are required per hybridisation (Duggan *et al*., 1999), most applications involve amplification of DNA or RNA by PCR or linear amplification steps prior to the microarray hybridisation (Anthony *et al*., 2001). After hybridisation, computer-assisted analysis is performed using a scanner and appropriate software.

## 5.5. Immunoassays

The highly specific interaction of viral components with anti-viral antibodies is the basis for many diagnostic tests, either for virus or for viral antibodies.

In the case of tests for the presence of virus, an anti-viral antibody may be used to trap and/or to detect viral antigens, whilst tests for antibodies usually employ a synthetic or natural viral component as the diagnostic antigen. A variety of immuno-assays are applied for routine diagnostic purposes. These include direct and indirect immunofluorescence, immunoperoxidase, virus neutralisation, agar gel immuno-precipitation, and ELISAs. They are all based on the common principle of specific interaction (binding) of antibodies with their respective antigen; the main difference between the test categories is the method of visualising the antigen-antibody binding reaction. ELISAs are most widely used and will therefore be described in detail. Other indicators of infection, e.g. T-lymphocyte, cytokine or chemokine responses, also have potential for future application.

### 5.5.1. ELISA

ELISA is now the most widely used form of immunoassay for the rapid detection of viral antigens and antibodies (for general information see Wardley and Crowther, 1982). The ELISA is based on the following principle: Antigens (e.g. inactivated viruses or individual recombinant viral proteins) are immobilised on a solid plastic surface, usually a 96-well polystyrene microtitre plate. The sample is then added into the well, allowing antibodies directed against the immobilised antigens to specifically bind, thereby being trapped by the antigen. A second antibody, usually directed against antibodies of the animal species from which the diagnostic sample originates, which is coupled to an enzyme such as peroxidase, is allowed to bind to the antibody trapped during the previous step. The entire binding reaction is finally visualised by adding a colourless substrate into the well that will be chemically modified by the enzyme on the second antibody, thereby causing a colour change. The test can be performed within a few hours, and does not require any high-tech equipment, since the results can be read either manually, i.e. by the naked eye (semi-quantitatively), or in a quantitative way by spectrophotometry. Alternatively, the ELISA can be performed in a fully automated way using robots, allowing the testing of numerous samples within a short time. The ELISA is by far the most widely used serological method for antibody screening purposes and there are many different formats available.

#### 5.5.1.1. ELISA for the detection of antibody

Whereas in the past most ELISAs used for the detection of anti-virus antibodies employed complete virus in its native (infectious) or inactivated form, nowadays recombinant viral structural polypeptides known to contain important immunogenic domains may be used, either in the form of authentic single viral proteins, as fusion proteins containing tags for efficient purification, or as peptides representing just the crucial antigenic domains of a viral protein. Therefore such ELISAs no longer have to be handled in biosecure facilities.

The vast majority of ELISAs described for antibody detection are designed to detect IgG antibodies. In principle, IgM-specific ELISAs can also be used, particularly in cases when an early immune response is to be detected. However, since IgM titres - in contrast to IgG levels – fall below detection limits soon after seroconversion, and furthermore are often not indicative of

a protective immunity (Mayr *et al*., 2001), IgM-detecting ELISAs are not suitable for routine diagnostic use unless combined with IgG detection. On the other hand, IgM detection can be used to detect a chronic but asymptomatic infection (Alem *et al*., 2002).

IgA detection by ELISA has also been described. However, IgA levels in blood are significantly lower than those of IgG, hence such ELISAs suffer from a reduced sensitivity. IgA are mainly secretory antibodies present on mucosal surfaces, which allows samples such as saliva to be used as diagnostic specimens. IgA is only produced upon infection or immunisation with a live vaccine. Therefore detection of IgA could also be used to differentiate infected from vaccinated individuals in cases where inactivated vaccines had been used (Amadori *et al*., 2000; Herremanns *et al*., 2002).

Two main test principles are employed:

(i) In the indirect ELISA virus-specific antibodies are captured by the immobilised antigen on the surface of the test plate. Captured antibodies are subsequently detected by an anti-immunoglobulin antibody conjugated to an enzyme which in turn causes a colour change after addition of a substrate, which serves as the readout of the reaction,

(ii) In the blocking ELISA virus-specific antibodies are again allowed to bind to the antigen. However, the presence of these antibodies is detected based on their ability to compete with a known antigen-specific indicator antibody (usually monoclonal) that can no longer bind to its corresponding epitope if the site has been previously occupied by the antibody in the test serum.

ELISAs detecting antibodies to viral components that are present in wild-type viruses, but not in vaccines, can be used in a number of ways for the differentiation of infected and vaccinated animals. If highly purified forms of inactivated vaccines are used, they will elicit antibodies directed against the viral structural proteins present in the vaccine, but not antibodies against certain non-structural proteins, which are only made during virus replication (following infection or use of a live vaccine) (Bergmann *et al*., 1993; Brocchi *et al.,* 1998; Lubroth *et al.,* 1998; Mackay *et al*., 1998a). Alternatively, live vaccines may be used which lack certain proteins or epitopes that are non-essential for replication (Quint *et al*., 1987; Mettenleiter *et al*., 1994), or in which particular proteins or epitopes have been substituted by distinguishable counterparts originating from a different virus (de Smit *et al*., 2001).

As with any diagnostic test, there is always a trade-off between sensitivity and specificity. Whereas some antibody detecting ELISAs are quite sensitive, they may suffer from an unsatisfactory specificity, requiring the use of labour-intensive secondary tests for confirmatory purposes. In general however, currently available ELISAs are still considered less sensitive than virus neutralisation tests (VNT), which are often considered as gold standards. However, comparison of ELISA and VNT titres is problematic, since the ELISA may be designed to detect any antibody, including isotype-specific antibodies, whereas the VNT measures any neutralising antibody

irrespective of its isotype. Furthermore, VNT does not discriminate between infected and vaccinated animals.

Future developments are likely to aim at increasing specificity as well as sensitivity, improving differentiation of infected and vaccinated (conventional or marker vaccines) individuals, and streamlining the test procedure, e.g. by using novel techniques such as fluorescence readout for detecting the antigen-antibody binding (Komatsu *et al*., 2002), and applying a homogeneous assay format which would simplify automation.

5.5.1.2. ELISA for antigen detection

Typically, ELISA for antigen detection involves a double antibody sandwich (Ferris and Dawson, 1988). The trapping antibody is used to coat a solid support and captures any viral antigens present in the sample. The second antibody is used to detect the presence of bound antigen by catalysing a colour change. This is brought about by means of an enzyme such as horse-radish peroxidase which is linked to the second antibody either directly or via a third anti-antibody. Alternative formats are possible, for example measuring the ability of sample antigens to compete with *in vitro* prepared enzyme-labelled viral antigens for binding to an appropriate capture antibody.

Antigen detection by ELISA is increasingly used as an alternative or adjunct to virus isolation and immunostaining of organ sections. ELISAs are rapid and can be completed within half a day. In the laboratory they can be readily automated to enable high sample throughputs. The samples can be treated at an early stage to inactivate live virus and reduce the need for biohazard containment. Monoclonal antibodies and more recently recombinant antibodies have been introduced to increase or tailor the sensitivity, specificity and reproducibility of the tests. Tests for different virus proteins can be used to provide independent confirmation of positive results. The main drawback to the techniques is that they are generally less sensitive and specific than those based on amplification of live virus or viral nucleic acids. Therefore, confirmatory tests are often required, particularly in the case of primary disease outbreaks. Further simplification and standardisation of the assays is possible.

5.5.2. Chemiluminescent and fluorometric immunoassay procedures

ELISAs employ various enzyme labels and chromogenic substrates to produce a colour reaction that indicates the presence of the target analyte. A number of variations on this approach are possible; for example substrates can be used that become fluorescent or chemiluminescent rather than coloured (Rongen *et al*., 1994; Hengen, 1997), or enzyme labels can be replaced by radioisotopes or fluorescent dyes (Schmidt, 1999; Cortese, 2000). Due to their considerably higher (about 100-1,000-fold) sensitivity, fluorescent and chemiluminescent substrates are replacing chromogenic substrates for applications such as immunoblots and solid phase nucleic acid hybridization techniques. In contrast, despite the growing availability of microplate luminometers, few ELISAs are on the market that use fluorometric or chemiluminescent substrates for the detection of enzyme-labelled antibodies. They are mainly designed for the detection of substances

like TNFα (tumour necrosis factor alpha), human gamma interferon and some human interleukins. Recently, a luminescence immunoassay for the diagnosis of bovine spongiform encephalopathy (BSE) using an HRP (horse-radish peroxidase) labelled antibody has been described (Biffiger *et al*., 2002). The use of 1,2-dioxetane chemiluminescent substrates with AP (alkaline phosphatase) enzyme labels provides highly sensitive detection for numerous immunoassay and nucleic acid detection formats. Current applications include chemical compound screening (Olesen *et al*., 2000), membrane-based detection of proteins and nucleic acids, immunoassays, microplate-based nucleic acid detection and, increasingly, array-based detection (Olesen *et al*., 2001).

### 5.5.3. Other immunoassays

Various non-enzymatic immunoassays have been developed to detect the interaction of viral components with a ligand such as a monoclonal antibody. A very simple approach is the chromatographic strip test, which has already been described for the on-farm detection of rinderpest and FMD (Bruning *et al*., 1999; Reid *et al*., 2001). In the strip test, tissue suspensions are prepared 'pen-side' by simple macerating devices and applied to a diffusion membrane impregnated with antibody-coated microparticles. Specific viral antigens present in the sample are bound by the antibody-coated particles and subsequently cross-linked by a line of immobilised antibody to form a visible reaction line. The whole process can be completed within fifteen minutes and similar principles are applicable to the development of on-farm antibody tests and a test for the detection of either FMD virus or antibody is close to being marketed. However, there have been no field trials performed to validate these tests. Another simple and rapid approach that has been available for many years is latex agglutination (Haikala *et al*. 1983; Al-Yousif *et al*. 2001). These types of rapid tests would be particularly useful for confirming secondary cases of exotic diseases.

In the future, colorimetric protein microarrays could become available to enable test sera to be examined simultaneously for the presence of antibodies to a range of different viruses and/or virus serotypes. For this purpose antigens (i.e. proteins or peptides) are fixed to solid supports and allowed to react with test sera. Antigen-antibody reactions are subsequently visualised using specific antibodies and chromogenic substances.

The term 'biosensor' is increasingly used to describe any analytical device incorporating a biorecognition system, of varying simplicity or sophistication, which is developed by interdisciplinary research involving physicists, engineers and other scientific fields. Biosensors rely on a wide range of approaches for detecting the specific interaction of a viral component and a ligand (often an antibody), which may be based on optical, electrochemical, thermometric, piezoelectric or magnetic principles (Morgan *et al*., 1996). There is the potential for the development of both laboratory-based and on-site applications and for the simultaneous detection of multiple pathogens, but further hardware developments are necessary. There is increasing interest in the use of biosensors, for example in clinical medicine and environmental monitoring, and their possible veterinary applications require further research. This is a very active area of high technology research, and considerable progress can be expected in the near future. At

present, methods for immediate and easy use in veterinary virology are not yet available and only preliminary reports have been published exploring such applications for veterinary purposes (Uttenthaler *et al*., 1998; Ditcham *et al*., 2001; Gajendragad *et al*., 2001; Feliu *et al*., 2002; Gomes and Andreu, 2002). Public sector support would facilitate the transfer of such technology to veterinary applications, since the industrial companies developing the technology are not likely to consider control of exotic animal disease viruses as an economically viable investment for them. If available these techniques would have an enormous advantage over existing methods with respect to the very high degree of automation and throughput possible.

## 6. STANDARDISING AND VALIDATING NEW TECHNIQUES

### 6.1. Current approaches

Various international guidelines for validation and standardisation of diagnostic methods exist, although the importance of these steps has sometimes been overlooked and further progress is needed on their general/widespread application. Furthermore, slow progress on development of reference standards has delayed the introduction of new techniques into routine use. A key stage in the development of a validated test is the definition of its planned usage and there is often a trade off between sensitivity and specificity (see Annex). High sensitivity is of paramount importance in the individual certification of animals for international trade, whereas specificity is important for large-scale, herd-based serosurveillance. In the case of surveillance the choice of the test's characteristics of either high sensitivity or high specificity may depend upon whether one is seeking to determine prevalence in an infected population, or to demonstrate freedom from infection following a disease control campaign.

Jacobson (1998) has provided a comprehensive description of the processes of validation for serological assays and defined assay validation in five stages:

- Step 1: feasibility (establish the basic technical procedures);
- Step 2: development and standardisation (standardise reagents, determine analytical sensitivity and specificity);
- Step 3: characterisation (establish reference samples, calculate precision and accuracy, determine diagnostic sensitivity and specificity);
- Step 4: monitoring (evaluation in use on the target population, determination of prevalence and predictive values);
- Step 5: maintenance and extension (ongoing assessment, interlaboratory proficiency testing, production and calibration of replacement reagents, extension to other populations).

In addressing and implementing these validation processes, the importance of steps 4 and 5 are crucial, ensuring that validation is seen as an ongoing

process that makes use of data from the routine application of the test. These parameters should always be applied as part of a quality assurance system.

The Office International des Epizooties (OIE) has developed, based on Jacobson (1998), an international standard for test validation as a chapter in its Manual of Standards for Diagnostic Tests and Vaccines (OIE, 2000), which has been formally adopted by the International Committee of the OIE. A version of it is also available separately as one of a series of OIE Guidelines (http://www.oie.int/eng/publicat/ouvrages/A_112.htm).

Many of the principles Jacobson described may be extrapolated and adapted to other types of assays such as those for antigen or nucleic acid detection. Belak and Thoren (2001) have begun to address this with respect to PCR, although there remains a need for a similarly definitive treatise on these topics. The OIE Standards Commission is planning to address this through a new chapter for the next edition of its Manual of Standards for Diagnostic Tests and Vaccines. Compared to the validation of serological tests, there is often a practical difficulty in obtaining sufficient samples of known positive or negative status. There is also a very real problem regarding defining what is meant by an ideal 'gold standard' test to determine the animal's true status. Given such difficulties, the outcome will be that estimates may be made for diagnostic sensitivity and specificity, but the confidence limits of those estimates will be much wider than if larger numbers of samples could be used for the validation. In this respect, it is important to recognise that deliberately contaminated 'spiked' samples, while useful for setting up the test in the first place and determining analytical sensitivity and specificity (step 2 above), should not be used for the full validation (step 3 of the process). The OIE Manual also provides a series of "prescribed tests" for individual diseases that are applicable to screening animals prior to international movement or trade. The international regulations that determine how prescribed tests are to be used are found in the companion OIE International Animal Health Code, which is updated annually (OIE, 2002). The principles underlying the definition of prescribed tests, as outlined in the OIE Manual, are that:

- they should be **validated** (although it is accepted that many historical tests lack formal validation data but are accepted on the basis of long experience in their use);

- the Manual should provide a **standard protocol** with sufficient information to enable any competent laboratory to carry out the test;

- diagnostic laboratories should calibrate the assay in their own laboratory using international **reference materials**;

- participation in **proficiency testing** (interlaboratory ring trials) is recommended where possible.

Laboratories need to perform well in ring tests and comply with quality assurance requirements. A second category of tests is referred to by the OIE as "alternative tests" for use in international trade. These include newer tests that show promise but are still lacking full validation, and in some cases older tests that are less than ideal but may be used by laboratories that lack

sophisticated equipment or other resources to provide high-tech services. It should be noted that the OIE Standards Commission regularly reviews the lists of prescribed and alternative tests and updates it in the light of new developments.

The OIE Manual describes many tests other than the "prescribed" and "alternative" lists. Some of these are well validated and are widely used, but are not relevant to international trade screening. The Standards Commission is planning to do further work on defining the preferred tests for purposes other than international trade, and the next edition of the Manual should provide better explanations of which tests are applicable to what purpose. For example, RT-PCR may be appropriate for confirming secondary disease outbreaks, but on its own may not be sufficient for diagnosing a primary case in a previously disease-free country. The categories would include, as well as international trade, diagnosis of suspect cases and surveillance. The role of "marker" tests for use in conjunction with DIVA vaccines will also need to be considered for specified diseases.

Where tests are considered adequate for herd level surveillance, e.g. non-structural protein (NSP) tests for FMD, but not for individual animal certification, it will be important to define what is meant by 'herd'. The key point is not so much the ownership of the animals but the extent to which they have contact with one another and constitute an epidemiological unit. For very small herds it may be necessary to consider wider populations of contiguous herds in order to include enough animals to have confidence in the significance of test results.

For serology, some progress has been made in the establishment of international reference sera, which can be used both to benchmark new tests and as performance checks on existing tests. The OIE has defined a basic minimum of three reference sera for any given disease (one negative, one weak positive and one strong positive). The rationale for this was described by Wright (1998) and again a set of guidelines is available (Anon, 1998a, OIE, 2002). For diseases such as FMD with multiple serotypes, such a set is required for each serotype, as well as specialised materials for use with companion tests to DIVA vaccines. Ideally reference sera derived from each of the main host species would be preferable, but this can multiply the task beyond what is realistically achievable. OIE has established the preparation of such reference standards as one of the tasks of its reference laboratories and those already adopted by OIE are listed on its website (www.oie.int). Unfortunately progress has been very slow, and reference materials are still not available for many important diseases. This is largely due to funding constraints rather than to any technical difficulty with the task. WHO has developed more elaborate guidelines for the preparation and establishment of Reference Materials (WHO, 1978). These can be regarded as a "gold standard" to which all should aspire, however the OIE has adopted a less onerous approach, recognising the resource constraints under which veterinary laboratories operate. There is also a more generic guide produced by ISO (ISO, 2000).

In addition to the basic set of international reference standards, the development of larger serum panels that represent a range of commonly found reactivities and cross-reactivities is required. These could be usefully

applied to international ring trials, organised by international reference laboratories, as well as providing additional information for the validation of new tests. OIE has issued guidelines on proficiency testing (Anon, 1998b; OIE, 2002), which are essentially based on the ISO Guide 43 (OIE, 1997).

Within the OIE Manual, new detection methods based on PCR technology are described for the detection of various infectious agents, including FMDV, CSFV and AIV, and in at least one case, bluetongue (BT), the RT-PCR is a prescribed test for international trade. As mentioned above, guidance is being developed on which situations these tests are suitable for. The OIE does not accept new tests into the Manual unless there is a reasonable consensus among international experts that they have attained at least a basic level of validation. However, there is a need to clarify what precisely this level of validation is. There are, as yet, no reference standards available to specify the minimum level of agent that should be detectable, whether by culture, immunoassay, or nucleic acid detection, and this can pose a difficulty with tests such as PCR that do not detect live organisms. An essential first task, which OIE is currently engaged in, is to draft guidelines for preparation of reference materials for this type of assay. Preparation of such standards could then be performed by an international reference laboratory. It should also be noted that even where such international designated reference materials exist there can be difficulties transporting them if they contain potentially viable organisms. Due to such obstacles in validation and acceptance there are uncertainties/difficulties regarding the application of RT-PCR.

Validation of a test such as RT-PCR involves two separate phases. In the first instance, the analytical sensitivity and specificity of the particular method need to be demonstrated. This must include the initial selection of primers and demonstration that they will reliably detect all strains of the organism likely to be circulating and this needs to be kept under review as new strains emerge. Secondly, it must be shown that the system of routine operation of the test is such that false negative or false positive results are minimised and that sufficient controls are in place to recognise any such results that may occur. Diagnostic manuals need to give guidelines on how such quality control is to be achieved. The main control on false positives, apart from use of negative control materials, is to maintain the most rigorous standards of laboratory practice to avoid cross-contamination. False negatives are most likely to arise (apart from pipetting errors) from inhibitory effects in the sample. They may be identified by use of internal controls, termed ‘mimics’, which have the same primer-binding characteristics as the target template, but generate a fragment of different size. For real-time PCR methods an internal control can be a suitable fragment of host animal genome which is detected by a specific colour reaction.

Robotic systems of sample preparation and test set-up are important new developments to minimise test variability and cross-contamination, but again there is little precedent on how to validate their use. Incorporation of the finalised procedure into an externally accredited test portfolio is highly desirable. This requires measures such as the development of detailed protocols to specify controls, internal test validation and interpretation

criteria, the provision of adequate facilities and adequately trained personnel and participation in external quality assurance exercises ('ring tests').

It is important that a system is developed to allow specified diagnostic tests to be scientifically evaluated and their use approved. To some extent this already happens for those diseases where tests are described in Appendices to Directives, or in Official Diagnostic Manuals (e.g. CEC, 1992a,b). In those cases a standard protocol is set out, but its performance should be evaluated on a regular basis by interlaboratory ring trials, as well as provision of officially designated standard reference materials. Failure by an individual laboratory to achieve the required standard of testing will require appropriate responsive action to resolve the variation.

As discussed above, standardisation is relatively straightforward for serology assays. However, this is more difficult for detection of live agents, or antigen in tissue sections or other materials, due to the difficulty in producing batches of uniform materials for distribution, as well as the expense and restrictions on international shipment of infectious materials. Proficiency testing for virus detection (cell culture/antigen detection/ nucleic acid detection) needs to be carried out, but the results are required to be assessed on a qualitative basis (positive/negative) due to the large number of confounding variables that could affect quantitative results.

International Reference Materials, where they are available, need to be used to ensure that test performance in laboratories meets the required criteria. Provision of working standards (calibrated against International Reference Materials) and conducting proficiency testing for participating laboratories are also important elements to be considered. These materials must also be available to regional laboratories in countries where testing is devolved from the national reference centre.

A more generic approach that can provide additional quality assurance is to require that laboratories be accredited to a third party quality assurance scheme. The most appropriate one for testing laboratories is ISO 17025:1999, or the OIE interpretation of that standard for veterinary laboratories for infectious diseases (OIE, 2002) and laboratories should aim to comply with these standards.

A system is needed to approve the use of commercial diagnostic kits for the diseases under discussion, including evaluation of their conformity with official guidelines (such as those of the OIE, ISO or WHO), the reproducibility of kit performance between laboratories and inter-batch repeatability. A procedure similar to that used for the evaluation and validation of alternative test systems for the potency testing of vaccines could be adopted (Hendriksen *et al.*, 1998). These guidelines propose five main stages in the evaluation process: (i) test development, (ii) in-house prevalidation, (iii) validation involving a formal inter-laboratory study, (iv) independent assessment by external expert panels from international organisations, and (v) acceptance and implementation by regulatory authorities. Such a procedure would become even more necessary if widespread use were to be made of 'pen-side' test kits that may be used in field conditions.

## 6.2. The present situation with regard to reference standards and proficiency testing

National reference laboratories (NRLs) are involved in monitoring the application of standards among regional laboratories. In cases where regional laboratories carry out testing for Class A Diseases, the NRL monitors the quality of work performed.

**FMD**

There is no Community Reference Laboratory (CRL) for FMD. The FAO/OIE World Reference Laboratory (WRL) for FMD in Pirbright has been involved in the production of reference standards and distribution of interlaboratory comparative test materials, which have so far been used only for serological standardisation. There is also a network of OIE Reference Laboratories that work *inter alia* on test standardisation, preparation of reference standards etc. They can be useful sources of local epidemiological information for FMD as well as other diseases.

The FAO European FMD Commission (EUFMD) has supported an initiative on harmonisation of FMD serology for many years (Kitching *et al*., 2000). This has been coordinated by the WRL and has involved:

- Preparation of primary reference standards by the WRL and comparative testing to agree on their validity.

- Test calibration using primary standards and preparation of secondary and tertiary reference standards by NRLs and their subsequent use as internal test standards.

- Proficiency testing on unknown test serum panels.

A set of bovine reference sera for FMD serotypes O, A and C have been adopted by the OIE. For each serotype these include a strong, weak and cut-off positive and there is also a single negative reference standard. It is planned to extend the range of serotypes covered and possibly the species of host from which the sera are derived. It is desirable that the standards could also be applicable to NSP tests, and this requires that they should be derived from infected rather than vaccinated animals. Within the EU there are proposals for a consortium of laboratories to develop a more extensive collection of reference sera.

There is a growing acceptance of the fact that different test thresholds are appropriate for different situations. For example, individual animal certification for international trade requires a lower cut-off threshold than herd-based serosurveillance. Consequently, there is a need for different reference sera to define these thresholds (Paton *et al*., 2002).

The WRL is engaged in a wide range of other activities including storage and distribution of viruses and diagnostic reagents, training, molecular epidemiology, etc.

**CSF**

The duties of the CRL at the School of Veterinary Medicine Hannover are specified in Annex IV of Council Directive 2001/89/EC (CEC, 2001a) with functions including:

(a) to coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing CSF, specifically by:

- storing and supplying cell cultures for use in diagnosis,
- typing, storing and supplying strains of CSFV for serological tests and the preparation of antisera,
- supplying standardised sera, conjugate sera and other reference reagents to the national laboratories in order to standardise the tests and reagents employed in the Member States,
- building up and holding a CSFV collection,
- organising periodic comparative tests of diagnostic procedures at Community level,
- collecting and collating data and information on the methods of diagnosis used and the results of tests carried out,
- characterising isolates of the virus by the most up-to-date methods available to allow greater understanding of the epizootiology of CSF,
- keeping abreast of developments in CSF surveillance, epizootiology and prevention throughout the world,
- retaining expertise on the virus causing CSF and other pertinent viruses to enable rapid differential diagnosis,
- acquiring a thorough knowledge of the preparation and use of the products of veterinary immunology used to eradicate and control classical swine fever;

(b) to make the necessary arrangements for training or re-training experts in laboratory diagnosis with a view to harmonising diagnostic techniques;

(c) to have trained personnel available for emergency situations occurring within the Community;

(d) to perform research activities and whenever possible coordinate research activities directed towards an improved control of CSF.

Proficiency testing has routinely been performed for approximately the last 15 years for Member States. During the last decade a similar annual inter-laboratory proficiency testing has been set up for EU Accession Candidate Countries and other Central and Eastern European Countries in cooperation with the OIE reference laboratory for CSF in Pulawy, Poland. Results of the proficiency testing are reported to the CRL and they are evaluated and

discussed at Annual Meetings of National CSF Reference Laboratories. These exercises have greatly improved the quality of CSF diagnosis in Member States and Candidate Countries.

Before the general introduction of new diagnostic techniques, such as PCR, workshops are held at the CRL in order to improve standardisation.

**AI**

The functions and duties of the CRL at the Veterinary Laboratories Agency (VLA) Weybridge are specified in Annex V of Council Directive 92/40/EEC (CEC, 1992a) and include:

(a) to coordinate, in consultation with the EC Commission, the methods employed in the Member States for diagnosing AI. Specifically by:

- typing, storing and supplying strains of AIV for serological tests and the preparation of antisera,
- supplying standard sera and other reference reagents to the National Reference Laboratories in order to standardise the tests and reagents used in the Member States,
- building up and retaining a collection of AIV strains and isolates,
- organising periodical comparative tests of diagnostic procedures at Community level,
- collecting and collating data and information on the methods of diagnosis used and the results of tests carried out in the Community,
- characterising isolates of AIVs by the most up-to-date methods available to allow greater understanding of the epizootiology of AI and to gain an insight into the epizootiology of the virus and the emergence of highly pathogenic and potentially pathogenic strains,
- keeping abreast of developments in AI surveillance, epizootiology and prevention throughout the world,
- retaining expertise on AIV and other pertinent viruses to enable rapid differential diagnosis,
- acquiring a thorough knowledge of the preparation and use of the products of veterinary immunology used to eradicate and control AI.

(b) to actively assist in the diagnosis of AI outbreaks in Member States by receiving virus isolates for confirmatory diagnosis, characterisation and epizootiological studies. In particular, the laboratory should be able to carry out nucleotide sequencing analysis to allow determination of the deduced amino acid sequence at the cleavage site of the haemagglutin molecule of AIVs of H5 or H7 subtpye,

(c) to facilitate the training or retraining of experts in laboratory diagnosis with a view to the harmonisation of techniques throughout the Community.

Sessions on proficiency testing and 'reagents' are included at an annual meeting of National AI laboratories. These discussions include identifying other aspects of testing that may require some occasional exercises for proficiency testing, i.e. antibody detection assays as well as analysing the annual test for virus characterisation.

## 7. SOME ADVANCES IN THE DEVELOPMENT OF VETERINARY VACCINOLOGY

Since the 1980s many new approaches have been applied to improve existing conventional vaccines and to develop novel vaccines. The rapid progress made in biotechnology has also enabled researchers to design a broad range of different biotechnological candidate vaccines. As with classical vaccines, two main categories can be distinguished: vaccines that replicate in the host and those that do not replicate (see Table 7).

**Table 7. Types of candidate vaccines**

| Non-replicating | Replicating |
|---|---|
| Sub-unit vaccines i.e. ISCOMS<br>Recombinant subunit vaccines produced in bacteria, yeast, baculovirus, cells<br>Peptide vaccines<br>DNA vaccines<br>Vector vaccines e.g. avipox, adenovirus | Attenuated (deletion) mutants<br>Reassortants e.g. influenza virus<br>Vector vaccines e.g. poxvirus, herpesvirus* |

* Some poxvirus, herpesvirus and adenovirus vectors replicate and produce infectious progeny virus, while others do not

A trend is noticeable of gradually moving towards the use of non-replicating vaccines, because of safety issues. In the framework of this report so called 'DIVA' (Differentiating infected from vaccinated animals) or marker vaccines deserve special attention. These were first recognised and developed in conjunction with a companion differential ELISA for use in the control of Aujeszky's disease in the 1980s (van Oirschot *et al*., 1986; Quint *et al*., 1987). It was subsequently demonstrated that FMD vaccines can act as DIVA vaccines since they contain no, or low amounts of, NSP and thus do not stimulate the production of antibodies to NSPs (Neitzert *et al*., 1991). In the 1990s DIVA vaccines against infectious bovine rhinotracheitis (van Oirschot *et al*., 1996) and CSF (Lütticken *et al*., 1998; Moormann *et al*., 2000) were developed. Vaccines have primarily been developed to prevent disease and in addition vaccination often reduced the replication of the virus when vaccinated animals became infected. This and other effects of vaccines may result in a lower spread of field virus in a vaccinated population, an effect of major importance when emergency vaccination is applied. In transmission experiments the level of this so-called herd immunity have been semi-quantified for various DIVA vaccines (De Jong and Kimman, 1994; Bouma *et al*., 2000). Various approaches to vaccine development as well as biotechnological vaccines already being marketed have been considered in some recent reviews (van Oirschot, 2001; Babiuk, 2002; Babiuk *et al*., 2002).

## 8. FOOT-AND-MOUTH DISEASE

FMD is a highly contagious viral infection of cattle, pigs, sheep, goats, buffalo, and artiodactyl wildlife species. FMDV is a member of the genus *Aphthovirus* in the family *Picornaviridae*. They are small viruses (27–30 nm in diameter), non-enveloped with an icosahedral capsid and contain a single strand of positive sense RNA about 8.5 kilobases in size. The FMDV genome of 8,400 nucleotides encodes for four structural proteins (SP), such as VP1, which form the virion, as well as for NSPs such as polyprotein 3ABC and 3D, which play an important role in virus replication- 3D acting as viral polymerase (Sobrino *et al*., 2001; Mason *et al*., 2003). There are seven serotypes of FMDV, namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals and infection with one serotype does not confer immunity against another serotype. Within the FMDV serotypes, over 60 subtypes have been described, and new subtypes occasionally arise spontaneously. Therefore, a number of vaccine strains for each serotype, particularly O and A, are required to cover the antigenic diversity. Today, FMDV strains are characterised by their genomic relationships and their antigenic similarities with established vaccine strains.

FMDV produces an acute, systemic vesicular disease and the main route by which animals naturally become infected is via the respiratory tract or through ingestion of the virus (Kitching, 2002a; Kitching and Alexandersen, 2002; Kitching and Hughes, 2002). Initial virus replication usually occurs in the pharyngeal epithelium manifesting in the formation of primary vesicles. Fever and viraemia can occur within 1-2 days after infection resulting in virus excretion from the respiratory tract, faeces, urine, saliva, milk and semen. The virus, which enters the blood circulatory system, disseminates to various organs and tissues, establishing itself into predilection sites to develop secondary vesicles in the mouth and nose, hooves and also sometimes teats and udder, as a result of infection of the cells of the epithelial stratum espinosum. Secondary infections are not uncommon, particularly in the feet, causing chronic lameness and delayed healing. An acute phase lasts for approximately a week receding in the face of a mounting humoral response. Mortality can occur in young animals, often through infection of the myocardium. Vesicles harbour the multiplying virus, and when they rupture virus particles are released to enhance further spread.

Cattle, sheep and goats are very susceptible to FMDV infection via the respiratory route, with as little as 20 tissue culture infectious doses 50% ($TCID_{50}$) required to establish an infection. Pigs are considerably less susceptible to aerosol infection: they may require as much as 600 times more $TCID_{50}$ of virus than ruminants to become infected. Infected cattle can produce up to 5.1 $\log_{10}$ $TCID_{50}$ of aerosol virus per day and also shed high titres of virus in milk, semen, urine and faeces. Infected sheep produce considerably less aerosol virus than cattle, but pigs produce more aerosol virus than cattle. The aerosol production of infectious FMDV by pigs differs considerably per virus strain (Donaldson and Alexandersen, 2002; Kitching 2002a,c; Kitching and Alexandersen, 2002).

An asymptomatic persistent infection is a common sequel following the exposure of ruminants to the FMDV (Salt, 1998). Where persistence continues beyond 28 days after the initial exposure it is termed the ‘carrier state’. A similar proportion of ruminant animals exposed to FMDV have been shown to become carriers, regardless of whether or not they have been vaccinated, and without ever having necessarily developed any clinical disease. The titre of carrier virus in the oesophageal-pharyngeal region is low, intermittently recoverable, and declines over time. The longest recorded carriage has been for 5 years in buffalo, more than 3 years in cattle, 12 months in sheep and 4 months in goats (Salt, 1998). Pigs have never been shown to become carriers (Sutmoller and

Casas Olascoaga, 2002), although evidence of the FMDV genome, but not live virus, in serum has been reported (Mezencio *et al.,* 1999) while others have never found evidence for this (Kitching, 2002c). The carrier state frequently occurs in vaccinated ruminants and there is a perceived low risk that such animals could transmit disease to susceptible livestock. There are several, mainly anecdotal, reports of carriers being responsible for field outbreaks of FMD (Salt, 1998), but in Europe and South America new cases of FMD could never be linked to the existence of carriers (Sutmoller and Casas Olascoaga, 2002). In addition, conclusive evidence of spread has not been demonstrated under controlled, experimental conditions (Sutmoller and McVicar, 1972; Kitching, 2002b). Nonetheless, the potential risk is sufficient to have had a major impact on international trade in livestock and their products and on the decision whether or not to use vaccines to assist in the control of an FMD outbreak. A number of EU funded research projects[2] are underway regarding the development of improved methods for the diagnosis of FMD (FAIR-CT98-4032) investigating the molecular basis of tissue tropism and persistence (QLK2-CT-2002-01719), optimising DNA based vaccination against FMDV in sheep and pigs (QLK2-CT-2002-01304) and biosafe coronavirus vector-based vaccine for the prevention of FMD (QLK2-CT-2002-00825).

## 8.1. Diagnostic methods that are currently used

### 8.1.1. Investigation of Suspect Cases

Primary outbreak

Animals showing clinical signs suspicious of FMD may be investigated by means of a variety of diagnostic tests for the presence of the FMDV or of FMDV-specific antibodies (OIE, 2000). If there are vesicular lesions then samples of epithelium are collected and submitted to a laboratory for virus detection. In the absence of vesicular lesions, samples of blood, milk, bone marrow or oropharyngeal fluids (probangs) may be submitted for virus detection. Epithelial samples are examined by antigen detection ELISA (Ag ELISA) and virus isolation, whilst other samples are examined by virus isolation. Successful virus isolation is recognised by the appearance of characteristic cytopathic effects, which can be observed in susceptible cell cultures inoculated with virus-containing suspensions. Where a cytopathic effect is observed, Ag ELISA is used to confirm that it is due to FMD virus. Whereas antigen ELISA can be completed within 4 hours, up to 4 days is necessary before a sample can be confirmed negative by virus isolation. The Ag ELISA procedure also identifies the serotype of FMD virus involved and, in the case of pig samples, provides a differential diagnosis for swine vesicular disease virus. Although much quicker than virus isolation, Ag ELISA has a relative sensitivity of only 70-80% (perhaps less in sheep) and cannot be applied to samples other than vesicular epithelia (Reid *et al*., 2002). It is therefore evident that neither Ag ELISA nor virus isolation is wholly satisfactory, since the former lacks sensitivity and cannot be applied to all sample types, whilst the latter is slow and labour-intensive to perform.

Once a positive virus identification has been made, partial genomic sequencing and antigenic analysis with reference antisera can be used to further characterise the strain of virus involved in a primary outbreak and

[2] Details of EU funded research projects may be found at the website http://www.cordis.lu/en/home.html

provide useful information on the likely origin of the outbreak. Antigenic analysis in *in vitro* and *in vivo* tests guide the selection of an appropriate vaccine.

If it is suspected that animals may have been infected some time previously, then blood samples are examined for the presence of FMDV-specific antibodies (IgG), which appear from around 5 days after infection. The most sensitive serological tests are serotype-specific and take the form of VNTs or ELISAs. ELISA has the advantage of being simpler and quicker and not reliant on the presence of live virus. It may be used as a screening test with confirmation of doubtful or positive results by VNT.

In the case of a suspected primary outbreak, in a previously disease-free region or country, the widest possible range of tests are used for confirmation, although secondary outbreaks may be confirmed without using the full range of available tests.

Follow-up outbreak

8.1.2. Tracing

Epidemiological tracing is used to identify herds that might have had some form of contact with an FMD-infected premises. If no disease is present this might be due to animals having already recovered or being still in the pre-clinical, incubating stage of the disease. Serological surveillance is carried out to look for virus-specific antibodies, indicative of convalescence, whilst blood or milk are analysed for the presence of a silent or early viral infection. Whereas ELISA readily accomplishes large-scale serological surveillance, neither virus isolation nor Ag ELISA is suited to large-scale pre-clinical diagnostic testing, the former being too labour-intensive and the latter lacking sensitivity.

8.1.3. Freedom of Infection

Ruminants that recover from FMD can carry the virus for many months in their oropharynx. The epidemiological significance of such animals is controversial since, despite some field evidence to the contrary, transmission of virus from carriers has not been demonstrated experimentally using domestic livestock species. Serological surveillance is used to screen populations within or outside control zones, in order to demonstrate that no virus-infected animals are still at large. Since very large numbers of animals may have to be screened, it is important to have tests that can be automated and have a high specificity. Confirmatory tests are needed to deal with inconclusive reactors.

Following a ‘protective’ vaccination strategy (where animals are not required to be subsequently culled, ‘vaccinate-to-live’ policy), it is necessary to be able to detect carrier or potential carrier animals amongst a vaccinated population. This is problematic since the virus is present in carriers at a very low level in the oropharynx only. Furthermore, there is no reliable serological difference between carrier and fully recovered animals. Direct detection of carriers is usually accomplished by collecting oropharyngeal samples (using a probang) and using virus isolation to look for the presence

of virus. Vaccinated animals that have been exposed to infection may be detected serologically using ELISA for antibody to NSP. This exploits the fact that only replicating virus will induce antibodies to NSPs, as opposed to inactivated virus.

## 8.2. New and emerging diagnostic techniques

RT-PCR and real time RT-PCR offer the potential of both rapid and high sensitivity testing for virus in a wide range of sample types, including lesion materials, probang fluids and milk. These methods can therefore be applied to all categories of virological diagnosis. New advances in RNA extraction techniques enable robotic handling within the laboratory to achieve contamination-free scale-up and the prospect of using portable systems for on-farm or near-farm use. Since the sample material can be treated to inactivate live virus, without affecting RT-PCR, the method could be used in regional laboratories that do not have full containment facilities. The high sensitivity of RT-PCR makes it applicable to preclinical diagnosis and detection of carriers. Multiplex RT-PCR would facilitate differential diagnosis at the same time as checking for FMDV. Microarrays would offer the ultimate flexibility for differential diagnosis.

On-farm antigen detection is possible using existing assays and will be further facilitated by new biosensor technologies. Chromatographic strip tests have been developed offering sensitivity equivalent to laboratory-based antigen ELISA, but giving results in the field within fifteen minutes (Reid *et al*., 2001). Similar devices can also be configured for the detection of antibodies.

New types of ELISA based on homogeneous assays (without a solid phase) may be used for FMD serology in the future. These types of ELISA systems are more suited to performance by robots than conventional ELISAs with multiple plate washing steps.

## 8.3. Types of vaccine

Both conventional and emergency vaccines are generally manufactured by the same basic methodology, as outlined in the OIE Manual (OIE, 2000). They are based on cell culture derived preparations of whole virus, which are chemically inactivated by the use of aziridines such as binary ethylenimine (Bahnemann, 1990; Barteling and Vreeswijk, 1992), and blended with a suitable adjuvant or adjuvants. Typically, vaccines formulated with the adjuvants aluminium hydroxide and/or saponin provide protective immunity in the three main ruminant species (cattle, sheep and goats), but are poor at conferring a similar response in pigs. However, mineral oil adjuvanted vaccines, in various emulsification forms, which were successfully developed for use in swine, afford protection in all target species. FMD vaccines can be monovalent, i.e. formulated to contain one virus strain that is, antigenically, as closely related to the field virus as possible. However these vaccines are frequently multivalent, including viruses of different serotypes. There is a constant requirement to monitor contemporary outbreak strains of FMDV, to check the suitability of the available vaccines and to identify the appearance of antigenically novel strains (Barnett *et al*., 2001).

## 8.4. Efficacy

The efficacy of any given FMD vaccine is established by quantifying its ability to protect against specific clinical signs of the disease, principally the prevention of lesions at the secondary sites (feet) following challenge. This is calculated as a potency of the vaccine and is expressed as the number of 50% cattle protective doses ($PD_{50}$) contained in the dose stated on the vaccine label (European Pharmocopoeia, 1997). To evaluate the potency of a specific strain of FMDV in a vaccine in accordance with current European regulatory and licensing requirements, groups of 5 cattle, no less than 6 months old, are vaccinated with reduced dose volumes of vaccine. Two unvaccinated individual control animals are also challenged 21 days later with an inoculation on the surface of the tongue of 10,000 $ID_{50}$ virulent bovine FMDV of the same type and subtype as the one contained in the vaccine. Animals are then closely monitored for 8 days for the appearance of FMD lesions on the feet and mouth. The proportion of animals vaccinated with all dilutions that do not succumb to a generalised infection and develop lesions is used to calculate the potency of the vaccine by the Kärber method (Kärber, 1931) or a similar technique. Potency is not synonymous with efficacy (in the European Pharmocopoeia only potency testing is described), efficacy being defined in clinical epidemiology "as the extent to which a specific intervention, procedure, regimen, or service produces a beneficial result under ideal conditions; the benefit or utility to the individual or the population of the service, treatment regimen or intervention" (Last *et al*., 2001). Potency is strictly meant as an indicator of the quality/efficacy of the vaccine and as such is a test for its ability to initiate immunity. The FDA defines potency as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" (FDA, 1998). Although not currently endorsed by the monograph there have been circumstances in which potency has been assessed using other species such as pigs.

Numerous research experiments have also been conducted to evaluate the efficacy of emergency FMD vaccines at protecting against disease at earlier time points following vaccination, using infected pigs to simulate indirect contact (airborne) (Barnett and Carabin, 2002). These experiments conclude that for the 3 species tested, cattle, sheep and pigs, the vaccines could be effective in protecting against clinical signs of disease within 4 to 5 days of vaccination under an indirect aerosol challenge under experimental conditions.

Whilst efficacy is only measured by the ability of the vaccine to protect against clinical signs, no allowance is made for any ability of the vaccine to prevent virus infection and local replication in the oropharynx. Although there is evidence to suggest that conventional FMD vaccination of cattle can reduce post-infection virus excretion (McVicar and Sutmoller, 1976; Sellers *et al*., 1977; Doel *et al*., 1993) and transmission to susceptible in-contact cattle (Donaldson and Kitching, 1989), there has been no unequivocal experimental evidence that routine FMD vaccination reduces the establishment or the duration of persistent infection. Moreover, it is very important to note that, quite separately from any consideration of the carrier state, transmission can occur from vaccinates to non-vaccinates in direct

contact for a period after vaccination, through the replication and excretion of virus before full immunity develops (Donaldson and Kitching 1989) and through contact with surviving excreted virus which has contaminated the animal or the environment (Bartley *et al*., 2002).

It is not yet clear whether there is a consistent relationship between potency and the ability of emergency FMD vaccines to reduce or prevent viral excretion and persistence. The underlying mechanisms of immunity, including cellular, humoral and mucosal elements, need to be elucidated to provide insights into ways of improving current vaccines. The ultimate objective of such investigations would be the development of vaccines that induce a sterile immunity, i.e. that are capable not only of preventing clinical disease but also infection and the possibly arising carrier state, thus blocking the further transmission of infection. Such a vaccine could dramatically reduce the current restrictions on animal movement and international trading applied in the aftermath of an outbreak of FMD.

## 8.5. Safety

Safety is required to be ensured both during the manufacture of the vaccine and after production of the final product. In addition, there is the need to produce FMD vaccines based on live FMDV under strict biocontainment, to exclude the risk of spreading virus from manufacturing plants.

In-process assessment includes sterility testing, and an *in vitro* test for freedom from infectious FMDV in the inactivated antigen (European Pharmocopoeia, 1997) by inoculation onto sensitive cell cultures. Ultimately however, the final product must be tested *in vivo* to show that it is not unduly toxic and to provide further evidence that it contains only non-infectious FMDV. For the latter it is generally agreed that the *in vitro* test is a more sensitive and reliable measure for the presence of infectious virus than the *in vivo* test, particularly as it accommodates a higher number of doses than the *in vivo* method. In accordance with the current European Pharmacopoeia monograph for FMD vaccines, a group of 3 cattle, no less than 6 months old, are inoculated intradermally in the tongue (while under sedation) at not fewer than twenty points, using 0.1ml of the vaccine at each point and observed for not less than 4 days for any signs of FMD. At the end of this observation period a further 3 doses are administered by the prescribed route and the animals observed for a further 6 days for signs of FMD, reactions at the site of injection or any undue toxicity. However, in recent years this has been superseded by that prescribed in the general monograph on (inactivated) vaccines for veterinary use, thus making allowance for other target species. It is anticipated that in the near future this will be accepted into a revised FMD vaccine monograph.

## 8.6. Quality

A battery of in-process and final product quality control testing is carried out, including: raw material testing, viral identity testing, infectivity assay, 146S assay (intact FMD virions have a sedimentation coefficient (S) of 146, thus the 146S assay is a measure of antigenic mass), inactivation kinetics, sterility, innocuity and potency testing (OIE, 2000). Given satisfactory testing the inactivated, concentrated and purified viral antigen can be

formulated directly as complete vaccine (with either mono- or polyvalent antigen incorporation and either aluminium hydroxide/saponin or oil adjuvants), bottled, and labelled.

In the past 20 years great advances have not only been made in the safety and efficacy of FMD antigens and vaccines available from appropriately licensed manufacturers, but also in their quality. Improvements in the technology for vaccine production have included virus inactivation (Bahnemann, 1990), innocuity testing (Anderson *et al*., 1970), antigen payloads (Doel and Collen, 1982), antigen stability (Doel, 1985), vaccine immunogenicity (Doel *et al*., 1993), antigen purification (Doel, 1999), improved adjuvants (Barnett *et al*., 1996; Barteling *et al*., 1997; Doel, 1999) and better quality control (Pay and Hingley, 1992), all within the framework of a full quality assurance system. Also, the continuous upgrading of plant and equipment has meant that high quality antigens and vaccines are available from those manufacturers that are in compliance with Good Manufacturing Practice (GMP) and pharmacopoeial requirements (OIE, 2000). Furthermore, in recent years more consideration has been placed on the potential contamination of the final product with adventitious agents and the risk of TSE contaminants. Overall, the obstacle has been to ensure the release of a high quality product suitably licensed by the necessary regulatory authority to have full market authorisation.

## 8.7. Emergency vaccine banks

The development of FMD vaccine banks has recently been reviewed (Forman and Garland, 2002). Whereas FMD in disease free countries has traditionally been controlled and eradicated by the stamping-out policy, the potential supporting role of vaccination is recognised. Initially, vaccine banks were composed of bulk reserves of conventional, formulated vaccine. However, the shelf-life of formulated vaccine was no more than 18 months, and constant replacement became expensive. In 1976, Denmark established the principle of storing concentrated FMDV antigen in liquid nitrogen for formulation into vaccine if required. This effectively extended the shelf life of the antigen indefinitely. In 1980, the US established an FMD antigen reserve using this principle, to which Canada and Mexico later subscribed to constitute the North American Vaccine Bank (NAVB), with the intention that if vaccine were needed the concentrates would be formulated in a commercial facility. In 1985 the International Vaccine Bank (IVB) was established at Pirbright by a consortium consisting of the UK, Australia, New Zealand, Finland, Ireland, Norway and Sweden. Malta later joined the IVB as an associate member in 1995. Since then the EU has established antigen banks (the EUVB centred in Lyon, Brescia and Pirbright), and a number of other countries maintain their own national banks (as reviewed by Ryan, 2001), the most recent example being in Argentina (Anon., 1999).

The IVB maintains its own facility for the formulation of emergency vaccine and the EUVB also has such facilities at its disposal through contractual arrangements. The distribution of antigen reserve between antigen banks was detailed in Commission Decision of 2000/112/EC (CEC, 2000a) and in its antigen stocks it now aims to hold around a total of 39.2 million bovine doses of antigen made up of the strains $O_1$ Manisa, OBFS, $A_{24}$ Cruzeiro, $A_{22}$Iraq, A Iran 96, A Iran 99, A Malaysia 97, C Noville, Asia1 Shamir,

SAT1 SAT2 East Africa, SAT2 South Africa and SAT3. The bulk of these antigens, representing all the strains listed, will be held within the commercial sector. It should be noted that Article 8 of Directive 2001/82/EC (CEC, 2001b) allows Member States to permit the release of an unlicensed product in the event of a "serious disease epidemic", provided that no authorised product is available for the disease concerned, and provided that the Commission is informed of the detailed conditions of its application.

The need to have immediate access to FMD vaccine, as specified in many contingency plans for use in an otherwise uncontrollable FMD outbreak, has seen an escalation in the establishment of banks of antigen and/or of formulated vaccines for immediate use. The term 'vaccine bank' is used in this report to encompass those facilities that may store antigen or ready-to-use vaccine. It has recently been reported that 26 European countries have made at least one arrangement for the supply of emergency FMD vaccine, and that the total stockpile of concentrated antigen or formulated vaccine in FAO-EUFMD member countries was equivalent to approximately 70 million monovalent cattle doses encompassing various serotypes and subtypes. This was made up of some 20.5 million bovine doses of the O strain, 25 million bovine doses of various A strains, 10 million bovine doses of C strains, 8 million doses of Asia strains and 2 million, 1.5 million and 0.5 million bovine doses of diferent SAT2, SAT1 and SAT3 strains respectively (Ryan, 2001). Some banks are held on the territory of one or several of its members or are retained in the commercial sector, and, if held as antigen, would be formulated for use either by the manufacturer, or in a dedicated facility maintained by the bank members. The location of stored antigens is therefore of vital importance since the need to formulate vaccine may require antigen to be returned to the original manufacturer, incurring a delay in supply. The presence of adequate infrastructure for vaccine formulation and bottling is a fundamental requirement for vaccine banks.

It is true to say that vaccine banks have only rarely been called upon to provide emergency vaccines (Ryan, 2001). However, it is significant that in more recent years banks have been increasingly activated to supply vaccine to various countries (e.g. the Balkan countries, Japan, South Korea, Turkey-Thrace region, the UK and Argentina), even though the vaccine was not always subsequently utilised.

In addition, vaccine banks in isolation may not suffice since there is also the need for operational vaccine manufacturing plants to ensure the fast and high quality production of new FMD vaccines in sufficient amounts.

In the establishment and supply of emergency vaccines, decisions on the quantity and potency of the product inevitably involve a compromise between the cost of purchase and the likely number of doses required. However, a minimum vaccine requirement might be based on the supply of the number of doses which could, in practice, be distributed and applied in the first week of vaccination, the expectation being that additional supplies could by then have been procured, either from other banks or from commercial sources.

There would also be advantages in making formal reciprocal supply arrangements with other banks, and/or enlarging the size of such banks.

However, it should also be noted that the exceedingly high potencies of many of the stored antigens could potentially allow the option of producing doses in excess of the nominal capability (Barnett and Statham, 1995; Barteling *et al*., 1997).

## 8.8. Differentiation of infected from vaccinated animals

One of the principal objections to the use of emergency FMD vaccine is the subsequent difficulty in differentiating between animals which have been vaccinated and animals which have either recovered from infection or which have acquired sub-clinical infection post vaccination. The detection of antibodies to the NSP 3ABC of FMDV has been shown to be a sensitive and specific method to differentiate between infection and vaccination (Bergmann *et al*., 1993, Mackay *et al*., 1998a, Bergmann *et al*., 2002)). However, where conventional vaccines which contain traces of NSP are used repeatedly, some animals can develop specific NSP antibody (Mackay *et al*., 1998b). Manufacturing methods have been reported whereby the NSP component can be reduced to a level that will not cause detectable sero-conversion following vaccination (Doel, 2001) thereby minimising this difficulty, but not all vaccines are purified to this degree. Moreover, it has been observed that some vaccinated animals exposed to infection can become asymptomatic carriers without seroconverting to 3ABC NSP, especially in animals with low level viral replication in the oropharynx (Mackay *et al*., 1998b; Sørensen *et al*., 1998a). Additionally, although the antibody response against NSP has been shown to persist for a year or so following infection (Mezencio *et al*., 1998), the longer term duration has yet to be established and investigations are complicated by the fact that detection of NSP antibody is very much dependent on the assay system used. However, although probang sampling and testing is a very specific method for the detection of carriers, its sensitivity is far lower than NSP serology (Haas and Sørensen, 2002).

FMD vaccinated and clinically protected animals may still support FMDV replication after challenge, albeit at a reduced level. A proportion of vaccinated ruminants that are infected following vaccination can become virus carriers in which live virus is found in the oropharynx at 28 days or more after infection (Salt, 1998). There is very little information available regarding the effect that the route of challenge has upon the development of FMD persistence. The possibility that vaccinated and subsequently challenged animals may be clinically protected but may nevertheless harbour live virus has important consequences for eradication strategies and regaining official virus-free status for international trade. Providing that the vaccines used have been highly purified to exclude the presence of NSP, then NSP protein serology can be used for serosurveillance in order to identify challenged animals, regardless of their vaccination status. Since NSP tests are not serotype-specific, they also have the advantage of being able to detect antibodies induced by all FMDV serotypes. However, since not all infected animals become carriers, animals found seropositive in NSP tests may or may not actually be virus carriers. Development of better NSP serology tests is a very active area of current research. One aim is to be able to improve the potential for using emergency vaccination to help control incursions of FMD in previously virus-free regions or countries. According to this paradigm, local vaccination programmes would contain a disease

outbreak or outbreaks and reduce the need for slaughtering large numbers of uninfected animals. Thereafter, serosurveillance using NSP serology would enable the selective identification and elimination of carriers followed by the rapid lifting of trade restrictions. NSP serology also has great potential as a means to monitor the continued circulation of virus in countries using mass vaccination programmes.

If NSP serology is used for large-scale, post-vaccination serosurveillance, to identify potential carriers, then a certain level of inconclusive, false negative and false positive results are inevitable. It is necessary to quantify what these levels would actually be and then to devise strategies to try and overcome the problems that they pose. However, problems arise due to a lack of data on the performance of the available tests and uncertainty about the effectiveness of novel control strategies that may only have been evaluated in countries where different husbandry practices prevail.

In conventional serosurveillance, screening of sera is carried out with very sensitive ELISAs that detect antibody to structural proteins, and positive and inconclusive results may be retested by VNT. Animals confirmed as seropositive can be tested for virus carriage by collection of probang samples and subsequent virus isolation testing. By comparison, NSP serology is less sensitive than conventional ELISA (Mackay *et al*., 1998a,b). Furthermore, VNT cannot be used to verify the results of NSP tests, since VNT also detects antibodies to structural proteins. An alternative but more cumbersome confirmatory technique that could be applied to NSP serology is Western blotting (Silberstein *et al.,* 1997). In practice, the lack of sensitivity of NSP serology increases the risk that some carriers will not be detected. Compensatory measures, such as testing a higher proportion of animals or reducing the test cut-off, will increase the number of false positive results, and depending on the accuracy of available confirmatory tests, is likely to greatly increase the amount of virological probang testing needed.

There is no evidence that pigs become carriers after recovery from FMD (Kitching, 2002b; Sutmoller and Casas Olascoaga, 2002). However, following vaccination, a method of serosurveillance to ensure that FMD virus was not circulating at a low level, especially in large herds, would still be desirable.

### 8.8.1. The current status of NSP serology

The concept of measuring antibodies to NSP as a means of distinguishing FMDV infected from FMD vaccinated animals is more than ten years old (Neitzert *et al*., 1991) and can even be dated back over thirty years (Rowlands *et al*., 1969). The approach has been most widely used in South America. Here, NSP serology has been used to monitor the effectiveness of vaccination, rather than to specifically eliminate carriers (Bergmann *et al*., 2000). The consequences of carriers for international trade is instead mitigated by the export of deboned meat rather than live animals. Improved methods of vaccine preparation have reduced the amount of residual NSP present, greatly reducing the risk that vaccination and especially multiple vaccination will lead to an anti-NSP antibody response.

A variety of recombinant NSP have been expressed for use as serological antigens in ELISA assays (Bergmann *et al*., 1993, Mackay *et al*., 1998b) and various studies have shown that, among NSP, 3ABC elicits the most reliable antibody response following infection.

The 2000 edition of the OIE Manual lists the NSP-ELISA assays currently developed and reports the detailed protocol of a screening and a confirmatory test for NSP serology (OIE, 2000). The methods described have been widely used in South America, to monitor virus circulation amongst vaccinated populations of cattle. The screening test is an indirect ELISA detecting antibody binding to bacterially-expressed 3ABC protein. The confirmatory test is a form of Western blot to measure the binding of antibodies to a range of NSP.

Also in Europe national laboratories have developed and validated "in house" tests, based on different principles (Veterinary Quarterly, 1998). In Italy, an indirect-trapping ELISA using a bacterially expressed 3ABC polypeptide captured by a monoclonal antibody coated to plates has been developed (De Diego *et al*., 1997), while in Denmark, a blocking test has been designed that measures the ability of antibodies to bind to a baculovirus expressed 3ABC protein and to thereby block the reaction of an anti-3ABC polyclonal or monoclonal antibody (Sørensen *et al*., 1998b; Haas and Sørensen 2002). Both assays have been validated on either experimental and field cases (Berlinzani *et al*., 1998; Brocchi *et al*., 1998; Sørensen *et al*., 1998a; Wen-Bin *et al*., 2002).

Also commercial ELISAs are currently or will shortly be available with kits developed in national laboratories: a European Company (Schalch *et al*., 2002) and a South American company market an indirect ELISA based on the 3ABC polypeptide, chemically or affinity purified and adsorbed directly to the plate and a North American company is developing a peptide based assay (Liu *et al*., 2002). The test has three components: Sera are screened for antibodies to a bound peptide representing a fragment of the NSP 3B, a proportion of false positive results are excluded by checking that the reaction can be blocked by soluble 3B, and a final confirmation is achieved by testing for antibodies to a second peptide representing part of NSP 3A.

The various NSP tests are at different stages of validation. Several seem to be quite specific for infection regardless of vaccination status. In unvaccinated, but virus-challenged animals, the NSP tests are less sensitive at detecting infection than tests for anti-structural protein antibodies (Mackay *et al*., 1998a,b). However there is evidence that NSP antibodies remain detectable for more than 1 year after infection (De Diego *et al*., 1997; Berlinzani *et al*., 1998; Bergmann *et al*., 1998; Sørensen, 1998; Sørensen *et al*., 1998b; Bergmann *et al.,* 2000). Experimental and field data show the ability of these tests to identify most, even if not all, vaccinated and subsequently infected animals (Brocchi *et al*., 1998; Mackay *et al*., 1998; Sørensen *et al*., 1998b; Bergmann *et al*., 2000), although preliminary results suggest that some tests are certainly more sensitive than others (Haas and Sørensen, 2002). Moreover, it is difficult to estimate how many carriers may fail to be detected. The lack of data is because vaccination-and-challenge experiments for FMD are very expensive and animals are not generally kept for long periods after protection has been assessed and field testing rarely

provides sufficient certainty as to the true status of animals that are assessed. It is noteworthy, that NSP tests have not yet been validated for species other than cattle.

An important variable is likely to be the potency of the vaccine used. It is known that higher potency vaccines are more effective at reducing virus replication following challenge, but it is yet to be determined whether this diminishes the likelihood of subsequent virus persistence, or affects the NSP antibody response. The nature of the viral exposure, including dose and route of exposure, may also be significant. It might be expected that killed vaccines would elicit a different type of immune response to that induced by infection with a live virus. Some studies have attempted to separate vaccinated and infected animals on the basis of the classes of antibody produced systemically and in various body fluids (Salt *et al*., 1996). This is a promising approach worthy of further study (Amadori *et al*., 2000). In addition, different cell-mediated immune responses are to be expected and this could be exploited by future tests.

The OIE health code is being redrafted to accommodate the principle of using NSP tests, post-vaccination, in order to help regain FMD free status.

It has been frequently stated that because of the risk of false negative results, NSP testing for this purpose needs to be used on a herd basis rather than for individual certification. However, the definition of what constitutes a 'herd' needs to be clarified. Another area of uncertainty is over how many vaccinated animals are likely to become infected in a herd that undergoes virus challenge after vaccination. This will clearly be affected by many variables, but the likely range and most probable outcomes are needed in order to estimate sampling rates for assured detection of a given prevalence of infection.

In the longer term, novel marked vaccines may be produced that offer additional alternatives for the serological differentiation of infection from vaccination.

## 8.9. Application of vaccine in the field

Since 1992 preventive annual vaccination against FMD has not been used in the EU. However, emergency vaccination may be used in a variety of different situations and in a number of different ways (SCAHAW, 1999a), including the possibility that animals might be vaccinated to live rather than being subsequently slaughtered when circumstances and resources allow. The following are key examples of how vaccine might be applied in the field:

- Against an outbreak of disease in a country, which is free of FMD and which, does not normally vaccinate. Emergency vaccination is usually applied as Ring Vaccination or Barrier Vaccination, outside of and around a focus of disease to try to prevent outward spread.

- Against an outbreak of disease in a neighbouring country or region when emergency Barrier Vaccination may be applied along the border in the country or region which is at risk. Notable examples include the

application of vaccine in parts of Bulgaria and Greece bordering on Turkey and in the Turkish Thrace itself against the threat of the spread of both endemic and exotic FMD across the Bosporus from Asia into Europe on several occasions since the 1960s.

- Against an outbreak of disease when emergency vaccination is applied both around and within the outbreak in so-called Suppressive or Dampening Down Vaccination (which includes ring vaccination). This is often followed by the culling of the vaccinated animals. This type of emergency vaccination was applied during the outbreak of FMD in The Netherlands in 2001. The applied monovalent vaccine was formulated as a double-oil emulsion containing 3 $PD_{50}$ of the vaccine strain O1 Manisa, and it was originally administered to all FMDV susceptible animals within a 1km ring around FMD outbreaks. However, since new outbreaks of disease occurred in or around the vaccinated area, the ring was extended to 2km and shortly thereafter an entire region was involved in emergency vaccination. A further 7 disease outbreaks occurred outside this area and an emergency ring vaccination with a diameter of 2km was immediately applied around these outbreaks also. In total approximately 200,000 animals in 1,800 herds were vaccinated and culling of the vaccinated herds commenced 2 weeks after vaccination had been performed. In approximately 2,700 herds, 270,000 animals were eventually culled and in total The Netherlands experienced 26 outbreaks, 9 of which were diagnosed before the start of the vaccination campaign.

- Against an outbreak of disease in a country which does normally vaccinate but where emergency vaccine is applied to boost existing immunity.

- Against an outbreak of disease in a country which does normally practice preventive vaccination, but where the vaccine(s) employed do not provide protection against the different serotype or strain involved in the outbreak.

An emergency FMD vaccine is ideally formulated to contain higher levels of antigen than conventional, routine vaccines with the objectives of it being more potent, creating immunity more rapidly and giving a wider spectrum of immunity than conventional vaccines. Incorporation of higher concentrations of antigen decreases the number of vaccine doses available from a given quantity of antigen. Conventional vaccines may also be used in an emergency when vaccine of appropriate strain composition is immediately available, as was the case in The Netherlands in 2001.

## 8.10. Future candidate vaccines

While existing, vaccines have been associated with notable success, there are a number of target areas for their potential improvement. These include the development of vaccines that:

- protect against the establishment of local virus replication in the oropharynx and possible acquisition of the carrier state, thus preventing potential viral transmission,

- allow simple and definitive differentiation between infected and vaccinated animals,

- are thermostable (avoiding the current necessity for a cold chain),

- have a broader immunogenic spectrum (conferring immunity against several, and ideally all, epidemiologically important serotypes and strains),

- engender a rapid onset (within days) and extended duration of immunity (ideally lifelong) following a single application,

- are unaffected by the presence of maternal immunity,

- can be administered topically (rather than parenterally), to stimulate the primary sites of infection, through a novel application that allows simultaneous immunisation of large numbers of animals with minimal effort, time and risk to animals and handlers and satisfies safety requirements.

The issue of vaccinated animals becoming carriers and thereby having the potential to spread the disease further is arguably the most important one, as this has undoubtedly inhibited the use of vaccines as a primary control measure. For this reason, perhaps the most significant points of this list are the first two. Undeniably, a vaccine that could offer immunity that abolishes the concern of carrier status would have radical significance to current policy and in particular to the export and free trade of animals and animal products. However, although there have been many relevant research developments (see later), the reality may be that such a vaccine is some years away. Pragmatically therefore, the greatest prospect for more immediate progress lies with the ability to differentiate between vaccinated and infected animals either through the advancement of diagnostic tests or by manipulation of the vaccine to assist such differentiation, the so-called 'marker vaccines'.

New molecular approaches to FMD vaccination have been followed since the mid 1970s when proteins, protein fragments and viral subunits – principally isolated VP1 or fragments of VP1 - produced in bacteria, baculovirus, and transgenic plants have been investigated (Brown, 1999). Other avenues have since been pursued, including: synthetic peptide vaccines; the use of replicating vectors; genetically engineered attenuated strains; and DNA vaccines (Sobrino *et al*., 2001). Although some early promise has emerged from these experimental studies, none have, as yet, progressed to the point at which they might offer advantages over existing, conventional vaccines. The following sections summaries the current state of art.

8.10.1. Inactivated antigens/subunit/peptide

The current vaccine relies on an integral whole (146S) virus particle that is chemically inactivated but retains the important epitopes to stimulate a protective immune response. Little has changed to this principal component of the vaccine apart from the quality standards by which it is produced.

Perhaps the most significant development, in recent years, concerning the production of the antigen constituent has been the improvement in its purity. The driving force for this was to remove as much of the NSP constituents produced during large-scale production of the virus as possible. This would alleviate any immune response to these NSPs following single or multiple administration of the vaccine and thus supplement the use of a diagnostic test that can detect an antibody response to such proteins following live FMDV infection, to determine whether a vaccinated animal has harboured infectious FMDV. This is clearly achievable with current technologies (Anon., 2001)

There have been approaches to use parts of the 146S particle and early studies revealed that one protein, VP1, and fragments from its carboxyterminal–half, were capable of eliciting a virus neutralising antibody response which conferred protection in some cases (Laporte *et al*., 1973; Bachrach *et al*., 1975; Strohmaier *et al*., 1982; Meloen and Barteling, 1986). Indeed, this led to a major impetus of research (reviewed in Cheung and Kűpper, 1984; Brown, 1988, 1992; Domingo *et al*., 1990) but despite the ability to express VP1 from various sources such as bacteria, the immunogenicity of the product fell far short of the same protein integrated within the virus particle (Brown, 1988, 1992; Domingo *et al*., 1990). This was probably due to inefficient folding as a separate entity and limiting exposure of important immunogenic sites to the hosts immune system. Studies exploring this area still continue, and strong antibody responses have been observed in mice that were administered transgenic plants expressing either the VP1 or the epitope VP 135-160. Additionally these mice were completely protected against experimental challenge with the virulent virus (Carrillo *et al*., 1998; Dus Santos *et al*., 2002). Nevertheless, the appreciation that VP1 alone is not as good immunogenically as that constrained within the capsid has led to researchers developing methods of producing the integral empty capsid, i.e. a virus particle containing no RNA, using various recombinant vectors. Baculovirus and *E. coli* expression systems have been shown to retain both conformational and linear epitopes present in the viral capsid and to induce protective immunity (Grubman *et al*., 1985). The major obstacle of this route appears to be the limited and inefficient amounts of antigen such systems produce.

The early studies which highlighted the importance of VP1, or fragments therein, involved the use of trypsin-treated virus or proteins isolated by chemical or enzymic treatment of intact virus particles and the available knowledge on the antigenic structure of the virus allowed the design and synthesis of peptide vaccines. It is fair to say that the majority of this work focussed on a continuous B cell epitope located in the G-H loop, around residues 140-160 of VP1. Uncoupled versions of this peptide induced virus neutralising antibodies in guinea pigs and mice and protection in swine (Bittle *et al*., 1982; Pfaff *et al*., 1982; Francis *et al*., 1990). A modified version of this peptide co-linearly synthesised with residues 200-213 representing the carboxy terminus of VP1 resulted in cattle being protected from virus challenge. There has also been a report that this construct could provide broader heterologous cross-protection in the guinea pig model (Doel *et al*., 1990). Again however, the immunogenicity of such constructs was poor compared with the conventional vaccine, requiring high payloads of peptide and repeated immunisations to achieve the desirable outcome.

Recognising this, some constraint was placed on the orientation of such peptides through co-expression as part of a hepatitis B core structure to a configuration similar to that found on the virus, and there were clear indications that such a constraint did indeed improve the immunogenicity of the peptide (Clarke *et al*., 1987). A significant inhibitory factor to the progress of peptide vaccines, in any given form, was the recognition that such constructs required the inclusion of T cell epitopes in order to promote suitable cooperation with the appropriate B cell lymphocytes for an effective immune response, and that ideally these should be identified from the infectious agent of concern in order to provide sufficient memory and recognition by T cells in the context of alleles of MHC class II frequently represented in the population of the natural host (Sobrino *et al*., 2001). There is also an appreciation that the administration of simple peptide antigens, particular those designed from a highly antigenically variable RNA virus, may induce the selection of a significant proportion of FMDV mutants which could continue to manifest the disease. There is therefore the need to consider more multi-faceted peptide constructs mimicking more complex conformational or discontinuous sites or using a cocktail of peptides covering different variant sequences (Gras-Masse *et al*., 1997).

8.10.2. DNA vaccines

In recent years the potential of using naked DNA as a means of eliciting humoral and cellular immunity to confer protection has been explored using a number of pathogens including FMDV. A neutralising antibody response was detected in mice following administration of a DNA vaccine expressing empty FMDV capsids (Chinsangaram *et al*., 1998) and it has also been reported that a DNA vaccine incorporating an FMDV attenuated full length infectious clone could induce protection in swine (Ward *et al*., 1997; Cedillo-Barrón *et al*., 2001). The concept of DNA vaccination offers several potential advantages for FMD vaccines. For example, aside from its stability, it would avoid manipulation of the infectious virus reducing production costs and the risk of potential escape of the virus and also by using an incomplete virus genome as the immunogen would potentially allow discrimination between infected and vaccinated animals. However, this avenue of research is only at its infancy and one of its limitations is the requirement for multiple administration of this type of vaccine. There is therefore a need to examine further DNA vaccines, using alternative routes of administration, either alone or as hybrid molecules using other delivery systems and with the inclusion of other adjuvants. Co-expression of such immunogens with specific cytokines, for example, may promote greater response efficiency (Lai and Bennett, 1998).

8.10.3. Live attenuated viruses and live viral vectors

It is well-established that the use of vaccines incorporating a live pathogen is more efficient at eliciting a protective immune response than an inactivated version of the same candidate, and this includes examples from the Picornavirus family. However, such an option relies on the attenuation of the pathogen to make it as innocuous as possible, through adaptation and continuous passage of the virulent form in non-susceptible hosts (Sagedahl *et al*., 1987). However, the high potential for variation in small RNA viruses such as FMDV makes this approach inherently dangerous given the likely

reversion to a virulent form or the possibility that the virus may not be attenuated in all target species (Sagedahl *et al*., 1987). The development and availability of modern molecular techniques and a greater understanding of the replication cycle of the virus and factors relating to its virulence may provide further means to explore this area- for example, the availability of infectious FMDV clones that allow the design of attenuated strains through deletion of a receptor binding site or the L gene (Mason *et al*., 1997; McKenna *et al*., 1995). However, the possible difficulties surrounding the requirement for a wide host range and the potential for virulent variance and therefore the need to perform extensive studies to assure both stability and safety makes this a less attractive option.

An alternative approach to achieving similarly efficient immunity to that provided by an attenuated live vaccine strain is the presentation of the required antigen/s in a replicative or limited replicative form, expressed by recombinant viral vectors. Molecular biology techniques allow more detailed characterisation of the genetic organisation of many viruses to such an extent that regions suitable for insertion of foreign genetic material have been identified. This has resulted in the development of numerous types of viral vectors from a wide variety of DNA and RNA viral families including adenoviruses, herpes viruses, poxviruses and retroviruses. In some cases this has offered the potential for delivering and expressing antigenically important gene/s from a foreign pathogen, thus acting as a vaccine vector. The chosen viral vector is often genetically attenuated or is incapable of completing its replication cycle in the target host, thus avoiding the manifestations of clinical disease, and it must also not pose a threat to the person administering the vaccine.

Live viral vectors offer several advantages for vaccine delivery compared to inactivated, subunit, or conventional modified or attenuated vaccine strains. The possibility of delivering multivalent vaccines using a single vector form resulting from a single manufacturing process and possibly a single administration into the host presents advantages for both manufacturer and end-user. Foreign gene expression in the cells of its natural host should ensure that post-translational modifications will be correct and produce an authentic antigen. It should also be possible to construct such vectors to simultaneously deliver immuno-modulators, such as cytokines, which could modify the immune response in a positive way. It may offer the potential to deliver the vector more conveniently and less intrusively, for example in a spray form, and it practically eliminates the possibility of disease following exposure to the candidate pathogen. Finally, and probably most importantly, using the appropriate viral vector will elicit both cellular and humoral arms of the immune response and may in some cases be applicable for inducing a more suitable response at the primary site of infection such as at the mucosal surface. However consideration must be taken of the possibility of pre-existing immunity in the target host against the candidate vector, the limitation on the insertions that can be incorporated and, like many other vaccines, the problem of stability. Furthermore, immunity against the viral vectors following the first application could influence the efficacy of further applications using the same vector system.

Although it is possible to generate live attenuated vector viruses with precise genetic changes either through deletion of non-essential or essential genes,

another alternative which has been exploited successfully, particularly in the FMD field, is the use of a virus that cannot complete an entire cycle of replication but is sufficient to allow expression of the foreign gene/s. For FMD, the human adenovirus type 5 (HAV-5) has so far been the primary vector exploited in this way. Preliminary studies using wild type HAV-5 that expressed the P1 protein provided evidence of partial protection against FMDV challenge in cattle (Sanz-Parra *et al*., 1999a) and pigs (Sanz-Parra *et al*., 1999b). An interesting insight from these studies, however, was the absence of detectable FMD specific antibodies in the vaccinated animals, suggesting a greater role for cell-mediated immunity in the protection observed. However, the awareness of the disadvantage of using replication-competent human pathogen also led to the development of a replication-defective variant of HAV-5 through the deletion of E1 to ensure a measure of safety to both target and vaccine deliverer. Studies in pigs using this vector, which expressed the FMDV capsid and the 3C proteinase, provided evidence of partial protection against FMDV challenge after a single immunisation and full protection after a booster immunisation in five of the six swine (Mayr *et al*., 2001). In addition, it has been demonstrated that an HAV-5-A24 Cruzeiro empty capsid candidate vaccine protected pigs and cattle against a virulent challenge (Grubman and Mason, 2002). A recent paper describes that pigs that have were inoculated with a recombinant, replication-defective HAV-5 vector containing porcine interferon-alpha were completely protected when challenged 24 hours later with FMDV (Chinsangaram *et al*., 2003).

Currently, there is an opinion, particularly among some FMD vaccine researchers, that the use of a host-specific vector with a localised but more efficient ability to replicate, would result in a highly effective immune response. Attention therefore is now being directed at the development of host-specific adenoviruses such as those from pigs which have been shown to confer protection against CSF (Hammond *et al*., 2000)

The development of these types of vaccine will clearly be hampered by safety issues at both developmental and field application, limiting the commercial possibilities, but despite this there are already commercially available viral vectored vaccines for veterinary application developed from the use of poxviruses.

### 8.10.4. Adjuvants

Vaccine adjuvants enhance the magnitude and duration of immune responses. From their initial introduction, adjuvants have been developed both by empirical observation and by application of a rational design based on analysis of the immune system and in the early days of vaccine development adjuvants were seen as additional chemical compounds in a formulation to facilitate this immune enhancement. However, this simple view has had to be radically reviewed in consideration of the multiple and overlapping biological effects of many adjuvants and the manner in which vaccine can be potentially manipulated at the molecular level to provide this effect. In realisation of this problem Edelman and Tacket (1990) devised a categorisation of the different types of adjuvants or immunostimulators under three broad headings of adjuvants, carriers and vehicles. However, a more recent categorisation of adjuvants proposed by Cox and Coulter

(1997), was based on five potential modes of action, immunomodulation (modification of cytokine networks for example), presentation (maintaining antigen conformation), induction of cytotoxic lymphocytes, targeting and depot generation. In truth such categorisations have drawbacks, not least the possibility of overlap. The reality is that there are now many forms of adjuvant available to the vaccine developer and their appropriateness depends greatly on the immunogen used, formulation, route of administration, required response and their safety for vaccinated animal and consumer.

Inactivated FMDV antigen, being a particularly poor immunogen, is dependent on the use of adjuvants when incorporated into vaccines. Although a variety of adjuvants have been investigated with the classical inactivated virus antigen, only two basic types are generally used. The well-established aluminium hydroxide gel, supplemented with another adjuvant saponin, has been used for many decades in vaccines destined for ruminants. However, this adjuvant combination worked poorly in another important target species (pigs), and led to the use of mineral oil emulsions. While the constituents of these oil adjuvants can vary significantly, they essentially have been preferred in either of two forms following emulsification. Either a single oil formulation, broadly similar to those involving Freund's incomplete oil adjuvant, or the more popular double oil formulation based on the resulting droplet separation of the aqueous phase in the oil.

Whilst these remain the stalwarts of FMD vaccination, some developmental progress has been made on oil adjuvants and there are now commercially available alternative 'ready-to-formulate' oil adjuvants, including a mineral-based oil containing esters of octadecanoic acid and anhydromannitol, which readily forms a water-in-oil-in-water emulsion, and one composed of oleic esters of anhydrous mannitol, which forms a single oil-in-water emulsion (Barnett *et al*., 1996). Their simplicity for producing stable emulsions along with the required efficacy in all the common target species without untoward local reactions, has led to at least one being incorporated into commercially produced FMD vaccines. Moreover, it has recently been shown that by using a novel formulation procedure such oil adjuvanted vaccines can be stored at ultra-low temperature to extend their normal shelf-life and be readily available for 'emergency' use and thus avoid the unnecessary delays required for manufacture (Barnett and Statham, 2002). This concept may equally have benefits to other emergency type vaccines that are required at short notice.

The many avenues of investigation aimed at improving the performance of conventional FMD vaccines, including manipulation of the immunogen or its route of administration, has led to many further adjuvants being examined. This has included a more purified form of saponin, immunostimulating complexes (ISCOMS), liposomes, cholera toxin, and metabolisable oils. Although naked DNA has been shown to be efficient at inducing immunity after systemic inoculation, here too adjuvants can play an important role to significantly improve the immune response. Co-delivery of DNA vectors encoding cytokines has been shown to increase the efficacy of DNA vaccines in rodents (Leitner *et al*., 2000) and pigs (Somasundaram *et al*., 1999) including FMDV (Cedillo-Barrón *et al*., 2001). Other conventional adjuvants such as aluminium salts, cationic liposomes,

ISCOMS and cationic poly-lactic co-glycolide (Singh *et al*., 2000; Ulmer *et al*., 2000) have also been shown to increase immune response to DNA vaccine in the rodent model.

In the mid 1980s the immunostimulatory properties of bacterial DNA was reported (Tokunaga *et al*., 1984) followed by a later report (Krieg *et al*., 1995) that showed that this was primarily due to the presence of unmethylated CpG dinucleotides. Importantly, this activity was specific for bacterial DNA, and it was found that short synthetic oligonucleotides were also potent inducers of B cell proliferation provided that they contained an unmethylated CpG in a particular stimulatory sequence context (CpG motif). This explained the enigmatic potency of some DNA vaccines as a result of the high frequency of unmethylated CpG motifs in the bacterial DNA backbones present in the plasmids, and the realisation that the unmethylated CpG motif in bacterial DNA is one of the molecular patterns recognised by the innate immune system. As a result of this, incorporation of CpG motifs as an adjuvant is now very much an active field in investigative vaccine research and will undoubtedly encompass FMD in one form or another.

8.10.5. Marker vaccines

Marker or DIVA vaccines can be defined as vaccines that allow the differentiation of infected animals from vaccinated animals. These vaccines contain at least one immunogenic peptide or protein less than the corresponding field microbe and they are consequently sometimes referred to as 'negative' marker vaccines. 'Positive' marker vaccines, which are not currently available, would contain an additional immunogenic substance compared to the corresponding field microbe. Such a vaccine may allow the detection of vaccinated animals, but there are currently no practical applications of this type of vaccine.

Despite the various comprehensive studies that have been undertaken in regard to FMD vaccines, some of the goals of this work still remain elusive. However, if emergency vaccination is to become a primary means of controlling an FMD epidemic, it is fundamental that some means is established of unequivocally differentiating infected animals from those that are vaccinated. This would undoubtedly lead to a more rapid means of returning to the desired 'disease-free status'. It would also make the option of vaccinating animals with the intention of allowing them to live and continue their productivity a realistic and practical possibility.

As previously mentioned it is now possible to differentiate between vaccinated animals and those that become infected with FMDV on the basis of testing for the presence of NSPs which are unique markers in infected animals, provided that a high degree of purity is assured in the production of conventional FMD vaccines, which can be achieved by current manufacturing technologies. However, there is clearly a need to ascertain the many factors that might affect the reliability of this approach, including the kinetics and duration of an NSP response, particularly in relation to sampling time points, and the level of viral infection that can occur in animals vaccinated at different time points. It is conceivable that with the desired vaccine and an appropriate assay system, whose sensitivity and specificity is

suitably validated, it should be possible to achieve such differentiation in the short-term.

Nevertheless, it is recognised that it is desirable to have further supportive tests, such as PCR, or other means of immunologically discriminating vaccinated from infected animals such as the disproportional stimulation of CD8+ T cells or detection of a particular cytokine/chemokine or other immunological parameter.

However, the concept of a marker vaccine in which the vaccine is in some way altered to further improve the means by which vaccinated and infected animals can be differentiated must be the ultimate goal. Such a vaccine must not only encompass all the attributes of a desirable vaccine listed in chapter 8.10, but also have the capability of being formulated simply and rapidly at a reasonable cost to allow ready availability when required. Given the partial success of peptide and subunit vaccines, which would provide a limited structural antibody response compared to that observed in infected animals and thereby achieve an obvious means of differentiation, the principle avenue for marker vaccine development appears to be the removal or replacement of a portion or portions of the capsid, an avenue easily attainable with current molecular biology technology.

Inactivated chimeric viruses are an example, in which the immunodominant G-H loop has been replaced either by an unrelated sequence or a sequence from an FMD strain of different serotype (Reider *et al*., 1994; Baxt *et al*., 1998). However, it is fair to say that the major reasons for developing such a vaccine with the ability to protect animals, in the absence of this important epitope, seems to have led to an oversight on the additional potential it may have toward differentiation. Thus, a detailed profiling of the humoral responses from such animals compared to those of infected animals has never been undertaken. With an appropriately developed assay, the structural antibody responses from such chimeric vaccines may potentially offer an additional, and perhaps more reliable, means of discriminating vaccinated and infected animals. This could be an even more practicable approach if a more conserved region of the virus capsid, akin to that which binds certain cross-serotype specific monoclonal antibodies, could be identified that is applicable to all vaccine strains.

## 9. CLASSICAL SWINE FEVER

CSFV is a member of the *Pestivirus* genus, along with the closely related viruses of bovine viral diarrhoea (BVDV types 1 and 2) and ovine border disease (BDV). The *Pestiviruses* are in the family *Flaviviridae* which also contains the *Flavivirus* and *Hepacivirus* genera. CSFV is an enveloped virus with a single stranded positive sense RNA genome approximately 12.5 kilobases long, which encodes a single large open reading frame, flanked by short non-coding regions (NCR).

The major envelope glycoprotein of CSFV (E2) is thought to be involved in binding the virus to cellular receptors. This protein is also the main target for virus neutralising antibodies, and is able to induce a protective immunity. Another glycoprotein ($E^{rns}$) also binds to cell surfaces. Although CSFV does not readily infect ruminants or ruminant cell

cultures, host specificity of pestiviruses does not appear to be absolute- BVDV and BDV can infect pigs and various ruminants.

CSFV replicates in cell culture without inducing a cytopathic effect. Depending on the virus strain and the host, it can be highly virulent *in vivo*, leading to a fatal disease characterised by high fever, leucopenia, thrombocytopenia, haemorrhages and secondary bacterial infection. CSFV has a predilection for cells of the reticuloendothelial system. Although, the mechanisms whereby CSFV causes damage *in vivo* are poorly understood, it is becoming clear that indirect mechanisms are important in the pathogenesis, since cells can be damaged without actually becoming infected.

Since 1980 the control of CSF in the European Union has been based on a policy of non-vaccination and stamping-out. Recent outbreaks have shown that the control of CSF in non-vaccinated populations through stamping out may be very expensive, particularly in areas with high pig densities (Koenen *et al*., 1996; Meuwissen *et al*., 1999). This is partially due to the large number of animals that have to be pre-emptively culled in order to minimise the virus spread in the vicinity of infected herds. This contiguous spread contributes to a large extent to the total number of infected herds during an outbreak. However, besides the financial implications, this pre-emptive cull strategy has also become ethically more and more debatable (Terpstra, 1978).

Although it is still not fully understood which routes of transmission are actually responsible for the infection of neighbouring herds, it has become clear that the pre-emptive culling of herds in the neighbourhood of an infected herd is an effective and even indispensable measure in the control of a CSF epidemic in areas with high pig densities (Koenen *et al*., 1996; Staubach *et al*., 1997; Elbers *et al*., 1999). The purpose of this measure is to prevent infection of new herds, which would generate massive infectious virus production, and thus to reduce the virus infection load in an area. This reduced infection load subsequently results in a reduction of the between-herd virus transmission.

Theoretically, this same goal can be achieved by emergency vaccination instead of the culling of neighbouring herds. Vaccination should result in:

- a decreased virus excretion by vaccinated pigs once they become infected, and
- a lower susceptibility of pigs to infection.

Both of these effects underly the reduced transmission often observed in vaccinated populations (De Jong and Kimman, 1994). In order to be equally as efficient as the pre-emptive culling strategy, it is important that the interval between vaccination and onset of immunity (reduction of infectivity and susceptibility) is as short as possible. An EU funded project is at present investigating the immunological mechanisms of protection against CSFV, towards the development of new efficacious marker vaccines (QLK2-CT-2001-01374) and another project is investigating the identification of efficacious delivery systems for recombinant and nucleic acid construct vaccines (QLK2-CT-2001-01346).

## 9.1. Diagnostic methods that are currently used

### 9.1.1. Investigation of Suspect Cases

Primary outbreak

Whenever pigs or wild boars showing suspicious clinical and/or pathological signs of CSF are reported, it is mandatory to collect a variety of suitable organ samples for virus as well as for antibody detection, at least including tonsils, spleen, several lymph nodes, and kidney, as well as coagulated and EDTA uncoagulated blood samples. These samples must then be differentially processed in the laboratory for the detection of infectious virus, viral antigen, or viral nucleic acid (Carbrey, 1988; Pearson, 1992). Virus detection is carried out by inoculating suitable cell cultures with clarified organ suspension or with whole blood, buffy coats, plasma or serum. Since CSFV does not cause a cytopathic effect in infected cells, the presence of infectious virus must be subsequently visualised by immunofluorescence or immunoperoxidase staining of viral proteins in these cells, either using CSFV-specific polyclonal sera or monoclonal antibodies (mabs).

Direct antigen detection may also be carried out on fixed cryosections of organs, preferably tonsils. Viral antigen is usually detected by direct immunofluorescence using a polyclonal antibody coupled to a fluorescent dye such as fluoresceine isothiocyanate (FITC), or by direct immunoperoxidase staining, using either polyclonal or monoclonal peroxidase-conjugated CSFV-specific antibodies. Viral antigen in whole blood, serum or plasma can also be detected by antigen ELISA. However, this method suffers from a low sensitivity, especially if infected animals are to be detected during the incubation phase or in a chronic state of the disease when viraemia is low or intermittent (Kaden *et al*., 1999a).

Detection of viral RNA after nucleic acid extraction from diagnostic specimens by RT-PCR has only been introduced relatively recently into diagnostic laboratories (Katz *et al*., 1993; Wirz *et al*., 1993; Harding *et al*., 1994). This method is considered quite sensitive, but is prone to false positive results due to contamination (Paton *et al*., 2000a). In addition, the cDNA fragments amplified by RT-PCR can be used as substrates for the characterisation of newly isolated CSFV strains by nucleotide sequencing (Hofmann *et al*., 1994; Lowings *et al*., 1996; Paton *et al*., 2000b).

Whenever possible, more than one detection method is applied, before a definitive result is issued. The antigen ELISA and RT-PCR can yield results within 4 to 6 hours, and allow the detection of viral antigen and/or nucleic acid even in samples that no longer contain any infectious CSFV. This absence of infectious CSFV may be due to prolonged storage of samples, inadequate shipping conditions, or the simultaneous presence of anti-CSFV antibodies which neutralise the infectivity of the virus (as observed in blood samples from CSFV-infected wild boars). However, cell culture isolation is considered a sensitive backup method and is required in order to confirm earlier results obtained by antigen ELISA and/or RT-PCR and, particularly in primary outbreaks, to isolate the virus responsible for subsequent typing (CEC, 2002a).

Typing of a new CSFV isolate was previously performed by determining the reactivity pattern with panels of mabs directed against immunogenic viral structural proteins such as E2 or $E^{rns}$ (Kosmidou *et al*, 1995). More recently mab typing has been replaced or is at least complemented by determining the genotype, based on nucleotide sequence comparison of suitable genome areas (5' non-translated region, E2). Genotyping provides the most detailed

information about the relationship of CSFV, and hence is considered the state-of-the-art method for epidemiological investigations (Greiser-Wilke *et al*., 2000).

If CSFV is suspected to have been present in a pig herd for a prolonged period, blood samples can be examined for the presence of antibodies which generally appear approximately 14 days post-infection. Ab ELISA, either in the form of a blocking (Have, 1984; Colijn *et al*., 1997) or an indirect (Moser *et al*., 1996) test, are most widely used. However, because some of these tests show a limited specificity in discriminating CSFV antibodies from antibodies against other pestiviruses, such as BVDV and BDV, additional testing by serum neutralisation assay may be necessary to differentiate CSFV antibodies from non-CSFV pestivirus antibodies. This differentiation is mandatory, since a herd with CSFV antibody-positive pigs is considered a CSF outbreak (irrespective of the presence of clinical signs resulting in stamping out measures, whereas no disease control measures are prescribed in the case of BVDV and BDV infections in pigs.

Secondary outbreaks

If an obvious epidemiological link is found between preceding outbreaks (primary or secondary outbreak) and a secondary outbreak, the presence of CSFV may be confirmed by positive results in two independent tests such as antigen ELISA and RT-PCR, without the necessity to wait for the result of virus isolation in cell culture. Confirmation of CSF within one day upon receipt of the samples provides the basis for a rapid decision to cull the suspected herd.

However, in order to establish a definite link between the primary and secondary outbreaks of CSF, genotyping of the virus strain involved may still be necessary.

### 9.1.2. Tracing

Epidemiological tracing is needed following a primary outbreak of CSF in order to check for additional herds where pigs might have been infected due to direct or indirect transmission of the virus to or from the diseased herd. Even though no clinical signs may be present, careful clinical inspection followed by sampling of several animals (EDTA blood and coagulated blood) must be performed. For blind sampling, a large number of animals would need to be sampled to have any chance of detecting virus, unless a high risk group can be identified by epidemiological means or by measurement of temperatures.

Virological as well as serological examination of samples need to be carried out, since pigs might still be in the preclinical incubation phase, have recovered already from CSF, or be infected with a virus strain of low virulence and not show distinct clinical signs throughout the course of the infection. Antibody detection can identify pigs that have recovered from CSF, and a positive result indicates that the virus has been present on the premises for at least 2 to 3 weeks. For virus detection, tests with a high sensitivity must be applied since the amount of virus present in the blood

during the incubation phase is usually low, as is the case if the infection is caused by a moderate or low virulent virus strain.

9.1.3. Freedom of Infection

Pigs which have recovered from CSF mount a protective humoral immune response, and do not shed infectious virus for a prolonged period.

An exception is piglets born from sows infected during pregnancy which can be persistently infected, shedding high levels of virus, yet surviving for weeks or months with or without retarded growth and wasting signs (van Oirschot, 1983). Serological screening of a statistically significant sample of pigs or wild boar by ELISA is therefore used to prove the absence of the disease in the pig population investigated.

## 9.2. New and emerging diagnostic techniques

Essentially the same developments concerning new and emerging diagnostic techniques as have been already described for FMDV (chapter 8.2) also apply for CSFV. New techniques such as an automated real time RT-PCR could lead to a faster, more sensitive and more specific diagnosis of CSF, and recently a portable PCR assay has been described that can potentially be used under field conditions (Risatti *et al*., 2003).

## 9.3. Types of vaccine

9.3.1. Live attenuated

Classical live vaccines are used worldwide, and are based on different attenuated virus strains. The most widely used vaccine strain appears to be the Chinese (C) strain, but there is a lot of confusion about the origin of the C-strain and there may be several of them in existence, with different histories. Most, if not all, C-strains have been attenuated by hundreds of serial passages in rabbits. (Aynaud, 1988). Other vaccine strains are the Japanese GPE-negative strain, the Thiverval strain, and the Mexican PAV strains.

9.3.2. E2 subunit marker vaccines

During the development of marker vaccines it became clear that the E2 glycoprotein in a purified form was capable of inducing a protective immunity (Rümenapf *et al*., 1991; Van Zijl *et al*., 1991; Hulst *et al*., 1993; König *et al*., 1995; Van Rijn *et al*., 1996; Peeters *et al*., 1997). This finding was the basis for the development of an E2 subunit vaccine that contains as an antigen only the E2 glycoprotein. The E2 glycoprotein is produced in cultures of insect cells infected with the baculovirus vector (Hulst *et al*., 1993). Pigs vaccinated with a sub-unit marker vaccine only develop antibodies against the E2 glycoprotein, whereas pigs that are naturally infected develop antibodies against different viral proteins (e.g. E2, $E^{rns}$, NS3). Consequently, it is possible to distinguish between an infected and a vaccinated pig by means of an ELISA test that only detects antibodies against the $E^{rns}$ glycoproteins (Moormann *et al*., 2000). Currently, there are two E2 sub-unit marker vaccines commercially available and both are

licensed via a European procedure. Also two similar differential diagnostic antibody ELISA tests are available.

## 9.4. Efficacy

### 9.4.1. Live attenuated vaccines

It is no longer sufficient that a CSF vaccine can protect pigs from possible signs of disease since vaccination should also prevent the animals from becoming carriers. In this case, carrier means a vaccinated animal in which the wild type virus can still multiply and spread to other animals without causing any clinical signs in the vaccinated animal. Two of the main factors that determine the efficacy of the live attenuated vaccine are the virus strain used and the virus titre. In order to determine the potency, according to the European Pharmacopoeia, CSF-vaccines with at least 100 $PD_{50}$ per dose are titrated in pigs. A number of pigs (6 to 8 weeks old) are divided into 3 groups. The first group of 5 pigs is vaccinated with 1/40 vaccine dose; the second group of 5 pigs is vaccinated with 1/160 vaccine dose and the third group of at least 3 pigs is an unvaccinated control group. Fourteen days later the animals are challenged intramuscularly with $10^4$ $ID_{50}$ of a virulent CSF-strain and are clinically observed for 14 days. Challenge infections after 14 days give the opportunity of a good differentiation between vaccines with diverse potencies. To evaluate the potency of CSFV vaccines for emergency usage, even earlier challenge infections are conceivable. Since clinical signs can be mild, it is also not always easy to interpret them. Additionally the age of the challenged animals is very important since the susceptibility to CSFV decreases with the age of the animals (Bironf and Leunen, 1988). The animals of the control group should die between 4 to 10 days after infection. Of the vaccinated animals, the following should be regarded as unprotected: those who die as a result of the challenge and those who show clinical signs. In addition, the tonsils of the infected animals can be examined for the presence of virus. There is a good correlation between the presence of viral antigen in the tonsils and the appearance of clinical signs. This is a more objective criterion than the interpretation of clinical signs (Biront and Leunen, 1988). In the model with intra-muscular challenge the vaccine should contain at least 100 $PD_{50}$ to prevent carriers (Leunen and Strobbe, 1977). A report using an oronasal challenge one week after vaccination demonstrated protection with a CL-vaccine containing 160 $PD_{50}$ (Biront and Leunen, 1988). For the lapinised C-strain a model on rabbits was also available for efficacy testing (Desmecht *et al*., 1977).

As described above, the efficacy of a vaccine is assessed by vaccination-challenge experiments in the target host. The C-strain has been found to be highly efficacious, in that in most studies it induced a virtually complete protection against challenge. From around 4 days after vaccination challenged pigs did not show clinical signs and replication of challenge virus, measured by shedding in oral swabs or by viraemia, was hardly detectable. This solid protection has been demonstrated to last more than a year, probably even lifelong (Biront *et al*., 1987; Aynaud, 1988; Terpstra *et al*., 1990). As with all vaccines, maternal antibodies inhibit the induction of vaccinal immunity: the higher the maternal antibody titre at vaccination the stronger the inhibition (Vandeputte *et al*., 2001).

With regard to emergency vaccination, it is of relevance whether, and how early, virus excretion in vaccinated pigs is reduced or prevented and whether and how early vaccinated pigs become less susceptible to CSFV infection. These effects will result in reduction or prevention of transmission of challenge virus, which can be examined in so-called transmission experiments (Bouma *et al*., 2000). It has been found that the C-strain is able to block transmission of virulent challenge virus to vaccinated in-contact pigs from at least 7 days after vaccination (de Smit *et al*., 2001; Dewulf *et al*., 2002), and possibly earlier (Koenen *et al*., unpublished observations).

Efficacious CSFV vaccines must also prevent congenital infections with field virus, since these may result in a variety of abnormalities in the foetuses. From an eradication point of view, the most insidious is the birth of persistently infected immunotolerant healthy piglets that survive for months and continuously shed virus (van Oirschot and Terpstra, 1977). Data on this efficacy aspect of the C-strain are not available or have not or rarely been documented. It may be anticipated, however, that the C-strain will prevent such congenital infections, since it appears to prevent challenge virus replication virtually completely. Nevertheless, it would be worthwhile to demonstrate that the C-strain is able to completely prevent congenital infections.

### 9.4.2. E2 subunit marker vaccine

During the development of the E2 subunit vaccine several experiments were carried out in which it was demonstrated that specific pathogen free (SPF) piglets were protected against the clinical course of the disease two weeks after double vaccination or 6 weeks after single vaccination (Hulst *et al*., 1993; König *et al*., 1995; Van Rijn *et al*., 1996; Peeters *et al*., 1997). More recently it was demonstrated that, with 32 micrograms E2 in a water-oil-water adjuvant, a protective immunity was conferred as early as 3 weeks after a single vaccination (Bouma *et al*., 1999). However, in order to prevent or minimise the spread of the virus in case of an outbreak, the efficacy of the vaccine to reduce replication and shedding is obviously more relevant than the clinical protection induced (van Oirschot, 1999a).

Several experiments have studied the horizontal transmission of the virus. In an experiment where, in a group of vaccinated SPF pigs, some were infected with the homologous CSF-strain, it was found that horizontal transmission within the vaccinated group was prevented by 10 days after a single vaccination (Bouma *et al*., 2000). In similar experiments, with conventional piglets and a recent field isolate as challenge virus, performed in several reference laboratories, it was shown that even after 21 days post vaccination a limited transmission was still possible (Uttenthal *et al*., 2001). In another experiment where SPF pigs were infected 3 weeks post vaccination and subsequently were brought into contact with susceptible piglets, in 1 group out of 8 the vaccinated piglets infected the susceptible piglets by shedding the virus (Bouma *et al*., 1999). In addition, it has been shown that virus infection by contact was delayed but not prevented in twice vaccinated pigs (Dewulf *et al*., 2000).

In experiments evaluating the vertical transmission of the virus, variable results were also obtained. Some reports describe that double or even single

vaccination of pregnant sows was capable of preventing transplacental infection of the foetuses when using as challenge the strain Zoelen, subtype 2 CSFV strain (de Smit *et al*., 2000) or the homologous Brescia strain (Ahrens *et al*., 2000). On the other hand, in the report of the experiments conducted by the EU reference laboratories it is reported that in pregnant sows infected with a recent CSFV-field isolate, Paderborn, a subtype 2 CSFV strain, at 2 weeks post vaccination, the transplacental infection of the offspring occurred in 100% of the cases (Depner *et al*., 2001). In sows infected after a double vaccination, the transplacental infection occurred in 5 out of the 12 sows (Dewulf *et al*. 2001). The authors concluded that double vaccination with an E2 sub-unit marker vaccine only protects pregnant gilts from the clinical course of the disease but prevents neither horizontal nor vertical spread of the CSF virus when a heterologous field virus was used for challenge.

A recent comparative study for emergency vaccination against CSF with an E2 sub-unit marker vaccine and a C-strain vaccine demonstrated that in a vaccinated population, the conventional C-strain vaccine prevents virus transmission from the day of vaccination and that the E2 sub-unit vaccine can prevent virus transmission with an interval of 14 days (Dewulf *et al*., 2003).

## 9.5. Safety

### 9.5.1. Live attenuated vaccines

In no case should the vaccine virus itself cause any damage. Some cell culture viruses as well as some rabbit-adapted viruses can indeed cause intrauterine infections (Bironf and Leunen, 1988), while others, C4, CL, CR20, Thiverval and GPE (-), appear to have lost all virulence for the animal type concerned. (Bironf and Leunen, 1988). It has been reported that the C-strain can pass the placental barrier of pregnant sows but does not seem to produce any abnormality in infected foetuses (Bran *et al*., 1971; Tesmer *et al*., 1973).

When evaluating safety, extra attention needs to be paid to the effects of the virus on the foetus. Pregnant non-immune sows, between the 25$^{th}$ and 35$^{th}$ day of gestation receive a double vaccine dose in one injection. The vaccine should not interfere with gestation or be harmful for the foetuses. The offspring need to be observed for several weeks, not only clinically but also virologically, to check for 'persistently infected piglets' which will only develop clinical signs when they are older (Liess, 1984). Inoculation of 10 doses of vaccine per animal at one time as well as inoculation of animals after treatment with immunosuppressive drugs (cortisone) were reported. The Thiverval strain, GPE (-), CR20, C4 and Cl strain appeared to be safe, even in immunosuppressed pigs (Bironf and Leunen, 1988). Finally, the absence of leucopenia after vaccination was also tested (Swangard *et al*., 1969). When using CSF strains attenuated on rabbits, serious anaphylactic reactions have been described. The allergens are thought to be built up by the sow following repeated vaccination and transmitted to the suckling piglets (Bironf and Leunen, 1988).

Genetic stability of the attenuated virus vaccine is very important. If the vaccine virus is capable of spreading from a vaccinated to a non-vaccinated animal, a selection of more virulent variants, by means of several passages in pigs, can take place. If pigs are inoculated with the CL, C4, CR20, GPE (-) or Thiverval strain under laboratory conditions no serological evidence for transfer of vaccine virus to non-vaccinated contact animals was reported (Biront and Leunen, 1988). However, under normal field conditions C virus spreading has been noticed (Terpstra and Tielen, 1976). The GPE(-) strain can spread more easily from vaccinated animals with respiratory problems to non-vaccinated animals. In the reports concerning genetic stability, more importance is attached to the return of virulence than to the eventual number of passages possible in pigs. With the C-virus strains 30 passages can be carried out (Bran *et al*., 1971) but with the CL strain only 3 passages can be performed before virulence is lost (Precausta *et al*., 1975). No increase of virulence was reported. It has to be emphasised that in most cases the regaining of virulence was tested only in piglets and not in pregnant sows.

The C-strain is the most extensively used vaccine. The replication of the C-strain appeared to be mainly restricted to lymphoid tissues, especially the tonsils, although C-viral antigens have occasionally been detected in kidneys. The strain appears not to persist in pigs for more than 2-3 weeks (Terpstra, 1978; Lorena *et al*., 2001). Chromosomal aberrations have been reported to be associated with C-strain vaccination (Genghini *et al*., 2002), but the consequences of these aberrations are not clear.

Concerning the contamination with other viruses, the recommendations of the European Pharmacopeia are followed with special emphasis on possible contamination with other pestiviruses. Since all these vaccines are relatively old, data concerning molecular differentiation between possible pestivirus contaminants are lacking. Unfortunately, this contamination also occurred with a C-strain vaccine batch, which was found to be contaminated with another pestivirus (Wensvoort and Terpstra, 1988).

#### 9.5.2. E2 subunit marker vaccines

The E2 subunit vaccines have the general safety advantages of inactivated vaccines and have indeed been shown to be highly safe, apart from some local tissue reactions at the injection site (Bouma *et al*., 1999; Lipowski *et al*., 2000; Depner *et al*., 2001).

## 9.6. Differentiation of infected from vaccinated animals

Several CSFV strains (e.g. C-strain, GPE-) have been attenuated by multiple passaging in rabbits or heterologous cell lines. These virus strains have been shown to be completely avirulent, and hence have been used extensively in the past to vaccinate pigs against CSF. Even today the C-strain is used as a live vaccine in areas outside Europe, where CSF is still endemic. However, whereas these vaccine strains induce a protective immunity, the antibody response after vaccination cannot be distinguished from the response after infection. Hence, it is impossible to serologically monitor the introduction and spread of a wild-type CSFV strain in a vaccinated population. For this reason, there is a great need for the development of antibody detection tests which allow an unambiguous serological differentiation of infected from

vacccinated animals. For CSF, several types of marker vaccines have been described:

(i) E2, the most immunogenic structural protein, can be expressed as a single viral protein in a heterologous expression system, such as in recombinant baculoviruses (Moormann *et al*., 2000). When pigs are immunised with this so-called subunit vaccine, they only produce antibodies against E2, but not against one of the other immunogenic proteins, namely $E^{rns}$. Therefore, serological tests intended to be used to differentiate infected from E2-vaccinated animals are based on the specific detection of $E^{rns}$ antibodies (Moormann *et al*., 1996; de Smit *et al*., 2000; Floegel-Niesmann, 2001). Therefore, any positive result implies a previous CSFV infection, whereas a negative result can either be caused by seronegative, naive pigs, or by E2-vaccinated pigs. However, these tests do not allow a differentiation of E2-vaccinated from naive animals. E2 vaccines are the first generation and the only category of CSF marker vaccines that have been licensed in Europe.

(ii) Second generation marker vaccines, which are still in the development phase and have only been used experimentally, consist of a genetically modified C-strain CSFV in which the authentic E2 has been replaced by the respective genome fragment from BVDV (de Smit *et al*., 2001). Serological differentiation of pigs vaccinated with such marker vaccines from wild-type CSFV-infected animals is based on the detection of CSFV-specific E2 antibodies which are absent due to the replacement of the E2 gene of BVDV. Another genetically modified pestivirus that can be used as a marker vaccine for CSF has recently been described, and consists of a recombinant BVDV containing the E2 gene from the CSFV strain Alfort/187 instead of the authentic E2 gene (Reimann *et al*., 2002). In this case, the same principle as for E2 subunit vaccines, namely the detection of antibodies against the CSFV $E^{rns}$ can be applied.

In contrast to subunit vaccines, genetically modified CSFV strains used as vaccine viruses still bear the risk of horizontal or vertical spread within a pig herd. To exclude this risk a new type of CSFV marker vaccine has been described recently based on the concept of a so-called ‘replicon’ vaccine (Widjojoatmodjo *et al*., 2000; Stettler *et al.*, 2002). Replicons are genetically modified ‘live’ viral particles containing an incomplete genome, still allowing them to replicate in the initially infected target cell but preventing them from producing new virions. Therefore, no progeny virus is produced in the vaccinated pig, and hence no virus shedding can occur. The accompanying test used to differentiate replicon-vaccinated from infected animals would again be based on the verification of absence of antibodies against the structural protein whose genetic information has been deleted in the replicon, and hence is not produced by the replicon upon vaccination.

Several other potential marker vaccines have also been produced by inserting the CSFV E2 gene into a heterologous virus genome used as a vector, such as porcine adenovirus (Hammond *et al*., 2000) or pseudorabies virus (Peeters *et al*., 1997). For these vaccines, differentiation of vaccinated from CSFV-infected pigs would be again based on the presence of E2-specific antibodies and the simultaneous absence of $E^{rns}$-specific antibodies.

Since the E2 subunit vaccine is the only type of marker vaccine which has been licensed in Europe so far, comprehensive validation data on the accompanying serological tests (ELISA) used to differentiate vaccinated from infected pigs are only available for this type of vaccine. Currently, two ELISAs are commercially available and they have both been shown to be capable of discriminating infected from vaccinated animals (Van Rijn *et al*., 1999). However, in a report of the experiments conducted by the EU reference laboratories it was shown that both the sensitivity and specificity of these two differential diagnostic antibody ELISAs are considerably lower than that of conventional tests (Floegel-Niesmann, 2001). One of the $E^{rns}$ ELISAs has a low specificity, because it appeared to detect antibodies against the two other pestiviruses, yet it proved to be more sensitive than the other differentiating ELISA, and vice versa. It can be concluded that the current $E^{rns}$ ELISAs are not sufficiently sensitive and specific to reliably identify infected pigs in vaccinated herds. Due to the acknowledged need for the improvement of these tests, one of the manufacturers has already modified the original test and an improved test is currently under evaluation by a network of Community and National Reference Laboratories. Another attempt to develop a more sensitive and specific $E^{rns}$ ELISA is based on a virus type-specific peptide ELISA (Langedijk *et al*., 2001).

## 9.7. Application of vaccine in the field

### 9.7.1. Domestic pigs

In countries where CSF is enzootic vaccination is often practised, and in some countries vaccination is used in addition to the killing of infected herds. Vaccination usually ceases when no more outbreaks occur or when a stage is reached where destruction of infected herds alone may eliminate the residual virus (van Oirschot, 1999a). In The Netherlands, a strict vaccination regimen pursued for 1 year and supported by the appropriate veterinary control measures succeeded in eradicating CSF from three enzootic areas. The programme consisted of mass vaccination of all pigs over 2 weeks of age and supplementary vaccination of previously unvaccinated 6 to 8 week old pigs and newly introduced stock at monthly intervals. The vaccination was compulsory and all vaccinated animals were identified by ear-tagging. The number of outbreaks declined in the vaccinated areas within 2 weeks, and the area was free of CSF from the fifth month after the start of the programme (Terpstra and Robijns, 1977). In order to boost the herd immunity, supplementary vaccination of all piglets born from vaccinated sows at the age of 8-9 weeks, and breeding gilts born from vaccinated sows were revaccinated when 6-7 months old (Terpstra and Wensvoort, 1987).

Recent comparative studies for emergency vaccination against CSF with an E2 sub-unit marker vaccine and a C-strain vaccine demonstrated that in a vaccinated population, the conventional C-strain vaccine prevents virus transmission from the day of vaccination and that the E2 sub-unit vaccine can prevent virus transmission with an interval of 14 days (Dewulf *et al*., 2003).

### 9.7.2. Wild boar

Wild boar play a major role as a reservoir for CSFV in many countries in Europe (Chenut *et al*., 1999; Laddomada, 2000). Therefore, in some areas vaccination programmes are used to reduce the number of susceptible animals below the threshold of transmission (Laddomada, 2000; Kaden *et al.*, 2002). However, the required percentage of the population vaccinated is a function of the estimated population and depends on the area and concentration of animals. As a simple example, if 1,000 wild boar are present it has been reported that a seroconversion rate in 80% of the animals might be needed, depending on the area and density of the animal population. If only 500 wild boar are present the rate might need to be at least 60%. In addition to the number of animals in an area the threshold for persistence depends of the virulence of the virus (Hone *et al*., 1992; Guberti *et al*., 1998). Vaccination programmes should be continued for at least two years after the last reported case (Rutili *et al*., 1998; SCAHAW, 1999b).

Field trials on oral vaccination of wild boar in infected areas using baits containing live attenuated CSFV vaccine strain C commenced in Germany in 1994 (Kaden *et al.*, 2000; Kern and Lahrmann, 2000; Kaden *et al.*, 2001; Kaden *et al.*, 2002). It could be experimentally demonstrated that oral immunisation using the C strain vaccine protected pigs from day 4 post immunisation against a lethal CSFV challenge (Kaden and Lange, 2001) and that the C strain is apathogenic (Chenut *et al.*, 1999)

The immunisation procedure in the field trials included two immunisations 14 days apart and re-vaccination every 6 months (Kaden *et al.*, 2000; Kaden *et al.*, 2002).

As a result of the trials in Germany, it was concluded that:

- Oral immunisation of wild boar can be an additional effective tool for CSF control in Germany.

- The success of oral immunisation is based on the increase of the herd immunity and the decrease of the virus incidence. However, access of young boar to baits is often sub-optimal resulting in low seroconversion rates.

- The manual application of vaccine baits is the method of choice.

- A single application is less effective than a double application.

- The biotope, the population density and the offer of food strongly influence the efficacy of the oral immunisation.

- The vaccinated zone should never be less than 20km wide and fixed on the basis of an intensive serological and virological monitoring.

- Oral immunisation should be continued for 2 years after the last reported case of CSF.

- Further testing of different oral immunisation procedures under defined and comparable conditions are necessary to improve the herd immunity, especially for young boars.

The actual conclusions are published in the working document of the Standing Veterinary Committee, SANCO/387/2002 and based on the research by Kaden *et al.* (1999a,b , 2000, 2001). It should be noted that the use of vaccination is foreseen in Community legislation (CEC, 2001a) and has recently beeen authorised in four German Länder and in Luxembourg) (CEC, 2003a,b).

Since a C-strain vaccine is used, serological monitoring of the wild boar population in order to detect infection becomes impossible and also the risk of carrier animals remains.

Up to now, no marker vaccines have been tested for the oral immunisation of wild boar. Since inactivated preparations (e.g. E2 subunit vaccines) and DNA vaccines may not be suitable to induce a protective immunity after oral application, modified live marker vaccines or 'defective in second cycle' (DISC) vaccines could be potent candidates for the oral CSFV vaccination of wild boar.

Therefore, improved immunisation procedures as well as new vaccines are required. New procedures and novel vaccines need to allow the efficient immunisation of young animals, and enable a discrimination of vaccinated from infected wild boar (marker vaccines).

## 9.8. Future candidate vaccines

The reported conventional modified live CSFV vaccines are safe and highly efficacious and novel vaccines should, in principle, have the same efficacy as that of the classical modified live CSFV vaccines. However, following vaccination with the conventional vaccines, no serological discrimination between vaccinated animals and those reconvalescent from natural infection would be possible. Serological differentiation of infections with different pestivirus species is in general based on monoclonal antibodies directed against the envelope proteins $E^{RNS}$ or E2 (van Rijn *et al.*, 1999; de Smit, 2000; van Gennip *et al.*, 2000; Widjojoatmodjo *et al.*, 2000; de Smit *et al.*, 2001; Floegel-Niesmann, 2001). Due to the high genetic stability of NS3 and the prevalence of non-CSFV pestiviruses in swine, the detection of NS3 specific antibodies seems to be not suitable for a CSFV specific serological marker test. Since E2 is a major immunogen, and part of many CSF vaccines, recently developed CSFV marker assays were designed for the detection of $E^{RNS}$-specific antibodies (Floegel-Niesmann, 2001). So called marker or DIVA vaccines in combination with sensitive and specific discriminating antibody assays would allow differentiation of infected from vaccinated animals (van Oirschot, 1999b). As a first step, subunit marker vaccines based on baculovirus-expressed E2 glycoprotein of CSFV have been developed. However, the immune response develops slowly and is less protective when compared with conventional live attenuated CSFV vaccines.

Taking these points into account, the development of novel CSFV vaccines concentrated on five different strategies, each based on genetically engineered constructs:

(1) viral vectors expressing CSFV proteins,

(2) DNA vaccines,

(3) mutated full length CSFV genomes,

(4) chimeric pestiviruses, and

(5) *trans*-complemented deleted CSFV genomes (replicons).

Most of the new CSF vaccines have been considerably facilitated by c-DNA clones. Infectious cDNA clones of CSFV or BVDV allow the directed manipulation of the genome (Meyers *et al.*, 1996 a,b; Moormann *et al.*, 1996; Ruggli *et al.*, 1996) and the deletion of genomic regions coding for one or more CSFV proteins.

### 9.8.1. Viral vector vaccines

The application of viral vectors for the immunisation against CSF infection has been reported for more than a decade. Using *vaccinia* virus as a viral vector, protection against lethal CSFV challenge could be demonstrated for *vaccinia* recombinants expressing envelope protein E2 and/or $E^{RNS}$. However, vector virus with a high titre had to be administered intravenously. In recent years additional viral vector systems, based on Pseudorabies virus (PRV) or *porcine Adenovirus* (PAV) (Hammond *et al.*, 2000, 2001a,b) expressing CSFV E2 (rPRV-E2 or rPAV-E2), were tested. It could be shown that pigs immunised with rPRV-E2 or rPAV-E2 were protected from CSF challenge infection. Full protection from clinical disease could be achieved early after immunisation using a single shot application (Hooft-van Iddekinge *et al.*, 1996; Hammond *et al.*, 2000). With rPAV-E2 protection rates of 100% were demonstrated after sub-cutaneous application, and of 60% following oral application. Interestingly, oral application of rPAV-E2 did not result in detectable neutralising antibodies (Hammond *et al.*, 2001b). Furthermore, a gD-deleted and gD-*trans*-complemented rPRV-E2 was described as a safe, non-transmissible DISC vector vaccine (Peeters *et al.*, 1997). Although rPRV-E2 and rPAV-E2 were reported to be avirulent (Mulder *et al.*, 1994; Hammond *et al.*, 2000) and efficacious, neither detailed vaccination trials nor data about field trials are available. Furthermore, there are no detailed data presented concerning immunity versus the viral vectors, which might influence re-vaccination. It has to be mentioned that PRV-based vectors are not suitable in regions free of Aujeszky´s Disease since the antibody response to PRV following vaccination could affect serology-based surveillance programmes.

### 9.8.2. DNA vaccines

The administration of naked DNA encoding CSFV envelope protein E2 (DNA-E2) was less efficient than immunisation with the described viral vector vaccines. Recently it was demonstrated that pigs immunised intramuscularly with a single dose of 200 µg DNA-E2 or two shots of 25 µg

DNA-E2 were clinically protected against CSF infection (Andrew *et al.*, 2000). Yu *et al.* (2001) also describe DNA-mediated protection against CSF. In contrast, Markowska-Daniel *et al.* (2001) reported on DNA-E2 immunised pigs clinically diseased post CSFV challenge infection. In addition, a prime-boost vaccination strategy using naked DNA-E2 and rPAV-E2 protected 100% of weaned pigs from disease, whereas in the same experiment rPAV-E2 immunised pigs of the control group had a slight increase in body temperature post challenge (Hammond *et al.*, 2001a,b). The reported CSFV DNA vaccines were safe, although efficacy is limited and high dosages of recombinant DNA are necessary for a complete protection from clinical disease after a single shot immunisation (Andrew *et al.*, 2000).

### 9.8.3. Genetically engineered live CSFV vaccines

Since 1996, infectious cDNA clones of CSFV and BVDV have been available (Meyers *et al.*, 1996b; Moormann *et al.*, 1996; Ruggli *et al.*, 1996). The c-DNA clones of pestiviruses facilitates the construction of deletion mutants and chimeric viruses. Meyers *et al.* (1999) constructed CSFV mutants with an abolished RNase activity due to deletions or amino acid exchanges within the $E^{RNS}$ encoding genomic region. Replication of the RNase negative virus mutants was indistinguishable in tissue culture from wild-type virus and most of the mutant viruses were attenuated *in vivo*. Furthermore, animals immunised with the RNase negative CSFV mutants were completely protected from challenge infection with highly pathogenic CSFV strain Eystrup at 10 weeks post immunisation (Meyers *et al.*, 1999). In contrast to the E2-expressing vector or DNA vaccines, CSFV full length mutants do not enable marker diagnostics yet, e.g. using $E^{RNS}$-blocking ELISA systems.

### 9.8.4. Chimeric pestiviruses

Therefore, chimeric pestiviruses based on the infectious DNA copy of the CSFV vaccine strain C (van Gennip *et al.*, 2000) or the BVDV strain CP7 (Reimann *et al.*, 2002) were constructed. Replacement of the antigenic region of E2 or of the complete $E^{RNS}$ gene of CSFV strain C by the analogous sequences of BVDV type II strain 5250 resulted in viable chimeric viruses. Pigs immunised with the CSFV/BVDV chimeras were completely protected against a lethal CSFV infection, and immunisation prevented CSFV infection of contact animals (de Smit *et al.*, 2001). Furthermore, $E^{RNS}$ and E2 specific antibodies induced by the CSFV/BVDV chimeras could be discriminated from those induced following wild-type infection using CSFV specific $E^{RNS}$ and E2 antibody ELISAs (van Gennip *et al.*, 2000). An additional chimeric pestivirus based on the infectious DNA copy of BVDV strain CP7 was constructed exchanging BVDV E2 with CSFV E2 from strain Alfort 187 (CP7_E2alf) (Reimann *et al.*, 2002). Following intramuscular inoculation, CP7_E2alf proved to be completely avirulent. Neither viraemia nor virus transmission to contact animals were detected. CSFV-specific neutralising antibodies were detected from day 11 post-inoculation and all animals were positive in an E2-specific CSFV-antibody ELISA, but clearly negative for CSFV-$E^{RNS}$-specific antibodies as determined with a CSFV marker ELISA. After challenge infection with highly virulent CSFV strain Eystrup, all pigs immunised with CP7_E2alf were fully protected against clinical signs of CSFV infection, viraemia and

shedding of challenge virus. All challenged pigs were positive in the CSFV marker ELISA at day 21 post challenge (Reimann *et al.*, 2002).

9.8.5. *Trans*-complemented CSFV deletion mutants (replicons)

Due to the discussion that replicating chimeric Pestiviruses might revert to virulent viruses or show unexpected negative features such as a change in tissue or species tropism, *trans*-complemented CSFV $E^{RNS}$ or E2 deletion mutants were constructed (Widjojoatmodjo *et al.*, 2000; van Gennip *et al.*, 2002). The $E^{RNS}$ and E2 deletion mutants autonomously replicated after RNA transfection into SK 6 cells without the production of virus progeny (CSFV replicons). The replicons were *trans*-complemented after transfection into $E^{RNS}$ or E2 expressing recombinant cell lines. Pigs immunised with the *trans*-complemented DISC virions could be protected against a lethal CSFV challenge infection. It was shown that the level of protection using *trans*-complemented replicons depends on the application route. Best results were demonstrated after intradermal inoculation of complemented viruses. No protective immune responses could be demonstrated using intranasal application (van Gennip *et al.*, 2002). The serological data suggested that animals vaccinated with the complemented mutants could be differentiated from wild-type infected animals using $E^{RNS}$ or E2 specific antibody assays (van Gennip *et al.*, 2002). No revertant viruses could be detected directly after *trans*-complementation and after several passages using *trans*-complementing cell lines (Widjojoatmodjo *et al.*, 2000).

It is difficult to compare the efficacy of these candidate vaccines, since the design of the vaccination-challenge experiments differed considerably. In addition, none of the new vaccine types seems to have been tested for its ability to prevent congenital infections. The safety of the various candidate vaccines has not been thoroughly investigated. It is anticipated, at least for the live (replicating) candidate vaccines, that many safety tests will need to be performed before they will be licenced for market use. Consequently, none of these candidate vaccines may be expected to be launched on the market in the next 5-10 years due to various factors, including a lack of support for emergency vaccine research from industrial and governmental bodies.

## 10. AVIAN INFLUENZA

AIVs belong to the *Influenzavirus A* genus of the *Orthomyxoviridae* family and are negative stranded, segmented RNA viruses. The influenza A viruses, can be divided into 15 subtypes on the basis of haemagglutinin antigens (HA). In addition to the HA antigen, influenza viruses possess one of nine neuraminidase antigens (NA). Virtually all H and N combinations have been isolated from birds, thus indicating the extreme antigenic variability that is the hallmark of these viruses. Changes in the HA and NA composition of a virus may occur following genetic reassortment in host cells. One of the consequences of genomic segmentation is that if co-infection by different viruses occurs in the same cell, progeny viruses may originate from the reassortment of parental genes originating from different viruses. Thus, since the influenza A virus genome consists of eight segments from two parental viruses, 256 different combinations of progeny viruses may theoretically arise. A wide variety of domestic and wild avian species are susceptible to AI infections, however on the basis of field evidence, among domestic

poultry, turkeys appear to be the most susceptible and are efficient hosts for virus amplification (Capua and Mutinelli, 2001).

HPAI, which in the past was known as 'fowl plague', is caused by infection with certain influenza A viruses. None of the disease signs can be considered pathognomonic but the incubation period is generally short, followed by high mortality. Wild birds, particularly those associated with aquatic environments, are the reservoirs of viruses of low virulence for poultry and are therefore risk factors for spread of viruses that may become virulent following transmission to poultry hosts.

The potential zoonotic aspects of avian influenza viruses have been recognised since the demonstration that the 1968 H3N2 pandemic virus had arisen as a result or reassortment between a virus of avian origin, donating the haemagglutinin gene, and a virus of human origin, donating most of the maternal genes (Scholtissek *et al*., 1978). This resulted in a virus that was able to spread among humans, but with a haemagglutinin to which the population was immunologically naïve. However, since 1996 it has become clear that avian influenza viruses may be important pathogens capable of infecting humans directly without reassortment. There has been a report of a single case of conjunctivitis from which a H7N7 virus was isolated in England in 1996 (Kurtz *et al*., 1996). In Hong Kong in 1997 H5N1 virus infected 18 people causing flu-like illnesses resulting in 6 deaths (Shortridge *et al*., 1998). In 1999, again in Hong Kong, 2 children were shown to be infected with H9N2 virus (Peiris *et al*., 1999) and there were possibly 5 other H9N2 virus infections in mainland China (Subbaro and Katz, 2000). In 2003 there were 2 deaths in Hong Kong of people infected with H5N1 virus. In The Netherlands during the outbreak of HPAI in poultry in 2003 caused by a virus of H7N7 subtype over 80 people in contact with infected poultry experienced conjunctivitis from which samples were positive for H7N7 virus. A small number of these also had flu-like illnesses and at least 3 appeared to be the result of human-to-human spread (among family members). In April 2003 a Dutch veterinarian developed respiratory signs shortly after participating in the depopulation of a farm of infected poultry. His condition deteriorated to pneumonia and he died approximately 2 weeks after the initial clinical signs. H7N7 virus was shown to be present in his lungs. Zoonotic aspects of the influenza viruses are reviewed in a number of publications (Alexander and Brown, 2000; Capua and Alexander, 2002; Horimoto and Kawaoka, 2001; Subbaro and Katz, 2000).

Diagnosis of AI in poultry depends on the isolation of the virus and a demonstration of its virulence for chickens (OIE, 2000). As the term HPAI refers to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. Whereas all truly virulent strains isolated to date have been either of the H5 or H7 subtype (at least two isolates both of H10 subtype fulfilled some of the criteria for pathogenicity defined below), most H5 or H7 isolates have been of low virulence (Alexander, 1993). The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity, but still primarily involve the inoculation of susceptible chickens with infectious virus; strains are considered to be highly pathogenic if they cause 75% or greater mortality within 10 days (OIE, 2000) or give an intravenous pathogenicity index (IVPI) of >1.2 (CEC, 1992a).

Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity, according to *in vivo* tests or based on molecular determinants (i.e. the presence of multiple amino acids at the cleavage site of the HA gene). These definitions evolve as scientific knowledge of the disease increases and presently there are no other validated methods that fulfil the official criteria laid down in Community

legislation (CEC, 1992a) and for the purposes of confirming disease and implementing the control measures in the EU the following definition applies (CEC, 1992a):

> *"an infection of poultry caused by any influenza A virus that has an intravenous pathogenicity index in 6-week-old chickens >1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin".*

However, recently the SCAHAW (2000a) recommended that the following definition of AI be applied for the purpose of diagnostic procedures for confirmation and differential diagnosis:

*" 'Avian influenza' means an infection of poultry[3] caused by any influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype".*

A new definition for AI similar to this SCAHAW wording has been proposed by the OIE to its member countries. If adopted, this change in definition would have some impact on the diagnostic techniques used since there may be greater use of serological tests for detection of antibodies to H5 or H7 viruses, particularly in primary and secondary outbreaks when involvement of these virus subtypes is suspected. However, in making their recommendation the SCAHAW (2000a) were concerned by the current lack of knowledge on the prevalence of LPAI viruses of H5 and H7 subtypes in poultry populations. Before the recommendation is implemented serological surveys of poultry populations in Member States will be undertaken to determine this prevalence and the likely economic impact that would be involved (CEC, 2002b).

Diagnostic procedures are therefore applied that address the requirements of the definition and rely on virus isolation and the characterisation of the pathogenicity of viruses isolated. However in view of the potential change to the definition of AI, with reference to the present document, the term AI applies to all AIVs of the H5 and H7 subtype, regardless of their virulence. It should be noted that an EU funded research project is investigating the pathogenesis of AI, improved diagnostics and control of AI infections (QLK2-CT-2002-01454).

## 10.1. Diagnostic methods that are currently used

### 10.1.1. Investigation of Suspect Cases

Compliant with the definition above the approach is to isolate the virus, confirm the presence of influenza A virus and identify the subtype. If an influenza A virus is detected it is necessary to assess the virulence of the isolate for poultry. Viruses of H5 or H7 subtype are also subject to nucleotide sequencing to determine the amino acid sequence at the cleavage site of the HA.

[3] It was recommended that 'poultry' be defined as birds that are reared or kept in captivity for the production of meat or eggs for consumption, the production of other commercial products, for restocking supplies of game or for breeding these categories of birds (SCAHAW, 2000a).

Primary outbreak

Clinical specimens from sick or recently dead birds are inoculated into the allantoic cavity of 9-11 day-old embryonated hen's eggs and the eggs are incubated at 35-37°C for 4-7 days (OIE, 2000). The allantoic fluid of any eggs containing dead or dying embryos as they arise, and all eggs at the end of the incubation period, are tested for the presence of haemagglutinating activity using the haemagglutination assay with chicken red blood cells. The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleoprotein or matrix antigens, both of which are common to all influenza A viruses.

The subtype of the newly isolated virus is determined using haemagglutination and neuraminidase inhibition tests against a battery of polyclonal antisera to a wide range of strains covering all the influenza A virus subtypes. As a minimum requirement all national laboratories in Member States need to be able to determine whether an isolate is of H5 or H7 subtype.

The virulence for poultry of all influenza A viruses is determined primarily by the inoculation of 10 susceptible 6-week-old chickens with infectious virus (OIE, 2000). Strains are considered to be highly pathogenic if they produce an index score of greater than 1.2 based on clinical signs seen in a 10 day observation period. An IVPI=0 means no signs were observed during the period whilst an IVPI=3 means that all birds died within 24 hours. Alternatively, for viruses of H5 or H7 subtype the nucleotide sequence of a portion of the HA gene coding for the cleavage site region is determined, thereby enabling deduction of the amino acid sequence. The most commonly used method for nucleotide sequencing is RT-PCR using oligonucleotide primers complementary to areas of the gene flanking the cleavage site coding region followed by sequencing. The sequencing process can take less than 24 hours with the use of automated sequencers.

Once positive virus identification has been made, genetic characterisation of the hypervariable region of the HA gene can provide useful information for identifying the likely origin of the infection and the precise relationship between viruses in the same epizootic.

All influenza A viruses have antigenically similar nucleoprotein and matrix antigens. This fact enables the presence or absence of antibodies to any influenza A virus to be detected by agar gel immunodiffusion (AGID) tests (Beard, 1970) or NP-ELISA (De Boer *et al*., 1990; Zhou *et al*., 1998a). AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection. The haemagglutination inhibition (HI) test is used as the standard for detecting strain-specific antibodies to AI (OIE, 2000) and can be useful in epizootiological studies. Individual sera from some avian species may cause non-specific agglutination of chicken red blood cells, so the presence of this characteristic should first be determined and then removed by adsorption of the serum with chicken red blood cells.

Follow-up outbreak

In the case of second and subsequent outbreaks preliminary diagnosis can be based upon clinical signs or post mortem lesions consistent with AI. Confirmatory laboratory diagnosis is as described for a primary outbreak.

10.1.2. Tracing

Epidemiological tracing is used to identify flocks with any form of contact with an infected premises. This is usually based on clinical signs or post mortem lesions consistent with AI supported by laboratory diagnosis.

Virus

Procedures are as described above to include virus isolation, type and subtype confirmation using standard serological assays and virulence determination using *in vivo* and/or *in vitro* methods.

Antibodies

During eradication programmes where the HA subtype of the virus responsible is already known, or by using the homologous virus as antigen, serological monitoring for evidence of infection may be performed using HI tests.

10.1.3. Freedom of Infection

In the absence of a vaccination policy, in the surveillance zones around an infected premises, statistical sampling of other flocks is based on collection of blood samples and cloacal swabs from the same birds. Freedom from infection is demonstrated by negative HI serology results on blood samples and failure to isolate AIV from cloacal swabs. Virus isolates obtained are characterised as described previously for primary outbreaks. Detection of AI antibody in the absence of isolation of virus is dealt with by policy at national level, i.e. there is no standard EU policy for Member States to follow. This issue can be complicated by birds being immune as a result of previous infection with LPAI strains of H5/H7 excreting HPAI in the absence of clinical signs.

## 10.2. New and emerging diagnostic techniques

Clearly future approaches for diagnosis of AI will be dependent on the adoption of the proposed change to the definition of AI with respect to statutory control. If the proposed changes are adopted there will be greater application of serological techniques with their use in primary and secondary investigations, in addition to tracing as currently occurs. Furthermore, these proposed changes would lead to an increased requirement for active surveillance with implications for the application of appropriate tests on a large scale. There are a number of technologies that have potential to be applied in the future to the diagnosis of AI:

(i) On-farm antigen detection using new technology: For example a chromatographic strip test containing three tests- a) antibody to influenza matrix or nucleoprotein for generic detection of influenza A virus, b)

antibody to H5 virus subtype, and c) antibody to H7 virus subtype. These devices can also be configured for antibody detection. These assays could provide rapid results in the field at relatively low cost. Detection of influenza antigen that is not H5/H7 will require further laboratory examination (as will H5 or H7 positives if the definition remains unchanged).

(ii) Antigen detection ELISAs are widely reported (De Boer *et al*., 1990; Chan *et al*., 2002) and well-established but require validation with clinical specimens from poultry and could be based on systems without a solid phase that are more suited to automation using robots. However, it is unlikely that large-scale throughput for virus detection will be required for AI given the normal epidemiological characteristics of the disease. Non-automated assays in kit form may be suitable for use in laboratories with basic facilities or even in the field.

(iii) RT-PCR offers high sensitivity and specificity combined with rapid throughput including automation using robots and could be applicable to preclinical diagnosis. Current limitations relate to validation with sample types obtained from poultry since these usually contain faecal material that can contain substances inhibitory to the PCR. Possibilities exist to use portable systems for on-farm or near-farm use. Multiplex PCR would enable differential diagnosis for Newcastle disease virus to be examined at the same time.

(iv) NASBA based assays have been developed for the detection of H5 (Collins *et al*., 2002) although they have not yet been validated with respect to their ability to detect every H5 virus.

(v) DNA microarrays have been reported for subtyping influenza and may be applicable to AI particularly if applied to differential diagnosis. This technology is largely in the early developmental phase with currently reported sensitivity being lower than other more widely used methods (Li *et al*., 2001)

(vi) Antibody detection ELISAs have been reported but are either generally targeted towards conserved influenza virus antigens (Zhou *et al*., 1998b), and therefore are not specific for H5 or H7 viruses, or have not been validated. In addition, these assays can now be applied to the detection of antibodies in meat juice, thereby providing a testing capability to examine poultry meat and associated products imported into the EU. Assay design should be compatible with use in a robotic system for application to large-scale testing either for surveillance or disease outbreak investigations.

(vii) Indirect immunofluorescence assays have been developed for differential detection of antibodies to 'field' or vaccine virus based on a heterologous neuraminidase subtype (Capua *et al*., 2003). The opportunity exists to develop alternative automated assays to apply DIVA strategies for AI, should a contingency to control by vaccination be employed in the future.

## 10.3. Types of vaccine

### 10.3.1. Inactivated homologous vaccines

These vaccines were originally prepared as 'autogenous' vaccines, i.e, vaccines that contain the same AI strain as the one causing the disease outbreak in the field. They contain an oil emulsion adjuvant.

The disadvantage of this system is the difficulty of detecting infection in a vaccinated population since the antibody response after infection cannot be differentiated from that after vaccination. This problem may be partly compensated for by the use of unvaccinated sentinel birds in the poultry house. However, sentinel birds may be identified only with difficulty, and there is a risk of substitution of birds.

### 10.3.2. Inactivated heterologous vaccines

This vaccine contains the same HA subtype as the field virus causing the outbreak but has a heterologous NA. In the case of field exposure, clinical protection and reduction of viral shedding are ensured by the immune reaction induced by the homologous HA subtype while the antibodies against the NA induced by the field virus can be used as a marker of natural infection.

### 10.3.3. Live vaccines

Due to the possibility of virus mutation of H5 and H7 subtype viruses no such vaccines are licensed for use in poultry.

### 10.3.4. Recombinant vaccines

A recombinant fowlpox virus expressing the H5 antigen has been licensed in Mexico and is currently being used there (Villareal-Chavez and Rivera Cruz, 2002). Experimental data have also been obtained for fowlpox virus recombinants expressing the H7 antigen (Boyle *et al.*, 2000). Experimental work has also been successfully undertaken on constructs using infectious laryngotracheitis virus (ILTV) as a vector (Lüschow *et al*., 2001). No such product has been licensed in the EU.

## 10.4. Efficacy

Inactivated oil emulsion homologous or heterologous vaccines are known to ensure clinical protection and a reduction of the amount of virus shed by field infected birds (Halvorson, 2002).

A significant amount of experimental work (Swayne *et al*., 1999; Capua *et al*., 2003) has been performed on the use of inactivated oil emulsion vaccines for AI. Generally, the results of experimental work, clearly indicate that, regardless of the use of a homologous (same HA and NA as the challenge virus) or heterologous (same HA but different NA to the challenge virus) strain, in the vaccine:

i) Clinical protection is obtained.

ii) The amount of virus shed into the environment is significantly less in the vaccinated birds than in the unvaccinated controls.

iii) The degree of clinical protection and the reduction of shedding are positively correlated with the antigen mass in the vaccine,

iv) The degree of cross-protection and the reduction of shedding is not correlated to the degree of homology of the HA genes between the vaccine strain and the challenge virus.

In none of the experimental trials was 'sterile immunity' achieved. With reference to the induction of rapid immunity no information is currently available, since all challenge experiments were performed at least 2 weeks after vaccination.

European Pharmacopoeia guidelines should be followed for the manufacturing, potency and safety of inactivated AI vaccines, although they do not currently specify the antigenic content. There is however evidence that higher antigenic mass can influence the immune response and the degree of viral shedding from challenged, vaccinated birds (Swayne *et al*, 1999), so it would be important to determine the minimum HA concentration for protection in these preparations.

10.4.1. Live recombinant vaccines

Similarly, a significant amount of work has been carried out for recombinant vaccines expressing the H5 or H7 antigens (Beard *et al*., 1991, 1992; Webster *et al*., 1996; Swayne *et al*., 1997; Swayne, *et al*., 2000a; Boyle *et al*. 2000; Lüschow *et al*., 2001), with clinical protection being achieved together with a reduction in the amount of virus shed into the environment.

A significant limitation is the failure of these vaccines to replicate and induce protective immunity in birds that have had field exposure to the vector, i.e. fowlpox or ILTV (Swayne *et al*., 2000b). Since serological positivity to these viruses is widespread (due to field exposure and vaccination) in the poultry population and can be in some instances unpredictable, the use of these vector vaccines in case of an emergency restricts their use to a seronegative population to the vector virus. In addition, their use is restricted to species in which the vector virus replicates. For example the ILTV construct could not be used for the immunisation of turkeys since this virus does not replicate in turkeys (Lüschow *et al*., 2001). Insufficient data is available on the immunogenicity of fowlpox virus constructs for species other than the chicken.

## 10.5. Safety

Inactivated oil emulsion vaccines have been proven to be completely safe for poultry, provided they are prepared following the guidelines of the European Pharmacopoeia. However, these vaccines employ mineral oil, which represents a hazard if accidentally injected into humans.

No recombinant vectored vaccines have been licensed in the EU and before this occurs, the issue of introducing a live genetically modified organism in the environment must be addressed, especially with regard to such an organism coming in contact with non-target species and wild birds.

## 10.6. Differentiation of infected from vaccinated animals

With currently available diagnostic methods, inactivated oil emulsion homologous vaccines do not allow the differentiation between field infected and vaccinated birds. The only strategy that can be applied is to leave unvaccinated sentinel birds in each poultry house containing vaccinated birds.

Currently the only approach in Europe that can be used to differentiate infected from vaccinated birds was developed and utilised recently as a strategy to control infection with LPAI H7N1 virus in Italy. The advantage of this method is that a vaccine bank of inactivated oil emulsion heterologous vaccines could be established relatively easily. These vaccines have the advantage that they enable the differentiation between field-infected birds and vaccinated birds through the application of a diagnostic test aimed at the detection of antibodies to the neuraminidase antigen of the field virus.

Vaccine banks have the added advantage that they can be prepared as a contingency for use when an outbreak occurs. It would be necessary to have stocks of vaccine available for two H5 viruses and two H7 viruses from the Eurasian 'lineage' of viruses possessing different neuraminidases. The current immunofluorescence assay to detect antibody to 'wild type' virus has been shown to be relatively robust, specific and sensitive but alternative systems that use automation may enhance throughput and reduce costs.

NS1 of influenza virus is only produced during active replication of the virus thereby the detection of antibodies to this protein could be used as a 'marker' of infection, since there is no active viral replication with conventional vaccines. Therefore both homologous and heterologous vaccination would theoretically allow the use of anti-NS1 antibodies to be used as a 'DIVA' tool. Active studies with avian influenza have not been published using this approach (however some work is currently being undertaken- Capua, pers. comm.) although the ability to detect antibodies to this protein has been demonstrated experimentally with equine influenza (Ozaki *et al*., 2001). If developed, tests to detect antibodies to NS1 could be applied to general serosurveillance programmes and to aid DIVA strategies. These tests would complement the established AGID test that can be applied specifically to determining freedom from infection (Beard, 1970).

Recombinant live vector vaccines also enable the differentiation between infected and vaccinated birds, since they do not induce the production of antibodies against the nucleoprotein antigen, which is common to all AI viruses. Therefore, only naturally infected birds will exhibit antibodies in the AGID or ELISA tests that are directed towards the detection of group A (nucleoprotein) antibodies. However, such studies have been largely experimental and do not offer a real prospect in the medium term for application in the EU.

## 10.7. Application of vaccine in the field.

In recent times inactivated homologous vaccines have been used to try and control HPAI infections in Pakistan and in Mexico (Swayne and Suarez, 2000), but, in the specific conditions under which they were used, they have not have been successful in eradicating the infection. In contrast, in one instance, in Utah (Frame *et al*., 1996), the use of this vaccination strategy, this time against LPAI H7 virus, was successful. The reason for the discrepancy between the results probably lies in the efficacy of the direct control measures, which must be implemented to support a vaccination campaign.

Inactivated heterologous vaccines have been used successfully in Italy during 2000 to 2002 to supplement control measures for the eradication of the H7N1 virus (CEC, 2000b). During 1999 to 2001 Italy was affected by four subsequent epidemic waves of AI caused by viruses of the H7N1 subtype. The first epidemic wave was caused by a LPAI virus of H7N1 subtype that subsequently mutated into a HPAI virus, having circulated in the industrial poultry population for approximately nine months. Following the emergence of the HPAI virus, which caused the death or culling of over 13 million birds, and the implementation of the measures indicated in Council Directive 92/40/CE, the H7N1 LPAI virus re-emerged twice (Capua *et al*., 2002).

In order to control the re-emergence of LPAI virus and to develop a novel control strategy, a coordinated set of measures, including strict biosecurity, a serological monitoring programme and a 'DIVA' strategy were enforced (CEC, 2001c). The 'DIVA' strategy was based on the use of an inactivated oil emulsion vaccine containing the same HA subtype as the field virus, but a different NA subtype, in this case an H7N3 strain. The possibility of using the diverse NA group, to differentiate between vaccinated and naturally infected birds, was achieved through the development of an *ad hoc* serological test based on the detection of specific anti-N1 antibodies (Capua *et al*. 2003).

The test is based on the expression of the N1 gene in a baculovirus system and on the use of the recombinant baculovirus in H5® cells as an antigen for an indirect immunofluorescent antibody test assay (iIFA) (Cattoli *et al*., 2002). The test was validated with 608 turkey field sera derived from: H7N1 naturally infected birds; H7 negative birds from negative flocks; H7N3 vaccinated birds. The agreement between the HI result and the iIFA was assessed by statistical analysis which was defined as 'almost perfect agreement' between the two tests. The relative sensitivity and specificity of the iIfa compared to HI were calculated as 99.1 and 95.7 % respectively.

The control of the field situation was ensured through an intensive sero-surveillance programme aiming at the detection of the LPAI virus, through the regular testing of sentinel birds in vaccinated flocks and through the application of the anti-N1 antibody detection test. Serological monitoring was also enforced in unvaccinated flocks, located both inside and outside the vaccination area. In addition, the efficacy of the vaccination schemes were evaluated in the field through regular testing of selected flocks.

After the first year of vaccination the epidemiological data collected indicated that the H7N1 virus was no longer circulating and the marketing restrictions on fresh meat obtained from vaccinated poultry were lifted (CEC, 2001c).

The experience gathered during the Italian 1999 to 2001 LPAI epidemics suggests that it is possible to use a heterologous vaccine as a 'DIVA' vaccine for the control of AI in poultry. The sensitivity and specificity of the test validated on field sera was satisfactory in discriminating infected from vaccinated birds. The results suggest that this test can be used in the framework of a control policy for avian influenza at a population level. In fact, this system overcomes the problems already described linked to the use of sentinels. The implementation of the discriminatory test allows the official veterinarian to sample directly the vaccinated birds and on the basis of the results to establish whether the flocks are infected or not, thus allowing an accurate assessment of the field situation.

It therefore appears that the combination of a 'DIVA' control strategy with a territorial monitoring system under official control may represent an effective tool for the control of avian influenza infections in poultry, particularly in densely populated poultry areas. In addition, the application of a 'DIVA' vaccination policy, as opposed to a conventional policy, enabled the authorities to establish that infection was no longer circulating and ultimately resulted in the possibility of marketing meat obtained from animals vaccinated against an OIE List A disease.

During 2002-2003 Italy has again experienced outbreaks of AI involving an H7N3 subtype influenza A virus of low pathogenicity (LPAI). Since the outbreak occurred in an area with a high density poultry production and consequent risk of massive spread, vaccination was considered in order to control the spread of infection. On the basis of experience gained in 2000-2002, a DIVA vaccination strategy was considered a feasible option. A vaccination programme based on heterologous vaccination and the development of a discriminatory test to detect field exposure were outlined and approved by the European Commission. In December 2002 the Commission authorised the use of vaccination with the only product immediately available, which contained an H7N3 strain (therefore homologous to the field strain). This product was that used during the 2000-2002 vaccination campaign against the H7N1 virus, and the monitoring of the epidemiological situation was based on the regular (every 28 days) testing of unvaccinated sentinel birds. Since homologous vaccination does not allow a DIVA strategy, meat from those birds vaccinated with the homologous vaccine could not be marketed for intracommunity trade. The experience gained in the 2002-2003 H7N3 AI epidemic indicates that at present, although vaccination represents an option for the control of AI, availability of appropriate vaccines is a major obstacle There are currently no fully licensed H5 or H7 vaccines in the EU and the provision of vaccine banks containing at least 2 different H5 and 2 different H7 strains would be required for the effective application of a vaccination strategy.

The only field experience with recombinant vaccines has been gained in Mexico, where these vaccines have been used in the vaccination campaign against an H5N2 virus. AI has not been eradicated in Mexico, probably

because an eradication programme based on a territorial strategy to include monitoring, vaccination and controlled marketing of infected birds was not established.

It should be noted that in countries where vaccination against HPAI has been applied (Mexico and Pakistan) the virus continued to be isolated from poultry.

## 10.8. Future candidate vaccines

### 10.8.1. Subunit vaccines

Limited studies have been performed on investigating the potential of conventional and recombinant HA to be used as antigens in subunit preparations for vaccines in poultry. As expected both of these preparations do induce protective immunity, however the nature of the immune response is greatly influenced by the content of viral protein and by the presence of an adjuvant or of an antigen presenting system in the vaccine preparation (Kodihalli *et al*., 1994; Crawford *et al*., 1999; Rimmelzwaan *et al*., 1999; Swayne *et al*., 2001).

On the basis of the work that has been undertaken to date, very high antigenic concentrations are necessary to induce protective immunity and the costs of manufacturing such vaccines limit their use in the control of poultry diseases. However, it should be mentioned that recombinant subunit vaccines have the advantage that they can also be used as 'DIVA' vaccines since they do not contain most of the influenza viral antigens.

### 10.8.2. DNA vaccines

DNA-based vaccines represent one of the most recent approaches to vaccine development. This technology has several advantages over conventional vaccines in that they are genetically stable, heat stable and free of contaminants. They can potentially encode several antigens derived from multiple pathogens and have been shown to be effective in the presence of maternal antibodies. In addition, they can induce both humoral and cellular immune responses including at mucosal surfaces (Van Drunen Little-Van den Hurk *et al*., 2000). Early *in vivo* studies demonstrated that plasmids persisted for an extended period of time using intramuscular administration and induced an immune response to the protein encoded by the plasmids (Wolff *et al*., 1992; Davis *et al*., 1997).

One of the first reports was published in 1992 and it demonstrated that vaccination of chickens with plasmid DNA encoding H7-HA resulted in partial protection against lethal viral challenge. In that study, chickens were immunised intravenously, subcutaneously or intraperitoneally and 50% of the vaccinated birds survived challenge 4 weeks after vaccination (Robinson *et al*., 1993). Fynan *et al*. (1993) demonstrated that vaccination by multiple routes i.e. intravenously, intramuscular and intraperitoneally, was more efficient than by the singular administrations via the intramuscular, intrabursal or intratracheal routes. The use of a 'gene gun' (a special injector) to immunise chickens with plasmid DNA encoding the H5-HA was evaluated by Kodihalli *et al*. (1997). The authors concluded that the levels

of protection after DNA vaccination with the 'gene gun' were as good as those achieved with a conventional whole virus vaccine, conferring 95% protection against a challenge with lethal antigenic variants.

Other studies indicated that the nucleoprotein (NP) antigen is able to induce a good cytotoxic T-lymphocyte response with wide cross-reactivity between various influenza A subtypes. Recently, Kodihalli *et al*. (2000) evaluated the ability of a combined DNA vaccine consisting of two plasmids encoding the HA gene of different subtypes (H5 and H7) and a DNA vaccine encoding the viral NP gene derived from H5 subtype to induce protection against highly lethal infection caused by H5 and H7 influenza viruses. Birds immunised with NP-DNA based vaccine only were poorly protected and less than 50% survived challenge, indicating that immunisation with DNA encoding a type-specific gene may not be effective against either homologous or heterologous strains. In contrast, they demonstrated that a vaccine containing a combination of HA subtypes can be effective against lethal infection with viruses expressing any of the HA subtypes used in the preparation.

10.8.3. Virus like particles vaccines

Delivering plasmids encoding antigens of various pathogens by using influenza virus like particles (VLPs) appears to be an effective tool for targeting and gene delivery, providing a novel and promising approach for the development of efficacious vaccines (Cusi and Gluck, 2000). Studies on the feasibility of this kind of vaccination in veterinary medicine are still limited and confined to laboratory trials. Watanabe *et al*. (2002) published a study on the immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. In this research mice were vaccinated with NS2- knockout VLPs and the animals were then challenged three months post-infection with a lethal dose of H1N1 mouse-adapted strain (WSN). After challenge, 94% of vaccinated mice survived demonstrating the potential of replication incompetent NS2-knockout VLPs as novel influenza vaccines. However the safety of these preparations, bearing in mind the capacity of influenza viruses to reassort must be investigated thoroughly.

## 11. SOME OTHER IMPORTANT OIE LIST A DISEASES

The Committee considered it important to briefly consider diagnostic techniques and vaccines for some other OIE List A diseases that are encountered in Europe and that may be important with regard to differential diagnosis for the main diseases considered in this report (FMD, CSF and AI).

### 11.1. African Swine Fever

African Swine Fever (ASF) is one of the most complex viral animal diseases that affects livestock. It was first described by Montgomery in Kenya in 1921, is still endemic in many African countries, and within the EU occurs in Sardinia. It is caused by an icosahedral complex DNA virus, recently classified as a member of the *Asfavirus* genus of the family *Asfaviridae.*

The porcine species is the only one found to be naturally susceptible to this virus which produces clinical signs and lesions, ranging from an acute form to a subacute, chronic and/or inapparent form. It is characterised by high fever, high mortality (in acute form), extensive haemorrhages, pulmonary oedema and intensive necrosis of lymphoid tissue. Clinically, ASF may resemble a variety of other swine haemorrhage diseases and specifically, it can be easily confused with CSF and swine erysipelas. Laboratory tests are required to establish a correct diagnosis. An EU funded research project is currently investigating virus epidemiology, virus-host interactions and improved diagnostic methods for ASF (QLK2-CT-2001-02216).

11.1.1. Diagnostic methods that are currently used

Clinical signs and pathological signs of ASF cannot be differentiated from these of CSF and both diseases should be considered in the diagnosis of any acute febrile haemorrhagic syndrome of pigs. Laboratory tests are essential to distinguish between these diseases.

When ASF is suspected, samples of EDTA blood, spleen, tonsils, kidney and lymph nodes have to be analysed for the detection of virus, virus antigen or viral nucleic acid and simultaneously the presence of antibodies needs to be checked either in serum or in tissue fluids.

Infectious virus can be detected by inoculating pig leucocytes with blood or clarified tissue suspensions. Tests are read by examining the cultures daily under a microscope for 7-10 days for cytopathic effects (CPE) and haemadsorption. Pig erythrocytes will adhere to the surface of pig monocytes or macrophages infected with ASFV producing characteristic haemadsoption rosettes. If no change is observed, a second passage on fresh leucocyte cultures should be performed. There are a small number of "nonhaemadsorbing", mostly avirulent ASFV strains. In those cases confirmation of the specificity of CPE must be performed by viral antigen or nucleic acid detection tests (OIE, 2000).

Viral antigen can be rapidly detected (within approximately 2 hours) on cryostat sections or impression smears of tissues by a direct immunofluorescence (DIF) test using ASFV specific polyclonal antibodies FITC conjugated. Acute cases can be detected with high sensitivity by DIF but in subacute or chronic forms, a sensitivity of only 40% is achieved, probably due to the presence in the animal of antigen-antibody ASFV immunocomplexes from about nine days after infection when high antibody titres are produced, that inhibit viral detection using direct immunofluorescence. Viral antigen can also be detected by ELISA using ASFV polyclonal antiserum but, as in DIF, low sensitivity is found in subacute or chronic forms (Pastor and Escribano, 1990). An ELISA test using monoclonal antibodies for ASFV VP72 protein detection has been described (Vidal *et al.*, 1997) that improves detection limits.

Rapid and sensitive viral nucleic acid detection in blood or tissue samples is now possible through PCR. This technique is particularly useful for identifying virus DNA in samples that are unsuitable for virus isolation or antigen detection (i.e. putrid tissues), but due to the high sensitivity false positive results are possible due to contamination.

Antibodies are not usually detected in pigs infected with virulent ASFV, since they usually die rapidly from peracute infections, but high levels of antibodies can be detected in pigs infected with low or moderately virulent ASF viruses. Commonly ASFV-specific antibodies are detected in an ELISA assay using extracts from infected cells. Confirmatory tests used are indirect immunofluorescence and immunoblotting. Large-scale serological surveillance by ELISA has been used extensively as part of ASF eradication programmes (Sánchez Vizcaíno, 1999; OIE, 2000).

Restriction enzyme analysis of complete virus genomes from different geographical areas over a long period of time has allowed the classification of five distinct groups, four groups containing only African viruses and one group with European and American viruses (Blasco *et al.*, 1989).

11.1.2. Vaccine

No effective vaccine is available and in practice ASF eradication is based on the detection of ASF positive pigs by laboratory diagnosis and the enforcement of strict control measures.

Current attempts to characterise and delete ASF viral genes coding for host range and virulence might open an opportunity for future vaccine development (Tulman and Rock, 2001; Neilan *et al*., 2002). Genes that are responsible for virulence characteristics in swine have been detected and localised in the genome. Such deletion mutants of ASFV may eventually form the basis of live attenuated vaccines against ASF (Neilan *et al*., 2002).

## 11.2. Bluetongue

BT is an infectious, non-contagious, insect-borne viral disease that affects sheep, goats, cattle and wild ruminants. The disease is produced by a segmented double-stranded RNA virus of the *Orbivirus* genus of the family *Reoviridae* that is transmitted by various *Culicoides* species, and has a seasonal incidence. Clinical signs include a febrile response, facial oedema and haemorrhages, and ulceration of the mucous membranes. In some cases the tongue may show intense hyperaemia and there can be lameness as a result of coronitis. At present BT occurs in Southern European countries with a tendency to move northwards.

Genetic studies indicate that BTV tends to exist in discrete, stable ecosystems. For example, the BTV serotypes that circulate in the Caribbean are largely different from those found in North America, probably due to the co-evolution of different strains of virus within each of the two ecosystems (topotypes). Topotyping may be used to monitor the introduction and long-term survival of BTV in different ecosystems (SCAHAW, 2000b).

11.2.1. Diagnostic methods that are currently used

BT is as yet the only disease for which OIE has adopted PCR as the prescribed test (OIE, 2000). Traditional approaches for virus detection in blood or tissue (spleen and lymph nodes) samples from domestic or wild ruminants suspected to be infected with BT virus (BTV) are based on virus

isolation followed by virus identification (serogrouping and serotyping) (OIE, 2000):

- Virus isolation with *in vivo* systems: a) Intravenous inoculation of samples intravascularly on embryonated hens' eggs and analysis of dead embryos by BTV antigen capture ELISA or other antigen detection method (if no embryos are killed a re-inoculation in embryonated hens' eggs or in cell culture with material from the first egg must be performed). b) Inoculation of samples in sheep and checking for the appearance of BTV specific antibodies.

- Virus isolation by inoculation of samples in susceptible cell cultures and monitoring the appearance of CPE whose identity needs to be confirmed by a specific antigen detection method.

After virus isolation a BT serogroup-specific monoclonal antibody (polyclonal anti-BTV antiserum would cross-react with Epizootic haemorrhagic disease serogroup of *Orbivirus*) can be used with different immunological methods (direct immunofluorescence, antigen capture ELISA, immunoperoxidase in microplates) for serogrouping the virus. Serotyping is performed by VNTs type-specific for the recognised 24 BTV serotypes. Different tissue-culture-based methods can be used to detect the presence of neutralising anti-BTV antibodies: plaque reduction, plaque inhibition, microtitre neutralisation or fluorescence inhibition test. Serotype identification is important to the epidemiology of the virus and also to establish vaccination strategies.

Virus isolation followed by virus identification will delay definitive diagnosis by several weeks. Recently several PCR procedures have been described that allow detection of the presence of BTV nucleic acid in blood or tissue samples within a few hours with a high sensitivity and specificity. In addition sequence analysis of known BTV conserved genes amplified by PCR can be used to establish the likely geographical origin of field strains of virus (Zhang *et al.,* 1999a). Serotype-specific primers have been described for several BTV serotypes that allow rapid typing of the isolated virus in a RT-PCR or multiplex RT-PCR assay (Johnson *et al.,* 2000; Zientara *et al.,* 2002).

Serological tests based on the use of monoclonal antibodies in competitive ELISAs that specifically detect anti-BTV antibodies are commercially available and laboratory diagnosis is essential to establish the serotype causing the outbreak, in order to match with the corresponding live attenuated vaccine.

11.2.2. Vaccine

At present only some attenuated virus vaccines are used and the SCAHAW has already produced a report concerning the possible use of vaccination against BT in Europe (SCAHAW, 2000b). This report highlighted various aspects related to the use of the vaccine (difficulty in accessing serotype-specific vaccine, absence of vaccine having been studied in ruminants other than sheep, teratogenic aspects etc.). At the research level other recombinant and inactivated virus vaccines have been evaluated in several laboratories

and under experimental field conditions. Experimentally developed sub-unit vaccines and inactivated vaccines would theoretically allow the differentiation of infected from vaccinated animals (Anderson *et al.,* 1993; SCAHAW, 2000b). From the results obtained until now the best protection is obtained with the virus-like particles (VLP) vaccine generated in infected insect cells by recombinant baculovirus expression of the proteins VP2, VP3, VP5 and VP7 (Roy, 1993; SCAHAW, 2000b). However, more studies should be performed with VLP and inactivated virus vaccine. An EU funded project is underway regarding the development of a safe, efficacious BTV vaccination strategy (QLK2-2001-01722), as well as projects concerning identification of vulnerable areas by surveillance and GIS modelling (including vector distribution) to aid risk assessment (QLK2-CT-2000-00611) and phylogenetic sequence analysis and improved diagnostic assay systems for viruses of the family Reoviridae (QLK2-CT-2000-00143).

## 11.3. Swine Vesicular Disease

Swine vesicular disease (SVD) is a viral disease of pigs caused by a positive single-stranded RNA virus classified in the genus *Enterovirus*, classified in the family *Picornaviridae*.The disease course can vary from a severe form to a subclinical infection depending on virus virulence and infectious dose. The acute form is clinically characterised by vesicles on the coronary bands, heels of the feet and more rarely on the lips and tongue. Laboratory diagnosis is essential since SVD is clinically indistinguishable from FMD.

### 11.3.1. Diagnostic methods that are currently used

The main importance of SVD is that it is clinically indistinguishable from FMD. Any outbreak of vesicular disease in pigs must be assumed to be FMD and samples for differential diagnosis should be obtained immediately. Diagnosis of SVD is based on the demonstration of SVD infective virus, viral antigen or viral nucleic acid in epithelium of vesicles, vesicular fluid or faecal samples or on the presence in serum of specific SVD viral antibodies (OIE, 2000).

Where a vesicular condition is seen in pigs, the detection of SVD antigen by ELISA in a sample of lesion material is sufficient for a positive diagnosis. If the test result is negative virus isolation, a more sensitive detection method, may be performed by inoculating samples in susceptible porcine cell cultures and examining for the appearance of CPE for 2-3 days. Identification of the virus in cultures with CPE must be carried out by ELISA or PCR. Two or three blind passages may be necessary before a CPE is detected in samples with a low viral titre.

Within the EU the disease occurs in Southern Italy and it mainly appears as a subclinical infection of pigs. For this reason, diagnosis based on recognition of clinical signs is not adequate and a different approach based on serology and pen-floor faecal screening test (immunocapture RT-PCR is the method of choice) is applied.

Recently methods of detection of viral nucleic acid by RT-PCR (Lin *et al.*, 1997; Callens and De Clercq, 1999) or Immuno RT-PCR (Fallacara *et al.*, 2000) that improves sensitivity and/or speed of diagnosis have been

introduced to diagnostic laboratories. Immuno RT-PCR test has been shown to be more sensitive than standard direct RT-PCR with faecal samples (possible inhibitors of the reaction are eliminated in the immunocapture process), while higher sensitivity is obtained with direct RT-PCR using epithelial samples. By sequencing RT-PCR products useful information about possible origin of the outbreak can be obtained (Zhang *et al.*, 1999b). Multiplex RT-PCR procedures that allow performing a differential diagnosis of swine vesicular diseases with only one reaction have also been described (Nuñez *et al.*, 1998).

Serological tests are routinely used in disease surveillance. A competition ELISA using mab and VNTs tests are the most widely used. ELISA is rapid and easy to perform, but a small proportion (1%) of sera give 'false' positive results. These sera derive from animals called 'singleton reactors' (SRs). SRs are pigs that yield a positive result in serological tests for SVD but they have no history of contact with SVDV and from which there is no evidence of spread of infection to in-contact animals. SRs occur at a prevalence of approximately 1 per 1,000 animals and may have titres by the VN test ranging from borderline to strongly positive. The following characteristics will help to detect SR: a) On re-sampling SR may show a decreasing or constant titre, b) Sera from SRs generally contain IgM only, c) No positive sera are found from in-contact pigs after first detection of SR. The factor(s) responsible for the appearance of the SR are unknown, they could derive from serological cross-reactivity with another picornavirus (not yet identified) or may be due to other non-specific factor(s) present in the serum.

11.3.2. Vaccine

At present no commercial vaccine is available.

## 11.4. Newcastle Disease

Newcastle disease (ND) is caused by negative single-stranded RNA viruses of avian paramyxovirus serotype 1 (APMV1), which are placed in the *Avulavirus* genus of the *Paramyxoviridae* family (SCAHAW, 1998; Mayo, 2002). Isolates of ND virus (NDV) show marked variation in virulence for chickens and can also infect a wide range of other bird species. Most isolates obtained from naturally infected birds have been shown to be of low virulence for chickens. There is evidence that some field isolates of low virulence have become highly virulent for poultry by mutation (Collins *et al*., 1998; Gould *et al*., 2001).

11.4.1. Diagnostic methods that are currently used

Diagnostic tests for use in virus isolation, virus identification, virus characterisation and serology are specified in the OIE Manual of Standards (OIE, 2000) and in EU Directive 92/66 (CEC, 1992b). Due to the variable virulence of different NDV isolates, diagnosis of ND is not straightforward. Essentially it involves isolation of the virus, its identification as NDV, followed by an assessment of virulence. The procedures used are essentially similar to those for AI (see chapter 10). The ability of various laboratories around the world to carry out full and prompt characterisation is extremely varied. Even within Europe comparative test exercises have demonstrated

the difficulty in achieving inter-laboratory consistency in results (Alexander, 1996; Alexander and Manvell, 1999, 2000). A problem in diagnosis is defining precisely what constitutes notifiable ND. Currently the accepted definitions refer to infections with specified viruses and allow both molecular definition and *in vivo* assessment of virulence (OIE, 2000). The EU definition in Directive 92/66/EEC states:

*"'Newcastle disease' means an infection of poultry caused by any avian strain of the paramyxovirus 1 with an intracerebral pathogenicity index (ICPI) in day-old chicks greater than 0.7".*

In addition, the definition adopted at the 67th General Session of the OIE held in Paris in May 1999 contained a second component (OIE, 2000):

*"Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:*

*a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater*

*or*

*b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113-116. Failure to demonstrate the characteristic pattern of amino acid residues described above would require characterisation of the isolated virus by an ICPI test".*

Many laboratories have been applying modern genetic and antigenic techniques in routine diagnosis in an effort to speed up diagnosis and obtain more information on the origins and epidemiology of ND (reviewed by Aldous and Alexander, 2001). Essentially all of the shortcomings of diagnostic tests related to virus detection and characterisation are as listed for AI (see chapter 10.1). In addition, it is not possible to use serology to assess whether birds have been exposed to field virus or not in countries that apply a vaccination policy.

### 11.4.2. Vaccines

Emergency vaccination in response to new epizootics of disease is usually employed in countries not maintaining an ND vaccinating status but the normal use of ND vaccines is as a prophylactic measure. In countries where ND is endemic and the social infrastructure (e.g. structure of holdings, backyard flocks etc.) is such that biosecurity measures are unlikely to be feasible there is little alternative. The presence of live vaccinal strains may compromise current diagnostic techniques due to the detection of avirulent virus that may exist in a mixed population with virulent viruses. As a result, *in vitro* techniques based on PCR and sequencing may give rise to false results due to primers binding preferentially to viruses of low virulence in a mixed population.

At least two candidate vaccines that basically allow serological differentiation of infected poultry from vaccinated poultry have been developed. One recombinant chimeric vaccine was generated by using a reverse genetics system (Peeters *et al*., 2001). The other candidate vaccine consisted of a recombinant vaccine virus with a deletion of an immunodominant epitope in the nucleoprotein gene. This epitope could also be replaced by a foreign epitope (Mebatsion *et al*., 2002). Both groups also developed a differential diagnostic test. However, it is not clear whether or when these vaccines will be submitted for registration.

## 12. CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH[4]

Although this report describes in particular recent developments in diagnostic techniques and vaccines for the diseases under consideration, some aspects dealing with the potential use of these techniques in the event of an emergency are also mentioned, as requested in the mandate. Some general conclusions and recommendations are listed below that apply to the various diseases under consideration, and disease-specific issues are listed under the headings of the respective diseases. These conclusions and recommendations focus on areas that may be improved. For a comprehensive description of all relevant diagnostic techniques and vaccines the reader is referred to the main text including disease-specific chapters. The Committee has already produced several reports on aspects relevant to the diseases under consideration in this paper[5], and the findings of these reports should be borne in mind in the context of the present report.

### General

Pre-clinical screening

C1: For all infections dealt with in this paper a major impediment for early diagnosis is the fact that there are no effective methods available allowing large-scale preclinical diagnosis (e.g. virological screening).

R1: In order to minimise the time for the primary detection of exotic disease agents following their introduction, it is of major importance to have surveillance systems in place and to carry out continued awareness programmes for veterinary practitioners, farmers and others involved in agri-business. These programmes should emphasise the early clinical signs of these diseases in order to maintain and improve awareness of these diseases.

Laboratory-based methods

C2: A general disadvantage of current diagnostic regimens requiring laboratory-based methods is the inevitable delay caused by the time needed for samples to reach the laboratory. In addition some tests are time-consuming and labour-intensive and not suitable for mass screening. This is especially true for virus isolation from clinical samples which is for all viruses under consideration a highly sensitive and specific diagnostic method.

R2: Therefore pen-side tests should be developed and validated, also in field trials and where possible during disease outbreaks in order to establish a 'first line' of diagnosis. It will be necessary to draw up clear guidance on how such tests should be used (e.g. only by properly trained staff) and in any case, a preliminary diagnosis must be confirmed in the laboratory.

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[4] The notation 'C' is used to identify conclusions and 'R' to indicate recommendations and these are numbered sequentially .

[5] Reports of the Committee are available at http://europa.eu.int/comm/food/fs/sc/scah/outcome_en.html

C3: There are inherent difficulties in sampling and testing large consignments of imported animal products (lack of sensitivity, diagnostic methods unsuited for large-scale application etc.).

R3: Consequently, priority should be given to other preventive methods for detecting virus introduction in a country (e.g. preventing the illegal import of potentially infected materials).

**Future Research**

The development of highly reliable novel formats of pen-side tests is required for the diseases under consideration in this paper. In addition, similar tests should be developed for clinically similar diseases to facilitate a rapid differential diagnosis. The potential for the development of pen-side tests based on nucleic acid amplification/detection methods should also be explored.

**Nucleic acid amplification methods**

C4: In general, nucleic acid amplification/detection methods have high specificity and high sensitivity compared to other diagnostic methods, and they allow a diagnosis within a few hours. However, the sensitivity of these methods can be further enhanced (e.g. by immunocapture).

R4: The potential of nucleic acid amplification/detection methods to become new diagnostic tools should be further explored and considerable efforts should be made to overcome the present difficulties hampering the routine application of nucleic acid amplification/detection methods. Reverse transcription (RT) polymerase chain reaction (PCR)-based methods and similar test protocols (amplification primers, detection or hybridisation probes) should be developed.

C5: Real-time PCR allows amplification of nucleic acids, detection of the amplified products and validation of the process at the same time. Real-time PCR is less prone to contamination compared to other PCR methods and can be automated.

R5: Real-time RT-PCR protocols should be validated and compared by interlaboratory tests. The ultimate goal should be to select the best approach for standardisation.

C6: Multiplex PCR allows the detection of nucleic acids from several pathogens in a single assay, but at present it is too labour-intensive for use in mass screening. Automation of the evaluation using multiplex real-time PCR could overcome this problem. The methods used for direct RNA amplification, especially nucleic acid sequence-based amplification (NASBA), may be more sensitive than PCR or RT-PCR. The drawback is that they use RNA, which is very sensitive to degradation and therefore difficult to handle. This makes the method unsuitable for mass screening. In addition, the probe material must be fresh and appropriately handled.

R6: The development of alternative techniques such as NASBA should be kept under continuous review and pursued if it becomes apparent that they have clear advantages over established protocols.

C7: The greatest drawback of all nucleic amplification and detection techniques is the need to isolate the nucleic acid and to remove potential inhibitors of the enzymes (reverse transcriptase and DNA polymerase) before starting the test. These procedures currently require sophisticated equipment and high laboratory standards to prevent cross-

contamination with the amplified products (amplicons), and a specialised laboratory environment. For the time being this hampers large-scale application and presents difficulties for the development of pen-side tests based on these techniques. Robotic systems are already routinely used for the isolation of DNA from blood.

R7: Development and validation of commercial robots and devices for effective nucleic acid extraction is therefore of crucial importance and should be encouraged. In order to make them useful for mass screening they need to be suitable for handling large numbers of samples without increasing the probability of cross-contamination of samples. After validation, guidelines should be produced as to which standard methods should be used for nucleic acid extraction from different matrices (tissue, blood with EDTA or heparin, clotted blood, serum, faeces etc.).

**Future Research**

The development of multiplex methods to detect several pathogens in one assay should be encouraged. The development of new ligase chain reaction (LCR) methods should be pursued when single (or more) base substitutions have significance for diagnosis, as the method can identify single nucleotide polymorphisms within known sequences.

**Microarrays**

C8: Microarray technology has the potential to revolutionise clinical diagnosis in veterinary and human medicine, especially as the costs for preparing the chips are continuously falling. DNA arrays allow multiple hybridisation tests to be carried out simultaneously, allowing a clinical sample to be screened for a series of pathogens in a single test. This makes them an ideal choice for differential diagnosis. A significant advantage of microarrays is the need for very small sample volumes, resulting in extremely short hybridisation times. Another important potential application is their use for genetic typing of viruses; this method could supplement the currently used sequencing of RT-PCR products. The biggest disadvantage of DNA microarrays is their lack of sensitivity, requiring large amounts of pure nucleic acid for hybridisation. Isolating the nucleic acid and amplifying it, generally by PCR, currently overcomes this. In addition, the PCR products have to be labelled, either radioactively or with a fluorescent marker. Presently, this technology is unsuitable for routine diagnosis and mass screening.

R8: Great effort is needed to develop more sensitive detection methods.

**Future Research**

The development of chips for differential diagnosis of several viral diseases in one assay should be pursued; e.g. a chip that detects CSFV, differentiating it from other pestiviruses, and from unrelated viruses causing similar diseases. This would greatly simplify initial diagnosis.

**Immunoassays**

C9: ELISAs are used to detect infected animals and also to differentiate infected from vaccinated animals (‘DIVA’). ELISAs usually require only a few hours for completion and can be integrated into an automated sample analysis process for high sample throughput. They are also inexpensive and do not require highly technical equipment.

R9: If not already in place, fully automated test procedures and standardised formats should be developed.

C10: The disadvantage of ELISAs is their limited specificity (particularly antibody ELISA) and limited sensitivity (particularly antigen ELISA). In some cases antigen ELISAs depend on the use of infectious virus as antigen, thereby requiring biocontainment laboratory facilities, although in most cases samples can be inactivated before testing (e.g. by heating). Pen-side tests give quick results, since the tests are themselves rapid and delays due to transportation to central laboratories are avoided. Also, if field or regional laboratory operatives have the appropriate communication links, test results can be transmitted immediately to co-ordinating disease control centres. On-the-spot testing also enables any additional/follow-up sampling and testing to be performed immediately, for example, in the case of inconclusive test results or an inconclusive overall assessment of the situation. Pen-side tests can be robust and very simple to perform. This type of test is particularly suited to dealing with suspected secondary cases, but could play a role in the initial monitoring for primary cases. The main problem with pen-side antigen-detection tests is that they are likely to be less sensitive than virus isolation or RT-PCR and therefore there is the relatively high potential for false negative results.

R10: No specific cell-mediated immunity (CMI) based assays, involving chemokines, cytokines etc., are currently available for the diseases under consideration and these should be further developed and their potential assessed. The development of chemiluminiscent tests should also be pursued and if possible the sensitivity and specificity of existing ELISA methods should be improved.

**Future Research**

Continued research is needed to improve the sensitivity of antigen detection ELISAs and to develop tests that can differentiate closely related viruses and their antibodies. Further work is needed to develop more ELISAs that use individual recombinant viral proteins or chemically synthesised peptides instead of complete virus as antigen and to develop ELISAs for the reliable detection of infected animals within vaccinated populations.

Luminescent and chemiluminescent substrates and suitable luminometers are under constant development. Their use in veterinary diagnostics should be further evaluated. It is recommended that the public sector should support collaborative research between veterinary laboratories and companies engaged in biosensor development, in order to develop applications for important animal diseases.

The immunological responses post-infection, including chemokine, cytokine and cell mediated responses, merit further examination, which could facilitate the application of cell-mediated immunity based assays for these diseases.

**Standardisation and validation of new techniques**

C11: There are formal test designations issued by the OIE that are primarily designated for international trade

R11: Minimum test performance criteria should be established for use in diagnosis and surveillance.

C12: In the EU there is a Community Reference Laboratory (CRL) for almost all OIE List A diseases, although there is currently none for FMD. Reference laboratories are

crucial to making progress in international test standardisation and harmonisation and can also collaborate with national/regional laboratories.

R12: Appropriate funding needs to be provided in order to enable reference laboratories to overcome present shortcomings and fulfil their functions properly to facilitate:

- Development, production and distribution of formally designated international reference materials for both serology and virus/antigen/nucleic acid detection for the diseases under discussion.
- Performance and coordination of tasks relating to test standardisation, harmonisation and validation.
- Organisation of proficiency testing for serology and virus detection systems and steps taken to resolve any inter-laboratory variation that emerges.
- Coordination of the establishment of large serum panels that should be shared between a number of different laboratories.

For validation of diagnostic tests OIE, WHO, and ISO reference standards should be used and compliance with corresponding testing recommendations demonstrated. Commercial kits for OIE List A diseases need to be evaluated and validated so that they meet internationally accepted criteria. Laboratories carrying out diagnostic tests for OIE List A diseases should operate in the framework of an international quality system (e.g. EN 17025) and such accreditation would help to safeguard quality assurance in laboratory testing. This would include diagnostic manuals with detailed quality control protocols.

**Vaccines**

C13: New vaccination approaches are currently adopting the potential of genetic manipulation and use of 'vectors', but research is hampered by lack of resources, and the concerns and constraints associated with this type of work.

R13: Vaccines nominated for emergency use should be maintained and produced to a quality acceptable to the national licensing authority of the user-countries. Further assessment should be made of the potential of cryogenically storing fully formulated 'emergency' vaccines.

C14: There are limited scientific data available on the immune responses underlying the early immunity after emergency vaccination. Insight into these processes will lead to improved vaccines and diagnostic tests.

R14: More work should be supported on marker-specific diagnostic tests linked to DIVA vaccines, to improve their sensitivity and/or specificity. More samples should be collected from animal experiments and during field outbreaks to better validate the tests.

**Future Research**

A general priority is that the immunological processes underlying the early onset of immunity after vaccination and challenge should be investigated, in order to improve existing vaccination or to develop improved novel vaccines and associated diagnostics. Future research should focus on resolving the major problems of viral persistence, carrier status and differentiation of infected from vaccinated animals.

**Foot-and-Mouth Disease (FMD)**

**Diagnostic strategies**

C15: Diagnosis of FMD is complicated by the large number of existing serotypes and subtypes of the virus. Matching of field isolates to available vaccines is time-consuming in the case of *in vivo* tests. *In vitro* tests provide only crude estimates of antigenic similarity between field isolates and vaccine viruses and are diffiult to standardise. There is insufficient data on whether these test results correlate to cross-protection, and this probably depends on other factors, such as the potency of a particular vaccine strain. Antigen detection ELISA is not suitable for all kinds of samples and also lacks sensitivity.

R15: Simplified and serotype-independent, e.g. non-structural protein (NSP), serological assays should be developed, for example those using synthetic or recombinant proteins and monoclonal antibodies.

C16: Detection of carrier animals by virus isolation is labour-intensive and unreliable due to the fact that the virus is detectable only on an intermittent basis in such animals. Progressive development of real time RT-PCR tests to detect specific viral RNA in clinical samples offers the possibility of supplementary confirmation by direct detection of carriers, although the method is likely to be less sensitive than NSP serology.

R16: Field and laboratory services should prepare contingency plans for:

- large-scale serology using both highly specific tests for structural antibodies, such as the solid phase competition ELISA, and in case of emergency vaccination, NSP tests

- regional and/or mobile laboratories to carry out testing to confirm secondary cases. This should include provision of ELISAs for detection of both antigen and antibody, and possibly RT-PCR or preferably real time RT-PCR

- the use of RT-PCR or preferably real time RT-PCR as a method to produce rapid diagnostic results, especially for the confirmation of secondary cases.

**Future Research**

Research is needed to develop simple and more reliable methods for the detection of carrier animals, for example the applicability of nasal or saliva swabs. More research is needed to establish the importance of carrier animals and the methods by which virus is able to persist, and the risks arising from the uncertain detection of carrier animals need to be assessed. Novel approaches should be developed for the sensitive detection of FMDV, e.g. in carrier animals, and all technological possibilities should be explored in order to facilitate advances in this area.

Work should be continued to develop marker tests with improved sensitivity and specificity using sera collected from different types of animals, and promising tests need to be commercialised as kits. The confirmatory test used in South America (a form of Western blotting) should be evaluated for more general application. Simplified, homogeneous assay formats of serological tests need to be developed, which are more suited to performance by robotic instruments.

Field tests should be performed to evaluate pen-side tests (e.g. chromatographic strip tests).

Further research is needed to enable antigenicity to be predicted from nucleotide sequence information (which can now be rapidly determined) and to develop well characterised monoclonal antibody panels for rapid antigenic matching of field and vaccine strains.

**Vaccines**

C17: Currently, there are safe, high quality, licensed FMD vaccines available for use in the field that are based on chemically inactivated virus adjuvanted with either aluminium hydroxide/saponin, or a mineral oil. However, in emergency situations there may be insufficient antigen reserves available for immediate incorporation in vaccines to be used in the field.

R17: For contingency plans involving the use of emergency vaccination, oil adjuvanted vaccines should be the principal formulation available. These promote immunity in all main target species (large and small ruminants and pigs) and, principally based on the prevention of clinical signs, a potency value of 3 $PD_{50}$ or greater is necessary. However, for vaccines required in cases of emergency, where a more rapid and possibly an antigenically broader immunity is needed, a potency of $\geq 6$ $PD_{50}$ is required.

C18: The need to have immediate access to such vaccines has led to the establishment of emergency vaccine banks, providing economic and logistical benefits by cryogenically storing concentrated antigen for formulation. The presence of adequate infrastructure for vaccine formulation and bottling is a fundamental requirement for vaccine banks.

R18: There should be close collaboration between vaccine banks. This would allow, for example, extended strain coverage and provide cover whilst the stock of a bank is replaced. In order to avoid delays in the availability of vaccines for use in emergency situations, vaccine banks should have adequate infrastructure readily available for the final formulation of vaccines and vaccine bottling.

C19: Efficacy testing of FMD vaccines is primarily concerned with the ability to prevent clinical signs of the disease. There is limited data relating to the vaccine's ability to inhibit virus excretion and subsequent transmission to in-contact susceptible animals, duration of protection following single administration, rate of protection under different challenge scenarios and use in other species, such as goats.

R19: From limited field evidence, the 'carrier', be it vaccinated or unvaccinated, is still perceived as a risk of further disease spread, and this needs to be substantiated under controlled conditions.

**Future Research**

More applied research should be undertaken on high potency FMD vaccines to determine their efficacy at controlling virus replication and persistence under different circumstances and in different species. More studies, including epidemiological modelling studies, are needed, supported by extensive virological investigations, to validate the approach of using emergency vaccination.

Future 'emergency' vaccine research should address thermostability, broader immunogenicity, topical administration, possible application in the presence of maternal

immunity and also the importance of single application, and an early onset of immunity. Promising new vaccine candidates that adopt the potential of genetic manipulation and 'vectors' should be further investigated.

**Marker vaccination and accompanying tests**

C20: Several NSP ELISAs are already commercially available in simple kit forms.

R20: Development of novel assays should be encouraged so that these kits are widely available where they have the potential to be used.

C21: NSP serology is currently relatively insensitive and therefore cannot be used to certify individual animals.

R21: There is a need to improve the sensitivity of NSP tests to decrease the risk of failing to identify carrier animals. In order to prevent false-positive reactions in NSP serology, vaccines should be manufactured in such a way, that the amount of NSP component is as low as possible.

**Future Research**

Existing commercially available (marker) tests need to be assessed under carefully controlled field trials during disease outbreaks (using uninfected, infected-vaccinated and convalescent animals. More research is needed to develop improved non-structural protein (NSP) tests, including confirmatory assays. A variety of experimental vaccination and challenge studies are needed using different methods and timings of virus exposure, different host species, and different virus serotypes, in order to provide sera and other body fluids for NSP test development and validation. In this framework, the collection of the maximum amount of data and samples during disease outbreaks is crucial very important for the purposes of validation. Acceptance criteria for the use of NSP serology should be established and harmonised as soon as possible.

## Classical Swine Fever (CSF)

### Diagnostic strategies

C22: CSF is usually suspected on clinical grounds, but clinical evidence may be non-specific. Serological differentiation of pestivirus infections still poses a major problem due to the need to perform multiple neutralisation tests on every serum under investigation, especially if large sample numbers have to be examined. Commercial ELISAs are less sensitive than virus neutralisation tests. In addition, no currently available ELISA is fully capable of distinguishing between CSFV-specific antibodies and antibodies to other pestiviruses.

R22: Suitable CSF viral genome areas should be selected in order to harmonise the sequence analysis and comparison of genotyping data. If possible, new diagnostic techniques (e.g. RT-PCR) should be used to check C-strain vaccinated pigs for wild type CSFV.

### Future Research

Research priority should be given to the differentiation of antibodies to CSFV from ruminant pestiviruses and of CSF-vaccinated from infected pigs, respectively. Improvement of automation is also needed to allow testing of high numbers of samples within a short time, both in the event of a massive CSF outbreak, as well as for epidemiological surveillance.

Automation and standardisation of viral genome sequencing for subtyping CSFV strains is required as well as the development of novel alternative tests (e.g. ELISAs) both for antigen and antibody detection.

Assessment is needed of novel techniques such as fluorescence readout for detecting antigen-antibody binding and other detection signals and ELISAs are required for the differentiation of pestivirus antibodies as replacements for the currently used, time- and labour-intensive VNT.

Diagnostic methods such as RT-PCR, antibody titres and inclusion of sentinels should be evaluated under experimental “emergency vaccination” conditions with the aim to demonstrate that pigs and herds are free of CSFV.

Methods to facilitate the early diagnosis of CSFV should also be further investigated (e.g. remote body temperature sensing devices, either by surface temperature determination- infrared, or by using implanted microchips).

### Vaccines

C23: Vaccines based on the C-strain are the most extensively used, and the experimental data as well as the practical experiences are substantial compared to other CSF vaccines. They are highly efficacious and prevent virus excretion, even shortly after vaccination. Therefore they appear to be the most suitable for use in emergency vaccination.

R23: The availability of sufficient dosages of tested and licensed, C-strain vaccine should be ensured by a vaccine bank.

C24: Contrasting results have been found on the efficacy of E2 subunit ‘DIVA’ vaccines. C-strain vaccines were shown to induce a protective immunity much more rapidly than E2 subunit vaccines. Additionally, C-strain vaccines have been successfully used to

support eradication programmes. Both C-strain vaccines and E2 subunit vaccines comply with safety requirements. In a 'suppressive' vaccination strategy (where vaccinated animals are required to be subsequently culled, 'vaccination-to-die' strategy) the C-strain vaccine is undoubtedly the emergency vaccine of choice. The use of the C-strain vaccine excludes the possibility to serologically detect infected pigs in a vaccinated population. Therefore, in a 'protective' vaccination strategy (where vaccinated animals are not required to be subsequently culled, 'vaccination-to-live' strategy) there may, under certain conditions, be some possibility to use the E2 subunit vaccine as an emergency vaccine.

There are still many uncertainties concerning the distribution and the transmission of the virus and the risk factors involved, in pigs, herds and regions under the various conditions of emergency vaccination. Oral vaccination of wild boar has been conducted in field trials although immunisation has been shown to be insufficient in young boar and the immunisation status of vaccinated wild boar is difficult to estimate due to the above mentioned lack of differentiation of orally vaccinated from naturally infected wild boar.

Recently developed experimental vaccines open new perspectives of CSFV vaccination. In addition, some of the candidates are highly efficacious and would allow the differentiation of infected from vaccinated pigs. Despite these promising advances, the perceived limited commercial viability of such vaccines is deterring further uptake of such candidate vaccines by pharmaceutical companies. For these reasons and the necessary compliance with registration requirements, candidate vaccines will not be available in the next five years.

R24: New efficacious and safe candidate marker vaccines should be further developed for registration and commercial distribution. Due to the possibly small commercial market of such vaccines, some public funding may be required to achieve these objectives. Baits should also be developed that could improve the immunisation of young wild boar.

**Future Research**

Studies should be performed on the efficacy of C-strain based vaccines to prevent congenital infections and virus carriers. For both C-strain and E2 subunit vaccines the effect of vaccination on the development of virus carriers should be investigated.

The epidemiology of CSF in emergency vaccination programmes should be observed, analysed and modelled on the level of the individual pig, the herd and the region. Based on these data, risk assessments should be performed with regard to confinement or spread of CSFV.

Since no differentiation by antibody detection can be made using the C-strain vaccine, new methods (such as a RT-PCR) to prove that pigs are free of CSF virus should be tested and evaluated.

The epidemiological evaluation of wild boar vaccination needs to be carried out in order to improve the efficacy of wild boar oral vaccination programmes and research is needed on the development of modified live marker vaccines that can also be used as an oral marker vaccine.

**Marker vaccination and accompanying tests**

C25: When marker vaccines are to be used, the companion serological tests are required to allow a reliable differentiation of infected from vaccinated pigs. The necessity of having to discriminate between CSF and the other pestiviruses compounds the difficulty.

R25: The sensitivity and specificity of currently available ELISAs used to differentiate CSFV-infected from vaccinated pigs need to be improved.

**Future Research**

Epidemiological and experimental virological research should be performed on the implementation of emergency vaccination and the use of 'DIVA' and other diagnostic tests to detect infected pigs in vaccinated populations.

Research is needed to improve the specificity and sensitivity of currently available ELISAs for the differentiation of infected from (marker-) vaccinated pigs. In addition, novel types of 'DIVA' vaccines and accompanying tests should be developed.

**Avian Influenza (AI)**

**Diagnostic strategies**

C26: For the purpose of confirming disease and implementing the control measures in the EU, diagnostic techniques for AI are selected to fulfil the requirements of the present definition (Directive 92/40/EEC). Although it has been proposed to extend the definition to include infection of poultry with any virus of H5 or H7 subtype, a number of recommendations are relevant to either the existing or proposed definition. For current application and subject to a change in the definition of AI for control purposes, current serological tests as specified (Directive 92/40/EEC) can be applied in confirming infection with H5 or H7 influenza virus in primary or subsequent outbreaks. Application of antibody detection ELISA systems to automated testing of serum or meat juice samples could be used if the present definition is revised to include all viruses of H5 or H7 subtype. NP-ELISAs can be used for screening flocks for antibodies against all subtypes of influenza virus and also for showing freedom of infection in the aftermath of an outbreak.

R26: In order to facilitate the application of serological testing on a large-scale, present test formats need to be evaluated for the application of this approach. The NP-ELISAs should be properly validated. For example, the use of these tests in current disease outbreaks should provide invaluable data for such validation. Rapid alternative tests for the detection of H5 and H7 influenza virus subtypes that can also be applied in the field should be developed.

C27: *In vitro* tests for the rapid determination of virulence of AI viruses are very important. The present technique contributes to prolonging the time taken to reach a diagnosis, whereas nucleotide sequencing alone for the detection of virulence can only be used to demonstrate the presence of virulent virus. Serology is useful for detection of H5 or H7 antibodies and could be used more widely if the present definition is changed and the use of large-scale serology could be facilitated by robots.

R27: The Committee reiterates the recommendation of its previous report (SCAHAW, 2000a) concerning amending the definition of avian influenza.

**Future Research**

*Based on current definition of AI for statutory control*

Development of a 'pen-side' test to detect AI virus (NP), H5 and H7 subtypes would be beneficial.

Research is needed aimed at investigating the use of *in vitro* systems for rapid determination of virulence of AI isolates ensuring complete correlation with *in vivo* tests

Antigen detection ELISA needs to be validated for use with clinical specimens from poultry that can be performed in a laboratory with basic facilities

Further research is required to examine the potential of NASBA as an alternative to RT-PCR for the detection of influenza A virus in specimens collected in pre- and post-clinical phases.

*Subject to modification of definition of AI (see above)*

Development of 'pen-side' tests to detect antibodies to influenza A virus (NP), H5 and H7 virus subtypes would be needed.

Tests should be developed for large-scale serology based on ELISA and automated by robots.

**Vaccines**

C28: At present, only inactivated oil emulsion vaccines are licensed in the EU, and therefore these represent the only candidates for emergency vaccination. Both homologous and neuraminidase-heterologous inactivated vaccines are efficacious in controlling the clinical condition and in reducing the amount of virus shed into the environment and therefore both are theoretically applicable.

R28: The use of emergency vaccination should be seriously considered when there is evidence of the introduction of a highly transmissible virus in a densely populated poultry area, or whenever the epidemiological situation indicates that there could be massive and rapid spread of infection. Since monitoring tests are based on the detection of antibodies, an additional method for detecting the early stages of infection should be considered as supplementary to the vaccination programme, such as the use of sentinel birds.

C29: The possible use of recombinant vectored live viruses in emergency vaccination can be complicated by the presence of pre-existing antibodies against the vaccine-vector in the population to be vaccinated. The degree of cross-protection and reduction of virus shedding are influenced by the antigen mass in the vaccine preparation.

R29: The establishment of licensed vaccine banks for AI should be a priority and in particular, the possibility of relying on vaccine preparations for immediate use should be evaluated. The viral strains to be used in the formulations could be either natural isolates or natural reassortants, on the basis of which strains are circulating in the field. In all cases, however, it would be advisable to have stocks of vaccine available produced from two viruses of H5 subtype with different neuraminidase subtypes, and two viruses of H7 with different neuraminidase subtypes, so that virus with a heterologous neuraminidase to the field strain could be used to produce vaccine.

**Future Research**

Identification and generation of appropriate strains to be used in vaccines leading to the establishment of an EU vaccine bank for AI are required.

Research is needed to determine the minimum antigen content of HA for vaccines in different avian species to guarantee adequate protection and significant reduction of viral shedding and the time interval between vaccination and the development of protective immunity.

**Marker vaccination and accompanying tests**

C30: In the case of emergency vaccination, consideration should be given to the use of neuraminidase heterologous vaccination rather than homologous vaccination, mainly because it would enable the differentiation of vaccinated from naturally exposed birds, through the development and application of an appropriate test. The experience gathered during the Italian 1999 to 2001 LPAI epidemics, suggests that it is possible to effectively use a heterologous vaccine as a 'DIVA' vaccine for the control of AI in poultry. The use

of a discriminatory test based on neuraminidase offers the potential to sample vaccinated birds to establish whether a flock is infected. The combination of a 'DIVA' control strategy with a territorial monitoring system under official control may represent an effective tool for the control of AI infections in poultry.

R30: Contingencies should be put in place for the development, validation and use of additional and alternative DIVA tests.

**Future Research**

Recombinant neuraminidase proteins (N1-N9) need to be produced that can be used to differentiate antibodies induced by 'vaccine' strains established in any EU vaccine bank from antibodies against wild type viruses.

New tests (e.g. ELISA-neuraminidase inhibition test) should be developed for the detection of antibodies to the neuraminidase proteins (N1-N9) to facilitate automation and reduce costs.

Assays need to be developed to measure antibodies directed to the NS1 of influenza virus and to evaluate the potential of this approach both as a tool in a 'DIVA' strategy (including use of conventional inactivated oil emulsion vaccines) and for surveillance purposes. Validation should be carried out for the former with serum specimens from epidemics where vaccination has been part of the control strategy and for the latter with samples that have already been tested with a validated method.

Research focused on alternative vaccine design to include subunit, DNA and VLP based vaccines and of companion tests, particularly in relation to the duration of immunity induced by these systems and on the protective effects in a range of poultry hosts.

**African Swine Fever (ASF)**

C31: There is currently no method to differentiate closely related isolates of the virus, which causes difficulty in locating the origin of an outbreak.

R31: Therefore epidemiological investigations are required to identify genome regions containing more variable sequences which can be used to distinguish between closely related isolates of the virus. There is a need to develop a sensitive and specific ELISA test to detect virus antigen for rapid diagnosis of ASF. The usefulness of described ELISA methods for antigen detection that employ monoclonal antibodies should be properly evaluated, including the use of field studies.

**Future Research**

A test needs to be developed to enable rapid differential diagnosis with CSF and efforts should be made to further elucidate the genome organisation of ASFV with the ultimate aim of developing a deletion mutant live vaccine for ASF.

**Bluetongue (BT)**

C32: The development of rapid methods for the diagnosis of BT is needed.

R32: Methods for the typing of BTV based on viral nucleic acid amplification and sequencing should be validated using known BTV serotypes from different geographical areas. BTV involved in new outbreaks should then be sequenced, in order to gain information about the genotype (topotype) of the virus, as a tool for the identification of the possible geographical origin of the virus.

C33: Live attenuated vaccines are available and are licensed for use in sheep. Safe vaccines with distinct efficacy, either based on DNA recombinant techniques or inactivated preparations, have been developed.

R33: The safety and efficacy of live attenuated vaccines should be evaluated in cattle and goats. New efficacious and safe vaccines with marker properties should be further developed for their possible application in the field. Vaccines effective against the appropriate serotypes and suitable for use in all ruminant species need to be developed, tested and held in a vaccine bank to facilitate their use when required.

**Future Research**

Reliable and specific procedures for serotyping, based on RT-PCR, multiplex RT-PCR or microarray technology, need to be developed that allow rapid typing of the virus in clinical samples. Research should be supported to evaluate the quality of alternatives to the present live vaccines, and also to investigate virus-vector interactions and vector distribution.

**Swine Vesicular Disease (SVD)**

C34: Immunocapture RT-PCR has advantages over other methods for detection of SVD virus in faecal samples because possible inhibitors of reaction are eliminated (advantage in comparison with direct RT-PCR), and results are not altered by the presence of other enteroviruses in the sample (advantage over virus isolation).

R34: Among current methods immunocapture RT-PCR should be the method of choice for the diagnosis of SVD.

**Future Research**

Development of multiplex RT-PCR and multiplex real-time RT-PCR methods are needed to simplify diagnosis of diseases with identical clinical signs (FMD/SVD).

More research is needed to identify the origin of the phenomenon of 'singleton reactors' and to develop new ELISA procedures that allow more specific serological tests of SVDV.

**Newcastle Disease (ND)**

**Diagnostic strategies**

C35: Test advantages and disadvantages are as identified for AI since the procedures are very similar.

R35: Tests should be developed for the rapid *in vitro* determination of virulence of ND viruses.

**Future Research**

Pen-side tests to detect APMV1 isolates using conventional or novel technology, possibly in combination with influenza virus antigens to aid differential diagnosis, need to be developed.

Research is needed aimed at investigating the use of *in vitro* systems for rapid determination of virulence of NDV isolatesensuring complete correlation with *in vivo* tests. Specifically LCR offers potential and has the advantage that low levels of virus can be detected in mixed populations of virus.

Evaluation is required of RT-PCR for use on clinical specimens collected in the preclinical phase with an option for use in portable systems for on-farm or near-farm application. The test should also be able to establish whether the viral RNA originates from virulent or avirulent isolates.

**Marker vaccination and accompanying tests**

C36: Vaccination is usually based on the use of live virus strains of low virulence. However vaccine strains cannot be distinguished serologically from naturally occurring field viruses and can sometimes complicate diagnosis when mixed populations of viruses of different pathotype are present in the same clinical sample. Candidate DIVA vaccine have been developed in the laboratory, but are not (yet) licensed for use in the field.

R36: Contingencies should be put in place for the application of a DIVA strategy to control ND and to include the development of a marker vaccine and associated tests for differentiation. The possibility of applying this strategy in a country that was formerly free from ND and had to implement an emergency vaccination policy following the introduction of the disease, would aid the regaining of free status in a shorter time span. More efforts should be put into development, registration and marketing of DIVA vaccines and accompanying tests.

**Future Research**

The further development of marker vaccines and accompanying diagnostic tests are urgently needed.

## 13. CO-ORDINATION OF RESEARCH

The Committee members wish to highlight the need to promote the more effective use of available resources within the European Union concerning research on OIE List A diseases as well as associated vaccines and diagnostic tests. Currently, individual EU Member States are developing contingencies to deal with various OIE List A diseases based on EU established guidelines (the term 'Class A' diseases is used in the EU legislative context). Since the last outbreak of FMD in Europe, governments have increased investment on the prevention of and research into this particular disease. This response raises the likelihood that efforts could be duplicated in separate Member States, and this may also apply to research on other OIE List A diseases, with closely-comparable research, tests, procedures etc. being performed in parallel. Furthermore, standardisation and harmonisation of tests and procedures are required, which is a time-consuming and labour-intensive task. In some Member States the number of researchers working on such tasks is limited and may not be sufficient to form a critical mass to optimise progress in these areas.

In order to carry out research on such OIE List A diseases, expensive high-containment facilities are required, and the concentration of research activities involving such diseases in a limited number of facilities would lead to cost-savings and more efficient use of resources. In order to coordinate and facilitate such research in the short term a 'virtual' laboratory structure could readily be organised between European laboratories. However, an ultimate long-term goal could be the establishment of an EU Research Institute to perform research on OIE List A diseases, with national laboratories continuing to carry out 'first line' diagnosis of these diseases. Scientists from different Member States and with different expertise could work together closely in order to execute research that could possibly prevent, or help to more efficiently handle, outbreaks of these highly contagious infectious diseases. Such a collaborative network could include Community Reference Laboratories and laboratories involved in the diagnosis of these diseases in individual Member States.

Collaboration already occurs on a limited scale in the context of EU funded Concerted Actions, FAIR and Quality of Life projects, COST activities, coordination of work in EU Joint Research Centres (e.g. ISPRA), ERA-NET scheme etc. However, such collaboration is often transient and of limited duration linked to specific projects, with the collaboration and the skills and expertise of personnel being lost upon completion of the project in question. It should also be noted that some projects involving major developments in areas related to diagnostics and disease control often cannot be completed within a short time-frame, such as 3 years for example, and a longer-term perspective could maintain continuity and facilitate continued and accelerated research progress.

To facilitate the implementation of durable and sustained cooperation between laboratories a different approach to funding may be required, with emphasis on longer-term commitments and secure funding provisions being needed. Such sustained cooperation and collaboration could be facilitated in the context of the 'virtual' laboratory structure proposed or the ultimate goal of an EU Research institute. Furthermore, cooperation with researchers in countries outside the EU would also be mutually beneficial.

## 14. EXECUTIVE SUMMARY

In the last decade Europe has experienced a number of epidemics of highly contagious OIE List A animal diseases, such as foot-and-mouth disease (FMD), classical swine fever (CSF) and avian influenza (AI). The threat of infection of European livestock with such diseases has increased due to greater worldwide travel, illegal importation of meat and other animal products and the risks posed by migratory birds carrying exotic diseases. Consequently, improvements in diagnostic techniques and vaccines for such diseases are crucial. As a consequence of disease outbreaks and the EU non-vaccination policy, many millions of animals were destroyed and the economic damage amounted to billions of euros in the outbreaks of FMD in the EU in 2001, as only one example. Apart from the economical dimensions, the mass killing of animals gives rise to animal welfare problems and considerable public concern.

Against this background, the Scientific Committee on Animal Health and Animal Welfare was requested to provide an update on available diagnostic techniques, including consideration of their rapid standardisation and validation, and methods of discriminating infected from vaccinated animals.

A general disadvantage of all laboratory-based methods is the inevitable delay caused by the time needed for samples to reach the laboratory. In order to stop the infection at the earliest possible time the availability and use of pen-side tests by the official veterinarian for a first line of diagnosis should be encouraged. Technologies for nucleic acid amplification offer great potential for the improved speed, sensitivity and specificity of viral diagnosis, including rapid detection of apparently healthy but infected animals and great effort should focus on optimising these technologies for routine diagnosis.

Standardisation, validation and reliability of laboratory diagnostic procedures are crucial. For this purpose, properly funded reference laboratories are essential. At the international level, the OIE provides formal test designations for international trade. However, in the EU there is not a Community Reference Laboratory for all OIE List A diseases (there is none for FMD). Reference laboratories should specify minimum test performance for tests used in diagnosis and surveillance, develop and provide reference materials, carry out proficiency testing, perform and coordinate tasks relating to test standardisation and validation, and evaluate the use of commercial kits for OIE List A diseases. Diagnostic manuals with detailed quality control protocols are also required. Funding constraints often impair performance of these essential tasks, i.e. there is no uniform agreement on minimum detection levels appropriate for infectious agents, whether by culture or indirect detection methods and there is no Europe-wide, official system for evaluation and approval of diagnostic veterinary tests, including commercial kits. Laboratories carrying out diagnostic tests for OIE List A diseases should operate to a third party accredited quality system.

Emergency vaccination using marker vaccines and their accompanying diagnostic tests could be a suitable tool to rapidly interrupt the chain of infection, thereby allowing an early stamping-out of the disease outbreak and avoiding collateral mass culling.

The classical FMD inactivated vaccine (containing no or low quantities of non-structural proteins) is the first choice among vaccines available for use as an emergency vaccine and can be used as a marker vaccine. However, the quality of discriminatory serological tests should be improved in order to more reliably detect infected animals in vaccinated populations. More laboratory and field studies are needed, supported by extensive

virological investigations, to validate the approach of using emergency vaccination in conjunction with NSP serology.

For CSF, live vaccines based on the C-strain are much more efficacious than the newly developed E2 subunit marker vaccines, and therefore appear to be the first choice as emergency vaccine. However, C-strain vaccines do not allow the serological differentiation of infected from vaccinated pigs. Consequently, virus detection methods, such as PCR, have to be applied to demonstrate absence of infection in vaccinated populations. Research on this issue is urgently needed. The diagnostic test that accompanies the E2-subunit vaccines has some deficiencies with respect to sensitivity and specificity. Therefore more efforts should be put into the development of novel marker vaccines and companion diagnostic tests. Vaccine banks for CSF emergency vaccines should also be established.

For AI there are licensed inactivated oil emulsion vaccines only for emergency vaccination available in the EU. The experience gathered during the Italian 1999 to 2001 low pathogenic AI (LPAI) epidemics, suggests that it is possible to effectively use the heterologous vaccine approach in a differentiating infected from vaccinated animals ('DIVA') vaccination strategy for the control of AI in poultry. Research is required in order to determine the minimum antigen content of haemagglutinin antigen (HA) required in a vaccine for different avian species and to determine the time interval between vaccination and the development of protective immunity. Appropriate virus strains to be used in vaccines for AI should be identified and vaccine banks established, while recombinant neuraminidase proteins (N1-N9) are needed which can be used to differentiate antibodies induced by 'vaccine' strains from antibodies against wild type viruses.

Mathematical modelling of these highly contagious diseases is required, and in such studies the impact of various intervention strategies, such as emergency vaccination, can be evaluated. Such work will also take account of diagnostics, disease epidemiology and experimental testing of emergency vaccination. More research is needed to improve diagnostic tests, especially marker-specific tests linked to differentiating infected from vaccinated animals ('DIVA') vaccines, to improve their validation data and, where appropriate, improve sensitivity and/or specificity. Future 'emergency' vaccine developments should exploit all appropriate biotechnologies available and must also acknowledge the importance of single application, and an early onset of immunity.

New vaccination approaches and new vaccine candidates are currently adopting the potential of genetic manipulation and use of 'vectors', although this area of research is hampered by the concerns and constraints related to this type of approach, by limited funding and by barriers to the registration and marketing of such vaccines. In any case it will require many years and the involvement of industry for the development and commercialisation of such vaccines as well as fulfilling licensing and authorisation requirements before such vaccines are available for use in the field.

## 15. ANNEX: APPLICATION OF DIAGNOSTIC TESTS

### Test evaluation

Diagnostic tests are commonly used for detection of a disease, for declaring a herd or region to be free from a certain disease, for screening programmes or for eradication programmes. Diagnostic tests are imperfect, the outcomes are not always correct. One should realise that the classification of animals into diseased and non-diseased is also imperfect, resulting in false-positive and false-negative test results (Noordhuizen *et al.,* 1997). Test evaluation is therefore highly important.

The validity of a diagnostic test for use in the field is represented by characteristics like sensitivity and specificity, and parameters like predictive value positive and negative[6].

Sensitivity and specificity depend on chosen cut-off values; the choice of the cut-off value depends on the purpose of the testing. For example, in case of zoonoses or highly contagious diseases a diagnostic test with high sensitivity is warranted. Sensitivity and specificity are more or less fixed (test feature), while predictive value may alter with the disease prevalence figures (population feature).

A 2x2 Table (Table 8) to calculate sensitivity and specificity of a diagnostic test is reported below.

**Table 8. Model for calculation of sensitivity and specificity of a diagnostic test.**

| | **Reference test ('gold standard')** | | Totals |
|---|---|---|---|
| **Test result** | Diseased | Non-diseased | |
| Diseased | a | b | a+b |
| Non-diseased | c | d | c+d |
| Totals | a+c | b+d | N |

Sensitivity (%) = a x 100 / (a+c)

Specificity (%) = d x100 / (b+d)

Predictive value (%) of negative result = d x 100/(c+d)

Predictive value (%) of positive result = a x 100/(a+b)

A diagnostic test with high sensitivity is indicated:

- In early phases of the infection;
- When truly diseased animals should not escape testing (no false-negatives desired, e.g. epizootic zoonoses);
- When disease probability is low;

[6]The public domain software programme (WIN)EPISCOPE for calculating different epidemiological parameters as named above can be downloaded from http://www.zod.wau.nl//qve (Frankena *et al.,* 1990; Thrusfield *et al.,* 2001)

A diagnostic test with high specificity is indicated:

- For confirming a diagnosis which was set earlier; and

- If false-positives would have a large effect (e.g. when test positive animals would be killed).

- If a disease has a low prevalence, the predictive value positive depends largely on the test specificity. Alternative for the parameter “predictive value” is the likelihood ratio, which is not dependent on prevalence (Noordhuizen *et al.,* 1997).

## 16. REFERENCES


Afshari, C.A., (2002). Perspective: Microarray technology, seeing more than spots. Endocrinology, 143: 1983-1989.

Ahrens, U., Kaden, V., Drexler, C., and Visser, N., (2000). Efficacy of the classical swine fever (CSF) marker vaccine Porcilis Pesti in pregnant sows. Veterinary Microbiology, 77: 83-97.

Al-Yousif, Y., Anderson, J., Chard-Bergstrom, C., Bustamante, A., Muenzenberger, M., Austin, K., and Kapil, S., (2001). Evaluation of a latex agglutination kit (Virogen Rotatest) for detection of bovine rotavirus in fecal samples. Clinical and Diagnostic Laboratory Immunology, 8: 496-498.

Aldous, E.W., and Alexander, D.J., (2001). Technical Review: Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathology, 30 (2): 117-128.

Alem, M., Alem, N., Cohen, H., England, T., Hamedi, N., Moussazadeh, M., Roth, J.A., and Shen, G.Q., (2002). Diagnostic value of detection of IgM antibodies *to Helicobacter pylori*. Experimental and Molecular Pathology, 72: 77-83.

Alexander, D.J., (1993). Orthomyxovirus infections. In*:* Viral Infections of Vertebrates, Volume 3: Viral Infections of Birds, McFerran, J.B., and McNulty, M.S., (Eds.). Horzinek, M.C., (Series editor). Elsevier, Amsterdam, The Netherlands, pp. 287-316.

Alexander, D.J., (1996). Newcastle disease haemagglutination inhibition tests in National Laboratories- Results of comparison of test reproducibility in different laboratories. In: Proceedings of the Joint Third Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of Countries of the European Union, Brussels 1995: pp. 71-84.

Alexander, D.J., and Brown, I.H., (2000). Recent zoonoses caused by influenza A viruses. OIE Scientific and Technical Review, 19: 197-225.

Alexander, D.J., and Manvell, R.J., (1999). Comparative tests for antigen identification in different EU National Laboratories. Proceedings of the Joint Fifth Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of Countries of the European Union, Vienna 1998: pp. 71-77.

Alexander, D.J., and Manvell, R.J., (2000). Comparative tests for antigen identification in different EU National Laboratories. Proceedings of the Joint Sixth Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of Countries of the European Union, Brussels 1999: pp. 47-52.

Amadori, M., Haas, B., Moss, A., and Zebrini, I., (2000). IgA response of cattle to FMDV infection in probang and saliva samples. Report of the Research Group of the Standing Technical Committee of the EU FMD Commission for the control of FMD, Borovets, Bulgaria, 5-8 September 2000. Appendix 9: pp. 88-106.

Anderson, E.C., Capstick, P.B., Mowat, G.N., and Leech, F.B., (1970). *In vitro* method for safety testing of foot-and-mouth disease vaccines. Journal of Hygiene, 68: 159-172.

Anderson, J., Mertens, P.P.C., and Herniman, K.A.J., (1993). A competitive ELISA for the detection of anti-tubule antibodies using a monoclonal antibody against bluetongue virus non-structural protein NS1. Journal of Virological Methods, 43: 167-176.

Andrew, M.E., Morrissy, C.J., Lenghaus, C., Oke, P.G., Sproat, K.W., Hodgson, A.L., Johnson, M.A., and Coupar, B.E., (2000). Protection of pigs against classical swine fever with DNA-delivered gp55. Vaccine, 18: 1932-1938.

Anon., (1998a). OIE Guidelines for International Reference Standards for Antibody Assays. Revue Scientifique et Technique Office International des Epizooties, 17: 602-605.

Anon., (1998b). OIE Guidelines for Laboratory Proficiency Testing. Revue Scientifique et Technique Office International des Epizooties, 17: 605-608.

Anon., (1998c). Proceedings of the final meeting of concerted action CT 93 0909. Special Issue of the Veterinary Quarterly, Volume 20, Supplement 2.

Anon., (1999). Argentina building FMD vaccine bank. Animal Pharm- World Animal Health and Nutrition News, 416: 11.

Anon., (2001). Repeated administration of maximum payload emergency vaccines made from inactivated purified antigen concentrates do not induce significant tires of antibodies against non-structural proteins of foot-and-mouth disease virus. Published in the Report of the European Commission for the Control of Foot-and-Mouth Disease, Session of the Research Group of the Standing Technical Committee. Island of Moen, Denmark, 12-15 September 2001 Rome 2001, Appendix 18: pp. 88-93.

Anthony, R.M., Brown, T.J., and French, G.L., (2001). DNA array technology and diagnostic microbiology. Expert Review of Molecular Diagnostics, 1: 30-38.

Aynaud, J.M., (1988). Principles of vaccination. In: Classical swine fever and related viral infections. Liess, B., (Ed.), Martinus Nijhoff Publishing, Dordrecht, The Netherlands, pp. 165-180.

Babiuk, L.A., (2002). Review: Vaccination- a management tool in veterinary medicine. The Veterinary Journal, 164: 188-201.

Babiuk, L.A., Babuik, S.L., and Baca-Estrada, M.E., (2002). Novel vaccine strategies. Advances in Virus Research, 58: 29-80.

Bachrach, H.L., Moore, D.M., McKercher, P.D., and Polatnick J., (1975). Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. Journal of Immunology, 115: 1636-1641.

Bahnemann, H.G., (1990). Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethyleneimine, Vaccine, 8: 299-303.

Barnett, P.V., and Carabin, H., (2002). A review of emergency foot-and-mouth disease (FMD) vaccines. Vaccine, 20: 1505-1514.

Barnett, P.V., and Statham, R.J., (1995). Long term stability and potency of antigen concentrates held by the international vaccine bank (IVB). Report of the 31st Session of

the European Commission for the Control of Foot-and-Mouth Disase. Rome, Italy. 5-7 April 1995. Appendix 9: pp. 60-65.

Barnett, P.V., and Statham, R.J., (2002). Stratified and cryogenically stored (SACS) vaccines: a new concept in emergency foot-and-mouth disease vaccine formulation and storage. Vaccine, 20: 2060-2064.

Barnett, P.V., Pullen, L., Williams, L., and Doel, T.R., (1996). International vaccine bank for foot-and-mouth disease: assessment of Montanide ISA 25 and ISA 206, two commercially available oil adjuvants. Vaccine, 14: 1187-1198.

Barnett, P.V., Samuel, A.R., and Statham, R.J., (2001). The suitability of the 'emergency' foot-and-mouth disease antigens held by the International Vaccine Bank within a global context. Vaccine, 19: 2107-2117.

Barteling, S.J., and Vreeswijk, J., (1992). Developments in foot-and-mouth disease vaccines. Vaccine, 9: 75-88.

Barteling, S.J., Swam, H., Anemaet, D.A.J., Tuyn, C., and Vreeswijk, J., (1997). Foot-and-mouth disease vaccines from the European bank seem to be sufficiently stable for emergency vaccination. Session of the Research Group of the Standing Technical Committee. European Commission for the Control of Foot and Mouth Disease; Poiana-Brasov, Romania. 1997. FAO, Rome. Appendix 12: pp. 68-70.

Bartley, L.M., Donnelly, C.A., and Anderson, R.M., (2002). Review of foot-and-mouth disease virus survival in animal excretions and on fomites. Veterinary Record, 151: 667-669.

Baxt, B., Neff, S., Reider, E., and Mason, P.W., (1998). Change in receptor specificity of FMDV type A12 by alteration of the VP1 G-H loop. Meeting of the European Study Group on the Molecular Biology of Picornaviruses. Fredrich Schiller University, Jena, Germany, 5-11 September 1998.

Beard, C.W., (1970). Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bulletin of the World Health Organization, 42: 779-785.

Beard, C.W., Schnitzlein, W.M., and Tripathy, D.N., (1991). Protection of chickens against highly pathogenic avian influenza virus (H5N2) by recombinant fowlpox viruses. Avian Diseases, 35: 356–359.

Beard, C.W., Schnitzlein, W.M., and Tripathy, D.N., (1992). Effect of administration on the efficacy of a recombinant fowlpox virus against H5N2 avian influenza. Avian Diseases, 36: 1052–1055.

Belak, S., and Thoren, P., (2001). Molecular diagnosis of animal diseases: some experiences over the past decade. Expert Review of Molecular Diagnostics, 1: 89-98, 434-443.

Bergmann, I.E., Augé de Mello, P., Neitzert, E., Beck, E., and Gomes, I. (1993). Diagnosis of persistent aphtovirus infection and its differentiation from vaccination response in cattle by use of enzyme-linked immunoelectrotransfer blot analysis with bioengineered nonstructural viral antigens. American Journal of Veterinary Research, 54: 825-831.

Bergmann, I., Astudillo, V., Malirat, V., and Neitzert, E., (1998). Serodiagnostic strategy for estimation of FMD viral activity through highly sensitive immunoassays using bioingeneered nonstructural proteins. Veterinary Quarterly, 20 S2: 6-9.

Bergmann, I.E., Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sanchez, C., and Falczuk, A., (2000). Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. Archives of Virology, 145 (3): 473-489.

Bergmann, I.E., Malirat, V., Neitzert, E., Correa Melo, E., (2002). In: Foot-and-mouth diseases: control strategies. Dodet., B. (Ed.). Éditions scientifiques et médicales, Elsevier SAS, pp.1-9.

Berlinzani, A., Brocchi, E., and De Simone, F., (1998). Performances of the 3ABC-trapping ELISA to differentiate infected from vaccinated animals in a field situation: experiences following FMD outbreaks in Albania. Report of the Sess. Res. Gr. St. Tech. Committee of the Europ. Comm. Control FMD, UK, 14-18 Sept. 1998, pp. 166-175.

Biffiger, K., Zwald, D., Kaufmann, L., Briner, A., Nayki, I., Purro, M., Bottcher, S., Struckmeyer, T., Schaller, O., Meyer, R., Fatzer, R., Zurbriggen, A., Stack, M., Moser, M., Oesch, B., and Kubler, E., (2002). Validation of a luminescence immunoassay for the detection of PrP(Sc) in brain homogenate. Journal of Virological Methods, 101: 79-84.

Biront, P., and Leunen, J., (1988). Vaccines. In: CSF and related Viral infections. Liess, B., (Ed.), Martinus Nijhoff Publishing, Dordrecht, The Netherlands, pp. 181-198.

Biront, P., Leunen, J., and Vandeputte, J., (1987). Inhibition of virus replication in the tonsils of pigs previously vaccinated with a Chinese strain vaccine and challenged oronasally with a virulent strain of classical swine fever virus. Veterinary Microbiology, 14: 105-113.

Bittle, J.L., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J., and Brown, F., (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence, Nature, 298: 30-33.

Blasco, R., Agüero, M., Almendral, J.M., and Viñuela, E., (1989). Variable and constant regions of African Swine Fever Virus DNA. Virology, 168: 330-338.

Bouma, A., de Smit, A.J., De Kluijver, E.P., Terpstra, C., and Moormann, R.J.M., (1999). Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. Veterinary Microbiology, 66: 101-114.

Bouma, A., de Smit, A.J., de Jong, M.C., de Kluijver, E.P., and Moormann, R.J., (2000) Determination of the onset of the herd-immunity induced by the E2 sub-unit vaccine against classical swine fever virus. Vaccine, 18: 1374-1381.

Boyle, D.B., Selleck, P., and Heine, H.G., (2000). Vaccinating chickens against avian influenza with fowlpox recombinants expressing the H7 haemagglutinin. Australian Veterinary Journal, 78: 44–48.

Bran, L., Mihaita, S., Popa, M., and Totorcea, N., (1971). Trans-uterine and transplacentar transmission of attenuated rabbit-adapted swine fever virus strains, in pregnant sows. Arch. Vet. Bucuresti, 7: 11-20.

Brocchi, E., De Diego, M., Berlinzani, A., Gamba, D., and De Simone, F., (1998). Diagnostic potential of Mab-based ELISAs for antibodies to non-structural proteins of foot-and-mouth disease virus to differentiate infection from vaccination. Veterinary Quarterly, 20 S2: 20-24.

Brown, F., (1988). Use of peptides for immunization against foot and mouth disease, Vaccine, 6: 180-183.

Brown, F., (1992). New approaches to vaccination against foot-and-mouth disease, Vaccine, 10: 1022-1026.

Brown, F., (1999). Foot-and-mouth disease: vaccine design, past, present and future. Archives of Virology Suppl., 15: 179-188.

Bruning, A., Bellamy, K., Talbot, D., and Anderson, J., (1999). A rapid chromatographic strip test for the pen-side diagnosis of rinderpest virus. Journal of Virological Methods, 81: 143-154.

Callahan, J.D., Brown, F., Osorio, F.A., Sur, J.H., Kramer, E., Long, G.W., Lubroth, J., Ellis, S.J., Shoulars, K.S., Gaffney, K.L., Rock, D.L., and Nelson, W.M., (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. Journal of the American Veterinary Medical Association, 220: 1636-42.

Callens, M., and De Clercq, K., (1999). Highly sensitive detection of swine vesicular disease virus based on a single tube RT-PCR system and DIG-ELISA detection. Journal of Virological Methods, 77 (1): 87-99.

Capua, I., and Alexander, D.J., (2002). Avian influenza and human health. Acta Tropica, 83: 1-6.

Capua, I., and Mutinelli, F., (2001). A colour atlas and text on avian influenza. Papi Editore, Casalecchio di Reno, Bologna, Italy.

Capua, I., Mutinelli, F., Marangon, S. and Alexander, D.J., (2000). H7N1 avian influenza in Italy (1999 to 2000) in intensively reared chickens and turkeys. Avian Pathology, 29: 537-543.

Capua, I., Cattoli, G., Marangon, S., Bortolotti, L., and Ortali, G., (2002). Strategies for the control of avian influenza in Italy. Veterinary Record, 150 (7): 223.

Capua, I., Terregino C., Cattoli G., Mutinelli F., and Rodriguez, J.F., (2003). Development of a DIVA-Differentiating infected from vaccinated animals- strategy using a vaccine containing a heterologous neruraminidase for the control of avian influenza. Avian Pathology, 32: 47-55.

Carbrey, E.A., (1988). Diagnostic procedures. In: Classical swine fever and related viral infections, Liess, B., (Ed.). Martinus Nijhoff Publishing, Boston, Dordrecht, Lancaster.

Carrillo, C., Wigdorovitz, A., Oliveros, J.C., Zamorano, P.I., Sadir, A.M., Gomez, N., Salinas, J., Escribano, J.M., and Borca, M.V., (1998). Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants, Journal of Virology, 72: 1688-1690.

Carrino, J.J., and Lee, H.H., (1995). Nucleic acid amplification methods. Journal of Microbiological Methods, 23: 3-20.

Carvalho, R., Passos, L.M., and Martins, A.S., (2000). Development of a differential multiplex PCR assay for equine herpesvirus 1 and 4 as a diagnostic tool. Journal of Veterinary Medicine B: Infectious Diseases and Veterinary Public Health. 47: 351-359.

Cattoli, G., Terregino, C., Brasola, V., Rodriguez, J.F., and Capua, I., (2002). Development and preliminary validation of an "ad hoc" N1-N3 discriminatory test for the control of Avian Influenza in Italy. In: Proceedings of the 5th International Symposium on Avian Influenza. Georgia Center for Continuing Education, The University of Georgia, Athens Georgia USA, 14-17 April 2002, p. 28.

CEC (Commission of the European Communities), (1986). Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for other scientific purposes. Official Journal of the European Communities.

CEC (Commission of the European Communities), (1990). Council Directive 90/539/EEC of 15 October 1990 on animal health conditions governing intra-Community trade in, and imports from third countries of, poultry and hatching eggs.

CEC (Commission of the European Communities), (1992a). Council Directive 92/40/EEC of 19 May 1992 introducing Community measures for the control of avian influenza.

CEC (Commission of the European Communities), (1992b). Council Directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease.

CEC (Commission of the European Communities), (2000a). Commission Decision 2000/112/EC of 14 January 2000 detailing the distribution between antigen banks of antigen reserve established within the framework of the Community action concerning reserves of foot-and-mouth disease vaccines and amending Commission Decisions 93/590/EC and 97/348/EC.

CEC (Commission of the European Communities), (2000b). Commission Decision 2000/721/EC of 7 November 2000 on introducing vaccination to supplement the measures to control avian influenza in Italy and on specific movement control measures.

CEC (Commission of the European Communities), (2001a). Council Directive 2001/89/EC of 23 October 2001 on Community measures for the control of classical swine fever.

CEC (Commission of the European Communities), (2001b). Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products.

CEC (Commission of the European Communities), (2001c). Commission Decision 2001/847/CE of 30 November 2001 amending for the third time Decision 2000/721/EC to modify the Italian avian influenza vaccination programme and current trade restrictions for fresh meat originating from vaccinated turkeys.

CEC (Commission of the European Communities), (2002a). Commission Decision 2002/106/EC of 1 February 2002 approving a Diagnostic Manual establishing diagnostic procedures, sampling methods and criteria for evaluation of the laboratory tests for the confirmation of classical swine fever.

CEC (Commission of the European Communities), (2002b). Commission Decision 2002/649/EC of 5 August 2002 on the implementation of surveys for avian influenza in poultry and wild birds in the Member States.

CEC (Commission of the European Communities), (2003a). Commission Decision 2003/135/EC of 27 February 2003 on the approval of the plans for the eradication of classical swine fever and the emergency vaccination of feral pigs against classical swine fever in Germany, in the federal states of Lower Saxony, North Rhine-Westphalia, Rhineland-Palatinate and Saarland.

CEC (Commission of the European Communities), (2003b). Commission Decision 2003/136/EC of 27 February 2003 on the approval of the plans for the eradication of classical swine fever in feral pigs and emergency vaccination of feral pigs against classical swine fever in Luxembourg.

Cedillo-Barrón, L., Foster-Cuevas, M., Belsham, G.J., Lefèand, F.R., and Parkhouse, M.E., (2001). Induction of a protective response in swine vaccinated with DNA encoding foot-and-mouth disease virus empty capsid proteins and the 3D RNA polymerase. Journal of General Virology, 82: 1713-1724.

Chan, K.H., Maldeis, N., Pope, W., Yup, A., Ozinskas, A., Gill, J., Seto, W.H., Shortridge, K.F. and Peiris, J.S.M., (2002). Evaluation of the Directigen FluA+B test for rapid diagnosis of influenza virus type A and B infections. Journal of Clinical Microbiology, 40 (5): 1675-1680.

Charleston, B., Brackenbury, L.S., Carr, B.V., Fray, M.D., Hope, J.C., Howard, C.J., and Morrison, W.I., (2002). Alpha/beta and gamma interferons are induced by infection with noncytopathic bovine viral diarrhea virus *in vivo*. Journal of Virology, 76: 923-927.

Chenut, G., Saintilan, A.F., Burger, C., Rosenthal, F., Cruciere, C., Picard, M., Bruyere, V., and Albina, E., (1999). Oral immunisation of swine with a classical swine fever vaccine (Chinese strain) and transmission studies in rabbits and sheep. Veterinary Microbiology, 64: 265-276.

Cheung, A.K., and Kűpper, H., (1984). Biotechnological approach to a new foot-and-mouth disease vaccine, Biotechnology and Genetic Engineering Reviews, 1: 223-259.

Chinsangaram, J., Beard, C., Mason, P.W., Zellner, M.K., Ward, G., and Grubman M.J., (1998). Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. Journal of Virology, 72: 4454-4457.

Chinsangaram, J., Moraes, M.P., Koster, M., and Grubman, M.J. (2003). Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. Journal of Virology, 77: 1621-1625.

Chomczynski, P., and Sacchi, N., (1987). Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. Analytical Biochemistry, 162: 156-159.

Clarke, B.E., Newton, S.E., Carroll, A.R., Francis, M.J., Appleyard, G., Syred, A.D., Highfield, P.E., Rowlands, D.J., and Brown, F., (1987). Immproved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. Nature, 330: 381-384.

Colijn, E.O., Bloemraad, M., and Wensvoort, G., (1997). An improved ELISA for the detection of serum antibodies directed against classical swine fever virus. Veterinary Microbiology, 59: 15-25.

Collins, M.S., Franklin, S., Strong, I., Meulemans, G., and Alexander, D.J., (1998). Antigenic and phylogenetic studies on a variant Newcastle Disease virus using anti-fusion protein monoclonal antibodies and partial sequencing of the fusion protein gene. Avian Pathology, 27: 90-96.

Collins, R.A., Ko, L.S., So, K.L., Ellis, T., Lau, L.T., and Hoi Yu, A.C., (2002). Detection of highly pathogenic and low pathogenic avian influenza subtype H5 (Eurasian lineage) using NASBA. Journal of Virological Methods, 103: 213-225.

Collins, M.S., Govey, S.J., and Alexander, D.J., (2003). Rapid *in vitro* assessment of the virulence of Newcastle disease viruses using the ligase chain reaction. Archives of Virology (in press).

Compton, J., (1991). Nucleic-acid sequence-based amplification. Nature, 350: 91-92.

Cortese, D., (2000). Technological improvements are pushing microplate readers into the 21st century's high speed computerized world. The Scientist, 14: 24-30.

Council of Europe, (1986). European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific purposes. European Treaty Series No 123.

Council of Europe, (1997). European Pharmacopoeia, 3rd Edition, Supplement 1998.

Cox J.C., and Coulter A.R., (1997). Adjuvants– a classification and review of their modes of action. Vaccine, 15: 248-56.

Crawford, J., Wilkinson, B., Vosnesensky, A., Smith, G., Garcia, M., Stone, H., and Perdue, M., (1999). Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine, 17: 2265-2274.

Crowther, J.R., (1998). Detection of viruses in livestock. Parasitology, 117: Suppl. S29-40.

Cusi, M.G., and Gluck, R., (2000). Potential of DNA vaccines delivered by influenza viromes, Vaccine, 18: 1435.

Cussler, K., Morton, DB & Hendriksen, CFM (1999a) Humane endpoints in vaccine research and quality control. In: Humane Endpoints in Animal Experiments for Biomedical Research pp 95-101. Proceedings of the International Conference, 22-25 Nov 1998 Zeist, The Netherlands. Eds. CFM Hendriksen & DB Morton. ISBN 1-85315-429-6 Publrs Royal Soc Med. London WIM 8AE

Cussler, K. Possibilities for the use of humane endpoints in vaccine potency tests. In: Proceedings of the 4th World Congress on Alternatives and Animal Use in the Life Sciences. New Orleans 2002 (In press, ATLA 2003/2004).

Davis, H.L., Brazolot-Millan, C.L., and Watkins, S.C., (1997). Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. Gene Therapy, 4: 181.

De Boer, G.F., Back, W., and Osterhaus, A.D.M.E., (1990). An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species. Archives of Virology, 115: 47-61.

De Diego, M., Brocchi, E., Mackay, D., and De Simone, F., (1997). The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Archives of Virology, 142: 2021-2033.

De Jong, M.C., and Kimman, T.G., (1994). Experimental quantification of vaccine-induced reduction in virus transmission. Vaccine, 12: 761-766.

de Smit, A.J., (2000). Laboratory diagnosis, epizootiology, and efficacy of marker vaccines in classical swine fever: a review. Veterinary Quarterly, 22: 182-188.

de Smit, A.J., Van de Wetering, G., Colijn, E.C., Hulst, M.M., Kramps, J.A., Van den Blink, A., and Moormann, R.J.M., (2000). Evaluation of an ELISA for the detection of antibodies against the $E^{rns}$ envelope protein of classical swine fever virus. In: PhD thesis: de Smit, A.J. Classical swine fever. Efficacy of marker vaccines and laboratory diagnosis. Utrecht University: pp. 117-131.

de Smit, A.J., Bouma, A., van Gennip, H.G., de Kluijver, E.P., and Moormann, R.J., (2001). Chimeric (marker) C-strain viruses induce clinical protection against virulent classical swine fever virus (CSFV) and reduce transmission of CSFV between vaccinated pigs, Vaccine, 19: 1467-1476.

Debouck, C., and Goodfellow, P.N., (1999). DNA microarrays in drug discovery and development. Nature, Genetics Supplement 21: 48-50.

DEFRA (Department for Environment Food and Rural Affairs), (2003). Statistics on foot and mouth disease. Data drawn from DEFRA Disease Control System Database. http://www.defra.gov.uk/animalh/diseases/fmd/cases/statistics/breakdownstats.asp

Deiman, B., Van Aarle, P., and Sillekens, P., (2002). Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Molecular Biotechnology, 20: 163-179.

Depner, K.R., Bouma, A., Koenen, F., Klinkenberg, D., Lange, E., de Smit, J.A. and Vanderhallen, H., (2001). Classical swine fever (CSF) marker vaccine. Trial II. Challenge study in pregnant sows. Veterinary Microbiology, 83: 107-120.

Desmecht, M., Charlie, G., Van Lierde, H., and Leunen, J., (1977). Contrôle d'activité du vaccin lapinisé de la peste porcine. Ann. Méd Vét., 121: 567-572.

Dewulf, J., Laevens, H., Koenen, F., Vanderhallen, H., Mintiens, K., Deluyker, H., de Kruif, A., (2000). An experimental infection with classical swine fever in E2 sub-unit marker-vaccine vaccinated and in non-vaccinated pigs. Vaccine, 19: 475-482.

Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., and de Kruif, A., (2001). An E2 sub-unit marker vaccine does not prevent horizontal or vertical transmission of classical swine fever virus. Vaccine, 20: 86-91.

Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., and De Kruif, A., (2002). A comparitive study for emergency vaccination against classical swine fever with an E2 sub-unit marker-vaccine and a C-strain vaccine. Proceedings of the International Pig Veterinary Society, Ames, p. 325.

Dewulf, J., Laevens, H., Koenen, F., Mintiens, K., and de Kruif, A., (2003). A comparative study for emergency vaccination against CSF with an E2 sub-unit vaccine and a C-strain vaccine. Vaccine (In press).

Ditcham, W.G., Al-Obaidi, A.H., McStay, D., Mottram, T.T., Brownlie, J., and Thompson, I., (2001). An immunosensor with potential for the detection of viral antigens in body fluids, based on surface second harmonic generation. Biosensors and Bioelectronics, 16: 221-224.

Doel, T.R., (1985). Prospects for improved vaccines. Vaccine, 3: 35-36.

Doel, T.R., (1999). Optimisation of the immune response to foot-and-mouth disease vaccine. Vaccine, 17: 1767-1771.

Doel, T.R., (2001). Repeated administration of maximum payload emergency vaccines made from inactivated purified antigen concentrates do not induce significant titres of antibodies against non-structural proteins of foot-and-mouth disease virus. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and -Mouth Disease. Island of Moen, Denmark, 12-15 September 2001. Appendix 18: pp. 88-92.

Doel, T.R., and Collen, T., (1982). Quantitative assessment of 146s particles of foot-and-mouth disease virus in preparations destined for vaccine. Journal of Biological Standardization, 10: 69-81.

Doel, T.R., Gale, C., Do Amarel, C.M.C.F., Mulcahy, C., and Dimarchi, R., (1990). Heterotypic protection induced by synthetic peptides corresponding to three serotypes of foot-and-mouth disease virus. Journal of Virology, 64 (5): 2260-2264.

Doel, T.R., Williams, L., and Barnett, P.V., (1993). Emergency vaccination against foot-and-mouth disease: The rate of development of immunity and its implications for the carrier state. Vaccine, 12: 592-600.

Domingo, E., Mateu, M.G., Martínez, M.A., Dopazo, J., Moya, A., and Sobrino, F., (1990). Genetic variability and antigenic diversity of foot-and-mouth disease virus. In: Applied virology research, Vol. 2, Virus variation and epidemiology, Kurstak, E., Marusyk, R.G., Murphy, S.A., and Van-Regenmortel, M.H.V., (Eds), Plenum Publishing Corp., New York, pp. 233-266.

Donaldson, A.I., and Kitching, R.P., (1989). Transmission of foot and mouth disease by vaccinated cattle following natural challenge. Research in Veterinary Science, 46: 9-14.

Donaldson, A.I., and Alexandersen, S., (2002). Predicting the spread of foot and mouth disease by airborne virus. Revue Scientifique et Technique Office International des Epizooties, 21: 569-575.

Duggan, D.J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J.M., (1999). Expression profiling using cDNA microarrays. Nature Genetics Supplement, 21: 10-14.

Dus Santos, M.J., Wigdorovitz, A., Trono, K., Rios, R.D., Franzone, P.M., Gil, F., Moreno, J., Carrillo, C., Escribano, J.M., and Borca, M.V., (2002). A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. Vaccine, 20: 1141-1147.

Edelman, R., and Tacket, C.O., (1990). Adjuvants. International Reviews of Immunology, 51-66.

Elbers, A.R., Stegeman, A., Moser, H., Ekker, H.M., Smak, J.A., and Pluimers, F.H., (1999). The classical swine fever epidemic 1997-1998 in The Netherlands: descriptive epidemiology. Preventive Veterinary Medicine, 42: 157-184.

European Pharmacopoeia, (1997). Foot-and-Mouth Disease (Ruminants) Vaccine (Inactivated) 1997; 0063: 875-876.

Fallacara, F., Pacciarini, M., Bugnetti, M., Berlinzani, A., and Brocchi, E., (2000). Detection of swine vesicular disease virus in faeces samples by immune-PCR assay. Annex 9 of Annual Meeting of the Swine Vesicular Disease National Reference Laboratories. Brussels, 10th October 2000.

FDA (Food and Drug Administration), (1998). Federal Register July 31 1998 (Vol 63, number 147).

Feliu, J.X., Ferrer-Miralles, N., Blanco, E., Cazorla, D., Sobrino, F., and Villaverde, A., (2002). Enhanced response to antibody binding in engineered beta-galactosidase enzymatic sensors. Biochemica et Biophysica Acta, 1596: 212-224.

Ferris, N.P., and Dawson, M., (1988). Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. Veterinary Microbiology, 16: 201-209.

Floegel, G., Wehrend, A., Depner, K.R., Fritzemeier, J., Waberski, D., and Moennig, V., (2000). Detection of classical swine fever virus in semen of infected boars. Veterinary Microbiology 77: 109-116.

Floegel-Niesmann, G. (2001). Classical swine fever (CSF) marker vaccine. Trial III. Evaluation of discriminatory ELISAs. Veterinary Microbiology, 83: 121-136.

Forman, A.J., and Garland, A.J.M., (2002). Foot-and-mouth disease: the future of vaccine banks. Revue Scientifique et Technique Office International des Epizooties, 21: 2.

Frame, D.D., McCluskey, B.J., Buckner, R.E., and Halls, F.D., (1996). Results of an H7N3 avian influenza vaccination program in commercial meat turkeys. Proceedings 45th Western Poultry Disease Conference, Cancun, Mexico, 1996.

Francis, M.J., Hastings, G.Z., Clarke, B.E., Brown, A.L., Beddell, C.R., Rowlands, D.J., and Brown, F., (1990). Neutralizing antibodies to all seven serotypes of foot-and-mouth disease virus elicited by synthetic peptides, Immunology, 69: 171-176.

Frankena, K., Noordhuizen, J.P.T.M., Willeberg, P., Voorthuijsen, P., and van Goelema, J.O., (1990). EPISCOPE: computer programs in veterinary epidemiology. Veterinary Record, 126: 573-576.

Fritzemeier, J., Teuffert, J. Greiser-Wilke, I, Staubach, C., Schlüter, H., and Moennig, V., (2000). Epidemiology of classical swine fever in the nineties. Veterinary Microbiology 77: 1-2, 29-41.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., and Robinson, H.L., (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proceedings of the National Academy of Science, USA, 90: 11478-11482.

Gajendragad, M.R., Kamath, K.N., Anil, P.Y., Prabhudas, K., and Natarajan, C., (2001). Development and standardization of a piezoelectric immunobiosensor for foot and mouth disease virus typing. Veterinary Microbiology, 78: 319-330.

Galvin, P., (2002). A nanobiotechnology roadmap for high-throughput single nucleotide polymorphism analysis. Psychiatric Genetics, 12: 75-82.

Genghini, R., Tiranti, I., and Wittouck, P., (2002). Pig chromosome abberations after vaccination against classical swine fever in field trials. Vaccine, 20: 2873-2877.

Gibbens, J.C., Sharpe, C.E., Wilesmith, J.W., Mansley, L.M., Michalopoulou, E., and Ryan, J.B., (2001). Descriptive epidemiology of the 2001 foot-and-mouth disease epidemic in Great Britain: the first 5 months. Veterinary Record, 149: 729-743.

Gibbens, J.C., and Wilesmith, J.W., (2002). Temporal and geographical distribution of cases of foot-and-mouth disease during the early weeks of the 2001 epidemic in Great Britain. Veterinary Record, 151 (14): 407-412.

Gomes, P., and Andreu, D., (2002). Direct kinetic assay of interactions between small peptides and immobilized antibodies using a surface plasmon resonance biosensor. Journal of Immunological Methods, 259: 217-230.

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della Porta, A., and Westbury, H.A., (2001). Virulent Newcastle Disease in Australia: Molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. Virus Research, 77: 51-60.

Grant, D.M., and Phillips, M.S., (2001). Technologies for the Analysis of Single-Nucleotide Polymorphisms: An Overview. Pharmacogenomics, Kalow, Meyer and Tyndale (Eds.), Vol. 113, Published by Marcel Dekker, Inc. New York, pp. 183-190.

Gras-Masse, H., Boutillon, C., Diesis, E., Deprez, B., and Tartar, A., (1997). Confronting the degeneracy of convergent combinatorial immunogens, or “mixotopes”, with the specificity of recognition of the target sequences, Vaccine, 15: 1568-1578.

Greiner, L.L., Stahly, T.S., and Stabel, T., (1998). Quantitative effect of porcine reproductive respiratory syndrome virus on pig growth and immune response. Iowa State

University (ISU) Extension, ISU Swine Research Report: Nutrition ASL-R1566. http://www.extension.iastate.edu/Pages/ansci/swinereports/asl-1566.pdf

Greiser-Wilke, I., Zimmermann, B., Fritzemeier, J., Floegel, G., and Moennig, V., (2000). Structure and presentation of a World Wide Web database of CSF virus isolates held at the EU Reference Laboratory. Veterinary Microbiology, 73: 131-136.

Grubman, M.J., and Mason, P.A., (2002). Prospects, including time-frames for improved foot and mouth disease vaccines. Revue Scientifique et Technique Office International des Epizooties, 21: 589-600.

Grubman, M.J., Morgan, D.O., Kendall, J., and Baxt, B., (1985). Capsid intermediates assembled in a foot-and-mouth disease genome RNA-programmed cell-free translation system and in infected cells, Journal of Virology, 56: 120-126

Guberti, V., Rutili, D., Ferrari, G., Patta, C., and Oggiano, A., (1998). Estimate of the threshold abundance for the persistence of the classical swine fever in the wild boar population of eastern Sardinia. In: Report on measures to control classical swine fever in European wild boar, Perugia, Italy, Commission of the European Communities, Document VI/7196/98-AL, pp. 54-61.

Haas, B., and Sørensen, K., (2002). Comparsion of ELISAs for the differentiation of infection from vaccination by detection of antibodies to the non-structural 3ABC protein of FMDV. Session of the Research Group of the Standing Technical Committee of the EUFMD. Izmir, Turkey, 17-20 September 2002.

Haikala, O.J., Kokkonen, J.O., and Leinonen, M.K., (1983). Rapid detection of rotavirus in stool by latex agglutination: comparison with radioimmunoassayand electron microscopy and clinical evaluation of the test. Journal of Medical Virology, 11: 91-97.

Halvorson, D.A., (2002). The control of mildly pathogenic avian influenza: a role for inactivated vaccine. Avian Pathology, 31: 5-12.

Hammond, J.M., McCoy, R.J., Jansen, E.S., Morrissy, C.J., Hodgson, A.L.M., Johnson, M.A., (2000). Vaccination with a single dose of a recombinant porcine adenovirus expressing the classical swine fever virus gp55 (E2) gene protects pigs against classical swine fever. Vaccine, 18: 1040-1050.

Hammond, J.M., Jansen, E.S., Morrissy, C.J., Goff, W.V., Meehan, G.C., Williamson, M.M., Lenghaus, C., Sproat, K.W., Andrew, M.E., Coupar, B.E., and Johnson, M.A., (2001a). A prime-boost vaccination strategy using naked DNA followed by recombinant porcine adenovirus protects pigs from classical swine fever. Veterinary Microbiology, 80: 101-119.

Hammond, J.M., Jansen, E.S., Morrissy, C.J., Williamson, M.M., Hodgson, A.L., and Johnson, M.A. (2001b). Oral and sub-cutaneous vaccination of commercial pigs with a recombinant porcine adenovirus expressing the classical swine fever virus gp55 gene. Archives of Virology, 146: 1787-1793.

Harding, M., Lutzewallace, C., Prudhomme, I., Zhong, X.H., and Rola, J., (1994). Reverse transcriptase PCR assay for detection of hog cholera virus. Journal of Clinical Microbiology, 32: 2600-2602.

Have, P., (1984). Detection of antibodies against swine fever virus by enzyme-linked immunosorbent assay (ELISA). Acta Veterinaria Scandinavica, 25: 462-465.

Hendriksen, C.F.M., and Morton, D.B. (Eds.), (1999). Humane Endpoints in Animal Experiments for Biomedical Research. Proceedings of the International Conference, 22-25 Nov. 1998 Zeist, The Netherlands. pp 150. Royal Soc. Med., London WIM 8AE. http://www.lal.org.uk/endpoint.htm

Hendriksen, C., Spieser, J.M., Akkermans, A., Balls, M., Bruckner, L., Cussler, K., Daas, A., Descamps, J., Dobbelaer, R., Fentem, J., Halder, M., van der Kamp, M., Lucken, R., Milstien, J., Sesardic, D., Straughan, D., and Valadares, A., (1998). Validation of alternative methods for the potency testing of vaccines. The Report and Recommendations of ECVAM Workshop 31, ATLA, 26, 747-761. http://altweb.jhsph.edu/publications/ECVAM/ecvam31.htm

Hengen, P.N., (1997). Methods and reagents. Chemiluminescent detection methods. Trends Biochemical Science, 22: 313-314.

Herremanns, T., Kimman, T.G., Conyn-Van Spaendonck, M.A., Buisman, A., de Melker, H., Abbink, F., Bijkerk, P., and Koopmans, M.P., (2002). Immunoglobulin a as a serological marker for the (silent) circulation of poliovirus in an inactivated poliovirus-vaccinated population. Clinical Infectious Diseases, 34 : 1067-1075.

Hofmann, M.A., Brechtbuhl, K., and Stauber, N., (1994). Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5' noncoding region. Archives of Virology, 139: 217-229.

Hone, J., Pech, R.P., and Yip, P., (1992). Estimation of dynamics and rate of transmission of classical swine fever (hog cholera) in wild pigs. Epidemiology and Infection, 108: 377-386.

Hooft-van Iddekinge, B.J., de Wind, N., Wensvoort, G., Kimman, T.G., Gielkens, A.L., and Moormann, R.J., (1996). Comparison of the protective efficacy of recombinant pseudorabies viruses against pseudorabies and classical swine fever in pigs; influence of different promoters on gene expression and on protection. Vaccine, 14: 6-12.

Horimoto, T., and Kawaoka, Y., (2001). Pandemic threat posed by avian influenza A viruses. Clinical Microbiology Reviews, 14: 128-149.

Hulst, M.M., Westra, D.F., Wensvoort, G., and Moormann, R.J.M., (1993). Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine fever from hog cholera. Journal of Virology, 67: 5435-5442.

ISO (International Organization for Standardization), (1997). ISO/IEC Guide 43: Proficiency testing by interlaboratory comparisons.

ISO (International Organization for Standardization), (2000). ISO Guide 34: General requirements for the competence of reference material producers.

Jacobson, R.H., (1998). Validation of serological assays for diagnosis of infectious diseases. Revue Scientifique et Technique de l'Office International des Epizooties, 17: 469-486.

Johnson D.J., Wilson, W.C., and Paul, P.S., (2000). Validation of a reverse transcriptase multiplex PCR test for the serotype determination of U.S. isolates of bluetongue virus. Veterinary Microbiology, 76 (2): 105-115.

Jordan, H.L., Scappino, L.A., Moscardini, M., and Pistello, M., (2002). Detection of feline immunodeficiency RNA by two nucleic-acid sequence-based amplification (NASBA) formats. Journal of Virological Methods 103: 1-13.

Kaden, V., and Lange, B., (2001). Oral immunisation against classical swine fever (CSF): onset and duration of immunity. Veterinary Microbiology, 82: 301-310.

Kaden, V., Hubert, P., Strebelow, G., Lange, E., Steyer, H., and Steinhagen, P., (1999a). Comparison of different diagnostic methods for the detection of the classical swine fever virus (CSFV) in the early infection period. Berliner und Munchener Tierarztliche Wochenschrift, 112: 52-57.

Kaden, V., Lange, E., and Steyer, H., (1999b). Orale Immunisierung gegen Klassische Schweinepest beim Schwarzwild: Bewertung maternaler Antikörper (Oral immunisation against CSF: Evaluation of maternal antibodies). Amtstierärztl. Dienst und Lebensmittelkontrolle, 6: 302-305.

Kaden, V., Lange, E., Fischer, U., and Strebelow, G., (2000). Oral immunisation of wild boar against classical swine fever: evaluation of the first field study in Germany. Veterinary Microbiology, 73: 239-252.

Kaden, V., Schurig, U., and Steyer, H., (2001). Oral immunization of pigs against classical swine fever. Course of the disease and virus transmission after simultaneous vaccination and infection. Acta Virologica, 45: 23-29.

Kaden, V., Heyne, H., Kiupel, H., Letz, W., Kern, B., Lemmer, U., Gossger, K., Rothe, A., Bohme, H., and Tyrpe, P., (2002). Oral immunisation of wild boar against classical swine fever: concluding analysis of the recent field trials in Germany. Berl. Münch. Tierärztl. Wochenschr., 115: 179-185.

Kärber, G., (1931). Beitrag zur kollekiven Behandlung pharmakologischer Reihenversuche. Arche. Exp. Pathol. Pharmakol., 162: 480.

Katz, J.B., Ridpath, J.F., and Bolin, S.R., (1993). Presumptive diagnostic differentiation of hog cholera virus from bovine viral diarrhea and border disease viruses by using a cDNA nested-amplification approach. Journal of Clinical Microbiology, 31: 565-568.

Kern, B., and Lahrmann, K.H., (2000). Oral immunization against classical swine fever (CSF) in the Federal State of Brandenburg from 1995 to 1997. Dtsch. Tierärztl. Wochenschr., 107: 490-495.

Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., and Lens, P.J., (1991). NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. Virological Methods, 35: 273-86.

Kim, O., Choi, C., Kim, B., and Chae C. (2000). Detection and differentiation of porcine epidemic diarrhea virus and transmissible gastroenteritis virus in clinical samples by multiplex RT-PCR. Veterinary Record, 146: 637-640.

Kitching, R.P., (2002a). Clinical variation in foot and mouth disease: cattle. Revue Scientifique et Technique de l'Office International des Epizooties, 21: 499-504.

Kitching, R.P., (2002b). Identification of foot and mouth disease virus carrier and subclinically infected animals and differentiation from vaccinated animals. Revue Scientifique et Technique de l'Office International des Epizooties, 21: 531-538.

Kitching, R.P., (2002c). Clinical variation in foot and mouth disease: pigs. Revue Scientifique et Technique de l'Office International des Epizooties, 21: 513-518.

Kitching, R.P., and Alexandersen, S., (2002). Clinical variation in foot and mouth disease: pigs. Revue Scientifique et Technique de l'Office International des Epizooties, 21: 513-518.

Kitching, R.P., and Hughes, G.J., (2002). Clinical variation in foot and mouth disease: sheep. Revue Scientifique et Technique de l'Office International des Epizooties, 21: 505-512.

Kitching, P., Rendle, T., and Newman, B., (2000). FAO collaborative study Phase XVI: Establishing reference standards. Report of the Research Group of the Standing Technical Committee of the EU FMD Commission for the control of FMD, Borovets, Bulgaria, 5-8 September 2000. Appendix 29: pp. 224-240.

Kodihalli, S., Sivanandan, V., Nagaraja, K.V., Shaw, D., and Halvorson, D.A., (1994). A type specific avian influenza subunit vaccine for turkeys: induction of protective immunity to challenge infection. Vaccine, 12: 1467-1472.

Kodihalli, S., Haynes, J.R., Robinson, H.L., and Webster, R.G., (1997). Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. Journal of Virology, 71: 3391-3396.

Kodihalli, S., Kobasa, D.L., and Webster, R.G., (2000). Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine, 18: 2592-2599.

Koenen, F., van Caenegem, G., Vermeersch, J.P, Vandenheede, J., and Deluyker, H., (1996). Epidemiological characteristics of an outbreak of classical swine fever in an area of high pig density. Veterinary Record, 139 (15): 367-371.

Komatsu, N., Shichijo, S., Maeda, Y., and Itoh, K., (2002). Measurement of interferon-gamma by high-throughput fluorometric microvolume assay technology system. Journal of Immunological Methods, 263: 169-176.

König, M., Lengsfeld, T., Pauly, T., Stark, R., and Thiel, H.J., (1995). Classical swine fever virus: independent induction of protective immunity by two structural glycoproteins. Journal of Virology, 69: 6479-6486.

Kosmidou, A., Ahl, R., Thiel, H.J., and Weiland, E., (1995). Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. Veterinary Microbiology, 47: 111-118.

Krieg, A.M. Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., and Klinman, D.M., (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature, 374: 546-549.

Kurtz, J., Manvell, R.J., and Banks, J., (1996). Avian influenza virus isolated from a woman with conjunctivitis. Lancet, 348: 901-902.

Laddomada, A., (2000). Incidence and control of CSF in wild boar in Europe. Veterinary Microbiology, 73: 121-130.

Lai, W.C., and Bennett, M., (1998). DNA vaccines, Critical Reviews in Immunology, 18: 449-484.

Lanciotti, R.S., and Kerst, A.J., (2001). Nucleic-acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. Journal of Clinical Microbiology, 39: 4506-4513.

Lander, E.S., (1999). Array of hope. Nature: Genetics Supplement, 21: 3-4.

Langedijk, J.P.M., Middel, W.G.J., Meloen, R.H., Kramps, J.A, and de Smit, J.A., (2001). Enzyme-linked immunosorbent assay using a virus type-specific peptide based on a subdomain of envelope protein $E^{rns}$ for serologic diagnosis of pestivirus infections in swine. Journal of Clinical Microbiology, 39: 906-912.

Laporte, J., Grosclaude, J., Wantyghem, J., Bernard, S., and Rouze, P., (1973). Neutralisation en culture cellulaire du pouvoir infectieux du virus de la Fievre aphteuse par des serums provenant de porcs immunises a l'aide d'une proteine virale purifiee, C.R. Hebd, Seances Acad. Sci. Paris, 276: 3390-3401.

Last, J.M., Spasoff, R.A., Harris, S.S., and Thuriaux, M.C., (2001). A dictionary of Epidemiology. 4th Ed. Oxford University Press, Oxford.

Leitner, W.W., Ying, H., and Restifo, N.P., (2000). DNA and RNA based vaccines: principles, progress and prospects, Vaccine, 18: 765-777.

Lemieux, B., (2000). High throughput single nucleotide polymorphism genotyping technology. Current Genomics, 1: 301-311.

Leunen, J., and Strobbe, R., (1977). Capacity of attenuated swine fever vaccines to prevent virus carriers in the vaccinated pigs after contact with field virus. Archiv fur Experimentelle Veterinarmedizin, 31: 533-536.

Leutenegger, C.M., (2001). The Real-Time Taqman PCR and Applications in Veterinary Medicine. Veterinary Sciences Tomorrow, 1: 1-15.

Li, J., Chen, S., and Evans, D.H., (2001). Typing and subtyping influenza virus using DNA microarrays and multiplex reverse transcriptase PCR. Journal of Clinical Microbiology, 39 (2): 696-704.

Liess, B., (1984). Persistent infections of hog cholera: a review. Preventive Veterinary Medicine, 2: 109-113.

Lin, F., Mackay, D.K.J., and Knowles, N.J. (1997). Detection of swine vesicular disease virus RNA by reverse transcription-polymerase chain reaction. Journal of Virological Methods, 65 (1): 111-121.

Lipowski, A., Drexler, C., and Pejsak, Z., (2000). Safety and efficacy of a classical swine fever subunit vaccine in pregnant sows and their offspring. Veterinary Microbiology, 77: 99-108.

Liu, S., Chang, T.Y., Walfield, A.M., Zhang, M.L., Sokoll, K.K., Chen, S.P., Li, M.C., Lin, Y.L., Jong, M.H., Yang, D., Chyr, N., and Wang, C.Y., (2002). Synthetic peptide-based serosurveillance and vaccine system for FMD. Session of the Research Group of the Standing Technical Committee of the EUFMD. Izmir, Turkey, 17-20 September 2002.

Lockhart, D.J., and Winzeler, E.A., (2000). Genomics, gene expression and DNA arrays. Nature, 405: 827-836.

Lorena, J., Barlic-Maganja, D., Lojkic, M., Madic, J., Grom, J., Cac, Z., Roic, B., Terzic, S., Lojkic, I., Polancec, D., and Cajavec, S., (2001). Classical swine fever virus (C strain) distribution in organ samples of inoculated piglets. Veterinary Microbiology, 81: 1-8.

Lowings, P., Ibata, G., Needham, J., and Paton, D., (1996). Classical swine fever virus diversity and evolution. Journal of General Virology, 77: 1311-1321.

Lubroth, J., López, A., Ramalho, A.K., Meyer, R.F., Brown, F., and Darsie, G.C., (1998). Cattle response to foot-and-mouth disease virus nonstructural proteins as antigens within vaccines produced using different concentrations. Veterinary Quarterly, 20, Suppl. 2: S13-17.

Lüschow, D., Werner, O., Mettenleiter, T.C., and Fuchs, W., (2001). Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine, 19: 4249–4259.

Lütticken, D., Drexler, C., Visser, N., and Kaden, V., (1998). The relevance of CSF marker vaccines for field use. In: Proceedings OIE Symposium, Classical Swine Fever, Birmingham United Kingdom, July 9-10. Summaries, p. 17.

Mackay, D.K., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M., and Ryan, M.D., (1998a). Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine, 16: 446-459.

Mackay, D.K., Forsyth, M.A., Davies, P.R., and Salt, J.S., (1998b). Antibody to the nonstructural proteins of foot-and-mouth disease virus in vaccinated animals exposed to infection. Veterinary Quarterly, 20, Suppl. 2: S9-11.

Mackay, I.M., Arden, K.E., and Nitsche, A., (2002). Real-time PCR in virology. Nucleic Acids Research, 30: 1292-1305.

Markowska-Daniel, I., Collins, R.A., and Pejsak, Z., (2001). Evaluation of genetic vaccine against classical swine fever. Vaccine, 19: 2480-2484.

Mason, P.W., Piccone, M.E., McKenna, T.S.C., Chinsangaram, J., and Grubman, M.J., (1997). Evaluation of live-attenuated foot-and-mouth disease virus as a vaccine candidate, Virology, 227: 96-102.

Mason, P.W., Grubman, M.J., and Baxt, B., (2003). Molecular basis of pathogenesis of FMDV. Virus Research, 91 (1): 9-32.

Mayo, M.A., (2002). A summary of the changes recently approved by ICTV. Archives of Virology, 147: 1655-1656.

Mayr, G.A., O'Donnell, V., Chinsangaram, J., Mason, P.W., and Grubman, M.J., (2001). Immune responses and protection against foot-and-mouth disease virus (FMDV) challenge in swine vaccinated with adenovirus-FMDV constructs. Vaccine, 19: 2152-2162.

McGoldrick, A., Bensaude, E., Ibata, G., Sharp, G., Paton, D.J., (1999). Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. Journal of Virological Methods, 79: 85-95.

McKenna, T.S.C., Lubroth, J., Rieder, E., Baxt, B., and Mason, P.W., (1995). Receptor binding site-deleted foot-and-mouth disease (FMD) virus protects cattle from FMD, Journal of Virology, 69: 5787-5790.

McVicar, J.W., and Sutmoller, P., (1976). Growth of foot-and-mouth disease virus in the upper respiratory tract of non-immunised, vaccinated and recovered cattle after intranasal inoculation. Journal of Hygiene, Camb., 76: 467-481.

Mebatsion, T., Koolen, M.J.M., De Vaan, L.T.C., De Haas, N., Braber, M., Römer-Oberdörfer, A., Van den Elzen, P., and Van der Marel., P., (2002). Newcastle disease virus (NDV) marker vaccine: an immunodominant epitope on the nucleoprotein gene of NDV can be deleted or replaced by a foreign epitope. Journal of Virology, 76: 10138-10146.

Meloen, R.H., and Barteling, S.J., (1986). An epitope located at the C-terminus of isolated VP1 of foot-and-mouth disease virus O induces neutralizing activity but poor protection. Journal of General Virology, 67: 289-294.

Mettenleiter, T.C., Klupp, B.G., Weiland, F., and Visser, N., (1994). Characterization of a quadruple glycoprotein-deleted pseudorabies virus mutant for use as a biologically safe live virus vaccine. Journal of General Virology, 75 : 1723-1733.

Meuwissen, M.P., Horst, S.H., Huirne, R.B., and Dijkhuizen, A.A., (1999). A model to estimate the financial consequences of classical swine fever outbreaks: principles and outcomes. Preventive Veterinary Medicine, 42: 249-270.

Meyers, G., Tautz, N., Becher, P., Thiel, H.J., and Kümmerer, B.M., (1996a). Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. Journal of Virology, 70: 8606-8613.

Meyers, G., Thiel, H.J., and Rümenapf, T., (1996b). Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. Journal of Virology, 70: 1588-1595.

Meyers, G., Saalmüller, A., and Büttner, M., (1999). Mutations abrogating the RNase activity in glycoprotein E(rns) of the pestivirus classical swine fever virus lead to virus attenuation. Journal of Virology, 73: 10224-10235.

Mezencio, J.M.S., Babcock, G.D., Meyer, R.F., Lubroth, J., Salt, J.S., Newman, J.F.E., and Brown, F., (1998). Differentiating foot-and-mouth disease virus-infected from vaccinated animals with baculo virus-expressed specific proteins. In: Proceedings of the Final meeting of Concerted Action CT 93 0909, Lelystadt, 28-29 April 1997. Veterinary Quarterly, 20 (Supplement 2): S11-13.

Mezencio, J.M.S., Babcock, G.D., Kramer, E., and Brown, F., (1999). Evidence for the persistence of foot-and-mouth disease virus in pigs. Veterinary Journal, 157: 213-217.

Moormann, R.J., van Gennip, H.G., Miedema, G.K., Hulst, M.M., and van Rijn, P.A., (1996). Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. Journal of Virology, 70: 763-770.

Moormann, R.J.M., Bouma, A., Kramps, J.A., Terpstra, C., and de Smit, H.J., (2000). Development of a classical swine fever subunit marker vaccine and companion diagnostic test. Veterinary Microbiology, 73: 209-219.

Morgan, C.L., Newman, D.J., and Price, C.P., (1996). Immunosensors: technology and opportunities in laboratory medicine. Clinical Chemistry, 42: 193-209.

Mortensen, S., Strandbygaard, B., Botner, A., Feld, N., and Willeberg, P., (2001). Monitoring porcine reproductive and respiratory syndrome virus infection status in swine herds based on analysis of antibodies in meat juice samples. Veterinary Research, 32 (5): 441-453.

Moser, C., Ruggli, N., Tratschin, J.D., and Hofmann, M.A., (1996). Detection of antibodies against classical swine fever virus in swine sera by indirect ELISA using recombinant envelope glycoprotein E2. Veterinary Microbiology, 51: 41-53.

Mulder, W.A., Priem, J., Glazenburg, K.L., Wagenaar, F., Gruys, E., Gielkens, A.L., Pol, J.M., and Kimman, T.G., (1994). Virulence and pathogenesis of non-virulent and virulent strains of pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus. Journal of General Virology, 75: 117-124.

Murphy, F.A., Gibbs, E.P.J., Horzinek, M.C., and Studdert, M.J., (1999). Laboratory Diagnosis of Viral Diseases, In: Veterinary Virology, Academic Press, 3rd edition, Chapter 12, pp. 193-224.

Murphy, C.M., Zhang, Z., Quan, M., Knight, J., and Alexandersen, S., (2002). Expression of inflammatory and antiviral cytokines in pigs experimentally infected with Foot-and-Mouth Disease. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the control of foot-and-mouth disease, held at Izmir, Turkey, 17-20 September 2002.

NAO (National Audit Office), (2002). The 2001 Outbreak of Foot and Mouth Disease. Report by the Comptroller and Auditor General. HC 939 Session 2001-2002: 21 June 2002. http://www.nao.gov.uk/pn/01-02/0102939.htm

Neilan, J.G., Zsak, L., Lu, Z., Kutish, G.F., Afonso, C.L., and Rock, D.L., (2002). Novel swine virulence determinant in the left variable region of the African swine fever virus genome. Journal of Virology, 76: 3095-3104.

Neitzert, E., Beck, E., de Mello, P.A., Gomes, I., and Bergmann, I.E., (1991). Expression of the aphthovirus RNA polymerase gene in *Escherichia coli* and its use together with

other bioengineered non-structural antigens in detection of late persistent infections. Virology, 184: 799-804.

Noordhuizen, J.P.T.M., Frankena, K., Thrusfield, M.V., and Graat, E.A.M., (1997). Application of quantitative methods in veterinary epidemiology. Wageningen Pers. Publ., 2nd ed., Wageningen, The Netherlands.

Nuñez, J.I., Blanco, E., Hernández, T., Gómez-Tejedor, C., Martín, M.J., Dopazo, J., and Sobrino, F., (1998). A RT-PCR assay for the differential diagnosis of vesicular viral diseases of swine. Journal of Virological Methods, 72 (2): 227-235.

OECD (Organisation for Economic Co-operation and Economic Development), (2001) Environmental Health and Safety Publications Series on Testing and Assessment No. 19 Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation Environment Directorate. Organisation for Economic Co-operation and Economic Development, Paris November 2000. http://www.oecd.org/ehs/test/mono19.pdf

OIE, (2000). Manual of Standards for Diagnostic Tests and Vaccines. 4th Edition. Office International des Epizooties, 12 rue de Prony, 75017 Paris, France. www.oie.int

OIE, (2002). International Animal Health Code, 11th edition. Office International des Epizooties, 2002, 12 rue de Prony, 75017 Paris, France. www.oie.int

Olesen, C.E., Yan, Y.X., Liu, B., Martin, D., D'Eon, B., Judware, R., Martin, C., Voyta, J.C., and Bronstein, I., (2000). Novel methods for chemiluminiscent detection of reporter enzymes. Methods in Enzymology, 326: 175-202.

Olesen, C.E.M., Kricka, L.J., Edwards, B., Juo, R.-R., Voyta, J.C., and Bronstein, I., (2001). Chemiluminescent detection with 1,2-dioxetane substrates: Substrates for alkaline phosphatase can be variously enhanced so as to provide highly sensitive detection with many assay formats and instrumentation platforms. IVD Technology: pp. 45; http://www.devicelink.com/ivdt/archive/01/04/002.html

Ozaki, H., Suguira T., Sugita, S., Imagawa, H., and Kida, H., (2001) Detection of antibodies to the non structural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. Veterinary Microbiology, 82: 111-119.

Pang, Y., Wang, H., Girshick, T., Xie, Z., and Khan, M.I., (2002). Development and application of a multiplex polymerase chain reaction for avian respiratory agents. Avian Diseases, 46: 691-699.

Pastor, M.J., and Escribano, J.M., (1990). Evaluation of sensitivity of different antigen and DNA-hybridisation methods in African Swine Fever Virus detection. Journal of Virological Methods, 28: 67-78.

Paton, D., (2002). The reappearance of classical swine fever in England in 2000. In: Trends in Emerging Viral Infections of Swine. Iowa State Press, Ames Iowa. ISBN 0-8138-0383-7, pp. 153-158.

Paton, D.J., McGoldrick, A., Belak, S., Mittelholzer, C., Koenen, F., Vanderhallen, H., Biagetti, M., DeMia, G.M., Stadejek, T., Hofmann, M.A., and Thuer, B., (2000a). Classical swine fever virus: a ring test to evaluate RT-PCR detection methods. Veterinary Microbiology, 73: 159-174.

Paton, D.J., McGoldrick, A., Greiser-Wilke, I., Parchariyanon, S., Song, J.Y., Liou, P.P., Stadejek, T., Lowings, J.P., Bjorklund, H., and Belak, S., (2000b). Genetic typing of classical swine fever virus. Veterinary Microbiology, 73: 137-157.

Paton, D.J., Armstrong, R.M., Turner, L.S., Hamblin, P.A., Corteyn, M., and Anderson, J., (2002). FAO Collaborative Study Phase XVII: Standardisation of FMD Antibody Detection. Session of the Research Group of the Standing Technical Committee of the EUFMD. Izmir, Turkey, 17-20 September 2002.

Pay, T.W.F., and Hingley, P.J., (1992). Foot and mouth disease vaccine potency tests in cattle: the relationship of antigen dose, serum neutralising antibody response and protection from challenge. Vaccine, 10: 699-706.

Pearson, J.E., (1992). Hog cholera diagnostic techniques. Comparative Immunology Microbiology and Infectious Diseases, 15: 213-219.

Peeters, B., Bienkowskaszewczyk, K., Hulst, M., Gielkens, A., and Kimman, T., (1997) Biologically safe, non-transmissible pseudorabies virus vector vaccine protects pigs against both aujeszky's disease and classical swine fever. Journal of General Virology, 78: 3311-3315.

Peeters, B.P.H., De Leeuw, O.S., Verstegen, I., Koch, G., and Gielkens, A.L.J., (2001). Generation of a recombinant chimeric Newcastle disease virus vaccine that allows serological differentiation between vaccinated and infected animals. Vaccine, 19: 1616-1627.

Peiris, M., Yam, Y.C., Chan, K.H., Ghose, P., and Shortridge, K.F., (1999). Influenza A H9N2: aspects of laboratory diagnosis. Journal of Clinical Microbiology, 37: 3426-3427.

Pfaff, E., Mussgay, M., Bóhm, H.O., Schultz, G.E., and Schaller, H., (1982). Antibodies against a preselected peptide recognize and neutralize foot-and-mouth disease virus, EMBO Journal, 1: 869-874.

Pfeffer, M., Meyer, H., and Wiedmann, M., (1994). A ligase chain reaction targeting two adjacent nucleotides allows the differentiation of cowpox virus from other Orthopoxvirus species. Journal of Virological Methods, 49: 353-360.

Pfeffer, M., Wiedmann, M., and Batt, C.A., (1995). Applications of DNA amplification techniques in veterinary diagnostics. Veterinary Research Communications, 19: 375-407.

Precausta, P., Brun, A., Kato, F., Terré, J., and Marcon, Ch., (1975). Peste Porcine Classique. Etude d'un vaccin preparé à partir de la souche chinoise CL adaptée à la culture cellulaire . Revue de Médecine Vétérinaire, 126 (7): 969-981.

Quint, W.G.V, Gielkens, A.L.J., van Oirschot, J.T., Berns, A.J.M., and Cuypers, H.T., (1987). Construction and characterization of deletion mutants of pseudorabies virus: a new generation of 'live' vaccines. Journal of General Virology, 68: 523-534.

Read, S.J., Mitchell, J.L., and Fink, C.G., (2001). LightCycler multiplex PCR for the laboratory diagnosis of common viral infections of the central nervous system. Journal of Clinical Microbiology, 39: 3056-3059.

Reid, S.M., Ferris, N.P., Bruning, A., Hutchings, G.H., Kowalska, Z., Akerblom, L., (2001). Development of a rapid chromatographic strip test for the pen-side detection of foot-and-mouth disease virus antigen. Journal of Virological Methods, 96: 189-202.

Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z., Belsham, G.J., and Alexandersen, S., (2002). Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. Journal of Virological Methods, 105: 67-80.

Reider, E., Baxt B., Lubroth J., and Mason P.W., (1994). Vaccines prepared from chimeras of foot-and-mouth disease virus (FMDV) induce neutralizing antibodies and protective immunity to multiple serotypes of FMDV Journal of Virology, 7092-7098.

Reimann, I., Depner, K., Trapp, S., and Beer, M., (2002). An avirulent chimeric pestivirus with an altered cell tropism protects pigs from lethal infection with classical swine fever virus. Abstracts Jahrestagung Deutsche Gesellschaft für Virologie, Erlangen, Germany, April 8-11, p. 109.

Rimmelzwaan, G.F., Class, E.C.J., van Amerongen, G., de Jong, J.C., and Osterhaus, A.D.M.E., (1999). ISCOM vaccine induced protection against a lethal challemge with a human H5N1 influenza virus. Vaccine, 17: 1355–1358.

Risatti, G.R., Callahan, J.D., Nelson, W.M., and Borca, M.V., (2003). Rapid detection of classical swine fever virus by a portable real-time reverse transcriptase PCR assay. Journal of Clinical Microbiology, 41: 500-505.

Robinson, H.L., Hunt, L.A., and Webster, R.G., (1993). Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine, 11: 957-960.

Romano, J.W., van Gemen, B., and Kievits, T., (1996). NASBA: a novel, isothermal detection technology for qualitative and quantitative HIV-RNA measurements. Clinics in Labarotory Medicine, 16 (1): 89-103.

Rongen, H.A., Hoetelmans, R.M., Bult, A., and van Bennekom, W.P., (1994). Chemiluminescence and immunoassays. Journal of Pharmaceutical Biomedical Analysis, 12: 433-462.

Rowlands, D.J., Cartwright B., and Brown, B., (1969). Evidence for an internal antigen in foot-and-mouth disease virus. Journal of General Virology, 4: 479-487.

Roy, P., (1993). Genetically engineered bluetongue virus-like particles and their potential for vaccine in sheep. In: Veterinary Vaccines, (Hogland *et al*., eds), Progress in Vaccinology 4: 29-53. Springer-Verlag, Heidelberg.

Ruggli, N., Tratschin, J.D., Mittelholzer, C., and Hofmann, M.A., (1996). Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. Journal of Virology, 70: 3478-3487.

Rümenapf, T., Stark, R., Meyers, G., and Thiel, H.J., (1991). Structural proteins of hog cholera virus expressed by vaccinia virus: further characterization and induction of protective immunity. Journal of Virology, 65: 589-597.

Rutili, D., Guberti, V., and Ferrari, G., (1998). Classical Swine Fever in wild boar. Evaluation of control measures applied in Italy and proposal for the future. In: Measures to control Classical Swine Fever in European Wild Boar. Perugia, Italy, 6-7 April 1998. European Union. Commission des Communautes Européennes. Direction Generale VI Agriculture, pp. 135-137.

Ryan, J., (2001). The availability of foot and mouth disease vaccine for emergency vaccination in Europe. Report of the 34th Session of the European Commission for the Control of Foot and Mouth Disease. Food and Agriculture Organization of the United Nations, Rome, Italy. 21-23 March 2001. Appendix 12: pp. 117-127.

Sagedahl, A., Giraudo, A.T., Auge de Mello, P., Bergmann, I.E., La Torre, J.L., and Scodeller, E.A., (1987). Biochemical characterization of an aphthovirus type C3 strain Resende attenuated for cattle by serial passages in chicken embryos. Virology, 157: 366-374.

Salt, J.S., (1998). Persistent infections with foot-and-mouth disease virus. Topics in Tropical Virology, 1: 77-128.

Salt, J.S., Mulcahy, G., and Kitching, R.P., (1996). Isotype-specific responses to foot-and-mouth disease virus in sera and secretions of 'carrier' and 'non-carrier' cattle. Epidemiology and Infection, 117: 349-360.

Sánchez-Vizcaíno, J.M., (1999). African swine fever. In: Diseases of Swine, 8th Edition, Leman, A.D., Straw, B.E., Mengeling, W.L., Dallaire, S., and Taylor, D.L., (Eds.). Iowa State University Press, Ames: pp. 93-102

Sanz-Parra, A., Jimenez-Clavero, M.A., García-Briones, M., Blanco, E., Sobrino, F., and Ley, V., (1999a). Recombinant adenovirus expressing the foot-and-mouth disease virus P1 precursor polypeptide induces cellular but not humoral antiviral immunity and partial protection in pigs. Virology, 259: 129-134.

Sanz-Parra, A., Vázquez, B., Sobrino, F., Cox, S.J., Ley, V., and Salt J., (1999b). Evidence of partial protection against foot-and-mouth disease in cattle immunised with a recombinant adenovirus vector expressing the precursor polypeptide of FMDV capsid protein (P1), Journal of General Virology, 80: 671-679.

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (1998). The definition of Newcastle Disease. Report of the Scientific Committee on Animal Health and Animal Welfare, adopted 24 March 1998. http://europa.eu.int/comm/food/fs/sc/scah/out04_en.html

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (1999a). Strategy for emergency vaccination against foot-and-mouth disease (FMD). Report of the Scientific Committee on Animal Health and Animal Welfare, adopted 10 March 1999. http://europa.eu.int/comm/food/fs/sc/scah/out22_en.pdf

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (1999b). Classical swine fever in wild boar. Report of the Scientific Committee on Animal Health and Animal Welfare, adopted 10 August 1999. http://europa.eu.int/comm/food/fs/sc/scah/out24_en.pdf

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (2000a). The definition of avian influenza. The use of vaccination against avian influenza. Report of

the Scientific Committee on Animal Health and Animal Welfare. http://europa.eu.int/comm/food/fs/sc/scah/out45_en.pdf

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (2000b). Possible use of vaccination against Bluetongue in Europe. Report of the Scientific Committee on Animal Health and Animal Welfare, adopted on 27 June 2000. http://europa.eu.int/comm/food/fs/sc/scah/out47_en.pdf

SCAHAW (Scientific Committee on Animal Health and Animal Welfare), (2001). Considerations of the Scientific Committee on Animal Health and Animal Welfare on Foot and Mouth Disease outbreaks. Summary Record of the Meeting of the Scientific Committee on Animal Health and Animal Welfare held on 25 April 2001. http://europa.eu.int/comm/food/fs/sc/scah/out58_en.pdf

Schalch, L., Rebeski, D.E., Samaras, H., Lozano, G., Thuer, B., and Schelp, C., (2002) Recently generated data with the CHEKIT-FMD-3ABC ELISA kit and methods to monitor the operational performance of a 3ABC ELISA. Session of the Research Group of the Standing Technical Committee of the EUFMD. Izmir, Turkey, 17-20 September 2002.

Scheibner, H., Frey, H.-R., Eicken, K., Meyer, H., and Greiser-Wilke, I., (2000). Nachweis des Genoms des Virus der Bovinen Virus diarrhoe (BVDV) mittels Polymerasekettenreaktion nach reverser Transkription (RT-PCR): Vergleich von Methoden zur Isolierung von Ribonukleinsäure (RNA) aus klinischen Probenmaterialien. Dtsch. Tierärztl. Wschr., 107: 431-437.

Schmidt, B., (1999). Light minded: Instruments for chemiluminescence and fluorescence measurements. The Scientist, 13: 18.

Scholtissek, C., Rohde, W., von Hoyningen, V., and Rott, R., (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology, 87: 13-20.

Sellers, R.F., Herniman, K.A.J., and Gumm, I.D., (1977). The airborne dispersal of foot-and-mouth disease virus from vaccinated and recovered pigs, cattle and sheep after exposure to infection. Research in Veterinary Science, 23: 70-75.

Shoemaker, D.D., and Linsley, P.S., (2002). Recent developments in DNA microarrays. Current Opinion in Microbiology, 5: 334-337.

Shortridge, K.F., Zhou, N.N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K.G., Norwood, M., Senne, D.A., Sims, L., Takada, A., and Webster, R.G., (1998). Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology, 252: 331-342.

Silberstein, E., Kaplan, G., Taboga, O., Duffy, S., and Palma, E., (1997). Foot-and-mouth disease virus-infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. Archives of Virology, 142: 795-805.

Singh, M., Briones, M., Ott, G., and O' Hagan, D., (2000). Cationic microparticles: a potent delivery system for DNA vaccines. Proceedings of the National Academy of Sciences of the United States of America, 97: 811-816.

Sobrino, F., Sáiz, M., Jiminéz-Clavero, M.A., Nūňuz, J.I., Rosas, M.F., Baranowski, E., and Ley, V., (2001). Foot-and-mouth disease: a long known virus, but a current threat. Veterinary Research, 32: 1-30.

Somasundaram, C., Takamatsu, H., Andreoni, C., Audonnet, J.C., Fischer, L., Lefevre, F., and Charley, B., (1999). Enhanced protective response and immuno-adjuvant effects of porcine GM-CSF on DNA vaccination of pigs against Aujeszky's disease virus. Veterinary Immunology and Immunopathology, 70: 277-287.

Sørensen, K.J., (1998). Experiences from the serological survey for antibody to FMDV in the former Republic of Macedonia. Report of the Sess. Res. Gr. St. Tech. Committee of the Europ. Comm. Control FMD, UK, 14-18 Sept. 1998, pp. 166-175.

Sørensen, K.J., Hansen, C.M., Madsen, E.S., and Madsen, K.G., (1998a). Blocking ELISA's using the FMDV non-structural proteins 3D, 3AB, and 3ABC produced in the baculovirus expression system. Veterinary Quarterly, 20 S2: S17-S20.

Sørensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J., and Mackay D.K.J., (1998b). Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. Archives of Virology, 143: 1461-1476

Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Lohman, K., Daum, L.T., and Suarez, D.L., (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology, 40: 3256-3260.

Staubach, C.,Teuffert, J., Thulke, H.-H., (1997). Risk analysis and local spread mechanisms of Classical Swine Fever. Epidémiologie et Santé Animale, 31-32, 06.12.1-06.12.3, ISSN 0754-2186.

Stettler, P., Devos, R., Moser, C., Tratschin, J.D., and Hofmann, M.A., (2002). Establishment and application of bicistronic classical swine fever virus genomes for foreign gene expression and complementation of E2 deletion mutants. Virus Research, 85: 173-185.

Strohmaier, K., Franze, R., and Adam, K.H., (1982). Location and characterization of the antigenic portion of the FMDV immunizing protein, Journal of General Virology, 59: 295-306.

Subbarao, K., and Katz, J., (2000). Avian influenza viruses infecting humans. Cell and Molecular Life Sciences, 57: 1770-1784.

Suradhat, S., Intrakamhaeng, M., and Damrongwatanapokin, S., (2001). The correlation of virus-specific interferon gamma production and protection against classical swine fever virus infection. Veterinary Immunology and Immunopathology, 83: 177-189.

Sutmoller, P., and Casas Olascoaga, R., (2002). Unapparent foot and mouth disease infection (subclinical infections and carriers): implications for control. Revue Scientifique et Technique Office International des Epizooties, 21: 519-529.

Sutmoller, P., and McVicar, J.W., (1972). The epizootiological importance of foot-and-mouth disease carriers. III. Exposure of pigs to bovine carriers. Arch. Ges. Virusforsch., 37: 78-84.

Swangard, W.M., Armbuster, O., and Carmenes, P., (1969). Viruman, eine Vakzine gegen die klassische Schweinepest auf der Basis von Zellkulturen aus Wiederkäuerzellen. Veterinär-Medizinische Nachrichten, 4, 255-271.

Swayne, D.E., and Suarez, D.L., (2000). Highly pathogenic avian influenza. Revue Scientifique et Technique Office International des Epizooties, 20 : 463-482.

Swayne, D.E., Beck, J.R., and Mickle, T.R., (1997). Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. Avian Diseases, 41: 910–922.

Swayne, D.E., Beck, J.R., Garcia, M., and Stone, H.D., (1999). Influence of virus strain and antigen mass on the efficacy of H5 avian influenza inactivated vaccines. Avian Pathology, 28: 245-255.

Swayne, D.E., Garcia, M., Beck, J.R., Kinney, N., and Suarez, D.L., (2000a). Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine, 18: 1088-1095.

Swayne, D.E., Beck, J.R., and Kinney, N., (2000b). Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. Avian Diseases, 44: 132-137.

Swayne, D.E., Beck, J.R., Perdue, M.L., and Beard, C.W., (2001). Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Diseases, 45: 355-365.

Sykes, J.E., Allen, J.L., Studdert, V.P., and Browning, G.F., (2001). Detection of feline calicivirus, feline herpesvirus 1 and Chlamidia psittaci mucosal swabs by multiplex RT-PCR/PCR. Veterinary Microbiology, 81: 95-108.

Terpstra, C., (1978). Aantonen van C-virus in varkens na vaccinatie tegen varkenspest. Tijdschr. Diergeneesk., 102: 678-684.

Terpstra, C., and Robijns, K.G., (1977). Experience with regional vaccination against swine fever in enzootic areas for limited periods using C-strain virus. Tijdschr Diergeneesk, 102: 106-112.

Terpstra, C., and Tielen, M.J.M., (1976). Antibody response agaist swine fever following vaccination with C-strain virus. Zentralblatt fur Veterinar-Medizin, Reihe B, 23, 809-821.

Terpstra, C., and Wensvoort, G., (1987). Influence of the vaccination regime on the herd immune response for swin fever. Veterinary Microbiology, 13: 143-151.

Terpstra, C., Woortmeyer, R., and Barteling S.J., (1990). Development and properties of a cell culture produced vaccine for hog cholera based on the Chinese strain. Dtsch. Tierärztl. Wschr., 97: 77-79.

Tesmer, S., Urbaneck, D., Kaden, V., Wittmann, W., and Hahnefeld, H., (1973). Effect of attenuated hog cholera virus vaccine from the inoculation virus strain "C" on pregnant sows and their progeny. Monatsheft Veterinarmed., 28 (7): 251-254.

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J.P., and Frankena, K., (2001). WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Veterinary Record, 148: 567-572.

Thuer, B., and Hofmann, M.A., (1998). Comparative detection of classical swine fever virus in striated muscle from experimentally infected pigs by reverse transcription polymerase chain reaction, cell culture isolation and immunohistochemistry. Journal of Virological Methods, 74: 47-56.

Tokunaga, T., Yamamoto, H., Shimada, S., Abe, H., Fukuda, T., Fujisawa, Y., Furutani, Y., Yano, O., Katooka, T., Sudo, T., *et al*., (1984). Anti-tumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG: I: Isolation, physicochemical characterization and anti-tumor activity. Journal of the National Cancer Institute, 72: 955-962.

Tulman E.R., and Rock D.L. (2001). Novel virulence and host range genes of African swine fever virus. Current Opinion in Microbiology, 4: 456-461

Ulmer, J.B., De Witt, C.M., Chastain, M., Friedman, A., Donnelly, J.J., McClements, W.L., Caulfiels, M.J., Bohannon, K.E., Volkin, D.B., and Evans, R.K., (2000). Enhancement of DNA vaccine potency using conventional aluminium adjuvants. Vaccine, 18: 18-28.

Uttenthal, A., Le Potier, M.-F., Romero, L., Mario de Mia, G., and Floegel-Niesmann, G., (2001). Classical swine fever (CSF) marker vaccine. Trial 1- Challenge studies in weaner pigs. Veterinary Microbiology, 83: 85-106.

Uttenthaler, E., Kosslinger, C., and Drost, S., (1998). Characterization of immobilization methods for African swine fever virus protein and antibodies with a piezoelectric immunosensor. Biosensors and Bioelectronics, 13: 1279-1286.

van Drunen Littel-van den Hurk, S., Gerdts, V., Loehr, B.I., Pontarollo, R., Rankin, R., Uwiera, R., and Babiuk, L.A., (2000). Recent advances in the use of DNA vaccines for the treatment of diseases of farmed animals. Advanced Drug Delivery Reviews, 43: 13-28.

van Gennip, H.G., van Rijn, P.A., Widjojoatmodjo, M.N., de Smit, A.J., and Moormann, R.J., (2000). Chimeric classical swine fever viruses containing envelope protein E(RNS) or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. Vaccine, 19: 447-459.

van Gennip, H.G., Bouma, A., van Rijn, P.A., Widjojoatmodjo, M.N., and Moormann, R.J., (2002). Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E(rns) or E2 of CSFV. Vaccine, 20: 1544-1556.

van Oirschot, J.T., (1983) Congenital infections with non-arbo togaviruses. Veterinary Microbiology, 8: 321-361.

van Oirschot, J.T., (1999a). Classical Swine Fever. In: Diseases of swine, 8th edition, Blackwell Science, UK.

van Oirschot, J.T., (1999b). Diva vaccines that reduce virus transmission. Journal of Biotechnology, 73: 195-205.

van Oirschot, J.T., (2001). Present and future of veterinary viral vaccinology: a review. Veterinary Quarterly, 23: 100-108.

van Oirschot, J.T., and Terpstra, C., (1977). A congenital persistent swine fever infection. I. Clinical and virological observations. Veterinary Microbiology, 2: 121-138.

van Oirschot, J.T., Rziha, H.J., Moonen, P.J.L.M., Pol, J.M.A., and van Zaane, D., (1986). Differentiation of serum antibodies from pigs vaccinated or infected with Aujeszky's disease virus by a competitive enzyme immunoassay. Journal of General Virology, 67: 1179-1182.

van Oirschot, J.T., Kaashoek, M.J., and Rijsewijk, F.A.M., (1996). Advances in the development of bovine herpesvirus 1 vaccines. Veterinary Microbiology, 53: 43-54.

van Rijn, P.A., Bossers, A., Wensvoort, G., and Moormann, R.J.M., (1996). Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. Journal of General Virology, 77: 2737-2745.

van Rijn, P.A., van Gennip, H.G.P., and Moormann, R.J.M., (1999). An experimental marker vaccine and accompanying serological diagnostic test both based on envelope glycoprotein E2 of classical swine fever virus (CSFV). Vaccine, 17: 433-440.

van Zijl, M., Wensvoort, G., de-Kluyver, E., Hulst, M., van-der-Gulden, H., Gielkens, A., Berns, A., and Moormann, R., (1991). Live attenuated pseudorabies virus expressing envelope glycoprotein E1 of hog cholera virus protects swine against both pseudorabies and hog cholera. Journal of Virology, 65: 2761-2765.

Vandeputte, J., Too, H.L., Fook, K., Chen, C., Chai, K.K., and Liao, G.A., (2001). Adsorption of colostral antibodies against classical swine fever, persistence of maternal antibodies, and effect on response to vaccination in baby pigs. American Journal of Veterinary Research, 62: 1805-1811.

Veterinary Quarterly, (1998). Foot-and-mouth Disease Special issue 20: pp. S1-40.

Vidal, M.I., Stiene, M., Henkel, J., Bilitewski, U., Costa, J., and Oliva, A., (1997). A solid-phase enzyme linked immunosorbent assay using monoclonal antibodies for the detection of African Swine Fever Virus antigens and antibodies. Journal of Virological Methods, 66: 211-218.

Villareal-Chavez, C., and Rivera Cruz, E., (2002). An update on avian influenza in Mexico. In: Proceedings of the 5th International Symposium on Avian Influenza.Georgia Center for Continuing Education, The University of Georgia, Athens Georgia USA.

Ward, G., Rieder, E., and Mason, P.W., (1997). Plasmid DNA encoding replicating foot-and-mouth disease virus genomes induces antiviral imune response in swine, Journal of Virology, 71: 7442-7447.

Wardley, R.C., and Crowther, J.R., (1982). The ELISA: Enzympe-linked immunosorbent assay in veterinary research and diagnosis. Current Topics in Veterinary Medicine and Animal Science, Vol. 22; Martinus Nijhoff Publishers, The Hague, Boston, London.

Watanabe, T., Watanabe, S., Neumann, G., Kida, H., and Kawaoka, Y., (2002). Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. Journal of Virology, 76: 767-773.

Webster, R.G., Taylor, J., Pearson, J., Rivera E., and Paoletti, E., (1996). Immunity to Mexican H5N2 avian influenza viruses induced by a fowl pox- H5 recombinant. Avian Diseases, 40: 461–465.

Weidmann, M., Czajka, C., Wilson, W., Luo, L., Barany, F., and Batt, C.A., (1994). LCR – Overview and applications. PCR Methods and Applicationa, 3: S51-S64.

Wen-Bin, C., Sørensen K.J., Pei-Chih, L., Pin-Cheng, Y., and Ming-Hwa, J., (2002). Differentiating foot-and-mouth disease virus infected from vaccinated pigs by blocking ELISA using non-structural protein 3AB as antigen and its application to an eradication program. Journal of Clinical Microbiology, 40: 2843-2848.

Wensvoort, G., and Terpstra, C., (1988). Bovine viral diarrhoea infections in piglets born to sows vaccinated against swine fever with contaminated vaccine. Research in Veterinary Science, 45, 143-148.

WHO (World Health Organization), (1978) Guidelines for the preparation and establishment of reference materials and reference reagents for biological substances. 29th report of the World Health Organization.

Widjojoatmodjo, M.N., van Gennip, H.G.P., Bouma, A., van Rijn, P.A., Moormann, R.J.M., (2000) Classical swine fever virus E-rns deletion mutants: trans-complementation and potential use as nontransmissible, modified, live-attenuated marker vaccines. Journal of Virology, 74: 2973-2980.

Win-Deen, E.S., (1996). Automation of molecular genetic methods. Part 2: DNA amplification techniques. J. Clin. Ligand Assay, 19: 21-26.

Wirz, B., Tratschin, J.D., Muller, H.K., and Mitchell, D.B., (1993). Detection of hog cholera virus and differentiation from other pestiviruses by polymerase chain reaction. Journal of Clinical Microbiology, 31: 1148-1154.

Wolff, J.A., Ludtke, J.J., Acsadi, G., Williams, P., and Jani, A., (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Human Molecular Genetic: 1: 363.

Wright, P.F., (1998). International standards for test methods and reference sera for diagnostic tests for antibody detection. Revue Scientifique et Technique Office International des Epizooties, 17: 527-533.

Yu, X., Tu, C., Li, H., Hu, R., Chen, C., Li, Z., Zhang, M., and Yin, Z., (2001). DNA-mediated protection against classical swine fever virus. Vaccine, 19: 1520-1525.

Zhang, N, Maclachlan, N.J., Bonneau, K.R., Zhu, J., Li, Z., Zhang, K., Zhang, F., Xia, L., and Xiang, W., (1999a). Identification of seven serotypes of bluetongue virus from the People's Republic of China. Veterinary Record, 145: 427-429.

Zhang, G., Haydon, D.T., Knowles, N.J., and McCauley, J.W., (1999b). Molecular evolution of swine vesicular disease virus. Journal of General Virology, 80: 639-651.

Zhou, E.M., Chan, M., McIsaac, M., and Heckert, R.A., (1998a). Evaluation of antibody response of emus (*Dromaius novaehollandiae*) to avian influenza virus infection. Avian Diseases, 42: 757-761.

Zhou, E.M., Chan, M., Heckert, R.A., Riva, J., and Cantin, M.F., (1998b). Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Diseases, 42: 517-522.

Zientara, S, Sailleau, C., Dauphin, G., Roquier, C., Rémond, E.M., Lebreton, F., Hammoumi, S., Dubois, E., Agier, C., Merle, G., and Bréard, E., (2002). Identification of bluetongue virus serotype 2 (Corsican strain) by reverse-transcriptase PCR reaction analysis of segment 2 of the genome. Veterinary Record, 150 (19): 598-601.

## 17. ACKNOWLEDGEMENTS

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