SCIENTIFIC REPORT submitted to EFSA

Scientific review on Classical Swine Fever[1]

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Scientific reviews on Classical Swine Fever (CSF), African Swine Fever (ASF) and African Horse Sickness (AHS), and evaluation of the distribution of arthropod vectors and their potential for transmitting exotic or emerging vector-borne animal diseases and zoonoses

Scientific review on Classical Swine Fever

CFP/EFSA/AHAW/2007/02

CONTENTS

1 Aetiology

1.1 GENOME AND VIRION ORGANIZATION

Classical swine fever virus (CSFV), is a member of the *Flaviviridae* family, genus Pestivirus (Van Regenmortel et al., 2000). The species consist of small, spherical enveloped viruses with an approximate diameter of 40-60nm based around an electron-dense inner core structure of about 30 nm (Moennig et al., 2003). The viral genome, single stranded RNA molecule with a positive polarity, spans approximately 12.5 kbp and is made up of a single open reading frame (ORF) flanked by a 3prime and 5prime untranslated region (UTR), the latter contains conserved regions implicated in the translational events (Sizova et al., 1998, Fletcher et al., 2002). Notwithstanding the fact that virus has a RNA genome, it is reported to be relatively stable (Vanderhallen et al., 1999). Nevertheless, a recent study (He et al., 2007) indicated that recombination between strains is possible. The ORF is translated into a single polypeptide, 3900 amino acids, and is co-and post-translationally processed into mature peptide by a number of virus and host encoded proteases (Rümenapf et al 1993, Falgout et al., 1995; Elbers et al., 1996; Heimann et al., 2006). The virion is made up by 4 structural proteins (C, Erns, E1 and E2) which are encoded at the 5prime end of the genome. Although the exact virion structure is up until now not known in detail, it consists out a spherical nucleocapsid and coat, which is composed of numerous proteins C while the surface is made out of Erns, E1 and E2 in homodimeric (Erns, E2) or heterodimeric (E1E2) form (Thiel et al. 1991; König et al., 1995; Weiland et al 1990, 1992 and 1999). In contrast to E1 and E2, Erns has no transmembrane spanning domain and its attachment to the virion is rather tenuous and currently not well known. In addition to the structural proteins, the CSFV viral

genome encodes further 8 non-structural proteins, including an N-terminal proteinase (Npro), p7, the non-structural proteins (NS) 2, 3, 4A, 4B, 5A and finally 5B.

Mutagenesis studies, performed on the CSFV genome, have identified several regions which are associated with virus virulence although the underlying molecular mechanism remains elusive. Insertion of 19 amino acids into the carboxyl terminus of the E1 region of Brescia resulted in attenuation of the virus and a reduced viremia, spreading to the different tissues and viral shedding (Risatti et al., 2005). Similar studies, in which genetic regions of different CSFV strains have been exchanged or mutated, resulted in the link between virulence in swine and the E2 region (Rissati et al., 2005b). Three different regions in the E2 have been identified as virulence determinants: glycosylation site at position 805 (Risatti et al., 2007b); a region between 805 and 837 (Risatti et al., 2006) and a stretch of 12 amino acids substitutions in the carboxyl terminus (882 to 1032) (Risatti et al., 2007). Van Gennip et al. (2004) also identified a determinant in E2 (position 710) but a decrease in virulence was only found in conjunction with mutations in the Erns region (position 276, 476 and 477). Similar to E2, glycosylation sites (position 269) in the Erns have been found to have an influence on virulence in swine (Sainz et al., 2008). Abrogation of the RNAse activity of Erns by mutating codons 297 and 346 of the Erns protein resulted in a changed virulence of the virus (Meyers et al., 1999). In addition the the structural proteins, a virulence determinant has also been identified in one of the non-structural proteins, namely Npro (Mayer et al., 2004), using Npro deletion mutants. In addition, recent findings show that the 3'UTR region has also an effect on the virulence of CSFV as was demonstrated by the attenuation of the highly virulent strain Shimen by swapping the 3'UTR with the corresponding region of the HCLV strain. This mechanism of attenuation is probably caused by the presence of a 12 nt sequence (CUUUUUUCUUUU) in the 3'UTR of HCLV as introduction of that sequence in Shimen also resulted in attenuation (Wang et al., 2008).

Genome sequence analysis of specific viral regions and subsequent phylogenetic analysis allows the division of CSFV in three phylogenetic groups, each containing three to four subgroups. The phylogenetic typing and groupings are in more detail discussed in section 2.1.3.

Summary as provided by the authors:

- CSFV genome is relative stable.
- There are 3 mayor genotypes, with 3 to 4 sub groups.
- Several virulence determinations have been identified in: E1, E2, Erns and Npro.

Future research identified by the authors:

- The possibility of recombination and its potential impact on phylogenetic typing needs to be further clarified.
- The identification of virulence determinants could help to better understand the disease which in turn could promote more efficient treatment and control strategies.
- Future characterisation of the virion structure and its constituents.

1.2 SURVIVABILITY AND RESISTANCE OF THE VIRUS TO EXOGENIC FACTORS

An important viral property is the capacity of the virus to survive in the environment and to withstand disinfecting agents. Both aspects are of great importance in any CSF preventive measures and control and management strategies as is it influences the possibility of re-emergence. A reference table is available detailing the survivability and inactivation conditions (see Excel files provided with the scientific review).

1.2.1 Survivability

In addition to the intrinsic virion properties, such as the presence of an viral envelope, the survival of the virus is highly influenced by the environmental temperature and matrix wherein it is situated as could be seen by a review in 2000 (Edwards 2000). Therefore it is very difficult to provide general guidelines for inactivation (disinfection). In general it can be stated that survival of the virus increases when the temperature is low and where the environment is rich in proteins (such as in meat) and moist. This also includes the pH sensitivity of the virus which is relative stabile between pH5 to 10. Typical stability is extended to lower pH values but the degree of stability becomes strain dependent (Depner et al., 1992). Notwithstanding the stability at lower temperatures, subsequent freeze-thawing cycles have a deleterious effect on the virus. However, certain chemical compounds, such as dimethyl sulfoxide, seem to stabilise the virus during such cyclic events (Tessler et al., 1975)

Meat and meat products

The increased stability in low temperatures, even at low pH (pH4), and in protein rich environments is important as they are encountered during storage. For example; pH values of semi membranous and longissimus dorsi muscle post mortem ranges from 6,17 to 6,71. During the commercial production of pork and pork products, the time and temperature of storage seldom allow the pH to fall below 5,7 (Farez et al., 1997) and provide therefore ideal surviving conditions. Survival rates up to 4.5 years for frozen meat have been reported (Edgar, 1949). Treatments, as curing and smoking on the other hand, have little effect on the survivability of the virus. The most important factor is the temperature, duration and height, applied during the processing stage (Edwards, 2000). Survival rates in processed meat products of for example 90 days in salami (Savi et al., 1962) and 126 days in Iberian loins (Mebus et al., 1993) have also

been reported. For a more extensive review of the survival rates in processed meat products see Farez et al. (1997).

Waste and pig slurry

Animal excretions (urine, faeces) or secretions (saliva) can become contaminated with CSFV due to an infection. Although the amount of virus excreted/secreted is highly influenced by a number of factors, such as the virulence of the virus and the vireamic period (Weesendorp et al., 2008b); it can be the cause of virus transmission (Ribbens et al., 2004b). Furthermore, it has been reported that the virus can survive in contaminated pens for at least several days (Artois et al., 2002) and up to 4 weeks in winter conditions (Harkness, 1985). This data is in agreement with findings in pig slurry where CSFV can survive up to: 84 days at 4°C, 70 days at 17°C; 14 days at 20°C (Haas et al 1995). In temperatures higher or equal to 35°C the survival time reduces dramatically and the virus is inactivated in matters of hours or even minutes when temperatures reaches 50°C or higher. The latter is of considerable interest for efficient treatment of biological waste in cases of outbreaks. Turner (2000) analysed the inactivation kinetics of CSFv in commercial pig slurry and compared it to the kinetics in Glasgow Eagles medium. Minimal conditions for complete inactivation under lab conditions (starting titre:10^7 $TCID_{50}$/ml) was found to be 60°C for three minutes for pig slurry and 65°C for 2 minutes in Glasgow Eagles medium. Higher temperatures were necessary for the latter probably due to the presence of ammonium compounds in the pig slurry which enhanced the inactivation process. However, operational temperatures of 65°C are recommended for treatment of farm-scale quantities of contaminated slurry due to the variability in its composition whereby a reasonable margin of error during operation is incorporated in order to provide confidence in the treatment process. During the decontamination process, the homogeneity of the target temperature in mixture that is to be inactivated is crucial as regions where this temperature is not reached can provide the virus with an escape

route. This greatly reduces the efficacy of the whole process and poses a significant risk (Gale et al., 2004)

Summary as provided by the authors:

- CSFV is relatively stable.
- In a pH range of 5 to 10.
- Temperatures up to 20°C.

- Factors promoting stability
  - Protein rich environments (as for example muscle)
  - Dimethylsulfoxide
  - Low temperatures (<4°C)

- Factors promoting destruction
  - Rising temperature above 37°C reduces dramatically the survivability
  - Ammonium compounds (cfr pig slurry)
  - Changing pH below pH 4 or above pH 10

1.2.2. Resistance of the virus to exogenous factors

As enveloped virus, CSFV is inactivated by organic solvents (ether or chloroform), detergents (Nonident P40, deoxycholate, saponin), chlorine-based disinfectants, phenolics and quaternary ammoniums aldehydes (formaldehyde, glutaraldehyde) (reviewed by Edwards, 2000). High alcohol containing mixtures (> 80%) have also proven to be effective in inactivating lipid enveloped viruses. Four to six log reductions have been reported within 15 to 20 seconds following application using ethanol or propanol based disinfectants. Complete inactivation viral suspensions (5-10% virus added) took around 1 minute (Kampf et al., 2007; Van Engelenburg et al., 2002). However, effectiveness was significantly reduced for surface-dried viral suspensions and this was even more pronounced in cases where the virus was air dried

in the presence of high protein containing materials. This was illustrated by the findings that 80% Ethanol (or even 0,1% Sodium hypochlorite) could not complete inactivate virus that was air-dried in the presence of plasma even after 10 minute exposure. In contrast, when the air dried material was first rehydrated and than subjected to disinfection with 80% Ethanol, complete inactivation was obtained after 1 minute (Terpstra et al., 2007). These findings have important consequences on disinfecting protocols as materials used, stables, transport vehicles… as they often contain dried material that can be exposed to virus. Rehydration before application of the disinfectants combined with longer exposure is recommended in these cases. For exo-transplantation purposes (pig heart tissue) treatment of pig aortic valve units with 0.2% glutaraldehyde during 11 days was sufficient to inactivate CSFV (Cunliffe et al., 1979). Furthermore, TRIzol® LS Reagent (Invitrogen Corp.,Carlsbad, CA) and AVL Buffer (Qiagen, Valencia, CA), frequently used during RNA extraction protocols, render the virus inactive in the samples, probably due to the chaotropic components in the respective reagents (Le Dimna et al, 2008). This is not only relevant for lab level biosecurity but also for sample handing under field or low level containment (Blow et al., 2004).

As previously mentioned the thermal inactivation is strongly influenced by the matrix wherein the virus is situated. However, in vitro studies indicated that the inactivation at 100°C take less then 1 minute, 2 minutes at 90°C, 3 minutes at 80°C and 5 minutes at 70°C. Inactivation at temperatures at 56°C was should to be more heterogeneous and strain dependent as for some only a small drop in titres was observed while for others a complete inactivation was found (reviewed by Edwards, 2000). In blood on the other hand inactivation at 69°C took at least 30 minutes.

Alternative methods of inactivation have also been developed. Ultra-violet light, hydrostatic pressure or a combination of both has been shown to be able to effectively

inactivate CSFV (Freitas et al., 2003). Similarly, Vapour-phase hydrogen peroxide has been shown to be an efficacious way for decontamination of material (Heckert et al., 1997). The latter was confirmed during a bio-decontamination trial with hydrogen peroxide vapour in a materials airlock (20m^3) showed that a total dosage of 12 - 15 g/m3 of H2O2 during 1hour exposure at a temperature of 26.4 °C drastically reduced the infectivity of CSFV (personal communication, 2008).

Summary as provided by the authors:

- Decontamination chemicals/methods:
  - Organic solvents
  - Chaotropic agents
  - UV
  - Hydrostatic pressure
  - High alcohol containing mixtures (> 80%)
  - Hydrogen peroxide
  - Chlorine-based disinfectants (0,1% Sodium hypochlorite)
  - Phenolics
  - Quaternary ammoniums aldehydes (formaldehyde, glutaraldehyde)
  - Temperatures above 70°C
  - Sodium hydroxide 2%
  - Vapour-phase hydrogen peroxide

- Factors negatively influencing decontamination:
  - High protein content
  - Air dried virus
  - Organic contaminants

Future research identified by the authors:

- Although general information is available concerning disinfectants more data should be generated in regards to adequate disinfectant protocols detailing factors as: contact time, renewing the disinfectants, application methodology (avoiding aerosols).
- In depth evaluation of the potential of new inactivation techniques/methods such as UV and H_2O_2 treatments.

1.3 STRAINS, GENETIC TYPING AND VIRULENCE

1.3.1. Genetic typing and the virus and sequence database at the European Reference Laboratory for CSF, Hannover, Germany

Between 1970 and until the late 1990ies, Germany was struck by several severe and less severe epidemics of CSF (Fritzemeier et al., 2000; Moennig and Plagemann, 1992; Wachendörfer et al., 1978). Since the Institute of Virology became European Reference Laboratory for CSF almost 30 years ago Council Decisions 80/217/EEC and 81/859/EEC), the virus isolates involved were collected and stored. The idea was to keep them to solve the many open questions concerning the virus, of which many still remain without conclusive answers. One aim was to find methods that would allow to distinguish isolates from individual outbreaks. This was a significant issue, because such information would be an invaluable tool for epidemiologists to trace primary and secondary outbreaks. First success was achieved using monoclonal antibodies against viral proteins for differentiating between *Pestiviruses* (Greiser-Wilke et al., 1990; Paton et al., 1995; Wensvoort et al., 1989). In addition, monoclonal antibodies (mabs) were successfully used for typing CSF virus isolates and other *Pestiviruses* (Kosmidou et al., 1995; Paton et al., 1995). This method is work-intensive and was found to be closely correlated to the availability of the mabs. At that time, technological advances led to the implementation of the polymerase chain reaction (PCR) in most laboratories, and automated DNA sequencing became practicable and affordable. It was then realized that isolates from individual outbreaks could be discriminated by genetic typing. For this, several different regions of the viral genome were used, and it was recognized that genetic typing had to be harmonised to ensure that results from different laboratories are comparable.

Therefore, the three most widely used genomic fragments were evaluated, namely fragments of the 3' end of the polymerase gene (NS5B), (Bjorklund et al., 1999; Lowings et al., 1994), 150 nt of the 5'NTR (Greiser-Wilke et al., 1998; Hofmann et al., 1994; Stadejek et al., 1996) and a fragment (190 nt) of the gene coding for the E2 glycoprotein (Díaz de Arce et al., 1999; Lowings et al., 1996). A standardised protocol was designed for typing new CSF virus isolates, fixing the three genomic fragments to be used, the algorithms for calculation of the phylogenetic trees, and the nomenclature of the genetic groups (Lowings et al., 1996; Paton et al., 2000). The CSF viruses were divided into three groups with three or four subgroups each, namely 1.1- 1.3, 2.1- 2.3, and 3.1-3.4 (Paton et al., 2000). Geographical distribution of the subgroups has been reviewed previously (Frias-Lepoureau and Greiser-Wilke; Moennig et al., 2003).

At the same time, it was decided to store the available epidemiological data (host, year of isolation, country and region) and the nucleotide sequences of the three genomic fragments in a CSF virus database, which was to be accessible online (http://viro08.tiho-hannover.de/eg/csf). It is held at the European Community Reference Laboratory for CSF in Hannover, Germany, and it was designed to aid genetic typing of new CSF virus isolates (Greiser-Wilke et al., 2000).

Phylogenetic analyses performed in different parts of the world confirmed that CSF virus isolates that differ by genetic typing seem to be characteristic for certain geographic regions (Bartak and Greiser-Wilke, 2000; Blacksell et al., 2005; Chen et al., 2008; Diaz de Arce et al., 2005; Kamakawa et al., 2006; Li et al., 2006; Pereda et al., 2005; Sabogal et al., 2006; Stadejek et al., 1997; Vilcek et al., 1997).

Extensive use of the database and an increasing number of records, including isolates with identical sequences from related outbreaks in different regions, made it difficult

for the user to select a standard dataset for genotyping new isolates. As a consequence, the database was supplemented with a module for searching for identical sequences, performing the alignment with a standard set of sequences, and calculating and graphically displaying the Neighbor-Joining phylogenetic tree (Dreier et al., 2007).

Summary as provided by the authors:

- Genetic typing is essential for epidemiological evaluation
- Standardization/harmonization of sequencing and phylogentic typing protocols resulted in defining:
  - the three genomic regions for typing
    - 3'end of NS5B gene
    - Part of the 5'NTR
    - Part of the E2 gene
  - the algorithms for calculation
  - the nomenclature for the genetic groups

- CSFV is divided in the following genetic groups:
  Genotype 1: with 3 sub genotypes (1.1 / 1.2 / 1.3)
  Genotype 2: with 3 sub genotypes (2.1 / 2.2 / 2.3)
  Genotype 3: with 4 sub genotypes (3.1 / 3.2 / 3.3 / 3.4))
- Online database, containing epidemiological data and sequence information, and associated tools for searching and aligning are accessible

Future research identified by the authors:

- Evaluation impact of recombination on genetic typing
- Incorporation new sequencing techniques
- Biological relevance of phylogenetic (sub)groups
- Continued collection of new isolates form new outbreaks

2 Pathobiology of Classical Swine Fever

2.1 CLINICAL SIGNS

Although classical swine fever (CSF) has been well known for decades and epidemics still occur, clinical diagnosis continues to cause problems for veterinary practitioners. This is due to the extensive differential diagnosis, further complicated by the emergence of new diseases such as porcine reproductive and respiratory syndrome (PRRS) and porcine dermatitis and nephropathy syndrome (PDNS). As a cause of considerable economical losses within the EU, control of CSF requires knowledge of the primary outbreaks and spread of the disease . Recent outbreaks have shown how a prolonged silent circulation of CSFV in pig herds may result in a large epizootic when vague and non-specific disease symptoms allow virus spreading while remaining unnoticed , .

Historically, different levels of virulence have been reported from peracute, acute, chronic or prenatal forms of CSF. The virulence of a strain is difficult to establish as the same isolate can induce different signs depending on the age, the breeding, the health status and immune status of the inoculated pigs. .

The piglets develop more evident clinical signs than the adults. The constant symptom is hyperthermia , usually superior to 40°C, the piglets pile in corner, but in adults it can be lower (39,5°C-40°C). The first usual signs in acute form are anorexia, lethargy, conjunctivitis, respiratory signs and constipation followed by diarrhea . During a chronic course of the disease, the issue is generally fatal. After displaying at first same clinical signs as in an acute form, the pigs survived for two to three months but never

more. They display non-specific signs as intermittent hyperthermia, chronic enteritis and wasting.

CSFV is able to cross the placenta of pregnant sows and infect the foetuses during all the stages of pregnancy, but depending on the strain virulence and the time of gestation, the infection can result in abortion and stillbirths in early pregnancy, when can lead to the birth of persistently viraemic piglets if infection occurs during the 50-70 days period of gestation. These piglets seem normal but rapidly waste or present congenital tremor . This course of infection was reported as "late onset CSF" **.** These animals shed a lot of virus for several months and are very dangerous reservoirs of virus as it is described for BVBV in ruminants.

Other pestiviruses can also infect pigs but natural infection with BVDV usually occurs without clinical signs . However, in some cases, natural infection of pig herds with pestiviruses other than CSFV is associated with breeding problems like poor conception, small litters, and a few abortions. Hyperthermia and colic spasms have also been described .

Domestic Boars

According to Wehrend et al , (2006), experimental infection with the CSFV virus has no evident effect on libido and ejaculate parameters of adult boars. The only clinical signs in the boars were an increase in body temperature, but never above 39.9°C and a temporally anorexia. The libido was always good, so semen collection was performed in three boars without difficulty and the semen quality was always in the range of the minimum requirements for sperm that is used for artificial insemination. Although one boar had a good libido only a sperm free ejaculate could be collected on one day. Insemination boars may thus be of special epidemiological relevance for the dissemination of the CSF virus.

Feral pigs or Wild boars:

After experimental CSFV infection in a pregnant wild boar and two wild boar weaners, the clinical, pathological and haematological findings noted in the young wild boars were comparable to those in domestic weaner pigs inoculated with the same virus isolate . Both wild boars showed the acute haemorrhagic form of CSF, one animal died 18 days post inoculation (pi) and the second one had to be euthanized when moribund two days later. The wild boar sow did not show any signs of illness pi but seroconversion was noticed. Twenty-eight days pi. birth was given to six clinically healthy offsprings. One of the newborn proved to be viraemic until death when 39 days of age. Except for poor growth no other symptoms were noticed in this piglet. The non-viraemic litter mates remained healthy, although they had close contact to the persistently infected piglet. High titres of neutralizing antibodies against CSFV were measured in the serum samples of these offsprings. All findings were more or less in accordance with observations previously made in domestic pigs when infected with CSFV around 85 to 90 days of gestation. The wild boar was calculated to have been inoculated at about 87 to 92 days of gestation.

Moreover, in an area where the virus has already spread, maternal antibodies can partially protect the wild boars piglets. Instead of an acute and fatal course, the disease is transient, as it was shown during an experimental study conducted to investigate the clinical course of classical swine fever (CSF) in wild boar piglets partially protected by maternal antibodies. Five healthy wild boar piglets with a low serum titre of colostral antibodies against CSF virus were challenged with virulent CSF virus at the age of three months. Apart of reduced food intake and diarrhea no major clinical symptoms were noticed after challenge. These signs were seen during the second and third week of infection, afterwards the piglets recovered completely. CSF virus could be re-isolated from blood samples taken on day 12 and day 19 post

challenge. From blood samples taken later on and from the organ material taken at post mortem examinations no CSF virus could be isolated anymore. It can be concluded that the presence of maternal antibodies influences the clinical course of CSF in terms that the outcome is rather transient than lethal. Such wild boar could play a crucial role in the spread of CSF virus and might contribute to the maintenance of long lasting epizootics..

A classical swine fever virus (CSFV) field isolate originating from wild boar was investigated on its virulence in domestic pigs and wild boar. Three weaner pigs and two wild boars (yearlings) were intranasally inoculated with the isolate "Spante" and tested for clinical, virological, hematological and serological findings until day 31 after infection (p. i.). One day p.i. the piglets were put in contact to three sentinel pigs. During a period of 31 d neither the domestic pigs nor the wild boars showed clinical signs specific for CSF. Two infected weaner pigs became transiently viraemic, transmitted CSFV in nasal secretions, showed a slight leucopenia and reacted serologically positive. The contact infection resulted in a viremia in two sentinel piglets on day 30. Only one contact animal developed antibodies. None of the wild boars became viraemic, excreted CSFV in nasal secretions or developed antibodies..

Even if experimental infection in domestic or wild pigs gives similar disease, it is more difficult to identify classical swine fever in the wild as found dead animals are the main alert sign. These carcasses cannot be found easily as they are most of the time eaten by other animals or hidden by high grass during the summer. At post mortem examination, the most frequent gross lesions seen are on the skin : round lesions similar to scabies, and ulcers on the intestine .

Summary as provided by the authors:

- Clinical diagnosis of CSF is not evident as clinical signs are highly variable,
- Differential clinical diagnosis is unreliable and laboratory confirmation is needed
- Early detection of CSFV circulation is crucial for minimizing epizootic size and economic consequences. Training courses should be proposed to veterinarians and farmers to help them to submit samples for differential diagnosis including CSF detection.

2.2 PATHOGENESIS OF THE DISEASE IN PIGS

CSFV is known as immunosuppressive however, neutralizing antibodies appear usually after two weeks post infection in recovering pigs. Recently, different teams have attempted to understand the mechanisms of the CSFV–host interactions that lead up to the innate immune response evasion and delay the onset of acquired immunity and produce its pathogenic effects. As other pestiviruses, CSFV grows in cell culture without any cytopathogenic effect, preventing the antiviral effect of INFα and apoptosis . Even if the majority of pestiviruses are noncytophatic in vitro, some BVD viruses from Mucosal disease cases or some CSFV strains are also cytopathogenic in vitro, and this cytopathogenicity of BVDV is correlated with a higher expression of the nonstructural protein NS3, which can be generated by processing of a fusion protein termed NS2-3 .

Since CSFV is noncytopathic in vitro, it has been suspected that the serious lesions seen in vivo were linked to immunopathological damages. The usual entry site is the oronasal route, the first site of virus replication is the tonsils, then the virus spread to the regional lymph nodes, before reaching, via the peripheral blood, bone marrow, visceral lymph nodes and lymphoid structures linked to the small intestine lymphoid, and spleen. The spread of the virus within the pig organism is usually completed in

less than 6 days. During infection, severe changes occur in the bone marrow and in the circulating white cell population, suggesting an indirect cytopathic effect induced in non infected cells either by a soluble factor, or by cell to cell contact. Interestingly, CSFV replicates in monocytes–macrophages and vascular endothelial cells in pigs. Leucopenia, in particular lymphopenia, is a characteristic early event during CSF . The leucopenia involves leukocyte sub-populations in a disparate manner, with B-lymphocytes, helper T-cells and cytotoxic T-cells being the most affected. Depletion of lymphocyte sub-populations occurs shortly before or at the time virus can be detected by RT-PCR in the serum. The pathogenic mechanism therein would involve indirect virus-host interactions, probably originating from the site of primary infection, rather than a direct effect of the virus or viral protein. Furthermore, these characteristics offer an explanation for the retardation of the cellular and humoral immune response observed during classical swine fever . Erns at high concentrations has been pointed out as an apoptosis inductor on lymphocytes in vitro, but its implication has been discussed as addition of infected cells supernatant did not induce apoptosis in target cells. The interactions between both viruses and the monocyte-macrophage-system result in the release of mediator molecules, which are important for the further progression of the disease. The changes in the haemostatic balance are thought to be caused by pro-inflammatory and antiviral actors, causes of the thrombocytopenia and the mechanisms of the haemorrhages, which are characteristic in the infection. The production of inflammatory cytokines by infected endothelial cells could play a role in the immunosuppression, as well facilitating virus dissemination by attracting monocytic cells . The question of the CSFV presentation by dendritic cells has been recently attempted, leading to the observations that CSFV can replicate in dendritic cells (DCs). CFSV could use these highly migrating cells as a vehicle to different sites in the body, especially to lymphoid tissues. However, the interaction between CSFV infected DCs and lymphocytes is not sufficient to induce the lymphocyte depletion, without another interaction with the particular environment

of the lymphoid follicles . demonstrated that CSFV infection induced DC recruitment and activation in secondary lymphoid organ T-cell areas during the first hours post-infection, leading to initiation of innate immune response, via IFN-α and TNF-α production. The uncommonly high levels of cytokines would also play a role in disruption of immune system cells, either inducing apoptosis and/or impairing DC maturation and T cell priming.

However, in clinically diseased pigs, CSFV and CSFV RNA can be normally detected from day 2 to 4 on. Duration of viremia depends on the clinical situation and is very short in subclinical infections e.g. of sows (1 to 2 days) or can be very long lasting e.g. during chronic or persistent infection.

Studies focussing on viral and host determined virulence have been limited by the past by the lack of a well established definition of the meaning of virulence in CSF. Three levels of virulence are generally mentioned : the highly virulent strains that kill most of the affected pigs, moderately virulent strains to which pigs can recover and avirulent strains that are most of the time attenuated vaccine strains. Nevertheless, it has been shown that some strains were more virulent than others but this virulence was depending on age and breeds of pigs . In the last years, a scoring system for clinical symptoms and gross lesions were described by , but no reliable in vitro parameters correlating with the virulence of a CSFV strain in its natural host have been identified. All viral virulence factors identified so far reduce the virulence of the virus if mutated or deleted but none of them is able to convert any avirulent parent virus into a virulent progeny upon insertion, (see 2.1.1). Furthermore, many of the described virulence factors seems to be strain specific.

2.3 IMMUNOLOGICAL RESPONSE OF THE HOST AND ACQUIRED IMMUNITY MECHANISMS

When pigs recover from infection, the immune response against infection with CSFV is quite effective. After ten to 14 days neutralising antibodies can be detected and it was shown that nearly the complete induction of neutralising activity depends on the envelope protein E2 (Reimann et al., 2003). However, non neutralising antibodies are also developed against envelope protein ERNS and non-structural protein NS3 . In addition, for all three types of antibodies ELISA-systems are available. However, only the E2-blocking–ELISAs and one of the available ERNS-ELISAs is CSFV-specific . In contrast, detection of NS3-antibodies as well as one of the ERNS-ELISAs is panpesti-virus specific.

Generation of cell-mediated and humoral immune responses was investigated in d/d histocompatible pigs following CSFV infection or vaccination. Viral infection induced high T cell responses with high primary and secondary CTL activity correlated with high IFN-gamma production, whereas vaccination with a live vaccine followed by infection mainly induced neutralizing antibody but low cell-mediated responses. Moreover, high IgG1 response was associated with high IFN-gamma response following infection whereas a weak IFN-gamma response was related to a good IgG2 response but a low IgG1 production. These data could reflect Th1/Th2-like balance of immune responses depending upon immunization protocols, which has not yet been described in the pig. T-cell responses to CSFV were evidenced by CSFV-specific CD25 up regulation on CD4-CD8+, but not on CD4+CD8- cells, which further illustrated the importance of CTL responses after infection. These results indicated that generation of cell-mediated immune responses was much higher following intranasal/oral CSFV infection than after intramuscular vaccination, which

implies that the capacity of new CSFV vaccines to induce higher T-cell responses should be considered

Very new results could demonstrate that the innate immunity modulating function of Npro is not relevant for the virulence of CSFV (Nicolas Ruggli, Poster at the GfV meeting, Heidelberg 2008).

In conclusion, both CSFV-specific neutralising activity as well as a specific killer cell activity is most important for an effective immune response. However, both parts alone have also the potential to protect pigs from a lethal CSFV-infection. It was demonstrated that E2-subunit vaccines can protect pigs on the basis of high titres of neutralising antibodies while experiments with related recombinant vectors , pestiviruses or chimeric constructs were efficient without detectable neutralising activity (Reimann et al., 2003; Beer et al., 2007). However, the combination of both cellular immunity and neutralising antibody response is obviously crucial for an optimized immunity allowing fast and complete protection with a kind of “sterile immunity”.

Summary as provided by the authors

- CSFV is an immunosuppressive virus
- The monocyte-macrophage line is the first target of the virus
- CSFV prevents the apoptosis of its host cells,
- CSFV circumvents the antiviral effect of INF alpha and can infect dendritic cells,
- CSFV induces the apoptosis of uninfected cells as endothelial cells leading to haemorrhages, the mechanisms of this indirect effect is still unknown,
- CSFV induces severe lymphopenia, most of the B cells are depleted.
- Depending on the strain virulence, infected pig dyes rapidly or can recover.
- In recovering pigs :

  CFSV infection induces humoral and cellular immune responses

  Induction of cellular response is quicker than humoral response (INF gamma detected before neutralising antibodies)

  B cells are not responding for some days to 1 week after their number comes back to a physiological level, delaying the humoral response
- Recommendations:

  Basic research is important and a prerequisite for further developments of rational intervention strategies (vaccine, anti-viral) and diagnostic methods; Viral protein functions, pathogenesis and host responses are still unclear

Future research identified by the authors:

- Identification of reliable in vitro parameters for assessing the virulence of CSFV in its natural porcine host

- Identification of viral virulence factors that acts as virulence factors per se, their presence or absence will definitively render the virus virulent or not
- Understanding the mechanisms of the virus-host relations is needed to develop more targeted intervention strategies (vaccine or anti-viral), special during the first hours after infection when the virus starts to circumvent the host immune defence.

3 Epidemiology

3.1 NATURAL HOST RANGE AND VECTORS

The domestic pig appears to be the only domestic animal species naturally infected by the CSFV (Terpstra, 1987). All breeds and ages are considered susceptible, although adults generally stand a better chance of surviving the infection. Natural outbreaks of CSF may also occur in European wild boar (*Sus scrofa ferus*) (Terpstra, 1987). Natural hosts of CSFV are members of the family Suidae (Moennig, 2000). Peccaries (Tayassu tajacu and T. pecari) may also become naturally infected by CSFV (Vargas Teran et al., 2004). Natural cases of CSF have never been reported in ruminants. However, CSFV can replicate in experimentally inoculated cattle and cause reproductive disease in experimentally infected goats (Paton 1995b).

Although of minor importance haematophagous arthropods may contribute under certain conditions to the spread of CSFV. Despite biological transmission has never been shown, it is conceivable that certain Diptera species e.g. of the genus Tabanus, Aedes and Musca could occasionally play a role in the mechanical dissemination of the virus from infected to susceptible herds, especially there these are in the close proximity and where the potential vector species is in abundance. Persistently infected pigs with a lifelong high-titred viremia constitute an ideal reservoir for vector transmission (Terpstra, 1987; Dahle and Liess, 1992).

Experimental studies to determine whether CSFV can infect pets or rodents revealed that dogs, cats and rats are unlikely to represent significant biological vectors of the CSF virus. (Dewulf et al., 2001b). CSFV transmission from lungworms to earthworms and back to swine could not be verified (Dahle and Liess, 1992)

3.2 DESCRIPTION OF THE ROUTES FOR VIRUS TRANSMISSION

Under experimental conditions, pigs have been infected by oral, nasal , conjunctival, genital and various parenteral routes. Infection via most of these routes is likely to occur in one way or another under natural conditions as well (Terpstra, 1987). Infected pigs may already shed virus during incubation period or the prodromal phase (Terpstra, 1987; Edwards, 2000). The most frequent natural route by which CSFV enters the host is oronasal (Liess, 1987; Terpstra, 1991).

Direct contact between pigs is the principal means of transmission (Terpstra, 1991). There is an ample field evidence to indicate that the major route of transmission of CSF is directly from pig to pig (Edwards 2000). Infections with virulent strains of CSFV gives rise to high levels of virus in blood and other tissues. Large amounts of virus are then excreted in saliva and smaller quantities in urine, nasal and lachrymal fluids. Virus excretion continues until death or, in pigs which survive, until antibodies have developed (Terpstra, 1991). Thus, direct transmission of CSF is undoubtedly the most efficient way of CSFV transmission (Ribbens et al., 2004b). It was recently evidenced that the speed and rate of virus transmission (R0) depends more on the virulence of the strain than on the between- or within- pens transmission (Durand et al, 2008).

Movement of pigs is the most common way in which CSF is spread. Most feared is road transportation of weaners collected from different breeding farms and sorted and regrouped in markets, and then reloaded and transported to various fattening farms (Terpstra 1991, Dahle and Liess 1992).

Transmission experiments clearly indicated that also indirect transmission of CSFV via secretions and excretions, even after depopulation of pens with CSFV infected animals, can be of importance in a late clinical stage of disease (Ribbens et al., 2004a).

Airborne spread of the virus can occur, especially where are large numbers of infected pigs, but is usually limited to local areas (Edwards 2000). If it occurs, airborne transmission of virus is probably only over short distances and mainly within the holding (Paton and Greiser-Wilke, 2003). Experimental studies of airborne transmission demonstrated that CSFV may be transmitted following the prevalent air currents within domestic pig herds (Dewulf et al., 2000).

Congenital infection of CSF are also described, where CSFV may transmitted from CSFV infected sows transplacentally to the fetuses where disease symptoms of apparently healthy piglets born were not observed before the age of 9-28 weeks after birth (Van Oirschot and Terpstra, 1977).

CSFV can survive in pork and pork products. Survival can be prolonged for months when meat is stored cool or even years when stored frozen (Terpstra, 1991; Dahle and Liess, 1992).

Mechanical transmission by man is of great significance in areas with a high density of pigs and pig herds (Terpstra 1991, Dahle and Liess 1992). Transmission by contaminated clothes and footwear is rare since the amount of virus thus transferred is usually below the minimum infective dose for pigs (Terpstra 1991). The mechanical transmission of CSFV by pets and rodents remains a possibility although transmission experiments have failed. However, the likelihood of mechanical spread is difficult to

assess (Dewulf et al., 2001b). The role of arthropods, birds, rodents and other animals in the spread of CSFV remains doubtful (Ribbens et al., 2004b, Kaden et al 2003).

3.3 DIFFERENT VIRUS SOURCES AND RESERVOIRS

Although, there are reports of susceptibility for CSFV of various animal species, domestic pig seem the major source of virus after multiplication in several organs and excretion by nasal, oral and intestinal route at high titers of virus (Liess, 1987).

In Europe, wild boar can also act as a reservoir under certain conditions (Laddomada, 2000, Rossi et al., 2005). Contacts to wild boar may occur directly, when domestic pigs are kept outside. Direct or indirect contacts between domestic pigs and wild boar caused a considerable number of outbreaks in the past (Dahle and Liess, 1992). However, the epidemiological role of wild boar as a reservoir is questionable because serological investigations have shown that under natural conditions, the wild boar population is free of antibodies (Dahle and Liess,1992).

Assumed that the virus contained in one of the sources comes into contact with susceptible pigs, it depends on the virus-host system whether infection takes place and disease occurs or whether merely antibody development can be demonstrated (Liess 1987).

Persistently infected pigs can serve as a highly potent source of CSF. If such pigs are slaughtered, offals and fluids if fed untreated to susceptible pigs can initiate CSF outbreaks (Liess, 1987; Terpstra, 1991). However one of the most important reservoirs of CSFV is the infected pregnant sow. As mentioned before, after primary

infection and vireamia, a sow transmits the virus transplacentally to the fetuses while the sow develops immunity (Liess, 1987).

3.4 AVAILABLE EPIDEMIOLOGICAL TOOLS SUPPORTING CONTROL AND MANAGEMENT STRATEGIES

By using descriptive methods in veterinary epidemiology, epidemics can be analyzed and conclusions can be drawn for the further control of the disease. As an example, the CSF epidemic 1997-1998 in the Netherlands was preciously analyzed with methods of the descriptive epidemiology (Elbers et al., 1999). It could be demonstrated that two major factors were responsible for the epidemic to be become a catastrophe. Firstly, it was the extended period of time between introduction of the virus in the region and the detection of the outbreak. As a result, CSFV had opportunities to spread from one herd to another during this period. Secondly, the measures initially taken did not prove sufficient in the swine- and herd-dense region involved (Elbers et al., 1999).

Decision tree analysis tool were used within the analysis of the CSF epidemic in Spain during 2001 -2002 (Allepuz et al., 2007).

On the basis of SIR-models, the transmission of CSFV can be quantified and the effectiveness of disease control measures during the CSF outbreak can be evaluated. This could be demonstrated by using the disease outbreak data of the CSF epidemic 1997-1998 in the Netherlands as well (Stegeman et al., 1999). From these data the infection-rate parameter (the average number of herds infected by one infectious herd during one week) and the herd reproduction ratio (the average total number of secondary outbreaks caused by one infectious herd) was estimated using a SIR-model (Stegeman et al., 1999).

Simulation models are useful tools supporting control and management strategies in CSF epidemics. A spatial, temporal and stochastic simulation model was developed to simulate the Dutch CSF epidemic of 1997-98 as closely as possible (Jalvingh et al., 1999; Nielen et al., 1999; Mangen et al., 2001). The simulation tool (InterCSF) simulates disease spread from an infected farm to other farms through three contact types (animals, vehicles, persons) and through local spread up to a specified distance. The main disease control mechanisms that influence the disease spread in InterCSF are diagnosis of infected farms, depopulation of infected farms, movement-control areas, tracing and pre-emptive slaughter (Jalvingh et al., 1999). Other models for CSF have been described by Stärk, 1998; Karsten et al., 2005; Milne et al., 2008, for Germany, The Netherlands and Australia, respectively.

In case of disease outbreaks, a science-based forecast of the further development of the epidemic is important in order to plan or adjust disease control measures. A new method to analyse outbreak data of infectious diseases such as CSF is described by Meester et al., 2002. The described method leads to prediction of the future course of the epidemic and hence can be used as a basis for control policy decisions (Meester et al., 2002).

A method to determine the rate of inter-herd transmission of CSF by different types of contact is described by Stegeman et al., 2002. These transmission rates can be used to optimize to stop future epidemics of CSF (Stegeman et al., 2002).

Tracing, a procedure that begins with a known infected individual, herd or flock, and which traces all possible local and interactive exposures in both directions, back towards the source and forward to the contacts, is the backbone of the disease emergency management (Elbers et al., 2001b). Elbers et al. (2001b) provided an

introduction to, and general overview of, tracking and tracing systems used during the Dutch CSF epidemic of 1997-1998.

A risk analysis tool is described by Mintiens et al., (2003) to assess the spread of CSFV through 'neighbourhood infections' for different regions. Using the disease outbreak data of a CSF epidemic in Belgium 1994, it could be demonstrated that the only risk factor that was associated with the occurrence of 'neighbourhood infections' was a kernel estimation of the intensity of neighbouring herds. Additionally, as a result of the risk analysis, different areas of higher and lower risk for CSFV spread through 'neighbourhood infections' could be spatially identified (Mintiens et al., 2003).

4 CSF occurrence world wide during the last 5 years

According the disease information available on the WAHI interface of the OIE http://www.oie.int/eng/en_index.htm, following countries reported the confirmation of CSF in their country during 2005-2008, either in domestic pigs or wild boars

Armenia (2005-2006), Bhutan (2005 and 2007), Bolivia (2005-2007), Bosnia and Herzegovina (2005-2007), Brazil (2006), Bulgaria (2005-2007), China (Peoples Rep. of) (2006), Chinese Taipei (2005), Colombia (2005-2007), Croatia (2006), Cuba (2005-2007), Dominican Republic (2005-2007), Ecuador (2006-2007), Former Yug. Rep. of Macedonia (2005), France (2005-2006), Germany (2005-2007), Guatemala (2006-2007), Haiti (2005-2006), Honduras (2007), India (2005-2007), Indonesia (2005-2006), Rep. of Korea (2005-2006), Laos (2005), Madagascar (2005-2007), Mexico (2005 and 2007), Myanmar (2005-2007), Nepal (2005 and 2007), Nicaragua (2005-2007), Peru (2005-2007), Philippines (2005-2007), Romania (2005-2007), Russia (2005), Serbia (2005-2007), Slovakia (2005-2006), South Africa (2005-2007), Thailand (2005-2007), Venezuela (2005) and Vietnam (2005-2007).

Following countries are registered as “current unresolved disease events” (Date: 15 June 2008): Bolivia, Bulgaria, Croatia, Russia and Slovakia.

5 Available diagnostic tools (description, performance and differentiation potential)

The clinical signs of CSF are extremely variable and it may be confused with many other diseases. Clinical signs can therefore only lead to a clinical suspicion of the disease and any suspicion of CSFV has to be confirmed by laboratory diagnosis. Laboratory diagnosis relies on either agent detection (detecting either viral proteins or genome) or antibody detection. The choice of the laboratory tests applied in a lab depend mainly on the goal (i.e. surveillance vs. confirmation of suspicions), but also on the infrastructure and experience of a lab. The technical annexes of EU legislation as well as the OIE Manual of Standards for Diagnostic Tests and Vaccines provide useful details on the laboratory procedures for diagnosis of CSF. Recent reviews give additional information on most of the tests (Blome et al., 2006; Greiser-Wilke et al., 2006a)

5.1 AGENT DETECTION

Depending on the virulence of the strain, the tests and samples used, virus can already be detected from 24 hours after an infection. Animals that die from the infection will usually be viraemic until the time of death, whether this is during the acute phase, or after going through a chronic infection that may last up to several months. Immunotolerant pigs are also viraemic during their whole life, which may last up to nine months. Pigs that recover from the infection are usually only viraemic for a short period, from only a few days up to two weeks, after which the virus is no longer detectable in the blood.

5.1.1 Virus isolation

Virus isolation is based on the incubation of sample material on susceptible cell cultures of porcine origin. If CSF virus is present in the sample, it will replicate in the cells to an amount that can be detected, by immunostaining of the infected cells with conjugated antibodies. Classical swine fever specific antibodies are required to differentiate between CSFV and other pestiviruses.

Suitable samples for isolation of CSF virus from live pigs are leukocytes, plasma or whole blood obtained from non-coagulated blood samples. Suitable tissue samples include tonsil, kidney, spleen ileum and different lymph nodes.

Virus isolation is best suited for the investigation of samples from small numbers of animals rather than mass surveillance. The virus isolation procedure is labour intensive and requires at least three days before results are available. Two further cell culture passages may be necessary to detect very small amounts of virus in the sample is detected. This may lead to an investigation time of more than 10 days before a final result is obtained. Samples that suffer from autolysis can be cytotoxic to the cell culture and consequently have limited value.

Virus isolation is still considered the gold standard, even though by now the PCR-based detection methods are recognized as more sensitive tests . The sensitivity of the VI is usually thought to be high, and in experimental infections, up to 95% sensitivity is reported . However, an evaluation of the VI during the 1997/98 outbreak in the Netherlands, showed that the diagnostic sensitivity of the VI on tonsils in the field was only approximately 77%, which was comparable to the sensitivity of the FAT . The sensitivity of the VI on blood samples may also be hampered by the presence of

antibodies, although no quantitative data, especially from the field, is available on this.

A positive VI is proof for the presence of infectious virus and any animal, tissue or blood sample being VI positive is assumed to be infectious to other pigs. A negative VI on the other hand does not proof infectious virus is absent

5.1.2 RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is based on the amplification and subsequent detection of genome fragments. Small fragments of viral RNA are transcribed into cDNA fragments during a RT-step, which are subsequently amplified by PCR to detectable quantities. Detection of amplicons is possible by gel electrophoresis, but nowadays mainly real-time RT-PCR's are being used. These PCR's use either SYBR green to detect amplicons, or, for enhanced specificity, hydrolysis of hybridization probes (Liu et al., 1991; Roehe and Woodward, 1991; Katz et al., 1993; Diaz et al., 1998; McGoldrick et al., 1998; Aguero et al., 2004; Belak, 2005).

A wide variety of samples are suitable for the PCR, but mainly whole blood samples and tissue samples will be used for the diagnosis of CSF. Beside whole blood, also serum, plasma or isolated leucocytes can be used. Tissue samples of preference are the same as for VI: tonsil, spleen, ileum, lymph nodes. Kidney samples may be less suitable.

The (real-time) RT-PCR requires appropriate laboratory equipment and skilled staff. For both RNA isolation and RT-PCR fully robotized solutions are available

nowadays. Furthermore, separated facilities are needed for different steps in the process, as well as strict protocols on sample treatment and transport, and movement of personnel and materials to avoid (cross-) contamination of samples or reagents. An RT-PCR can be performed within several hours, but for high-throughput 24-48 hours between receiving samples and sending out results is more realistic.

RT-PCR has been found to be the most sensitive method for detection of CSFV, . In carcasses from wild boar it is the method of choice, especially if the material is subject to autolysis and virus is either inactivated or virus isolation is not possible any more due to cytotoxicity of the sample . With the RT-PCR, viral genome can be detected for a long time in certain tissue samples from animals that are fully recovered from an infection. In tonsils from pigs recovered after an infection, viral genome was detectable for at least 9 weeks (Loeffen et al., 2005). An RT-PCR positive result does not necessarily mean that infectious virus particles are present (Dewulf et al., 2005; Haegeman et al., 2006). This situation is seen with several other viruses.

Due to its high sensitivity, and the amplification of huge amounts of amplicons, the RT-PCR is also very sensitive to contamination or cross-contamination of samples, reagents or other materials. As stated above, separate rooms should be used for separate steps in the PCR diagnostics, for instance pre-treatment of samples, preparing buffers and stock-reagents, RNA-isolation, and RT-PCR. Strict protocols should be in place with respect to movement of people, materials and samples between these rooms, or between these rooms and other rooms in the laboratory. Furthermore, retesting or independent confirmation of positive samples may be necessary if in doubt.

RT-PCR is also highly specific, especially if specific probes are being used. Hybridization probes may be slightly more specific than hydrolysis probes, as the

latter may be subject to non-specific degradation during high cycle numbers and therefore cause very weak-positive or doubtful results.

In general it can be said that from a RT-PCR negative result it can be concluded with a very high confidence that the tested animal or tissue sample is not infectious to other pigs, while on the other hand a sample that is RT-PCR positive, is not necessarily infectious.

Depending on the vaccine, and the sample to be tested, RT-PCR can also be used as a DIVA test ('genetic' DIVA). If the vaccine does not contain any CSFV genomic fragments (i.e. E2-subunit vaccines, chimerical constructs using non pestivirus vectors) or if the vaccine has deletions or substitutions on the primer sites (i.e. deletion mutants or chimaeric vaccines), an RT-PCR positive result would be proof for an infection with field virus (Koenig et al., 2007).

5.1.3 Immunohistochemistry

The immunofluorescence test (IFT) or fluorescent antibody test (FAT) is based on the detection of viral proteins with FITC-conjugated antibodies (Robertson et al., 1965). The immunoperoxidase test (IPT) is based on the detection of viral proteins with HRPO-conjugated antibodies. The most commonly used test is the IFT/FAT, while the IPT is or was used by some labs for confirmation of IFT/FAT-positive results (Wensvoort et al., 1986; De Smit et al., 1999, 2000).

The test can only be carried out post-mortem and the organs of preference are the tonsil, spleen, kidney, ileum, and several lymph nodes. From these organs, cryosections are cut for staining. A smear of bone marrow cells might also be used, for instance in case of feral pigs, if organs are not available or are subject to autolysis.

The test is relatively easy to perform, but requires an experience staff because interpretation of staining is not fully objective. Furthermore a cryostat is needed to cut the cryosections. The test can be performed within a few hours, but, as for PCR, for high-throughput testing 24-48 hours between receiving samples and sending out results is more realistic.

The IFT/FAT is often considered as less sensitive than VI, but an evaluation of the FAT and VI during the 1997/98 CSF outbreak in the Netherlands showed that in the field the sensitivity of both tests on tonsils is almost equal (Bouma et al., 2001).

The specificity of the test depends on the antiserum used. If polyclonal sera are used, positive samples need to be confirmed in a second test, especially to differentiate between CSFV and other pestiviruses. With monoclonal antibodies, the test is, however, highly specific.

Due to the introduction and implementation of the RT-PCR in many diagnostic labs, this test is not very commonly used anymore.

5.1.3. Antigen ELISA

The antigen ELISA is based on the detection of viral proteins, binding to antibodies in an ELISA plate (Shannon et al., 1993; Depner et al., 1995). The test is easy to perform and is relatively cheap and fast. A low sensitivity and specificity compared to most of the other diagnostic tests, especially RT-PCR, means that the added value of the test is rather limited (Dewulf et al., 2004; Depner et al., 2006; Depner et al., 2007). With the availability of the other tests, the use of the antigen ELISA is being discouraged more and more lately.

5.1.3. Sequence analysis

Genetic typing of classical swine fever virus isolates is achieved by determining the nucleotide sequence of portions of the virus genome, especially parts of the 5'noncoding region and/or of the E2 glycoprotein gene (Paton et al., 2000a). This can hardly be considered a routine diagnostic test, but is very valuable for molecular epidemiology (Greiser-Wilke et al., 2006b). The similarity of the sequence from an isolated virus with those already obtained from previous virus isolates can indicate whether or not outbreaks of disease are caused by new or already recognised strains. This can support or refute hypotheses on transmission routes that have been provided by epidemiological tracing. Genetic typing of classical swine fever virus isolates is of major importance to determine the source of disease. However, a close relationship between viruses obtained from different outbreaks is not an absolute proof for a direct epidemiological link.

5.2 ANTIBODY DETECTION

In classical swine fever virus infected pigs, antibodies are usually detectable in serum samples from one to three weeks after infection. In pigs that have recovered from the disease, protective neutralising antibodies can be detected for several years or even for their lifetime. Antibodies are also sporadically detectable in the terminal stage of lethally diseased animals. In some pigs with chronic form of classical swine fever, antibodies might be detectable for a few days at the end of the first month post-infection. (Liess et al., 1976). Pigs infected in uterus may be immunotolerant against the homologue classical swine fever virus and produce no specific antibodies (Terpstra, 1987). However, maternal antibodies can be detected during the first weeks of life. The half-life of maternal antibodies against several viruses in non-viraemic healthy piglets can vary from approximately 8 days, found for CSF (Vandeputte et al.,

2001), 12 days for swine influenza (Loeffen et al., 2003) to 3 weeks for porcine parvo and foot-and-mouth disease (Francis and Black, 1984; Fenati et al., 2008). In wild boar, maternal antibodies against CSFV were detectable up to 22 weeks of age, suggesting a quite high half life value. Half life values of maternal antibodies seem to be determined mainly by the increase in blood volume anyway (Francis and Black, 1984). Because domestic pigs grow much faster than wild boar, this would explain why maternal antibodies in wild boar can be detected much longer than in domestic pigs.

5.2.1. E2-ELISA

Several ELISA techniques using specific monoclonal antibodies have been developed, mainly competitive or blocking ELISA and non-competitive ELISA's (Moser et al., 1996; Colijn et al., 1997; Clavijo et al., 2001).

The competitive or blocking ELISA is usually based on monoclonal antibodies. If the serum sample contains antibodies to classical swine fever virus, the binding of a selected peroxidase-conjugated monoclonal antibody to virus antigen will be inhibited resulting in a reduced signal.

In general only serum samples will be used in ELISA's, although meat juice is also being used for several other infections, including Salmonella and Aujeszky's disease (Nielsen et al., 1998; De Lange et al., 2003). ELISA's are relatively easy to perform, with minimum demands of facilities and personnel. ELISA's can be fully robotized and automated for high throughput and most can be performed within several hours. However, for high-throughput testing 24-48 hours between receiving samples and sending out results is more realistic.

The sensitivity of the E2-ELISA is in general comparable to that of the virus neutralization test (VNT), although the latter is more sensitive in samples obtained within 3 weeks after infection. If no antibodies can be detected in infected pigs, it is usually because they are chronically infected, with a persistent viraemie. The specificity is usually also high, in the range of 98 to >99.5%. Part of the specificity problems may be caused by infections with other pestiviruses.

Detection of antibodies doesn't necessarily mean that the animal is infectious. On the contrary, in most cases where antibodies are present, infectious virus will no longer be detectable.

The E2-ELISA can be used as a DIVA test for vaccines that do not contain the E2 of CSFV. Such vaccines can either have the E2 replaced by that of another pestivirus (Van Gennip et al., 2000; De Smit et al., 2001) or have it deleted (Van Gennip et al., 2002)

5.2.1. E^{rns}-ELISA

The Erns-ELISA is based on the same principle as the E2-ELISA's, but instead detects antibodies against the Erns -protein. The Erns -ELISA's were developed as companion tests for the E2-subunit vaccine (Van Rijn et al., 1999). Two commercially available Erns-ELISA's, by Bommeli and Cedi-Diagnostics, were evaluated in a large EU-trial in the late 1990's (Floegel-Niesmann, 2001). At that time the ELISA from Cedi-Diagnostics lacked sensitivity, while the one from Bommeli was deemed not to be specific enough. A new evaluation by the EU community reference lab in 2003,

together with 15 national reference labs from the EU, concluded that an improved version of the Bommeli test was suitable as a DIVA test in combination with the E2-subunit vaccine.

The sensitivity of the Erns-ELISA from Bommeli is in general somewhat lower than that of E2-ELISA's. Furthermore, it is not CSF-specific, but detects also antibodies against other pestiviruses. For a population where non-CSF pestivirus infections occur, the test is therefore less useful. While this test is developed in combination with the E2-subunit vaccine, it can be used as a DIVA test with any vaccine that does not contain Erns, including live deletion mutants (Widjojoatmodjo et al., 2000). For chimaeric vaccines, that contain Erns from a non-CSF pestivirus (Van Gennip et al., 2000; Reimann et al., 2004), the test can, however, not be used as a DIVA test. In these cases the Erns-test from Cedi-Diagnostic could be used, as it is CSF-specific, but this test lacks sensitivity (Floegel-Niesmann, 2001).

5.2.1. Virus neutralisation test (VNT)

The virus neutralisation test (VNT) is carried out by incubating serum samples in several two-fold dilutions with a known amount of virus together with a susceptible cell culture. In the absence of neutralizing antibodies, these cells will get infected and virus replication will take place to detetable amounts of virus. In the presence of neutralizing antibodies, the virus will be neutralized and no virus will grow. Detection of virus is usually done with an immunoperoxidase monolayer assay (IPMA).

The VNT is a laborious and time-consuming test. Furthermore, because virus is replicated, hygiene and containment procedures should be in place. Requirements for facilities, but also personnel are therefore much higher than for an ELISA.

The VNT is considered to be the gold standard of antibody detection. It is regarded as the most sensitive antibody test, but lacks specificity by itself. Cross-neutralizing antibodies against non-CSF pestiviruses will readily be detected as well. To solve this problem, the VNT for CSFV antibodies is usually carried out in parallel with a VNT for BVDV antibodies and sometimes also a VNT for BDV antibodies. The VNT for the detection of antibodies against BVDV and BDV follows the same principals mentioned above for CSFV. If the CSF-titre is equal to or higher than the BVDV/BDV-titre, the presence of CSF antibodies is confirmed. If the CSF-titre is one-third or less compared to the BVDV/BDV-titre, the presence of CSF antibodies is ruled out. This procedure results in a highly specific test, but this will be at the expense of the sensitivity. CSF infections in the presence of BVD antibodies will result in false-negative test results (Wieringa-Jelsma et al., 2006).

The procedure of choice in diagnostic of CSF is summarised in the following table. Usually , the diagnostic, either for routine surveillance, clinical suspicions or for lift-up control measures is based on a two steps procedure tat is described in the figure 1.

Table - procedures of choice for CSFV diagnosis

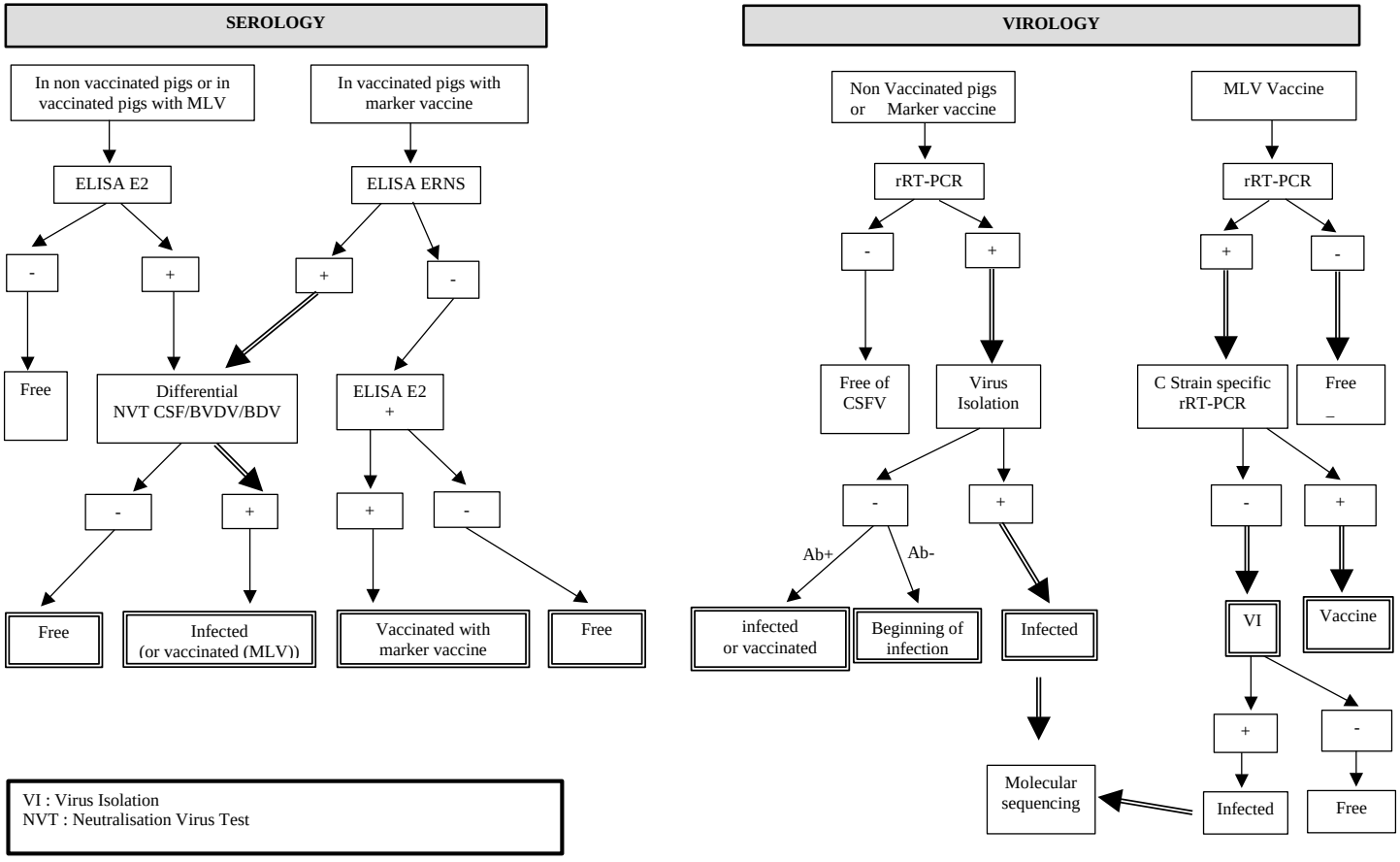
Test	Sample type	Sensitivity	Specificity	Feasibility	Post infection time detection	Disadvantages	Advantages	Reference
IFT/ IPT	Organ cryostat sections	Medium	High with Mabs	Medium - High	Post mortem (from 4-5 p.i.d.)	Equipment Experience	Short time	OIE Manual of Diagnostic Tests and Vaccine for Terrestrial Animals, Fifh Edition, 2004. EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June 2007). Bouma et al., J Vet Diagn Invest, 2001, 13, 383-388
CSF antigen ELISA	Serum Plasma Blood Homog enate	Low	Low	High	7-12 p.i.d	Specificity Sensitivity Not for individual diagnosis	Short time Automated systems Cost	Depner K. et al. Rev. sci.tech.Off.Int.Epiz., 14, 677-689, 1995 EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June 2007)
Virus isolation *	Leukoc ytes, plasma, whole blood, organs	Medium	High	Medium	5 p.i.d	Time consuming Cell culture facilities Cost Autolysed sample Up 10 days for results	Strain recovery Useful for genetic typing and molecular epidemiology Antigenic typing of isolates	OIE Manual of Diagnostic Tests and Vaccine for Terrestrial Animals, Fifth edition, 2004. EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June 2007). Bouma et al., J Vet Diagn Invest, 2001, 13, 383-388
RT-PCR	Blood, organ, serum	High	High	High	3-5 p.i.d	Detection of uninfectious virus The need of skilled staff Contaminations	Results after few hours Useful for genetic typing and molecular	Paton D.J. et al. Veterinary Microbiology, 77,71-81, 2000. Aguero et al. Vet. Res., 35, 551-563, 2004. Belak S. Acta Vet. Hung., 53, 113-124, 2005. EU Diagnostic Manual for Classical Swine Fever

Test	Sample type	Sensitivity	Specificity	Feasibility	Post infection time detection	Disadvantages	Advantages	Reference
						Stringent quality control procedure are essential	epidemiology Suitable for carcasses from wild boar	(CSF) Diagnosis: technical part (Third Draft June 2007).
Real Time – PCR	Blood, organ, serum	Very high:	High	High	2 p.i.d	Detection of uninfectious virus The need of skilled staff Stringent quality control procedure are essential Expensive automated equipment	Results after few hours Quantitative results Automated equipment Differential detection of wild-type from vaccine viruses Suitable for wild boar carcasses	Depner K. et al. Veterinary Microbiology, 121,338-343,2007 Zhao J.J. et al. Veterinary Microbiology, 126, 1-10, 2008. EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June 2007).
Antibody ELISA	Serum	High	Medium - High	High	12-21 p.i.d	Screening test Qualitative results Cross reactivity resulting in false positive or questionable results	Fast Automated systems DIVA	Colijn E.O. et al. Veterinary Microbiology, 59, 15-25, 1997. Langedijk J.P. et al. J. Clin. Microbiol., 39, 906-912, 2001. EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June 2007).
VNT *	Serum	High	Low/High	Medium	12.14 p.i.d	Cross-neutralising antibodies Time consuming	Quantitative Differential diagnosis	Liess B. et al. EUR 5486, 187-197, 1974 EU Diagnostic Manual for Classical Swine Fever (CSF) Diagnosis: technical part (Third Draft June

Test	Sample type	Sensitivity	Specificity	Feasibility	Post infection time detection	Disadvantages	Advantages	Reference
								2007).

* Gold standard

Figure 1 – Two steps diagnostic procedures for lift-up surveillance



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Summary as provided by the authors:

- In general highly sensitive and specific diagnostic assays are available to diagnose CSF.
- Agent detection tests are mainly suitable to detect infected animals during early stages of infection.
- Infectious virus can be detected for a longer time in chronically infected and immunotolerant pigs.
- By testing tissue samples (especially tonsils) viral genome may be detected for 9 weeks or sometimes longer in previously infected but recovered animals. These kinds of animals/samples are often not infectious to other animals.
- Antibody detection tests are mainly suitable for monitoring and surveillance purposes, but not for diagnosis of suspicions. When non-Csf antibodies are present in a pig population, the VNT may give false-negative results. An another test to discriminate between CSF and non-CSF pestivirus antibodies and to correctly diagnose double infections would be welcome.
- The main problem with respect to antibody detection is however DIVA diagnostic tests, especially those that rely on the detection of Erns antibodies. Currently available tests lack either sensitivity or specificity, although the latter will not be a problem in populations with a low prevalence of non-CSF pestivirus antibodies. Even so, serological confirmation tests do not exist and will be needed in case of large-scale use of these tests. DIVA vaccines that rely on E2 for differentiation, and for which the already available E2-ELISA's can be used as DIVA tests therefore offer a better perspective for the future.
- Virus isolation and PCR detection should be used for different purposes.

- Recommendations

  Additional research is needed to determine the exact nature of positive RT-PCR test results.

  Additional research is needed to permit the confirmation of serological DIVA tests

6 Prevention and control

6.1 BIOSECURITY

Biosecurity measures are not only important for the protection of the animals at local level, i.e. the herd, but also on a nationwide level in order to prevent the introduction of exotic disease. Although the term is used vaguely and the exact impact of the individual measures is rarely quantified, their importance is ever increasing due to the industrialisation of the pork husbandry resulting in increased pig densities. Nevertheless, the importance of such biosecurity measures was clearly demonstrate in a study preformed by Elbers (2001), following the 1997/98 epidemic in The Netherlands, were factors, such as the availability of clothing, presence of other animals and disinfection of transport vehicles, were linked with increased risk of infection. Furthermore, a recent survey in the EU demonstrate the high prioritisation on preventive measures as it was deemed more important to prevent an outbreak than to eradicate it (Cohen et al., 2007).

Biosecurity is a multi-domain aspect that needs to be addressed at different levels. The geographical localisation of a farm (including factors as: distances to the nearest pig farm (de Gante et al., 2003), the vicinity of major traffic lanes…) need to be considered (Pritchard et al., 2005; Moore et al., 1992) as they can have important implications. The later was demonstrated during the outbreak in Belgian in 1993-94 and in The Netherlands 1997-98 were respectively 50% (Roberts, 1995; Koenen et al., 1996) and 54% (Stegeman et al., 2000) of infected herds were situated within a range of 1 km of the primary outbreak. Biosecurity measures on a working farm need to be implemented on several levels. First of all, direct access to the pig pens and preferentially the farm as a whole needs to be limited and restricted. This can be

achieved by adequate fencing and a gate blocking the entrance to the farm. Furthermore, locking mechanisms situated on the access points of the pens should prevent unseen or impromptu visitors (Moore et al., 1992). In order to facilitate traceability, registration of each visitor is recommended.

Although unavoidable, human contact with the animals poses a potential risk. Although not a host himself, human visitors / personal can act as a mechanical vector. Clothing, for example, can become contaminated during hunting or visiting/working other farms and present therefore a physical means to transmit the virus. Although Laevens et al. (1998) did not find an increased risk of transmission, compared to airborne transmission alone, from adjacent infected pens by additional contacts with contaminated clothing/footwear, a recent study, employing a worst-case scenario, transmission to susceptible pigs after contact with people who did not change clothing after visiting infected animals (Ribbens et al., 2004b).The potential risk is further substantiated by the linking of not changing clothing with an increased risk of becoming infected (Elbers et al. 2001) during the epidemic of 1997-98. Therefore, separate clothing and a changing room should be available for each person who enters the animal pens/farm. Used clothing should be properly cleaned in order to assure destruction of the virus. The later is not evident for small scale or "hobby farms" but disposable overalls can provide an alternative. Disinfectant baths at the entrance of the pens for boots / shoes further enhances biosecurity. However, the uses of disinfectant baths are only effective if the baths are regularly cleaned (and the disinfectant renewed as organic matter reduces effectiveness) and the contact time with the boots is long enough. Because improper used of disinfectant bath can render it a source of infection rather than preventing it (Pritchard et al., 2005). Similar comments apply to material used in the animal pens. Material should be restricted to one pen if the farm contains multi-sites. Veterinary equipment should be specific to the farm and should not be re-used on other farms.

Similar to human – pig contact, animal – pig contact is similarly a risk factor. A special annotation needs to be made for farms with free ranging pigs as contact with other animals is more likely. In these situations, physical barriers separating the farm from its surroundings become even more critical. Although it has been demonstrated that CSFV has a limited host range and that other animals as cats, dogs , rats and birds (Dewulf et al., 2001; Kaden et al., 2003b) are no biological hosts, the transmission by mechanical means (for example, contaminated blood or faeces on legs, muzzle..) remains possible. Transmission by mosquitoes (Stewart et al., 1975) and horseflies (Tidwell et al., 1972) has been reported but this data is relative old and no epidemiological data is available concerning the role of arthropods. Nevertheless, adequate insect and rodent control programs or counter measures need to be implemented and the presence of other animals on the premises should be limited. Replacement animals can also present a risk for CSFV introduction, especially if the health status of the animals is unsure. The possibility to place "new" animals under quarantine, isolated from the rest of the pigs is therefore recommended. Similar isolation can be warranted for sows which are artificially inseminated as CSFV has been detected in semen (Floegel et al., 2000) between 5 to 11 dpi (de Smit et al., 1999). Infected semen has also been shown to transmit the disease to artificially inseminated sows (de Smit et al., 1999). The importance is further demonstrated in the outbreak 1997-98 in The Netherlands were a number of farms became infected after using contaminated semen (Elbers et al., 1996). A register (or log book) should in both latter cases also be implemented (Moore et al., 1992; Pritchard et al., 2005).

A number of factors pose an indirect risk. A clear example of that are (transport) vehicles on the farm. The importance of this factor has demonstrated during the outbreak 1997-98 in The Netherlands were live stock trucks were one of the most important routes of transmission (Stegeman et al., 2002). Similar to the entrance of

the animals, disinfectant baths/installations can provide a solution. Another risk issue is the transmission of CSFV by air or aerosols. It has been demonstrated that CSFV survives aerosolisation and remains infectious at least for 30 minutes in that state (Weesendorp et al., 2008). Furthermore, a positive link between aerosols, produced during cleaning of electrocution equipment used during depopulation of a neighbouring farm, and infection has been found (Elbers et al., 2001). Therefore, aerosol production during cleaning/disinfecting should be minimised. Although the transmission by air under natural circumstances is not yet clearly proven (Crauwels et al., 2003), CSFV has been detected in the air origination from infected pigs (Weesendorp et al., 20008). Furthermore, short distances transmission and under experimental conditions (mechanical air flow) (Koenen et al., 1996; Dewulf et al., 2000, Laevens et al., 1998, Gonzalez et al., 2001) has been shown. The epidemiological significance, however, is unclear as conflicting data can be found. Data based upon the 1993-94 outbreaks in Belgian a wind effect was found (Laevens, 1999; Mintiens et al., 2000.) but this was not the in case during the Dutch outbreak in 1997-98 outbreak. The latter should be addressed during the design of the animal pens (Moore et al., 1992).

In several studies the implementation of different biosecurity related measures has been studied (Pinto et al., 2003; Casal et al., 2007; Ribbens et al., 2008; Boklund et al. 2004). The type of biosecurity measure implemented varied form country to country. Remarkably certain measures that are imposed by law (2001/89/EC), as for example the ban on swill feeding was not 100% followed (Ribbens et al., 2008) notwithstanding the proven risks for introduction of the infection by improperly inactivated meat (Gale 2004; Kim 2008) and its implication CSFV epidemics (Williams et al., 1988). No recent data is available concerning the risk posed by parasites residing in meat products of animals which have been infected. However, a study from 1966 by Zimmermann et al. (1966) showed the transmission of the

infection using Trichinella Spiralis larvae collected from pigs which had been infected with CSFV.

In general, it can be stated that out-side contact (meaning all material and persons out side the holding pens) with the animals need to be limited and restricted, as much as possible. Measures need to be implemented on all levels to be effective and should be supported by management (follow up, registration, disinfecting protocols…). Although large scale production farms are starting to implement different biosecurity features, the mayor problem lies with small scale or hobby farms as the financial investments needed is not always possible. Financial support by governmental instances could provide a solution. In addition, a better picture on the impact of individual measures could help to prioritise the implementation of different biosecurity measures.

Summary as provided by the authors:

Important Biosecurity related measures

Restriction Important Biosecurity related measures

Restriction of the access to the farm / pens (fencing, gates, warning signs)

Hygienic measures

- Separate clothing/footwear
- Disinfectant baths before entrance (personnel / vehicles)
- Separate materials

Building design

- Clothing / washing rooms
- Geographical location (local spreading)
- Quarantine / isolation facilities

Management of the access to the farm / pens (fencing, gates, warning signs)

- Registration (Ingoing / outgoing)
  - Personal / visitor
  - Material (food, equipment)
  - New animals (including semen for insemination)
- Availability of Biosecurity protocols, such as
  - Disinfecting (duration, when, renewing baths)
  - Entering (personnel, visitors)

Hygienic measures

- Separate clothing/footwear
  - Desinfectant baths before entrance (personnel / vehicles)
  - Separate materials

Building design

- Clothing / washing rooms

Geographical location (local spreading)

Quarantine / isolation facilities

Management

Registration (Ingoing / outgoing)

Personal / visitor

Material (food, equipment)

New animals (including semen for insemination)

Availability of Biosecurity protocols, such as

Disinfecting (duration, when, renewing baths)

Entering (personnel, visitors)

Future research identified by the authors:

- The epidemiological impact of air transmission needs to be clarified.
- Evaluation of the role of arthropods in transmission needs to be re-evaluated.
- Impact of individual Biosecurity measures on transmission.

6.2 VACCINES

Accompanying this section is a reference table detailing vaccine and vaccination associated parameters, with or without subsequent challenge, such as viraemia (first detection, duration), antibody response, horizontal and vertical transmission, clinical symptoms following vaccination and/or challenge.

6.2.1 Types of currently available commercial vaccines

There are only two relevant types of CSFV-vaccines on the market: live attenuated (modified live vaccines = MLV) and E2 subunit vaccines.

6.2.1.1 Live attenuated/modified Vaccine (MLV)

The first group of available vaccines are the live CSFVs that have been attenuated by multiple passages in cell culture or in rabbit (lapinised) (Aynaud, 1988). In addition to the most widely used "Chinese (C)-strain", a number of other MLVs are produced, namely ATCC 131-VR (Argentina), TVM-1 (Czech Republic), GPE (Japan, Singapore), RP 93 (Romania), Thiverval (France; sequence: Fan et al., 2008) and PAV (Mexico) (Roth and Spickler, 2003). Notwithstanding its world wide application there is some confusion about the origin of the C-strain and there are several strains with different histories. Most, if not all, C-strains have been attenuated by hundreds of serial passages in rabbits. However, Lorena et al (2001) found differences in Erns and NS3 antigenic distribution in blood and brain tissue between C-strain propagated in rabbit and on cell culture, indicating different replication behaviour and neurotrophic affinity.

Following oral immunization with C-strain using a vaccine dose of 10^6 to 10^7 PD50, the virus could be isolated from tonsil at 9 dpv for wild boars and up to 8 dpv in domestic pigs. For the latter the virus could also be isolated from spleen and the mandibularis lymph node during that period (Kaden et al., 2004). Using real-time RT-PCR, however, C-strain RNA could be detected in tonsil samples at least for 42 days post vaccination (Koenig et al., 2007), but no infectious virus could be isolated on those time points. In blood, RNA was detected by nested RT-PCR at least until 16 dpv (Lorena et al., 2001). However, further analyses have to be performed using additional PCRs differentiating C-strain from wild type CSFV as well as new

techniques for the demonstration of the presence of full-length CSFV genomes (Haegeman et al., 2006).

6.2.1.2 E2 subunit marker vaccines

In order to address the problem of the MLVs in regards to the differentiation between infected and vaccination animals (DIVA-principle), a new generation of vaccines was developed. During this development, it became clear that the E2-glycoprotein of CSFV in a purified form was capable of inducing a protective immunity (Rümenapf et al., 1991; Van Zijl *et al*., 1991; König et al., 1995; Van Rijn et al., 1996; Peeters et al., 1997) and this subsequently formed the basis for the E2 subunit vaccine. This vaccine contains the E2 glycoprotein of CSFV as an antigen. The E2 glycoprotein is produced in cultures of insect cells infected with the baculovirus vector (Hulst et al., 1993). Two commercial E2-sub unit vaccines have been licensed, via an European procedure, namely from Intervet BV and Bayer (patent; Kretzdorn, 2005). However, only the one of Intervet is currently available.

6.2.2 Current vaccine field application

Live attenuated/modified vaccines have the advantage that they can be administrated by parenteral or oral means, allowing it not only to be used on farms with domestic pigs but also for vaccination programs for wild boars using baits (Kaden et al, 2000, 2003). The possibility to lyophilize C-strain before putting in the baits and thereby providing additional stability to the vaccine (Faust et al, 2007), further supports vaccination strategies in the wild. However, the bait uptake by younger animals is problematic. Although new smaller baits have been developed, they are still not picked up by animals younger then 3 months (FP6 “CSFVACCINE &WILD BOAR” annual report). The latter indicates that vaccination with baits for that age class is

probably not possible. In order to better follow and study oral vaccination using baits, iophenoxic acid (IPA) has been successfully used as biomarker (Cowled et al., 2008). In contrast to the MLVs, both E2-subunit vaccines have to be administered parenterally while oral immunization is not possible, limiting its use for field vaccinations.

6.2.3 Vaccine differentiation potential

Due to fact that MLVs are in essence wild type viruses which have undergone natural adaptations to the method of passaging, i.e. no introduction of "foreign" or deletions of "own" elements by genetic engineering, they cannot be distinguished, with the current available validated diagnostics, from the wild type virus, such as virus isolation or antigen/ antibody detections systems. The latter inhibits the general and even prophylactic use of MLVs in control en management strategies. However, there are differences on the genomic level that can be exploited by methods such as PCR-technologies which rely on the detection of specific nucleotide sequences. In this regard, several PCR-based techniques have been developed which are able to differentiate between C-strain and wild type viruses from China (genotypes: 1.1, 2.1, 2.2, and 2.3) (Li et al., 2007c; Zhao et al., 2008) or differentiate between the LOM vaccine and wild types from Korea (Cho et al., 2006). Although these are promising assays, additional evaluation and validation in regards to sensitivity and specificity (testing of additional genotypes) is necessary if they are to be used on a global scale.

Pigs vaccinated with a E2 sub-unit marker vaccine, however, only develop antibodies against the E2 glycoprotein, whereas pigs that are naturally infected develop antibodies against different viral proteins (e.g. E2, ERNS, NS3) (Weiland et al., 1992, van Rijn et al., 1994). 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 Erns glycoprotein (Moormann et al., 2000). Furthermore, as the vaccine is an E2 sub unit protein, systems based upon detecting nucleotide sequences, as PCR and its derivatives, can be easily used for discriminatory purposes.

6.2.4 Performance (efficacy)

For any efficient eradication program as well as for emergency use it is not sufficient for a vaccine to protect the vaccinated pigs from possible signs of disease but should also prevent viremia and any form of transmission (horizontal and vertical) as they could perpetuated the infection as carriers (e.g. in lymphatic organs).

6.2.4.1 Live attenuated/modified live (MLV)

The C-strain has been found to be highly efficacious inducing a virtually complete protection against the challenge infections. This protection starts very quick after vaccination (orally and intramuscularly) and is complete at least 4-7 days dpv as challenged pigs did not show any clinical signs, replication of challenge virus, measured by shedding in oral swabs or by detection of viremia, and did not transmit the challenge virus to susceptible contact animals (Terpstra et al., 1990; Dewulf et al., 2004, Kaden and Lange., 2001). Partial protection can even be observed as soon as 0-2 dpv (Kaden et al., 2001; Kaden et al, 2001b) and horizontal transmission was prevented when the challenge was performed at the some moment as the vaccination (0 dpv) (Dewulf et al., 2004). Furthermore, the duration of this protection lasts more than a year, probably even lifelong (Terpstra *et al*., 1990; Kaden and Lange 2001; Müller et al. 2005). As with many modified live vaccines, maternal antibodies have a deleterious effect on the induction of vaccinal immunity: the higher the maternal antibody titre (neutralizing titre: >65) at vaccination the stronger the effect (Suradhat et al., 2003). This is not surprising as a neutralizing antibody titre of ≥1/64 is viewed

as protective against a CSFV infection (Terpstra and Wensvoort, 1988). However, the latter is not always the case as demonstrated by Kaden et al., (2006). Although the impact of maternal derived antibodies (MDAs) has important implications in any vaccination strategy as it can reduce the clinical signs (making it thereby more difficult to detect) while at the same time viremia remains (Depner et al., 2000). However, the MDAs usually diminish very quickly within 3 months of birth (Kaden and Lange, 2004; Soos et al, 2001) and wild boar piglets, before that age do not pig up the vaccine baits (Brauer et al., 2006). However, persistent low levels of MDAs have been reported (Depner et al., 1995, Müller et al., 2005). A less well studied factor in vaccine efficacy is the health status of the animal and the relationship with the vaccine induced immunity, certainly in the presence of a pre-dating infection with immune modulating properties. The effect varies from a well less established immunity, as seen by a reduced antibody response and increased fever and leucopoenia (Holland et al., 2003, Li et al., 2003) to a complete failure of the vaccine in PRRS-infected pigs as they were not protected against the challenge and the viremia was not reduced (Suradhat et al., 2006).

Efficacious CSFV vaccines must also prevent congenital infections with field virus, since these may result in a variety of abnormalities in the foetuses or even result in persistently infected immuno-tolerant healthy piglets that survive for months and continuously shed virus (van Oirschot and Terpstra, 1977). It could be shown that pigs orally immunized with C-strain were completely protected from transplacental infection. In addition, vaccine virus was not detected in any of the piglets from immunized sows (Kaden et al., 2008).

6.2.4.2 E2 subunit marker vaccines

In contrast to the data of MLV, the situation regarding the efficacy of the E2 subunit vaccine is less homogenous. Protection against the clinical course of disease starts from 10 dpv onwards with a complete protection from 14 dpv (Bouma et al., 2000, Dortmans et al. 2008, Uttenthal 2001). The latter was confirmed during a comparison trail between C-strain and the intervet E2 sub unit vaccine. Using identical experimental conditions, it was noted that C-strain protected the animals against the clinical signs starting from 7 dpv. In comparison, the protection against clinical course and horizontal transmissoin was delayed until 14 dpv for the E2 sub unit vaccine (Intervet) (Dewulf et al., 2004). In 2 comparative trails with the 2 commercial E2-subunit vaccines (Bayovac and Intervet) no significant difference was noted in the protective capacity against the clinical course of the disease (Utthenthal et al., 2001; Depner et al., 2001). Notwithstanding the protection granted by both commercial E2 subunit vaccines against the course of the disease, they failed to protect the animals against the infection itself. This is substantiated by Dewulf et al. (2001) where it is shown that virus infection by contact was delayed, but not prevented in twice vaccinated pigs using the intervet vaccine. Following vaccination, viremia is significantly shortened if the infection takes place starting form 10 dpv but the chance that an animal becomes viremic decreases only starting from 14 dpv (Bouma et al., 2000; Dewulf et al., 2004; Uttenthal et al., 2001; Dortmans et al., 2008). Furthermore, virus could be isolated form leucocytes even when challenge was performed at 21 dpv following a single intramuscular vaccination and horizontal transmission was not completely blocked at this time point as suggested by the Erns seroconversion in one of the contact animals. Although both vaccines reduced the probability of transmission significantly, a small preference was found for the Bayer vaccine (Uttenthal et al., 2001). In contrast, no horizontal transmission was found by Dewulf et al (2004), using the Intervet vaccine, at 14 dpv and by Bouma et al (2000), using SubV-E2-Brescia, at 10 dpi. A comparison of two E2-sub unit vaccines whereby the E2 originated either form Brescia or Alfort, resulted in a complete blocking of

horizontal transmission, at 10 dpv for the vaccine containing the E2 from Brescia but was delayed until 14dpv for the vaccine containing the E2 from Alfort. Even then the blocking was only complete when the vaccine was applied intramuscularly but not when it was given intradermally (Dortmans et al., 2008). A single vaccination of a E2 subunit vaccine (32 µg) in a water-oil-water emulsion protected against a 28 dpv CSFV challenged and blocked transmission in all cases except in 1/3 groups which received a vaccine with a shelf life of 18 months (Bouma et al., 1999). The duration of the protection granted by this vaccine following a single vaccination can go up to 13 months. However, a limited transmission was found at this time point (de Smit et al., 2001)

In the second comparative trial, a large scale transplacental transmission experiment (Depner et al. 2001), single vaccination of pregnant sows did not block the virus transmission to the piglets following a challenge at 14 post-vaccination. Although double vaccination did not succeed either in blocking 100% the vertical transmission, as viremic piglets were obtained for both vaccines, a significant reduction was found in the transmission probability for both vaccines. However, the protection (against clinical course and against foetal infections) granted by the Bayovac seemed to be more potent than the one given by the Intervet vaccine which was reflected by a more pronounced antibody response against the Bayovac vaccine (Depner et al., 2001). The inability to block completely the vertical transmission was also reported by Dewulf in 2002 using the Intervet vaccine. Notwithstanding that double vaccination (with a 4 week interval) protected the animals from the clinical course of the contact infection (at least 46 dpv), vertical transmission was not prevented as 37.5% of the litters had viraemic piglets. In addition, virus genome could be detected in the faeces (40%) and nasal swabs (60%) between 9-12 dpi and 15-18 dpi respectively. A limited vertical transmission (1/10 litters) was also reported by Ahrens et al. (2000) following a double vaccination with the Intervet vaccine and challenge at 126 dpv. However, in

a similar study but with a water-oil-water emulsion (double oil in water (DOE)) of an E2-sub unit vaccine whereby the E2 originated form Brescia (= SubV-E2-Brescia; Hulst et al.1993), prevented vertical transmission after double vaccination but not after single one (11% positive) (de Smit et al., 2000).

Similar to vaccination with MLV, an effect, although less pronounced, of MDAs was observed when vaccinating 2 weeks old piglets. The antibodies titres induced by the vaccine were lower at 3 and 6 months post vaccination in piglets with MDA potentially reducing the efficacy of the vaccine at those time points (Klinkenberg et al., 2002).

6.2.5 Safety

In general, safety issues are more often discussed for MLVs while subunit vaccines are normally accepted as innocuous. However, not only for E2subV but also for most of the CSFV MLV only very few cases with side effects were reported

6.2.5.1 Live attenuated/modified live (MLV)

In no case should the vaccine virus itself cause any damage in the host or in other species which come into contact with it, as for example during field vaccination with baits. 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 was also reported that C-strain vaccine virus 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). The C-strain, Thiverval strain, GPE (-), CR20, C4 and Cl strain appeared to be safe, even in immuno-

suppressed pigs (Bironi and Leunen, 1988; Suradhat et al., 2006). Upon administration to pigs, orally or intramuscularly, neither significant clinical signs nor CSFV-associated signs or pathology have been reported. Similarly no adverse effects on the pregnancy have been reported (Soos et al., 2001). Finally, the absence of leucopoenia 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 (Bironi and Leunen, 1988).

Genetic stability of the attenuated virus vaccine is also 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. Although Terpstra and Tielen (1976) noticed that under normal field conditions C virus spreading was possible, this is not collaborated with recent data. Also, using pigs 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 (Bironi and Leunen, 1988). In addition, C-strain is the most used CSF vaccine and up to now no reports have been submitted showing C-strain spreading to contact animals. Furthermore, no evidence for nasal secretion or excretion by faeces was found in domestic animals (Kaden et al., 2004b).

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. However, no increase of virulence was reported up to now, but it has to be emphasised that in most cases the regaining of virulence was tested in piglets only and not in pregnant sows. C strain appears not to persist in pigs for more than 2-3 weeks (Terpstra, 1978; Lorena *et al*., 2001, Kaden et al. 2004b), however,

recent real-time PCR data demonstrated the detection of C-strain RNA for at least 42 days post intramuscular vaccination in the tonsils, but no infectious vaccine virus could be isolated (Koenig et al., 2007).

Oral administration of C-strain to sheep and hares revealed no pathogenicity while rabbits displayed a moderate hypothermia and growth retardation. None of the three species shedded the virus but the hares and rabbits did seroconvert (Chenut et al., 1999)

In addition, chromosomal aberrations have been reported to be associated with C-strain vaccination (Genghini *et al.*, 1988 and 2002), but the consequences of these aberrations are not clear.

6.5.2.2 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. Furthermore no effect on the course of the pregnancy or birth performance has been reported (Bouma et al., 1999; Lipowski etal., 2000; Depner et al., 2001, Ahrens et al. 2000)

6.2.6. Other types of vaccines and other future developments

6.2.6.1 Vaccines

In order to address the short-comings of the current available vaccines in regards to DIVA potential and protection efficacy, a number of approaches have been followed. This has resulted in new types of vaccines and the development of antiviral agents.

Two extensive reviews of the new vaccines and obtained initial results have been published in 2007 (Beer et al., 2007; Dong et al., 2007). Dependent on the author the new vaccines types can be grouped in different categories. Basically, these vaccines are modified viral genomes, DNA expression plasmids, sub-unit / peptide vaccines.

Firstly, the modified viral genomes are group of vaccines that use a backbone from an avirulent or attenuated virus which has been modified in order to facilitate DIVA potential. This is clearly demonstrated by the chimerical constructs named, Flc9 and Flc 11 where respectively parts of the E2 (5'end) or the complete Erns have been swapped with the complementary regions from a BVDV type II strain. Both constructs replicated well on porcine cells and no cell tropism was noted. Furthermore, both constructs protected pigs from a lethal challenge and induced an antibody response that was distinguishable from a wild type infection using existing ELISA systems (Van Gennip et al., 2001). Protection against the clinical course disease was noted with both constructs as well as a significant reduction in transmission probability. However, the blockage of transmission was only found to be complete with the Flc 11 (de Smit et al., 2001b). The vaccine strain pRiems has also been used as backbone in a chimerical construct (pRIEMS-ABC-Gif) whereby E2 has been exchanged with the BDV strain Gifhorn. Full protection was obtained following intramuscular vaccination but only partial following oral application. The lack of E2 antibodies before challenge, using commercial ELISAs, confirmed the DIVA potential of the construct (Wehrle et al., 2007). In a similar approach, the E2 region of a BVDV genome was swapped with the E2 of CSFV Alfort187, resulting in CP7_E2alf (Reimann et al., 2004) or with the E2 of Gifhorn (CP7_E2gif: Rasmussen et al., 2007). Interestingly, CP7_E2alf has a cell tropism that reflects its donor Alfort, namely porcine and not its BVDV backbone. A similar shift in cell tropism was found for the CP7-E2gif: from bovine to ovine. However, bovine and porcine cells remain receptive for this construct but to a lesser degree. This indicates the involvement of E2

in cell tropism. Both constructs were found to be complete avirulent upon intramuscular administration in pigs and no viremia or shedding was observed. Furthermore, complete sterile immunity (no viremia or shedding) is achieved as was seen after challenge with the strain Eystrup (Reimann et al., 2004; Rasmussen et al., 2007). The excellent protective capacity of CP7_E2alfwas not only noted when the chimera was administrated intramuscularly but also when given orally (Koenig et al., 2007b). In both studies, the DIVA potential of CP7_E2Alf was confirmed using commercial Erns-Elisa systems. Aside pesitivruses other viral genomes have been used for introduction of CSFV specific proteins such as swinepox virus (Hahn et al., 2001). Similarly, the insertion of E2 of strain Weybridge in a porcine adenovirus has been reported (rPAV-gp55). Not only were no adverse effect reported upon single subcutaneous administration of the vaccine, complete protection against a lethal challenge, using strain Weybridge and administrated subcutaneously, was observed (Hammond et al., 2000 and 2001). However, the protection was only partial (only 60% survived) following oral challenge at 42 dpv (Hammond et al., 2001) and no neutralizing antibodies were detected at the time of challenge in these animals. Using contact infection, at 43 dpv using animals infected with Weybridge, single subcutaneously vaccinated pigs (rPAV-gp55) were protected while oral vaccinated ones (similar dose) were not. However, double orally vaccinated (3 week interval) were protected although neutralizing were still not detected (Hammond et al., 2003). Not only E2 or Erns have been used for the development of chimerical constructs. A pseudorabies virus has been used as carrier for the CSFV E1 protein (Mulder et al., 1994). A complete protection and blocking of transmission was observed, following double intramuscular vaccination for the constructs containing the transmembrane region (TMR) of E1 while only partial protection was observed with those without the TMR (van Zijl et al., 1991). Complete production using CSFV E1 in a pseudorabies vector was also reported by Hooft van Iddekinge (1996) following a single intramuscular vaccination and challenge at 42 dpv A parapoxvirus in which the

E2 of CSFV was cloned was found to be able to protect pigs following single shot application and challenge at 21 dpv. Interestingly multi-application was more effective in protecting against the clinical course than single site. Neutralizing antibodies titres of > 1:100 were already observed starting form 7 dpv (Voigt et al., 2007). The pseudorabies virus has also been used as a viral vector for CSFV E2 protein (Peeters et al., 1997). Exchanging the pseudovirus envelope protein D for the CSFV E2 protein resulted in construct capable of eliciting an anti E2 response in all the animals at 42 dpv and protecting them against a lethal CSFv challenge. A different approach within this group of vaccines is the deletion of a structural protein which gives rise to non-transmissible viruses (replicons). This can be achieved either be creating bicistronic genomes (Stettler et al., 2002) or deleting parts of the viral genome. The vaccination with C-strain where the complete or partial Erns (Flc 22 and Flc 23) was deleted, resulted upon administration in a complete protection against a lethal CSFV challenge when the vaccine was given intradermally. Partial and no immunity was seen when the vaccine was administrated intramuscularly and intranasal respectively (Widjojoatmodjo et al., 2000; van Gennip et al., 2002). The importance of administration route of the vaccine was also demonstrated with another Erns replicon (VRP A187delErns: 227 amino acid deletion). Single intradermal application resulted in complete protection against a lethal Eystrup, combined with E2 antibody and cellular response. In contrast, oral immunization only resulted in partial protection with an impeded immune response (humeral and cellular) (Frey et al., 2006). Complete protection was also found using a construct based upon a deletion of the B/C domain of C-strain E2 (Flc4). Removing the A domain or the entire E2 protein, resulted in only a partial protection (van Gennip et al., 2002). In a study of Maurer et al (2005) 2 Alfort 187 E2-deletion mutants were evaluated (partial deletion: VRP A187-E2del68; complete deletion: VRP A187-E2del373). Oronasal vaccination resulted in immunity (Eystrup challenge) with both chimeras while intradermally

vaccination only resulted in full protection with the construction containing the partial deletion.

A second group of vaccines is formed by the DNA plasmids containing mammalian expression cassettes allowing expression of the inserted sequence which can be a complete or partial protein. Plasmids expressing E2 have shown to be able to provide protection against the clinical course of the disease after single or double vaccination (Andrew et al., 2000; Markowska-Daniel et al., 2001; Yu et al., 2001). Importantly Ganges et al; (2005) found complete protection against severe CSF challenge in the absence of CSFV specific antibodies. This clearly demonstrates the importance of cellular responses in vaccine induced immunity. Upon challenge the neutralizing CSF titers rose very quickly in 2 of the 3 vaccinated animals. . Co-expression with cytokines has been shown to augment the immune response of the host and thereby achieving a higher vaccine efficacy: namely interleukin (IL)-3 (Andrew et al., 2006), interleukin-18 and CD154 (Wienhold et al., 2005). For the last two, in combination with an E2 DNA vaccine, have induced an earlier antibody response and reduce B-cell deficiency upon challenge with Eystrup. In contrast, co-delivery of IL-12 resulted in a reduced antibody response and protected compared to the E2 DNA vaccine alone (Wienhold et al., 2005). In addition to a mammalian expression cassettes, DNA vaccines have also be developed using a viral replicon expression system. A Semliki Forest virus (SFV) replicon DNA vaccine (pSFV1CS-E2) expressing the E2 (Li et al 2007 and 2007b) has been shown to protect the animals against a lethal challenge with Shimen at 28 dpv following multiple prime boost vaccinations (3 times 600 µg; interval 3 weeks) (Li et al., 2007). This protection was also found using double vaccination using 100 µg and challenge at 42 dpv (Li et al., 2007b).

The last group of vaccines are a continuance of the existing E2 subunit vaccines. On the one hand this has led to new production systems of E2 proteins. Adenovirus

transduced mammary glands of goats, has led to the purification of E2 proteins (on the g/L level) from goats milk. The latter protected the animal, following a double vaccination (3 week interval) with 25 or 50 µg, from a lethal challenge 21 dpv (last vaccination). Neutralizing antibodies could be detected starting from the second week following vaccination (Toledo et al., 2008). Similar results were obtained with adenoviral transduced PK-15 cells and production concentration reached 120-150 µg/ml. E2 purified fro cell culture supernatants granted protection against challenge using 15 to 30 µg and intramuscular prime boost vaccination (3 week interval) (Sanchez et al., 2008). Similar to E2 sub unit vaccines, NS3 sub unit vaccine has been developed in view as it shown to stimulate an immune response by the host. However, the NS3 sub unit vaccine was not able to protect the animals against a lethal challenge notwithstanding a stimulatory effect on the cellular and humoral branch of the immune system (Voigt et al., 2007b). Similarly, when NS3 is co-delivered with E2 sub unit, no significant enhancement in immunity was found (Rau et al. 2006). Further development in this group of vaccines has led to the administration of peptides in stead of proteins. A multipeptide vaccine (5 peptides), covering the E2 amino acids (aa) 693 tot 777 (B/C domain) elicited a strong antibody response and conferred protection against a lethal challenge with Shimen following a double vaccination (interval 2 weeks) with 50ug (Dong et al., 2002 and 2005). Subsequent evaluation showed that the peptide covering aa 693-716 (PV-BC1) was the most potent and the one covering aa 712-722 (PV-BC2) had no protective effect (Dong et al., 2006). Further refinement of the 693-716 region, allowed the identification of highly neutralizing epitope, which covers the aa 693-699 (Dong et al., 2006c). Similarly, peptides covering the A domain were evaluated for the protective capacity. Two peptides were found to be have potent protective properties following double immunization protocol (20 µg/pig), covering respectively an amino acid region of 844-865 and 792-814 (Dong et al., 2006b). An additional B-cell epitope was identified by Liu et al (2006) spanning aa . Double vaccination, using 500 and 250

ug/animal, resulting in a strong humeral response and a partial resistance to a lethal challenge (40%) (Liu et al., 2006).

Type	Description	DIVA (serology)	Reference
A/ Modified viral genomes			
- Chimerical pestiviruses	CSFV backbone + exchange of E2 region (complete or parts) with BVDV or BDV	E2-CSFV specific ELISA	Van Gennip et al., 2001; de Smit et al., 2001b; Wehrle et al., 2007)
	CSFV backbone + exchange of Erns region (complete or parts) with BVDV	Erns-CSFV specific ELISA	
	BVDV backbone + exchange of E2 region with CSFV	Erns-CSFV specific ELISA	Reimann et al., 2004; Koenig et al., 2007b
- Other viral vectors	Expression of E2 (complete or partial), integrated into the genome of other viruses (viral vectors),	Erns-CSFV specific ELISA or NS3 pestivirus specific ELISA	Hahn et al., 2001; Hammond et al., 2000, 2001, 2003; Voigt et al., 2007 ; Peeters et al., 1997
	Expression of E1 (complete or partial), integrated into the genome of other viruses (viral vectors),	NS3 pestivirus specific ELISA	Van Zijl et al., 1991, Hooft van Iddekinge et al., 1996.
- Trans-complemented replicons	Packaged replicons with a deletion in the ERNS-encoding region	Erns-CSFV specific ELISA	Van Gennip et al., 2002; Widjojoatmodjo et al., 2000; Frey et al., 2006

	Packaged replicons with a deletion in the E2-encoding region	E2-CSFV specific ELISA	Van Gennip et al., 2002; Maurer et al., 1995
B/ DNA vaccines			
	Mammalian expression plasmids, containing CSFV E2 (complete or partial)	Erns- or NS3 CSFv or pesitivrus specific f ELISA	Andrew et al., 2000, 2006 ; Markowska-Daniel et al., 2001 ; Yu et al., 2001 ; Ganges et al., 2005, Wienhold et al., 2005
	Viral expression system containing CSFV E2	Erns- or NS3 CSFv or pesitivrus specific f ELISA	Li et al., 2007, 2007b
C/ Protein vaccines	Sub unit E2: alternative production methods	Erns- or NS3 CSFv or pesitivrus specific f ELISA	Toledo et al., 2008; Sanchez et al., 2008
	NS3 protein with or without E2	Erns CSFV Specific ELISA	Voigt et al., 2007b; Rau et al., 2006
	Single peptides or a mixture of peptides from antigenic domains BC or A of CSFV E2	Detection of ERNS- or NS3-antibodies specific for CSVF or pestiviruses, e.g. using blocking ELISAs Detection of CSFV-E2-domain-sepcific antibodies using immunogenic peptides, not present in the vaccine	Dong et al., 2002, 2005, 2006

6.2.6.2 Antivirals

Although vaccination has been the method of choice in the prevention of CSF, alternative/additional control methods are under development. Antivirals against CSFV, which consist of a wide variety of agents are recently described in vitro. Small molecules like the triazino[5,6-b]indole-derivate VP32947 showed to inhibit the pestiviral replication by specifically targeting the RNA-dependent polymerase (RdRp) of BVDV (Baginski et al., 2000). The mechanism of action was confirmed in CSFV and yet another class of molecules belonging to the imidazo[4,5-c]pyridines was recently developed with an analogue mechanism of action proving the viral polymerase to be an important potential target for antiviral agents against CSFV (Vrancken et al. 2008). The importance of the polymerase as a potent antiviral target was substantiated by the activity of the pyrrolo[2,3-c]pyridine analogue AG110, a structurally unrelated compound displaying a high antiviral activity against CSFV-replication (Paeshuyse et al., 2007). Other target proteins have been described for RNA viruses, but have not yet for CSFV (Leyssen et al., 2008). An alternative strategy for CSF prevention could be the repression of gene expression. This can be achieved by RNA interference as was demonstrated for FMDV (Chen et al., 2004).The complete inhibition of the expression of the RdRp was recently achieved by Xu et al. (2007) by the construction of small interfering RNAs corresponding to the highly conservative Motif V of the polymerase. A combined approach by targeting simultaneously regions within Npro and NS5B (coding for the RdRp) proved to inhibit viral growth (Xu et al., 2008) demonstrating their potential as a therapeutic tool.

Summary as provided by the authors

The advantages posed by the MLV vaccines

- Very quick and complete protection of the animals against clinical the course of the disease (4-7 dpv) and partial protection can even be observed when vaccination and challenge is simultaneous

- C-strain prevents viremia starting form 2 to 4 dpv. Viremia is significantly reduced if challenge is earlier.
- Horizontal transmission is blocked starting from 0 dpv (vaccination directly followed by challenge)
- Even though there are no published data, it can be concluded from long term experience that after intramuscular vaccination transplacental infection is blocked.
- Current MLV do not appear to cross the placenta and infect the foetus, which is supported by many years experience with prophylactic vaccination. These observations contrast with a few earlier uncertain data suggesting fetal infection.
- Limited detection of the vaccine by VI after vaccination (10 dpv). Although viral RNA has been detected in organs at least until 42 dpv, especially in the tonsils and up to 16 dpv in blood.
- Oral application, using baits is possible, allowing field application to control CSFV in wild boar populations. However vaccination in animals younger then 3 months using baits is not possible
- Very long protection is granted by C-strain (minimal 1 year, probably life long)
- Carcasses of animals vaccinated with a MLV and subsequently infected after more than 2 to 4 days post vaccination have a negligible risk to carry infectious CSFV
- No adverse effects have been reported upon single or multiple application for

  young and adult animals

  pregnant sows and their offspring: A degree of protection is granted to piglets from vaccinated sows.
- Innocuous for other animals
- Although massive use world wide, no evidence for reversal to a pathogenic state

The disadvantages posed by the MLV vaccines

- Currently no validated technique is available that allows the differentiation of vaccinated (C-strain) from infected (wild type) animals.

- MDAs reduces vaccine efficacy, resulting in incomplete protection in young animal.
- Oral vaccination using baits is problematic for animals younger then 3 months as they do not take up the baits. The two points last points need to be incorporated in any vaccinated strategy in the field.

The advantages posed by the E2 subunit vaccines

- No adverse effects have been reported upon application even on pregnant sows.
- Vaccine has a high shelf live of at least 13 months
- Although the available data is limited, MDAs seem to have a lesser impact on the vaccine induced immunity compared to on C-strain
- Quick protection against the clinical course of the disease (10 dpv) but not as fast C-strain
- Decreased viraemic period: starting from 10 dpv onwards. However doesn't prevent it as C-strain
- The chance of animal becoming viraemic decreases from 14 dpv onwards.
- The vaccine has DIVA-properties, using the presence/absence of Erns antibodies as discriminatory factor with the wild type CSFV and PCR can be readily used for detected in subunit vaccinated animals
- Very safe vaccine: It is inactive vaccine, meaning it does not infect cells or replicates in the host and enhances thereby the safety of the vaccine.
- Apart from local reactions no adverse reactions have been reported (same as with C-strain)

The disadvantages posed by the E2 subunit vaccines

- The vaccine needs to be administered parentally which makes it unsuited for field vaccination using baits.

- Reduced robustness in preventing horizontal and vertical transmission. Although significant reduction in transmission probability, starting from 10 dpv to 21 dpv, it efficacy is highly influenced by the genotype of the challenge strain in comparison to the origin of the E2 sub unit. Incomplete blocking has been reported in several cases even after double vaccination.
- Carcasses of animals vaccinated with a E2subV and subsequently infected with wild type CSFV have a reduced risk to carry infectious CSFV but these carcasses may be able to have infectious CSFV.
- Duration of the protection is not as long as C-strain (6 months) and re-vaccination should be performed at 6 months

Advantages to the new vaccines

- Enhanced DIVA potential
- Increased safety potential (replicons, peptide vaccines)

Advantages of antivirals

- Fast action (bridging the immunity gap)

Recommendations

- In domestic pigs testing of blood samples should not take place until at least 14 after vaccination (only for PCR)
- The characteristics of both MLV and E2subV have to be carefully taken into consideration for emergency vaccination plans
- MLV could be used as an additional tool for the control of CSFV outbreaks in wild boar
- PCR can be used as a diagnostic tool in vaccinated population
- A positive PCR diagnosis should be considered to indicate that an animal has been in contact with infectious virus

- The novel marker vaccine strategies should be further evaluated and the most promising strategies should be further promoted (e.g. concerning licensing).

Future research identified by the authors:

- The significance of the prolonged detection of the viral genome (C-strain) by real-time PCR needs to be future evaluated in regards to its infectivity
- The impact of MDAs and the health status on vaccination efficacy needs to be further addresses.
- The background of the current C-strain needs to be clarified.
- Impact of administration route on the efficacy of the new generation of chimerical vaccines
- Additional information for the chimerical vaccines regarding
  - transmission (horizontal and vertical)
  - efficacy against highly virulent strains
  - onset of immunity
  - duration of protection
  - Safety (pregnant sows, other species)
  - Protection after challenge wit heterogeneous strains
  - Efficacy after single application
- Antivirals

  Although in vitro inhibition has been demonstrated for different strategies, no published data of in vivo performance are available to date. Further in vivo evaluation has to be performed including safety and efficacy assessment. The applicability of antivirals in the field do not only rely on the in vivo performance, but drug residues in edible tissues and legal hurdles still have to be taken to allow an alternative approach to the control and prevention of CSF.

6.3 MEASURES IN RESPONSE TO AN OUTBREAK

In order to be able to respond quicker and more efficiently, each EU Member state has to submit a contingency plan to the EU Commission, explaining how they plan to deal with a CSFV outbreak. The approvals for the different plans are listed in the Commission Decision 1999/246/EC, 2000/113/EC and 2004/431/EC and the accompanying corrigenda. The notification system and measures to be taken in case of a suspension of infection or a confirmed infection are laid down in the Council Directive 2001/89/EC and Commission Decision 2006/911/EC. Briefly In case of outbreaks in the EU, the main policy is based on stamping out the infection, i.e. slaughtering of all pigs in the infected farms and the destruction of cadavers, and pre-emptive culling of potentially infected herds. A protection zone (3 km radius) and surveillance zone (10 km radius) are established around each outbreak, with restrictions on pig movements. The diagnostics to be used, sampling methods and evaluation criteria are described in a CSF Diagnostic manual laid down in the Commission Decision 2002/106/EC. In order to develop an effective control and management strategy the source of the infection and possible spread of the virus needs to be determined using epidemiological. Under specific circumstances emergency vaccination can be applied, using a conventional live attenuated vaccine or a marker vaccine. This has been accommodated by the adaptation of the above mentioned Commission Decision 2002/106/EC by Commission Decision 2003/859/EC as regards to the establishment of a classical swine fever discriminatory test. Finally, dependent on the situation, the commission reserves the privilege to adopt additional measures.

Following the confirmation of an infection in wild boar, the EU Member state in question must submit, for approval by the EU commission, a plan detailing the eradication or vaccination. Notwithstanding the fact that CSF is has been eradicated in feral pigs in many EU Member states, control measures are still in place in some areas. A complete listing of the European directives, concerning these regions and their respective control programs, can be found at: http://ec.europa.eu/food/animal/diseases/controlmeasures/csf_en.htm.

6.4 MONITORING AND SURVEILLANCE SYSTEMS

Monitoring and surveillance systems are essential activities for official veterinary services because of the increased trade in animals and animal products and the increased importance of international disease reporting (Salman et al., 2003). Within the European Union, the surveillance and monitoring programmes are regulated in the EC legislation. Consequences of CSF epidemics depend on the control measures but also on the number of infected herds at the end of the high risk period (Klinkenberg et al., 2005). A method of the evaluation of the effectiveness of the Dutch surveillance system concerning CSF is presented by Klinkenberg et al., 2005. Different types of CSF surveillance, like routine gross pathology of severely diseased pigs, routine virological test of tonsils, daily clinical observation by the farmer, periodic clinical inspection by the veterinarian, leucocyte counts in blood samples from diseased animals on a herd where antimicrobial group therapy was started, were evaluated by modelling study (Klinkenberg et al., 2005). Certain surveillance measures have also an effect on the progress of disease control measures. As an example, the late detection of the first CSF infection in an area, the organisation of pig farming. Especially in highly densely populated livestock areas and during running epidemics a decreasing sensitivity of clinical inspections may complicate eradication campaigns (Pluimers et al., 1999).

As wild boar can act as a reservoir (Laddomada, 2000), therefore monitoring measures is needed as overpopulation increases the probability of the occurrence of the disease (Vengust et al., 2006). However, the incidence and persistence of CSF in free-ranging wild boar varies considerably apparently dependent on environmental conditions. For example, epidemiological studies among wild boar in CSF affected regions of France revealed that higher CSF incidences were found in large forest compared to smaller ones (Rossi et al., 2005).

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