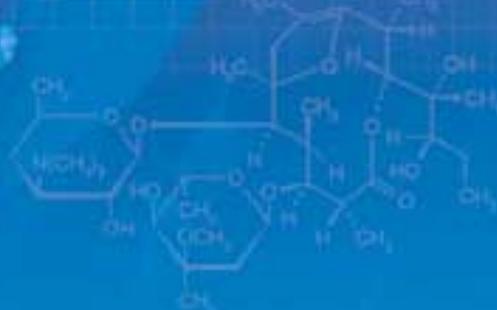


*Pestivirus contamination  
of bovine sera and other  
bovine virus contamination*

Paris, 29-30 March 2001

**PROCEEDINGS**





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**OPENING REMARKS**

Prof. D. H. Calam, Chair of the European Pharmacopoeia Commission

It is a great pleasure to welcome all of you, participants and speakers, on behalf of the European Pharmacopoeia Commission to this International meeting on Pestivirus contamination.

The Commission relies heavily on its Groups of Experts for technical advice and recommendations about publication of monographs in the European Pharmacopoeia. These Groups include those responsible for work on vaccines for human and for veterinary use.

This meeting will provide the current scientific basis for decisions that the relevant experts will have to take to address the issue of pestivirus contamination as it affects veterinary, and to some extent human, medicines.

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

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**Discussion**

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

## **THE BIOLOGY OF THE INFECTION BY RUMINANT PESTIVIRUSES**

Prof. P.-P. Pastoret

Dr C. Hamers and Dr P. Dehan (University of Liège, B)

### **SUMMARY**

This contribution will mainly focus on the biology of the infection by bovine pestivirus responsible for the complex bovine viral diarrhoea/mucosal disease in cattle.

Bovine viral diarrhoea virus (BVDV) is a member of the genus pestivirus within the family Flaviviridae. It shares common antigenic determinants with other pestiviruses notably responsible for classical swine fever and Border disease in sheep. Nevertheless the virus is highly variable and two biotypes exist, the non-cytopathic and the cytopathic. Cytopathogenicity is defined in cell culture and does not relate to their pathogenic potential in cattle. In fact two main antigenic and genotypic variants exist type I and type II. Some non-cytopathic type II viruses are responsible for an haemorrhagic syndrome found in Northern America; European type II strains differ from these isolated in North America in this regard. Cytopathic strains derive by random mutation from non-cytopathic strains provoking the emergence of pairs of non-cytopathic/cytopathic strains antigenically similar. The reservoir of the infection consists of permanently infected animals immunotolerant against their infecting strain which is always non-cytopathic. Those permanently infected animals are generated by infection of foetuses during the first trimester of pregnancy. The infecting BVDV strains remain stable both genotypically and phenotypically in the permanently infected animals and are responsible for the horizontal transmission of the infection since cytopathic strains seem to be dead-end in virgin adult animals. Therefore non-cytopathic strains induce a better immune response than cytopathic ones. Following horizontal transmission strain variation occurs. Herd related strains can be described due to the presence of permanently infected animals. Bovine viral diarrhoea is a contagious and mild infection whilst mucosal disease is a sporadic and highly fatal disease. Mucosal disease is the result of the superinfection of cattle permanently infected with a non-cytopathic strain by a cytopathic strain antigenically similar.

The percentage of permanently infected animals in the general cattle population is around 1-2% but some herds may show a higher percentage.

Border disease virus infection in sheep shares the same biological characteristics.

### **INTRODUCTION**

Pestiviruses are a genus of the family *Flaviviridae*. They infect numerous animal species among which cattle, sheep and pigs producing bovine viral diarrhoea/mucosal disease in cattle, border disease in sheep and classical swine fever in pigs. This last disease did give the name to the genus (Peste porcine classique in French). Researches on bovine viral diarrhoea/mucosal disease virus did permit to clarify many aspects of those infections (Laude, 1987).

It took time to understand the link between Bovine Viral Diarrhoea (BVD) and mucosal disease (MD) in cattle. In 1946 Olafson and co-workers described a contagious gastro-enteritis in cattle: bovine viral diarrhoea and in 1953 Ramsey and Chivers described a fatal disease in cattle: mucosal disease. The antigenic relationship between the viruses responsible for those two entities was later on demonstrated by Gillespie and co-workers in 1961 and the two infections were associated (Pritchard, 1963). The role of the two biotypes of BVD virus was demonstrated by the experimental reproduction of the fatal mucosal disease by Brownlie and co-workers

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in 1984 and Bolin and co-workers in 1985. The other breakthrough was the first sequencing of the complete BVD virus genome by Renard and co-workers in 1985. Bovine viral diarrhoea virus (BVDV), Border disease virus (BDV) and Classical Swine Fever virus (CSFV) are antigenically related and cross the host specific barrier (Wensvoort *et al.*, 1989). In fact, BVDV is a ubiquitous agent which infects mainly cattle but is able to infect pigs, sheep, goats and other ungulates (Nettleton, 1990).

### **THE BVD/MD PESTIVIRUS**

Bovine viral diarrhoea is a disease with a high morbidity and low mortality whereas mucosal disease is sporadic and highly fatal. BVD virus shows two distinct biotypes cytopathic and non-cytopathic with antigenically similar pairs.

Cytopathogenicity is an *in vitro* characteristic and does not relate to *in vivo* pathogenic effect; in fact non-cytopathic strains are the most frequent and are associated with the most dramatic syndrome following horizontal transmission.

Cytopathic strains differ from non-cytopathic ones by the presence in cell culture of a non-structural protein (NS3) (80 kDa) which results from the cleavage of a precursor protein (NS2-3) (125 kDa) which can be found in cell lysates infected with both biotypes. The reservoir of the infection is immunotolerant animals persistently infected with a non-cytopathic strain of BVD virus, and which excrete it permanently (mean prevalence: 1-2% of cattle population). These reservoir animals result from the infection of pregnant females during pregnancy with a non-cytopathic strain of BVD virus and the resulting infection of the foetus during the period of tolerance acquisition. Calves infected during that period are born persistently infected with a non-cytopathic BVD virus strain, which is immunologically tolerated by the animal. Cytopathic strains derive by mutation from the non-cytopathic strains reservoir. Those mutations are random, but localised within the NS2-NS3 region of the genome. If a cytopathic strain superinfects an animal already persistently infected with a non-cytopathic strain antigenically similar (pair of non-cytopathic/cytopathic strains) it provokes a fatal mucosal disease. The genome of BVDV consists of a single stranded RNA molecule of positive polarity, about 12,5 kb long devoid of 3' poly A (Dehan *et al.*, 2001). There is a single large ORF, which encodes a polyprotein of about 4000 amino acids. Four structural proteins are encoded towards the 5' terminus (C, E0, E1, and E2). At the extremity of the 5' region there is a non-coding one approximately 400 nucleotides long preceding the aforementioned ORF and a 3' terminal non-coding region of approximately 200 nucleotides. Two third of the ORF code for 8 non-structural proteins among which NS3.

Comparison of nucleotides sequence show that 5' non coding region is highly conserved and is therefore an ideal tool to identify and differentiate pestivirus strains by polymerase chain reaction. On the basis on the 5' non coding region sequence and the sequence coding for NS3 two distinct BVDV genotypes can be distinguished, type I and type II. Among type II strains one can find the highly virulent thrombocytopenic strains responsible for the haemorrhagic syndrome observed in Northern America.

### **BVDV VARIABILITY**

As already mentioned BVD virus strains share common antigens with other pestiviruses such as Border disease virus and Classical swine fever virus and cross contaminations do occur. Cross contaminations induce serological responses against the shared antigens; for instance whenever a pig is contaminated with BVD virus it will be serologically positive against classical swine fever

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virus. Nevertheless, Bovine viral diarrhoea virus is genetically highly variable and its variability may be influencing its biological and antigenic properties.

First of all as already mentioned BVDV can be divided in two biotypes cytopathic and non-cytopathic. Due to the fact that cytopathic strains derive by random mutations from non-cytopathic ones, there exist pairs of cytopathic/non-cytopathic strains antigenically similar leading to the emergence of mucosal disease. Those biotypes are classified according to their growth characteristics in cell culture but other *ex vivo* and *in vivo* biological differences do exist. First of all only cytopathic strains are able to induce apoptosis (Lambot *et al.*, 1998b). They behave differently after horizontal transmission in naïve animals. Non-cytopathic strains produce viraemia and are excreted by the animal, whereas cytopathic ones do not produce viraemia, do not infect the foetus, and are poorly excreted (Lambot *et al.*, 1998a); cytopathic strains seem to consist epidemiological dead end. Non-cytopathic strains induce a stronger immune response than cytopathic ones (Lambot *et al.*, 1997). Cytopathic and non-cytopathic strains infect different tissues and only non-cytopathic strains are isolated from the lungs. As a consequence only non-cytopathic strains circulate horizontally among naïve animals and this horizontal transmission is responsible for the genetic drift which is observed (Hamers *et al.*, 2000b) and persistently infected immunotolerant animals carry and excrete non-cytopathic strains only.

In a persistently infected animal the non-cytopathic strain which is carried is stabilised both genetically and antigenically (Hamers *et al.*, 1998 a and b) leading to herd specific strains. This explains why several animals may be persistently infected with the same strain within the same herd, even if the prevalence of persistently infected animals is no more than 1 or 2% in the general population. When a persistently infected animal is superinfected with a cytopathic strain it excretes it and may infect other persistently infected ones with the same strain, within the same herd, leading to several cases of mucosal disease. There are also differences in virulence among BVD type II non-cytopathic strains after horizontal transmission among naïve animals. Some type II strains can produce a fatal haemorrhagic syndrome as observed in North America (Corapi *et al.*, 1989) and recently imported in Europe through a contaminated vaccine (Falcone *et al.*, 2000). Haemorrhagic syndrome was also been previously observed in Europe (Lecomte *et al.*, 1996) but the disease is rather sporadic contrarily to what is observed in North America where it is epizootic. Moreover one cannot reproduce the syndrome with BVD virus strains isolated in Europe from such cases (Hamers *et al.*, 2000a). Due to horizontal circulation and genetic drift (Hamers *et al.*, 2000b) BVD virus strains show antigenic variations and strains antigenically different may sometimes be isolated in the same herd. This antigenic variability is found at the level of structural glycoprotein (E0, E1, E2) where one can find protective antigens (especially E2) (Boulangier *et al.*, 1991) whereas NS3 is antigenically stable and can be used as a tool for the diagnosis (Lecomte *et al.*, 1990). Antigenic variability may have important consequences for vaccines by limiting the protective spectrum of monovalent vaccines. It is becoming obvious that future vaccines should contain at least both type I and type II strains.

### **CONSEQUENCE OF THE BIOLOGY OF THE INFECTION BY BOVINE PESTIVIRUSES**

After horizontal primary infection of naïve animals, virus excretion of non-cytopathic strains of BVD virus is relatively low (5 to 500 CCID<sub>50</sub>/ml) whereas titres are more elevated in the blood ( $\pm 10^{4.0}$  CCID<sub>50</sub>/ml). In persistently infected immunotolerant animals the amount of virus is far greater. There is permanently more than  $10^{4.0}$  CCID<sub>50</sub>/ml in the blood and some tissues may contain more than  $10^{7.0}$  CCID<sub>50</sub>/g.

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

Persistently infected animal is the key reservoir of the infection, their prevalence being more or less 1-2% in the overall population. This percentage is higher in immunotolerant foetuses. If foetuses are infected later during pregnancy they are able to mount a sterilising immune response. Therefore, before colostrum ingestion, new-born calves may be either virologically positive and serologically negative against BVD virus or virologically negative and serologically positive against BVD virus. In most cases the mother was naïve and immuno competent when infected and therefore is able to produce an immune response which will be transmitted to the new-born through the colostrum. New-born calves born from seropositive dams will therefore be serologically positive and either persistently infected or not. Therefore foetal calf sera may be contaminated either by BVD virus, BVD virus specific antibodies or both. When collecting large batches of foetal calf sera for the production of pharmaceuticals (as much as 2000 litres) it is practically impossible to avoid contamination by BVD virus and anti BVD virus antibodies.

This symposium will discuss the inactivation of bovine foetal calf sera and the detection of BVD virus or anti BVD virus antibodies.

A special emphasis should be given on the use of either isolation or polymerase chain reaction for the detection of BVD virus contamination.

It must always be kept on mind that bovine foetuses are only infected by non-cytopathic strains of BVDV and therefore those strains cannot be detected directly in cell culture through cytopathic effect, but well indirectly by immunofluorescence for instance.

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## **HOST RANGE, DIVERSITY AND CLASSIFICATION OF PESTIVIRUSES**

Dr D. Paton (VLA, Weybridge, UK)

The genus *Pestivirus* is in the family *Flaviviridae*, which includes human pathogens within the genera *Flavivirus* and *Hepacivirus*. However, Pestiviruses are not known to infect man. The principal Pestivirus genotypes are the two types of bovine viral diarrhoea virus (BVDV 1 and 2), classical swine fever virus (CSFV) and ovine border disease virus (BDV).

Although the virus nomenclature implies host specificity, this is not absolute. As well as CSFV, pigs are occasionally infected with all of the genotypes more normally associated with ruminants. Cattle can be infected with BVDV types 1 and 2, but do not seem to be very susceptible to CSFV or BDV. Sheep readily acquire BVDV from cattle and these infections may be more common than those due to “ovine” BDV. A growing range of free-living and exotic ruminants has been shown to be susceptible to infection with the “cattle” viruses. Unusual genotypes have also been reported in giraffe and buffalo suggesting the existence of “type-specific” virus varieties.

Differentiation between infection with the different Pestivirus genotypes can be achieved by a variety of antigenic and genetic means. Studies of the viral diversity in different parts of the world have revealed that different genotypes and subtypes predominate in different locations. Molecular epidemiology can therefore be used to trace incursions of new variants at both the national and the herd level.

### **PESTIVIRUS STRUCTURE**

Meyers and Thiel (1996) have reviewed the molecular characteristics of Pestiviruses. Pestiviruses have enveloped virions approximately 45 nm in diameter. They bear three virally-encoded envelope proteins, termed E<sup>ms</sup>, E1 and E2. E<sup>ms</sup> exists as a homodimer and as well as being found in virions and infected cells, is released extracellularly. Antibodies raised against it may be virus neutralising and it is able to elicit a protective immunity against disease. As a virion protein, it may have a role in cellular attachment. It is known to bind to cell surface peptidoglycans such as heparan sulphate. It is also unusual in possessing RNase activity and being able to pass across cell membranes. It can induce apoptosis in T cells and mutations in E<sup>ms</sup> can lead to viral attenuation. The second glycoprotein E1 is poorly studied. It seems not to be immunogenic and may be largely retained within the envelope membrane. The E2 glycoprotein is highly immunogenic and elicits strong neutralising antibodies and disease-protective immunity. It exists as a homodimer or as a heterodimer with E1. The gene for E2 is one of the least conserved in the pestivirus genome and genetic and antigenic diversity may relate to immune evasion and/or adaptations to different host cell targets. It is likely that the N terminus of E2 interacts with one or more unidentified cell receptors leading to viral entry. There is an unglycosylated core protein that encapsidates the genome.

The viral genome is a single-stranded, positive sense RNA molecule of at least 12.3 kb, with untranslated regions at either end. It has an internal ribosome entry site (IRES) near the 5' end, leading to the translation of a single large open reading frame. The first protein to be produced is the non-structural autoprotease N<sup>pro</sup>. The structural and then the remaining non-structural proteins follow this. The form of expression of the non-structural protein NS23 seems to play a key role in determining whether a pestivirus is of a non-cytopathogenic or a cytopathogenic biotype.

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### **CLASSIFICATION WITHIN THE *FLAVIVIRIDAE***

The *Pestiviruses* are one of three genera within the family *Flaviviridae*, the other two being the *Hepaciviruses* and the *Flaviviruses* (van Regenmortel *et al.*, 2000). The viruses of all three genera have a similar morphology and a similar genomic organisation. The viruses multiply in the cytoplasm of infected cells in association with cell membranes, maturing in vesicles. However, the biological properties are quite distinct. Most *Flaviviruses* are arthropod borne and some have a wide host range. Several of these viruses cause serious human diseases. Some cause cytopathic effects in cell cultures, but persistent infections are also common. The *Hepaciviruses* are represented by Hepatitis C virus. This affects only humans and chimpanzees, causing a serious and sometimes fatal illness as a result of persistent infection. The virus does not grow in cell cultures. There is no serological cross-reactivity between the *Pestiviruses* and the other genera.

### **PESTIVIRUS HOST SPECIFICITY AND CLASSIFICATION**

The *Pestiviruses* infect cloven-hoofed animals of the order *Artiodactyla*. They were first described as diseases of pigs and cattle – hog cholera (later classical swine fever) and bovine viral diarrhoea-mucosal disease. Serological cross-reactivity between antisera to classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV) provided the first indication that the viruses were related. The traditional view has been that cattle are infected by BVDV, pigs by CSFV and sheep by Border disease virus (BDV). This remains broadly true, but it is now clear that although there are different *Pestiviruses* adapted to each of these main hosts, the viruses can also be transmitted between host species, particularly from cattle to sheep, from cattle and sheep to pigs and from cattle to wild ruminants (Paton, 1995).

Cross-neutralisation studies, typing with monoclonal antibodies (mAbs) and genetic sequence comparisons all lead to the same principle divisions amongst the *Pestivirus* genus. Four main “viral species” or “genotypes” can be identified, namely types 1 and 2 BVDV, CSFV and BDV. There is variable, but weak cross-neutralisation between the different viral species. Mabs can be raised against both conserved and variable viral epitopes. The most conserved epitopes are to be found on the NS23 non-structural protein and all known *Pestiviruses* share some of these pan-*pestivirus*-specific epitopes. By contrast, mAbs raised to the structural proteins E2 and E<sup>ns</sup> are often type-specific or even strain-specific. The pan-*pestivirus* reactive and the type-specific mAbs, in particular, are very useful diagnostic and typing reagents. The most recent tool for typing *Pestiviruses* is the method of comparing all, or more often, part of the genomic sequence. A similar classification, with respect to genotype, is obtained when different regions of the viral genome are compared, although some areas have greater or lesser sequence variability. At the genome level, viruses of different genotype such as BVDV 1 and CSFV share about 65% nucleotide and 85% amino acid identity.

### **CROSS-SPECIES INFECTIONS**

A growing number of *Pestiviruses* are being typed from different hosts. In pigs it has been found that as well as infection with CSFV, there are occasional infections with *pestiviruses* that normally circulate in cattle and sheep, principally BVDV type 1 and BDV. These “ruminant *pestiviruses*” are transmitted to pigs via contact with infected ruminants or ruminant products. The latter include feed-stuffs and vaccines contaminated with viruses present in foetal calf serum. The ruminant *pestiviruses* cause transient and clinically inapparent post-natal infections in pigs, and the viruses do not readily spread and persist in the pig population. However, transplacental transmissions can cause illness and lead to the birth of persistently infected

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offspring. The main importance of these infections is that they cause problems in the diagnosis of CSF. This is most significant in countries where CSFV has been eradicated, but BVDV and/or BDV remain endemic.

Cattle are principally infected with BVDV types 1 and 2 and the two viruses cause an essentially similar range of disease. In most parts of the world BVDV type 1 either predominates or is the only virus found in cattle. BVDV type 2 seems to have emerged in North America and to be gradually spreading elsewhere. There is little evidence that cattle are naturally infected with either CSFV or BDV, although experimentally, CSFV infection has been demonstrated. Although it might be anticipated that cattle would become infected by exposure to other pestivirus-shedding ruminants such as sheep and wild ruminants, there is little direct evidence for this.

As well as their own pestivirus – BDV, sheep are readily infected by BVDV type 1 from cattle (Vilcek *et al.*, 1999). There is also some evidence that they can be infected with BVDV type 2. Naturally occurring infections with CSFV have not been described. The predominant pestivirus in sheep differs in different parts of the world and may be either BDV or BVDV type 1.

### **SUB-TYPES WITHIN THE PESTIVIRUS GENOTYPES**

Antigenic and especially genetic typing can be used to define additional sub-divisions below the genotype or viral species level. An extensive database of CSFV sequence data has been established for typing purposes and to help to trace the origins of new outbreaks. At least three genetic subtypes can be distinguished (e.g. CSFV type 1), each with a further three to four sub-subtypes (e.g. CSFV type 1.1) (Paton *et al.*, 2000). Similarly, for BVDV type 1, at least eleven genetic subtypes have recently been defined (Vilcek *et al.*, 2001).

### **IMMUNOLOGICAL CROSS-PROTECTION BETWEEN PESTIVIRUSES**

Animals that have recovered from an acute pestivirus infection develop a strong and usually long-lasting immunity (Fredriksen *et al.*, 1999) that protects them against re-challenge by the same virus. The degree of protection afforded against re-challenge by a heterologous virus depends on the antigenic relatedness to the initial immunising strain, and the time that has elapsed since immunisation. However, even when the re-challenge is with a virus from another pestivirus genotype some protection against disease is likely to be afforded. A similar situation applies to vaccines, although many commercially available products induce a weaker and more short-lived immune response than natural infection. Unfortunately, partial protection may not be sufficient to protect against transplacental transfer and halt virus transmission in the field. There is considerable interest, but little data on the degree of protection afforded between infection with viruses of the same genotype but differing sub-type.

### **EVIDENCE OF INFECTION IN OTHER SPECIES:**

In all studies involving the isolation of pestiviruses in cell cultures, the risks of adventitious contamination by foetal calf serum have to be carefully avoided.

Wild boar are of the same species (*Sus scrofa*) as domestic pigs and appear similarly susceptible to CSFV. CSFV-infected wild boar constitute an important reservoir for the disease in parts of Europe (Laddomada, 2000). In contrast, New World pigs such as peccaries are not susceptible. Pestivirus infections of goats are rather poorly documented, but naturally and experimentally induced infections have been described. Pestiviruses have been isolated from many different free-living and captive wild ruminants, including deer, bison, buffalo, eland and giraffe (Nettleton, 1990). Most of these viruses are closely related to cattle pestiviruses, however,

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unique genotypes have been reported from giraffe and bison. Pestivirus antibodies have been found in an even wider range of ruminant species. More recently pestiviruses have also been isolated from old and New World camelids (Belknap *et al.*, 2000).

Both BVDV and CSFV have been adapted to growth in rabbits (Koprowski *et al.*, 1946; Fernelius *et al.*, 1969). Experimental infections of birds and cats have not shown any evidence of viral replication. There is a single report of an equine isolate (Sockett *et al.*, 1997), but it is generally believed that horses are not susceptible. The data purporting to show evidence of human infections are so far not convincing. Some authors have found low levels of pestivirus antibodies in sera taken from various categories of sick and normal patients. Another study found viral antigens in faecal specimens from young children with gastro-enteritis, although the test used is of uncertain specificity. A BVD virus was reported to have been isolated from the buffy coat of a Belgian patient (Giangaspero *et al.*, 1997).

### BASIS OF HOST SPECIFICITY

The ability of a virus to enter into and replicate within host cells is one primary requirement, that is likely to limit its adaptability to different host species. Pestiviruses usually grow readily, if at low titre, in cell cultures that are derived from the same host species from which the virus was isolated. For example, BVDV will grow in a variety of bovine cell cultures as well as in cells from other ruminants. However, most ruminant pestivirus isolates do not grow well in pig cells, unlike BVDV and BDV isolates obtained from pigs. Certain isolates will grow in cell cultures from other species, possibly after a period of adaptation, and the list of permissive types described includes cells from pigs, horses, rabbits, hamsters, cats, non-human primates (Vero cells) and even birds (chick embryo fibroblasts).

Work on the basis for cell type specificity for Pestiviruses is still in its infancy. Both the E2 and E<sup>ms</sup> proteins bind to cell surface molecules and are likely to be involved in attachment and/or virus entry. Heparan sulphate has been identified as a cell surface target for the E<sup>ms</sup> protein, but it seems that this may be more important for *in vitro* than *in vivo* virus replication (Hulst *et al.*, 2000). The E2 receptor may be a 50 kDa protein found on a wide range of bovine cell types. Anti-idiotypic antibodies to BVDV E2 react with such a protein and partially block infectivity (Minocha *et al.*, 1997). Antigenically diverse BVDVs do not all compete with this anti-idiotypic antibody for binding to cells. However, different pestivirus genotypes seem to compete for binding to the same receptor, although possibly via different sites (Flores *et al.*, 1996; Hulst and Moormann, 1997). It has also been reported that BVDV, and also hepatitis C virus can enter cells by means of low density lipoprotein receptors, possibly mediated by an association between virions and lipoproteins (Agnello *et al.*, 1999). Finally, monoclonal antibodies to a 93/60 kDa cell surface molecule also block entry of pestiviruses into permissive cells (Schelp *et al.*, 1995).

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### DETECTION AND MOLECULAR TYPING OF PESTIVIRUSES

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Bovine viral diarrhoea viruses (BVDV) are classified as members of the pestivirus genus within the flavivirus family (Wengler *et al.*, 1995). In addition to BVDV, the pestivirus genus includes hog cholera virus [HCV, also known as classical swine fever virus (CSFV)] and border disease virus (BDV) of sheep. Pestiviruses are small-enveloped viruses with a single stranded RNA genome. Viruses from the Pestivirus genus were originally classified as BVDV, CSFV or BDV based on the animal host of origin. This classification proved problematic because some pestiviruses are not restricted to a single host. BVDV, for example, has been shown to infect cattle, sheep and swine (Paton *et al.*, 1995). Further, while it appeared that all pestiviruses possessed some cross-reactive epitopes, differences in biotype, virulence and protective epitopes suggested that viruses isolated from the same animal species were highly heterogeneous. The heterogeneity observed among pestiviruses is due, in large part, to the nature of the pestiviral genome. Single stranded RNA genomes tend to accumulate mutations at a high rate because there is no proof reading function that accompanies transcription (Domingo, 1989; Domingo and Holland, 1997; Domingo *et al.*, 1985; Duarte *et al.*, 1994). Recently phylogenetic analysis has been used as to subdivide pestiviruses into at least six different groups, or genotypes (Becher *et al.*, 1997; Giangaspero *et al.*, 1997; Harasawa and Giangaspero, 1998; Ridpath and Bolin, 1997; Ridpath *et al.*, 1994; Sandvik *et al.*, 1997; van Rijn *et al.*, 1997). Viruses previously known as CSFV and BDV each make up a distinct genotype. While viruses that had been labelled BVDV belong to two different genotypes, which are now referred to as BVDV1 and BVDV2. Only one viral strain each has been identified for the fifth and sixth pestiviral genotypes. These strains were isolated from a deer and a giraffe respectively.

Prior to the discovery of two distinct BVDV genotypes, attempts were made to subdivide BVDV by monoclonal antibody binding (Bolin *et al.*, 1988; Corapi *et al.*, 1988; Corapi *et al.*, 1990; Donis *et al.*, 1991; Moennig *et al.*, 1987). This method of classification resulted in a large number of groups that could not be characterized by any centripetal phenotype. Things became a little clearer when Mab binding patterns of viruses from different genotypes were compared. While antigenic variation was observed among viruses within a genotype, comparison of Mab binding patterns between viruses from different genotypes revealed that panels of monoclonal could be used to differentiate between viruses from the two genotypes (Bolin and Ridpath, 1998; Ridpath *et al.*, 1994). Subsequently it was found that once viruses were grouped by genotype, neutralization using polyclonal sera could also be used to differentiate between genotypes (Ridpath *et al.*, 2000). However the differences in serum neutralization are not large enough to state that the two genotypes belong to different serotypes using the classic definition of serotype. Antigenic differences between the two genotypes have practical significance. Viruses that belong to the BVDV1 and BVDV2 genotypes are sufficiently different at the antigenic level that a vaccine that protects against one genotype may not protect against the other genotype (Ridpath *et al.*, 1994). Further differences observed between strains from the two genotypes include virulence and recombination tendencies.

Outbreaks of a clinically severe disease, termed haemorrhagic syndrome (HS), have been reported in the U.S. and Canada. Thus far, all HS outbreaks have been associated with acute uncomplicated infections with BVDV2 strains. However, not all BVDV2 strains cause clinically severe disease (Ridpath *et al.*, 2000). Avirulent BVDV2 isolates do exist and may predominate over virulent BVDV2 in nature. When virulent BVDV2 viruses are inoculated into calves they induce a disease characterized by fever, diarrhoea, leukopenia, lymphopenia, neutropenia,

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thrombocytopenia, and death. Infection with avirulent BVDV2 results in a reduction of leukocytes that may be accompanied by a low-grade fever. These viruses do not cause clinical disease or a clinical leukopenia. Clinically severe disease as the result of infection with BVDV1 strains has been reported. However such outbreaks appear to be rare and the disease syndrome has not been replicated under laboratory conditions. Severity of clinical disease is a function of not only viral strain but also immune status, reproductive status and the presence of secondary pathogens. Thus differentiation of BVDV1 and BVDV2 must never be based on the observation of clinical signs alone.

The BVDV1 genotype can be further divided into the subgenotypes BVDV1a and BVDV1b (Harasawa, 1994; Harasawa and Sasaki, 1995; Pellerin *et al.*, 1994; Ridpath and Bolin, 1998). These two subgenotypes may be differentiated by Mab binding and differential polymerase chain reaction (PCR) amplification. While the practical significance of this subdivision has not been established, BVDV1b field strains have been isolated from herds vaccinated with BVDV1a vaccines (Hana Van Campen, Colorado State University, personal communication).

The methods used for detection of BVDV infections are the same regardless of BVDV genotype. BVDV infections are difficult to diagnose based solely on clinical signs because clinical manifestations such as reproductive failure, birth of weak, malformed calves, respiratory disease, diarrhoea and increased susceptibility to other pathogens are not unique to BVDV (Houe, 1999). BVDV may cause both acute and persistent infections. Persistently infected (PI) animals are born as the result of in utero infection. PI animals are immunotolerant of the virus they carry and shed this virus through out their lifetime. Acute outbreaks that do not result in clinically severe disease may pass unnoticed until there is a spate of persistently infected animals born into a herd. Acute outbreaks may last from a few weeks to several months or may appear sporadically in a herd over several years depending on whether or not a PI animal is present, the virulence of the BVDV strain and management and housing of cattle herds. Direct contact with PI animals is probably the most frequently recognized method of transmission of infection; however, field studies have shown that spread of infection also occurs in the absence of PI animals. Most diagnostic tests have been designed to detect PI animals (Baszler *et al.*, 1995; Deregt and Prins, 1998; Radwan *et al.*, 1995; Saliki *et al.*, 1997; Thur *et al.*, 1996). A number of different technologies have been used in these tests including immunohistochemistry, ELISA and PCR. Virus isolation remains the gold standard. It is not known how reliably tests designed to screen for PI animals detect acute BVDV infections or conversely whether acute BVDV infections are confused with persistent infections in surveys for PI animals.

In addition to causing problems for the cattle industry, BVDV are also frequent contaminants of cultured cells and viral stocks (Bolin *et al.*, 1994a; Bolin *et al.*, 1994b; Erickson *et al.*, 1991; Levings and Wessman, 1991; Wensvoort and Terpstra, 1988). BVDV has been detected as a contaminant in modified live vaccines (Wensvoort and Terpstra, 1988). Contamination of research reagents and veterinary biologics most frequently is the result of using foetal bovine serum, derived from BVDV infected animals, as a cell culture supplement. Detection in FBS and cultured cell lines can be difficult due to the low level of virus present and in the case of FBS the presence of antibodies against BVDV. Amplification of the number of viral particles present by serial passage in BVDV permissive cells greatly increases the rate of detection (Bolin *et al.*, 1994a; Bolin *et al.*, 1991; Bolin and Ridpath, 1998; Bolin *et al.*, 1994b).

The method used for differentiating between BVDV strains depends on the purpose of the analysis. Genomic differences can be exploited for differentiation of genotype by PCR. Genomic differences are seen throughout the genome and thus many different regions have been used for differential PCR (Canal *et al.*, 1996; Katz *et al.*, 1993; Ridpath and Bolin, 1998;

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Vilcek *et al.*, 1994; Wirz *et al.*, 1993). However, PCR tests based on highly conserved region are more likely to pick up out lying variants. While very useful for differentiation, use of PCR for primary detection has practical limitations in many diagnostic laboratories. PCR based tests require specialized equipment. Because cross contamination must be rigorously avoided laboratory space and equipment may need to be dedicated to performing these tests. This is not practical in many diagnostic laboratories. Antibody based techniques can be used to detect and differentiate between BVDV1 and BVDV2. Such tests are comparatively cheap and use technologies and equipment available to most laboratories. However, caution is advised when using these techniques because of the variability observed among BVDV isolates within any genotype (Ridpath *et al.*, 1994). This is particularly important when using antibodies made against the E2 viral polypeptide (Ridpath *et al.*, 1994). Even cloned BVDV populations quickly evolve into viral swarms due to the variability inherent in the BVDV genome. Studies examining antigenic shift in BVDV populations in response to Mab binding have shown that plaque-purified BVDV stocks contain neutralization escape mutants with a frequency of 10(-2.47) (Donis *et al.*, 1991). Direct sequence comparison, while impractical for detection and routine differentiation, is an indispensable tool for epidemiology and for identification and characterization of new pestivirus genotypes and subgenotypes.

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## GEOGRAPHICAL DISTRIBUTION AND PREVALENCE OF BOVINE PESTIVIRUS INFECTIONS TYPES I AND II

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Bovine viral diarrhoea virus (BVDV) infections occur world-wide causing a number of diverse diseases in cattle including enteritis, reproductive and respiratory disorders, hemorrhagic syndrome, persistent infections and mucosal disease (3). Its importance as a pathogen is indicated by the large number of vaccines (>140), which exist for the virus and the announcement in recent years of BVDV eradication programs in some European countries (6,40). Whether controlled by vaccination or eradication (without vaccination), an important element in the control of BVDV is the identification and removal of persistently infected (PI) animals from infected herds.

BVDV is now recognized as comprising two distinct genotypes or species, type 1 and type 2 (BVDV I and BVDV II) (36,38). Virulent strains of BVDV II have been associated with severe disease outbreaks of hemorrhagic syndrome and acute BVD in the United States and Canada in recent years (13,36,38).

Prevalence data for BVDV infections can be expressed as the prevalence of animals with antibodies to BVDV (seroprevalence) or as the prevalence of PI or viremic animals. Since most countries vaccinate their herds for BVDV, vaccination complicates using seroprevalence data as an indicator of BVDV infection. Thus, seroprevalence data are most useful for countries, which do not vaccinate. Several large surveys for seroprevalence of BVDV were taken in the mid-1980s to the early 1990s in several European countries and the United States. Table 1 shows the results for some of the largest surveys taken during this period. These surveys show a high seroprevalence of 89% for a survey of 66 herds in the United States to a low seroprevalence of 19% for a survey of 187 herds in Norway.

*Table 1. Seroprevalence of BVDV in several large surveys of the mid-1980s and early 1990s.*

Country	Year survey was reported	No. of animals†	Antibody positive	Reference
United Kingdom	1987	>18,000	65%	17
United States	1985	>3,000	89%	8
Denmark	1991	>2,500	64%	21
Sweden	1986	>700	41%	1
Norway	1991	>1,000	19%	31

† Or number of samples tested.

More recent surveys of seroprevalence (Table 2) show similar figures for seroprevalence of BVDV when compared to those of older surveys. A seroprevalence between 60% and 80% is quite common for surveys conducted in various countries.

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Table 2. Seroprevalence of BVDV in more recent surveys.

Country	Year survey was reported	No. of animals	Antibody positive	Reference
The Netherlands	1999	>1,700	65%	27
United States	1996	>1,700†	57%	33
United States	1996	>2,100*	78%	33
Brazil	1998	>400	56%	12
Zimbabwe	1995	>400	79%	29
Belgium	1999	>9,600	66%	43
Switzerland	1997	>2,800	84%	10
New Zealand	1994	140	63%	37
Sweden	2000	>700	32%	11

† 119 unvaccinated cow-calf operations.

\* 137 vaccinated cow-calf operations.

The prevalence of viremic and persistently infected (PI) cattle in several large surveys reported since the mid-1980s is shown in Table 3. An animal is identified as PI when two samples taken three weeks or more apart are both positive for BVDV. Thus, cattle are classified as viremic if they have been tested only once or were found to be negative for BVDV on a second tested sample. These surveys show that the prevalence of cattle, which are persistently infected or viremic, is generally between 0.5% and 2%. However, herd health improvement programs can result in a much lower prevalence of viremic/PI animals as indicated by the 0.1% prevalence of PI cattle in twenty Michigan dairy herds reported in the 1995 U.S. survey (Table 3).

Table 3. Prevalence of viremic and persistently infected (PI) cattle.

Country	Year survey was reported	No. of animals	Viremic	PI	Reference
United Kingdom	1986	>900	0.8%	0.4%	23
United Kingdom	1987	>3,000	1.8%	ND*	17
United States	1985	>3,000	1.9%	1.7%	8
United States	1995	>5,000†	0.1%	0.1%	22
United States	1996	>1,200	0.7%	ND	33
Belgium	1999	>9,600	ND	0.8%	43
Germany	1987	>2,000	0.9%	ND	30
Denmark	1991	>2,500	1.4%	1.1%	21
Sweden	1986	>700	1.7%	1.3%	1

† Twenty Michigan herds enrolled in a dairy herd health improvement program.

\* Not determined or not stated.

Prevalence may also be defined in terms of the percentage of herds, which are positive for BVDV antibodies, or the percentage of infected herds. Herd prevalence data are especially important for countries such as Sweden, Norway, Finland, and Denmark, which have had BVDV control and eradication programs since 1993-1994 (6). These programs are conducted

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without vaccination and involve classification of herds as to their BVDV status, removal of PI animals from infected herds, monitoring herd status and preventing infection in BVDV-free herds (6). Bulk milk testing of dairy herds for BVDV antibodies is performed in these countries. The results are used to classify herds as to their BVDV status and to estimate the percentage of dairy herds with PI cattle or with active infection.

*Table 4. Prevalence of positive herds†*

Country	Year	No. of herds (type)	Prevalence	Reference
United Kingdom	1998	1,070 (dairy)	95%	35
United States	1996	256 (beef)	91%	33
Italy	1999	174 (mixed)*	56%	24
Sweden	1993	14,463 (dairy)	51%	2
Sweden	1995	14,463 (dairy)	24%	2
Denmark	1994	16,113 (dairy)	39%	6,7
Denmark	1999	11,500 (dairy) <sup>+</sup>	9%	7
Norway	1993	26,401 (dairy)	23.0%	48
Norway	1996	all (dairy)	14.4%	48
Finland	1994	34,169 (dairy)§	1%	32
Finland	1997	28,455 (dairy) §	0.4%	32

† Herds with BVDV antibodies or estimated percentage of herds with PI cattle/active infections based on antibody testing (Sweden, Denmark, and Norway).

\* Herds enrolled in a BVDV control program in the Rome province.

<sup>+</sup> Number of herds in 1998.

§ Number of bulk milk samples tested.

As indicated in Table 4, the prevalence of herds with BVDV seropositive animals may exceed 90% in some countries. In sharp contrast, the prevalence of BVD antibody-positive herds in Finland was only 0.4% in 1997, a drop from 1% in 1994, the year it began its control and eradication program (32). In a similar fashion, Sweden, Denmark and Norway have observed a significant decrease in the prevalence of BVDV-infected herds due to their control and eradication programs (Table 4).

In recent years there have been outbreaks of severe disease caused by virulent BVDV II, (13,36,38). However, retrospective typing of BVDV isolates has shown that BVDV II had been circulating in North American cattle since at least the early 1980s (13). Since severe outbreaks did not occur until the 1990s, we can assume that earlier BVDV II were non-virulent and that later some BVDV II acquired virulence factors. Although the molecular basis of virulence in BVDV II is not understood, it is clear that both virulent and non-virulent BVDV II circulate in today's environment.

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*Table 5. Prevalence of type 1 and type 2 BVDV isolations in United States and Canada.*

Country	Year†	No. of isolates typed	Type 1	Type 2	Ref.
U.S. (Midwest)	1998	203	57%	43%	9
U.S. (Southwest)	2000	105	61%	39%	26
U.S. (Northwest)	2001	52	60%	40%	41
U.S. & Canada	1998	320	53%	47%	39,40
Canada (Manitoba)	1993-2000	150	71%	29%	20
Canada (Ontario)	1998-2000	487	48.5%	51.1%	14

† Year study was reported or years for which isolates were typed.

Prevalence of type 2 BVDV isolations generally occurs between about 30% and 50% in the United States and Canada (Table 5). We have been typing BVDV isolated at the Animal Health Laboratory in Ontario, Canada for the past three years using type-specific monoclonal antibodies and a nested multiplex PCR assay (15,16,18). We used a direct method without RNA extraction for PCR typing of cell culture isolates which can also be used for whole blood samples (18).

For all isolations at the Animal Health Laboratory (Ontario, Canada) for the years 1998 to 2000, the percentage of BVDV I to BVDV II was 48.5% to 51.1% (0.4% of the isolates were either not typed or showed a mixture of BVDV I and BVDV II) (Table 5).

*Table 6. Prevalence of Type 1 and Type 2 BVDV isolations in Ontario, Canada by year†*

Year:	1998	1999	2000
Total isolations	199	166	126
Type 1	111 (55.8%)	83 (50.0%)	42 (33.3%)
Type 2	86 (43.2%)	83 (50.0%)	80 (63.5%)
Mixture	2 (1%)	0	0
Not Done	0	0	4 (3.2%)

† Reference 14.

In table 6, the typing data for each year are shown. Although the number of BVDV isolations have decreased from 199 to 166 to 126, for the years 1998 to 2000, respectively, it is interesting to note that BVDV II as a percentage of BVDV isolated has dramatically increased in Ontario. In 1998, the percentage of BVDV I to BVDV II isolations was 55.8% to 43.2%, respectively (1% was a mixture of both types). In 1999, the percentages were 50% to 50%; and, in 2000, 33.3% BVDV I to 63.5% BVDV II (3.2% were not typed). Since most BVDV vaccines still only contain BVDV I, the increased percentage of BVDV II isolations suggests that these vaccines may be less protective against BVDV II and may have selected for the current predominance of BVDV II in Ontario clinical cases.

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Table 7. Prevalence of type 1 and type 2 isolations in Europe.

Country	Year†	No. of isolates typed	Type 1	Type 2	Ref.
Europe*	2001	78	97.4%	2.6%	47
Germany	1999	505	93.5%	6.5%	5
Germany (Bavaria)	1997	96	89%	11%	49
Germany (L. Saxony)	1960-2000	61	97%	3%	19
Belgium	1999	107	88%	12%	28
Sweden	1999	42	100%	0%	45
United Kingdom	1996-1997	62	100%	0%	46

† Year study was reported or years of original isolation for viruses typed.

\*Seven countries: Austria, France, Spain, United Kingdom, Italy, Hungary and Slovakia. Two of twenty five isolates from France were BVDV II.

The present BVDV situation in Europe appears to be quite different from that in the United States and Canada. Recent published surveys have shown BVDV II isolations in some European countries: Germany, Belgium, and France (Table 7). However, for other European countries, BVDV II appears to be absent or rare. In a survey of seven countries, in which 78 isolates were typed, only two isolates (2.6%) from France were BVDV II (47). BVDV II isolations were highest in Belgium (12%) and in Bavaria, Germany (11%) (28,49).

BVDV II isolations have also been observed in Brazil and Japan with a prevalence of 21% and 7%, respectively (25,42) (Table 8). It should be noted, however, that since relatively few isolates were typed in these studies, the BVDV II prevalence for these countries may be overstated. BVDV II isolations have not been observed for Australia, New Zealand and Southern Africa (Table 8).

Table 8. Prevalence of type 1 and type 2 BVDV isolations in other countries.

Country	Year†	No. of isolates typed	Type 1	Type 2	Ref.
Brazil	2000	19	79%	21%	25
Japan	1999	28	93%	7%	42
Southern Africa*	1990-1996	73	100%	0%	4
Australia	2001	>20	100%	0%	34
New Zealand	1967-1997	17	100%	0%	44

† Year study was reported or years of original isolation for viruses typed.

\*South Africa and Mozambique.

In summary, BVDV occurs worldwide and a 60% to 80% prevalence of cattle with antibodies to BVDV is common. Prevalence of herds with BVDV antibodies can exceed 90%. Countries with control and eradication programs have seen the percentage of infected herds significantly decrease in the years since their programs began in 1993 and 1994. The country with the lowest prevalence of BVDV infected herds appears to be Finland with a herd prevalence of only

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0.4%. BVDV II has been circulating in North America since at least the early 1980s. In recent surveys in the United States and Canada, it comprised 30 to 50% of BVDV isolations. However, prevalence presently (for the year 2000) exceeds 60% of BVDV isolations in Ontario, Canada, suggesting that a selective force (e.g. vaccination) may be increasing the prevalence of BVDV II. In Europe, BVDV II occurs at a much lower level and in many countries BVDV II appears to be absent or rare. Brazil and Japan appear to have a medium and low prevalence of BVDV II, respectively. For Australia, New Zealand and Southern Africa, all BVDV isolations have typed to BVDV I.

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## **CONSEQUENCES OF BVDV INFECTION ON THE INNATE AND ADAPTATIVE IMMUNE RESPONSE**

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Bovine viral diarrhoea virus (BVDV) isolates can be discriminated in tissue culture into two biotypes, non-cytopathic (ncp) and cytopathic (cp). Infection of the bovine foetus with ncpBVDV during the first trimester of pregnancy usually results in the birth of immunotolerant, persistently infected (PI) animals. Superinfection of these PI animals with antigenically homologous cp virus causes mucosal disease, which is almost invariably fatal (1). The unique pathogenesis of mucosal disease has been elucidated and has spawned many detailed studies describing the molecular mechanisms that generate cytopathic virus. However, although mucosal disease is clinically dramatic it cannot be considered to be the major cause of economic loss due to BVDV. Rather, the persistently infected animals shedding ncp virus to infect herd mates is a cause of major insidious financial loss to the livestock producer. Most primary postnatal infections are subclinical but there are numerous reports that intercurrent BVDV infection seems to enhance the virulence of other pathogens or change the nature of the resulting pathology. Based on these observations it has been suggested that animals are transiently immunosuppressed after acute infection (2).

The capacity of the virus to cause immunosuppression may exacerbate secondary infections and result in a delay in detectable specific T cells responses after challenge with ncpBVDV (3). Available evidence suggests that cell-mediated immunity is required to resolve BVDV infection since antibody alone is not sufficient to clear persistent infection and *in vivo* depletion of CD4<sup>+</sup> T cells delays resolution of acute infection (4). Virus clearance, seroconversion, maturation of antigen-specific lymphoproliferative responses and the specificity of the CD4<sup>+</sup> T-cell component of the immune response to BVDV were determined for calves infected with NADL (cp), Pe515c (cp) and Pe515nc (ncp) BVDV. The rate of virus clearance and seroconversion (as determined by ELISA) was similar for infection with either biotype. However, virus-specific lymphoproliferation was detected earlier in the animals infected with cp virus than in those infected with ncp virus. CD4<sup>+</sup> and CD8<sup>+</sup> T-cells from recovered animals recognised virus-infected cells whereas non-infectious viral antigen was recognised only by CD4<sup>+</sup> T-cells. These responses were strain cross-reactive, MHC-restricted and identified the envelope protein E2 and the nonstructural (NS) protein NS3 as dominant T-cell determinants.

The CD4<sup>+</sup> T cell response to E2 and NS3 was examined using overlapping 18-mer synthetic peptides. The responses of three BoLA-DRB3\*2002 and three BoLA-DRB3\*0701 homozygous cattle that had been infected previously with Pe515nc virus were determined. The CD4<sup>+</sup> T cell repertoire was biased toward the recognition of conserved regions of NS3 and excluded the hypervariable regions of E2. The total number of peptides recognised varied between animals but patterns of responses could be clearly identified in those animals that shared the same DRB3\* haplotype. Animals bearing the DRB3\*2002 haplotype shared recognition of regions E2<sub>259-276</sub>, E2<sub>265-282</sub>, NS3<sub>145-162</sub>, NS3<sub>313-330</sub>, NS3<sub>325-342</sub> and NS3<sub>619-636</sub> and animals bearing the DRB3\*0701 haplotype shared recognition of regions E2<sub>13-30</sub>, E2<sub>109-126</sub>, E2<sub>259-276</sub>, NS3<sub>61-78</sub>, NS3<sub>397-414</sub> and NS3<sub>505-522</sub>. E2<sub>259-276</sub> was recognised by both of the haplotypes examined and NS3<sub>397-414</sub> was located in a region that is structurally analogous to a previously published hepatitis C virus CD4<sup>+</sup> T cell epitope. For one determinant (NS3<sub>397-414</sub>) the stimulatory sequence was located to residues NS3<sub>400-409</sub>. A specific pattern of BVDV peptides recognised by cattle during maturation of the immune response could be detected from 6 weeks post-challenge.

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Evidence from numerous *in vitro* studies has demonstrated that ncpBVDV does not induce type 1 interferon (IFN) (5)(6)(7). An example of the effect of ncpBVDV on the type-1 IFN response pathway *in vitro* has been demonstrated by the enhancement of Semilike Forest Virus (SFV) plaque size on ncpBVDV infected cells. Plaques are increased in size most probably because of a reduced capacity of cells to produce IFN in response to SFV, when they are infected with ncpBVDV. Additional evidence for the failure of ncpBVDV-infected cells to produce IFN was provided by examination of the expression of the type-1 IFN induced protein Mx, which is induced by SFV. Mx protein was not expressed in SFV infected cells when they were also infected with ncpBVDV. Based on these and other *in vitro* observations it has been speculated that impaired IFN responses *in vivo* could result in exacerbation of infections with IFN-sensitive viruses.

The establishment of persistent infections with ncpBVDV is crucial for the maintenance of BVDV in cattle populations. It has been shown previously that foetal infection with cpBVDV does not result in persistent infection (8). A study was performed where 60-day-old bovine foetuses were challenged directly with ncpBVDV, cpBVDV or mock antigen. IFN was not detected in the amniotic fluid of the ncpBVDV-infected foetuses 3, 5 or 7 days after challenge. However, IFN was detected in the amniotic fluid of the cpBVDV-infected pregnancies on day 5 and day 7 post-infection, but was undetectable on day 3. Western blot analyses of lysates from foetal spleens were also used to demonstrate activation of the type-1 IFN induction pathway. A strongly staining Mx-specific band was present in the samples from foetal spleens harvested 5 days and 7 days after challenge with cpBVDV, but was absent from the day 3 sample. In the samples from the ncp BVDV-challenged pregnancies faint Mx- specific bands were present in the day 5 and day 7 samples, but only a very faint band was present in the day 3 sample. These findings strongly suggest that the ability of ncpBVDV to inhibit the induction of type-1 IFN has evolved to enable the virus to establish persistent infection in the early foetus.

A series of experiments were performed in gnotobiotic calves to determine the early cytokine response to ncpBVDV and relate the response to the immunosuppression caused by the virus. Gnotobiotic calves infected intranasally with ncpBVDV produced strong type-1 and -2 IFN (IFN- $\gamma$ ) responses. These responses were also associated with depressed serum levels of TGF- $\beta$ . Acute ncpBVDV infection of gnotobiotic calves, previously vaccinated with bacille Calmette-Guerin (BCG), resulted in the temporary suppression of two *in vitro* assays used to monitor the immune response to BCG. Lymphocyte proliferation and IFN- $\gamma$  production by whole blood culture with purified protein derivatives prepared from *M. avium* (PPD-A) and *M. bovis* (PPD-B) were markedly suppressed. Therefore, during the period after acute infection when ncpBVDV causes immunosuppression, there is also an induction of strong type-1 and -2 IFN responses. Work is progressing to establish whether infection with BVDV has a direct effect on antigen presenting cell function.

It has been suggested that the immune response to acute ncpBVDV challenge results in a predominantly Th2 type response (9). It has been further speculated that a Th2 CD4<sup>+</sup> T cell response might interfere with protective Th1 responses to other pathogens. However, we have found no evidence of a Th2-like response during the acute phase of the infection. To extend these studies the memory response to ncpBVDV was examined. *In vitro* proliferation assays using whole peripheral blood mononuclear cells (PBMC) from cattle immunised with Pe515nc virus were analysed for cytokine production using bovine IL-4, IL-10 and IFN  $\gamma$  specific ELISAs. Analysis of the supernatants revealed high titres of IFN  $\gamma$  production on Day 2 and 3 after antigenic stimulation. In contrast, IL-10 was detected on Day 1 after antigenic stimulation but not on subsequent days. IL-4 was not detected in any of the supernatants. The

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proliferative response and cytokine profile of PBMC stimulated either live or killed antigen was similar. These data suggest the memory response to ncpBVDV is also predominantly Th1.

In conclusion, the ability of ncpBVDV to inhibit the induction of type-1 IFN has evolved to enable the virus to establish persistent infection in the early foetus. In contrast, infection of calves with ncpBVDV induces strong type-1 and -2 IFN responses and biases the immune response towards a Th1 phenotype. In spite of generating a Th1 immune response, acute infection of animals with ncpBVDV results in the temporary suppression of two *in vitro* assays used to monitor an established immune response to BCG.

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## **Pestivirus contamination of bovine sera and other bovine virus contamination**

**IMMUNITY TO BVDV IN THE ABSENCE OF ANTIBODY:  
CONSIDERATIONS FOR VACCINE EVALUATION**

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Co-investigators on this project were Drs. Janice Endsley (ISU), Julia Ridpath (USDA, National Animal Disease Center, Ames, Iowa), and John Neill (USDA, NADC)

**INTRODUCTION**

Young calves are protected from clinical signs due to BVDV infection by passively acquired antibody derived from colostrum. Maternally derived antibody to BVDV has a half life of approximately 3 weeks and is usually catabolized by approximately 6 months of age. High titers of maternal antibody are capable of preventing the induction of an active antibody response to vaccination in calves. This has led to the conclusion that it is necessary to wait until maternal antibody has waned to effectively induce protective immunity.

Stimulating an immune response to BVDV in the presence of maternal antibody could generate a protective memory response to this virus at a much earlier age. Cell mediated immune responses to some pathogens of domestic animals can occur in the presence of maternal antibody and in the absence of a detectable humoral response. Protective immune responses mediated by T lymphocytes may be overlooked because there is no simple test to detect them. The objective of this trial was to determine if calves with a high level of maternal antibody to BVDV develop CD4+, CD8+, or  $\gamma \delta$  T lymphocyte responses to BVDV in the absence of a humoral immune response, and to determine if the antibody negative, T cell positive calves were protected from challenge with virulent genotype 2 BVDV.

**EXPERIMENTAL DESIGN AND RESULTS**

Twelve calves were fed pooled colostrum containing a high titer against BVDV for 48 hrs postpartum and six calves were fed milk replacer instead of colostrum. Calves fed colostrum developed high serum antibody levels due to uptake of antibody from colostrum. Calves fed milk replacer instead of colostrum after birth did not have antibodies against BVDV. Half of the calves in each group were exposed intranasally to a virulent BVDV (strain 1373, type 2) at 1-4 weeks of age and half were not exposed to virus at this age. The milk replacer-fed calves exposed to BVDV all died from the infection. The colostrum-fed calves exposed to BVDV did not show any clinical signs of BVDV infection.

Antibodies to BVDV1 and BVDV2 from colostrum fed calves declined at similar rates for the challenged calves compared to the unchallenged calves. The calves in all groups were sampled for T lymphocyte subset responsiveness to four strains of BVDV *in vitro*, approximately monthly, until maternal antibodies were no longer detectable (between 7-9 months of age). Peripheral blood CD4+, CD8+, and  $\gamma \delta$  T cells from animals in all treatment groups responded to *in vitro* stimulation with Concanavalin A by increasing expression of CD25, indicating that all T cell subsets were capable of responding. CD25 is the high affinity subunit of the interleukin-2 receptor and serves as a marker for cell activation. Antigen specific CD4+, CD8+ and  $\gamma \delta$  T cell activation in response to both BVDV1 and BVDV2 *in vitro*, as indicated by CD25 expression, was detected in calves challenged with BVDV2 while they still had high titers of maternal antibody. Variation of the response of the three subsets of lymphocytes to the four strains of BVDV was observed. The T lymphocyte responses of the calves fed milk replacer and not exposed to BVDV were not significantly ( $P < 0.05$ ) different from the T lymphocyte responses of the calves fed colostrum and not exposed to BVDV.

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Calves from all remaining treatment groups were challenged intranasally with BVDV2-1373 when maternal antibodies were no longer detectable in peripheral blood. The calves that had previously been exposed to BVDV when maternal antibody levels were high were protected from challenge compared to the animals that had not been previously exposed.

### **DISCUSSION**

This report demonstrates that calves challenged with BVDV while maternal antibody levels are high can develop BVDV specific T lymphocyte subset responses to *in vitro* BVD antigen. This T lymphocyte response occurred in the absence of a detectable humoral immune response to BVDV in the challenged calves. The individual T cell subsets from colostrum fed, BVDV challenged calves were all activated to some degree in response to the homologous BVDV genotype. Detection of CD25 expression indicates activation by specific antigen, but does not give any information on the function of responding T cells (Minami *et al.*, 1993; Quade and Roth, 1999).

The development of T cell memory to pathogens in the absence of an antibody response has been described for other viruses in animals with circulating maternal antibody. Calves challenged with bovine herpesvirus 1 (BHV-1) while maternal antibody levels were high had T lymphocytes that proliferated and secreted IFN  $\gamma$  in response to *in vitro* BHV-1 antigen in the absence of a measurable antibody response (Ellis *et al.*, 1996; Lemaire *et al.*, 2000). Antigen specific T cell proliferation or cytokine secretion in the absence of an antibody response has also been described in cattle vaccinated against bovine respiratory syncytial virus (Ellis *et al.*, 1996).

The results of the current trial demonstrate that calves can be seronegative for BVDV and still have immune memory due to an exposure to the virus during the presence of maternal antibody. This conflicts with the current practice of identifying BVDV naïve and susceptible animals based on the absence of acquired antibody. Currently, a non-persistently infected calf that is seronegative after maternal antibody has waned is considered to be immunologically naïve to BVDV. The existence of seronegative calves that have T cell memory to BVDV could significantly impact interpretations of immune responses of calves to BVDV infection or vaccination.

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## **Pestivirus contamination of bovine sera and other bovine virus contamination**

# **THE ROLE OF ANIMALS PERSISTENTLY INFECTED WITH PESTIVIRUSES**

Prof. J.-C. Brownlie (The Royal Veterinary College, Hatfield, UK)

### **SUMMARY**

Bovine virus diarrhoea virus (BVDV) is, undeniably, one of the most important viral diseases of cattle. It is a pestivirus and frequently contaminates foetal calf serum. To understand why this occurs it is necessary to review the complex pathogenesis and clinical disease of the virus. The paradox for clinicians is that clinical signs range from the inapparent to either severe haemorrhagic disease or fatal mucosal disease whilst the immunosuppressive effect of acute BVDV infections can enhance clinical disease from other pathogens. In recent years, there has been a growing awareness for major role of BVDV in reproductive loss; causing early embryonic loss, abortions and the birth of **persistently infected (PI)** calves.

It is the persistently infected animal that is at the centre of the pathogenesis of BVDV infections. We know that in the pathogenesis of mucosal disease, the two biotypes of the virus, non-cytopathogenic and cytopathogenic, act sequentially. The initial transplacental infection of the early foetus, with the non-cytopathogenic virus, can result in the subsequent birth of calves persistently viraemic for life with this biotype alone. Experimental studies have shown that the cytopathogenic biotype does not establish persistent infections. Persistent infection with the non-cytopathogenic virus occurs with at least 1-2% of all live calves born but the real incidence of in utero BVDV infection could be considerably higher, possibly greater than 10%. Thus, if batches of foetal calf serum contain sera from more than 10 foetal calves, there is a real danger of pestivirus contamination. Furthermore, it is the live born PI calves that represents the major reservoirs for viral persistence and infection.

### **INTRODUCTION**

Bovine virus diarrhoea virus (BVDV) is one of three named pestiviruses and widely considered as being a major cause of cattle disease worldwide. In the same genus are classical swine fever virus (CSFV) and Border disease virus (BDV). However, it is BVDV that frequently contaminates foetal calf serum. To understand why this occurs it is necessary to understand the complex pathogenesis and clinical disease of the virus. The paradox for clinicians is that clinical signs range from the inapparent to either severe haemorrhagic disease or fatal mucosal disease whilst the immunosuppressive effect of acute BVDV infections can enhance clinical disease from other pathogens. In recent years, there has been a growing awareness for the major role of BVDV in reproductive loss; causing early embryonic loss, abortions and the birth of persistently infected calves.

It is the persistently infected animal that is at the centre of the pathogenesis of BVDV infections. We know that they represent the major reservoirs for viral persistence and infection. They are also the only animals that can develop mucosal disease. However, the real question for this paper is how the serum from persistently infected animals, particularly those of foetal age, contributes to the contamination of commercial foetal calf serum products.

### **IN UTERO AND CONGENITAL INFECTIONS**

BVDV rarely infects the foetuses of sero-positive cattle. It is only during the viraemia of acute or persistent infections in sero-negative dams that the virus invades the placentome and replicates in the trophoblast before crossing to the foetus. In sheep, BVDV has been shown to damage the maternal vascular endothelium within 10 days of infection and the resulting cellular debris is

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ingested by the foetal trophoblast (Barlow 1972). This was considered to be a mechanism of virus transfer from ewe to offspring but may also account for the placentitis that leads to the high level of abortion following pestivirus infection. The time taken for the passage of virus in cattle from dam to foetus is variable but it has been recorded that abortions due to BVDV can occur within 10-18 days after intramuscular infection. Our own experience has shown that abortions can take place several months after foetal infection.

It could also explain the early embryonic death, infertility and "repeat breeder" cows that are often the sequel to pestivirus infection during pregnancy. In a herd infected with BVDV, conception rates were reduced from 78.6% in the immune cows to 22.2% in infected cattle (Virakul *et al.*, 1988). In a further study, BVDV infection at the time of conception reduced pregnancy rates at 77 days from 79% in the control animals to 33% in the virus-challenged group (McGowan *et al.*, 1993).

Most, if not all, foetuses born of PI dams become likewise persistently infected. This near 100% vertical transmission from dam to foetus is an important concept for those investigating disease outbreaks. Thus, the question to be asked of all PI animals is the virus status of their dams. However, the proportion of PI calves that are born to PI dams is reported only 7% (Grotelueschen *et al.*, 1998) inferring that the remaining 93% arise as a result of acute infection of the sero-negative dam in early pregnancy.

The outcome of foetal infection is dependant on two main variables; the age of the foetus at the time of infection and the biotype of the infecting virus. Infection during the **first trimester** (0-110 days) of foetal life can result in abortions, congenital damage or the birth of PI calves; during the **second trimester** (110-180/200days), there can be congenital damage and foetal loss whereas, during the **third trimester**, the foetus is immunocompetant and able to mount an active immune response.

The biotype responsible for in utero infections is non-cytopathogenic. Experimental infections during the first trimester have shown that up to 30% of foetuses are aborted even though the majority of the surviving foetuses go to full term and are born persistently infected (*personal observation*). In contrast, no animal has yet been demonstrated persistently infected with the cytopathogenic virus. Experimental *in utero* infection with the cytopathogenic biotype does not result in abortions or PI calves thereby giving doubt whether this biotype can even establish in the early foetus (Brownlie *et al.*, 1989).

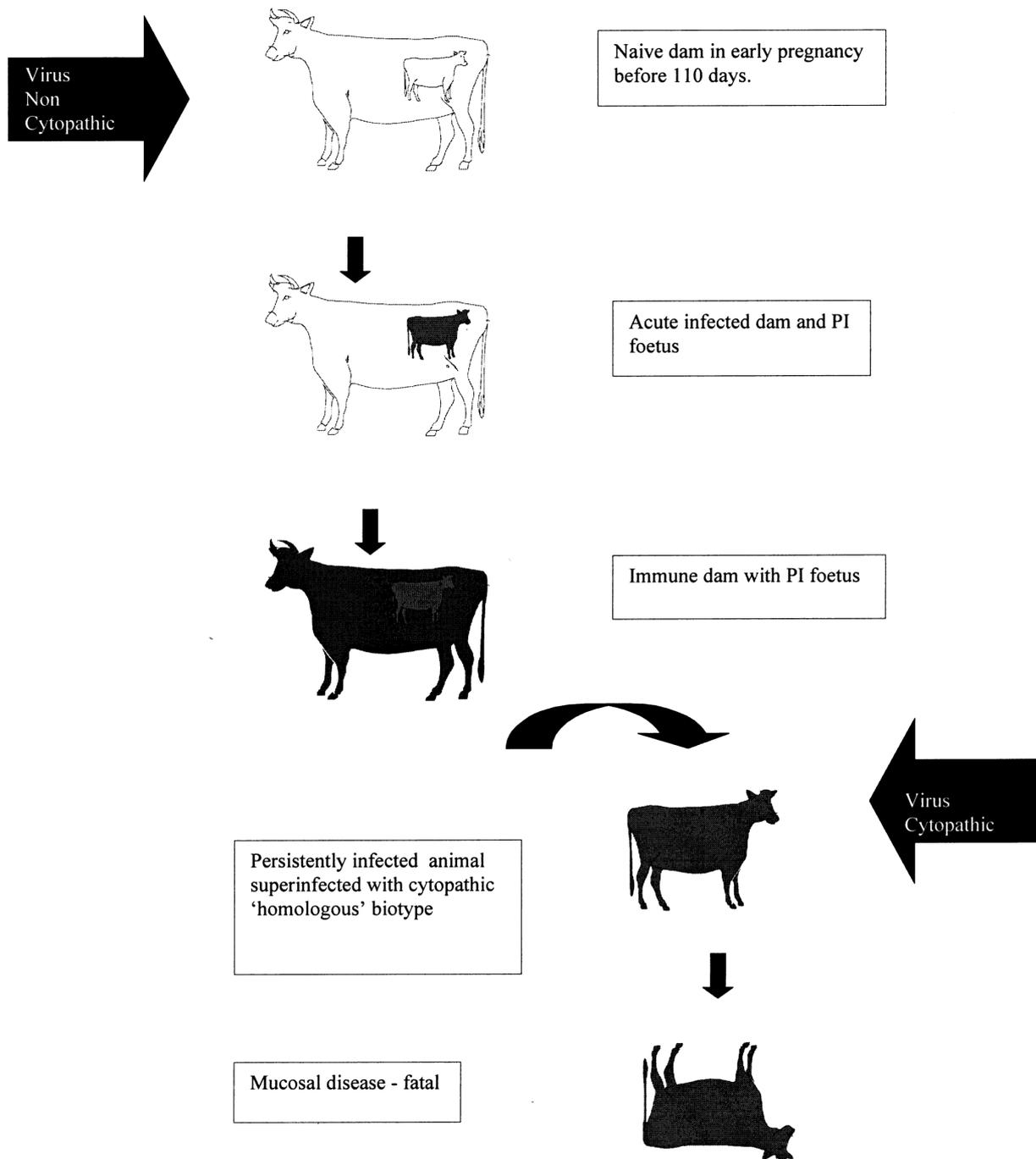
BVDV causes significant intrauterine growth retardation in many tissues of the foetus, particularly the CNS, the skeletal system and the thymus (Van Oirschot 1983). Hypomyelination in the CNS, associated with cerebellar hypoplasia, has also been observed. A further consistent finding is viral localisation in the vascular endothelium and, from the resulting vasculitis, there can be inflammation, oedema, hypoxia and cellular degeneration. Ocular lesions, primarily cataracts, has been observed in both field and experimental BVDV infections

### **MUCOSAL DISEASE**

Mucosal disease was first reported in 1953 and described as a fatal condition of cattle, characterised by severe erosive lesions in the oral and intestinal mucosa (Ramsey and Chivers 1953). Over the next thirty years a series of observations were made about the association of BVDV with mucosal disease.

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Figure 1 - Hypothesis for the pathogenesis of Mucosal Disease



These observations were finally refined into a hypothesis and proven experimentally (Figure 1) (Brownlie *et al.*, 1984). The hypothesis states that an initial transplacental infection of the early foetus, with the non-cytopathogenic virus, results in the birth of calf, which has a lifelong persistent viraemia. These calves (and only these calves) may later develop mucosal disease as a result of superinfection with a "homologous" cytopathogenic BVDV. In the field, mucosal disease usually affects animals of 6-18 months of age, although it has been reported in calves a few weeks old and adult cattle of 5-10 years.

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### **BVDV, GERMLINE CELLS AND THE BULL**

BVDV has a tropism for the germline cells of both sexes. The virus can infect ovarian tissues and has been demonstrated in oocytes within ovarian follicles (Brownlie *et al.*, 1997). Similarly, Border disease virus antigen has been found in the germinal cells of the sheep ovary. The longevity of viral infection in the ovary is unclear but viral antigen has been demonstrated in the ovaries of cattle for at least 60 days after intramuscular inoculation. The risks that germline cell infection will lead to vertical transmission of virus are, as yet, unproven but the implications are obvious.

The bull can play an important, if sometimes overlooked, role in BVDV disease. All PI bulls produce semen that is infected with BVDV and, therefore, it is inexcusable for any health check of the bull not to include a blood test to examine for persistent BVDV infection.

Acute infection of the sero-negative bull is not without risk. BVDV infects testicular tissues and virus can be recovered from semen for a limited period (Paton *et al.*, 1989). The semen is often of poor quality and has the potential to spread infection to sero-negative heifers.

Recently, a further potential consequence has been demonstrated following an acute infection in a young bull (Voges *et al.*, 1999). The bull appeared to become infected during adolescence (possibly at 6-9 months of age), at which time the virus crossed the blood/testes barrier to the testis. Although the bull made antibodies to the virus, they were unable to cross the testes barrier; thus the virus was able to establish a persistent infection in the seminiferous tubules. In this case, virus was continually shed in the semen over a prolonged period of time (between 7 and 22 months of age). On normal blood screening techniques, this bull would have been considered immune and therefore not to be shedding BVDV in semen. Although a further bull has been described (Bruschke 1999, personal communication), the incidence of virus persistence in the testes of seronegative bulls is presently unknown.

### **ESTABLISHING RISK FOR PESTIVIRUS CONTAMINATION OF COMMERCIAL BOVINE SERA**

With a better understanding of BVDV pathogenesis, it is now easier to establish the risk of viral contamination of bovine sera. It is widely acknowledged that persistent BVDV infection occurs in about 1-2% of all calves born live (Bolin *et al.* 1985; Howard *et al.*, 1986.). Most estimates of risk are based on this incidence (Houe 1995). However, when foetal infections, rather than post-natal infections, have been considered the incidence of persistent infections is significantly higher, possibly greater than 10% (Nettleton 1985). Thus, if batches of foetal calf serum contain sera from more than 10 foetal calves, there is a real danger of pestivirus contamination. Furthermore, it is the live born PI calf that represents the major reservoir for viral persistence thereby providing the source of BVDV acute infection and, as a consequence, 97% of foetal infections. This cycle has to be broken if we are to reduce or eliminate foetal BVDV infections.

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**Pestivirus contamination of bovine sera and other bovine virus contamination**  
**BACKGROUND KNOWLEDGE OF PESTIVIRUS CONTAMINATION OF**  
**VETERINARY VACCINES**

Dr P. Vannier (Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, F)

In spite of considerable progresses accomplished in the manufacture and control of vaccines, failure or iatrogenic infections can occur. The pestivirus contamination of vaccines remains the major problem faced by the manufacturers and the competent authorities involved in the production, the registration and control of vaccines.

**CONTAMINATION OF VACCINES BY PESTIVIRUSES**

Numerous cases of iatrogenic infections induced by pestivirus contamination of veterinary vaccines were described. A recent case (1999) showed that this problem remains a real and actual one.

**Contamination of live IBR marker vaccine by Bovine Viral Diarrhoea Virus (BVDV).**

This recent case appeared in different European countries and more acutely in the Netherlands, had dramatic effects as it occurred in the framework of a campaign of compulsory vaccination against Infectious Bovine Rhinotracheitis (Report from Food and Veterinary Office – D.G. Sanco – 12/07/00). On March 9, 1999, a rapid alert was activated in all European Union Member States concerning the occurrence of serious disease – symptoms Bovine Viral Diarrhoea (BVD) alike, following vaccination with batch WG 4622 of an IBR-Marker Vivum vaccine. This IBR Marker vaccine is a modified live marker vaccine intended for intranasal and intramuscular administration to cattle in order to actively immunise the animals against the respiratory disease caused by Bovine Herpes virus 1 (BHV1).

On a number of Dutch cattle farms, where the above-mentioned batch of the IBR Marker vaccine had been inoculated according to the manufacturer instructions, a drop in milk yield, diarrhoea and death cases had been recorded approximately 10-15 days after vaccination.

On February 26th, the Dutch Central Veterinary Institute (ID-DLO) had already detected the presence of a BVD virus (BVDV) in that particular batch; by the time the rapid alert system had been initiated, about 500 animals had either died or been slaughtered.

On March 13th, Italian Authorities were informed that two batches originating from the same bulk materials as WG 4622 (respectively batch n. VF 4456, and 02U056 as sister batch) had been distributed in Italy. From official inquiries, it resulted that only one vial of 02U056 batch of vaccine had been used, on March 12th, to vaccinate 10 out of 192 animals (142 adult cows and 50 heifers and calves) present in a beef farm in northern Italy.

Within 12-13 days, a number of animals (unfortunately, it was not possible to differentiate these animals in the whole vaccinated herd) showed symptoms of BVD disease. Temperature, reaching 41.5°C and foul copious greenish-yellow diarrhoea (blood tinged, in one case) was recorded in seven animals; nine animals showed a slight to severe depression (up to total immobility of five heifers sharing the same box), slight to copious mucopurulent nasal discharge and anorexia associated with heavy weight loss; agalaxia was present in three milking cows. Twenty days after vaccination, abortion of a dead calf occurred in a pregnant cow, which had not presented any further symptom of disease (Falcone and Tollis, 2000).

The detection and characterisation of a highly virulent BVDV type II (BVDV II) from three vials of batch 02U056 (received directly from the manufacturer) was completed within 10 days by two different methods based on genomic criteria.

In the first test, a RT-PCR assay (Sullivan and Akkina, 1995) based on the amplification of a conserved region of pestivirus genome was carried out; a 826-bp DNA product was detected in

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the vaccine sample indicating the presence of a pestivirus. A second round of amplification with type specific primers generating internal fragments whose size is characteristic for each virus type (BVDV I, BVDV II), allowed to allocate the vaccine contaminant to BVDV II genotype (Falcone and Tollis, 2000).

In the second test (Ridpath *et al.*, 1994), a nested RT-PCR reaction was performed to amplify a region from the 5' UTR.

The first-round primers allowed the authors to differentiate BVDV from other pestiviruses. The second round primers selectively amplified BVDV II but not BVDV I sequences, thus confirming the presence of a BVDV II in the vaccine preparation under test.

Confirmation of the results obtained in RT-PCR was further accomplished by direct sequencing the amplified product from partial 5' UTR (Ridpath *et al.*, 1994). A high level of homology in the sequence of the contaminant virus with BVDV II strains was demonstrated together with the evidence of the loss of an internal PstI restriction site (only present in all known BVDV I 5'UTR sequences (Harpin *et al.*, 1995). Finally, two specific nucleotide substitutions, characteristic of highly virulent BVDV II isolates (Topliff and Kelling, 1998), were detected in the sequenced 5'UTR region (Falcone and Tollis, 2000).

### **Contamination of a live classical swine fever vaccine by a BVD virus.**

In September and October 1984, an increased death rate was observed in piglets of up to five weeks old born to sows which had been vaccinated four months earlier against swine fever (SF). Since the observed signs resembled those of congenital SF infections, samples from eight affected herds were sent to the ID-DLO for laboratory diagnosis of swine fever virus (SFV). The immunofluorescence test (IFT) performed on frozen tissue sections was positive. The diagnosis of SF was supported by virus isolation in PK-15 cell cultures. However, the IFT, which uses conjugated anti-SFV immunoglobulins produced in pigs, does not discriminate between infections with SFV and with bovine viral diarrhoea virus (BVDV) or Border disease virus (Wensvoort and Terpstra, 1988).

Affected litters with IFT-positive piglets were encountered on eight breeding farms (1 to 8) between September 7 and October 12, 1984. All these litters were born to sows, which had been vaccinated with vaccine A, batch A-1, between May 28 and June 11, 1984.

On all eight farms affected piglets were found only in the offspring of sows vaccinated with vaccine A, batch A-1 in the first trimester of gestation. At birth many litters contained dead and mummified fetuses and often the living piglets showed alopecia, ascites and congenital tremors. In the affected litters, piglets up to five weeks old were found which showed diarrhoea, petechiae in the skin, blue eartips and retarded growth. At necropsy these piglets often had multiple petechiae, haemorrhagic lymph nodes and occasionally button ulcers in the colon (Wensvoort and Terpstra, 1988). When cultured in PK-15 cells both batch A-1 and batch A-2 were positive in the IFT after one passage.

The signs resembling congenital swine fever on the eight farms were caused by a pestivirus, which was not recognised by the two MCAs used in the differential diagnosis of SFV and BVDV infections in pigs. The isolates appeared to be different from the SFV strains causing the 1983 to 1984 epizootic. On all eight farms the sows had been vaccinated with SFV vaccine A, batch A-1, which was the reason why this batch was examined for possible contamination with BVDV or Border disease virus.

It was shown by rabbit inoculation that, batch A-1 did not contain the expected C strain virus in detectable amounts. The majority of the fattening pigs vaccinated with batch A-1 raised antibodies against BVDV, while fewer pigs raised antibodies against SFV, indicating that batch A-1 contained a virus which resembled BVDV more than SFV. From both batch A-1 and from

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another batch, a pestivirus could be isolated in PK-15 cells. This was detected by the IFT, using a swine anti-SFV conjugate. This positive virus isolation was expected since vaccine A is produced in secondary lamb kidney cells and has thus been adapted to cell culture. However, characterisation with monoclonal antibodies of the viruses isolated from the two A batches revealed a completely different pattern. The other batch was recognised by MCA 2, 3, 4, 7, 9, 10, 11 and 13, a pattern consistent with C strain virus (G. Wensvoort, unpublished data), while batch A-1 was not recognised by any MCA used. Thus, the pestivirus in batch A-1 was not the expected C strain but a contaminant.

This contaminant was shown to have an unexpectedly high virulence for sheep after postnatal infection. Three of the eight lambs infected with batch A-1 died spontaneously, and one was slaughtered when moribund. All four were positive by IFT and the antigen was not recognised by MCA1 and MCA2. All four lambs which did not die developed neutralising antibodies against BVDV.

The eight lambs inoculated with two other batches of vaccine virus did not show any clinical signs. Two lambs developed antibodies against SFV strain Brescia, indicating replication of the C strain virus, while none developed antibodies against BVDV. The field-isolate from farm 6 did not evoke clinical signs of SFV in for eight-to 10-week-old specific pathogen free pigs, but two developed antibodies to BVDV. From these data it is concluded that batch A-1 contained BVDV or Border disease virus (BD virus).

The BVDV or Border disease virus contaminant could have originated from the cultures of lamb kidney cells used to produce batch A-1. The virus might have originated from the serum used in the growth medium, or from cell cultures derived from kidney tissue of a lamb persistently infected with Border disease virus (Wensvoort and Terpstra, 1988).

### **Contamination of a live Aujeszky's disease vaccine by an ovine pestivirus.**

Since January 1984, various disorders occurred in piglets and breeders, in herds located in North and (more often) in West of France. In piglets, the symptoms were quite constant: eyelids oedema, locomotor disorders and signs of decay. The piglets were most often 10 to 15 days old, and the signs were sometimes associated with some variable symptoms, such as arthritis and diarrhoea (according to the herd). In sows, repeat-breeding was often observed.

At necropsy, the haemorrhagic lesions in piglets dead spontaneously, or killed, were similar to those observed in Classical Swine Fever (CSF) infection. The number of piglets affected was high and the mortality rate in the litters of each group of sows varied between 30 and 70 %.

In some herds, a virus was isolated, which reacted with CSF antibodies. However, the development of this virus in the cell line Pig Kidney 15 (PK15) was somewhat different of those of CSF strains isolated during outbreaks of the disease. Moreover, in most affected herds, CSF neutralising antibodies were observed in the blood of sows, although with antibodies titre relatively low and generally lower than observed in CSF outbreaks.

Two main hypothesis might explain these disorders:

- 1) CSF low virulence strains might have been responsible for these troubles,
- 2) all the herds concerned had been vaccinated against pseudorabies (Aujeszky's disease) with a live virus vaccine. The same batch of the vaccine production, batch 33D04, has been used in these herds. Moreover, the intensity of the symptoms was connected with the time of injection of the vaccine in pregnant sows: the clinical signs were more serious if the sows had been vaccinated during the first third of the pregnancy. The possible contamination of the vaccine with some pestivirus would then be tested.

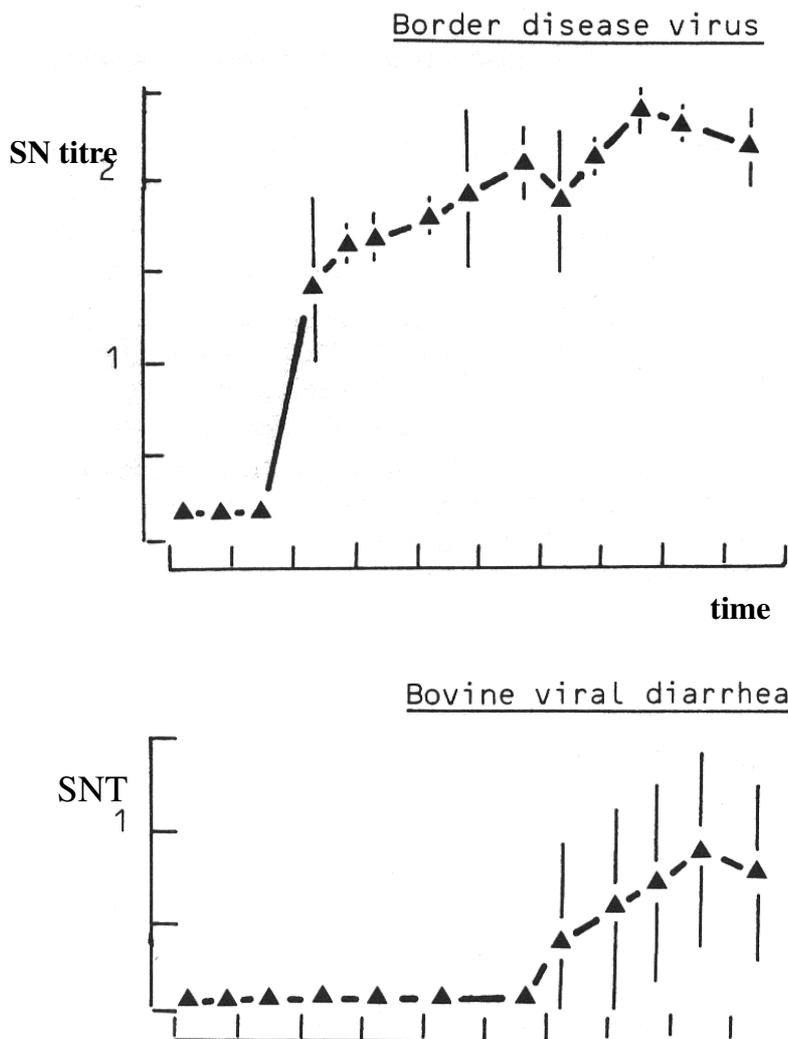
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These symptoms were also observed in herds closely isolated from outside contamination, thus, the epidemiological data did not favour the first hypothesis, and further investigations (experimental inoculations with the suspected vaccine) were necessary to test the second hypothesis (Vannier *et al.*, 1988).

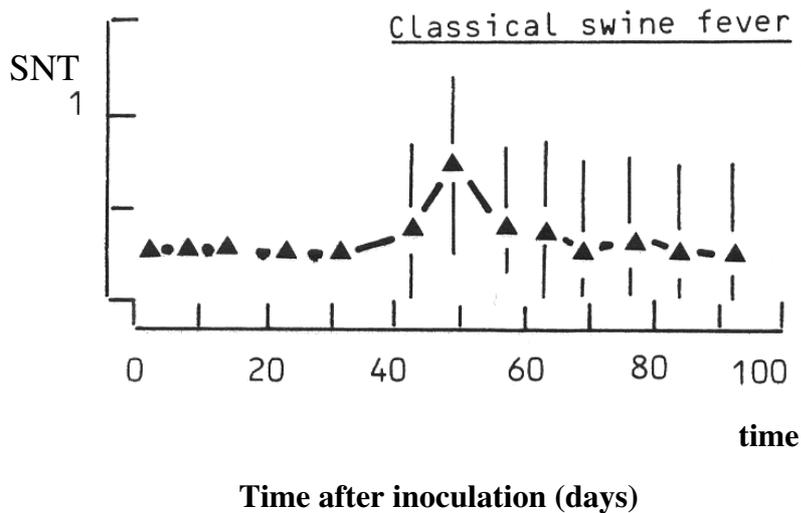
In all the experiments, after injection of the batch 33D04, a pestivirus seroconversion was observed, together with the expected pseudorabies seroconversion. A virus was isolated from the blood or spleen of piglets born from an inoculated sow. This virus was embryotoxic but has little pathogenicity for the pig. Although the lesions observed are similar to those of Classical Swine Fever, neither the seroconversion data nor the culture of the virus supported the identification with CSF. The immunological data suggested a close connection of the virus with Border disease virus and lesser relation to BVD virus.

The hypothesis of Border disease virus is the most consistent with the data, although it suffers also some difficulties. The immunological connection with BD virus of the antibodies found in vaccinated animals is certainly the stronger argument: the serological response obtained with the Moredun strain of Border Disease virus is early, homogenous and intense. Conversely, if tested against NADL strain of BVD virus and the Alfort strain of CSF virus, the neutralising antibodies titres are late, variable and low (Fig. 1).

**Figure 1: Kinetics of SN Antibodies to 3 pestiviruses in the serum of 4 piglets inoculated with virus isolated**



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As in the previous example the border disease contaminant could have originated from the cultures of lamb kidney cells used to produce the vaccine.

### Other examples of final batches of vaccines contaminated by pestiviruses.

Five dairy goat herds (1 to 5) within a district in south-western Norway suffered reproductive failures during the breeding season of 1983 to 84. The goats had been vaccinated in early pregnancy with an experimental Orf (contagious pustular dermatitis) vaccine. The vaccine, was later proved to be contaminated with ruminant pestivirus (Coken T., Krogsrud J. and Bjerkas I., 1991).

The Orf vaccine included infective orfvirus (ovine parapoxvirus) propagated in ovine CP cells grown in a medium containing commercial foetal calf serum. From controls performed on final batches of vaccines by National Veterinary Services Laboratories (APHIS, USDA) between 1981 and 1991 following results were obtained: (BVD positive tests/tested/eligible to be tested (% positive of those tested) : 6/169/281 (3.6 %), 1/61/299 (1.6 %), 2/66/409 (3 %), 0/55/436 (0 %), 0/48/354 (0 %), 0/41/NA (0 %), 0/43/384 (0 %), 0/32/265 (0 %), 0/25/161 (0 %), and 0/16/151 (0 %), respectively (Levings and Wessman, 1991).

From a significant number of batches tested, the number of infected ones remains low and the infected ones were detected between 1981 and 1983.

In another study, bovine live virus vaccines were examined included those against adenovirus infection, Akabane disease, Ibaraki disease and infectious bovine rhinotracheitis (IBR), and a combined vaccine against IBR, BVD and parainfluenza 3 virus infection (PI-3). Porcine live virus vaccines examined include those against CSF, Japanese encephalitis, parvovirus infection, Aujeszky's disease (pseudorabies), and transmissible gastroenteritis (TGE), and a combined vaccine against CSF and swine erysipelas. The vaccines were produced by several manufacturers in 1993 (Harasawa, 1995).

RNA extracted from the live virus vaccines was submitted to the RT-PCR. Among the virus vaccines that contain modified live pestivirus strains as principal components, such as the tripled combined vaccine against IBR-BVD and PI-3, the CSF vaccine and the combined vaccine against CSF and swine erysipelas were positive in the two rounds PCR. The bovine and swine live virus vaccines prepared by using porcine cell cultures were all positive for the pestivirus RNA in the RT-PCR. All the monovalent bovine live virus vaccines (except for the adenovirus vaccine) while they were not produced in bovine cell cultures were also positive for the

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pestivirus RNA. Among the monovalent swine live virus vaccines (except for the CSF vaccine) the Aujeszky's disease vaccine and the two Japanese encephalitis vaccines from different manufacturers were positive. The nucleotide sequences were homologous (over 75 % homology) but were not identical to those of the BVD vaccine strains NADL (Harasawa, 1995).

### **CONSEQUENCES OF PESTIVIRUSES CONTAMINATION OF VACCINES**

Consequences can be limited when there is no iatrogenic pathology induced by the contaminated vaccines. The absence of adverse effects depends, mainly, on the target species where vaccine is used, on the category of animals vaccinated; it is clear that dramatic effects can be induced when pregnant females are vaccinated with the contaminated vaccine. At the end, the pathogenicity of the pestivirus strain contaminating the vaccine plays a role in the intensity of the adverse effects, which are observed.

Nevertheless, even where there is no pathological consequences to the infection of the contaminated vaccine, the consequences can be dramatic when the contamination interferes with a prophylactic programme. Especially, in pigs, BVD or BD contamination induce antibodies cross-reacting with classical swine fever virus, which can compromise an eradication programme. Such a situation shows that the pestivirus contamination is not only a major problem for live virus vaccines, but also for inactivated vaccines. Indeed CSF antibodies can be induced after the 2<sup>nd</sup> or 3<sup>rd</sup> booster injection of the BVD contaminated vaccine (Holm-Jensen, 1981). The previous examples showed that the contamination can induce a real iatrogenic disease similar to an epizooty or an anademy. Using an Aujeszky's disease vaccine in pigs contaminated by BD virus, dramatic effects were observed in the breeders: transplacental infection, birth of immunotolerant piglets which spread the virus to other animals, mortality after birth, clinical signs and lesions similar to classical swine fever, appearance of CSF antibodies in the serum of vaccinated breeders (Vannier *et al.*, 1988).

Besides these direct effects, the psychological impact of such a situation is dramatic on the farmers and the situation becomes very difficult to manage by the competent authorities especially when the contaminated vaccine is used in a compulsory programme. The credibility of the programme is lost as well as the confidence in the competent authority. Moreover, such a situation creates a real psychosis among farmers who are associating strongly all types of pathology with the use of the contaminated vaccines: it was the case in France in 1984; it is the case in the Netherlands, at the moment, after the use of the IBR contaminated vaccine: a wasting syndrome occurring on the cows is associated systematically to the contaminant.

The consequences are also dramatic for the manufacturer who has to pay very high compensations and for which the marketing picture is seriously affected.

### **ORIGIN OF THE PESTIVIRUSES CONTAMINATION**

The cells are very often contaminated by the BVD virus. Wellemans and Van Opdenbosch, in 1987, showed that 5 out of 8 cell lines and 35 out of 158 primary testis or kidneys cells (from calves) were infected by the BVD virus. The (fatal or not) calf serum used as a nutritive component of cell medium coming from permanently infected immunotolerant calves is the main source of infection of the cells.

Master cell stocks tested in fiscal years 1986 through 1990 were: 16 (1 positive), 13, 11, 23 and 22, respectively. None were positive in fiscal years 1987 to 1990 (Levings and Wessman, 1991).

The inquiry performed by Levings and Wessman (1991) showed that: the frequency of BVD virus or antibody detection in 1 litre lots containing sera from no more than two calf foetuses in fiscal years (October 1 to September 31) from 1982 through 1990 were (positive/tested (%))

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positive)): 34/120 (28 %), 161/508 (31.7 %), 83/285 (29 %), 98/394 (25 %), 176/240 (73.3 %), 62/312 (20 %), 107/211 (50.7 %), 17/22 (77 %), and 115/132 (87.1 %), respectively.

The frequency of virus detection from ultra centrifuged pools of four virus and antibody-negative sera in fiscal years 1985 through 1990 were (positive/tested (% positive)): 25/48 (41.4 %), 11/53 (20.8 %), 8/22 (36.4 %), 0/0 (NA), 16/40 (40 %), 1/2 (50 %).

These results confirm that the frequency of BVD contamination of bovine serum is very high and explain the high probability of contamination of cells multiplied with nutritive medium added with bovine or calf serum.

Moreover even when irradiation, ultra filtration and treatment with  $\beta$  propiolactone have been used on commercially available foetal bovine serum to inactivate infectious viral contaminants, non-cytopathic BVD virus has been isolated (Bolin *et al.*, 1991).

All these information demonstrate the importance of the problem of the risk of pestiviruses contamination of vaccines and the difficulties to manage it.

### **CONCLUSION**

The risk of pestivirus contamination of vaccines is probably the highest one. When a contaminated vaccine is used on pregnant females, an iatrogenic pathology can be induced whereas breeders have pestivirus antibodies in their serum, which can interfere with prophylaxy such as classical swine fever.

Even if the level of requirements applied to the vaccines manufacture increased considerably in Europe these ten last years with the implementation of 92/18 directive, it is clear that accidents always occur mainly because of contamination of bovine serum and also because infected cells had been used to multiply vaccinal strains. Nevertheless, thanks to the requirements from E.U. and in the European Pharmacopoeia monographs, the use of primary cells to produce vaccine batches has been progressively banned when it was possible. Moreover, it seems the procedures used to inactivate bovine serum are not always efficiently applied or are not validated. Pestiviruses are certainly among the most difficult viruses to isolate, to identify and it can be supposed that vaccines manufacturers have difficulties to handle it when they have no sufficient experience about this viral genus.

### **ACKNOWLEDGEMENT:**

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### **VIRAL CONTAMINATIONS OF BOVINE SERUM**

Dr D. MacKay (VMD, Addlestone, U. K.)

#### **ABSTRACT:**

Although only rarely included in the formulation of the final product, bovine serum is widely used in the production of many veterinary medicinal products. Due to issues related to both human and animal health, there has been increasing pressure to reduce or eliminate the use of bovine serum in medical products. However, at the present time, many veterinary vaccines can still only be manufactured if bovine serum is incorporated into the media used in the production of the biological active ingredient. Therefore, whilst this remains the case, the quality of the serum used becomes an important criterion in the quality of the product as a whole. One of the most important elements of the quality of the serum is freedom from extraneous agents and ensuring the quality of serum used in vaccine production is a process of risk reduction rather than risk elimination. The three essential elements of risk reduction are control of the origin, control of the testing, and control of any inactivation procedures applied to the serum to eliminate or reduce any potential contaminants.

Of these three elements, control of the origin of the serum is the most important, as elimination of the possibility of contamination at source is obviously the most effective means of ensuring freedom from extraneous viruses. However, for some ubiquitous or widespread pathogens (e.g. BVD, IBR) it is difficult on practical or financial grounds to assure freedom at source and therefore measures have to be taken either to ensure freedom at a batch level and/or to treat the serum to reduce further the risk. Inevitably, attention is focussed on the presence, and inactivation, of known extraneous agents but it is important to bear in mind that unknown agents might also be present and, where possible, measures should be put in place to cover this contingency.

The starting point for attempting to minimise the risk of contamination by testing is the realisation that testing can never be carried out sufficiently stringently to assure complete freedom from infection. The risk of contamination therefore becomes a statistical probability that can be reduced in proportion to the measures taken. The reduction in risk depends on the performance of the test (sensitivity, specificity, reproducibility) and the sampling level used. Maximal assurance is obtained from the use of a fully validated test which is applied at an adequate sampling level before inactivation. Testing can either assure freedom from infection with the required degree of statistical probability or can be used to demonstrate the presence of a particular agent at a level below that which has been established as being completely inactivated by the inactivation procedure applied.

A wide variety of methods of treatment to reduce or remove potential contaminants in serum are available, from heat treatment to the addition of chemical inactivants. The method chosen will depend on the level of assurance required and the use to which the serum will subsequently be put. Any method is inevitably a compromise between effective inactivation and retention of the activity of the serum, as these two factors are inversely related. What is essential is that the method applied is fully validated against the range of viruses that might be present as potential pathogens and that sufficient controls are included to ensure that the technique has been applied correctly.

PRESENTATION NOT AVAILABLE

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### **DISCUSSION SESSION I**

Prof. J-C.Brownlie: When you look at the genetic diversity, it appears that the border disease is a long way away from BVD 1 but when you look at monoclonals the border disease was very close to BVD 1.

Dr D. Paton: Border disease is more closely related to classical swine fever and slightly less related to BVD 1 and 2.

Mr P. Castle: On the slide with the antigenic relationships it was surprising to see how close they were and how far away type 2 was.

Dr D. Paton: The BVD 1 cluster is quite a heterogeneous cluster and so there was a spread of isolates. Some of them are close to Border disease virus near the middle of the map. When you are doing a mapping study, with this computer programme, the viruses in the middle are the ones which do not react within any of the monoclonals, therefore, their position is more uncertain.

Dr M. Chudy: You mentioned BVD 2 strains with low and with high virulence. What is the view on the virulence hypothesis of the genetic determination in the 5' UTR of the virus, like regions of the D domain?

Dr J. Ridpath: We have found this not to be true. If you look at enough BVD you will find ones which do not follow this rule and we did find quite a few which did not. We have found both virulence viruses that have the change that would indicate virulence and high virulence viruses that did not have the changes.

Dr D. Paton: Is your prong horn isolate recognised by the pan pestivirus reactive monoclonal antibodies?

Dr J. Ridpath: I have not used the pan pestivirus, I have used Dr De Bovies' C15 and it does weakly react with it. It does seem that there is some antigen reactivity. How far out do we let these things go and we still call them Pestiviruses, are we going to go with antigenic cross-activity or genomic organisation, do you have an opinion?

Dr D. Paton: I would have thought that if it is still antigenically cross-reactive and it has the same genetic organisation, yes it is a Pestivirus.

Question from the floor: Do we have an explanation for these enormous differences in the prevalence of the BVD type 2 between north America and the rest of the world?

Dr D. Deregt: It seems to have originated in North America. However, the earliest reported BVD from France in pigs, was in the 1960's. We do not know the origin of type 2. Recent introductions in Europe may have originated from North America.

Dr Hamers: I believe that North America is vaccinating much more against BVDV than Europe and most vaccines only contain type 1, would this be an explanation of selection of the genotype 2?

Dr D. Deregt: The problems of isolations between 1980 and before the outbreaks was about 20% - since the severe outbreaks, they have been very diligent in vaccinating. There have been reports that reproductive disease is not being controlled adequately with predominantly type 1 vaccines. You see a lot of PI animals with type 2 in herds that have been vaccinated with type 1 and it is not being controlled.

Dr D. Paton: On the origin of BVD type 2 I think that a possibility has to be another introduction from one of these exotic species - another prong-horn antelope or something. One comment concerning Dr Deregt's presentation. If you are trying to prove persistent infection you have to wait a long time, may be more than three weeks. Certainly, our experience recently, is that if

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you are using very sensitive methods such as nested PCR, you may have to wait a very long time. From experimentally infected animals, which we have looked at, you can detect viraemia even up to 80 days after acute infection in the blood.

Dr R. Gudding: A comment to Dr Dereg on prevalence of positive herds. Your data from Sweden and Norway are a few years old, they are quite successful in their eradication programmes and both are approaching Finland in prevalence of positive herds. In Norway, it is approximately 1% of the herds being sera positive.

Dr D. Dereg: This is the most recent data, which I could find. When I first heard about these programmes, I wondered how well they would work and it seems that they can be successful. We would not probably want to attempt this in North America where we have type 2, some kind of eradication with vaccination might some day be attempted.

Prof. J. Van Oirschot: Did you challenge the animals intranasally?

Dr J. Roth: Both challenges were intranasal.

Prof. J. Van Oirschot: Were the challenge titres high?

Dr J. Roth: There were  $10^6$  virus in the challenge.

Prof. J. Van Oirschot: Am I correct in thinking that I saw quite a considerable level of non specific activation in the first 10 weeks in animals which did not get the virus but had a high index. Is this because of the colostrum, non-specific substances, which activate T cells?

Dr J. Roth: This may be one explanation, however, we did not have a control to be able to be sure. In the first ten weeks we were using a formalin to fix our cells that was not as pure as it should have been and so we switched to a different formalin and our backgrounds dropped. I think that this is an artefact, but I do not have the appropriate controls to know for sure. The point made concerning intranasal - it may be that vaccines given intra-muscularly in the presence of maternal antibody may not do this because it may not replicate well enough. Whereas, perhaps, intranasal or mucosal exposure may be more efficient in inducing this response in the presence of the maternal antibody.

Dr P. von Hoegen: Dr Roth suggested having standard T cell assay to monitor the immune response - how would you do that? Would you propose the facs analysis or simpler assays?

Dr J. Roth: The facs analysis is still quite cumbersome. We did not look at cytokine response at all. This is a very important component of cell mediated immunity. We are therefore, not evaluating the whole range of cell mediated immunity. We are trying to streamline this assay to make it easier - we can however, only do about 15 animals a day now and this assay has a fair amount of variability. Therefore, you have to repeat it in the same animals to make sure that you are not seeing artefact and then always run the control and the principle animals on the same day to avoid day-to-day variability. There are limitations to the assay but we think that we can streamline this further to make it more practical.

Dr P. von Hoegen: When looking for the T cell activation of the serum but we have heard that local mucosal immune response might be more relevant as it is at the site of infection. How would you trace these T cells as mucosal?

Dr J. Roth: We are looking at peripheral blood lymphocytes not the other lymphocytes and only 1 to 2% of the body's lymphocytes are in the blood. The ones in the tissues are probably even more important, we are using this only as a marker of T cell responsiveness. It would be very interesting to use tissue T cells and get information about how they are responding at mucosal surfaces - especially the gamma delta T cells that are prominent at mucosal surfaces. One of our

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goals is to have an assay that can be used almost like antibody titres to monitor response and the number of animals over periods of time.

Dr P. Marbehant: Does it appear that the strain 890 is a good candidate for vaccination, preferable to other strains of BVD 2?

Dr J. Roth: I do not want to extrapolate that far - it would seem to be better in vitro because in vitro it seems not to interfere with the lymphocyte responses as much, but I cannot extrapolate this for vaccine development.

Dr P. Marbehant: From your experiments, are you questioning the use of seronegative cows for evaluating the efficacy of vaccines?

Dr J. Roth: Yes, there is a danger of misinterpretation if there are a lot of seronegative calves with reactive T cells. It would be best to pick seronegative calves who have either, never received colostrum or come from herds that are known to be BVD negative, to be certain that you do not have any T cell responses. If you do not do this, as long as there are control animals from the same herd that are fully susceptible to BVD, it depends on your randomisation, to hope that the ones which were vaccinated were also fully susceptible.

Dr D. Paton: Your figures on transmission to cows in utero 7% being from PI dams and 93% being from acutely infected dams - what is the basis of this calculation?

Prof. J. Brownlie: This is someone else's fine data who reported at the Cornell symposium. This was looking at persistently infected calves and going back to bleed their mothers. This data therefore is pretty secure.

Dr M. Moos: It has been pointed out by different speakers that the persistently infected calf has a key role. In Group 15V of the European Pharmacopoeia, we have prepared a draft for inactivated BVD vaccines. The problem, which I have with this draft, is that in the potency test with 10 cows and their offspring, one persistently infected calf is seen as acceptable. On the other hand, two vaccine producers could demonstrate 100% protection of all 10 calves, with the help of their vaccines. Would you say that a registration authority should, under these circumstances, accept a persistently infected calf in the potency test?

Prof. J. Brownlie: You could answer this by saying that if you were a farmer and you had a big, clean herd, would you mind having just one persistently viraemic animal on that herd? The answer is no, as one persistently viraemic animal actually is the way that you get 100% persistently viraemic animals. This is however, not so simple.

Dr J. Ridpath: Do you know why the gamma radiation is failing, through poor practices or is the virus more refractive to inactivation?

Dr P. Vannier: The first problem is the validations of the inactivation process to be carried out. This is not so easy to get good validation done by manufacturers when they are treating the serum or sub-treating the serum by other companies. The problem is how to manage and to control the validity of these inactivation processes. You have to have the right dose in the mass of the products, sometimes your dose is not at the right level to be capable to induce inactivation.

Dr M. Tollis: Dr Vannier made an important point on the fact that through contaminated vaccines you spread diseases. This is under-estimated because, there are not many reports of contamination vaccines.

Dr P. Vannier: This is the reason why, even if there is a lot of controversy, in France we have decided not to use live virus vaccines in sows. The risk remains and recent experiences with some live vaccines prove this.

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Comment from the floor: Is this because the serum had not been irradiated properly, or was the cell bank that the manufacturer used already infected?

Dr P. Vannier: I do not have the information on this to reply.

Dr D. Kretzdorn: With regard to the question while the cell bank was contaminated or the foetal calf serum - it was the foetal calf serum. The cell banks were repeatedly tested and shown to be free. Very low contamination of the foetal calf serum containing relatively high levels of antibodies.

In answer to whether the antibodies would interfere with gamma radiation - we do not have enough information. It may be possible but there is no proof. High levels of antibodies in the foetal calf serum do interfere with detection of BVD virus. To check for antibodies in the foetal bovine serum has more importance and effect in connection with detectability of the BVD virus content in foetal calf serum.

Dr P. Vannier: Therefore, it is better to have antibodies in the serum and to remain capable to set up tests allowing to reject the batch or to have no antibodies and to have a higher probability to detect the virus because there is no interference between antibodies and virus?

Dr D. Kretzdorn: Difficult to answer! You may not run into problems if you have foetal bovine serum with a high level of antibodies, if you continuously use the same batch throughout production. Of course, you do not detect potential contamination in that batch - this is the risk, however, it may not cause problems at a later stage because the virus cannot multiply due to the antibody level in the foetal bovine serum. If you change during production, to a non-antibody containing foetal bovine serum, you will have problems.

Dr M. Chudy: From the results of studies, we cannot rule out that the incriminated foetal calf serum batch is the source of the contamination, however, we have isolated only one single BVDV 2 strain that is not fully identical to the strain from the vaccine.

Question from the floor: Do we have any information on the presence of BVD contamination in the virus seeds?

Dr P. Vannier: During the multiplication of the master seed, or the working seed, you use infected cells to amplify the vaccinal strains, you will contaminate your final batch. The virus and master seed have to be free of contaminants and be cautious during the various steps of production of the vaccine.

Dr C. Hamers: With viruses that are multiplying quickly such as IBR, as you need three or four days in order to detect BVD virus by immunofluorescence, when inoculated with viruses multiplying quickly such as IBR virus, the cell layer has disappeared before being able to detect BVD contaminant.

Comment from the floor: The low titres of BVD can also contribute to the problem. It may not be to the cell system but it may come out in vivo when you use the vaccine.

**SESSION II:**

**MEASURES TO LIMIT THE RISK THAT SERUM OBTAINED FROM BOVINES IS CONTAMINATED WITH PESTIVIRUSES:**

**Diagnosis of BVD virus infection in cattle**

Prof. V. Moennig (School of Veterinary Medicines, Hannover, D)

**Elimination of BVD virus infection in herds**

Prof. F. Schelcher (Ecole Nationale Vétérinaire, Toulouse, F)

**Vaccination of cattle against BVD/MD virus infection**

Dr P. Kerkhofs (Veterinary & Agrochemical Research Centre, Brussels, B)

**To present available methods to detect pestivirus in bovine serum and their sensitivity and validity:**

**Detection of pestiviruses in bovine serum**

Dr P. Nettleton (Moredun Research Institute, Edinburgh, UK)

**Detection of anti BVD antibodies**

Prof. F. Schelcher (Ecole Nationale Vétérinaire, Toulouse, F)

**Control and diagnosis of pestivirus infections in pigs**

Prof. J. van Oirschot (ID-DLO, Lelystad, NL)

**BVD virus detection and risk minimization in FBS production**

Dr P. Price (Life Technologies Inc., Rockville, USA)

**Serum free culture medium development for biologicals manufacturing**

Dr M. Gonze (GlaxoSmithkline Biologicals, Rixensaart, B)

**Discussion**

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### **DIAGNOSIS OF BVD VIRUS INFECTION IN CATTLE**

Prof. V. Moennig (School of Veterinary Medicine, Hannover, D)

Endemic infections of cattle caused by Bovine virus diarrhoea virus (BVDV) occur worldwide. Cattle of all ages are susceptible to infections with BVD virus. Acute postnatal infections are terminated by a potent and long lasting immune response of the host animal. The damage done by the infections is predominantly caused by intrauterine infections of susceptible pregnant cattle. BVDV interferes with fertility and induces a wide range of foetal lesions, ranging from abortions, stillbirths, a wide variety of malformations of predominantly the central nervous and skeletal systems and the birth of persistently infected (pi) calves, that are immunotolerant to the BVDV they are infected with. The latter are infected as foetuses at early stages of pregnancy in utero when the foetal immune system is not yet developed. The viraemia lasts for the rest of their lives. Pi animals continuously shed large amounts of infectious virus and they are ideal reservoirs for the perpetuation of the infection in the cattle population. The occurrence of pi cattle ranges between 0,5-2 %. The economic significance of BVD infections is enormous and BVDV is probably the economically most important cattle virus. Due to the ubiquitous nature of the viral infection until recently its control was thought to be impossible. However, due to considerable progress in the field of diagnostic tools and vaccines attempts are being made in several countries and regions of Europe to control and even eradicate BVDV. In addition to this background the sensitive and reliable diagnosis of BVD infections is becoming increasingly important, since the certification of a BVD-free status is important for a range of products derived from bovines designed for use in the pharmaceutical industry and biomedical research.

A proper diagnosis of BVD infection in cattle should be suited for the following situations:

#### *In vivo:*

Although persistently infected animals have the highest epidemiological impact, acutely and transiently infected animals also contribute to the spread of the infection. For this reason there may be situations where it might be desirable to diagnose the acute infection reliably. However, these animals display only a short viraemic period of approximately one week and virus titres vary dependent on stage of infection. Acutely infected animals shed only moderate or small quantities of virus with their excretions.

Persistently infected (PI) animals act as virus reservoirs in the cattle population and they are relatively easy targets for virus detection since they display a continuously high-titred viraemia. They shed massive amounts of virus with their excretions.

#### *Post mortem:*

The post mortem diagnosis of BVD infection is sometimes needed to clarify the cause of death and thereby collecting information about the status of a cattle herd. A separate and most important issue is of course the testing of the absence of BVD from bovine components, e.g. organs and serum that are designed for pharmaceutical or other biomedical purposes.

### **FREQUENTLY USED METHODS**

Isolation of BVD virus from peripheral blood leukocytes using cell culture is still considered as gold standard for the detection of BVDV. However, this technique is applied in several variations: For the classical approach buffy coat cells or other samples (nasal swabs, serum etc.) are co-cultured with susceptible bovine cells (non-permanent or permanent) for about 4-6 days. Thereafter the cells are subjected to a cycle of freeze thawing and their supernatant is passaged a second time using susceptible cells. In an attempt to increase the efficiency of this technique it has been adapted in various manners to micro titre plates using only one passage and

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visualising the viral antigen with immuno-enzymatic methods using either soluble or insoluble substrates. The downside of the latter approaches is a somewhat reduced sensitivity of detection.

Despite all variations virus isolation using cell culture still is laborious, time consuming and expensive. Therefore alternative methods have been developed: Several enzyme-linked immunosorbent assays (ELISA) designed for the capture of BVD viral antigen from peripheral blood cells, plasma or serum, respectively were introduced in the last decade. Targets for these tests are either viral NS2/3 or Erns proteins or whole virus, respectively. The following Table summarises the different formats of the available tests:

#### *Formats of Antigen Capture ELISAs for the detection of BVDV:*

<u>Capture Antibody</u>	<u>Detection Antibody</u>
1. polyclonal	polyclonal
2. monoclonal	monoclonal
3. monoclonal	polyclonal
4. polyclonal	monoclonal

In general these tests yield a sensitivity of >90% compared to sensitive versions of cultural virus isolation. Antigen capture ELISAs are widely used in programmes for the control of BVDV infections in cattle populations. These test are well suited for the detection of pi animals. However, due to low-level viraemia they often fail to detect transiently infected animals. A few laboratories routinely use flow cytometry (FACS) analysis for the detection of BVD infection. Sensitivity and specificity of this method are comparable to the ELISA.

A promising method that has been adapted to BVD diagnosis is the polymerase chain reaction after reverse transcription (RT PCR). Most frequently used viral genes are the NS3 (p80), E2, or the 5' nontranslated region (NTR). Depending on the genomic region used for amplification, diagnostic results are either BVDV- or panpesti-specific. In order to increase sensitivity and specificity some methods using two primer pairs (nested PCR) were developed. As samples individual or pooled blood, serum or milk are being used. Depending on the protocol used PCR may be superior to virus isolation. However, currently there are still some technical limitations of the technique, e.g., a lack of standardised, automatised and reliable RNA extraction procedure and the risks of contamination. Recent developments in the automatised of PCR may solve these problems in the near future.

Depending on the goal to be achieved, there are a number of tools for the reliable and sensitive diagnosis of BVD infections in cattle. Advances in the field of molecular diagnostic tools will soon expand the range of diagnostic methods.

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### **ELIMINATION OF BVD VIRUS INFECTION IN HERDS**

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#### **ABSTRACT:**

Practically there are two ways for eliminating BVD virus infection in cattle herds: vaccination and/ or test and cull chronic infected animals. For this purpose efficacy of vaccination depends essentially on foetal protection. Some proofs of this efficacy criteria were recently provided for vaccines in experimental conditions of challenge (ref). Nevertheless avoidance of vertical transmission was not demonstrated in field conditions.

The main way actually developed for eliminating BVD infection is a test and cull program. Chronically infected animals are identified in two steps. Animals are first tested for NS 2/3 antibodies and then, if seronegative results, virologically. Bovines are considered chronically infected if two tests one month apart give positive results, either by isolation or antigen detection. The test and cull program can be applied to all bovines in the herd, or only to a subpopulation consisting in the young animals between 6 months and 2 years old. In this case, the mothers of all chronically infected calves are later tested as well as the cows for which no calf was initially tested (calves sold). Whatever the method, it is necessary to undertake a second set of tests about 12 months later, for calves initially in utero or aged under 6 months.

This test and cull program must be completed by a rational and rigorous protection against reintroduction of the BVDV in the herd: avoidance of direct contacts with other ruminants and swine, systematic test of introduced cattle. Purchase of pregnant cows with potentially chronic infected foetus is particularly at risk.

The test and cull program must be rigorously applied to be efficacious. In field conditions absence of eradication is not infrequent.

PRESENTATION NOT AVAILABLE

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### **VACCINATION OF CATTLE AGAINST BVD/MD VIRUS INFECTION**

Dr P. Kerkhofs (Veterinary and Agrochemical Research Centre, Brussels, B)

Bovine viral diarrhoea virus (BVDV) is classified in the *Pestivirus* genus of the *Flaviviridae* family and is a cattle pathogen of worldwide economical importance.

Persistently infected immunotolerant (PI) animals which are the consequence of an *in vitro* infection with a non-cytopathic (ncp) strain of BVDV, play the most important epidemiological and economical roles in the course of BVDV infection.

To eradicate BVDV from a cattle population, these virus carriers have to be eliminated and congenital infection must be prevented. This objective can be reached by avoiding the exposure of pregnant cattle to the virus and by enhancing the immunity of the pregnant cows to the virus. The primary aim of BVDV vaccination is therefore the prevention of foetal infection. The development of vaccine against post-natal infection is now more and more stimulated due to the emergence of more virulent BVDV strains.

Commercially available inactivated and modified-live vaccines have been extensively used for more than 30 years without significant amelioration of the BVDV prevalence in countries or regions where they were used.

There is thus a real need for more efficacious and safe BVDV vaccines. The basis to enhance the performances of this vaccination programme is to understand the immune mechanisms that are involved in protection and to know which could be the impact on immune protection of the genetic and antigenic diversities among BVDV isolates.

Based on published results in peer-reviewed journals, this presentation focuses on the current status and prospects of BVDV vaccination.

Mechanisms of protection: far from clear!

Different immune processes are implicated in the protection conferred by vaccines against congenital and post-natal infections. The targets of the virus neutralizing response consist into the envelope proteins gp53 (E2) and gp48 (E0); non-neutralizing antibodies are also produced against the non-structural protein p80 –p125 (NS2-3). A vaccine containing only structural proteins will not induce an immune response against the NS2-3 protein whereas cattle infected by a wild-type virus will develop antibodies against this protein. This characteristic can be used for the discrimination between vaccinated and infected cattle.

The use of vaccines inducing cellular immune response indicates that this response is directed firstly against the capsid protein and secondly against gp53 (Elahy *et al.*, 1999 a).

For the prevention of postnatal infection, virus-neutralizing antibodies seem to be involved (Howard *et al.*, 1989) but these results are not confirmed by more recent vaccination experiments (Harpin *et al.*, 1999). After modified-live (Cortese *et al.*, 1998) and DNA (Harpin *et al.*, 1999) vaccines administration the protection against post-natal infection seems to be correlated to *in vitro* lymphoproliferation and IFN- $\gamma$  production after re-stimulation with BVDV. Any clear information is currently available concerning the implication of cytotoxic response and mucosal immunity in the virological protection after post-natal infection.

Even if some vaccines have already proven a certain level of efficacy against congenital infection, the mechanisms of this partial protection have not definitively been elucidated.

#### **DIVERSITY AMONG BVDV ISOLATES**

BVDV isolates are characterized by an important genetic, antigenic and pathogenic diversity. The emergence of new hypervirulent strains in North America has provided evidence of

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pathogenic differences between BVDV strains. The origin of this diversity is related to high mutation rate occurring in RNA viruses but the consequences of mutations obviously depend on the genes, which are involved. Mutation in genes encoding for structural proteins of immunological importance may have practical implications. Knowledge of BVDV diversity is important for understanding the wide variety of pathogenesis of diseases caused by the virus, for monitoring the epidemiology of the different types and for the design of optimum laboratory tests and vaccines (Hamers *et al.*, 2001). The BVDV strain variation has been suspected to cause vaccination failure (Meyling *et al.*, 1987; Bolin *et al.*, 1991). This point is, however, controversial as several studies (Bolin and Ridpath, 1995; Cortese *et al.*, 1998) have shown that a BVDV type I immunization induces a clinical protection against a type 2 challenge. This apparent contradiction is probably due to the degree, duration and type (clinical, foetal or epidemiological) of protection expected by the different authors as well as the vaccine type (van Oirschot *et al.*, 1999).

So, beside foetal protection, BVDV vaccination has also to induce a protective immunity against a wide diversity of viral strains. Indeed, based on the sequence of different regions of the genome, BVDV can be classified in two groups (I and II) and in subgroups (Ia, Ib, Id, If, ...). These genetic differences can be correlated with cross-neutralization tests revealing that antigenic differences exist between viral strains and principally between the groups I and II.

### **EFFICACY OF BVDV VACCINES**

In the case of BVDV infection, recent results on the vaccine efficacy were obtained by vaccination–challenge experiments. At this moment, there is no well-designed field trial demonstrating that the use of BVDV vaccine has any positive effect on herd health.

Vaccine experiments have been performed to evaluate the efficacy of live, killed and subunit vaccines against congenital infection (van Oirschot *et al.*, 1999). These studies were characterized by the differences of their parameters (duration of the immunity, challenge virus strain and dose, challenge route) leading to the difficulty to perform a real comparison. Even if some of the vaccines induced a good protection level, it is not known if these vaccines would also protect against a challenge with BVDV from another genetic and antigenic type.

The protection against post-natal infection is evaluated by measuring the clinical protection and the virological protection of the calves after administration of the challenge virus. A commercial live attenuated combined vaccine containing a BVDV-I strain was tested for protection against the BVDV-II 890 hypervirulent strain (Dean and Leyh, 1999). The data obtained indicate the utility of this kind of vaccine in the induction of a long-term immune response and a clinical and virological protection of the vaccinated calves against the infection by a BVDV-II hypervirulent strain.

Vaccination trials using a conventionally inactivated vaccine appeared to provide complete protection of the calves after challenge using homologous (genotype I) strains (Howard *et al.*, 1994). Beer *et al.* (2000) presented results of partial protection of calves after administration of a BVDV I and II combined killed vaccine and challenge with a BVDV-I strain).

The general conclusion concerning the review of the published data on BVDV vaccination efficacy is that so far, no vaccine has demonstrated to prevent congenital and postnatal infection by genotypes I and II. There is room enough for the improvement of this efficacy.

### **VACCINATION AGAINST BVDV: NOVEL DEVELOPMENTS**

These novel developments mainly focus on two aspects of BVDV vaccination:

I) the study of new antigen delivery of E2 and

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II) the study of other antigens and new antigen preparations.

Results of DNA vaccination were first presented by Harpin *et al.* (1999). They show that naked DNA (N-DNA) or DNA formulated in cationic liposomes induced after vaccination of calves both humoral (SN titers) and cellular (lymphoproliferatio). Partial protection was observed after challenge in N-DNA group.

The mouse model was used to study the humoral and cellular immune responses after immunization with recombinant fowlpox or adenoviruses (Elahi *et al.*, 1999 b, c).

The study of other immunogens concerns the nucleocapsid (C) protein expressed in recombinant adenovirus (Elahy *et al.*, 1999 a).

Full-length infectious DNA copies (Meyer *et al.*, 1996) can also be used in order to develop attenuated strains of BVDV.

### **CONCLUSION AND PERSPECTIVES**

To eradicate BVDV infection in countries or regions where BVDV prevalence is high, vaccination is to be advised as accompanying measure to the elimination of immunotolerant animals. So far, no vaccine has been demonstrated to prevent congenital infection with all antigenically divergent BVDV strains.

There is thus a need for efficacious and safe BVDV vaccine that protects against congenital infection and against postnatal infection with viruses from different antigenic groups. For control and eradication programmes marker vaccines should be preferred in a plasmidic formulation, the combination of different genes corresponding to different genotypes could be the basis of this vaccine.**REFERENCES**

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### **DETECTION OF PESTIVIRUSES IN BOVINE SERUM**

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#### **ABSTRACT**

Pestiviruses, particularly bovine virus diarrhoea virus (BVDV), have a remarkable record for contaminating veterinary viral vaccines. The risk of contamination is high, frequently through the use of contaminated batches of commercial foetal bovine serum (FBS). Between 1989 and 1995 102 batches of commercial FBS were tested for the presence of BVDV using cell culture isolation and for the presence of serum neutralising (SN) activity against BVDV. Virus was detected in 24 batches, 6 of which had detectable SN activity. A further 56 batches had some evidence of SN activity leaving only 22 batches suitable for the growth of pestiviruses. Between 1995 and 2000 31 batches of gamma-irradiated FBS were tested as before. No BVD virus was detected but 17 batches had SN activity.

RT-PCR has also been used for detecting pestivirus RNA in batches of FBS. Three laboratories detected RNA in 29 of 34 batches. No standard RT-PCR method has yet been developed and evaluated. Key stages of the method are discussed and an optimal method suggested.

Comparison of virus isolation and RT-PCR for detecting pestiviruses in bovine serum confirm that both have a place in the production of veterinary biologicals. Since no detection system is 100% effective, constant monitoring of cells used for viral vaccine production is essential to detect early pestivirus contamination.

#### **INTRODUCTION**

Pestivirus contaminants of modified live virus veterinary vaccines have been incriminated as the cause of serious disease following their use in cattle, pigs, sheep and goats. It is the insidious ability of non-cytopathic pestiviruses to cross the placenta and set up persistently infected animals that give them the potential to contaminate vaccines through the use of contaminated cells, serum used as medium supplement or seedstock virus. The purpose of this paper is to examine the best ways of ensuring that any serum used as medium supplement is free of contaminating pestivirus.

#### **SERUM CHOICE AND AVAILABILITY**

The ideal serum for viral vaccine production will have good cell growth properties, be free of contaminants and antibody against the vaccine virus, and be cheap and consistently available. Any serum collected from artiodactyls, particularly cattle and sheep, can be potentially contaminated with a pestivirus. Horse serum will not contain pestivirus and should be used if batches with consistent good cell growth properties can be sourced. Avoidance of the use of serum from cattle will reduce the risk of pestivirus contamination. Sheep, goat and pig sera pose less of a risk but can also be contaminated.

In practice, however, only bovine serum reliably fulfils the requirements for viral vaccine production. The best source will be a herd of known disease-free status. Failing that commercial sources provide foetal bovine serum (FBS), newborn calf serum (NBCS), calf and adult bovine serum. All 4 categories have a high risk of containing bovine virus diarrhoea virus (BVDV). In addition the NBCS, calf and adult bovine serum will contain neutralising antibody against BVDV, which can mask the presence of infectious virus. Practically FBS is preferred for its superior cell growth properties and its potential lack of neutralising activity against the vaccine virus. The techniques described below will relate to the testing of FBS but could equally well apply to any batch of bovine serum.

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DETECTION OF INFECTIOUS PESTIVIRUSES IN BOVINE SERUM USING VIRUS  
ISOLATION**

The risk of BVDV contamination of susceptible cell cultures through bovine serum used as a growth medium supplement has been recognised since 1975 (14, 15). Since then, there has been overwhelming evidence from the USA that between 20 and 50% of lots of FBS contain infectious BVDV (2, 3, 16, 25). In addition 11 to 13% of batches had detectable antibody to BVDV (2, 3).

In our laboratory batches of FBS are screened before purchase to ensure they support the growth of a range of cell cultures, that they are free of adventitious viruses particularly BVDV, and they have no neutralising activity against BVDV. All batches tested were from one of 26 suppliers and were offered for sale as screened to be free of contaminating viruses.

To test for virus a total of 19.5 ml of each batch of FBS is tested as a 15% supplement to growth medium used to grow a semi-continuous cell line of bovine turbinate (BT) cells over 4 passages. Two 80 cm<sup>2</sup> flasks are seeded out at 1 x 10<sup>5</sup> BT cells/ml in 20 mls IMDM medium supplemented with 15% test serum. Flasks are incubated at 37°C for a minimum of 3 days after which cells are trypsinised, pooled and used to seed a further 2 x 80 cm<sup>2</sup> flasks as before. A third passage is done the same way after which pooled cells are used to seed out ten cell culture tubes with cover slips at 1 x 10<sup>5</sup> cells in 1ml per tube. After 5 days duplicate cover slips are fixed in cold acetone and cells examined for the presence of noncytopathic BVDV using an indirect immunofluorescence test. The first stage serum is from a calf hyperimmunised with the NADL strain of BVDV and the second stage is a donkey anti-sheep/goat IgG serum conjugated to FITC.

To test for neutralising activity each batch of FBS is screened in a microneutralisation test against 30 TCID<sub>50</sub>/well of BVDV (NADL strain). Sera are tested from neat (25µl serum + 25µl virus) to 1:4 initially and titrated further if necessary.

Between 1989 and 1995 103 batches were tested only for pestivirus. Twenty-three batches (22%) were positive. During the same 7 years 102 different batches were tested for both virus and neutralising activity. The annual results are shown in Table 1.

*Table 1. FBS batches tested between 1989 and 1995 for neutralising (SN) activity against BVDV (NADL) and for the presence of BVDV*

Year	No tested	SN positive BVDV negative	SN positive BVDV positive	SN negative BVDV positive	SN negative BVDV negative
1989	13	6	1	5	1
1990	1	0	0	0	1
1991	7	2	0	1	4
1992	8	2	0	3	3
1993	12	4	2	1	5
1994	15	11	1	1	2
1995	46	31	2	7	6
Totals	102	56(55%)	6(5%)	18(18%)	22(22%)

BVD virus was detected in 24 batches but the principal reason for rejecting batches was the presence of some neutralising activity against BVD virus. Not all sera were titrated out but titres greater or equal to 1:6 were present in 29/56 SNT positive/BVDV negative sera and one batch

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of serum from which BVDV was isolated had an SN titre 1:22. Five other batches of virus positive sera had serum neutralising activities ranging from neat to 1:6.

In 1995 sporadic BVDV contamination of different susceptible cells was detected during weekly screening. The contamination was traced to a recently introduced batch of FBS, which had been pre-tested as described above, and which was heat inactivated at 56°C for 45 minutes before use. Following that experience only batches of gamma-irradiated sera have been screened before purchase. Between 1996 and 2000, 31 batches have been tested (Table 2).

*Table 2. Gamma-irradiated batches of FBS tested between 1996 and 2000 for neutralising (SN) activity against BVDV (NADL) and for the presence of BVDV*

Year	No tested	SN positive	SN positive	SN negative	SN negative
		BVDV negative	BVDV positive	BVDV positive	BVDV negative
1996	3	2	0	0	1
1997	4	3	0	0	1
1998	2	0	0	0	2
1999	7	3	0	0	4
2000	15	9	0	0	6
Totals	31	17(55%)	0	0	14(45%)

No BVD virus was isolated from any of the gamma-irradiated batches but again more than half of the batches were rejected on the basis of some neutralising activity against BVDV (NADL strain).

### **DETECTION OF PESTIVIRUS RNA IN BOVINE SERUM USING REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)**

In our laboratory experience of using RT-PCR to detect pestivirus in bovine sera has been restricted to testing foetal sera from aborted bovine foetuses (12) and to measuring viral loads in persistently infected cattle (Maley *et al.*, in preparation).

Several laboratories have described the use of RT-PCR for screening batches of FBS. For example, Yanagi *et al.* (25) detected BVDV in all 6 of the batches they tested while all of 15 batches tested by Sandvik *et al.* (20) were also positive. In a third study 8 of 13 batches were positive for the presence of viral RNA (13).

An optimum method for testing FBS by RT-PCR to detect pestivirus RNA will require the following stages:

Concentration of virus from at least 13 ml of FBS by ultra centrifugation

In addition, or alternatively, carrier RNA is added to FBS to obtain as much viral RNA as possible.

Total RNA is isolated by phenol-chloroform, TRIZOL, guanidine isothiocyanate (GITC), or a spin column method

Synthesis of cDNA using random hexamers or antisense primers

Use of panpestivirus primers from the 5'-UTR

A nested PCR employing around 25 cycles in the first amplification and 30-35 cycles in the second amplification

Use of a TaqMan or other probe system to identify the pestivirus product.

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Primer choice is critical. A number of panpestivirus primers from the 5'-UTR have been identified but their use do not always give consistent results between laboratories (19). If RT-PCR is to become a validated procedure for screening bovine sera for the presence of BVD-RNA agreement of the best primers will be required.

## **DISCUSSION**

The results of FBS testing from our laboratory are similar in terms of BVD virus isolation from non-irradiated batches to earlier American reports that detected virus in 20 to 50% of batches of commercial FBS (5, 16). Indeed, there seems to have been little change in the BVDV contamination rate of FBS batches since the earliest UK reports in the 1970s (14, 17). It was reassuring that none of the 31 gamma-irradiated batches of FBS tested positive for BVD virus. Gamma-irradiation of FBS for removal of contaminating viruses has been shown to be effective at a dose of 35kGY without any decline in cell growth properties (18). It would, therefore, seem sensible to use only gamma-irradiated serum for the production of veterinary biologicals.

The presence of neutralising activity in 55% of FBS batches using a simple micro neutralisation test against only one strain of BVD virus is a higher detection rate than those previously reported (2, 3). Whether this is because a lower TCID<sub>50</sub> per well was used or it is a property of the plaque purified stocked of cytopathic BVDV employed is not known. The presence of any neutralising activity against BVDV must be avoided in batches of FBS used for BVDV diagnosis. The importance of neutralising activity in masking the presence of live BVD in batches of FBS must also be recognised and could account for the very low sporadic BVDV contamination rates produced by occasional batches of FBS screened to be virus-free.

It is the exquisite sensitivity of the RT-PCR method that makes it attractive for screening batches of bovine sera for BVDV RNA. From already published reports (13, 20, 25) it appears that it is difficult to identify batches of FBS free of pestivirus RNA. The correct role of RT-PCR for screening for the presence of pestivirus RNA in bovine sera and veterinary biologicals has yet to be determined. Several laboratories have used RT-PCR to detect and/or type BVDV in FCS. Bolin and Ridpath (3) have done the most comprehensive study on the detection of BVDV contamination in FCS. They analysed one thousand pooled FBS samples for the presence of BVDV contaminant by virus isolation. BVDV was isolated from 203 FBS lots (20.3%). BVDV contaminants were typed using RT-PCR method employing BVDV1 or BVDV2 specific primers. BVDV type 1 was detected about twice as frequently as BVDV type 2, 115 and 65 viruses, respectively. Further segregation of 115 BVDV1 into subgenotypes yielded 51 as BVDV1a (NADL like type) and 64 as BVDV1b (Osloss like).

Yanagi *et al.* (25) also detected BVDV in bovine sera by RT-PCR. They concluded that most commercially available FBS are contaminated with BVDV. Although there is no evidence that such virus is infectious, FBS should always be screened by RT-PCR when sera are used for cultivation of hepatitis C virus in human medicine especially for the development or production of vaccines.

We also found during screening of human vaccines for pestivirus contamination using closed one tube nested Taq-Man RT-PCR method that no vaccines but the control FBS batch was contaminated with BVDV (22). In addition we typed some pestiviruses isolated from FBS batches as BVDV1a and BVDV1b (Vilcek *et al.*, in preparation).

Since FBS is used as nutrient for cultivation of cell lines it is not surprising that several cell lines have been found to be contaminated by BVDV.

Bolin *et al.* (4) have found that of 41 cell lines originating from cattle, sheep, goat, deer, bison, hamster, cat, monkey, human and mosquito obtained from ATCC13 were contaminated with BVDV. Immunoperoxidase technique and RT-PCR provided exactly the same results.

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Harasawa and Mizusawa (9) have examined 20 cell lines of various animal origin and found using RT-PCR that 15 were contaminated with pestiviruses. Sequencing of PCR amplicons revealed that bovine cell lines were contaminated with BVDV1 and BVDV2 while cell lines of canine, feline and primate origin were contaminated with BVDV2.

Most likely the contamination of vaccines with pestivirus RNA originates from contaminated batches of FBS used in the preparation of vaccines. Pestivirus RNA was detected by RT-PCR in live human vaccines against mumps, rubella and combined measles-mumps-rubella vaccines (10). Another group who tested 43 lots of human live viral vaccines produced by six manufacturers in Japan has also reported such contamination. Twelve lots of vaccines were contaminated by pestivirus RNA as detected by the RT-PCR technique (21). On the other hand no pestivirus RNA has not been detected in live human vaccines by other groups (1, 22). Live veterinary virus vaccines against Akabane disease, Ibaraki disease, IBR, porcine parvovirus infection, transmissible gastroenteritis and Japanese encephalitis produced in Japan have also been contaminated with pestivirus RNA (7). Recent detection of a BVDV contaminant in a live IBR 'marker' vaccine has again focussed attention on pestivirus contamination of live veterinary vaccines (6).

In general, the use of contaminated cells by pestivirus from FBS for vaccine production may result in contaminated vaccines. Their application may lead to seroconversion or disease in the vaccinated animal (24). The risk of pestivirus contaminated vaccines in human medicine is unpredictable at present.

A contradiction in the detection of pestivirus contaminants has also been reported in testing of interferon preparations. While Harasawa (8) has found contaminated preparations Audet *et al.* (1) published negative findings.

Contamination of virus stocks with pestiviruses (11) originated from FBS interferes in diagnostic tests or in genetic typing. Recently we found inconsistent results in the typing of an ovine pestivirus strain at the genetic level (23). The discrepancy was due to the contamination of host cell line by BVDV2 from FCS as indicated by the RT-PCR technique using pestivirus specific primers. While original noncontaminated viral strain belonged to BDV, BVDV2 contaminant became predominant in the third cell passage. As a result a BDV sample was erroneously typed as BVDV2.

In conclusion it is highly recommended to check commercial FBS lots for the presence of possible pestivirus contamination by the RT-PCR method as well as by virus isolation. The RT-PCR is increasingly being used routinely in many laboratories to control FBS batches for pestivirus contaminants, and must have a future place in the monitoring of all stages of vaccine production.

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**DETECTION OF ANTI BVD ANTIBODIES**

Prof. F. Schelcher (Ecole Nationale Vétérinaire, Toulouse, F)

**ABSTRACT:**

The seroneutralisation test for the detection and the titration of BVD-antibodies exist since the isolation of the first BVD cytopathic strain in 1953.

All serological surveys to determine the incidence of this virus in different Ungulates species (domestic or wild): bovine, ovine, caprine, porcine, etc. have been conducted in various parts around the world using the SN test, but using different “reference” strains on different cell systems.

This test was also used to determine the cross-relationship between strains on immune or hyperimmune bovine sera. Since the 1980' years and the development of new tools (sequencing and cloning of RNA viruses, monoclonal antibodies) and of new techniques (ELISA), some reagents were developed for a wider and easier use by laboratories.

This talk will try to present all the existing techniques with their advantages or disadvantages and how they contributed to a better knowledge for the diagnostic, the epidemiology and the control of the BVD disease.

PRESENTATION NOT AVAILABLE

**Pestivirus contamination of bovine sera and other bovine virus contamination**  
**CONTROL AND DIAGNOSIS OF PESTIVIRUS INFECTIONS IN PIGS**

Prof. J. van Oirschot (ID-DLO, Lelystad, NL)

**ABSTRACT**

Because the most typical of classical swine fever (CSF) is that it is so atypical, it is impossible to make a definite diagnosis in the field. A tentative diagnosis can be made based on a thorough anamnesis, the appearance of acute clinical signs and pathological lesions, of which haemorrhages are the most prominent. The other pestiviruses (bovine virus diarrhoea virus (BVDV) and border disease virus (BDV)), which are naturally occurring in cattle and sheep, respectively, only cause clinical signs when piglets become infected in foetal life. As a consequence of such congenital infection, these piglets may show CSF-like signs and poor growth, become persistently infected and immunotolerant, may shed BVDV/BDV continuously and thus may spread the virus in the population. The prevalence of antibodies against BVDV or BDV has been reported to be between 2 and 40% in some European pig populations. These viruses have probably been introduced into the pig populations by contact with cattle, feeding bovine milk or offal to pigs or by contaminated vaccines. Not much information is available about the prevalence of BVDV or BDV antibodies in pigs in other countries. Infections of pigs with BVDV or BDV strongly interfere with the diagnosis of CSF.

A definite diagnosis is usually made by detecting antigen in tonsil or other organs, or by virus isolation, followed by differentiation between CSFV and the two other pestiviruses. This can be performed with specific monoclonal antibodies or by (differential) reverse transcriptase-polymerase chain reaction (RT-PCR), and if adequate, followed by direct sequencing of the amplicon or identifying it by other molecular analyses. Direct genome detection in diagnostic samples, which basically is a very sensitive technique, is on the increase, but not yet used for routine diagnosis.

Antibodies to pestiviruses in pigs appear between 10 and 21 days after infection and probably persist lifelong. Tests to detect antibodies against CSFV are often used for serosurveillance and for screening herds for subclinical infections. ELISAs and virus neutralisation tests are most often used. Because BVDV and BDV have antigens in common with CSFV, antibodies against these viruses cross-react in antibody detection tests. To avoid this cross-reaction ELISAs have been developed that only detect antibodies against CSFV. However, such highly specific ELISAs still occasionally detect antibodies against BVDV/BDV. Therefore, sera that are positive in ELISAs must be retested in two neutralisation tests, one of which uses CSFV and the other BVDV/BDV as indicator virus. Higher neutralising antibody titres against CSFV than against BVDV/BDV indicate a CSFV infection.

In most European countries a stamping-out policy is implemented in case of a CSF outbreak. Vaccination measures are discouraged. However, it may be anticipated that CSF diva vaccines and accompanying diagnostic tests may be used in cases of emergency. No control measures are implemented to prevent introduction of BVDV or BDV in the pig population. It may be recommended to avoid direct or indirect contact with cattle or sheep. The risk of introducing BVDV or BVD in the pig population by vaccination is currently virtually nil.

This article also briefly considers an aspect of susceptibility of animals for pestiviruses and deals with a report of the European Commission on virus inactivation in bovine blood.

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### **INTRODUCTION**

Classical swine fever (CSF) is a highly contagious viral disease of pigs that can run a course varying from acute disease with a high mortality to a subclinical infection. The interplay between viral, host and environmental factors largely determine the outcome of the infection. Outbreaks in countries with industrialised pig holdings can have devastating economic, social and pig welfare consequences. For instance, the 1997-outbreak in the Netherlands led to a loss of approximately 2 billion Euro, and marked the beginning of a strong reduction in number of pig holdings. In addition, about 11 million pigs had to be killed and destroyed. An outbreak in one country jeopardizes the pig health status in other countries/continents as well. For example, the outbreak in the Netherlands in 1997 resulted in outbreaks in three other European countries. Classical swine fever is one of the prominent diseases on list A of the Office International des Epizooties. Because of the above, a rapid and reliable diagnosis of CSF is a prerequisite in countries that aim to control or eradicate the CSF virus (CSFV) infection. Because the most typical feature of CSF is that it often runs an atypical course, it is impossible to make a definite diagnosis in the field, even when combining a thorough anamnesis, the appearance of clinical signs and pathological lesions. Consequently, specimens of affected or dead pigs must be submitted to a specialized laboratory to confirm the tentative diagnosis. The genus pestivirus of the *Flaviridae* family comprises, besides CSFV, two other members: bovine virus diarrhoea virus (BVDV) and border disease virus (BDV). These ruminant pestiviruses can also infect pigs. The prevalence of antibodies against BVDV or BDV has been reported in several countries, e.g. Australia, Denmark, Germany, Ireland, and the Netherlands. However, most figures date from more than 10 years ago. A BDV/BVDV serum antibody prevalence of 11% has been found in samples from 12,000 sows from 215 herds that were collected in 1997-1998 in the Netherlands. Based on a small survey, more breeding pigs had antibodies against BDV than against BVDV (De Smit *et al.*, 1999).

Because BVDV and BDV are antigenically closely related to CSFV, infections caused by these viruses interfere with the laboratory diagnosis of CSF. This paper emphasizes the diagnosis of CSF and ways that have been developed to differentiate it from BVDV and BDV infections, and briefly outlines the control of pestivirus infections in pigs. In addition, an aspect of susceptibility of cattle and pigs for pestiviruses, and a report on virus inactivation in bovine blood will be dealt with.

### **CLINICAL SIGNS OF PESTIVIRUS INFECTIONS**

Acute CSF begins with fever, dullness, a reluctance to move and anorexia. These signs aggravate and peak temperatures above 42°C can be reached. Hyperaemia, blotting and purplish discoloration of the skin is common. Conjunctivitis, constipation, followed by diarrhoea, and vomiting is frequent signs. In terminal stages convulsions and posterior paresis are often seen. Most pigs die between 10-20 days after the first signs appeared.

Subacute CSF basically shows the same, but less severe, signs. Pigs that do not recover, and survive for more than 30 days are considered to be chronically infected. They can show intermittent disease periods of fever, anorexia, diarrhoea and dermatitis and often become runtig pigs. As a sequel of congenital infection, sows can deliver mummified, stillborn, malformed or weak piglets, but also piglets can be born that are persistently infected, remain healthy in the first weeks of their lives, but later develop signs resembling of chronic CSF, like runtig. This course is often described as late-onset CSF. Mild and subclinical CSFV infections are common (Van Oirschot, 1999). After experimental postnatal infections of pigs with ruminant pestiviruses mild signs of disease have been described (Fernelius *et al.*, 1973, Stewart *et al.*, 1971, Wensvoort and Terpstra, 1988, Edwards *et al.*, 1995). A recent article described

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experimental intranasal infections of pigs with various dilutions of BVDV type I or type II. None of the inoculated pigs developed clinical signs of disease. Type I BVDV was found to establish infection in pigs at lower doses than type II BVDV (Walz *et al.*, 1999). It is generally recognized that natural postnatal BVDV and BDV infections generally pass unnoticed. They only cause clinical signs when pregnant sows become infected and the virus is transmitted to the foetuses (Vannier and Albina, 1999). The sows do not show disease, but the piglets can be born dead or die within a couple of days after being farrowed. Piglets can remain alive for some weeks and then develop signs like anaemia, rough hair coat, growth retardation and runting. Conjunctivitis, diarrhoea and polyarthritis can also be observed (Terpstra and Wensvoort, 1988). Vaccines that were contaminated with ruminant pestiviruses and administered to pregnant sows induced dead and mummified foetuses, and alopecia, ascites, congenital tremors, diarrhoea, skin petechiae, blue eartips, growth retardation, eyelid oedema and arthritis in piglets born alive. Most piglets died shortly after birth (Vannier *et al.*, 1988, Wensvoort and Terpstra, 1988). The consequences of experimental infections of pregnant sows with ruminant pestiviruses appear to depend on the virus strain and on the stage of gestation. In general, experimental congenital infection led to similar signs in piglets as natural infection; in one study stunted growth was found to be striking (Paton and Done, 1994, Vannier and Albina, 1999). Signs in pigs congenitally infected with BVDV or BDV resemble those of chronic and late onset CSF. Congenitally infected pigs, as with congenital CSF, are immunotolerant and persistently infected, and consequently shed the virus continuously into the environment. Occasionally, such pigs may remain healthy for years. However, pigs with persistent viraemia can eventually eliminate the virus; this clearance was related to the appearance of neutralising antibodies (Paton and Done, 1994, Terpstra and Wensvoort, 1997).

### **ANAMNESIS**

When disease signs on a farm raise suspicion of CSF, the information that can be gathered from the farmer can support this suspicion, when pigs have recently been introduced into the farm, or swill has been fed. Prevalence of CSFV in the neighbourhood presents a high risk. During outbreaks neighbourhood infections are common, but precise routes of transmission have not yet well be elucidated (Elbers *et al.*, 1999).

In the case of BVDV or BDV infections recent purchase of pigs or swill feeding appears not of importance. The presence of cattle on a farm (virus may also be transmitted to pigs by the feeding of bovine milk or colostrum) and the use of pestivirus-contaminated vaccines have been reported as sources of pestivirus infections of pigs (Vannier *et al.*, 1988, Wensvoort and Terpstra, 1988, Terpstra and Wensvoort, 1991). It cannot be excluded that BVDV may be transmitted from persistently infected cattle to pigs if the same needles are used for injections.

### **PATHOLOGY**

At post mortem, CSF is characterised by widespread multiple haemorrhages of various sizes, particularly in lymph nodes and kidneys. Lymph nodes also become oedematous and consequently can have a 'marbled' appearance. Infarctions are often seen, especially in the spleen. There is disseminated intravascular coagulation with microthrombin in small blood vessels. Catarrhal, fibrinous and haemorrhagic inflammatory reactions can be observed in respiratory, digestive, and urogenital tracts. Pigs can show encephalitis with perivascular cuffing. In pigs that die from chronic or late-onset CSF, a general and severe depletion of the lymphoid system is the most outstanding feature. Foetal infection can result in stillborn piglets having excessive amounts of fluid in body cavities, cerebellar hypoplasia and hypomyelogenesis (Van Oirschot, 1999).

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When the pathology is indicative for CSF, and occurs together with clinical signs and an anamnesis that raises suspicion a tentative diagnosis can be made. However, for a diagnosis of CSF to be definitely made laboratory investigations are required.

Congenitally infected piglets that die from BVDV or BDV infections show lesions comparable to that of congenital CSF. In general, haemorrhages in lymph nodes and other lymphoid tissue and in kidneys are consistent lesions. Marked sub acute inflammatory lesions were seen in lymphoid tissues, and chronic gastroenteritis and cerebellar hypoplasia were also observed (Vannier and Albina, 1999).

### **LABORATORY DIAGNOSIS**

Samples of CSF-suspected cases should be submitted to the lab in cooled conditions. Samples to be collected from pigs that recently died, or from pigs that were killed when moribund include: tonsil, spleen, kidney and the distal part of the ileum. Preferably samples from more than one pig should be submitted. The methods for a laboratory diagnosis can be divided into tests that aim to detect virus, namely infectious virus (virus isolation), viral antigen (immunofluorescence tests on frozen tissues) or viral genomic sequences or tests that aim to detect antibodies against the virus. In the latter case neutralisation tests and enzyme-linked immunosorbent assays are mostly applied.

### **ANTIGEN DETECTION**

In the case of a CSF suspicion, a rapid diagnosis is of prime importance. Therefore, tests to detect antigen are the method of choice, because a diagnosis can be made in a few hours. In most laboratories the direct immunofluorescence or immunoperoxidase test on frozen tissue sections is used, whereby monospecific, polyclonal swine sera form the basis for the conjugate. The tonsil is the first organ to become positive at 48 hours after oral exposure (Ressang, 1973) and is the most important to detect antigen. An absence of viral antigen in tonsil and other organs in single pigs obviously does not exclude CSF as cause of the outbreak. A positive immunofluorescence test can be the result of a CSFV, a BVDV or a BDV infection. Therefore, an additional test has to be performed to differentiate between CSFV antigen on the one hand, and the two other pestivirus antigens on the other hand. For this purpose, monoclonal antibodies (Mabs) have been developed and selected that differentiate between these viruses (Wensvoort *et al.*, 1986, Hess *et al.*, 1988). For this second test, Mabs are used that recognize all field strains of CSFV, but not BVDV or BDV strains. A positive result indicates CSFV, a negative does not per se mean that BVDV or BDV is involved. This requires confirmation by antigen detection with monoclonal antibodies that detect ruminant pestiviruses only (Edwards *et al.*, 1991, Deregt *et al.*, 1994). However, no single Mab against BVDV appears to recognize all BVD/BD strains (Edwards *et al.*, 1991). In countries, where vaccination is routinely applied, it is necessary to carefully select Mabs that also differentiate between field virus and vaccine virus (Wensvoort *et al.*, 1986).

ELISAs have been developed that can detect CSFV antigen. Most of these use blood to detect antigen. Their sensitivity is, however, lower than that of virus isolation and PCR (Clavijo *et al.*, 1998, Kaden *et al.*, 1999). Antigen detection methods by ELISA are particularly suitable in situations where large number of samples have to be screened rapidly. These tests are not used for diagnosis of BVDV or BDV infections in pigs.

#### *Virus isolation*

Isolation of CSFV is best performed by inoculating a PK-15 cell line or other susceptible swine kidney cell line with a suspension comprising a ten percent homogenate of tonsillar and splenic tissues. These cell cultures are examined for antigen by the immunofluorescence test after 24 to 72 hours. Virus isolation is slightly more sensitive than the direct immunofluorescence on

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frozen tissues. To further characterize the virus and to apply molecular analyses it is necessary to have the virus isolated. A BVDV isolate from pigs has been described that contained an internal duplication in the 5' untranslated region. Such a rearrangement has never been reported before in pestiviruses (Van Gennip *et al.*, 1999). For isolation of BVDV or BDV either cell cultures of porcine or ruminant origin can be used. BVDV or BDV from pigs is reported to grow better in ruminant cells (Wensvoort *et al.*, 1989).

### *Genome detection*

The reverse transcriptase polymerase chain reaction (RT-PCR) in its various modifications is increasingly used in the diagnosis of CSF. Especially, parts of the 5' untranslated region has been amplified by the RT-PCR. Sequencing of the amplicons offers possibilities to subtype the strains and characterise them phylogenetically (Paton *et al.*, 2000a). This information is of importance to study the molecular epidemiology of outbreaks and may lead to finding associations between different outbreaks and tracing transmission routes of viruses in outbreaks. It is clear, that the detection and analysis of parts of the viral genome (the 5' untranslated region, the E2 gene and the NS5B gene are mostly used for this purpose) can form the basis for differentiation of CSFV from the ruminant pestiviruses (Paton, 1995). It can be done by using virus-specific primers in an RT-PCR, or by first amplifying with panpestivirus primers followed by further analysis or sequencing of the amplicons. For instance, RT-PCR of a part of the 5' untranslated region followed by restriction fragment length polymorphism analysis of the amplicons has shown that a pestivirus isolate of a congenitally infected pig was BDV (Vilcek and Belak, 1996). Advantages of the PCR are that it is generally more sensitive and rapid than virus isolation (Paton *et al.*, 2000b). A disadvantage is that it is prone to inaccuracy caused by contaminations and therefore stringent precautions must be taken to prevent false-positive results. Features of an ideal RT-PCR have been listed (Paton *et al.*, 2000b). The RT-PCR is not yet included in the routine diagnosis of pestivirus infection of pigs.

### *Antibody detection*

Serological surveys are often performed in case a country wants to show or confirm freedom of CSF. Also during or at the end of eradication/control programmes test to detect antibodies against CSFV are frequently used. Virus neutralisation tests and ELISAs are most often used; the neutralisation tests are much more laborious than the ELISAs. Several formats of ELISAs have been developed to detect antibodies against HCV, its structural proteins E2 and E<sup>ms</sup>, or against the non-structural protein NS3. Some of them have been developed and improved to specifically detect antibodies against CSFV, but not against BVDV or BDV infections (Wensvoort *et al.*, 1988, Muller *et al.*, 1996, Colijn *et al.*, 1997). For this purpose, E2 is used as antigen. However, it appeared still necessary to test sera that are positive in such an ELISA in parallel in two virus neutralisation tests: one to detect antibodies against CSFV and one for antibodies against BVDV or BDV. Higher antibody titres against CSFV than against BVDV or BDV indicate a CSFV infection and higher antibody titres against BVDV or BDV indicate an infection with these viruses. Two modifications of a virus neutralisation test have been described (OIE, 1996).

## **CONTROL**

In many countries that are endemically infected with CSFV, vaccination with live-attenuated vaccines is practiced on a large scale. These vaccines are basically highly efficacious and safe. A well-planned systematic vaccination programme should eventually result in freedom of CSF. Countries free of CSFV should take strict precautions to prevent the introduction of the virus. In most European countries a stamping-out policy is implemented in case of a CSF outbreak. This policy depends on the local situation and usually includes: destruction of infected herds, thorough cleaning and disinfection of these premises, a ban on movement of pigs, close

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surveillance of herds in a wide zone around the outbreak, tracing of the source of the infection and possible contacts, and financial compensation of the farmers. The member states of the European Union have implemented a nonvaccination policy. However, it may be anticipated that CSF diva (Differentiating Infected from Vaccinated Animals; also called marker vaccines) vaccines and accompanying diagnostic tests may be used in case an outbreak gets beyond control. For this purpose, E2 subunit vaccines and ELISAs to detect E<sup>ms</sup> have been developed and licensed (Ahrens *et al.*, 2000, Moormann *et al.*, 2000).

No control measures are implemented to prevent introduction of BVDV or BDV in the pig population. It may be recommended to avoid direct or indirect contact with cattle or sheep, and to attempt to recognize, diagnose and remove pigs congenitally infected with ruminant pestivirus, which are often runtling. The risk of introducing BVDV or BVD in the pig population by the use of contaminated vaccines appears currently virtually nil, because the vaccine producers are well aware of this danger.

### **ROUTE OF INFECTION AND SUSCEPTIBILITY**

Pestivirus contamination of cell lines, foetal bovine serum batches and vaccines is a great problem in veterinary virology. Vaccines are usually administered via the intramuscular or subcutaneous routes, which are not the natural routes of pestivirus infection. With regard to the chance of establishing a pestivirus infection through vaccination with a pestivirus-contaminated vaccine, it is of relevance to gain insight into the infective dose 50% (ID<sub>50</sub>) of pestivirus required to start an infection after parenteral or after (oro)nasal infection. The latter is considered the natural route of infection. Information on this issue is scarce. Cook *et al.* (1990) inoculated yearling heifers with 2 ml of tenfold dilutions of serum from a BVDV-carrier, using subcutaneous, conjunctival or intranasal routes and determined the infective doses, based on the number of heifers that had produced antibodies after inoculation. The virus titre of the undiluted serum was 10<sup>4.3</sup> cell culture ID<sub>50</sub> (CCID<sub>50</sub>) per ml. The highest dilutions of serum at which seroconversion occurred was for the conjunctival route undiluted, for the intranasal route 10<sup>-1</sup>, and for the subcutaneous route 10<sup>-5</sup>. All 4 heifers inoculated subcutaneously with the 10<sup>-3</sup> dilution produced antibodies. Hence, via the subcutaneous route a 1000-fold less virus was needed to establish a BVDV infection than via the intranasal route. A probit analysis indicated that a 99% infection rate could be expected, when a dose of 10<sup>1.6</sup> CCID<sub>50</sub> would be administered subcutaneously. An estimate of the heifer ID<sub>50</sub> required by subcutaneous injection was comparable to the CCID<sub>50</sub>. In this experiment none of the control heifers, which were in contact with the inoculated heifers, became infected, indicating the absence of secondary spread of BVDV.

Another study gave some indication of the ID<sub>50</sub> required to infect bovine foetuses, and hence indirectly pregnant heifers (Ficken *et al.*, 1996). They inoculated pregnant BVDV-seronegative heifers intranasally with approximately 10<sup>7.5, 3, 1</sup> CCID<sub>50</sub> of a noncytopathic type 2. Each dilution group contained 4 heifers. There were no differences in onset and duration of viraemia, and all foetuses became infected. Thus 10 CCID<sub>50</sub> of BVDV type 2 intranasally administered resulted in infection in 4 of 4 heifers. This contrasted with findings in the same study on BVDV type 1, where heifers were inoculated with approximately 10<sup>8, 6, 4, 2</sup> CCID<sub>50</sub>. All heifers that were inoculated with 10<sup>8</sup> or 10<sup>6</sup> CCID<sub>50</sub> became viraemic, one out of 4 animals inoculated with 10<sup>4</sup> CCID<sub>50</sub> became viraemic, and none of the heifers inoculated with 10<sup>2</sup> CCID<sub>50</sub>. BVDV antigen was found in all foetuses of heifers inoculated with 10<sup>8</sup> or 10<sup>6</sup> cell culture ID<sub>50</sub>, in 3 of 4 of the 10<sup>4</sup> group, and in 0 of 4 of the 10<sup>2</sup> group. This suggests that the threshold dose of BVDV required to infect the foetus is related to virus type or strain.

The findings of the two studies suggest that a low level of BVDV contamination in a vaccine would suffice to establish a BVDV infection in cattle that are vaccinated subcutaneously or

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intramuscularly, and that the characteristics of the BVDV type or strain involved may also play a role in this respect.

In addition, a study was performed on susceptibility of pigs to low doses of CSFV via the intranasal or intramuscular route (Terpstra, unpublished). For this purpose, 4 groups of 8, 21-week-old pigs were inoculated intramuscularly with either of tenfold dilutions starting with 7 CCID<sub>50</sub> of the Brescia strain. The strain used was derived from blood of a pig and has never been passaged in cell culture. In the first group all 8 pigs became infected, in the second 7, in the third 1 and in the fourth also 1. The pig ID<sub>50</sub> was estimated to be 0.17 CCID<sub>50</sub>. A similar experiment using the intranasal route resulted in a pig ID<sub>50</sub> of 74 CCID<sub>50</sub>. Hence, there was about 440 times less virus required to infect a pig via the intramuscular than via the intranasal route. This finding is in line with those of Cook *et al.* (1990) and both studies thus suggest that for pestiviruses the parenteral route is much more susceptible than the natural route for initiating an infection. This result is of importance with regard to application of vaccines accidentally contaminated with pestiviruses.

### **VIRUS INACTIVATION IN BOVINE BLOOD AND BLOOD PRODUCTS**

Because the chance that a batch of foetal bovine serum, which appears to be often collected from 100 to 1000, or even more, foetuses is contaminated with BVDV is very high, it is highly relevant to treat a contaminated batch in such a way that infectious BVDV and other viruses that are possibly present will be inactivated. There is EC legislation on this issue (Chapter 7 of Annex 1 of Directive 92/118/EEC), but the question has been put forward to the Scientific Committee on Animal Health and Animal Welfare whether this legislation is still the most appropriate. Answers to this question and several recommendations have been formulated, and can be found at Internet: [http://europa.eu.int/comm/food/fs/sc/scah/out50\\_en.pdf](http://europa.eu.int/comm/food/fs/sc/scah/out50_en.pdf)

This report deals with relevant parts of directive 92/118/EEC, lists the most important viruses that give rise to a viraemia in adult or foetal bovines, and states that ‘to guarantee absence of viruses the blood or blood product or serum must be treated in such a way that all relevant viruses are inactivated’. It concludes that the current legislation does not appear to be the most appropriate, because, amongst other things, the treatment must guarantee not only the absence of the five specific RNA viruses mentioned, but also that DNA virus. Hence, it has been recommended to use representatives of various virus families for the validation of methods aimed at inactivating virus infectivity in blood and blood products. The following viruses are proposed for this purpose: parvovirus, adenovirus, bovine herpesvirus1, foot-and-mouth disease virus, bluetongue virus, and bovine viral diarrhoea virus. Other recommendations are:

to check a serum batch after treatment for residual infectious virus, in many circumstances inoculation of young animals is preferred above cell cultures;

inactivation procedures that produce a linear inactivation curve are recommended, and ideally extrapolation of such a curve should indicate that there is less than one infectious unit per 10,000 litres of serum at the end of inactivation;

there should be further research on cell culture supplements that replace bovine blood or blood products.

The report also contains an example of risk assessment of bovine viral diarrhoea virus in foetal calf serum, which goes as follows:

‘Bovine viral diarrhoea virus (BVDV) is the virus most often contaminating foetal bovine serum. The existing EC legislation cannot prevent BVDV contaminated foetal bovine serum batches entering the market. In individual foetuses BVDV can be present in the blood up to titres of 10<sup>6</sup> TCID<sub>50</sub> per ml (Straver *et al.*, 1983). There is a prevalence of 0.5 to 2% of persistently infected

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calves (Houe, 1999), implying a somewhat higher percentage of persistently infected foetuses. If a batch is composed of blood from 100 bovine foetuses and assuming that 5 out of these 100 are persistently infected foetuses and assuming a titre for each of  $10^6$  TCID<sub>50</sub> per ml of foetal blood, then the BVDV titre in the batch will be  $10^{4.7}$  TCID<sub>50</sub> per ml.

It has been described that a dose of 25 kilogray (=2.5 Mrad) gamma-irradiation inactivated 6 logs of TCID<sub>50</sub> of BVDV when spiked serum in 500 ml bottles was irradiated (Hanson and Foster, 1997, Daley *et al.*, 1988). Based on the above, it may be stated that a commonly used virus inactivation procedure may profoundly minimise the risk for infectious BVDV contamination of foetal bovine serum. On the other hand, BVDV has still been found as contaminant of recent commercial vaccines, which indicates that absence of infectious BVDV cannot yet be fully guaranteed. The probable presence of antibodies against BVDV in most foetal bovine serum batches may influence the detection of infectious BVDV before or after the application of viral inactivation procedures.

The above illustrates that more safeguards are needed to prevent the risk of BVDV contaminated bovine blood acting as a source of BVDV spread in the animal population.

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**Pestivirus contamination of bovine sera and other bovine virus contamination**  
**BVD VIRUS DETECTION AND RISK MINIMIZATION IN FBS**  
**PRODUCTION**

Dr P. Price (Life Technologies Inc., Rockville, USA)

**ABSTRACT**

This article provides an overview of the relationship between BVDV and fetal bovine serum (FBS) production, including the ways of product testing and risk reduction of final product contamination.

It also discusses a possibility of using a new S-ELISA kit for testing of raw (unprocessed). In our evaluation study FBS pooled samples (n=84) were tested using the S-ELISA. Additionally, 30 serum specimens originating from persistently infected (PI) calves, that had been confirmed virus isolation (VI) positive, and another 30 previously confirmed VI negative samples were also evaluated. Of the 84 field samples the S-ELISA detected 13 (15.5%) BVDV-positive specimens.

When these positive samples (n=13) were tested for virus isolation and virus detection by immunofluorescent assay 11 (84.6%) were found positive, whereas 2 (15.4%) were negative. The S-ELISA was positive on all 30 PI samples (100%), and negative on all 30 negative samples (100%). These data indicate that the new kit is a relatively reliable laboratory diagnostic tool and can be considered for upstream detection of BVDV contaminated raw FBS pools.

**PRESENTATION NOT AVAILABLE**

**Pestivirus contamination of bovine sera and other bovine virus contamination**  
**SERUM FREE CULTURE MEDIUM DEVELOPMENT FOR BIOLOGICALS**  
**MANUFACTURING**

Dr M. Gonze (Smithkline Beecham, Rixensaart, B)

Serum-free media were developed in the early 90's due to undesirable variations in the quality, cost and availability of Bovine Serum (BS). More recently, the global awareness and concern regarding potential undetected adventitious contaminants (e.g. virus, prion) has further raised regulatory concerns about using animal - derived components in the production of human vaccines. There is therefore a great demand for the development of media devoid of components of animal origin to cultivate animal cells with and produce human vaccines.

Actually, we have designed cell culture media in which the animal components have been replaced with non - animal (vegetable, chemical, fermentation, recombinant...) in order to obtain a growth/production medium free of animal - derived elements. The media have also been supplemented with amino acids, vitamins, Fe carrier, lipids precursor, anti-shear and anti-oxidant agents to adapt animal cell lines to suspension culture and to develop batch production for viral vaccines. Cell growth profiles and high viability, as well as glycoprotein expression level can be maintained over a long - term propagation after cell cryopreservation without component of animal origin.

Of course, collaboration with media vendors is essential to ensuring a successful medium formulation and traceability of the raw material.

Some examples of serum-free medium developments are discussed (CHO, VERO, insects cells).

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### **DISCUSSION SESSION II**

Dr P. Vannier: What is the proportion of success for the people who have applied the eradication measures in different herds?

Prof. F. Schelcher: This question is difficult to answer. We have the example of the Scandinavian countries but the eradication there is not complete. In France, we have some examples but these are very limited in certain geographical areas like Brittany.

Dr M. Tollis: In relation to Dr Kerkhofs presentation: the 140 vaccines which are on the market are not worth anything, if I have understood your presentation! You mentioned that there is no vaccine capable of avoiding congenital infection, at present, this is clearly a general statement but, if true, what good is the BVD vaccine if we want to avoid transmission of the virus by transplacental infection of animals. Are we talking about vaccine against persistent infection or having vaccine against respiratory disease associated with BVD. This is very complex and we need clarification on what we are looking at.

Dr P. Kerkhofs: To create control programmes to prevent contamination of bovine product we have to look at a very long periods to have a good system in place. Therefore to control BVDV infection in one herd and more interestingly in a region we have to control the disease and we should look at a vaccine system which has to be associated with the elimination of the PI animals. This system should act for the protection against vertical transmission of the virus. There are not so many encouraging published results concerning this product. A good vaccine should induce a TH1 response, this needs to be worked on. If we look at the control of BVDV infection, we have the example of Scandinavian countries. There, it is easier to block the animal between herds and therefore easier to follow the incorporation of new animals in a herd. This is due to low trade levels between herds. Therefore, in Sweden, it should be possible to obtain eradication of BVDV without any vaccines. However, in countries like Belgium, for example, where the trade of animals is very high and there is a lot of animal traffic between herds, we need a control programme based on the detection of infected animals combined with the use of vaccines. We have to choose therefore the best vaccine, which is presently available.

Concerning the high prevalence strain, it would seem that the current vaccine Ia strain which is regularly used in the States is not totally effective against this strain. I also know that different vaccine companies are now testing their vaccine against this high prevalence strain. We may therefore, see a better product than that which we have a present.

Prof. J. Brownlie: I agree that the ideal vaccine will be one which will protect against both type I and type II but as the world is only really affected by type I and only North America and perhaps Brazil by type II, there is still a lot of advantage to be had from a vaccine that does protect from type I. There are inactivated vaccines which give you 100% protection against foetal infection, which is encouraging! Dr Kerkhofs comment on 140 vaccines in the States is that it is not a matter that some of the vaccines might be better than others but that they are not always used correctly. Particularly, when you are looking for inutero protection, you must ensure full protection before the cows are inseminated, that is not always undertaken. A survey in the USA shows that only 27% of vaccines are used correctly. There are vaccines available and good ones but we must use them correctly.

Dr P. Kerkhofs: Vaccination has to have a place in a global control programme. You have to follow all the steps.

Dr P. Marbehan: The debate between isolation through culture and PCR is becoming more frequent. When Dr P. Nettleton presented the advantages and disadvantages of the two methods what is the ratio between the sensitivity and the sample quantity for these methods? I have a

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particular batch of foetal calf serum in my laboratory where to demonstrate that it was contaminated I would have to test 1&1/2 litres every time.

Dr P. Nettleton: This is difficult to answer, someone made the point that with some batches, you have to test the whole batch to make sure that it is free and then you do not have any of the batch left!! The reason why we chose 20 ml of every batch to test was because we made some assumptions on how many foetal sera contributed to each batch of serum. You get different answers from different companies, however, we took the level that in any batch of foetal bovine serum there were probably sera in there from at least two hundred foetuses. If you assume that only one of those foetuses had virus circulating in its serum at the time of collection, and if you only assume that the titre was low at  $10^2$ , then you could test 20 ml of that serum and still detect virus from that batch, from 200 foetuses. The complication comes when some of those other foetuses have antibody and you start to lose some of your infectious virus. The point which I would like to make is that, a lot will depend on every batch of sera, especially the presence of the antibody, which is also in there and makes it very difficult for one to come up with the firm types of figures, that are wanted. We can get these figures from other studies where we are looking at the level of infectious virus in the serum of individual foetuses or in the serum of individual persistently infected animals and compare those with what we can grow in cell culture. The figures are of an extra log or two detectable by RT PCR that you cannot get by virus isolation. To do this in a milieu of a lot of batches, some with antibody and some virus this becomes very difficult to get a conclusion.

Dr P. Marbeant: I agree with your theoretical calculation. Where the problem lies is when you make an audit of the producer and they are below the theoretical titre in a normal batch, taking into account one IP, one infectious foetus. If you then do an inspection, you could also detect that several producers are not exactly working within the GMP, not rinsing the tank of the former batch, not making any distinctions on the foetal calf serum followed by a calf serum. This is where the problems are with very low titres of contaminants.

Dr P. Nettleton: I agree, another astonishing thing is receiving batches with foetal calf serum with neutralising titres in the thousands. How do you get that?!

Dr J. Ederveen: General question: has anyone screened gamma irradiated serum positive in RT PCR?

Dr P. Nettleton: People have, yes.

Dr P. Vannier: It is not clear for me if there is an opinion on the acceptability of the presence of antibodies for vaccine production - and this in the serum batches. This is to say, on one side people are saying that it is not realistic to ban antibody positive batches because we do not find any in the field, on the other side, it would seem that the presence of antibodies can mask the infection. The solution to this is not clear.

Dr P. van der Valk: The result of a survey that was done indicated that, at the request of the CVMP, the general attitude in industry was that low levels of antibodies should be acceptable otherwise, there would be nothing available. They would prefer to use batches of serum with low levels of antibodies. In specific cases, no antibodies are accepted when it is targeted for specific production methods. It is not specified what a low level is!

Dr P. Paton: It is very difficult to find antibody free batches but if you are a laboratory working with Pestiviruses, you have to find such batches if you are going to grow the viruses in cell culture, therefore these can be found. A comment on Dr P. Nettleton's presentation. He said that they did a neutralisation test on batches of serum to look for antibodies, we find it necessary to go further than that, and to do a Pestivirus growth index test to actually titrate the virus and look at how it is growing in the different batches of foetal calf serum. In our experience, even

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batches that have low or negative neutralisation titres will still sometimes interfere with the growth of Pestiviruses.

Dr J. Ederveen: It was shown that it was possible to produce infection in animals with only .17 TCID (tissue culture infective dose). Does this suggest that may be the infectivity *in vivo* in the animals is higher than *in vitro*?

Prof. Jan Oirschot: Yes, this is correct. The pig after intramuscular inoculation seems more susceptible than the PK50 cells used at that time.

Question from the floor: What is the internal temperature of the serum?

Dr P. Price: This is difficult to measure but somewhere around -20°C, -30°C range.

Dr H. Ovelgönne: Will inactivation become more efficient if it was warmed, or the contrary?

Dr P. Price: This has nothing to do with efficiency of virus kill, there are factors in there that would be destroyed at a higher temperature. You get free radicals forming during gamma irradiation and there would be things within the serum that would be destroyed so that the growth properties of the serum would be severely reduced.

Dr H. Ovelgönne: But there are no effects on the inactivation.

Dr Price: No negative effects - no.

Dr C. Lecomte: How do you proceed for sampling for the 9 CFR testing of the final product. Do you take a sample at the beginning?

Dr P. Price: There are small samples (100 ml). We start with 1600 litres so 100 ml at the beginning, 100 ml in the middle and 100 ml at the end. The three samples are pooled so you have three hundred ml and out of that pool, 100 ml is sent to Cornell for 9 CFR testing.

Dr L. Bruckner: (Question to Dr P. Price). Is it correction that you have small, intermediate sub-batches during the production of the huge batches?

Dr P. Price: Yes, we collect the serum and pool it. You get 300 to 500 ml from a foetus. We make 3 litre pools and the samples are taken from these 3 litre pools and then the serum is frozen. This is prior to the final pooling and filtration and then this is what is tested for haemoglobin and endotoxin and now BVDV, but not antibody.

Dr L. Bruckner: What would be the additional costs for testing these small pools for BVDV virus and BVDV antibodies in order to exclude them afterwards?

Dr P. Price: One of the problems is that no one has requested it. I will see if we can do that. This would add to the cost but if there is a demand for antibody free BVDV, this is about the only way that you are going to do it.

Dr P. Vannier: Concerning the conditions of irradiation of your batches of serum. How sure are you that the correct dose is administrated equally to all the bottles, and the gamma irradiation is performed to have the right dose of 35 kGy in the mass of the product and equally distributed?

Dr P. Price: With irradiation, you aim for 35 kGy but there is going to be a range which will be between 30 and 40. We try and get closer to 40 so that the range is higher. They have monitors within the batch that measures the amount of radiation getting to an area. A statistician worked out where you have to put each of these monitors in the batch and where you have to put your virus inoculated samples in the batch to validate it. The validation package is thick but it is available upon request, should you want it

Dr R. Levings: Question to Dr Gonze: When you have adapted one of these cell lines for a particular infection or recombinant production, does it maintain its original characteristics in

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terms of the range of viruses which it will produce, or do you have to adapt a medium for each purpose.

Dr M. Gonze: In a few cases you have the same and in other cases you have to adjust. For example with recombinant proteins, we always have the same process.

Dr M. Huether: We also have seen for recombinant proteins it does not seem to be a big issue, however, when you move to different types of viruses you have to read about the whole media to get the proper types of infections, proper titres and the proper immunogens produced.

Dr P. Price: I do virus titration whenever I develop a medium for specific cell line and in several of these media you get better growth than in serum supplemented but as long as you are getting equivalent growth, we tend to get equivalent virus titre. The medium is developed for the cells, and then you show that it will then grow virus. I cannot think of any of them where I got equivalent growth of serum where I did not get, at least, equivalent virus titre.

Dr R. Dobbelaer: I appreciated Dr M. Gonze's comments on traceability and the need to ensure traceability on the recombinant proteins. The mere fact of using a recombinant protein is not equal to no protein of animal origin because your recombinant may have been produced as well on a mammalian cells and have used serum in their preparation.

Dr R. Dobbelaer: Could someone comment on the difficulties related to the ability of PCR (NAT) to tell the difference between the replication competent virus and killed virus.

Dr H. Ovelgönne: I think that there are a lot of people experimenting at present with hybrid techniques in which viruses are cultured in tissue culture, not for three weeks but for one or two days and then the detection is done by PCR in order to get the best of both techniques.

Dr P. Vannier: We spoke of traceability for synthetic components but there is one point which is not clear that is the traceability for the sources of serum from animal origin. There are two types of collection: donors herds, where the traceability is very easy to take into consideration but in the second type when the serum is collected from the slaughter house, it seems to be impossible to have a good traceability to the herds providing the animals. This seems to be a consensus from the serum suppliers. I would like to have more data on this. Is it really impossible to set up a system to have quality assurance in order to ensure the traceability from the herds to the slaughter houses concerning the herd's health status, and especially in regard to BVD infection?

Dr P. Price: We and other companies can trace where each sample came from at the slaughter house. However, we cannot tell you which herd the mother came from as we do not trace back that far. We know and trace every single unit that goes into the pool and where it was from - what slaughter house, when it was collected etc. We do have that type of capability. If you are looking at 1600 litres and 3200 foetus in a single pool or higher, it would be difficult to be able to trace the heritage of the foetus back to the mother. We do know which herd it came from but not the specific animal within the herd.

Dr P. Vannier: There was an interesting idea from Dr L. Bruckner concerning the testing of mini pool intermediate batches for BVD virus or/and antibodies. Do you think that it is a practical way to discard some mini pools which could be infected to avoid mixing with large volumes from a known status with regard to BVD infection. Is this practical, feasible?

Dr R. Dobbelaer: The process is used in the preparation of plasma derivatives and hepatitis C PCR testing where mini pools are tested and potentially discarded, to avoid having to throw away large batches. It is used in other fields.

Dr P. Van der Valk: We have to be realistic. If we accept that irradiation when properly controlled and validated is capable of providing an inactivation of the doses of virus that might be present in foetal calf serum, why do we go so far as to test 3 litre sub-batches individually,

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which is quite an additional cost to the production of the batch? If you know what titre is present in a batch before irradiation and we know that the dose used is capable of inactivating, that can be confirmed by a test after irradiation if necessary. To sub-test 1200 sub-batches if you have a 3500 litre batch, it is extremely costly procedure.

Dr P. Vannier: Because you think that inactivation is always successful in eliminating all the infection?

Dr P. Van der Valk: If the evaluation procedure is properly validated, and I am sure that in the past there have been mistakes and it was difficult to get proper validation data, but if it has been properly validated, we know what can be inactivated by irradiation. The dosage is a fairly linear regression when you look at the titre. We know what a 30 kGy dose may do as a minimum guarantee dose and in general, your average dose given will be higher. At least, 30 kGy will be given. If you know the titre in the batch of serum before irradiation you know if that dose of irradiation you know if that dose is sufficient to inactivate and that can be confirmed after irradiation. If you ask for a single testing of every 3 litre batch that is too costly to the production of a batch of serum. Within the veterinary industry the margins are not that big in general therefore I am more pragmatic in this approach.

Dr P. Vannier: With whom are you sub-contracting the inactivation treatments? Are the safeguards of quality assurance applied by the sub-contractors or by with the sub-serum suppliers responsible for this inactivation treatment.

Dr P. Van der Valk: In our company we know where the evaluation is done. I agree that in the past there have been certain difficulties in getting the proper validation data. This has now changed and if we look at Dr Nettleton's presentation, he states that based on his finding of the last five years, when he checked irradiated batches of serum, they were found to be free of live virus. That agrees with the remarks made, that if you use a 30 kGy dose you will have at least a  $10^6$  reduction in virus. Whatever you do, when you mix a batch of virus from various foetuses, you will never get that amount of virus per ml in a total batch. You will have results that are lower than this. This can be confirmed by testing prior to the irradiation. The irradiation is then calculated and shown sufficiently high for the total inactivation. It is excessive to ask for every three litre batch to be tested on a routine basis. If you have specific requirements for specific batches this is different. If you want to have a batch free of antibodies, that is the only way to proceed, otherwise it is not necessary.

Mr M. Edginton: You must always bear in mind that with irradiation processes that these are evidence based processes. The sterilisation process should be evidence based. You should know what you are trying to eliminate before you irradiate. This is the way it is applied to the sterilisation of medical devices and other items. Knowledge of the bio burden first, then related it to the dose. If you do not have the evidence before you start there is no point.

Dr M. Schully: The situation with BVD is not equivalent to HCV. With BVD infection we know there is probably going to be contamination.

Dr P. Vannier: There are different opinions. Some people say that we do not mind that we have to control even if you have inactivated the serum. I would say that this is in opposition with the Directive because it is said that 'you control or you inactivate' but you do not do both. It is not useful and too expensive. We have to consider the results. Effectively, is it necessary to control before and after inactivation, or not? - the cost will be increased if both are done.

Dr D. Plancon: The question is not to know if the sera is contaminated or not because most of them are contaminated but, at what level are they contaminated. We think that we have to have a good level of insurance that the batch that we process, will have a level of contamination of various BVDV strains of virus, possibly uncontrolled in the batch, which is at a sufficiently

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low level to be fully inactivated by rigorous and fully validated gamma irradiation. We are in the position to control the batch before processing and to have insurance that the irradiation will be conducted in a correct way. It is possible to select companies who will do this correctly. Some problems may arise with the way to load the palette which will enter into the gamma irradiation source, we must be careful that not only the use of time of exposure as a proof of irradiation but also to use a dosage metre. You must know that a source of irradiation will decrease over time and therefore, should be changed every two to three years and the time of passage in the gamma ray depends on the activity of the source. It is important to select a strict and serious company to make the irradiation and to put dosage meters in the worst place of the load in order to avoid too high irradiation for the vials near the source which would lead to defects in the production batches. If the process has been fully validated and controlled I do not think, provided that the initial level of contamination is known, that an additional control after the process is necessary.

Dr P. Marbehan: We also have to measure the bio burden of our solution prior to sterilisation. It is a good assurance characteristic to know what is accepted as a preliminary titre of contamination to guarantee a successful inactivation during the gamma irradiation which should be properly validated. If all the steps have been properly carried out, there is no use in having a final test after irradiation.

Dr R. Dobbelaer: We have heard about the contamination of cell cultures of serum, we have heard about accidents with contaminated vaccines. I think that there must be incidents in between - during production for example? Any comments?

**SESSION III:**

**REGULATORY AND OTHER MEASURES TO PREVENT THE  
CONTAMINATION OF VETERINARY VACCINES WITH PESTIVIRUS**

**Contamination of bovine serum and veterinary vaccines with pestivirus:**

**Results of the EMEA/CVMP/FEDESA survey**

Dr P. van der Valk (Fedesa, Brussels, B)

**The TSE risk versus the BVD risk**

Dr L. Bruckner (Institute of Virology and Immunoprophylaxis Mittelhäusern, CH)

**Existing and proposed regulatory requirements:**

**IWP/CVMP/EMEA Guidelines on bovine sera**

Dr P. Vannier (Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, F)

**International cooperation on harmonization of technical requirements for registration of  
veterinary products VICH**

Dr O. Itoh (Ministry of Agriculture, Forestry & Fisheries, Tokyo, J)

**The European Pharmacopoeia monograph on BVD vaccines**

Prof. M. Pensaert (Faculty of Veterinary Medicine, Merelbeke, B)

**Risk assessment of vaccine contamination**

Dr M. Tollis (Istituto Superiore di Sanita, Roma, I)

**Discussion**

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

### **RESULTS OF THE EMEA/CVMP/FEDESA SURVEY**

Dr P. van der Valk (Fedesa, Brussels, B)

Bovine serum is an important raw material in the manufacturing of veterinary vaccines. The specifications of the serum used vary between foetal calf serum, calf serum, serum of adult cattle, fortified calf serum etc.

A survey carried out by FEDESA at the request of the CVMP provided the following data:

Serum is in general purchased in bottles containing 500 to 1000 ml, but 10 litre bottles are used as well.

Batch size varies between 300 and 2500 litres, on average it is 500 to 1000 litres,

The number of batches purchased per year varies between 1 and 25.

A conservative estimate on the total amount of serum used per year show it to be at least 53,000 litres. The supplier through his production process guarantees the homogeneity of a batch.

Each batch of serum is accompanied by a certificate of origin, of analyses and of irradiation (when applicable) provided by the supplier. The certificate of origin will list the type of animals used for collection of the serum and the collection sites, which in general will often be a slaughterhouse or in some cases a donor herd. The certificate of analysis will provide data that will specify the type of serum by its composition. It may also contain data on sterility testing, Mycoplasma and extraneous virus testing when done by the supplier. For these methods, the standard used is provided. The manufacturer may add certification for testing done in house. The extraneous virus tests done are cell culture and primary cell tests. One company uses an in-house PCR test as well, but indicates the BVDV genome to be present in every batch of serum examined.

The absence of extraneous agents in serum is thus guaranteed by tests, irradiation with at least 25 kGy, sometimes additional heat inactivation and in specific cases chemical inactivation.

The number of batches rejected is very limited. The main reasons for rejection in order of importance are insufficient growth characteristics, presence of mainly IBR or BVDV antibodies and viral contamination. Viral contamination found is mainly BVDV; bluetongue is mentioned once. The presence of BVDV antibodies is a reason for rejection when the serum is to be used for specific productions in which the presence of BVDV antibodies may interfere. In general low levels of antibodies will be accepted. It is worthwhile to notice that the frequency of detection of BVDV antibodies varies between the companies questioned. This might indicate the need for reference material to be made available for proper standardisation of test methods.

Taking into account the number of batches of vaccine made, the occurrence of contaminated batches is very low, indicating that the control system described works well.

In case of a contamination BVDV is the major cause found and in general is traced to contaminated serum. The incidence and duration of contaminated production will increase when a seed or cell stock has become infected. If contamination is not detected at that level several batches of vaccine may become contaminated. Detection of a contaminated batch will depend on the target species for the vaccine. It may be detected as part of final product release testing or because it results in disease: for BVDV this applies to ruminant and porcine vaccines. It also may go unnoticed because BVDV is not part of final product testing and it will also not cause disease in the target animal such as chickens, dogs or cats.

The European Pharmacopoeia, method 5.2.5, requires that substances of animal origin are shown to be free of contaminants by testing or are subjected to a validated inactivation procedure. When an inactivation procedure is used this must be capable of reducing the contaminant titre with

### **Pestivirus contamination of bovine sera and other bovine virus contamination**

$10^6$  or inactivation kinetics has been shown satisfactory for the level of contamination encountered. As material has to be sourced from healthy animals, the contaminant titre possibly present in the material can be assumed to be low. However reliable data are not available for the majority of possible contaminants listed in the table for extraneous agents.

For extraneous agent testing a solution containing 300 g of the substance per litre must be examined, using suitable sensitive cell cultures and primary cells of the species of origin. Depending on the sensitivity of the test method used, this amount of material may not be sufficient to detect low levels of contamination. However a choice has to be made to keep it workable.

The general method used for inactivation is irradiation. For most of the extraneous viruses listed in the table the guaranteed dose of 25 kGy is not sufficient to provide a  $10^6$  reduction in titre. Although data from literature vary slightly, in general this applies to BVDV. As the argument that inactivation kinetics may be expected to be sufficient to cope with the contamination encountered is difficult to defend, additional testing is required, either prior or post irradiation.

Industry is of the opinion that the scope of the note for guidance on Bovine serum should be restricted to BVDV only. As BVDV is the most important contaminant, industry proposes to increase the minimum guaranteed irradiation dose to 30 kGy. According to the available literature data, this will provide a  $10^6$  reduction in titre, thus validating the inactivation method in accordance with EP requirements. In addition industry is prepared to test the serum for absence of BVDV prior and after irradiation. This way a possible contamination titre is determined before irradiation, which allows assessment of the effect of irradiation and confirmation of its efficacy through the final test.

Assessment of the efficacy of irradiation should be possible as a linear regression in virus titre can be expected with an increase in the irradiation dose provided. This linear regression of about 0.95 has been demonstrated for viruses like the Akabane virus, bluetongue virus, Foot and Mouth Disease virus, Bovine Virus Diarrhoea virus and Infectious Bronchitis virus. Thus the irradiation dose necessary to inactivate  $10^6$  for each of the viruses listed in the table for Extraneous Agents can be calculated. The minimum dose necessary, around 50 kGy, would result in serum with greatly reduced growth characteristics. Such serum could no longer be used in vaccine manufacturing.

## **THE TSE RISK VERSUS THE BVD RISK**

Dr L. Bruckner (Institute of Virology and Immunoprophylaxis Mittelhäusern, CH)

In order to illustrate the risks of BSE and/or BVD on bovine serum and other biological materials of bovine origin and their implication on vaccines, I would like to follow two cows at different phases during their life. In a second part of my presentation I would like to look closer at some characteristics of the two agents causing BSE or BVD respectively in cattle.

### **SOURCING OF MATERIAL**

Let's have a look at two different cattle. The first might be born in UK, France, Portugal, Switzerland or in some other European country. Why do I mention these countries?

The risk to be infected and thus being able to contaminate raw material derived from cattle with either BSE (which is not a virus) or BVD, depends on the prevalence of the disease in the country where the animal is born or where it was or is actually living.

BSE occurs only in Europe, the incidence is different from country to country. It is still highest in Great Britain, where the disease was detected as early as the mid 1980's. Just recently indigenous cases were reported from nearly all western European countries.

Careful sourcing of biological raw material can efficiently reduce the risk of introduction of BSE contamination.

The situation is completely different concerning BVD. BVD occurs worldwide, the prevalence of different serotypes being different from country to country. The country of origin of bovine serum has no influence on the risk of contamination with BVD virus.

### **TISSUE TROPISM OF INFECTIOUS AGENTS**

Let's go back to the animals. Both were inseminated and now they are pregnant.

We assume they were infected; the first - most probably orally - with meat and bone meal containing the BSE agent. Which part of the body contains BSE agent? It is restricted to mainly the brain and the spinal cord. In these organs huge amounts of BSE agent may be demonstrated.

No infectivity is found in whole blood, serum, milk or meat. BSE agent was never transmitted vertically to the offspring. In some intestines a low infectivity could be detected.

The second cow was infected with BVD virus. It is hardly difficult to detect BVD virus in adult animals, but if the fetus has been infected through the dam in the first trimester of pregnancy the whole fetus and all its organs as well as the serum is then contaminated. A batch of fetal calf serum may be hundreds of litres and is a pool of serum of a large number of animals. One infected animal may thus contaminate a whole batch of serum.

If we look at the risk arising from the two agents BSE and BVD, the situation for TSE contamination seems to be manageable, whereas BVD looks rather disastrous.

### **DETECTION METHODS FOR THE INFECTIOUS AGENT**

BVD virus may easily be detected in raw materials used for the production of biologicals. PCR is only one method that can be used for the demonstration of BVD virus; you also may detect the virus in tissue culture or if you want to use an old fashioned method you may inject your material into a calf and look some weeks later if the calf seroconverts.

When we try to use the same approach to exclude the risk of TSE contamination, we have to realize that the situation is completely different. In order to check for the presence of TSE agent we need the brain of the animal which was used for the preparation of the material. There are

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different tests available for the detection of BSE, but none of them can be used for living animals or materials, such as serum, derived from animals. In addition these tests are not very sensitive and a diagnosis of BSE can only be made shortly before the first occurrence of clinical symptoms of the disease.

### **INACTIVATION OF THE INFECTIOUS AGENT**

There are also other methods to reduce the risk involved with BVD virus. You may decontaminate raw material, you may treat it chemically or with gamma-rays. You may validate your inactivation procedure, which gives you additional assurance of the validity of your inactivation procedure.

There is no such procedure to inactivate TSE agent. Even extremely harsh procedures, which usually destroy your material, are not capable to completely inactivate TSE agent.

### **MULTIPLICATION OF THE INFECTIOUS AGENT *IN VITRO***

While producing a vaccine you have to propagate a micro-organism. Preferably you do this *in vitro*, in tissue cultures, or in a specially prepared nutrient broth. This *in vitro* propagation is the origin of additional risks. In tissue culture you can multiply not only vaccine virus; it is very likely that you multiply contaminating BVD virus as well. It may happen that you start with a very low concentration of BVD contamination, but the contamination multiplies perfectly in cells.

In contrast, there is no possibility to multiply BSE agent *in vitro*. Even sophisticated tissue cultures are not able to propagate BSE agent; there is no risk to multiply TSE agent in cell cultures. The situation is different if you use animals for the multiplication of your vaccine organism. This is only one additional reason to leave such crude procedures of vaccine production, there is a huge number of other disadvantages and risks.

### **SUMMARY**

- BSE occurs only in Europe; by careful sourcing your primary substances you may minimize the risk.
- BSE agent is essentially restricted to the central nervous system; by eliminating brain and the spinal cord from your production systems you eliminate the risk involved with the use of these organs.
- By choosing tissue cultures or other *in vitro* systems for the propagation of micro organisms, which are used as vaccines, you do not multiply any contaminating TSE agent that might be present in a starting material.

If your material is contaminated with BSE agent there is no practical way to get rid of TSE agent, you cannot inactivate it without the destruction of your product.

There is no way to routinely check for the presence of TSE agent.

BVD occurs worldwide, if you buy fetal calf serum or other bovine material from your regular supplier, there is a great risk, that the batch you just bought is or was contaminated. If you want to go the safe way, you can grow your own BVD free herd, which serves as donor of different materials.

Restriction to the use material from specified organs to reduce the risk of BVD contamination is not feasible, because BVD virus infects the entire animal; all parts of a calf are contaminated. Serum for which we are particularly interested is probably the part of the body with the highest virus load.

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If you have introduced the contaminating BVD virus in your production it multiplies in cell cultures.

- BVD virus may be inactivated and thus the risk of contamination may be reduced.
- When even inactivation fails, there are tools available to detect the contamination.

The final evaluation is up to you. It is obvious that the risk of TSE contamination can be minimized, whereas the risk of BVD contamination is always present and can only be prevented by decontamination of the raw materials derived from animals.

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

## **EXISTING AND PROPOSED REGULATORY REQUIREMENTS**

### **IWP/CVMP/EMEA GUIDELINES ON BOVINE SERA**

Dr P. Vannier (Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, F)

#### **INTRODUCTION: SCOPE OF THE GUIDELINE**

Starting materials of animal origin are usually necessary for the production of immunological veterinary medicinal products. Substances of animal origin including bovine serum are essential ingredients of the cell culture media used in the production of many veterinary vaccines. Different risks are associated with the use of such starting materials. Indeed, the nature and quality of the bovine serum can profoundly influence the quality of the manufacturing process and of the finished product.

The Note for Guidance outlines the general principles, which should be applied to the controls, tests and inactivation treatments, applied to bovine serum for the purposes of ensuring acceptable quality and a minimisation of the risks of transmitting infectious disease.

The presence of extraneous agents in the bovine serum certainly represents the major risks to the quality of the finished product. Nevertheless, the global quality of the serum used has to be considered to detect other deleterious effects and more particularly on the growth of cells. Therefore the guideline will focus mainly on the risk due to extraneous agents. All the data requested in this guideline have to be submitted in the application file of the immunological veterinary medicinal products.

This guideline applies for serum batches used for production of vaccines intended to be administered to all target animals, irrespective of their species.

Testing before and after inactivation may be carried out by the serum supplier, by the manufacturer, by a contract laboratory or by more than one of these. This Note for Guidance is not intended to proscribe which parties should carry out the testing but rather to define the testing which should be done at each stage of processing and the relevant quality standards that apply. It is recognised that not all serum suppliers, contract laboratories or manufacturers will necessarily possess the expertise and facilities required to perform all of the testing specified. There is a legislative requirement that the testing is carried out to the principles of GMP; it is the responsibility of the manufacturer to ensure that the testing is carried out to the required standard. It is therefore strongly recommended that the manufacturer do testing themselves. Where this is not practical, testing can be devolved to the serum supplier or a contract laboratory; but responsibility for providing the necessary data to demonstrate compliance with the relevant quality standards rests with the manufacturer.

#### **1. SOURCE**

The health status of the herds of origin of cattle used as donors should be well defined and must be documented. Compliance with the Note for Guidance on Minimising the Risk of Transmission of Animal Spongiform Encephalopathy Agents via Veterinary Medicinal Products must be shown. As herd health status for exotic diseases is usually defined through the health status of the country of origin, the Office International des Epizooties (OIE) code should be followed to ensure freedom at source from exotic diseases (e.g. FMD, bluetongue). In relation to other diseases of cattle, such as IBR, BVD, the status of the herd should be known unless justified and, where necessary, serum should be sourced from herds free of these diseases. Traceability of serum from final flask to farm of origin is of prime importance and a clear audit trail must be demonstrable including records of volumes at each stage.

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### **2. PREPARATION AND MAXIMUM SIZE OF EACH BATCH**

Products of animal origin should be prepared in a homogeneous manner, designated with a batch number. A batch may contain material (raw serum) derived from as many animals as desired. Once designated and given a batch number, a batch shall not be mixed with other batches nor contaminated in any way. The serum supplier must describe the process for harvesting material, blending intermediate pools/bulks and producing the final batch of serum and must certify the key data of the serum batch. A specific procedure must be applied to ensure good homogeneity of the harvesting material, intermediate pools/bulks and the final batch. The serum batch test protocol shall contain the batch number and country of origin of the source animals. The total volume of the serum of one batch designated by one batch number cannot exceed 1500 litres.

### **3. SERUM SUPPLIER CERTIFICATE**

The certificate provided by the calf serum supplier must state precisely: the catalogue number, the batch number, the origin of the source animals, the total batch volume and the date of blending of the batch. The serum supplier should demonstrate and certify that the serum is exclusively of bovine origin. The content of serum proteins and the chemical properties of the serum should be indicated including the content of albumin, alpha globulin, beta globulin, gamma globulin, total protein, haemoglobin, the pH and the osmolality. The units of measurement have to be defined in the results shown on the certificate.

As a minimum, the serum must ultimately be certified as being free from bacteria, fungi, mycoplasma and relevant viruses. The stage at which the definitive testing is carried for the purposes of certification is discussed under Section 4 below. Manufacturers usually require the results of cell testing such as relative cloning efficiency, relative plating efficiency, relative diploid growth promotion, toxicity testing, relative myeloma/hybridoma growth. This may either be presented on the certificate or may be generated by the manufacturer. Chemical and physical properties such as pH and osmolality of the serum must be tested and the results indicated on the certificate.

All the controls performed by the supplier must be stated on the same certificate as the results. All tests should be performed preferably in compliance with the requirements of the European Pharmacopoeia and should also be validated according to the principles given in ISO 17025. The controls should preferably be carried out on a representative sample from each bulk. All operations performed by the serum supplier should be controlled by quality systems, which are compliant with the principles of Commission Directive 91/412/EEC (Good Manufacturing Practice). A suitably qualified person responsible for QA should sign the final batch certificates to indicate compliance with the appropriate quality system.

### **4. ASSAYS AND CONTROLS TO BE CARRIED OUT EITHER BY THE VACCINE MANUFACTURER OR UNDER THEIR RESPONSIBILITY.**

If one of the controls carried out by the vaccine manufacturer shows a result, which is not satisfactory, the test has to be repeated or the serum batch rejected. A representative sample from each batch of serum should be used for the following testing. The assays and controls on serum batches must be carried out before inactivation treatment; if the manufacturers receive treated serum batches, the assays and controls must be carried out on representative samples provided by the suppliers from each untreated serum batch before inactivation treatment. Some tests can be carried out after the inactivation treatment such as the tests for toxicity- cell growth.

#### **4.1. Bacterial and fungal sterility tests**

The serum batch complies with the tests for sterility of the European Pharmacopoeia Monograph.

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### **4.2. Tests for the presence of mollicutes**

The serum batch complies with the tests for the presence of mollicutes as described in the European Pharmacopoeia Monograph (tests for Mycoplasma) applied to the culture method. All the results must be satisfactory.

### **4.3. Tests for the presence of viral contaminants**

#### **4.3.1. General and Specific Tests**

All the tests performed must be validated and in compliance with guidelines for IVMPs (Rules Governing Medicinal Products in the European Union, Volume 7B) and with the European Pharmacopoeia Monograph "*Vaccina ad usum veterinarium*".

The tests to be carried out must be capable of detecting most of the known bovine contaminants, particularly those inducing viraemia (Volume 7B, page 99). In addition to the tests recommended in this note for guidance, other validated methods such as PCR and RT-PCR can be used usefully to increase the probability of detecting viral contaminants.

Control cells are used for each test, cultivated with a previously controlled and treated calf serum.

#### **4.3.2. Tests to detect Pestiviruses**

##### **4.3.2.1. Tests to detect Bovine Viral Diarrhoea Virus (BVDV)**

At the end of the observation period when the cells have been cultured with the serum tested, an immunostaining technique is applied to the cells with a conjugated (polyclonal or a pool of monoclonal antibodies) reference serum monospecific for BVDV. It is recommended to perform such an immunostaining technique on micro plates to increase the number of clones of cells, which are observed in the wells.

Representative samples of each cell type are frozen and thawed and inoculated to bovine cells sensitive to pestiviruses. After 2 multiplications of the cells inoculated, an immunostaining technique is applied on them as previously described. Control cells are used for each test, cultivated with a previously controlled and treated calf serum.

##### **4.3.2.2. Tests to detect BVD antibodies**

An appropriate volume of serum is collected from at least 5 different flasks of one serum batch. The sera are tested using a validated technique to detect BVD antibodies. A result is considered as satisfactory if no antibody is detected.

##### **4.3.2.3. Comparative Titration Tests**

The appropriate sensitive cells, which are grown in the presence of the serum tested, are used at the end of the observation period to titrate a reference strain of Pestiviruses. Positive controls are used in that testing on cells cultivated with a previously controlled and treated serum to compare the viral titres, which are obtained. The titres will not differ significantly.

##### **4.3.2.4 Tests to detect Bovine Polyomavirus**

It is recommended to use the most sensitive technique. It is recommended to use cells susceptible to polyomavirus and to reveal the presence of the virus by using an immunosorbent assay or an assay to detect specific sequences of nucleic acids, such as PCR. This technique may be most useful as a screening method and could replace more costly and time-consuming tests such as animal inoculation, mouse antibody production tests and viral isolation.

### **4.4. Tests for toxicity-cell growth**

#### **4.4.1. Cell lines**

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It is recommended to test, with the batch of serum, several cell lines (including, at least, one bovine cell line and cells sensitive to classical swine fever virus or Border Disease virus), the selection of which are dependent on the intended use of the serum.

At least, 10 cell culture flasks (of 25 cm<sup>2</sup>) containing 9 ml of medium and 1 ml of serum should be inoculated with the cells at a concentration of  $4 \times 10^6$  cells. Medium is changed one day later and replaced by medium containing 5% of the serum tested. The cells are multiplied after one week of cultivation with a ratio 1/6 (1 flask for 6 flasks).

At least 2 flasks per passage are kept for observation and cultivation with, in addition, all the flasks necessary for additional examination. The cells are observed, microscopically, at last the day after trypsination and their aspect is indicated. The cells are maintained in cultivation for 3 weeks and at last trypsination the cells are counted. These cells are also used for other controls such as viral contaminants.

#### **4.4.2. Primary cells**

After trypsination of a kidney, 10 flasks (of 25 cm<sup>2</sup>) per batch of serum tested are inoculated, each with 2 to 4 million cells; nutrient medium is added containing 10% of the serum tested. One day later, the medium is replaced with a new one containing 5% of the serum tested. After one week of cultivation, the cell culture is multiplied with a ratio 1/3 (1 flask to 3 flasks). At least 2 flasks per passage are kept for observation and cultivation with, in addition, all the flasks necessary for additional examination. The cells are maintained at least for 3 weeks. Twice a week they are observed and the appearance is noted.

### **5. INACTIVATION TREATMENT**

According to the risk associated with the use of bovine serum in contaminating the vaccine productions, it is absolutely necessary, in addition to controls performed on each batch of serum, to inactivate the serum by validated and efficacious treatments for increased reduction of potential undetectable organisms. For validation the rationale of the choice of the viral strains must be indicated, including representatives of different viral families (enveloped or naked viruses, DNA or RNA viruses) and representatives of viruses of different degrees of resistance to various types of treatments. The following viruses may be used for the validation of the inactivation procedure: BVD virus, IBR virus, one of the bovine enteroviruses, bovine adenovirus and one of the Reoviruses (REO), Porcine Parvovirus (PPV). A check for pestiviruses must be included.

The titration of the chosen viruses should be carried out (before inactivation treatment) after incubation at 37°C for 1 hour with the serum, which will be submitted to the inactivation treatment. It is recommended to use gamma radiation as a means of obtaining a safe but biologically active product. In consequence, the validation study has to determine the consistency, reproducibility, effectiveness, and efficiency of the process while maintaining the product performance.

For inactivation by irradiation, the validation study has to determine the optimal temperature, to establish a standard packaging configuration, to set specific time limits, to determine the minimum and maximum radiation exposure or dose with the product itself and to establish a radiation dose range that protects product integrity while maximising inactivation of microbial contaminants. The validation study must therefore demonstrate the actual dose received throughout the mass of the serum. It is recommended to apply a minimum ionisation of 35 kiloGray (kGy) at all points to vials of bovine serum making it possible to obtain a minimum value of 25 kGy in the vial (25-35 kGy is equivalent to 2.5-3.5 Mrad). For inactivation by means other than the application of minimum of 25 kGy to the serum in the bottle, the validation studies

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undertaken must be suitable to demonstrate the extent to which the process to be applied is appropriate, effective and reproducible.

All these data must be included in the IVMP application file.

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# **INTERNATIONAL COOPERATION ON HARMONIZATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF VETERINARY PRODUCTS VICH**

Dr O. Itoh (Ministry of Agriculture, Forestry & Fisheries, Tokyo, J)

I would like to explain VICH that stands for International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products. VICH was established in the 62nd OIE General Meeting held in May 1994. Japan, America and Europe have taken the cooperative and practical approach to achieve the international harmonization of approval standards since 1996.

All the countries require the pharmaceutical manufacturers to submit the data that assure the quality, safety and effectiveness when they make a new drug application. The contents of these data, however, are significantly different in each state or region because of the difference in systems, history and environment. In this situation, the establishment of the international harmonization of the standards for drug application is indispensable for promoting the development and international distribution of drugs and quickly providing high quality drugs for users. The success in the promotion of VICH activities has been expected because the data on drugs that are validated by an authority concerned in a region can be regarded as effective data in all the remaining regions and the adoption of this approval system may contribute toward saving human, equipment, financial resources, and time which are wasted in making a new drug application in different states.

Regarding ICH, which was used as a model to establish VICH, more than 40 topics have been evaluated since its introduction in 1990 and a large variety of guidelines have been practically applied in various regions since an agreement was reached. VICH was launched to effectively manage the expert working groups organized to cope with the high-priority assignments by utilizing as much agreement reached by ICH as possible.

THE SC REACHED AN AGREEMENT  
ON 5 TOP PRIORITIES TO START IN 1996  
Quality guidelines  
Safety guidelines  
Good clinical practice  
Efficacy requirements for anthelmintics  
Ecotoxicity/ environmental impact assessment

In these circumstances, VICH established five expert working groups in 1996.

TWO EXPERT WORKING GROUPS FOR THE FOLLOWING TOPICS WERE  
ESTABLISHED IN THE THIRD SC MEETING IN 1998

- 1) Biological Quality Monitoring
- 2) Pharmacovigilance

The biological quality monitoring expert working group and the pharmacovigilance expert working group were established as a part of the second project to promote VICH activities in 1998. The biological quality monitoring expert working group was to discuss with various problems related to the testing of residual moisture, the testing of residual formaldehyde, mycoplasma detection method and the test on the presence of extraneous agents. Therefore the staff was required to have a wide range of knowledge including chemistry, bacteriology virology and the others.

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### **TOPIC LEADERS OF THE BIOLOGICAL QUALITY MONITORING EXPERT WORKING GROUP (BQMEWG)**

Topics	Names of Leaders	Membership
Moisture and Formaldehyde	Dr. Hans DRAAYER	AHI
Mycoplasma	Dr. R. L. LEVINGS	USDA
Extraneous agents	Dr. Chris FOLKERS	FEDESA

The three internationally famous specialists, who selected as the leaders of each topic, reflect on agreement of the guidelines that each region is eager.

### **MEETINGS OF THE BQMEWG**

Meetings	Periods	Places
1st meeting:	February 24-26, 1999	Tokyo
2nd meeting:	November 14-16, 1999	Brussels
3rd meeting:	July 11-14, 2000	Ames
(4th meeting:	Summer, 2001	Tokyo)

Thanks to the cooperation and active participation of 17 regional representatives including regional staff and advisers, much progress has been made in the past three expert working group meetings.

### **PRESENT SITUATION OF THE TOPIC OF THE TESTING OF RESIDUAL MOISTURE AND RESIDUAL FORMALDEHYDE**

As for two guidelines, EWG has reached an agreement and is collecting public comments

The two guidelines that the testing of residual moisture and testing of residual formaldehyde were agreed in the last Ames Meeting. Through a laborious process, these guidelines were established on the basis of the results obtained in collaborative studies. In order to establish the guidelines for biologicals, which were not evaluated by ICH, we discussed the necessity to conduct these collaborative studies from the beginning because different methods of testing of residual moisture such as Karl Fischer method, Azeotropic method and gravimetric method have been adopted in different regions or states.

### **WIDELY USED TESTING FOR RESIDUAL MOISTURE AND TESTING FOR RESIDUAL FORMALDEHYDE**

#### **1. Testing for Residual Moisture**

Karl Fischer method

Azeotropic method

Gravimetric method

#### **2. Testing for Residual Formaldehyde**

Acetyl acetone method

Ferric chloride method

The basic fuchsine method

Acetyl acetone method, ferric chloride method and basic fuchsine method have frequently been used to measure residual formaldehyde in inactivated vaccine. Considering these backgrounds, I am sure that the sufficient evaluation of the whole contents of guidelines by the established institutes in various areas may contribute significantly toward making a final agreement. Important matters pointed out through the evaluation process are reviewed as necessary. The common approval standards are to be quickly established when the required procedures are accomplished. Two underlined parts of the testing methods shown in the tables are those proposed by the expert working group.

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PRESENT SITUATION OF THE TOPIC OF THE MYCOPLASMA DETECTION TEST  
METHOD

1. Continuation of discussion
2. Course of discussion
  - A. Common examination method  
Culture methods (Agar and liquid medium)
  - B. Examination method adopted only by a limited regions  
Indicator cell (DNA stain) method
  - C. Effective new technology  
PCR method *et al.*
  - D. Others

As for the topic of the detection of mycoplasma, many related problems have been pointed out for further discussion. I would like to propose the strategic orientations to discuss this topic. The first, A, we should quickly reach an agreement on the testing method using the culture medium which is commonly adopted in various regions. The second, B, is needed to determine whether the testing methods adopted in specific regions are to be included in the guidelines. The third, C, is need to discuss the appropriateness whether new technique, which is not currently applied to the quality assurance of preparations but may serve as a useful testing method. I have to admit that there exist many problems in the way of our discussion.

As Issue about, A, although I had regarded the culture medium method as a relatively easy common testing technique, I recognized two problems: the difficulty in obtaining the common reference strains or positive strains and the difference in the detailed test protocols between regions. The former problem arose because of the increase in the number of strains, which might cause the contamination in the manufacturing process and the belief in the theory of the selection of possibly unacclimated strains. Fortunately EDQM promotes the project study on this problem and the early solution is expected. The latter problem may arise in the situation of international negotiation and the topic leaders are expected to have sufficient endurance and tolerance to promote the discussion and obtain an agreement. As issue about, B, the dominant trend is that these testing methods should be included in the guidelines as long as they are covered by the currently effective regulatory standards. The trouble is that these testing methods should be carefully validated in the regions where they are to be introduced as new techniques. As issue about, C, the application of PCR method is expected. In May 1999, EDQM sponsored a symposium on mycoplasma detection method, which had significant effects on the expert working group. The working group seemed to reach a general agreement on the proposition that the detection methods, which might be effective in the future, should not be excluded. Regarding the section, which may include this statement and the contents, however, more detailed discussion is needed. We must watch it so that it is not strengthened regulation, the increase in manufacturing cost and the idealization of the test method by adoption of those new detection methods.

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### **PRESENT SITUATION OF THE TOPIC OF THE EXTRANEIOUS AGENT DETECTION TEST METHOD**

1. Continuation of discussion

2. The agreement on the following task should be quickly reached.

Live viral vaccines for mammals produced on established cell lines

3. Subjects to be discussed in the future

Poultry vaccine

Fish vaccine

Bacterial vaccine

Agents other than viruses

Others

I would like to explain the present condition of the most difficult problem, the topic of the extraneous agents detection test method. It is difficult to reach an agreement partly because of the difference in the target of test samples and the regulatory theory between Japan and the others. We understood different systems from the scientific viewpoints and discussed in order to make guideline on the harmonized examination methods while think over and over. We repeatedly remind ourselves to promote discussion on the basis of this essential principle. Consequently most of difficult problems lying in our way are being solved by the topic leader's excellent ability of negotiation. As a first step, we took a strategic approach to discuss of the mammalian live viral vaccines produced by established cell lines. We will soon complete the general review. Regarding the specified methods for the detection of Pestivirus and other possible extraneous agents mentioned in the theme of this symposium, however, continued discussion seems to be necessary before the establishment of these scientific techniques by the expert working group. Poultry vaccines are to be discussed in the next meeting. Extensive discussion is probably necessary to establish the testing methods for fish vaccines and bacterial vaccines and the test on the presence of extraneous agents other than viruses. When I address myself to a certain task, I make it a rule to reach a conclusion within the given time. For this purpose, I would like to propose the restriction of the vaccines and target antigens and would like to consult with the expert working group and the steering committee so that they may accept my proposal.

I have to mention an important matter, which is related to the establishment of the guideline. Careful consideration is necessary before the practical introduction of PCR method, which is regarded as a useful tool with excellent specificity and sensitivity. As you know, VICH guideline recommends the standard testing methods. Therefore it does not exclude the testing methods other than the standard ones which have proved to be equivalent. At present, PCR method is protected by patent and its free use is restricted to scientific purposes. If we use PCR method for purposes other than scientific use, we have to pay the licensing fee. In this situation, I wonder if we should add PCR method to the testing methods recommended by VICH. The PCR method is superior in sensitivity, but commonly detect it without concern in life-and-death of micro organisms. Regarding this matter, I would like to sum up the theoretical ideas in the process of reaching agreements on the two guidelines currently under consideration.

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### **SUGGESTED FUTURE TOPICS**

- 1) Inactivated testing and *in vitro* testing for killed products
- 2) Validation of potency tests
- 3) Safety tests
- 4) Test on the dissemination of the organism in the body
- 5) Test on the reversion to virulence
- 6) Product classification and nomenclature
- 7) Master seeds
- 8) Requirements for cell lines and primary cells
- 9) Stability testing
- 10) *In vitro* testing for killed products

I heard that there still remain ten more guidelines to be established by the biological quality monitoring expert working group. I have to thank you for your expectation but I am afraid that we cannot complete all the missions assigned to this expert working group. There are various limitations around this working group such as shortage of human and financial resources and lack of sufficient ability. The establishment of integrated testing methods and the assignments of high priority, which deserve to be discussed during the limited period, are to be considered.

### **TIME LIMIT OF VICH GOAL**

Implementation of major guidelines by 2005

I think, if VICH activities should be closed by the year of 2005, there is not much task, which can be discussed with this expert working group.

Dr. Boisseau chaired the first VICH open conference held in Brussels last year. He asked me what the next topic of high priority to be coped with by our expert-working group was and I could not give an appropriate answer at that time.

Today I would like to answer his question. I cannot determine the next topic by myself. But if I have the right to select the next topic, I suggest that the three topics, which seem to make the most of the merits of harmonization, should be selected. These three topics are summarized in the following table

### **NEXT TOPICS TO BE COPEDED WITH BY VICH / BQMEWG**

Safety tests for batch release

Requirements for the master seeds and the cell seeds

Stability tests

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**THE EUROPEAN PHARMACOPOEIA MONOGRAPH ON BOVINE  
DIARRHOEA VACCINES IN CATTLE**

Prof. M. Pensaert (Faculty of Veterinary Medicine, Merelbeke, B)

**1. INTRODUCTION**

For control of bovine viral diarrhoea (BVD) virus infection in cattle, different approaches are presently applied in Europe. One consists of attempts to eradicate the virus either on a regional or a national basis by detection of positive animals and the application of certain sanitary measures or restrictions (Bitsch *et al.*, 2000).

In most European countries to date, the virus is still widespread among cattle and eradication is not (yet) feasible. Artificial insemination centers are required to have negative bulls and some restrictions may be imposed on the sale of persistently infected animals. Detection and elimination of calves persistently infected with BVD virus (BVDV) markedly diminishes virus circulation but such measures should be accompanied by prevention of the occurrence of new transplacental infections. If foetal infections are prevented, then the postnatal mucosal disease form in immunotolerant animals will also disappear. This way, virus circulation in the cattle population can be diminished significantly. To avoid foetal infections, vaccination can be an important tool. Several vaccines are commercially available against bovine viral diarrhoea virus for application in cattle. Until recently, the European Pharmacopoeia had drafted no monograph.

**2. LIVE VERSUS INACTIVATED VACCINES**

As well live, as inactivated vaccines are commercially available. While some of the modified live vaccines were shown to induce a solid immunity similar to that obtained after BVDV infection, safety has been a major concern. Virulence factors and determinants for transplacental dissemination of BVDV have not been elucidated and the risk, therefore, remains that attenuated BVDV vaccines may cross the placenta in pregnant animals. Indeed, live BVDV vaccines have been shown to pass the placental barrier and fetuses could develop clinical signs (Liess *et al.*, 1984). Live BVDV vaccines have also been associated with adverse effects by causing a pathology resembling mucosal disease. Also, a possible immunosuppressive effect of the live attenuated virus is not excluded. While such effects may not be detected by experimental inoculation, some peculiar field situations may occur which allow some effects of live BVDV vaccines to come forward, certainly as long as the mechanisms of attenuation (or virulence) of BVDV are unknown. Pestiviruses have a variety of pathogenetic features and, therefore, attenuated strains always represent a risk. Another disadvantage of live BVDV vaccines is that they may be contaminated with field BVD viruses, usually derived from bovine sera, and detection of such homologous contaminant is highly problematic if the live vaccine has no marker (Van Oirschot *et al.*, 1999).

For all these reasons, vaccine research has mainly focused on the development of efficacious inactivated vaccines. Different strains have been used (NADL strain or Singer strain or field strains) and inactivation has been performed with different agents (e.g.  $\beta$ -propiolactone); adjuvants such as aluminium hydroxide or with Quil A have been used (Beer *et al.*, 2000; review see Van Oirschot *et al.*, 1999). Recently, experimental vaccines against BVDV infection have been developed consisting of the recombinant-baculovirus E2 protein (Bruschke *et al.*, 1997) that would allow a differentiation possibility between vaccinated and infected animals. The European Pharmacopoeia Commission has decided to draft a monograph for an inactivated BVD vaccine starting from whole virus strains. In the present communication, a few aspects of this newly drafted monograph will be discussed.

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### **3. MONOGRAPH ON BVD INACTIVATED VACCINE**

#### 3.1. Definition of the monograph

*“Bovine viral diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of BVD virus inactivated by a suitable method. This monograph applies to vaccines intended for vaccination of heifers and cows to protect the foetus against transplacental infection”*. The monograph clearly involves conventional inactivated vaccines, which may contain more than one strain. The issue of genotype I and II will be discussed later. The definition is clear: the vertical transmission should be prevented by vaccinating the mother (prior to pregnancy). This is indeed a well-defined claim and the potency test should, therefore, be clear-cut. Safety aspects should not present a problem. Efficacy, however, may be influenced by several factors such as strain genotype, antigenic variations of strains within a particular genotype, antigen quantity, adjuvant type, etc. The vaccine is indeed expected to contain an adjuvant.

#### 3.2. Immunogenicity and genotypes

The text reads as follows: *“The test for potency (section 3.6) is suitable to demonstrate the immunogenicity of the vaccine with respect to BVDV genotype 1; if protection against BVD genotype 2 is claimed, an additional test, similar to that described under Potency but using bovine diarrhoea virus of genotype 2 for challenge, is carried out”*.

Cross neutralisation tests have revealed that antigenic differences exist among strains of BVDV and this antigenic diversity may pose a problem in the development of vaccines. The primary aim of vaccination is to prevent congenital infection caused by BVDV field strains with antigenic diversity. Two genotypes (I and II) of BVDV exist and some diversity appears to occur within each genotype. So far, inactivated vaccines have tried to compensate for antigenic variability by including 2 or more strains of the same genotype. As far as the 2 BVDV genotypes is concerned, both are rather widespread in the cattle population in Europe and are able to cause transplacental infections. Also, experiments have shown that a marked difference -up to 35 fold- exists between the neutralising activity of defined homologous genotype-monospecific sera from experimentally infected cattle and the heterologous BVDV genotype (Wolfmeyer *et al.*, 1997). Therefore, it becomes more and more evident that novel BVDV vaccines should comprise both genotypes I and II (Beer *et al.*, 2000). Such possibility is, therefore, included in the section on immunogenicity of the monograph.

A question can be raised on whether the potency test for a vaccine containing both genotypes should be repeated with each of the genotypes. In such case, 2 groups of vaccinated animals need to be challenged, one with genotype I and one with genotype II. The group 15V which drafted the monograph has opted for separate challenge experiments because insufficient information is available on the issue whether a dual challenge, when administered at the same time, may lead to viral interference in which case the protective ability of one of the active components would not be tested. Also, transplacental infection in the control animals then needs to occur with both genotypes. In a recent experiment, pregnant heifers were challenged intranasally with a type I and a type II isolate of BVDV at 75 days of gestation and all foetuses became infected with both types (Brock and Chase, 2000). However, these studies were preliminary and involved only 4 challenged animals.

#### 3.3. Potency

The potency test is to be carried out in cattle. It is divided in 3 parts, which will be discussed consecutively.

##### 3.3.1. Vaccination and experimental design

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*“Use heifers free from bovine diarrhoea virus and that do not have neutralising antibodies against bovine diarrhoea virus. Vaccinate not fewer than thirteen animals using the recommended schedule. Keep not fewer than seven heifers as non-vaccinated controls. Keep all the animals as one group. Inseminate the heifers. Take a blood sample from non-vaccinated heifers shortly before challenge. The test is discontinued if fewer than ten vaccinated heifers or five non-vaccinated heifers are pregnant at the time of challenge.”*

The number of cattle at the start must be rather high so that sufficient animals remain at the time of challenge to obtain reliable results. Control animals first serve as sentinels to exclude that a possible infection with field virus has occurred during the prechallenge period. At challenge, they will serve as control animals to ensure that the challenge virus is able to cause transplacental infection in sufficient animals.

It was considered that the potency test should be performed in animals that are not immune to BVDV and that are not persistently infected (immunotolerant). Such negative animals can be obtained e.g. in certain farms particularly in countries where eradication programmes are running. The duration of the entire potency test is rather long and a strict isolation and follow-up of the animals prior to challenge remains important to exclude that a field infection has taken place during the period before the challenge.

#### **3.3.2. Challenge**

*“Between the 60<sup>th</sup> and 90<sup>th</sup> days of gestation, challenge the animals by the intranasal inoculation of a suitable quantity of a non-cytopathic strain of bovine diarrhoea virus or by contact with a persistently viraemic animal. Observe the animals clinically from challenge until the end of gestation. If abortion occurs, examine the aborted foetus for bovine diarrhoea virus by suitable methods. Immediately after birth and prior to ingestion of colostrum, examine all calves for viraemia and antibodies against bovine diarrhoea virus. Transplacental infection is considered to have occurred if virus is detected in foetal organs or in the blood of newborn calves or if antibodies are detected in precolostral sera of calves.”*

Two important issues in the potency test were “the challenge route” and “the time of detection of foetal infection”.

The intranasal challenge route was preferred in the monograph since a preset virus dose is administered at a predetermined stage of pregnancy. Also, this route of administration has successfully been used in several challenge experiments (Van Oirschot *et al.*, 1999). In other studies, however, challenge has been performed by allowing vaccinated and unvaccinated cattle to undergo a natural contact with persistently BVDV viraemic animals. It was considered that sufficient information is now available to consider the contact challenge as valuable, reproducible and reliable, if care is taken that the contact between the animals is sufficiently frequent and intense. Contact challenge was, therefore, included.

The stage of pregnancy at challenge ranges from 60 to 90 days, which is rather wide but universally accepted as optimal for transplacental dissemination to occur. A non-cytopathic strain (of genotype I and/or II of BVDV) is advised for challenge because such strains are known to cause transplacental spread and foetal infection.

Confirmation that transplacental infection has occurred upon challenge of the mother is obtained through examination of the newborn calf for virus or for antibody (precolostral) at birth. A proposal was made to harvest foetuses for virus examination during the pregnancy e.g. at 28 days after challenge. This method has the advantage of shortening the duration of the potency test and avoiding the possibility that intercurrent infections may occur with other viruses possibly leading to abortions. A report showed that this virus detection in foetuses at 28 days after challenge was reliable. However, the group responsible for drafting the monograph considered that the data,

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presently available, were insufficient. Also, nothing is known on whether it is possible that, in vaccinated cows, a delayed transplacental infection might occur which would remain unnoticed when foetuses are harvested soon after challenge. The proposal was, therefore, not accepted. Recently, a limited study was published in which 4 unvaccinated heifers were challenged at 75 days of pregnancy and all 4 foetuses collected at 60 days post challenge were positive for virus in spleen and blood (Brock and Chase, 2000).

#### **3.3.3. Criteria to satisfy the potency test**

The criteria of the potency test read as follows *“The test is invalid if any of the non-vaccinated heifers have neutralising antibody before challenge or if transplacental infection fails to occur in more than 10 per cent of non-vaccinated heifers. The vaccine complies with the test if there is no transplacental infection in 90 per cent of vaccinated animals.”*

The figures set forward for the rate of transplacental infection in unvaccinated animals (at least 4 of the 5 should have transplacental infection) and in vaccinated animals (not more than 1 of the 10 should have transplacental infection) were part of a long discussion.

It was considered that a 100 % transplacental infection is attainable in unvaccinated control heifers. However, it is recognised that transmission from the viraemic mother, through the placental barrier, to the foetus is a pathogenetic process in which several factors may play a role (degree and duration of viraemia, cell associated viraemia, strain virulence, ...). Therefore, a 100 % transplacental dissemination from the mother, even though feasible, cannot be guaranteed in every experiment. When such high requirement would not be reached, then the long lasting and expensive potency test must be repeated. Therefore, a margin was built in.

Also, the immunological processes, which underlie protection against transplacental infection, remain far from being clear. For the same reason, a margin was built in so that vertical transmission in one of the 10 vaccinated cows is allowed. It should be mentioned that, so far, a 100 % protection has been obtained in only one published experiment involving the use of inactivated vaccines and challenge, (Brownlie *et al.*, 1995). However, the decision on the rate of protection to be obtained in vaccinated animals is still pending as one of the European Pharmacology members insists on 100 % protection. More data showing that such high rate can be attained on a regular and reproductive basis are awaited.

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**RISK ASSESSMENT OF VACCINE CONTAMINATION**

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**KEYWORDS:** Vaccine Contamination, Bovine Viral Diarrhea Virus, Risk Assessment.

Despite improvements in manufacturing practices and quality control of biologicals, education of end-users and field monitoring, the large-scale use of veterinary vaccines may still result in a series of adverse outcomes. In particular, contamination of an otherwise safe vaccine with an extraneous agent is a potential risk. Although intensive quality control can reduce such a risk, the presence of these pathogens can hardly be totally excluded. Fungi, bacteria, mycoplasma and mainly viruses, can be responsible for contamination. A part from reducing quality and safety of vaccines, extraneous virus contamination can be responsible of severe diseases in vaccinated animals (implication on animal welfare ground and major cause of economic consequences), seroconversion (interfering with surveillance and eradication campaigns) and spreading of animal pathogens (introduction of novel, exotic, unknown pathogens). In recent years, an assessed-risk policy has been widely accepted in Europe and throughout the world, in order to identify, evaluate and document the potential risks involved in the manufacture, transport and use of veterinary biologicals. In this respect, a risk assessment (RA) of the hazards posed by these products is regarded as a specific process aimed to estimate either the likelihood that animals, humans or ecological systems will be adversely affected by the use of a veterinary biological product and the degrees of severity if a hazardous event should occur. In case of vaccine contamination, the extent of RA depends on the hazard posed by each specific agent (unless the hazard is zero, there will always be a potential risk; risk scores can vary from negligible to very high), the use of the product (if a contaminated vaccine is intended for use in a susceptible animal species, there will be a high risk probability with a low level of uncertainty for a major adverse event; if a contaminated vaccine is intended for use in a non susceptible animal species, there will be a small risk probability with high level of uncertainty of a major adverse event) and the risk reduction strategies (the processes and actions undertaken to reduce the risk associated with a given hazard).

A decision pathway for hazard identification and evaluation of diseases according to importance, prior to recommending risk management (RM) procedures should therefore include:

1) the formulation of a list of specific agents (for each animal species) as hypothetical contaminants of vaccines. For each agent the following factors must be considered:

- The aetiology (taxonomy and infectious status in country/region and source herd/animal);
- the epidemiology of the disease in animal species: where available, information regarding susceptibility to infection, rate of transmission between and among animal species, consequences on animal species becoming infected (including the likelihood of infection being present and transmitted by adult animals and vertically), survival of the pathogen should be provided;
- the ease of detection in animals with the available recommended diagnostic tests (these, capable of proving either the absence of the specific pathogen in source material and the actual disease status of source animals);

2) the assessment of the pathogenic significance of each agent. Such a process should evaluate:

- the likelihood of the transfer of infection and the establishment of infection/disease in animals vaccinated with a contaminated vaccine;
- the consequences of the establishment of the disease;

3) the examination of risk reduction options. A long list of accepted and validated control

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methods together with risk indicator systems, form part of suitable models of qualitative assessment of risk reduction strategy (RRS). This include:

- the use of disease –free sources (country/ region/ farm/herd/ animal/ SPF animals) of starting materials, raw materials of biological origin (sera, media, components), master cell/virus seeds; -
- where applicable, product treatments capable of inactivating extraneous agents;

- testing for ensuring freedom from extraneous agents on starting materials and raw materials of biological origin, in process (e.g. inactivation kinetics), on final product. Tests used to detect extraneous agents must be sensitive (adequate limit of detection), specific (possibility to detect range of strains), reliable and validated (for the purpose for which the test will be used). Despite the adequacy of RRS and safeguards adopted by RM, vaccines contamination can occur. The difficulty to establish a causal relationship between the use of a contaminated vaccine and the consequences, which can derive following administration to humans or target animal species, is widely recognized. In the attempt to establish a causal relationship between the use of contaminated veterinary vaccines and the appearance of disease problems, several data are needed. The crucial question, based on the assumption that hazard is distinct from risk, is whether or not vaccination with a contaminated product is the cause of the reported field problems. The answers to be provided rely on the demonstration of the effective risk posed by the contaminated vaccine to susceptible animal population and on the evidence for the infective nature of the contaminant agent. Although the degree of details required and the ground for a risk assessment will depend on the hazard involved, crucial information must necessarily include confirmation of the epidemiological association and the temporal relationship between vaccination and problems, unequivocal evidence of contaminant-related clinical signs, seroconversion to the contaminant agent and its detection after vaccination, demonstration of the homology between the contaminant virus detected in the animals after vaccination and the contaminant agent present in the vaccine, experimental reproduction of infection and/or disease. Following preliminary genomic characterization of a highly virulent strain of a type 2 Bovine Viral Diarrhoea Virus (BVDV) detected as a contaminant of a live vaccine against infectious bovine rhinotracheitis (IBR) and shown to be related with field problems, occurred in Italy in the beginning of 1999, sequential steps were carried out to demonstrate the infective nature of the contaminant virus. The infectivity studies included identification of a type 2 BVDV from susceptible cell cultures infected with an aliquot of the contaminated vaccine and from three months old type 1 and 2 BVDV seronegative calves experimentally infected with the contaminant BVDV isolated in cell cultures. The BVDV sequence of the virus isolated from animals, fully matched with the type 2 BVDV detected as contaminant of the live IBR vaccine or isolated from cell cultures, thus confirming that the contaminant virus was a live replicating type 2 BVDV, very likely responsible for the clinical symptoms and haematological changes reported in the field.

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### **DISCUSSION SESSION III**

Dr S. Gilliam: We test for bovine polyomavirus by PCR and we find that there is a presence of about 80% in the total foetal bovine serum that we test. What is the opinion on bovine polyoma?

Dr P. van der Valk: It is not a virus which is routinely tested. I know that one of the industries has done this recently because it was mentioned in the Netherlands where we had a contaminated vaccine and we had difficulties in linking the contamination with all the problems encountered in the field. I think that this is where the polyomavirus came from. The experience of this particular industry is that most of the sera examined is contaminated. We do not think that the polyomavirus will be part of list on virus assessment, as it is not linked to a disease, as far as we know, in cattle. Once it is mentioned, it appears in the Draft Guideline for extraneous testing for VICH.

Prof. J. van Oirschot: BVD virus testing: there are so many different sensitivity of different tests, different cell lines. Perhaps it would be worthwhile considering to use calves instead of cell lines as these may be more susceptible when injecting intra-muscularly.

Dr P. van der Valk: This has not been considered and one of the reasons is, of course, we would like to move away from animal testing. This applies to both the extraneous agent testing and antibody testing. We need standards and reference material to better harmonise this.

Dr P. Jones: In many ways TSE is more insidious than BVD but it would seem that it is easier to do a risk analysis with TSE, in as much as you identify the hazard, you characterise it and then you manage the risk by sourcing and by looking at where the material comes from. Whereas with BVD, applying risk analysis is the same principle, but as we saw in the Netherlands from 1999 to 2000, however many steps you take, the risk is sometimes unmanageable.

Prof. J. van Oirschot: Question to Dr Bruckner: You summarised in saying that BVD is easily detectable. If you have few infectious virus particles in a big batch (1 or 2 thousand litres), in my opinion, it is not easy to detect these infectious viruses. But as you know, a couple of virus particles are enough to start an infection in cell culture and then in vaccines.

Dr L. Bruckner: In comparison to TSE's, BVD is easily detectable! But concerning the problem of safety testing, you always have the problem that you look at a very small volume and make a larger conclusions for volume - a critical point, like for any other safety test.

Prof. J. van Oirschot: we have bought inactivated irradiated serum and after two, three or four passages we get the infected virus out.

Dr J. Ridpath: The problem is that while PCR can pick up one or two viral particles, you do not know if the 10 or 20 ml samples you are testing out of 40 litres lot has the one or two viral particles. This is why it is better to test larger sample volumes and go through the multiple tissue passages. It depends where the virus is, in comparison to dilution.

Dr P. van der Valk: (Comment following Dr P. Vannier's presentation) This is just a note for guidance! Within the regulatory bodies we often encounter notes for guidance to be obligatory however, this one is a note for guidance only. We would like to limit the scope of this note for guidance because, we would like to avoid a repetition of all kinds of requirements already given in the European Pharmacopoeia. When we talk about a list for viruses to be used for inactivation validation, this is not only applicable for serum. We would like to have a list like this for all the inactivation methods. We have to validate when we use raw materials for production of vaccines. This will be given in a different note than that of the European Pharmacopoeia, which you can use on a general scale, and not just for serum. Please try to limit the scope for BVD otherwise, a note for guidance is not just a note for guidance, it becomes obligatory.

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Dr W. Hesselink: Question to Dr P. Vannier: Why was polyoma also included in the draft note for guidance?

Dr P. Vannier: One of the reasons is that this contaminant is widely spread in the bovine serum batches. This was a group consensus and it was considered very important to know what was done and to be sure that, in the final product, the virus is not found. This is the major reason. Are we sure that if a virus is called specific for one species, when injected to other species, it will not induce some adverse effects? We have had bad experiences in the past with this.

Dr W. Hesselink: As we have heard, 80% of the serum batches are contaminated with both polyoma strains and this will probably not be killed by the 25 kGy irradiation given. In the past, millions of animals must have received polyomavirus. Is there any indication that this is harmful to any species?

Dr P. Vannier: This is a point to be considered.

Dr D. Kretzdorn: Possible concentration of polyomavirus should be considered in the serum batches - we say that these are not inactivated, but do we know about the content? How many logs are there per millilitre in the serum? We may not be inactivated as we may not achieve  $10^6$  log reduction, which is required for the polyomavirus with 25 kGy. It is assumed that the actual concentration in serum is lower than  $10^6$  log. But do we have that amount of virus in the serum to start with?

Why do we restrict the serum batch to a volume of 1500 litres? There are other measures to ensure that you do not get a high contamination level in the final serum batch, as it was shown by a serum supplier that you can pre-test the serum pre-pools in a much better way and then ensure that the level of contamination in the final batch is minimised. The general restriction to the volume of the batch is not justified. It would be better to do a pre-screening, or an evaluation of the actual titre of the contaminant in the serum batch, rather than just applying shear limits on the volume.

Why do you think that the information should be included in the application file? Is this because you may have different suppliers over the time of production run. This would be restrictive to the vaccine manufacturer. Such information, if generally demonstrated that the inactivation method is good and that the test methods are good, is sufficient. The rest should be in-house documentation under GMP guidance, not necessarily in the application file.

Dr P. Vannier: Why do we tend to limit the size of batches? This is because we can limit the volumes used to constitute one batch and in consequence, to limit or to minimise the risk of increasing the contamination by successive addition of serum from infected animals. This is a clear consensus from manufacturers and cell suppliers that size should not be limited but there is a certain contradiction. Previously, when people spoke about the pre-pool testing before blending it seems to be too expensive and too restrictive. This however, would be a good way of limiting pre-pools which are infected by BVD virus. The bigger the size of a batch, the more difficult it is to have a representative sampling to detect BVD virus because, how can you have a representative sampling on a batch of 5000 litres? These are arguments to take into consideration to find the right scientific and economic compromise.

Concerning the market authorisation file - two comments. Firstly, personally I am surprised that for the vaccine manufacturers the level of requirements is so high. There are inspections and for a key sensitive problem as bovine serum, which are universally used for the production of vaccines, there is no guarantee of the quality - there is only a contract between the vaccine manufacturer and the serum supplier. From laboratories doing research, we receive batches with perfect certificates, no BVD virus, no BVD antibodies, but after the controls, we find the batches contaminated by BVD virus or which contain BVD antibodies. There is also a problem there.

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The goal of the group is to include general data and not specific data for each batch of serum of course, and we have to be clear about this, if it is to be included in the marketing authorisation file, to give to the competent authorities some guarantees that the manufacturers such as these, at present, do not exist.

Prof. J. Brownlie: You look for antibodies to BVD, do you look for antibodies in any other agent? Do you think that this would be important? We are suffering an FMD (Food and mouth disease) outbreak, it would be valuable to know that there was no chance that any of the foetal calf serum could come from any country that had an epidemic or an outbreak of FMD.

Dr P. Vannier: We have said in the guideline, to have a global approach but FEDESA, is requesting us to limit the scope of the guideline to BVD virus. It is clearly said that for the source, the source has to be free of exotic diseases according to the OIE code. This means that the collection of serum must not be taken in the different parts of the world where there are exotic diseases, such as FMD. This is a difficult situation which has to be taken into consideration. The problem of not limiting too much the sources of serum but to give a guarantee of the quality of this. According to different regional sanitary situations in the world, this is a difficult problem.

Dr D. Kretzdorn: Question to Prof. M. Pensaert: Is it correct that the harvesting of foetuses pre-parturition is not acceptable in the new monograph?

Dr M. Pensaert: It is not accepted, that is correct.

Dr D. Kretzdorn: Question to Prof. M. Pensaert: Could you specify why there is concern that there is a delayed infection of the foetus? Is there any evidence that something like this could happen - e.g. several months after the infection that there is a real infection of the foetus occurring? We have some experience but we have never seen this.

Prof. M. Pensaert: There is no evidence that this occurs but there is no evidence that this does not happen. The group 15 V felt that, as longer it is not clear, delayed transplacental transmission is not excluded. Collection of foetuses was, therefore, not accepted.

Prof. J. Brownlie: Comment to Prof. M. Pensaert's presentation. I think that the numbers which you are proposing are quite low for setting up the experimental groups, only 13 and 7 were mentioned. My experience in this, and the two trials, which we have done, is that you do need bigger numbers as it is difficult to get the number of animals through insemination and keep them. I would suggest 15 per group. You seem to have lower controls too, these are as important as the vaccinated animals. If you are going to set standards for efficacy where you are going to expect 85 to 95 or 100% then you have to have enough numbers to make that more approachable.

Prof. M. Pensaert: We are also concerned about trying to keep the number of animals as low as possible.

Prof. J. van Oirschot: Prof. M. Pensaert proposes intranasal challenge or challenge by contact of a persistently infected animal. Concerning intranasal challenge, you did not mention the type of strain or dose, which you use. You may prefer to use a heterologous strain and not a homologous strain but in pestiviruses what is heterologous, how far must a challenge strain be away from the strain which is in the vaccine?

Prof. M. Pensaert: I always wondered about this when judging a monograph. Specific information on the challenge strain with regard to the strain itself or the dose are usually not given. The answer is that you take what you want, as long as you get the criteria in your controls, in this case with regard to the percentage of animals which undergo transplacental spread of the

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virus. It is up to the person or group who is carrying out the experiment to decide what strain to use and what dose to be used.

Prof. J. van Oirschot: Do you not think that manufacturers would choose the homologous strain? Normally, you do not expect in the field when the vaccines are used that these animals are challenged by homologous strains. You should use a heterologous strain and you can define what you mean by heterologous.

Prof. M. Pensaert: This is the difficulty with BVD virus! We are not talking about genotypes, which are clear cut.

Prof. J. van Oirschot: We have the experience with contact challenge where we had one persistently infected animal put into contact during one month with 10 other animals. Five of them were sero negative and five of them were sero positive, probably due to an infection four years prior to that time. The five sero negative became sero positive. Of the five sero positives that were put into contact with the persistently infected animal, only one had an increase in antibodies, the other four did not have any increase in antibodies. After one month in contact with the virus but they still did not show an increase. You cannot prove that these animals were exposed and this is a disadvantage if you use contact challenge. Intranasal challenge is therefore a better way of infection, even though this is not the natural choice.

Prof. M. Pensaert: I agree, and I think that the intranasal challenge is to be preferred as it is clear-cut. It has been proven that contact challenge can work but it is possible that contact infection occurs with a small amount of virus only and this may be of influence in determining the further course of the pathogenesis. We really do not know the mechanism by which BVD virus is able to cross the placenta. I would also use the intranasal challenge test to be sure that, at the end, the criteria is fulfilled.

Prof. J. Brownlie: It is not just a matter of dose, it is a matter of timing. These experiments are like a military manoeuvre to get them all tightly in time. If you are trying to challenge in 80 days or 90 days, you have only a very short period of time in which you need these animals to be infected before the foetus become immunocompetent. I would think that, as a national or international recommendation, the contact animal can be problematic. I would prefer to have an intranasal challenge of a set dose. The strain is difficult but the dose could be set.

Prof. V. Moennig: I agree that it might be difficult to reach 100% however, for a control scheme like we have in Germany which is test and removal first and the second step is vaccination of female animals in order to prevent the merging of new permanently infected animals, it is absolutely essential to have a very high potency vaccine available. If we look at the live swine fever vaccine, that has a 100% foetal protection - of course, one cannot compare swine fever as it is genetically more homogeneous, but what we are asking for is future vaccines that cover both BVD 1 and BVD 2. It must be possible to reach this 100%.

Prof. M. Pensaert: There is room for improvement of vaccines and we will be able to adapt the criteria in function of the developments. However, at present, with what we have and with the knowledge available, the 100% can be reached but cannot be reached routinely. When talking about swine fever, this was a live virus vaccine. When we, however, refer to the inactivated swine fever vaccines a long time ago, there was the problem with carrier sows. The sows themselves were protected by the inactivated vaccine but it was possible that, in such sows upon infection with virulent virus, transplacental infection still occurred with birth of infected pigs. With live vaccines, the immunity which is established may include the cell mediated immunity part. A live vaccine may induce an immunity which is more complete and which is more efficacious to prevent transplacental spread. Of course, with live BVDV vaccines, there may be problems with regard to safety.

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Comment from the floor: If we talk about a control programme including vaccination, the whole herd will be vaccinated or at least all the animals at risk, within the unit of the stable. It would not make sense to vaccinate part of the herd or individual animals. The pressure of infection that you would expect in this herd, is much lower than the one applied during challenge, which is an artificial model. Under field conditions, yes, in a vaccinated herd, you can get the 100% protection but not necessarily with very hard laboratory challenges.

Dr P. Vannier: Comment to Dr M. Tollis' presentation: I agree with your comments on the difficulty to temporarily link the problems appearing in the field and the use of the vaccine. The problem for the pharmacovigilance, which is set up in Europe, is much more designed for chemical products and adverse effects of chemical products. For the vaccines, it is much more difficult to establish this link and the approach from my point of view, and from our experience in this field, is that it is more an epidemiological enquiry to be set up to really show the link between vaccination and the adverse effects, especially when you vaccinate pregnant females. The adverse effects will appear some months later in certain cases. When field problems occur farmers say that there are two key causes: the feeds and the vaccine. Very often, this is evidently wrong - sometimes it could be true and the problem at that time is to demonstrate the link between the vaccine failure and the accident.

Dr P. Jones: Pharmacovigilance on this occasion worked extremely well, in the sense that the report first came from the Netherlands and the Dutch authorities which were very rapid to send an alert and information which was co-ordinated within the member states and action was taken from all the parties concerned. The analysis which the CVMP undertook, because the matter was referred for an opinion with a 24 step analysis of the risk and the conclusions which mirrored what you were doing in Italy with the situation which you found there.

Prof. J. van Oirschot: I agree with the difficulties in establishing a causal-relationship between contaminated vaccine and consequences of this, months later. Dr Tollis wanted to establish the reproduction of the infection of the disease by the vaccine, and if I have understood correctly, you used BVDV type 2 and grew the contaminant in cell culture and you put the contaminant into cattle. Why did you not use the contaminated vaccine to put into the cattle?

Dr M. Tollis: Because I did not have enough product. This has already been done, nevertheless, as other people have detected virus from the vaccinated animals. I could not do this for two reasons; I did not have enough product and because the animals which had been vaccinated in Italy, had been immediately cleared. What I wanted to demonstrate was the effective nature of the virus in a more detailed way, this was the only way that I had to recover the contaminant virus and to show the identity among the viruses isolated from the vaccine, the one isolated from cell culture and the one isolated once experimentally reproduced.

Question from the floor: How did the tests react which were performed on the vaccine before it was released and how did you test to detect the virus, and how did these differ? What had the manufacturer done before the release?

Dr M. Tollis: The manufacturer performed all kinds of tests but no PCR. They performed cell culture tests.

Prof. J. Brownlie: Following on the enquiry concerning the vaccine, with the vaccine that I am familiar with, every batch has to be tested for potency and it is tested in calves for an antibody response. I am sure that if you had had a vaccine contaminated with the type 2 you would have picked this up in calves. Are the manufacturers able to market vaccines without doing a potency test such as that?

Dr M. Tollis: Actually, the manufacturers perform some tests on cows, and how they justify the

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situation is by saying that they use sero positive animals. This is because it was an IBR vaccine so they did not use a negative to BVDV virus.

Dr D. Kretzdorn: Firstly, this is a live vaccine, therefore the potency test for the live vaccine is the tritration in cell cultures so there is no potency test in calves for batch release. The safety test is done in calves and possibly the risk perception was wrong. At the time when the safety test was established, there was no requirement and there is no requirement in the monograph for the live IBR vaccines that these calves have to be free of antibodies against BVDV, therefore, we never checked for that; actually, those calves came from a herd vaccinated against BVDV so they had maternal antibodies and these were sufficient to eliminate the relatively low virus, which did, nevertheless, cause disease in sero negative cattle.

Dr P. Jules: Sequencing results after animal studies; how long was the site of the sequence reagent? Is there 100% identity compared to the vaccine?

Dr M. Tollis: Yes, in the virus directly detected contaminated vaccine directly from the cell culture and taken from the animals.

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### **SESSION IV:**

#### **GENERAL DISCUSSION ON: HOW TO DETECT AND AVOID PESTIVIRUS CONTAMINATION AND POSSIBLE REGULATORY MEASURES AND CONCLUSIONS**

- Are the issues discussed over the two days applicable to all the products and for all the species or do you make differences according to the species, for instance in the veterinary field and taking into account the target animals?

- If we have to do something at the herd level, is this feasible? e.g. with TSE certification, most Europeans source their sera outside Europe, would a certification for BVD likewise the procedure established for TSE and operated by the EDQM level, be useful?

- Raw materials: what do we test? Antibodies and viruses?

- When do we test? Before or after production of the biologicals concerned?

- How do we test? Isolation v. PCR

- How do we inactivate?

- How do we validate an inactivation?

Regulatory issues: What is the contribution at the CVMP level within Europe? Do we need a certification for the raw materials as for TSE?

- What is the contribution of the EDQM? Do we need a specific monograph?

- International dimension: What are the consequences of the above on VICH procedure and on the globalisation of the market in general?

Dr D. Mackay: There is a need for a decision and a discussion within the Immunological Working Group of the EMEA (IWG) on:

- Limiting of the size of the batch - going through the guideline and see whether it is reasonable within the framework of that document to specify an upper limit of size for the batch of FCS or other culture media but which will obviously relate to statistical probabilities of contamination.

- Feasibility and desirability of testing pre-pools of sera before they are then used to create large bulks;

- The extent of what is reasonable to ask in terms of traceability. There have been two different lines which were presented by manufacturers of serum during this symposium. Some will say that they can trace right back to the farm of origin even, if not to the actual animal of origin, in other cases we were informed that this is not possible at the level of a slaughter-house. Before we include a statement in a guideline, we need to be clear that if anyone will comply with these traceability requirements!

- Homogeneity of a batch. In theory and in terms of a definition of a batch, a batch is considered to be a homogeneous volume of product, however, we have heard several times that this is not always the case. This may be because the contaminating agent is present at such a low level that it is impossible to get homogeneity. We have also heard that when testing several bottles from the same batch, for example for antibody, let alone for virus, different results can be obtained. Is there something that we can do at the level of the guideline to make more stringent the requirements for homogeneity.

- Testing: One of the fundamental questions is, is this guideline only to relate to BVDV or all other potential contaminants of bovine sera especially the virological ones?

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- When and how often to test? Should it be tested before and then be subjected to a validation inactivation procedure and then retested? Currently there is no absolute requirement for that second retesting. Should there be one? If you have one afterwards, should you have one before? We need a consensus of opinions.

- Polyoma virus: the practicality and desirability of whether we need to test for polyoma virus - if we do, what do we do with the results? How to interpret a positive contamination in terms of infection and for which species.

- Comparative titration test: this is written into the current guidelines - what is the general feeling on the value of that test? We have discussed the need for standardisation of testing and this is a good way of doing this, therefore, should it remain within the guidelines?

- Testing in live calves. We have discussed how to test for BVDV in terms of virus isolation and tissue culture, PCR and also the possibility of testing in calves. Should this be within the scope of the guideline?

- The role of PCR - this needs to be definitely decided. There have been a lot of discussions on its merits and demerits. Should the requirement be made for BVDV sero negative calves in the safety test for all bovine vaccines? At present these need to be sero negative for the agent for which one is doing safety testing. Should this be made a more global requirement for any product going into cattle?

- The requirement for testing sera for antibody to BVDV, it is very unlikely that you will get any batches satisfying such requirement unless they are from donor herds or very known and monitored health safety herds that are completely free from antibody. What should the requirement be? Freedom for antibody and acceptable level of titre? Or, that we do not consider this as important?

- GMP - documentation. The issue of cross contamination within the vaccine manufacturers. This should be dealt with by GMP but it is obviously a potential source of contamination of serum and should be addressed within the document.

- At what level should this documentation be presented? Should it be part of the GMP dossier for a manufacturer? Should it be part of a dossier he presents to a registration authority? Should certification of compliance of sera be a completely separate issue which can then be done on the basis of providing a certificate?

Prof. P. P. Pastoret: Do Regulations fit for all products and all species or are there differences between species, for example, would you prescribe the same requirement for vaccines intended for dogs as for that for ruminant?

Prof. J. van Oirschot: We talk about BVDV contamination and pestivirus contamination so we have a tendency to limit the issue to that only for animals which are susceptible for pestiviruses, which are pigs, cattle and sheep. The question is what about the other animals? How big is the chance that BVDV will affect dogs, cats, horses and man? There is no answer as how big is the chance that an animal, which is normally not susceptible, can start an infection after parenteral vaccination with a contaminated vaccine.

Dr R. Levings: The issue is valid to ask what is the risk to other species that are not known to be infected with pestiviruses, considering the evidence as well as, the shifts in production methods which have currently developed. I would like to back-up the susceptibility question one step; there are two places where you could make the cut off. The first is the susceptibility in the target animals species to a pestivirus and secondly, the susceptibility of the manufacturing cell line to pestiviruses. This is because we have much broader cell line susceptibility and that is certainly

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going to shift the characteristics of the final product if the foetal bovine serum contaminates that lot.

Prof. P.-P. Pastoret: So, it would be better to have the same rule for all the products.

J.-M. Spieser: In a scientific poster presented it illustrates that you can find in a certain number of batches of vaccines which are intended for human use, some of the cell lines used in the production of human biologicals that are susceptible. You have some cases shown which have 6 over 7 batches, which were positive. The Biotech Working Party has addressed the issue of the draft of the IWG and are waiting for the outcome of the present discussion to advance on this. As regards the non-use of animal tissues in growing cell culture, it appears that there is still a long way to go as there is still a great use of this media in the field of production of human biologicals and biotech products.

Prof. P.-P. Pastoret: We focus on BVD virus, however, there are other viral contaminants and therefore applying a simple rule for all products shall be an option.

Dr P. Van der Valk: The draft guideline deals with bovine serum and in my opinion, the BVDV contamination is the reason that we would accept a guideline on the contrary we are not in favour to extend this to everything, especially as product wise it needs a completely different guideline. I would like to see that the guideline is restricted to BVDV because the other provisions laid down in the European Pharmacopoeia for extraneous agent testing are still satisfactory.

Dr L. Bruckner: I think that it is short minded to restrict it to BVDV. We have had the dramatic experience of the BVDV contamination - tomorrow there will be something new. We have heard over the last two days a lot of concern over the polyomavirus. There might even be other contaminants for instance, rabies. We should try and get a general approach and not limit ourselves. Of course, the BVDV is a wide spread antigen, but other may also occur.

Dr P. van der Valk: This means that we put out all the present precautions which we have in the European Pharmacopoeia for extraneous agent, however, do we know that these tests are not sufficient. This puts a bomb under the VICH who are trying to harmonise the basic requirements at present. If these are not satisfactory, we will have to rediscuss all the extraneous agent tests!

Dr L. Bruckner: I do not think that the present requirements are not satisfactory, however, I think that the applications of the present requirements are not satisfactory. We have to be careful how these are applied.

Dr P. Jones: It seems that there is a potential for differences. We are aware in industry of the concerns about regulation of medicines, overregulation of medicines and availability of medicines. I think that Dr P. van der Valk's point is a valid one - notwithstanding the comments also by the experts who say 'look at what happened with BVDV' we saw with experience what the result of that was. Dr M. Tollis gave a presentation on risk assessments and I wonder if it is feasible to consider some of these agent, some of them are probably more likely to be contaminant than others, and should we try and accept that there is a need for a risk assessment on the host of these agents to try and define what would be embraced in a guideline, as opposed to saying that it is a blanket cover for all possibilities.

Dr D. Mackay: Is BVDV a special disease, which requires a special monograph? Or, is serum a special material that has special requirements over and above those other substances of animal origin? Arguments could be put forward for both. My personal feeling is that we are saying that it is BVDV, which is the particular problem rather than that serum is the particular material, which is a problem.

Prof. P.-P. Pastoret: We are talking about the human contamination by a bunyavirus. It is not BVDV but it is a viral contaminant. If we look at validation of inactivation, it could be that we

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would like to see it, not only from the point of view of BVDV, but also from the point of view of other viruses. For example, a choice of viruses to validate an inactivation procedure will depend on the fact that one only focuses on BVDV or also other viral contaminants - this is linked. I am not sure where to mark the consensus!

Dr R. Levings: Depending on how rushed you are - you could take a risk assessment, standpoint and still probably arrive for most agents at about the same position, whether you started off with BVDV barely worrying the others or worrying about all of them and then solving a country risk assessment because, I think, that several speakers have mentioned that while other agents that could be of concern, bluetongue of course raises my mind for the USA. If they do not reach viraemia in the foetus and are unlikely to be at such a titre that they would not be diluted out to the degree that the inactivation, that was already carried validated for all family of viruses, at least the index set of viruses, then, even if you crunch that through some risk assessment numbers, probably BVDV would still come out as the only one that would merit a specific assay. I might be wrong, but if a serum manufacturer sourcing mainly in certain herds in the Nebraska in the USA, bluetongue might rise up to the same level and have to be tested for. However, then we could require the serum manufacturers, based on their herd of origin, and based on the OIE list, and our experience, those numbers would move up and down in a very small number of equations that would yield then what you had to specially test for. Assuming, of course, that we had some of these general precautions such as the inactivation that cover the indexes. This would be a compromise that would not raise the number of tests to the expense of the serum unduly, and yet assure the consumer and regulatory authorities of the quality and safety of the product.

Dr M. Edgington: The way that I have been looking at this is on the way of pharmaceutical production and considering serum as an aseptically filled sterile product. There is a case for making serum a special material. Whatever you do in the line of biological testing, we all realise that there are severe limitations on the limit of detection. We know that serum can be contaminated, with a wide variety of agents, ranging from bacteria to mycoplasma, through to a whole range of viruses. We have to make a guideline, which covers all agents, and we should look at processing the serum in a way that it has a treatment that inactivates the agents. Gamma radiation looks to be a suitable process. We should not only concentrate on BVDV but serum as a special material.

Prof. P. -P. Pastoret: Our problem, however, is linked to BVD virus but I agree that we need to be more general than simply focusing on BVDV.

Dr M. Edgington: It did strike me that BVD virus might be a suitable candidate for validation experiments of the irradiation as it presents as a single stranded RNA, small target.

Prof. J. Brownlie: It does seem that serum is a special risk and within that BVDV is perhaps the most notorious of the pathogens. Also, it is sometimes at such a low concentration that it causes a special problem and you need the most sensitive detection system you can. It would seem that the one tested in the live calf is the most sensitive indicator. If you look on a world scale, it appears that 50 000 litres are produced yearly and if they were all put into 2 000 litre batches, that is only 25 calves, which is very trivial. You can tell a huge amount post challenge with foetal calf serum. You could put 100 ml intravenously into a calf without any problem, for subsequent testing. The whole batch could have a certificate that it was free from a series of immune responses or infection developments.

Dr M. Huether: The difficulty comes in finding sero negative cattle to every virus that we would want to go and test and that causes a large problem. It causes a large problem for us to be able to find that they are truly BVDV negative for type 1 and type 2, especially in the USA. It would incur a great deal of costs and it would be very difficult for us to do, especially as regards viral inhibition particularly when it comes to look at all the different species. When you are

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qualifying the serum, the serum is going to be used for production of a large majority of vaccines, whether it is for canine, feline, bovine etc., and we do perform viral inhibition on all those as well as antibody testing. Again, BVDV is a special consideration as I have seen people looking at antibody for BVDV, which I do not think it is the right way to go. Really, viral inhibition assays, showing that there are other components within the serum, besides the antibodies, which can specifically inhibit BVDV is very important. We should be setting limits on what that viral index is of that serum, knowing what the sensitivity of your assay, what you can and cannot reach. When we are specifically talking about BVDV, we should look at the serum and our requirements for making BVDV vaccines associated with potentially sero negative or low levels of antibodies against BVDV in that serum. As regards the others, again, I think that it is part of the risk assessment, it is for the user company to evaluate this risk in the context of a different product, based on the host animal that it is going into eventually.

Prof. J. Brownlie: Can I just come back on the comment that it would be impossible to get calves that were free from all diseases or have free antibodies from all disease. You could go a long way by using colostrum free calves. They are not so difficult to obtain or to raise.

Dr M. Huether: I would agree - the one thing that we have seen, and we have done the studies, when you look at colostrum deprived derived cattle when they are very young and you give them very low levels of BVD, they do not respond very well. You want to detect a contaminant that might be 2 logs in the serum, you will not pick it up in your antibody determination or in infection development. You are also looking at trying to standardise some tests, and no doubt animal tests have the greatest deviations. I think that these are not as sensitive as doing a cell-based assay.

J. -M. Spieser: Can we in 2001 still make a proposal based on animal testing when we know how much this is controversial. This is not needed now with the various alternative methods, which we have available, including PCR.

Dr L. Bruckner: I would strongly object to these animal tests. It is not for the reason of animal welfare but introducing a new animal test, which is not validated. This is unacceptable and scientifically incorrect. Any new test whether *in vivo* or *in vitro* methods to be used has to be validated.

Prof. J. Brownlie: All batches of vaccines have to be tested in animals before they are released. I have not heard of a more sensitive indicator, although I agree that we will reduce animal testing as much as possible in future. We are talking about a very small number of test animals if you test large batches of foetal calf sera.

Dr P. Vannier: BVDV antibodies - we are still not clear on this topic. It was clearly shown that if, in the guideline, it was proposed that the manufacturer will have to reject all the batches with antibodies, there is a majority of people saying, and I can believe this, that there will be no longer any batch available. On the other hand, it was clearly and convincingly shown, that a certain level of antibodies could mask the presence of viruses. Consequently, the use of such a serum is not appropriate because it will be impossible to detect pestiviruses by cultivation. FEDESA was proposing a low level of antibody but what does this mean? I am sure that the industry has data on the problem of validation of inactivation procedures, concerning the problematic of detecting some contaminants by using sera with low level of antibodies we need this data in order to set up a text, which is realistic but, at the same time, gives guarantees to prevent problems of BVDV contamination. In the absence of this data, it will be very difficult to properly advice.

Dr P. Marbeant: I have some difficulties in positioning myself as to what we are now debating. Of course, I am frustrated because I do not know which route was taken in contamination of

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the vaccine. I cannot assess if my testing programme is making more confident to avoid this contamination. I am afraid that we are too counter reactive at present and adding a lot of requirements which will make life very difficult for the producers as we have no evidence as to the errors made in the process that we could easily avoid. It is a more general question however, prior to elaborate to new things, have we made the completely route analysis and if the current norm and guidelines in default to considering what has been done.

Dr D. Kretzdorn: Comment on antibodies in the serum: Dr P. Vannier I think that you are right in saying that serum has to be antibody free if you use it for testing purposes because, there it could mask the detection of potential contaminants. This is completely different if you have a serum that was inactivated according to validated methods. We have discussed the validation and agree that in the past there may have been deficiencies in validation of the inactivation procedure. As long as you test the serum with the validated test, before the inactivation and you know the level of the potential contaminant, with an antibody free serum for cell culture, then low antibody titre in the actual production serum for vaccine manufacturing would not harm anything. Unless, you produce the vaccine virus like BVDV - this is something for the manufacturer as the titre for the virus harvest will be lower possibly - but this is not the safety aspect. We should differentiate between the sera used for vaccine production and the sera used for testing purposes.

Dr P. Von Hoegen: I would like to insist on risk assessment on serum.

Prof. P. -P. Pastoret: We have problems with BVDV type 2 in Europe, coming from USA, but we may also have problems with bluetongue. The risk assessment should take into account the country of origin.

Should we do something at the herd level? My feeling is that we have to take care of this within the risk assessment.

Question from the floor: If you look at the risk assessment, this depends on the *in vitro* situation as you use a serum to expand the virus in your vaccine in the cells. Even you have many different viruses in your serum, your culture condition and your source of cells is defining which viruses can replicate. At the outcome, one could say that the risk of viruses that are in the vaccine is an *in vitro* phenomenon. In this field, in staying *in vitro*, it would also be reasonable to only test with *in vitro* technologies the viral contaminations. There could be other viruses and they would not replicate in the system and then depends on which cell types you have, which virus could be replicated. At the end, the problem is *in vitro*. The ECVAM who are working on *in vitro* assays for these specific areas and in the field of vaccines, which is, still a little behind. Traditionally, there have been tests established but now we are in the phase of establishing new assays and I think that this should be the moment to go with the problem, which is an *in vitro* problem.

Prof. P.-P. Pastoret: If you focus on BVD there will be differences, for instance, serum from herds which have been shown to be free of BVD virus infection, and in the risk assessment this is different. Do we have to propose something at the herd level or do we take care of the herd level within the risk assessment? If you have a very good vaccine, able to prevent foetal contamination, you diminish the risk, is it a sound proposal to propose to vaccinate with a good vaccine or not? Or, do you deal with this within the risk assessment? I would favour the proposal to deal with this within the risk assessment.

Dr D. Mackay: Does this not depend on the question whether it is possible to have an audit trail that is clearly documented as far as the herd. Obviously, you can put in what risk assessments you like, but if there is not a clear audit trail, and it still seems unclear whether the manufacturers can audit it back to the herd.

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Dr P. Vannier: I have received a lot of comments from serum suppliers and manufacturers and it is clear that all serum suppliers say that it is impossible to trace back to the herd. You also have difficult organisation for the collection of serum as you have companies close to the slaughterhouses, collecting small pools and selling these small pools to the serum suppliers, blending these small pools for bulk. In fact, the organisation of the trade is relatively complicated, more complicated than we can suppose. It is clear that there are two different herds, donor herds - perfectly identified and defined, for which it is very easy to have a qualification of the health herd status. Beside this, there is collection at slaughterhouses, there is identification of cows or veals but they cannot trace back to the herd to have information on herd status.

J. -M. Spieser: We have heard that for the traceability in relation to TSE is absolute or nearly perfect and can contribute to the certification etc., and now, we hear the opposite. We should pursue this - are herds and collection of sera traceable or not?

Mr R. Festen: We are a serum manufacturer. When we talk about a donor herd you can certainly trace back to the specific animal of the day of collection, however, for most serum (and serum is just a by-product of the slaughter industry and that a cow could have been transported four or five times to many different farms during its life cycle) therefore, to keep the traceability is difficult. We can trace back to the slaughterhouse on the day of slaughter and depending on our supplier, we can trace back to the farm. This depends however, on the supplier and how well they monitor their herds.

Dr P. van der Valk: Sera is the second largest group, which have received TSE certificates. Therefore, EDQM must have information available as to what can be done and cannot be done. They have certified bovine sera.

Dr P. Price: If you can use donor animals, you have complete traceability. If you are only using foetal bovine serum, because of the amount of animals involved, it is difficult to control the collection as another company often does this. It is not easy to go back and do complete traceability to the cattle. We can say which country of origin it comes from, which slaughter house it came from, the day where it came from, when it was collected, exactly what went into the pool but, to go back and say where the mother came from - I do not think that this is reasonable.

Dr. L. Bruckner: What would be the advantage of herd identification? What will it help in preventing in the contamination? There is a difference between TSEs and BVD. TSE might be a notifiable disease but BVD is just a disease amongst others not submitted to any specific regulations.

Dr R. Levings: If you knew that the herd was vaccinated or that the herd was certified free, it would shift your risk assessment and you may be able to skip a step. What we are hearing is that the uncertainty is too high to give us the confidence to skip a step, but probably, it would be harder to get the information than to perform the step! That means that we have to do more testing upfront to qualify suitably the product.

Prof. P. -P. Pastoret: What do we test? Antibodies, viruses. There was a suggestion from Dr P. van der Valk to use sera with low antibody titres.

Dr P. van der Valk: In principle, the industry prefers to use low antibody titres when they use serum for production.

Dr P. Price: From the testing that we have done, nearly all the foetal bovine serum has a titre that is positive. It has a titre of less than 1/16. If you start vaccination of the animals would that not increase the antibody titre in the foetus? If you have a live vaccine, would not that cross the placenta in producing antibody?

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Prof. J. van Oirschot: From the safety point of view, this would not be acceptable.

Dr R. Levings: The practice recommendations in the USA are not to use modified live BVDV in pregnant cattle.

Prof. P.-P. Pastoret: We agree therefore, that we should check for the antibodies. Is it a consensus to use low antibody titres among the companies?

Dr M. Tollis: It is a consensus among the companies, but I do not think that we have reached the consensus with other involved parties, controllers etc. It is important to have serum completely free of antibodies. Having a level of contamination, either contamination or antibodies in the serum, this is a problem for the quality of the product which we are going to make. We should discuss if this is a basic quality issue or not.

Dr D. MacKay: It strikes me that what we are almost saying is that you would end up with two categories of serum - one of which comes from donor herds, whose BVDV status you know and which may be used for specific purposes such as diagnosis, which would obviously need to be BVD antibody free. The other for general vaccine production, whose original donor animals you would not know and whose you would not want to and could not certify as being BVD antibody free; maybe our guideline should be amended to reflect that.

Prof. P.-P. Pastoret: Concerning the size of the batch. Is it feasible to have smaller batches?

P. Castle: If you look at the proportion of sera that are BVDV positive, then limiting the size of the batch does not make sense. This is because there are batches which have several hundred units of sera in there and if you limit the size, you still are going to have a contaminated unit in there in the proportions. Unless you limit it to such a small size, statistically it does not make sense.

Dr R. Levings: How do vaccine manufacturers and serum producers in how they market their batches because, when we try to trace back on vaccine accidents, we get mixed answers on how serum lots are sold and how they are used by the vaccine companies. On the one hand you hear that 'we buy this huge batch and then use it in the next 20 serials of the next 5 licensed products and reserve a whole batch from the serum manufacturer'. On the other hand, from the serum manufacturer 'no one firm would buy a whole batch, we sold a quarter to this firm, a quarter to another etc'. Personally, to limit the batch would not be to improve your odds of originally being not contaminated, it would improve your odds if you had a mistake at the serum company or, a mistake in the manufacturing testing of the incoming batch.

Dr M. Edgington: From basic GMP, the maximum size for the batch should be defined, as this is an aseptically processed product. Validation and its purification are dependent on the amount that you treat and filter. If you move a batch of 1 500 litres up to 2 600 litres, what happens to the results from your filtration?

Dr M. Huether: When we look for BVDV free, this usually ranges from 300 to 500 litres for a batch. With that being said, when you have different research activities going on within the organisation, I do not control what my development group gets from another company which they can get a batch from them which, again, is not necessarily the same batch that we are using and they will bring that in. In development, we source our viral serum with manufacturing because, obviously we are making a product and we want to fall through when we do the validation of the process. We look at our growth parameters because we are developing master seeds and cell stocks etc. We have encouraged our development colleagues to go along in that same round because it makes good sense, but good sense does not always work. The other component is that it really goes back to the serum manufacturer because they can say what they feel that they have as a validation parameter. What is the maximum lot batch size that you can make, that you can validate? What you are going to find is that the different serum companies

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are going to have different batch sizes that they feel comfortable saying that this is truly validated. Who is going to check? Is this the responsibility of the serum company, the regulators, or the manufacturer? You would be safe to pick somewhere in the middle and the limit would have to be based on the smallest amount that all manufacturers can validate. Number and batches are mixed messages, for instance, we do more testing on it, even if we share the same lots with manufacturing, we do additional tests as we are an analytical laboratory and we have to make sure that it is free of everything else. For the testing, people have to realise that the majority of the analytical laboratories do not even use foetal calf serum for testing.

Dr C. Lecomte: We are involved in human vaccine and from our prospective, what is really important is the dilution factor that you have when you test your serum. Whatever size of the initial size of the batch. I was surprised to see the dilution factor, which was applied by the supplier, and obviously they could miss the possible contaminant with such a dilution factor.

Dr R. Fensten: Our batch sizes are based on validations for blending of particular serum lots. In terms of the maximum size, that is based on aseptic fill validations done every six months. There are, therefore, maximum limits.

Prof. P.-P. Pastoret: This is good compromise. No maximum limit, provided that the company can validate the process for the batch size. This should be included in the guideline.

Dr P. Vannier: Validation studies - I am surprised to receive certificates and to see certificates from serum suppliers, antibody free, virus free and to find antibody and/or viruses in a high proportion of batches.

Prof. P.-P. Pastoret: Probably, these were validated, not according to the contamination, but for other purposes.

Batch size - we must look at antibodies, but what about the virus? When?

Dr P. van der Valk: This linked back to our discussion what is the scope of the guideline. For BVDV, we have said that testing prior to inactivation should at least be done because, this gives you the certainty that the level of contamination should be taken care of by the inactivation method used. We have offered to retest after evaluation in order to confirm in principle validated test, which you should do, but in the case of BVDV this was the outcome of the discussion within the Immuno Biological Working Party. For other viruses, we would like to use the methods, which we have at present. This means, in principle, the method 5.2.5 in the European Pharmacopoeia states that you have either a validated inactivation method or you test for absence for extraneous agents. This is what we would like to remain valid for all the other extraneous agents.

Dr D. Paton: If I understand, the pretesting is only to show that you do not have a level of viruses that is extremely high,  $10^6$  for example. In this case, it would seem to me that a fairly simple assay could be used which would be much less difficult to use than the vigorous assays that would be required in post testing.

Dr P. Price: I agree that you have to know what virus you are looking for and what the titre is before you do the inactivation. For example if I am looking at data, and this is one the most difficult, it presents at the start a contamination level of over 9 logs of virus, (this is canine adenovirus in foetal bovine serum), but at 30 kGy we still had virus, whilst we had no virus at 35 kGy. We still show, even at 25, a better than 6 log reduction. You need to know the titre that you are starting with. The standard inactivation level has to be increased to about 35 kGy to be on the safe side.

Dr R. Levings: I heard a comment on where does the  $10^6$  come from. I heard that the  $10^6$  was

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based on  $10^4$  with a two times  $10^2$  overkill - is this what we would be testing for nothing more than  $10^4$ ? Nothing higher than  $10^6$  - what is the threshold?

Prof. J. van Oirschot: There is a publication concerning BVDV; in some foetuses  $10^6$  per ml of blood could be detected and this is the basis of the provisional risk assessment we made in the scientific group of animal health and welfare within the European Union. We also talked about validation of the inactivation methods. Either by gamma irradiation or by other methods. The proposal was to use 5 viruses from the different relevant families so that you cover the whole range of physical and physico-chemical properties of viruses. You have to validate the method once,  $10^6$  you can say for BVDV but for bluetongue we know that there could be titres as high as  $10^8$  in foetal serum. So, with bluetongue, you need to start with a higher titre.

Dr R. Levings: Therefore this means titre in individual slots, is that the starting point for our numbers and then you have to build in the dilution factor and then multiply it back for the small sample than what is actually used in vaccine production.

Prof. J. van Oirschot:  $10^8$  or  $10^6$  is more or less the worst-case scenario. You will never have titres higher than  $10^6$  for BVDV in foetal calf serum batch. You will never have titres higher than  $10^8$  for bluetongue, and you can put this into the risk assessment.

Dr P. van der Valk: I thought that the titre  $10^6$  applied to the individual calf.

Dr W. Hesselink: In the FEDESA proposal, there are a hundred fold safety masks. In general terms, we are talking about  $10^4$ .

Dr D. MacKay: We need to be clear as we are obviously putting in additional requirements for BVDV and are we going to put in additional requirements for other agents or are we happy with the requirements as they currently exist in the Pharmacopoeia? I have yet to see any reason to put in any additional requirements for other agents over and above those currently in the Pharmacopoeia, other than for BVDV.

Dr D. Kretzdorn: We are talking much about the maximum allowed titre for serum and the titre reduction, which we should achieve. We have also spoken about possibility increasing the gamma irradiation dose, to an extent where we know that foetal bovine serum may not be as good in growth improvement than in lower doses. This is a difficult issue. I would say, it should be considered whether we cannot say if the testing on the non-irradiated batch of foetal bovine serum should be validated thus, we should know the detection levels of at least, of known lead organisms and we have to know what titre of these organisms we actually can detect with our method so as to adjust the gamma irradiation accordingly in order to kill the dose contaminating and in addition add a safety margin. We are talking about  $10^6$  and  $10^8$  logs that may be present, but has ever anyone seen that level being present in the foetal bovine serum batch? Generally, the contaminations are much lower.

P. Castle: At present, in the European Pharmacopoeia requirements, we still allow the alternative, either inactivate or test. I wonder if this is a realistic option? Is this option of just testing acceptable for sera? Are there cases where you have to use non-inactivated serum for producing vaccines? If we turn this around and say, you test and then you inactivate, clearly the tests are designed to do something else. If you test after inactivation everything has to be negative. If you test before inactivation everything will not be negative and you will then have the difficulty of setting the levels, which you will accept, and what you are going to test for before inactivation. Is that the way this is going and requires testing before inactivation?

Prof. P.-P. Pastoret: In my opinion, I would prefer to have a common level of inactivation for the serum, and then check or test afterwards.

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Concerning BVD virus - unfortunately I was not present yesterday - but there was a discussion on isolation or PCR - I would like your comments on this.

Dr L. Bruckner: It is very difficult to decide which method should be used. We have no clear scale as to what these methods do. People use different methods as we have no standard methods and comparison is very difficult. I wonder if it is not wise to have some comparative testing between different laboratories, how do the different test behave in different test situations. This would give us the answer as to which is the most suitable method.

J.-M. Spieser: We can organise collaborative studies to validate and cross-validate but this is a lot of work we have firstly to examine what will be the added value of such studies.

Prof. P.-P. Pastoret: Are there companies who have the experience of doing both these methods? Both PCR and isolation?

J.-M. Spieser: If this is available, this would be the first step by sending this data to us for an assessment group who can look through the data and then propose what additional laboratory work should be done.

P. Castle: Some people were reporting 100% of batches PCR positive. Dr Nettleton reported 100% positive for PCR. This means 0% serum acceptable.

Dr D. Kretzdorn: I think that, based on experience, we can only use PCR as an indicator to qualify certain batches and this should not be over such or such a reaction level. You can now use PCR, at least if it is a well-established and very sensitive PCR, but you have to be aware that there are also differences in the field. If you have a particularly sensitive PCR against BVDV and you screen the serum batches, you will find almost all of them positive and therefore, it will make it impossible to use any foetal bovine serum, yet, you may find PCR reactions at a low level and still have infectious virus in there. It does not help to base only on PCR. The only relevant issue is the cell culture test.

Prof. J. van Oirschot: The critical issue is that you do not want to have infectious virus in your vaccine. The only way therefore, is to test this virus through isolation but you have to have the most sensitive virus isolation procedure which exists. I know that in our small country we had four or five laboratories doing BVDV virus isolation and we have the same titre sent to the different laboratories and asked to titrate this sample, we noticed differences of 100 fold! Big differences therefore exist in the sensitivity between laboratories. The suggestion of having a virus batch as a standard that you have to at least detect is a good reference approach.

Dr C. Lecomte: Of course, by PCR you can also catch iron or defective particles, which may not be infective. The only way would be to combine PCR and cell culture and make a PCR before cell passages. In this case, you need to develop a semi-quantitative PCR and this is technically highly demanding. It would be good to combine the two techniques.

Dr M. Chudy: I agree with the need of standardisation of PCR testing, however, the question is what do we want? We want qualitative test or quantitative test? It is possible to standardise a quantitative test therefore, we have a possible way to define a limit from which we can have sera deferred in combination with inactivation, I think, this is a good option for virus safety. Not PCR testing alone but using the combination.

Dr D. Mackay: There is one category of sera we were talking about where PCR would seem to be extremely useful and that was the case of donor herds and serum for special purposes, such as, diagnosis where we did want to be entirely sure that the donor animal had not had exposure to the agent at all, and in those cases, it would seem that we should include PCR within the context of the guideline. In the other context, where we have the problem of not being able to tell whether we are detecting replication competent, we would need to consider the possible role

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for Dr O. Itoh's test where he is looking for replication competent viruses by PCR in a combination with tissue culture.

Dr C. Lecomte: I agree, to put a threshold detection level for PCR is not enough, you have to know if your virus is able to replicate or not.

Dr M. Huether: What primers set would you use? Would it be multiple primer sets? What would you use as your reference standard for PCR? Would it be certain types of virus, which you then would have to test against four or five strains as far as your positive control? Quantitative PCR, you are going to have to validate and control reverse transcription. You would like to have an internal standard, which is another RNA template, you would like to put it into serum and show that you can quantitate and you do not have any inhibitors after inactivation because of the free radicals. I am supportive of the PCR but there are a lot of relative issues, and we have done it and looked at both methods, but I would say that the cell culture is true and we use PCR as a follow up. I think that it is up to the individual laboratories if they can take that on, in our manufacturing facilities, PCR would be very difficult.

J.-M. Spieser: A certain number of these issues which Dr M. Huether's mentioned, we had already discussed in the relation of making obligatory the HCV testing in human blood donations for plasma for fractionations. We have, in the European Pharmacopoeia, a general method on PCR and also a general guideline, which approaches the issues of inhibitors, run controls etc. If anyone uses a standard method and a standard guideline, which are already prescribed it, would probably save a lot of time in connection with the issues which Dr M. Huether's mentioned. You would then just need to have an appropriate run control which of course, you have to decide which strain it should be, how many times you would need to include a reference material, how many replicate best you run etc.

Prof. P.-P. Pastoret: Virus isolation - who is still in favour? We are trying to find infectious viruses and it is certain that we maintain virus isolation and not only rely on PCR findings. We now move to inactivation, please comment.

J.-M. Spieser: The issue of mini-pool testing or not? This may change the viewpoints of using isolations.

Dr D. Mackay: I would request comments from FEDESA and from the serum manufacturers. The proposal was that it would greatly help and assist risk control to have testing of mini-batches of up to around 3 litres before they are then pooled to create the greater batches. We have had various discussions about the relative costs and benefits.

Dr P. Price: We are now doing testing of 3 litre batches for live non-cytopathic BVDV by Elisa.

Dr P. Van der Valk: I am not aware of the entire costs of the tests, but if you have a batch of 1500 litres, this means that you have to do 500 extraneous agents tests, even if they are only for BVDV, the cost would be quite high. I will see what information I can get and inform the participants of the the Immuno Biological Working Party. Why should we go to the extent of testing every mini-pool and try to identify the ones, which may have a level of contamination, which is not acceptable. There are many questions to be looked at. In relation to the inactivation as such, I do not see the need for this.

Dr R. Fensten: A comment on the collection methods; I think that there are a variety of manufacturers out there who are producing serum products, some will produce small pools, such as the 3 litres and others bigger. You are having a non-standard pool size depending on the manufacturer and as such, you are not getting a homogenous mixture if that particular pool is not qualified for mixing. You can tend to question how well the testing would go - depending on the particular size of the serum pool for that day. I agree that gamma irradiation is a fairly robust

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process and we should be looking more towards the inactivation of the product, as opposed to testing of the pools. The cost is extraordinarily high.

Prof. P.-P. Pastoret: Inactivation, do we fix limits for the irradiation dose? I have been listening to someone who told us that you need 35 kGy for real inactivation.

Prof. J. van Oirschot: From what I heard from members of the Committee I referred to earlier, they said that if you irradiate with more than 25 kGy you lose a lot of cell growth promoting properties of your cell culture mediums. Therefore, 35 kGy is probably too high for keeping good cell growth promoting properties.

Dr R. Fensten: Regarding inactivation, certainly for a number of cell lines, you can inactivate serum, and we have done it up to 60 kGy and some cell lines will grow fine if your inactivation programme is well validated, on the contrary some other cell lines will not. Regarding fixing a particular limit for irradiation I wish to say that if you move up to 35 kGy as being your minimum, if you go in for radiation, you have to remember that there is going to be a range so you may have to inactivate in that range i.e. 30 to 45 kGy. The higher you go up in your minimum dose, the wider that range is going to be. There should be some specifics regarding the viruses, which you are going to be looking at for your model. Let me add that to get 6 log reductions, you would need a dose of irradiation greater than 100 kGy to inactivate something like PPV or MVM at 6 logs.

Dr P. Price: A clarification - you need 100 kGy to kill porcine parvo?

Dr R. Fensten: Yes, if you wish a 6 logs reduction.

Dr P. Price: Our data shows that this is not necessary.

Dr R. Fensten: There are differences between validation studies with the viruses and that is probably depends on titration method and a number of ways that the process is performed. We have seen ranges between 70 and 120 kGy depending on the virus and the size.

Dr P. Price: Our validation study, which is available, with porcine parvo shows that 30 kGy gave you more than a 6-log reduction.

Prof. P. -P. Pastoret: If you have validated data, which you could make available, this would be very useful for us in order to decide whether to fix a limit in the guideline and at which level.

Dr P. Marbehant: Once again, if you test prior to inactivation it makes different results. If you only source sera batches which are tested negative for testing up to 32 TCID<sub>50</sub> you could have lower doses for irradiation.

Prof. P. -P. Pastoret: The problem is contaminants - you know the one you have, but you do not know the other. I would prefer to have clear-cut level in order to have the maximum precautionary safety. We should not only focus on BVDV but on other viruses also. For Bunyaviruses no one knows information on this and therefore you find it only when it is too late. If you fix a limit for irradiation, which should not be lower than a given figure, at least you have the best safety precautions.

Dr D. Kretzdorn: If we test before the gamma irradiation, the picture is completely different. If we go into irradiating a serum where we do not know the content of the possible contaminants, then of course, there is a justification for a higher dose, but if we can quantify the possible contamination that we know as being the lead organisms which we look for, then I think that there is a justification to apply a more reasonable dose. I think that there is no one here saying that we should go below 25 kGy but the question is, whether we go above 35 kGy - this is a big difference. If we do not test before the gamma irradiation, then we have to apply much stricter measures and we can understand that therefore, we need a higher irradiation dose.

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Dr C. Lecomte: Regarding the safety margin and the reasonable level. What do you mean when you speak about your safety margin, what is this representing?

Dr D. Kretzdorn: I cannot say that I have one which is proven to be acceptable. As we discussed before, the safety margin of possibly 2 or 3 logs is acceptable, or should be acceptable. If you can show in your pre-inactivation testing that you are using validated methods showing that the lead organisms are not present, those would be the one which are considered to be the risk and the most prominent organisms which could contaminate the serum, that enables you to demonstrate the viral titre which is present (or its negativity serum already in your test before the inactivation which would be even better) and then you would only have to inactivate the small possibly remaining contamination. If we want to look for any potential contaminant, that we do not know of yet, we do not know if we can inactivate it, there is no chance of validating an inactivation procedure against this unknown potential future contaminant at present. We are always a little bit behind in the development in this field.

Prof. P.-P. Pastoret: If we have to validate, how would you follow the suggestion made by Prof. J. von Oirschot as to validate strictly to five type of viruses which will cover a complete range viruses? Shall I take it that you would agree?

Dr O. Itoh: In Japan it is not authorised to irradiate material for animal or human use.

Prof. P. -P. Pastoret: Regarding regulatory affairs: firstly we said that there are three different bodies which may be involved. At first, the CVMP, then the contribution of EDQM with a question mark concerning certification and last, the international dimension within the VICH procedure. The first step would be to discuss what to do at the CVMP level. From the discussions we had many suggestions to be included in the guideline, and we await comments from industry.

Dr D. Mackay: The suggestion which is starting to emerge in terms of having a specific monograph and a certificate of compliance to that monograph would seem to be a very useful way forward.

Concerning the guideline itself; there is a succession of points that we have come through over the discussions. The guideline will have to be modified in light of the discussions that have taken place.

Dr P. Jones: The consultation period has closed, however, after this meeting we still will take into account further comments and take these back to the Immunobiologicals Working Party.

P. Castle: Essentially, the Pharmacopoeia requirements are contained in the chapter 5.2.5, on substances of animal origin used for the production of vaccines, and then you find additional requirements in the individual monographs. We have begun to look again at the chapter 5.2.5 and I am sure that the discussions during this conference will affect the way in which we go about revising this chapter, as it will be revised. There is the other question as to whether we need to look for some monographs. Prof. J. Brownlie for example told us that the safety test would be better carried out in sero negative calves. There is a safety test in the monograph on live viral bovine vaccines, this is something, which we will look at. I think that we will have very long discussions on this!

When we are talking about BVDV contamination, I suppose that it is only the modified live vaccines, this does not concern the inactivated vaccines. It appears that no one has had a problem with an inactivated vaccine?

Dr P. Jones: This morning we heard about discussions from VICH and that there may be some changes or agreements on tripartite harmonisation on extraneous agents and moisture. If there were changes or recommendations coming those that were out with the current

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recommendations in the monographs of European Pharmacopoeia, how would we handle this in Europe?

P. Castle: As a general question, covering not only extraneous agents but also other subjects, these topics in the Biologicals Group of VICH, they are more than just licensing guidelines, they are affecting the regulations. EDQM is represented on the VICH group as an observer. Our intention is to keep the Pharmacopoeia in line with whatever is agreed in VICH. There is a European delegation at VICH. This means that we need to keep our consultative processes in step with the VICH ones. In the end, the normal course of things would be that we would align the Pharmacopoeia with whatever is agreed at VICH.

Prof. P. -P. Pastoret: Concerning certification and risk assessment - this has not yet been solved. Do you think that it is possible to certify foetal calf serum?

J.-M. Spieser: If you want to use the certification system as it exists for the time being in the European Pharmacopoeia, you would, of course, have to define in a monograph, what are the references against which you will certify; in both cases, which are at present using the certification procedure these references are set up in relevant monographs, the classical certification system for chemicals, APIs and excipients, are always referring to the individual specific monographs and for TSE the same approach has been used through a general monograph. For the issue of concerns you would have to define in a monograph, what the references are. You would then of course, have to immediately have to define certain number of things which are not yet clear cut and therefore you would not be able to certify through that procedure, you cannot use a guideline which leaves open some interpretation or some issues by using 'either' /'or'. This is something, which, between the Institutions responsible in E.U. for regulatory and standards issues and their relevant scientific committees has to be discussed as to whether the certification approach is feasible.

Prof. D. H. Calam: It may help on this issue of certification to explain about TSE. The Pharmacopoeia Commission were aware that the two directives relating to new European and existing products for human and veterinary medicines were going to be introduced. After a lot of discussion, the final outcome was that with the effect from 1 January 2000, a general monograph on products at risk of transmitting TSE was introduced into the Pharmacopoeia. It is a very short monograph, the first part is a definition saying that this applies to all substances of relevant animal origin, be they active substances, excipients, reagents, starting materials, media components etc., and of course there are no individual monographs for many of those things. The second part of the monograph had a production statement which said that all the things caught by the definition are produced in accordance to the General Chapter (chapter 5.2.8) and that chapter reproduces, in its entirety, the CPMP/CVMP Note for Guidance. Because this had been directed very much from the point of view of human medicines, that Note for Guidance has been modified to address the issues of materials derived from milk and wool and it is about to be changed again, in the light of the recent implementation of a single Note for Guidance, applying to both human and veterinary medicines, which was finally implemented in February. The advantage of this is that, having a general monograph, the reference to the General Chapter makes the Note for Guidance mandatory but not only within the EU, also within the 12 member countries of European Pharmacopoeia, that are not EU countries. This therefore extends the mandatory requirements of the Note for Guidance. A large number of certificates have been issued, a number of which relate to serum. In principle, it would be possible to extend that procedure if that is what people wanted to cover, maybe the new Note for Guidance, if it was finally decided that there was value in making it obligatory. This is a contentious issue. It has also been suggested that serum itself is a rather special case, and maybe there ought to be a monograph for it. This would represent a shift, but not a complete change in policy for the European Pharmacopoeia Commission because, there is in fact, a monograph on plasma for

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fractionation, which is in effect a starting material for blood products. It may be that the Commission, with enough influence from the regulatory authorities and possibly from industry, could be persuaded that there would be merit in having a monograph on bovine serum. In terms of the extension of the certification scheme, the scope already applies to general monographs in addition to specific monographs. The way the scheme operates, is that there is a certification unit in Strasbourg, the assessments are done by assessors drawn from licensing authorities within Europe (specialists) and the whole scheme is overseen by a steering committee, which has representatives of the Human Biotech Working Party, the Veterinary Immunologicals Working Party, the EMEA, the EU, the non-EU countries, the Chair of the European Pharmacopoeia Commission ; a large number of people who, between them, could take a decision in principle, that the extension of the scheme was appropriate and then the Pharmacopoeia would have to put into place the necessary requirements. It could be done; whether it is done is dependent on the view of all the parties who are involved in this; not least industry and certainly the regulatory authorities.

Dr R. Dobbelaer: To underline that indeed, under the European scale, the certification is an ideal system an ideal tool to have a unified approach and evaluation throughout Europe through a central procedure avoiding unnecessary duplication. Another potential tool or idea could be to think about a serum master file, which like the plasma master file, would more or less live an independent life. It would be a file where, a given manufacturer for the whole range of vaccines, would have data on the collection system and quality assurance as well as inactivation for the serum used for the production of the range of vaccines available in Europe. This would give a unified approach in evaluation in Europe.

Prof. P. -P. Pastoret: Regarding the international dimension within the VICH procedure.

Dr O. Itoh: I think that the two days of conference are very concentrated on virus contamination. When we work on the guidelines of this subject, we would take into account the discussion held during this symposium and the experts of the VICH would reconsider their position in line of this and so it would hopefully be reflected within the VICH meeting.

## **BIOGRAPHICAL NOTES**

## **Pestivirus contamination of bovine sera and other bovine virus contamination**

**Prof. Joe Brownlie** graduated in 1967 from Bristol with BVSc, and MRCVs from London and then gained his PhD from Reading University in 1972.

In 1995 he received the Diplomat Eur. Coll. Vet Path. From 1995 to present, he currently is Professor of Veterinary Pathology and Head of Department in the Department of Pathology & Infectious Diseases as well as Director of the Eur. Centre for Toxicologic Pathology at the Royal Veterinary College, London, UK.

He has gained numerous personal awards and honours and has held honorary and consultancy positions. He is expert consultant for industrial companies as well as international organisations. He has over 100 publications in scientific journals to his name.

**Dr Lukas Bruckner** obtained his degree in veterinary medicine from the University of Berne in Switzerland. He studied for his DVM at the Institute of Veterinary Virology at the University of Berne. He obtained his DVM in 1982, submitting a thesis on different aspects of BVD virus.

Since 1983 he is working at the Institute of Virology and Immunoprophylaxis (IVI), the Official Medicine Control Laboratory for biologicals for veterinary use in Switzerland. He started at the IVI as a research assistant. Meanwhile he is head of the Biologicals Department.

He is member of expert group 15 V (vaccines and sera for veterinary use) of the European Pharmacopoeia since 1989.

**Prof Derek Calam** obtained his degrees from Oxford University, UK. After posts with the UK Agricultural and Medical Research Councils, he joined the National Institute for Biological Standards and Control becoming Head of Chemistry and now European Co-ordinator. He is currently Chairman of the European Pharmacopoeia Commission. He is also Chairman of the British Pharmacopoeia Commission.

**Peter Castle** – is Secretary to the European Pharmacopoeia Commission and head of the division developing monographs and general chapters, graduated (1968) in biochemistry from Cambridge University, England. He worked for 3 years (1969–1972) at the Pharmaceutical Society of Great Britain on the publication ‘Identification and Isolation of Drugs in Body Fluids’. This was followed by 2 years in the veterinary division of Smith Kline & French (UK) working on licensing of veterinary products. Since 1974 he has been a member of the Technical Secretariat of the European Pharmacopoeia, Council of Europe, Strasbourg working particularly on the monographs on veterinary vaccines, vaccines for human use and blood products.

**Dr Bryan Charleston** obtained his degree in veterinary medicine from The Royal Veterinary College, London in 1982. After 5 years in cattle practice, including 2 years spent exclusively in the embryo transfer industry, he studied for an MSc in molecular biology at University College, London. Subsequently he gained his PhD, funded by the Wellcome trust, in 1991. After a Wellcome post-doctoral fellowship in London and Cambridge, he was appointed to the Institute for Animal Health, Compton. He is now a research group leader working on the immune response to bovine viral diarrhoea virus and follicular dendritic cell biology.

**Dr Dirk Deregt** obtained his degree in veterinary medicine from the University of Saskatchewan in Canada and also studied for his PhD at the University of Saskatchewan. He obtained his PhD in 1988. In 1987, he became employed as a research scientist with the Food Production and Inspection Branch of Agriculture Canada, which later became the Canadian Food Inspection Agency. He remains employed at the Canadian Food Inspection Agency and is the designated expert of two OIE (Office International des Epizooties) reference laboratories including one for bovine viral diarrhoea.

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**Dr Roland Dobbelaer** graduated in chemistry/biochemistry from Gent (Belgium) University in 1965. In 1986 he obtained his PhD at the faculty of Medicine of Louvain University while working at the Scientific Institute of Public Health - Louis Pasteur (SIPH) of the Belgian Ministry of Public Health. He is currently head of the Section for Biological Standardisation of the SIPH where he is responsible for the National Control Authority Batch Release of vaccines and plasma derivatives operating in the Official Medicines Control Laboratories (OMCL) network co-ordinated by the European Directorate for the Quality of Medicines (EDQM). He is a member of the WHO Expert Committee on Biological Standardisation (ECBS) and is currently chairing the European Pharmacopoeia Biological Standardisation Steering Committee. He is also involved in advising the Belgian Medicines Board and, as a member of the CPMP Biotechnology Working Party, also the European Authorities in matters concerning licensing and regulation of biologicals. As a member of the European Pharmacopoeia Expert Group N° 15 he is involved in drafting European monographs on vaccines. As a member of the Scientific Committee for Medicinal Products and Medical Devices (SCMPMD) he is advising the European Commission's DG Sanco in matters related to biological medicinal products and consumer protection.

**Dr Christiaan Folkers** obtained his degree in veterinary medicine from the State University of Utrecht in the Netherlands in 1956. He obtained his PhD in Veterinary Medicine at the same university in 1962. During the period of 1963 -1965 he was Senior Veterinary Officer at the Tsetse and Trypanosomiasis Division of the Ministry of Animal and Forest Resources in Northern Nigeria and became in 1965 Reader in veterinary parasitology at the Ahmadu Bello University in Zaria (Nigeria). In 1970 he became head of the Veterinary Vaccine Laboratory of Philips- Duphar in the Netherlands. In 1993 he retired as Director of Regulatory Affairs and Quality Assurance. He is now an independent consultant and represents FEDESA at the Working Group Biological Quality Monitoring of VICH, being the topic leader for the harmonisation of the test on extraneous agents."

**Dr M.-M. Gonze**, is associate Director in charge of the Manufacturing Process Development at GlaxoSmithKline. She obtained her PhD degree in the Department of Physiological Chemistry at the Medicine Faculty of University of Namur Belgium. From 1990 to 2000, Dr Gonze worked in RD, Process Development, at SmithKline Beecham Biologicals, to develop all the serum-free processes for viral vaccines. Since 2000 she has been in charge of developing the serum-free processes in the Manufacturing Department and of ensuring the traceability of the raw material used in the production of human vaccines.

**Dr Claude Hamers** graduated in veterinary medicine (University of Liège) in 1988. After an additional training of one year in tropical veterinary medicine (Institute for Tropical Medicine, Antwerp), he worked, for more than five years, as technical adviser in several developing countries in Africa. Among others, he worked for MSF in Zaire, for A.P.E.F.E. in the Republic of Comoros (2 years) and for UNDP and EDF-PARC in The Gambia (2 years). In 1995, he joined the Department of Immunology – Vaccinology of the Faculty of Veterinary Medicine, University of Liege, where he started a research and obtained a PhD degree on the variability of the bovine viral diarrhoea virus (BVDV).

**Dr Osamu Itoh** was born in Aichi Prefecture, Japan. He studied veterinary medicine at Nihon University in Japan, which granted him a DVM degree in 1969 and a M.Sc. degree in 1971. After graduation, he worked in a private vaccine maker Co. for one year. He has been working for National Veterinary Assay Laboratory. He has been in charge of national examination of the vaccines for animal use. He took the degree of a PhD in 1986 at the Nihon University. He wrote his doctoral thesis on the isolation of the new character virus and interferon production of bovine

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viral diarrhoea virus. He serves as chairman of the Biological Quality Monitoring Expert Working Group of VICH from 1998.

**Dr Peter Jones** graduated from the Faculty of Veterinary Science at Liverpool University in July 1971. After several years in general veterinary practice in the United Kingdom and Canada, Dr Jones joined the pharmaceutical industry in the animal health sector. He has held a number of appointments in research and regulatory affairs in multinational companies and, most recently as Senior Director of International Regulatory Affairs for Animal Health Products for Merck Sharp and Dohme in New Jersey, USA. He joined the European Agency for the Evaluation of Veterinary Medicines in June 1995, and was appointed Head of the Veterinary Unit in December of the same year. Since January 2000 Dr Jones has assumed the additional responsibility for the IT sector at the EMEA

**Dr Pierre Kerkhofs** graduated in 1986 from Veterinary Faculty, University of Liège. He obtained in 1992 a Master in Molecular Biology from Faculty of Science, University of Brussels and his PhD in 1996 from the same Faculty.

He joined, from 1986 the Veterinary and Agrochemical Research centre, and was head of the department of Virology from 1999.

From 1996, he also does teaching at Faculty of Agronomy, Catholic University of Louvain.

**Dr Randall L. Levings** received a BA degree in biological sciences from the University of Missouri in 1974 and joined the United States Department of Agriculture, Animal and Plant Health Inspection Service, in 1975. He received a DVM in 1984 and a M.S. in immunobiology in 1985, both from Iowa State University. He has been involved in temperature/identity telemetry of cattle, antigenic markers for killed IBR vaccines, testing the first live genetically engineered veterinary vaccines and companion diagnostic kits, production of heterohybridomas secreting bovine or porcine monoclonal antibodies to viral pathogens, and detection of extraneous or non inactivated agents in live and killed vaccines. He was selected Head of the Cytology Section in 1987, Head of the Large Animal Biologics Virology Section in 1989, and Chief of the Veterinary Biologics Laboratory in 1995. His current duties as Director of the Center for Veterinary Biologics (CVB) – Laboratory include providing overall program and administrative management for the Laboratory and partnering with the other CVB Directors in leading the Center as a whole. CVB activities include standard setting, licensing, inspection, testing, batch release, vaccinovigilance, and compliance for all veterinary biological products sold in the United States.

**Dr David Mackay** graduated from the Royal Veterinary College, London in 1981. He received an MSc in Immunology from Birmingham University in 1985 and a PhD in Veterinary Immunology from the University of London in 1993. After periods in general practice and industry, Dr Mackay worked as a research scientist on exotic diseases of livestock for nine years at the Pirbright Laboratory of the Institute for Animal Health. In 1999 Dr Mackay took up his current position as head of the Immunologicals Team at the Veterinary Medicines Directorate where he also acts as the UK representative on the CVMP Immunologicals Working Party.

**Prof. Volker Moennig** In 1965-70 he studied Veterinary Medicine at Veterinary Medicine School in Hanover. From 1970- to 71 he worked at the Federal Animal Virus Research Institute, Tübingen from there until 1974 he worked for Max-Planck-Institute for Virus Research, Tübingen. Until 1985 he was with the Institute of Virology, School of Veterinary Medicine Hanover and then became for one-year visiting Professor to the Department of Veterinary Microbiology and Preventive Medicine at the Iowa State University, Ames, Iowa. Working on research on pestiviruses. From 1987 to 1993 he worked at the Institute of Virology, School of Veterinary Medicine Hanover. Later as the President of the Federal Research Institute for

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Animal Virus Diseases (Tübingen, Wusterhausen, Island of Riems) for two years. In 1995 he gained full Professorship of Veterinary Virology, School of Veterinary Medicine Hanover, Director of the Institute for Virology, Head of the European Union Reference Laboratory for Classical Swine Fever. From 1997 to date he is Rector at the School of Veterinary Medicine in Hanover. 4.11.1997 he became member of the „Scientific Committee on Animal Health and Welfare“ of the Commission of the European Union

**Dr Manfred W.A. Moos** graduated in 1972 from the University of Giessen (Germany) in 1973, he started working at the Paul-Ehrlich-Institut (Federal Agency for Sera and Vaccines) in Germany. He received his PhD in 1975. From 1976-1985, he was Team Leader of the veterinary laboratory group “Virology” at the Paul-Ehrlich-Institut.

Since 1986 he has been Head of Department for Veterinary Medicine at the Paul-Ehrlich Institut. In 1987, he was appointed Director and Professor. Currently, he is member of the European Committee for Veterinary Medicinal Products (since 1988), German Representative at the Group of Experts n° 15V of the European Pharmacopoeia Commission (since 1989), member of the EMEA Inspection Control working Party (since 1997), and member of the Expert Committee “biological Standardisation” of the European Directorate for the Quality of Medicines at the Council of Europe (since 1999).

**Dr Peter Nettleton** graduated in 1970 from the Glasgow Veterinary School. After working in Africa he graduated in 1975 with an MSc in Virology from Birmingham University Medical School. From 1975-79 he worked for Wellcome on the commercial production of Foot-and-Mouth disease virus vaccine. Since 1979 he has worked at the Moredun Research Institute. He graduated PhD from Edinburgh University in 1985 for studies on pestiviruses. He is currently head of the virus Surveillance Unit with research interests in pestiviruses and parapox viruses.

**Prof. Paul-Pierre Pastoret** graduated as a doctor in Veterinary Medicine at the University of Liège in 1970. After a spell in rural practice, Paul-Pierre Pastoret became in 1972 an assistant at the Department of Infectious Diseases of the same university and at the “Office Vaccinogène” in charge of the production of the vaccine against smallpox. After completion of his PhD thesis in virology on the latency of Bovine herpes virus 1, he did post doctoral studies at the University of Saskatchewan, Saskatoon, Canada (Prof. L. Babiuk) and at the University of Cornell, USA (Prof. L. E. Carmichael).

Back in Belgium he became head of the Department of Virology – Immunology at the Faculty of Veterinary Medicine of the University of Liège in 1979. Since 1995 he has been a member of the CVMP Working Party at the European Agency for the Evaluation of Medicinal Products (EMA) based in London where he chairs the Immunologicals working party. He has been Chairman of the Veterinary Medicinal Products Evaluation Board in his country since 1998. Paul-Pierre Pastoret is the author or co-author of more than 700 publications in referred journals or books.

He is the first editor of several major textbooks including “Immunologie Animale” published in 1990 by Flammarion in Paris, “Veterinary Vaccinology” published in 1997 by Elsevier in Amsterdam, and “Handbook of Vertebrate Immunology” published in 1998 by Academic Press in London. He is a full member of the Royal Academy of Medicine in his country.

**Dr David Paton** graduated as a veterinarian in 1984 from Cambridge University and received his PhD in 1992 from the University of Surrey. His thesis was entitled “Bovine viral diarrhoea: studies of viral epitopes and of porcine infections”. After three years in veterinary practice, he joined the Virology Department of the Central Veterinary Laboratory as a research officer.

He has remained in the department up until the present, working on the diagnosis and control of

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a range of viral diseases, particularly RNA viruses of pigs and cattle. He has been head of the department since 1998.

**Prof. Maurice Pensaert** graduated in 1963 as doctor in Veterinary Medicine at the Ghent University, Belgium and received his Master of Science and PhD in Virology from the Faculty of Veterinary Medicine at Purdue University, USA in 1966 and 1968 respectively. He joined the Faculty of Veterinary Medicine at the Ghent University in 1968 and is currently department head of the Department of Virology, Parasitology and Immunology and Professor of Virology.

He is representing Belgium as a member of the group 15V of the European Pharmacopoeia and of the Immunological Working Party in EMEA. He is a member of the Chamber of Medicines in Belgium.

**Prof. Jean-Marc Person** graduated in 1974 Docteur-Vétérinaire from national veterinary school of Alfort and Paris XII University in France. He obtained his PhD in 1980. During the period 1975-1980, he was maitre-assistant at the national veterinary school of Alfort. From 1980, he was professor in microbiology and immunology at the national veterinary school of Alfort and full professor from 1988 in microbiology and immunology at the national veterinary school of Nantes (France). Since 1981, he was member of the group 15V of the European Pharmacopoeia and from 1995 chair of this group.

**Dr Paul Price** received a BS in Microbiology from the University of Maryland and a MS and PhD. From the George Washington University with majors in Cell Biology, Biochemistry and Virology. He joined Microbiological Associates while still an undergraduate student and spent 20 years with them as Director, Cell Culture Production and later Senior Scientist and Director of numerous NIH sponsored contracts dealing with the development of In Vitro systems. Dr. Price left Microbiological Associates to take the position of Branch Chief, Cell Culture and Media Branch, Center for Infectious Diseases, Centers for Disease Control. In 1983 he left the CDC to become founder and Executive Vice-President of Hycor Biomedical. Dr. Price has also held Director-level positions at Hazelton Research Products and Hana Biologics. In 1990 he was elected to return to the bench and joined the Cell Culture R&D Department of Life Technologies. Dr. Price is currently a Research Fellow. Dr. Price has been an active member of the Society for In Vitro Biology since 1959. From 1998 –2000, he was the vice-president of the Society for In Vitro Biology. He was also a founder and the first Vice President of the National Capitol Area Branch of the SIVB (then called the Tissue Culture Association) and their first Program Chair as well as Vice President and Program chair of the California Branch. He has also been a member of the Membership Committee, a reviewer for In Vitro and other journals and Co-Chair of the In Vitro Toxicology Section. Dr. Price was also a founder and first membership chairman of a biotechnology oriented society in the San Francisco bay area called NCA-SIM. Dr. Price has been an Adjunct Professor at several Universities. He was also a member of the Board of Directors of the National Institute for the Advancement of In Vitro Science and was on the Scientific Advisory Committee of the Cosmetic, Toiletry and Fragrance Association.

**Dr Julia Ridpath** graduated in 1977 from Doane College, Crete, Nebraska, U.S. and received her PhD in 1983 from Iowa State University, Ames, Iowa, U.S. in the Molecular, Cellular and Developmental Biology Program. She was a postdoctoral fellow for the Muscular Dystrophy Association before joining the National Animal Disease Center in 1984 as a Research Associate in the parvovirus Research Project. She joined the Detection and Control of bovine Viral Diarrhoea Research Project in 1986. Since 1998 she has been the lead scientist in that project.

**Dr James Roth** obtained his DVM degree in 1975 and his PhD degree in 1981 from Iowa State University. He is currently a Distinguished Professor of Immunology in the Department of Veterinary Microbiology and Preventive Medicine at the College of Veterinary Medicine, Iowa

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State University. Dr Roth is also the Executive Director of the Institute for International Cooperation in Animal Biologics (IICAB). The IICAB is a joint Institute involving Iowa State University and the USDA animal health facilities in Ames, Iowa. The IICAB is part of the OIE Collaborating Center for the Diagnosis of Animal Diseases and Vaccine Evaluation in the Americas.

**Professor F. Schelcher** Prof F. Schelcher graduated in 1983 Docteur-vétérinaire from the National Veterinary School of Toulouse (France). He obtained his DEA Economie et Droit Rural in 1984 (University of Toulouse) and in 1989 his Agrégation des Ecoles Vétérinaires (Paris). Diploma in Virology (1991) from the Institut Pasteur (Paris). Since 1994 he has been Professor in Veterinary Medicine in Toulouse (France). He is currently teacher and researcher, head of the Unité de Pathologie des Ruminants, Ecole Vétérinaire de Toulouse and Head of the joint research Unit INRA-ENVT 959. Prof Schelcher is the author reviews and original articles on pathology of ruminants, bovine respiratory syncytial virus infection and scrapie in sheep.

**Jean Marc Spieser** – obtained his degree in Pharmacy at the University of Strasbourg and his Masters Degree in Applied Industrial Pharmaceutics at the University of Montpellier, in 1973. In 1971 and 1973 he was a research assistant in enzymology at the INRA (Public Research Institute for Agriculture), having worked as an assistant in the Plasma Fractionation Unit of the Blood Transfusion Centre in Strasbourg in 1972. He joined the pharmaceutical industry in Germany in 1973 and was appointed as Assistant to the R & D Director in 1975. He moved in 1976 to join the Technical Secretariat of the European Pharmacopoeia Commission at the Council of Europe, Strasbourg, in 1976, where he was in charge of different Expert Working Groups in the field of biologicals. In 1985 he was appointed as Principal Scientific Officer and Liaison Officer with the EC Commission, DG III Division of Pharmacy (Biotech, Quality Inspection Working Groups), participating particularly in the elaboration of the rules (directives, guidelines, etc.) governing the Regulations of Biologicals since the early stage of the Biotech Working Group meetings, all of which he attended as an observer. In 1992 he developed the Biological Standardisation Programme (a joint contractual programme between the EC Commission and the Council of Europe) within the European Department for the Quality of Medicines (EDQM). In 1994 he developed the European Network of Official Medicines Control Laboratories (OMCL) in the areas of traditional pharmaceuticals and biologicals, for market surveillance and batch release activities. He is currently the Head of Division IV at the EDQM, an independent division which manages the activities of the Biological Standardisation Programme and the OMCL Network.

**Dr Maria Tollis** obtained her degree of Doctor in Veterinary Medicine from University of Naples in Italy. She first joined, from 1980 to 1984 as junior researcher, the laboratory of Veterinary Medicine at Istituto Superiore di Sanità (ISS) in Rome. During the period 1985-1987 she was visiting scientist at the Rabies Unit of the Wistar Institute in Philadelphia, USA, working with Prof. Tadeus Wiktor and Hilary Koprowski. Senior researcher at ISS up to the end of 1991, she was appointed director of research and chief of the department of viral diseases of domestic animals at ISS. She is in charge of testing viral veterinary immunological products before and after licensing and of giving scientific advice on their use. From 1990 up to 1994 in Brussels and since 1995 at EMEA in London, she has been member of veterinary immunologicals working party; since 1991 she has been member of group 15V of the European Pharmacopoeia.

**Dr Pieter Cornelis van der Valk** qualified as a veterinary surgeon in 1970 at the State University of Utrecht (the Netherlands) and obtained his PhD at the same university in 1979. He worked at the Veterinary Faculty from 1970 to 1985. In 1985 he joined the Veterinary Services of the Ministry of Agriculture and Fisheries. In 1989

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he was appointed by Solvay Duphar as Head of Clinical Research and in 1992 as Manager Regulatory Affairs for biologicals.

After the take over by Fort Dodge Animal Health he was appointed Regional Technical Manager for biologicals. Since 1970 he has been a member of the Royal Dutch Veterinary Society.

**Dr Philippe Vannier** obtained his degree in Veterinary medicine in 1973 from Maisons-Alfort Veterinary School (France). He obtained a Research Direction Habilitation in 1993 from Rennes I University. From 1973 to 1989, he has been in charge of a virology laboratory involved in the research on pig viruses, Classical Swine Fever, Aujeszky's disease, Parvovirus and also in the control of pig vaccines. He is now, from 1996, the director of AFSSA Ploufragan (Poultry and swine Research and studies laboratory) and research director in AFSSA (French Food Safety Agency). He is an expert in the immunological working group of the Committee for Veterinary Medicinal Products (in Brussels then in London). He is expert for porcine vaccines in the group 15V of the European Pharmacopoeia and expert for OIE as associate reference laboratory for Aujeszky's disease and in the biotechnology commission. He is expert for DG Sanco (EU) in the scientific Committee: Medicinal products and Medical devices.

**Prof. Dr J.T. van Oirschot** obtained his degree in veterinary medicine from Utrecht University in the Netherlands in 1973. Since that year he is doing research at ID-Lelystad, mainly on pestiviruses and herpes viruses. He obtained his PhD at the same University in 1980; his thesis dealt with classical swine fever. During the period 1981-1988 he was project leader and was appointed head of department at ID-Lelystad, and professor of veterinary vaccinology at Utrecht University in 1988. Since 2001, he is heading a virus discovery unit at ID-Lelystad.

**FINAL LIST OF PARTICIPANTS**

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## Pestivirus contamination of bovine sera and other bovine virus contamination

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