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Final policy review of the bluetongue virus risks associated with the importation of cattle, sheep, goat and deer semen and embryos from the European Union and cattle semen and embryos from Switzerland and Norway

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Contents

Tables

Figures

Glossary of terms and abbreviations

ABARE	Australian Bureau of Agricultural and Resource Economics
ADHIS	Australian Dairy Herd Improvement Scheme
AGID	Agar gel immunodiffusion test
ALOP	Appropriate level of protection
AQIS	Australian Quarantine and Inspection Service
Analytical sensitivity	Ability of a test to detect amounts of antigen or antibody
bELISA	Blocking enzyme-linked immunosorbent assay
BTV	Bluetongue virus
Case	An animal infected with BTV (OIE Terrestrial Animal Health Code 2010, Article 8.3.17.)
$CCID_{50}$	50% cell culture infective dose
CI	Confidence interval
cELISA	Competitive enzyme-linked immunosorbent assay
DEFRA	United Kingdom Department of Environment, Food and Rural Affairs
Diagnostic sensitivity	The proportion of true positive results that are detected by a test
Diagnostic specificity	The proportion of true negative results that are detected by a test
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EMAI	NSW Agriculture Elizabeth Macarthur Agriculture Institute
EU	European Union
HASAC	Health and Safety Advisory Committee of the International Embryo Transfer Society
Infection	BTV has been (1) isolated and identified as such from an animal or a product derived from that animal, or (2) viral antigen or viral ribonucleic acid (RNA) specific to one or more of the serotypes of BTV has been identified in samples from one or more animals showing clinical signs consistent with bluetongue, or epidemiologically linked to a confirmed or suspected case, or giving cause for suspicion of previous association or contact with BTV, or (3) antibodies to structural or nonstructural proteins of BTV that are not a consequence of vaccination have been identified in one or more animals that either show clinical signs consistent with bluetongue, or epidemiologically linked to a confirmed or suspected case, or give cause for suspicion of

	previous association or contact with BTV (OIE Terrestrial Animal Health Code 2010, Article 8.3.17.)
IETS	International Embryo Transfer Society
iELISA	Indirect enzyme-linked immunosorbent assay
in utero	In the uterus (during gestation)
in vitro derived embryo	Embryo produced by fertilisation and subsequent development outside of the body (IETS 2010b)
in vivo derived embryo	Embryo recovered after fertilisation and development occurred in the reproductive tract of the donor female (IETS 2010b)
MLV	Modified live virus vaccine
NAMP	National Arbovirus Monitoring Program
NSW	New South Wales
OIE	World Organisation for Animal Health
OIE Code	OIE Terrestrial Animal Health Code
Parous	Having produced offspring
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RIRDC	Rural Industries Research and Development Corporation
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SCFCAH	European Commission Standing Committee on the Food Chain and Animal Health
SPS	Sanitary and Phytosanitary, referring to the SPS Committee or the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organisation
TaqMan®	PCR methods which use TaqMan® probes use thermostable Taq polymerase derived from the thermophilic bacteria *Thermus aquaticus*. This method requires a single closed tube to be used and analysed in a machine for rapid, quantitative, real time PCR.
$TCID_{50}$	50% tissue culture infective dose
Topotype	Identical serotypes of bluetongue virus which have been isolated in different geographical areas and can be differentiated by genome sequencing.
TOV	Toggenburg Orbivirus
Viraemia	Circulation of virus in the blood
VNT	Virus neutralisation test
WTO	World Trade Organization

zona pellucida	The acellular, glycoproteinaceous coat which surrounds the mammalian oocyte and embryo at all stages prior to hatching of the blastocyst.

Summary

A new strain of bluetongue virus (BTV) serotype 8 (BTV8) that causes disease and deaths in cattle, as well as in sheep and goats, first appeared in the Netherlands and Belgium in 2006 and spread rapidly across Europe over 2007–2008, with severe economic consequences. After two breeding seasons in Europe, evidence emerged in early 2008 of transplacental transmission of virus in cows, with abortion and foetal malformation of calves. These characteristics appear to be unique to the strain of BTV8 in Europe. Subsequently, another four new serotypes of BTV (1, 6, 11 and Toggenburg Orbivirus) were detected in northern Europe over 2008–09. The latter three serotypes are also exotic to Australia. The BTV situation in Europe is dynamic. Further changes are anticipated, not only in the virus and its vectors, but also in European management and regulatory responses to bluetongue.

These issues raised concerns that the risk of BTV transmission via semen and embryos may have changed. Australia imports genetic material derived from several ruminant species from Member States of the European Union (EU) and cattle semen and embryos from two countries which are not EU members (Switzerland and Norway).

In July 2008, in response to new scientific information on the transmission of BTV8, Biosecurity Australia recommended to the Australian Quarantine and Inspection Service (AQIS) that interim quarantine requirements for imported embryos from the EU, Switzerland and Norway be introduced and that import permits for some commodities should be issued on a case-by-case basis, pending more detailed analysis. Interim risk management measures included BTV testing of donors of cattle embryos from the EU and Switzerland and case-by-case assessment of permits for cattle embryos from Norway. Australia notified trading partners of these changes.

The interim quarantine conditions introduced in July 2008 are set out in Appendix 3.

Australia has now completed the review of Australia's risk management for BTV for cattle, sheep, goat and deer semen and embryos from the EU and cattle semen and embryos from Switzerland and Norway. The first part of the review analyses the available information about BTV8 and other serotypes of BTV in Europe. The second part evaluates the risk presented by BTV8 compared with other serotypes of BTV in Europe at each stage of semen and embryo collection, and the likely consequences of entry, establishment or spread of BTV8 in Australia. Proposed quarantine risk management measures are set out in Chapter 7. Public comment was invited on this review, including the draft requirements.

The revised risk management measures outlined in this review address the risk of BTV8 to Australia with ruminant semen and embryo imports after the application of relevant EU legislation and recommendations of the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code (OIE Code).

The review found that:

- The EU, Switzerland and Norway are infected with BTV8.
- The strain of BTV8 in Europe presents a greater likelihood of transmission through insemination and embryo transfer than other serotypes of BTV:
  - BTV8 shares characteristics with laboratory-adapted strains of BTV, which research has shown are more likely to cause clinical signs in cattle, be shed in semen, transmitted transplacentally and cause foetal malformation.
  - There is published evidence of hydranencephaly in calves from BTV8 and transplacental transmission of BTV8 in up to 40% of calves. A small proportion of these calves are apparently immunotolerant.
  - Initial anecdotal reports suggested that BTV8 was present in semen of approximately 10% of clinically normal bulls. Subsequently, BTV8 was isolated from the semen of bulls with a history of clinical BTV8 infection.
  - Recent experiments have demonstrated BTV8 infection of goat embryos collected *in vivo* and cattle embryos at later stages of development which were collected *in vitro*.
- The emergence, circulation and reassortment of multiple BTV serotypes, including vaccine strains, in Europe is likely to be ongoing, requiring BTV tests capable of detecting all known serotypes.

The review concluded that the unrestricted risk of BTV8 in imported ruminant genetic material from the EU, Switzerland and Norway exceeded Australia's appropriate level of protection (ALOP) and proposed that the following risk management measures be applied:

- Embryos of cattle, sheep, goats and deer must continue to be collected *in vivo*, processed and washed ten times according to International Embryo Transfer Society (IETS) protocols.
- BTV testing of cattle embryo donors from the EU, Switzerland and Norway should remain for cattle embryos collected after 1 May 2006 (with modifications to BTV testing as outlined below).
- BTV testing should continue for donors of cattle, sheep, goat and deer semen and sheep and goat embryos from the EU and cattle semen from Switzerland and Norway (with modifications to BTV testing as outlined below).
- Deer embryo donors from the EU should be tested for BTV.
- BTV test requirements:
  - Donors to test negative to a competitive enzyme linked immunosorbent assay (cELISA) against BTV antibody, or a BTV virus isolation test, or a real time-reverse transcription polymerase chain reaction (RT-PCR) test.
  - Serological testing for BTV antibodies with agar gel immunodiffusion (AGID) tests should not be used.
  - Serological testing to occur at 60 day intervals during the semen collection period and at between 28 and 60 days after semen or embryo collection.
  - RT-PCR tests must be capable of detecting all serotypes of BTV, as well as BTV8, because of the need to exclude new isolates of field or attenuated vaccine strains of BTV.
- If used, vaccines against BTV administered to semen and embryo donors must be:
  - Inactivated, and
  - Approved by the competent authority in the exporting country, and
  - Administered more than 60 days before semen or embryo collection.

1 Introduction

Australia's biosecurity policy

Australia's biosecurity policies aim to protect Australia against risks that may arise from exotic diseases and pests entering, establishing or spreading in Australia, thereby threatening Australia's unique flora and fauna, as well as those agricultural industries that are relatively free from serious diseases and pests.

Biosecurity Australia is responsible for developing and reviewing quarantine policy for the import of animals and plants and their products. It does this through a science-based risk analysis process. At the completion of the process, Biosecurity Australia provides recommendations to Australia's Director of Animal and Plant Quarantine (the Secretary of the Australian Government Department of Agriculture, Fisheries and Forestry), who is responsible for determining whether or not an importation can be permitted under the Quarantine Act 1908, and if so, under what conditions. AQIS is responsible for implementing the import protocol, including any risk management measures.

Australia's science-based risk analysis process is consistent with Australian Government policy and Australia's rights and obligations under the World Trade Organisation's (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement).

Successive Australian Governments have maintained a conservative approach to the management of biosecurity risks. This approach is expressed in terms of Australia's ALOP, which reflects community expectations through government policy and is currently aimed at reducing these risks to a very low level, but not to zero.

If the risks exceed Australia's ALOP, risk management measures are proposed to reduce the risks to an acceptable level. However, if it is not possible to reduce the risks to an acceptable level, then no trade will be allowed.

This policy review

Background

The strain of bluetongue virus (BTV) serotype 8 (BTV8) that appeared in northern Europe in 2006 expressed characteristics which emerged over the following two breeding seasons. AQIS and Biosecurity Australia were monitoring the situation closely with regard to import conditions for ruminant semen and embryos from the EU, Switzerland and Norway. In early July 2008, pending a review of policy, Biosecurity Australia recommended interim measures to address the risk (Appendix 3).

Scope

The aims of this review are to:

- Evaluate risks posed by bluetongue virus in Europe, especially serotype 8, through the importation of genetic material from bluetongue-susceptible species.
- Ensure import conditions with regard to bluetongue for cattle, sheep, goat and deer semen and embryos from the EU, and cattle semen and embryos from Switzerland and Norway, adequately address risks in accordance with Australia's ALOP.

The review applies to embryos which are derived with *in vivo* methods, i.e. fertilised in, and collected from, living donors, rather than from unfertilised oocytes harvested from living or dead animals and fertilised outside the donor (*in vitro* collection). Australia does not have conditions that allow the import of ruminant embryos collected *in vitro* from the EU, Switzerland or Norway.

Existing policy

Prior to July 2008, Australia's import policy for cattle, sheep, goat and deer semen from the EU required negative serological testing or agent identification in blood of semen donors for BTV. Importation of cattle semen from Switzerland and Norway required these countries to meet the OIE Code requirements for freedom from bluetongue.

Import policy for cattle embryos from the EU, Switzerland and Norway required embryos to be collected, processed and stored in accordance with EU legislation. Negative serological testing or agent identification in blood of cattle embryo donors for BTV was not required. However, Australia required BTV testing of donors of sheep and goat embryos from the EU. Importation of deer embryos required the exporting country to meet OIE Code requirements for freedom from bluetongue.

Interim measures in place are as follows:

- Importation of cattle semen from the EU and Switzerland, subject to negative serological testing or agent identification in blood of all donors for BTV.
- Assessment of permit applications for cattle semen from Norway on a case-by-case basis.
- Importation of cattle embryos collected after 1 May 2006 from the EU, Norway or Switzerland, subject to negative serological testing or agent identification in blood of all donors for BTV.
- Assessment of permit applications for sheep, goat and deer semen and embryos from the EU on a case-by-case basis.

Details of these measures are provided in Appendices 3 and 4.

Assessment of import permit applications on a case-by-case basis was applied to genetic material that was imported infrequently. No permits have been issued since 1998 for sheep, goat or deer semen or embryos from Norway or Switzerland, bovine embryos from Norway or goat semen or embryos from the EU. Single permits for sheep semen and embryos from the United Kingdom expired in 2005 and 2006 respectively, and a single permit for deer semen from Poland expired in 2007. A single permit for bovine semen from Norway expired during the period of this review.

2 Method

This review is based on the principles of an import risk analysis outlined in the OIE Code. The review was conducted in three consecutive stages:

1. Hazard identification
2. Risk assessment
3. Risk management

Hazard identification

The hazard assessed in this review is the European strain of BTV8. Australia has several BTV serotypes which may occur on a seasonal basis in parts of northern Australia but BTV8 has never been detected.

Risk assessment

Risk assessment is defined in the OIE Code (OIE 2010d) as '*…the evaluation of the likelihood and the biological and economic consequences of entry, establishment and spread of a hazard within the territory of an importing country'*. In accordance with the OIE Code, the likelihood that a pathogenic agent will enter an importing country and the likelihood that susceptible animals will be exposed to the agent are determined in this review through a 'release assessment' and an 'exposure assessment' respectively. The likelihood of establishment or spread and the biological, environmental and economic consequences of introducing a pathogenic agent, are determined through a 'consequence assessment'. The risk assessment for an identified agent concludes with 'risk estimation' —the combination of the likelihood of entry and exposure and the likely consequences of establishment or spread— and yields the 'unrestricted risk estimate' (Table 1).

Table 1. Risk estimation matrix: estimation of unrestricted risk.

Likelihood of entry and exposure							
	High likelihood	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk	Extreme risk
	Moderate	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk	Extreme risk
	Low	Negligible risk	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk
	Very low	Negligible risk	Negligible risk	Negligible risk	**Very low risk**	Low risk	Moderate risk
	Extremely low	Negligible risk	Negligible risk	Negligible risk	Negligible risk	**Very low risk**	Low risk
	Negligible likelihood	Negligible risk	Negligible risk	Negligible risk	Negligible risk	Negligible risk	**Very low risk**
		Negligible impact	*Very low*	*Low*	*Moderate*	*High*	*Extreme impact*
		Consequences of entry and exposure					

Those risk estimates that are 'very low' or lower meet Australia's ALOP and no risk management measures are necessary. For those where the risk is greater than 'very low', risk management measures are recommended to reduce the risk to a level that meets Australia's ALOP.

In the case of BTV8, the likelihood of the pathogenic agent being released into Australia via contaminated semen or embryos follows the biological pathway in Figure 1. This includes consideration of the distribution and prevalence of BTV8 in the country of origin, farms and artificial breeding centres before transport to Australia. In this review the likelihood of release was assessed after taking into account relevant EU legislation for the collection and processing of ruminant semen and embryos (Appendix 2) and recommendations in the OIE Code and IETS.

In this review, the likelihood of introducing BTV8 in ruminant germplasm was assessed after compliance with OIE Code Articles on '*Collection and processing of bovine, small ruminant and porcine semen*' (Chapter 4.6) and '*Collection and processing of in vivo derived embryos from livestock and horses*' (Chapter 4.7), which include IETS recommendations for embryo collection, processing and storage.

IETS standards for the *in vivo* collection and processing of embryos require embryos to be free of debris, not subjected to micromanipulation, have an intact zona pellucida, washed ten times and sometimes treated with trypsin (OIE 2010c; Stringfellow 2010). In cattle, these standards have managed the risk of BTV contamination of embryos, and consequently, cattle embryo donors are not tested for BTV. Accordingly, any BTV8-specific controls would be in addition to these measures.

After entry of semen or embryos to Australia, the likelihood of exposure of recipient animals to BTV8 from imported contaminated semen or embryos is evaluated. Assuming infection of a recipient animal, the likelihood of establishment or spread of BTV8 to other livestock and susceptible species is considered as part of the likely consequences.

Imported genetic material is widely distributed in Australia to artificial breeding premises, technicians, veterinarians and producers. Distribution of semen and embryos and the structure of Australia's artificial breeding industry affect the likelihood of exposure, establishment or spread of BTV8. Thus many contaminated doses from a single infected donor could cause disease outbreaks in several locations and a range of environments.

Outbreaks could occur concurrently or over an extended period, depending on the length of time the semen or embryos were stored before use, and an incursion of BTV8 was detected. Tracing the location of imported genetic material once it has entered Australia is difficult because of the number of operators in the artificial breeding industry, the absence of centralised lists of operators, re-sale of genetic material and limited records of its storage or use, especially in the case of semen.

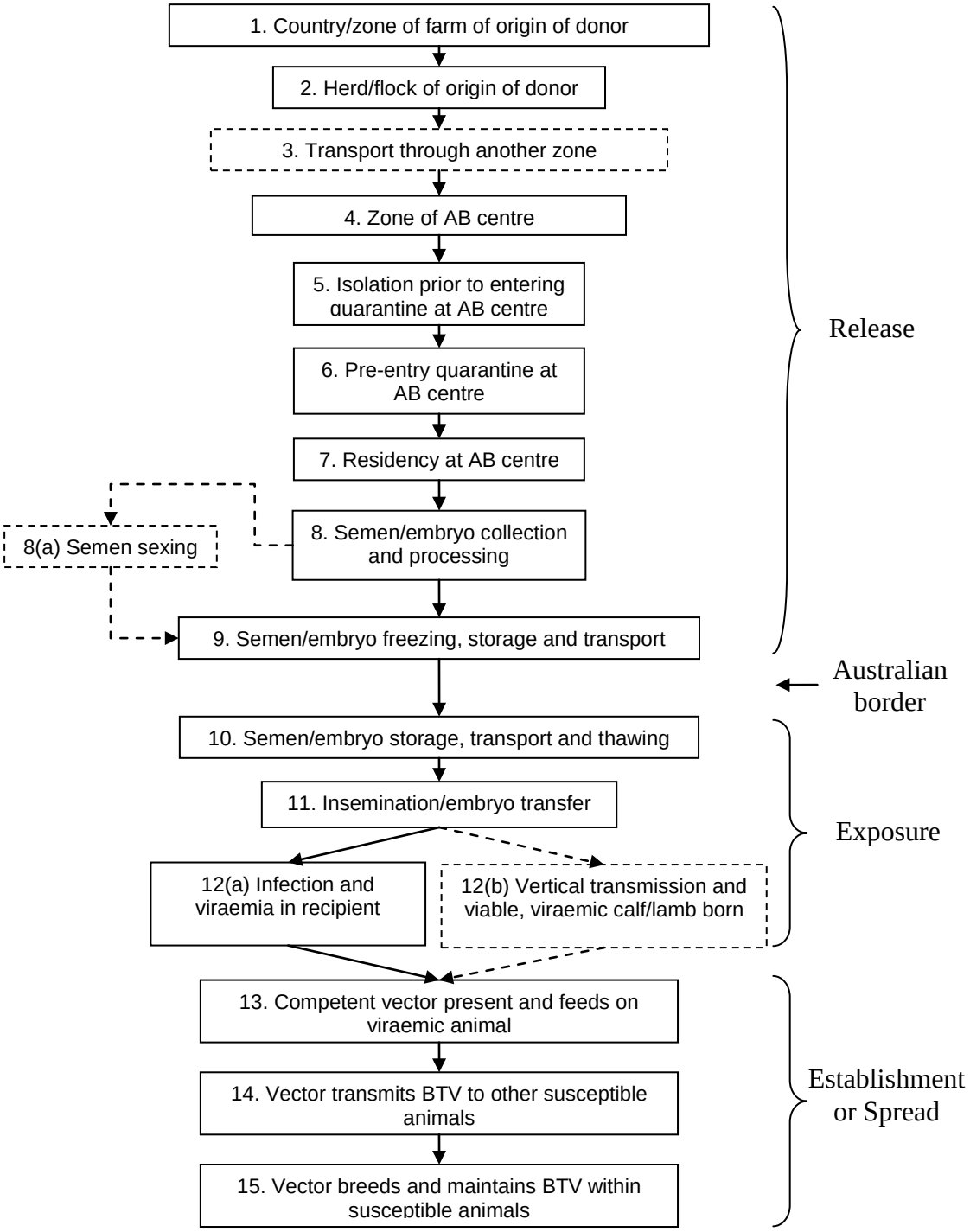

Figure 1. The biological pathways for the entry (in Europe) and exposure (in Australia) of BTV8 via infected semen or embryos. The probability of an infected donor depends on the BTV status of the country or zone in which the farm of origin and artificial breeding (AB) centre are located, and the incidence of BTV in the donor's herd/flock of origin and the AB centre. Steps 4-7 may take place on-farm for embryo donors. Dashed lines indicate additional steps, for example semen processing at a separate location to the centre or semen sexing (8a) or vertical transmission of BTV8 (transplacentally or orally), which can only infect vectors if viable and viraemic offspring are born (12b).

Volume of trade

The final level of risk is a function of the number of doses of semen and the number of embryos from the EU, Switzerland and Norway imported into Australia. The volume of trade in ruminant semen and embryos varies from year to year (Figure 2).

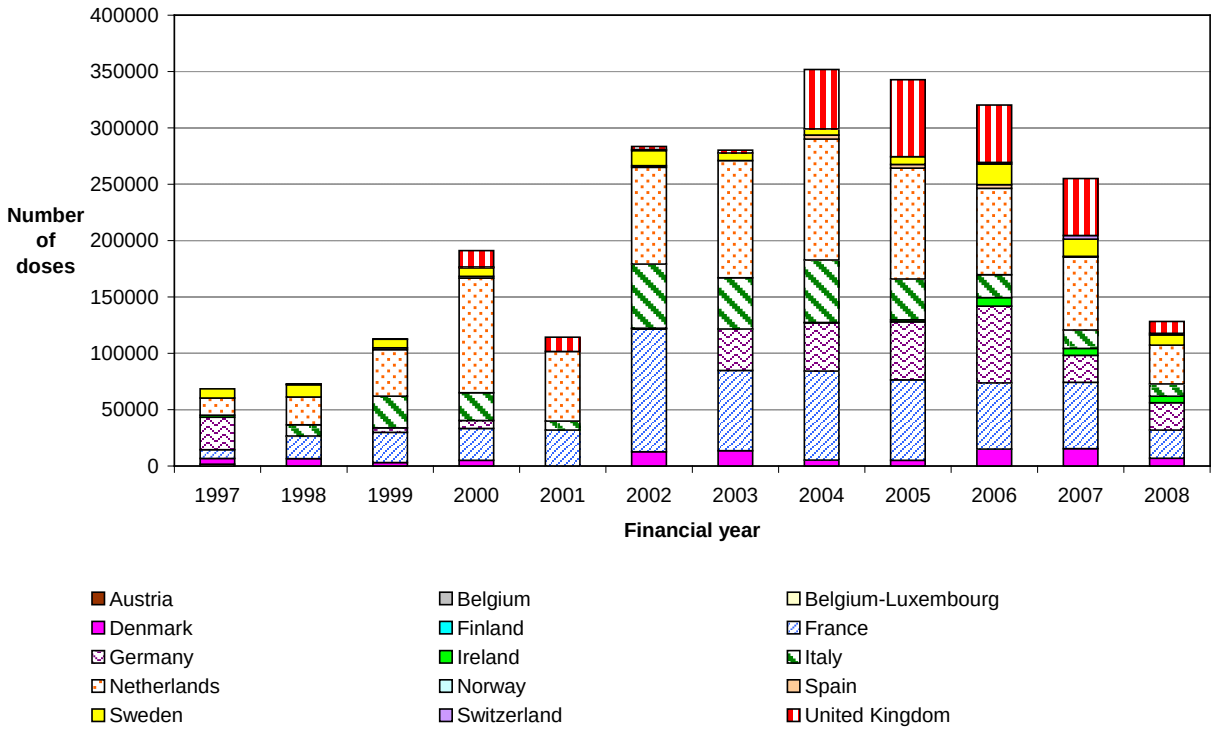

Figure 2. The number of doses of cattle semen imported into Australia from European countries (1 January 1997–31 December 2007).
Source: ILRIC (2008)

Australia imported approximately 750 000 doses of cattle semen from the EU, Norway and Switzerland between 1 January 2006 and 30 September 2008 (Australian Bureau of Statistics 2009; ILRIC 2008). From January to December 2008, the total number of cattle semen doses imported from these countries was about 400 000[1]. The amount of cattle semen imported from the EU, Norway and Switzerland is approximately one third of the total quantity imported into Australia. Most imported cattle semen originates from the United States, Canada and New Zealand.

The number of ruminant embryos imported into Australia is small. Over January and December 2008, a total of 229 cattle embryos were imported from the EU, Switzerland and Norway[2]. Several consignments can be imported under a single permit, with variable numbers of embryos in each consignment. From 2003 to 2008, the numbers of embryos in each of six consignments entering New South Wales (NSW) from the EU ranged from four to 1 000 (Table 2). The number of sheep, goat

[1] Data collated from import permits cleared by AQIS regional offices in Brisbane, Melbourne, Sydney and Perth.
[2] Data collated from import permits cleared by AQIS regional offices in Brisbane, Melbourne, Sydney and Perth.

and deer semen doses or embryos imported from the EU is very small (none were imported in 2008 and 2009).

Table 2. Cattle embryos imported into NSW from the EU in six consignments from 2003 to 2008.

Date of entry of consignment to Australia	Exporting country	Number of embryos in consignment
14/5/2003	Italy	4
2/3/2004	Italy	14
15/1/2007	Italy	25
19/1/2007	United Kingdom	1 000
26/2/2008	Germany	121
16/6/2008	Germany	7

Source: AQIS (unpublished)

These data do not necessarily indicate future volumes of semen and embryos. AQIS noted an increase in consignments of semen after improvement in seasonal conditions in southern Australia in 2008, in anticipation of the forthcoming breeding season for cattle[3].

[3] Y. Hill, AQIS (*personal communication*).

3 The artificial breeding industry

Artificial insemination and embryo transfer in cattle

For many years, cattle breeders have used artificial insemination to improve cattle production and to reduce the incidence of sexually transmissible diseases. Dairy production systems enable close observation and handling of cows required for artificial breeding. This contrasts with beef cow management which is more extensive and beef cows are not as conveniently managed for artificial insemination and embryo transfer (Macmillan and Tervit 1990).

Technology of semen handling and insemination of cows is similar for beef and dairy cattle. In semen collection centres, bulls are trained to serve an artificial vagina. Nearly all semen for artificial insemination is frozen to allow for transportation and long term storage. Increasingly, semen may undergo further processing for sex selection (Norman et al. 2010). An average dose consists of 20 $x10^6$ spermatozoa usually administered via intra-uterine insemination. However, high semen dilution rates can be used with as few as $2x10^6$ spermatozoa/insemination, particularly for sex-sorted semen (Garner and Seidel, Jr. 2008; Macmillan and Tervit 1990).

Embryo transfer in cattle is conducted on farm or at an artificial breeding centre by a team of technicians under the supervision of a veterinarian qualified in embryo transfer procedures. Embryo collection in cattle using *in vivo* methods requires multiple ovulation of a donor cow, fertilisation by natural service or artificial insemination, and collection of fertilised embryos. Most commercial embryo transfer programs use non-surgical techniques to flush embryos from the uterus using a catheter placed through the cervix (Selk 2007). Embryos are then evaluated for quality and washed before being transferred fresh or frozen in liquid nitrogen for storage and transport. Recipient cows are prepared by synchronising oestrus and embryos are transferred by passing a catheter though the cervix into the uterus.

Artificial insemination and embryo transfer in sheep, goats and deer

Techniques for semen collection and artificial insemination are similar for sheep and goats and have been described in detail by Evans and Maxwell (Evans and Maxwell 1987). Semen quality and breeding efficiency are affected by season (Foote 2002); however, particular breeds and individual animals can conceive all year. Both rams and bucks can be trained to serve an artificial vagina, but electroejaculation, pioneered by Gunn (1936), is used for field purposes.

Techniques and media for freezing sheep or goat semen were adapted from those developed for bull sperm (Davis et al. 1963). Buck sperm cryopreservation is more successful than the cryopreservation of ram sperm (Foote 2002). Similarly, intra-cervical artificial insemination using frozen-thawed semen results in satisfactory fertility in goats, but not in the ewe. Therefore, transabdominal insemination into the uterus with the aid of a laparoscope is required to achieve good fertility in the ewe and

is also used extensively in goats (Leboeuf et al. 2000). Laprascopic techniques present a greater risk of infection from contaminated instruments than non-surgical methods.

Development of the laparoscopic method for intra-uterine insemination (Killen and Caffery 1982) initiated a steady increase in the number of ewes inseminated in Australia, from approximately 20 000 in 1983 (Maxwell 1984) to 250 000 in 1988 (Maxwell and Wilson 1990) and around 500 000 in 1994–95 (Maxwell and Watson 1996). These figures were small compared with the total number of sheep; however they represented a significant proportion (~20 to 30%) of the stud ewe population (Maxwell and Watson 1996). Artificial insemination in commercial flocks is limited because of the poor fertility obtained after cervical insemination with frozen-thawed semen and the high cost of synchronisation of oestrus and laparoscopic insemination.

Techniques for *in vivo* embryo collection and transfer in sheep and goats were developed from methods in cattle. Embryos are collected surgically under anaesthesia, requiring exteriorisation of the uterus or use of a laprascope (Thibier and Guerin 2000). Embryo transfers in sheep and goats in Australia accounted for more than half of world transfers in these species in 2002 (Thibier 2003).

Artificial breeding techniques of deer have also been adapted from methods used in cattle. However, most farmed deer species — fallow deer (*Dama dama*), red deer (*Cervus elaphus*) and waipiti/elk (*Cervus elaphus canadensis*) — originate from temperate regions, and artificial breeding is restricted by highly seasonal oestrus and sperm production and difficulties in handling individual animals (Asher et al. 2000). Some of the less commonly farmed species in Australia which originated in the tropics, for example, rusa deer (*Cervus timorensis*) and chital deer (*Axis axis*) are less seasonal in their reproductive pattern.

Semen is usually collected by electroejaculation of sedated or anaesthetised stags. Laprascopic intrauterine insemination of does with fresh or frozen-thawed semen is the preferred method for commercial breeding programs (Morrow et al. 2009). Intravaginal and transcervical insemination can also be used but conception rates are lower. Surgical and laprascopic embryo transfer techniques in red deer achieve similar success rates to those in cattle (Fennessy et al. 1994) and have been developed for fallow deer (Morrow et al. 1994). Commercial application of non-surgical embryo transfer methods is also under investigation (Plante 2009).

Application of artificial breeding in Australian livestock industries

The Australian artificial breeding industry has developed as a diverse group of individually and corporately owned and operated artificial breeding centres and service facilities. These facilities are randomly scattered throughout rural Australia in areas of highest demand. Artificial breeding centres hold valuable elite breeding stock and stores of conserved genetic material, and serve as quarantine facilities for collection of export genetic material. A number of service providers operate out of licensed or approved artificial breeding centres. Licensing arrangements for these providers vary between states and territories. Some maintain registers of artificial breeding centres, but all have some legislative control through their stock diseases and stock (artificial breeding) acts and regulations.

There are also artificial breeding activities that operate outside the confines of licensed or approved artificial breeding centres. There are no legislative controls or

industry codes of practice covering unlicensed operations or services of 'on-farm' enterprises, which now account for a significant proportion of all artificial breeding operations in Australia. State/territory legislation and regulations pertaining to stock disease, stock artificial breeding and veterinary surgeons cover some of the procedures and activities.

Importation of semen or embryos is governed by the *Quarantine Act 1908* and regulations. Imported semen and embryos are not necessarily stored at licensed or approved artificial breeding centres, but may be stored at other centres or sub-centres or with commercial artificial insemination technicians (inseminators), veterinarians or livestock producers.

Use of artificial breeding in the Australian livestock industries increased as new technologies became available, reducing costs and improving fertilisation. It is difficult to determine the extent to which reproductive technology is used currently in Australia due to the fragmented nature of the artificial breeding industry. A study undertaken in 1991 of licensed breeding centres and artificial insemination and embryo transfer practitioners (Evans 1991) does not reflect the current situation but indicates the development of the reproductive technologies used widely today.

Cattle

Beef cattle greatly outnumber dairy cattle in Australia. Australia has approximately 12.5 million adult beef cows and 1.6 million dairy cows (ABARE 2009). Beef breeds consist of *Bos taurus*, *Bos indicus* (Brahman) and their crosses. About half of the national beef cattle herd is located in Queensland and around a quarter is in NSW. Most dairy herds are located in Victoria and high rainfall coastal regions of NSW, south west Western Australia and Queensland. The dominant dairy breeds are Holstein-Friesian and Jersey.

During 1989–90, artificial insemination was used for approximately 60% of the national dairy herd (1.7 million at the time), but only 1–2% of beef cows (Evans 1991). These figures were reflected in the breed of bulls used in artificial insemination centres; 74% of semen doses processed was from dairy breeds, predominantly Holstein-Friesian. The remaining semen was obtained from a variety of beef breeds, including Hereford (12%), Angus (6%) and Limousin (4%), much of which would have been used on dairy cows to produce first-cross calves.

Some industry organisations collect data on artificial breeding practices, but this is difficult to obtain and often not indicative of the industry overall. In 2007, Australian and New Zealand cattle embryo transfer programs using *in vivo* methods produced 10 800 transferrable embryos from 1 700 flushes, with 4 200 fresh and 3 700 frozen embryos transferred. However, these figures underestimate actual numbers (Thibier 2009). In 2006, a survey of dairy farms by the Australian Bureau of Agricultural and Resource Economics (ABARE) showed that 57% of dairy cows were calved using artificial insemination (Ashton and Mackinnon 2008). Similarly, recent data from the Australian Dairy Herd Improvement Scheme (ADHIS) indicated that 46% of dairy cows were herd recorded and of these, 74% of Holsteins and 70% of Jerseys were bred via artificial insemination (ADHIS 2008).

Activity in the artificial breeding industry is also indicated by the total amount of imported genetic material (from all countries, including those in Europe). In 2006, Australia imported approximately 1.14 million doses of cattle semen and exported about 570 000 doses of semen and 3 000 cattle embryos (Thibier 2009). Comparable

data on the number of imported embryos was not available. However, in 2008 about 4 000 cattle embryos were imported into Australia: Queensland (1 040), NSW (1 442) and Victoria (2 038)[4].

Sheep and goats

The total number of sheep in Australia halved from 167 million in 1990 to 80 million in 2008 (ABARE 2009). At the same time, the breed composition of the Australian sheep flock shifted, with an increase in the proportion of ewes joined to meat breed rams. Reflecting this change, lamb production has been increasing. While Merinos, crossbreds and other British sheep breeds dominate, dual purpose breeds (Dohne, South African Mutton Merino) and meat breeds (Dorper, Damara) are gaining popularity in Australia. Most of Australia's sheep are located in NSW (34%), Western Australia (23%) and Victoria (22%) (ABARE 2009).

Recent information on the number of farmed goats is difficult to obtain. In 1997, there were several thousand breeders of Angora goats and the Australian Angora flock was about 200 000 (NSW Department of Primary Industries 1997) but numbers have since declined. Recently meat breeds have increased, particularly Boer goats (Goat Industry Council of Australia 2009).

Current data on artificial insemination in sheep and goats is not available. It is believed between 400 000 to 500 000 ewes were inseminated annually in Australia since 2000, despite the declining sheep population. Almost all of the artificial insemination in Australia during 1989–90 was performed with Merino semen (94%), with a small proportion using Corriedale semen (4%) (Evans 1991). These figures would undoubtedly have changed as market demands and production systems have changed. Most semen used for goat artificial insemination came from Angora goats (93%) in 1989–90, but it was estimated that less than 1% of the national goat herd was artificially inseminated. In 2008, about 3 500 doses of sheep semen, 3 200 sheep embryos and 200 goat embryos were imported into NSW and Victoria[5].

Deer

The number of farmed deer in Australia increased rapidly from about 8 000 in 1980 to 200 000 in 1995 but decreased subsequently to an estimated 150 000 head in 2006 (RIRDC 2007). In 1997, the principal farmed species were fallow deer (49%), red deer (39%) and elk/wapiti (3%) followed by rusa deer (6.5%) and chital deer (2.5%) (Tuckwell 1998). Currently, red deer and their hybrids predominate because of their value as dual purpose animals for velvet and venison (RIRDC 2007).

Detailed information on the use of artificial breeding in deer in Australia is difficult to obtain. A report by Dixon (1991) described the successful transfer of about 250 imported frozen red deer embryos in 1991. In 2008, about 200 doses of deer semen and 23 deer embryos were imported into NSW and Victoria.

[4] AQIS data
[5] AQIS data

4 Technical background

Bluetongue – the disease

Bluetongue is a non-contagious, insect-borne viral disease of ruminants, characterised in sheep by cyanosis of mucous membranes with widespread haemorrhages and oedema. Bluetongue is enzootic in most countries between 53 °N and 34 °S with occasional epizootics outside these latitudes.

BTV is an Orbivirus of the family Reoviridae. There are 24 serotypes recognised worldwide. Identical serotypes have been isolated in different geographical areas and can be differentiated by genome sequencing (topotypes). Differences in pathogenicity and virulence between strains of the same BTV serotype or topotype are well recognised (Gard 1987; Waldvogel et al. 1986; Waldvogel et al. 1992). Virulence and pathogenicity can also differ between laboratory and wild strains of a topotype.

Epidemiology

All ruminant species are susceptible to BTV. Sheep and white-tailed deer are the most severely affected, while goats are occasionally clinically affected. Cattle and camelids are generally subclinically infected (Lager 2004; Rivera et al. 1987) but cattle are the major vertebrate amplifier of virus.

BTV is transmitted by biting midges (*Culicoides* spp.). Virus distribution depends on presence and density of animal reservoirs, amplifying hosts such as wild ruminants and cattle, and a sufficient population of competent *Culicoides* vectors. Although there are over 1 400 species of *Culicoides* worldwide, only 32 are known vectors of BTV (Meiswinkel et al. 2004). The range of serotypes in a region is considered to be the result of relatively stable relationships between local vector species and BTV serotypes or topotypes (Gibbs and Greiner 1994).

In cattle, infection is usually subclinical. The incubation period is four to eight days. Viraemia is detectable at three days post-infection and usually lasts less than four weeks but may vary in duration with virus serotype and ruminant host species and age. In exceptional cases, viraemia may persist for eight weeks (Anthony et al. 2007; Bonneau et al. 2002; Gard 1998; Gard and Melville 1992; Koumbati et al. 1999; Melville et al. 2005b; Melville et al. 2005c; Richards et al. 1988; Singer et al. 2001; Venter et al. 2007). The OIE Code (2010a) defines the infective period for BTV as 60 days (Article 8.3.1).

Virus is found transiently in serum and monocytes but persists within red blood cells in the presence of high antibody titres (Schwartz-Cornil et al. 2008). The appearance of antibody does not coincide with the removal of virus from blood (Richards et al. 1988). Antibodies are usually first detected around one to two weeks post-infection and remain detectable for at least 60 days. There is no cross protection between BTV serotypes and earlier reports of carrier states were probably re-infections of animals with different serotypes (Geering et al. 1995).

In sheep, infection with some serotypes causes fever, hyperaemia and cyanotic oedema of buccal and nasal mucosa, membranes, skin and coronary bands, muscle degeneration and/or foetal infection with abortion and congenital abnormalities. The incubation period generally ranges from four to seven days and viraemia lasts about

seven days, with a maximum of about 20 days. Breeds differ in the severity of clinical signs and length of the viraemic period. In goats, clinical signs are usually less severe than in sheep.

The role of wild and domestic carnivores and other wildlife in BTV transmission is unknown (Alexander et al. 1994; EFSA 2007d; EFSA 2008b). BTV antibodies have not been found in horses, donkeys, pigs, dogs, marsupials or humans in Australia (St George et al. 2001) but were present in about 20% of domestic dogs in Morocco (Oura and el Harrak 2010). A laboratory adapted strain of BTV contaminated canine cell culture lines for canine vaccines and resulted in the death of dogs (Akita et al. 1993).

Bluetongue in Australia

Ten serotypes (1, 2, 3, 7, 9, 15, 16, 20, 21 and 23) of BTV have been identified in Australia from insects or clinically healthy sentinel cattle[6]. Clinical bluetongue disease has not been reported in cattle in Australia. Moreover, clinical disease has not been reported in commercial sheep flocks or goats because they are largely separated from vectors which are typically distributed in the north of Australia and east of the Great Dividing Range (Geering et al. 1995). Australia conducts a national surveillance and monitoring program for BTV and its *Culicoides* vectors[7]. Of the approximately 180 *Culicoides* species reported in Australia, only eight have been shown to be capable of infection by BTV. *Culicoides brevitarsis* is the principal Australian vector of BTV (Bellis and Dyce 2005; Standfast et al. 1992).

Incursions of exotic serotypes of BTV carried by windborne vectors to northern Australia originate in South-East Asia and Papua. Ten of 13 serotypes of BTV identified in Indonesia (1, 2, 3, 5, 6, 7, 9, 12, 15, 16, 20, 21, 23) have been isolated in northern Australia. Serotypes 1, 16, 21 and 23 have been isolated in Papua and there is serological evidence of BTV in East Timor. BTV8 has been recorded in India but not Malaysia or Indonesia (Daniels et al. 2009; Daniels et al. 2004; Pritchard et al. 2004).

Bluetongue in Europe

Three significant events that affected the EU response to BT were the introduction of BTV serotypes in Mediterranean Europe since 1998, the introduction of BTV8 into northern Europe leading to the pan-European epidemic of 2006-08 and serotypes detected in northern Europe after 2008.

BTV serotypes 1, 2, 4, 9 and 16

Since 1998, there have been 11 introductions of BTV into Europe, mostly due to BTV serotypes 1, 2, 4, 9 and 16 in countries around the Mediterranean basin (Mellor et al. 2009). Between 1998 and 2005, over one million sheep died or were culled due to

[6] BTV2 was detected by routine surveillance of healthy cattle in two sentinel herds in the Northern Territory in April 2008.
[7] National Arbovirus Monitoring Program (NAMP). See www.animalhealthaustralia.com.au/aahc/programs/adsp/namp/namp_home.cfm

BTV outbreaks in 12 European and three North African countries and Israel (Purse et al. 2005).

- These BTV serotypes cause clinical disease in sheep and goats but not cattle. Severity of disease from BTV varies with serotype, breed and location of flocks, presence of local vectors and other infections (Panagiotatos 2004). Three bluetongue outbreaks involving BTV serotypes 2, 9, 4 and 16 occurred in Sicily over 2000–2003. Over this period, morbidity increased from 10 to 17%, while mortality was stable at about 7%. Case fatality rates ranged from 21 to 60% (Carcappa et al. 2004; Gomez-Tejedor 2004).
- BTV1 and BTV4 were detected in wild ruminants in Spain but the effect of wild ruminants in Europe on bluetongue control is not clear and likely to vary with species, serotype and region (Fernandez-Pacheco et al. 2008a; Garcia et al. 2008; López-Olvera et al. 2010).
- BTV serotypes 2, 4, 9 and 16 persist in Italy[8]. Spain and Portugal remain infected with BTV4 as well as BTV1.

BTV1 first appeared in Italy in 2006 and reached Spain and Portugal in 2007 and France in 2008 (OIE 2009f) (Figure 3).

- BTV1 accounted for about half of the 244 000 reported cases of BTV serotypes 1 and 8 in Europe between July 2006 and December 2008. Roughly 2% of BTV1 cases occurred in cattle (European Commission: Health & Consumer Protection Directorate-General 2011).
- BTV1 was considered endemic in Spain in 2007 (OIE 2008e).
- BTV1 continued to spread northwards in Portugal and France and, in late October 2008, a case was detected in Brittany (Figure 4). Compulsory vaccination against BTV1 (and BTV8) was required in all regions of France from December 2008 (SCFCAH 2008i).
- In November 2008, cattle infected with BTV1 were imported to the United Kingdom and Netherlands from France but local spread did not occur (Figure 3) (Promed Mail 2008x).
- Clinical disease, transplacental infection and deaths were reported in llamas in France infected with BTV1 (Meyer et al. 2009) and the first outbreak of BTV1 in France for 2009 was reported in alpacas (Promed Mail 2009e).

After 2008 the number of outbreaks of all serotypes of BTV in EU Member States decreased to 1 118 in 2009 and 120 in 2010 (European Commission 2010).

Novel BTV serotypes

New serotypes — BTV6 and 11 and Toggenburg Orbivirus (TOV) — were reported in late 2008 and early 2009. Separate outbreaks of BTV16 also occurred as mixed infections with BTV8 in Greece and caused mortalities in cattle in Israel (Brenner et al. 2010; OIE 2008e; Promed Mail 2009c; Promed Mail 2009f).

Vectors

There are four species of vectors of BTV known in southern Europe but historically the distribution of bluetongue corresponded to the distribution of *C. imicola*. For example, in Italy the distribution of BTV is largely confined to the distribution of

[8] Detailed OIE reports on worldwide outbreaks of bluetongue can be found at: www.reoviridae.org/dsRNA_virus_proteins/outbreaks.htm

C. imicola in the south (Goffredo et al. 2004; Meiswinkel et al. 2008a; Mellor et al. 2008; Mellor and Wittmann 2002). In the absence of *C. imicola*, BTV2 and 9 have been transmitted by *C. obsoletus*, *C. scotius* and *C. dewulfi* (Savini et al. 2005). Year-round transmission of BTV is possible in Italy because of the wide distribution and activity of *C. obsoletus* during winter, when *C. imicola* is absent (Ferrari et al. 2005; Monaco et al. 2004).

Vector ecology is also influenced by management practices, such as housing animals. Most *Culicoides* species, including *C. imicola,* are found in greater numbers outside rather than inside animal housing (Mellor and Wittmann 2002). However, *C. imicola* is abundant near livestock holdings where it feeds on cattle, sheep and horses, and breeds in damp or wet soil containing dung.

The distribution of *C. imicola* may also be expanding, for example, into northern Spain and the Mediterranean coast of France, where it has been found with vector species for BTV8 (Balenghien et al. 2008; Goldarazena et al. 2008; Promed Mail 2008g). Expansion of BTV serotypes northwards from the Mediterranean basin has largely been associated with other vectors which also transmit BTV8 (*C. obsoletus/scotius* and *C. pulicaris*) (Calvete et al. 2008; Goffredo et al. 2004; Mellor et al. 2008; Savini et al. 2005). In the absence of *C. imicola,* the presence of BTV1 in south-west France is associated with *C. obsoletus/scotius*, *C. dewulfi* and *C. chiopterus* (Balenghien et al. 2008).

Origins

Full sequencing of the BTV genome established the genetic origins of serotypes circulating in Europe prior to the detection of BTV6, BTV11 and TOV (Maan et al. 2008) (Table 3).

Table 3. Genetic origin of bluetongue serotypes in Europe

BTV serotype	Origin
9, 16, 1 (2001 incursion)	Middle East
2, 4, 8	sub-Saharan Africa
1 (2006 incursion)	northern Africa

Source: Maan *et al*. (2008)

The routes of introduction of BTV serotypes from North Africa and the Middle East into Europe are described in detail by Saegerman *et al*. (2008). However there is no definitive explanation for the appearance since 2006 of the three BTV serotypes (6, 8 and 11) which were first detected in the same geographical region of northern Europe (Eschbaumer et al. 2010a).

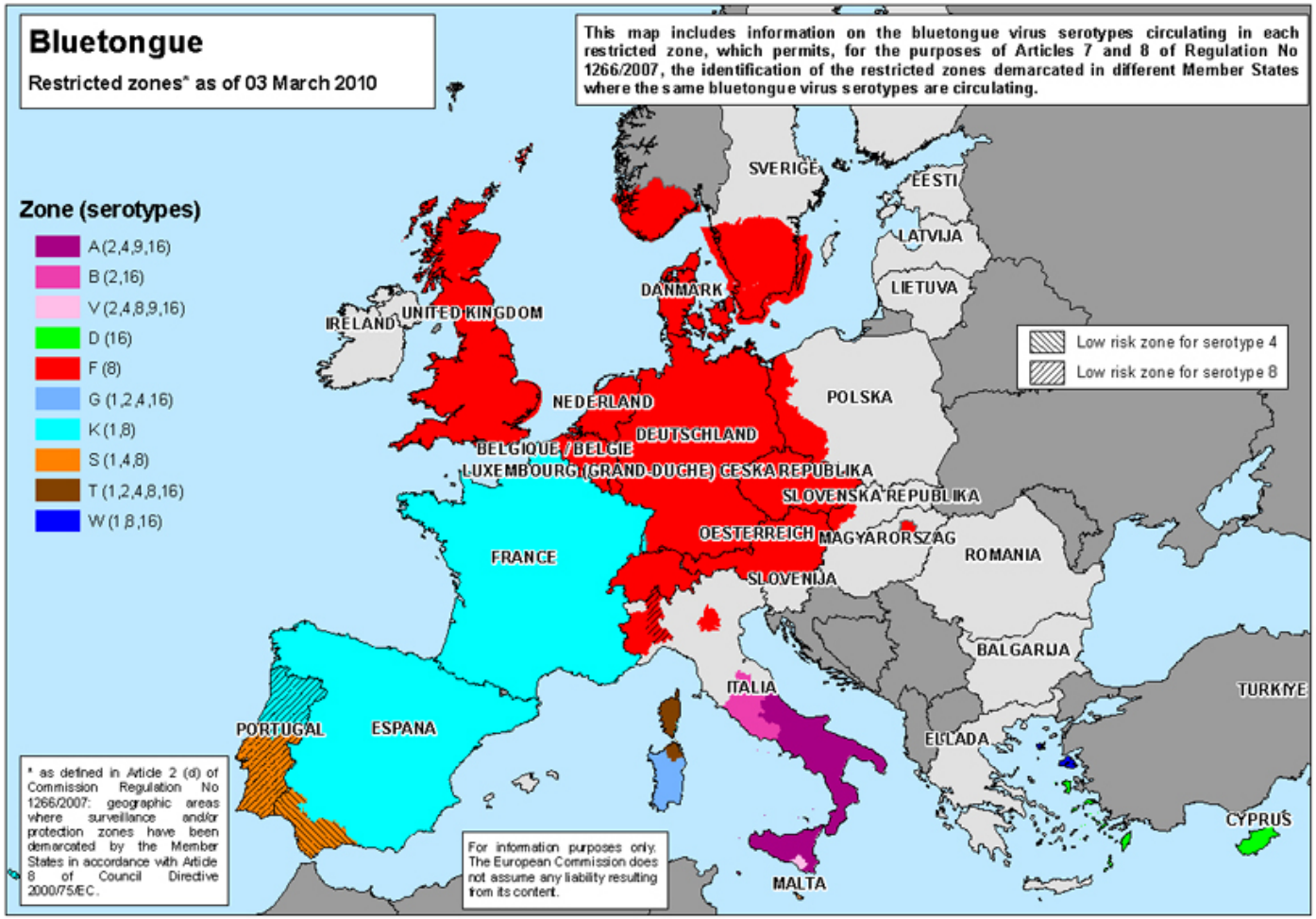

Figure 3. Map of bluetongue restricted zones in EU Member States showing the distribution of BTV serotypes.
Source: http://ec.europa.eu/food/animal/diseases/controlmeasures/bluetongue_en.htm (accessed 12 March 2010)

BTV1 **BTV8**

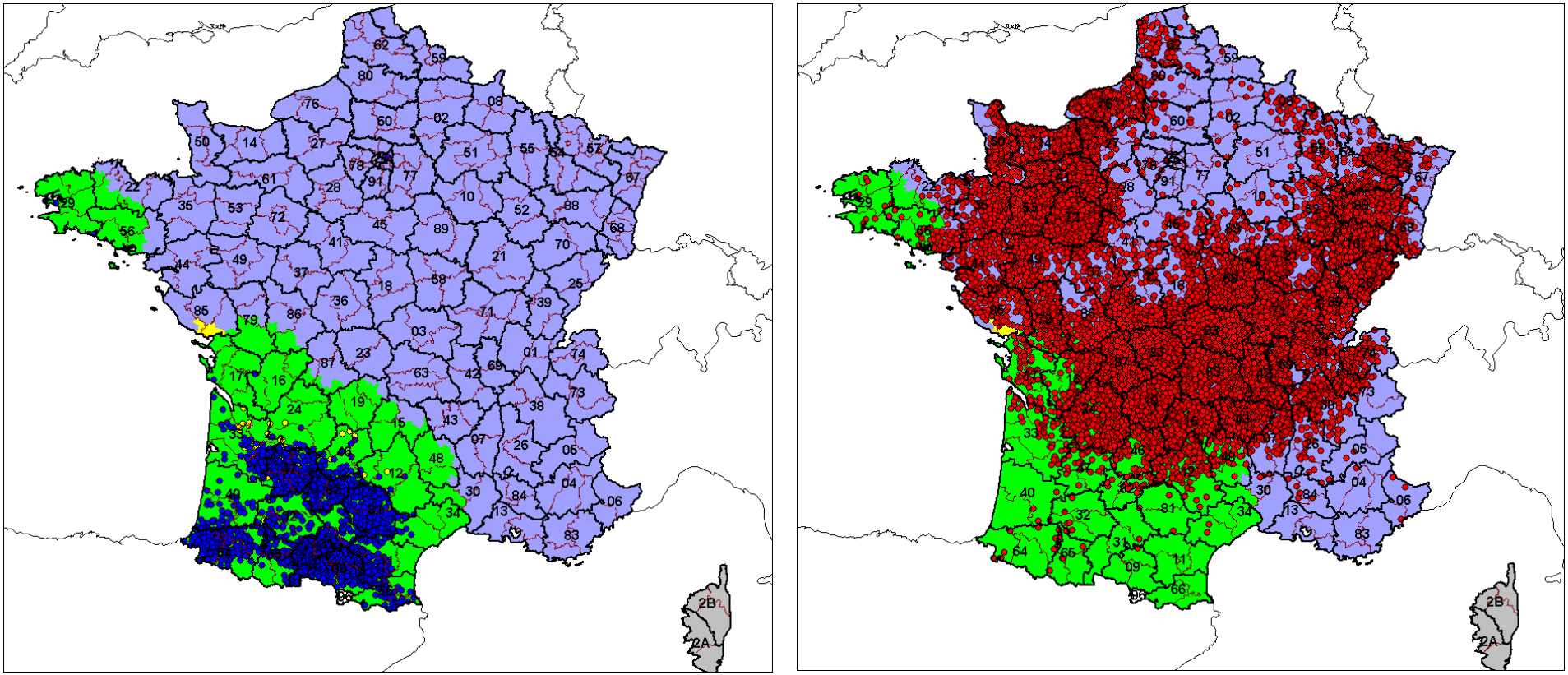

Figure 4. The distribution of new cases of BTV1 (blue dots), BTV8 (red dots) and mixed infections of BTV1 and BTV8 in the same animal (yellow dots) in the administrative districts of France in 2008. BTV 1 was confined to south-west France except for a small number of cases in Brittany.

Source: http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations/bt_23122008_fr.pdf

BTV serotype 8

Distribution

BTV8 first appeared in August 2006 in the Netherlands, Belgium, Germany and Luxembourg, and spread to northern France and Poland. Between July 2006 and December 2008, BTV8 accounted for about half the 244 000 cases of BTV in Europe due to serotypes 1 and 8 and infected over 60 000 holdings between 2006 and July 2009 (European Commission: Health & Consumer Protection Directorate-General 2011). Most BTV8 cases (70%) were reported in cattle. The spread of BTV8 decreased after 2008, with 350 cases reported in 2009 and 19 cases in 2010 (European Commission: Health & Consumer Protection Directorate-General 2011).

- In 2007, BTV8 reached the Czech Republic, Denmark, Switzerland and the United Kingdom.
- In 2008, BTV8 extended to the Mediterranean coast and southern France, southern Spain, Sweden, Hungary and Austria (OIE 2008a; OIE 2008b; OIE 2008d; OIE 2009e; Promed Mail 2008x).
- In early 2009, subclinical BTV8 was detected in cattle in Italy near the French border and in Sardinia, and in sheep in Greece on the island of Lesvos (OIE 2009a; OIE 2009c).
- In France and Spain, the distribution of BTV8 overlaps with BTV1 (Figures 3 and 4). In late 2008, BTV8 was detected in Andalusia in southern Spain (OIE 2008d). France reported more cases of BTV8 and BTV1 in 2008 than 2007, despite vaccination (Promed Mail 2008q).
- BTV8 was considered endemic in Belgium and the Netherlands in 2007 and in France, Germany and Switzerland in 2008 (OIE 2008e; Promed Mail 2008i).
- Sweden detected BTV8 in September 2008 in a limited number of holdings after surveillance testing of bulk milk (OIE 2009e; Promed Mail 2008k; Promed Mail 2008m; Promed Mail 2008q). Norway notified the OIE of BTV8 infection in cattle in February 2009 but spread was limited (OIE 2008e; OIE 2009d).

Origins

In the past, BTV8 has been recorded in Kenya, Nigeria, South Africa, South and Central America and India (Maan et al. 2008; Sreenivasulu et al. 1996).

Serological evidence of BTV8 infection in sentinel cattle in Bulgaria in 2006 raised concerns that BTV8 was associated with the use of modified live virus vaccine (MLV) against several serotypes during an outbreak of BTV9 over 1999 to 2000 (Maan et al. 2008). However, initial genetic analysis of viral ribonucleic acid (RNA) by Mertens *et al.* (2007b) showed conclusively that BTV8 in Europe was most closely related to a 1982 isolate of BTV8 from Nigeria. Complete genome analysis confirmed that the European strain of BTV8 was derived from ‘western’ topotypes of BTV present in sub-Saharan Africa and that it differed from the MLV strain (Maan et al. 2008; Nomikou et al. 2008).

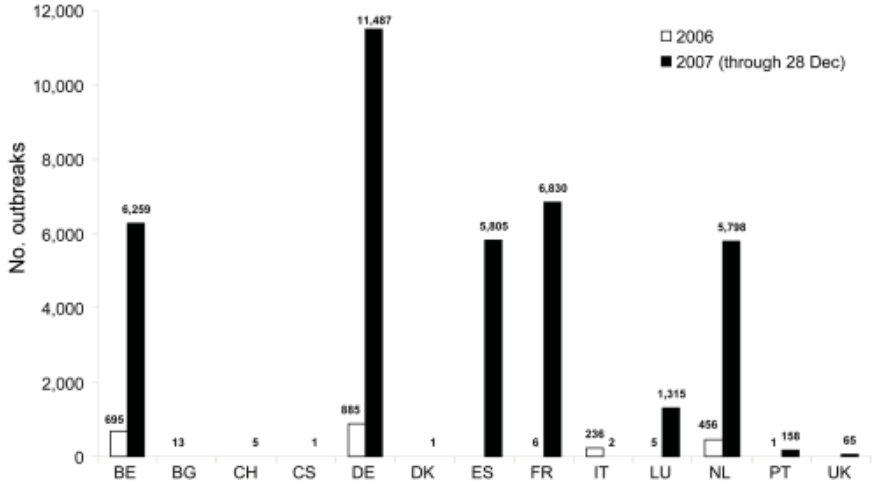

Figure 5. Number of bluetongue outbreaks in Europe in 2006 and 2007 (all serotypes). BE (Belgium); BG (Bulgaria); CH (Switzerland); CS (Czech Republic); DE (Germany); DK (Denmark); ES (Spain); FR (France); IT (Italy); LU (Luxembourg); NL (the Netherlands); PT (Portugal); UK (United Kingdom). Source: Saegerman (2008)

However, full genome sequencing also showed associations between BTV8 and South African vaccine strains of BTV serotypes 1, 2, 3 and 4 and isolates of BTV serotypes 2 and 4 from Corsica, which also originated in Africa (Maan et al. 2008). The strain of BTV8 in Europe also has features of a laboratory-adapted virus. BTV passaged repeatedly in mammalian cell lines may acquire undesirable characteristics, such as the ability to cross the placenta and appear in semen (Kirkland and Hawkes 2004). Reports that BTV8 is readily isolated in mammalian cells[9] also suggest laboratory adaptation. Other reports claim that the European strain of BTV8 required passage in insect cell culture before it could be isolated in mammalian cells and embryonated chicken eggs (Darpel et al. 2007; Maan et al. 2008). BTV field strains are generally more likely to grow and retain virulence in insect cell culture than in mammalian cell culture (Mertens et al. 2009b; Oura et al. 2009).

Previous work showed that biological characteristics of other BTV serotypes may alter after genetic reassortment between field isolates. For example, increased infectivity and length of viraemia in mammalian hosts were observed after reassortment of an Indonesian Java C strain with local isolates of BTV serotypes 20 and 21 in northern Australia (Melville et al. 2005c).

Epidemiological analysis of the outbreak of BTV8 in Europe resulted in nomination of 1 May 2006 as the date from which measures to manage the risk of BTV8 were justified (EFSA 2007a; Gerbier 2007). This date assumed a conservative incubation period of 100 days before the first possible date of infection (17 July 2006) plus one month's delay before bluetongue was diagnosed and the Netherlands notified the OIE (17 August 2006). There is no definitive explanation for the almost simultaneous appearance of BTV8 in Belgium, Germany and the Netherlands in 2006. This is despite investigation of several possible routes: wind events; transport and livestock

[9] P. Kirkland (*personal communication* 17 July 2008).

movement from eastern to western Europe; importation of cut flowers flown in daily from Africa and BTV8-infected vector larvae in horse manure at an international horse event (EFSA 2007c; Hendrickx et al. 2008; Mintiens et al. 2008b). An analysis of the first recorded outbreaks, stored milk samples, and surveys of farmers concluded that BTV8 was introduced into southern Belgium near a national park on the German border when vectors became active in the spring of 2006 (Saegerman et al. 2010).

Imported semen was used in some of the first 150 herds confirmed as bluetongue infected in Belgium. Introduction of BTV8 via germplasm was investigated by tracing registered imports of semen and embryos between 1 January and 18 August 2006 (Mintiens et al. 2008b). However, countries of origin were not infected with BTV8 at the time of semen collection (Germany, Italy, France, the Netherlands and the United Kingdom) or any period (United States and Canada). The study did not trace semen imported at an earlier date which was stored or semen transferred to the region from other areas. Other routes of BTV transmission (transplacental or oral) were not investigated.

The re-emergence and rapid spread of BTV8 in northern Europe after winter in 2007 were unexpected (Figure 5). Several factors are thought to affect the spread of BTV in Europe:

- Changes in distribution of BTV in Europe are attributed to climate driven factors, such as the extended distribution of vectors, increased vector capacity to transmit BTV and infection of novel vector species (Burgin et al. 2009; Carpenter et al. 2009; Gloster et al. 2007; Gloster et al. 2008; Mellor et al. 2008; Mellor and Wittmann 2002; Purse et al. 2008; Saegerman et al. 2008; Schwartz-Cornil et al. 2008).
- Non-climatic factors have also contributed to BTV spread, including the inherent susceptibility of vectors to remote strains of virus and overlap in distributions of vector species (Carpenter et al. 2009; Purse et al. 2008). Effects of human activities in providing micro-environments for vector survival and breeding, such as animal housing, feedlots and silage, are unknown.
- Long distance spread of vectors on the wind. The spread of BTV8 to the United Kingdom in September 2007 and to Sweden in August 2008 was most likely via infected *Culicoides* spp. borne on the wind from northern Europe (Agren et al. 2010; Burgin et al. 2009; Gloster et al. 2008; Hendrickx et al. 2008).

Mass livestock movement also introduced BTV8 into uninfected countries after virus first appeared in north-west Europe:

- Cattle imported from the Netherlands, France and Germany into Northern Ireland, England, Scotland, Italy, Spain, Portugal, the Czech Republic and the Slovak Republic tested positive for BTV8 with RT-PCR tests (Anonymous 2008; SCFCAH 2008f; SCFCAH 2008n; SCFCAH 2008o; SCFCAH 2008p; SCFCAH 2008q). Portugal reported that 15% of imported consignments contained at least one animal that was RT-PCR positive to BTV8 (SCFCAH 2008k). It is not known whether animals were also positive on virus isolation (and therefore infectious) but RT-PCR positive animals were usually destroyed to avoid local spread.
- Within herd spread was reported after Hungary's first case of BTV in September 2008 from imported PCR-positive cattle from France (Promed Mail 2008p; SCFCAH 2008d).
- In 2007, feedlots in northern Italy introduced BTV8 via imports of 57 500 cattle from infected zones in France. Post-movement testing from October 2007 to

January 2008 showed that 0.6% of imported cattle were seropositive, and of these, 80% were PCR positive. Surveillance between March and May 2008 showed limited seroconversion in the herds receiving imported animals but no spread to neighbouring herds (Cecchinato et al. 2008; Giovannini et al. 2008; SCFCAH 2008j; Tamba et al. 2008). Ongoing surveillance showed that between May and August 2008 BTV8 had not spread beyond five local herds in Verona province (European Commission 2008a), despite the presence of competent vector species for BTV8 in northern Italy in 2007 (Goffredo et al. 2008).

- In late 2008, surveillance detected BTV8 in Italy in clinically normal cattle, sheep and goats in Sardinia and cattle in Piemonte, near the French border (OIE 2009c; SCFCAH 2009d).

Disease in Europe

Clinical signs of infection with BTV8 in Europe in cattle include fever, conjunctivitis, oral mucosal congestion and ulceration, submandibular oedema and coronitis, with reduced conception rates and milk production in cows (Dal Pozzo et al. 2009b; Darpel et al. 2007; Elbers et al. 2008b; Santman-Berends et al. 2010a; Santman-Berends et al. 2011a). Transplacental infection with BTV8 in cattle may be associated with abortion and congenital hydranencephaly (Darpel et al. 2008; De Clercq et al. 2008b; De Clercq et al. 2008a; Desmecht et al. 2008; Méroc et al. 2009; Santman-Berends et al. 2010a; Vercauteren et al. 2008; Wouda et al. 2008). A small proportion of apparently healthy calves infected with BTV8 in late gestation have a transient corneal oedema (‘blue eye’), which is from deposition of antigen-antibody complexes after ingestion of colostrum (Holzhauer and Vos 2009; MacLachlan et al. 2009). Rarely, BTV8 causes oesophageal paresis and difficulty swallowing in cattle (Pardon et al. 2010).

Host species

Natural infection with BTV8 may cause clinical disease in cattle as well as sheep and goats (Darpel et al. 2007; Elbers et al. 2008b; Saegerman et al. 2008).

Clinical disease was also reported in alpaca, bison, mouflon, llama and yaks (Dercksen et al. 2007; EFSA 2008d; Elbers et al. 2008b; Elbers et al. 2008a; Henrich et al. 2007; Mauroy et al. 2008; Promed Mail 2006; SCFCAH 2007b; SCFCAH 2007a; SCFCAH 2008c; Thiry et al. 2006).

BTV8 can infect wild ruminants. Apparent seroprevalence of BTV in red deer in Belgium was zero in 2005 and increased to 0.9% in 2006 and 40.4% in 2007, without significant corresponding increases in mortality (Linden et al. 2008). Over 2000 to 2002, antibodies to BTV were not detected in any of 150 sera collected from red deer, roe deer and fallow deer submitted by hunters from six national parks in Germany (Frolich et al. 2006). However, in 2007 Germany reported BTV infection in mouflon, fallow deer, red deer and roe deer (SCFCAH 2007a; SCFCAH 2007b). BTV8 (and BTV1) RNA can be detected in the blood of asymptomatic red deer for up to 112 days after experimental infection (López-Olvera et al. 2010). Deaths of wild ruminants were reported from 59% of holdings in countries affected by BTV8 while no deaths were reported from countries affected by other serotypes (EFSA 2008d). In southern Spain there was no evidence of BTV8 infection in ibex that were seropositive for BTV serotypes 1 and 4 (Lorca-Oro et al. 2011).

Two captive Eurasian lynx (*Lynx lynx*) died from BTV8 after eating carcasses of ruminants sourced from BTV-infected areas in Belgium (Jauniaux et al. 2008).

Without further studies, the role of wildlife in the epidemiology and control of BTV8 is unknown (EFSA 2008d).

Prevalence

In Europe, many herds and flocks became infected with BTV8 but only a few animals showed clinical signs. However, up to half the clinically affected sheep died, while little mortality occurred in cattle.

Between herd prevalence: The few published cross sectional serological surveys suggest that a high proportion (up to 100%) of herds and flocks were infected with BTV8 after it had spread in a region (Durand et al. 2010; Elbers et al. 2007a; Méroc et al. 2008; van Schaik et al. 2008; Vandenbussche et al. 2008b).
Within herd prevalence: As for other serotypes of BTV, a high proportion of BTV8-infected animals occur in herds without clinical signs. In individual herds and flocks the number of infected animals ranged from 1 to 76% (Durand et al. 2010; Elbers et al. 2007a; Méroc et al. 2008; van Schaik et al. 2008; Williamson et al. 2008). In Belgium, a higher within herd prevalence of BTV8 in 2007 was associated with a lower morbidity than in 2006 due to protective immunity (Méroc et al. 2009).

Morbidity and mortality

In past outbreaks in the Caribbean, South-East Asia and Africa, infection with BTV8 caused either no clinical signs or very mild disease in cattle, with differences in morbidity observed between breeds of sheep. Similarly, previous studies with other serotypes of BTV showed that naïve livestock and introduced European breeds such as the Merino and poll Dorset were more likely to show clinical signs than indigenous breeds (Barnard et al. 1998; Barratt-Boyes and MacLachlan 1995; Elbers et al. 2008b; Gerdes 2004; MacLachlan et al. 2009; Mellor and Wittmann 2002; Mo et al. 1994; Parsonson 1993).

Table 4. Total number of cases of bluetongue in northern European countries in 2007.

Country	Number of cases
France	15 253
Germany	11 487
Belgium	6 661
The Netherlands	5 798
Luxembourg	1 315
Denmark	1
Switzerland	5
Czech Republic	1
United Kingdom	65
Total	**40 586**

Source: Schwartz-Cornil (2008).

The total number of cases of BTV8 in Belgium, the Netherlands, Germany and northern France increased from 2 300 in 2006 to 41 000 after the reappearance and rapid spread of virus in 2007 (Table 4) (Schwartz-Cornil et al. 2008). These figures differ from the number of cases for 2007 (101 400) recorded on the European

Commission surveillance and information system[10], which suggest a crude morbidity rate of less than 0.2%[11]. Bluetongue was more widespread in France in 2008 than in 2007, with 24 500 new outbreaks of BTV8 and 4 500 new outbreaks of BTV1 recorded in 2008 (SCFCAH 2008i).

Surveillance to demonstrate the absence of circulating BTV8 in 2009 and 2010 enabled Sweden and Austria to regain their BTV free status from the EC in December 2010 and March 2011 respectively (SCFCAH 2010e; SCFCAH 2011b; SCFCAH 2011c).

Published studies of morbidity and mortality rates for BTV8 in 2006-07 are shown in Table 5. Overall, the morbidity rate[12] in cattle from natural infections was low (2 to 3%) and mortality rates were less than 1%. Morbidity rates in sheep and goats were less than 8% but case fatality rates in these species (18 to 50%) were higher than those of cattle (0 to 18%).

Variation in severity of clinical signs makes detection of bluetongue by clinical signs alone unreliable. In the United Kingdom in 2007, single clinical cases were observed in 82% of cattle herds and 75% of sheep flocks with clinical bluetongue (Williamson et al. 2008). In 67% of seropositive herds in the Netherlands, only one or more cattle showed clinical signs of bluetongue (Elbers et al. 2008a). Clinical signs were observed more frequently in adult sheep and lactating dairy cattle than in lambs, beef cattle, calves and goats (Dercksen et al. 2007; Elbers et al. 2008c).

Most epidemiological data in Europe is presented as within herd morbidity or mortality, and small flock and herd sizes can cause highly skewed results. Half the flocks affected by BTV8 in France, Belgium and the Netherlands consisted of between one and ten animals (Elbers et al. 2007b; European Commission: Health & Consumer Protection Directorate-General 2011).

In the Netherlands BTV8 morbidity and mortality incidence in sheep flocks and cattle herds and infertility in cattle were greater in 2007 than 2006 (Elbers et al. 2009). Average case fatality rates for sheep decreased or increased in 2007 in different regions but halved in cattle over the same period (Figure 6) (Elbers et al. 2009; Szmaragd et al. 2007). In the month that herds detected BTV8 infection, mortality rates increased by 1.4 in cows and 1.3 and 1.2 in young cattle (3 days to 1 year old) and newborn calves respectively (Santman-Berends et al. 2011b).

Bluetongue was responsible for the death of 5 to 10% of the national sheep flock in Belgium (Saegerman et al. 2011). Analysis of rendering plant data from Belgium showed similar mortality rates in adult cattle in 2007 compared with previous years. However, the number of cattle and sheep foetuses and adult sheep carcasses submitted increased in 2007 (Méroc et al. 2009). In Germany, claims that the severity of clinical signs in cattle increased in 2007 were supported by an increase in case fatality rates in that year (Conraths et al. 2009; Promed Mail 2007b). In areas of France affected by BTV8 in 2007-08 cattle deaths were 26% higher than expected (Perrin et al. 2010).

[10] EU-BTNET (http://eubtnet.izs.it) was established in 2007 but was not immediately fully implemented by all Member States (Caporale, 2008).
[11] Calculated using the number of sheep and cattle in Germany, Belgium, the Netherlands and France in 2007 (51 million) (FAOSTAT, 2008).
[12] The morbidity rate is the proportion of animals at risk which show clinical signs.

Table 5. Published morbidity, mortality and case fatality rates for sheep and cattle from field and experimental infections of BTV8 in Europe in 2006-08.

Rate	Type of infection	Sheep (%)	Cattle (%)	Year	Country*	Source
Morbidity	Field	7.7	2.5	2006	NDR	Dercksen et al (2007), Elbers et al (2008c)
	Field	2.8 and 7.1	1.1 and 5.6	2006 and 2007	NDR	Elbers et al (2009)
	Field	1.7	1.0	2007	GDR	SCFCAH (2007b)
	Field	6.03 and 6.38	2.34 and 2.03	2006 and 2007	GDR	Conraths et al (2009)
	Field	-	1.8	2007	NDR	van Schaik et al (2008)
	Experimental	100	0	2007	UK	Darpel et al (2007)
	Experimental	66	-	2007	NDR	Backx et al (2007)
	Experimental	-	100	2008	FR	Dal Pozzo et al (2009b)
Mortality	Field	<3	0.2	2006 and 2007	BEL, FR, GDR, LU	Szmaragd et al (2007)
	Field	4.4	0.22	2006	NDR	Elbers et al (2008c)
	Field	1.1 and 0.8	0.08 and 0.2	2006 and 2007	NDR	Elbers et al (2009)
	Field	-	(3.2 times)	2007	NDR	van Schaik et al (2008)
	Field	2.26 and 2.65	0.25 and 0.27	2006 and 2007	GDR	Conraths et al (2009)
Case fatality	Field	32	20	2007	GDR	SCFCAH (2007b)
	Field	50**	0**	2006	NDR	Elbers et al (2008c)
	Field	51 and 22	11 and 5	2006 and 2007	NDR	Elbers et al (2009)
	Field	42	18	2007?	BEL	Vandenbussche et al (2008b)
	Field	18 and 28	9 and 5	2006 and 2007	BEL, FR, GDR, LU	Szmaragd et al (2007)
	Field	37.46 and 41.29	6.37 and 13.12	2006 and 2007	GDR	Conraths et al (2009)

*BEL (Belgium), FR (France), GDR (Germany), LU (Luxembourg), NDR (Netherlands), UK (United Kingdom). ** median values

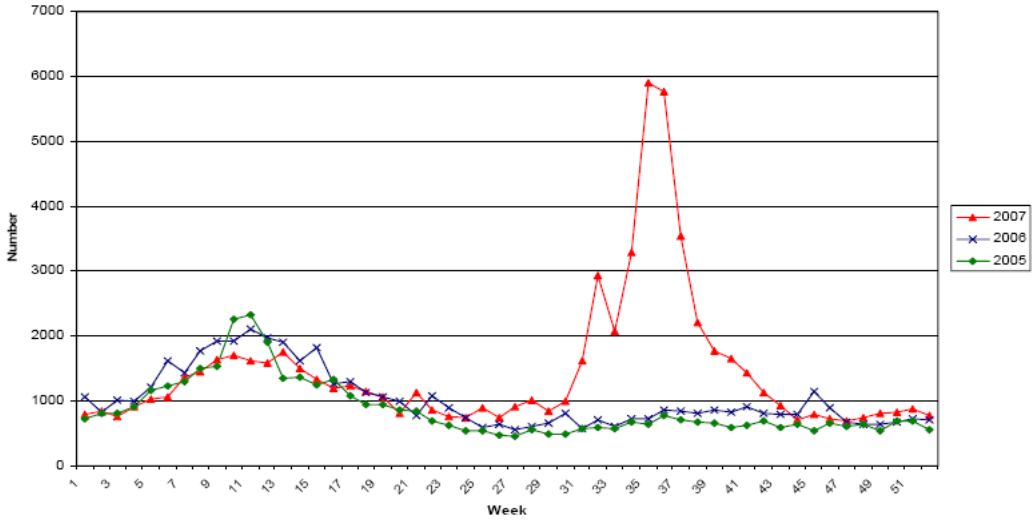

Figure 6. Weekly mortality of small ruminants in Belgium 2005–2007
Source: SCFCAH (2008c).

Experimental infection with BTV8

Experimental studies are limited (Table 5) but support field observations:

- Inoculation of BTV8 isolated from animals infected during the 2006 outbreak in the Netherlands caused severe clinical disease in two of four poll Dorset sheep (and moderate signs in the other two animals), while four Friesian calves were clinically unaffected despite high levels of viraemia detected by PCR testing (Darpel et al. 2007).
- A field strain of BTV8 caused clinical disease in two calves inoculated intravenously after limited passages of the virus in cell culture (Dal Pozzo et al. 2009b).
- Inoculation of BTV8 caused moderate clinical signs in two of three Texel sheep and mild signs in one of two Dutch dairy goats (Backx et al. 2007).

Experimental studies need to be conducted with field virus because culture of BTV in mammalian cells alters virulence (Oura et al. 2009).

Vectors

Persistence of bluetongue in a region requires the presence of active adult vectors throughout the year, naïve hosts or incursions of new serotypes. Vector species with different seasonal activity and habitats may increase the transmission period and geographic distribution of a particular BTV serotype. Rates of BTV replication within vectors depends on ambient temperature (Mellor et al. 2000; Veronesi et al. 2009). Below 15 °C, BTV replication in vectors and transmission stops, but resumes once temperatures exceed 20 °C for any period (Mellor et al. 2000). Transmission of BTV typically occurs at times favourable to adult vector activity, that is, in late summer and autumn.

Vector species

In general, BTV8 is transmitted by species of *Culicoides* vectors which differ from BTV vector species in southern Europe, where *Culicoides imicola* predominates. Transmission of BTV8 by *C. imicola* is yet to be verified (Balenghien et al. 2008).

- Principal vectors of BTV8 were initially thought to be *Culicoides* species of *obsoletus/scotius* and *pulicaris* complexes because BTV8 has expanded into areas where these are abundant (Calvete et al. 2008; Mehlhorn et al. 2007). *C. obsoletus* complex species are distributed widely in western Europe and the Mediterranean basin (Meiswinkel et al. 2008a) and experiments demonstrated replication of BTV8 in *C. scotius* (Carpenter et al. 2008a). *C. pulicaris* is also widely distributed and recorded as far north as Norway[13]. The importance of six species of the *C. pulicaris* complex in the transmission of BTV8 in northern Europe was subsequently questioned (Meiswinkel et al. 2008a) but a more recent study confirmed the presence of BTV8 in this species by RT-PCR in Belgium (Vanbinst et al. 2009).
- In 2006–2007 two new species in the *C. obsoletus/scotius* complex, were identified as *C. dewulfi* and *C. chiopterus* (Dijkstra et al. 2008; Meiswinkel et al. 2007). Both species feed on cattle and horses, and breed in their dung. BTV was detected by PCR in these species but laboratory and animal transmission studies are required to establish their competence as vectors. Their significance in disease transmission must be determined from vector species abundance, rates of BTV infection, feeding behaviour on ruminants and breeding sites. Obtaining this information is limited by difficulties in differentiating species within the *C. obsoletus/scotius* complex (Meiswinkel et al. 2008a). New quantitative PCR and other techniques may help identify vector species and determine their competence (Carpenter et al. 2008a; Veronesi et al. 2008).
- *C. nubeculosus* complex, which is present in northern Italy, has been shown to transmit BTV8 in the laboratory (Goffredo et al. 2008).

Vector surveillance

Recent reviews of BTV8 vectors recommended that countries conduct standardised, year-round entomological surveillance at permanent sampling sites, investigate the survival of adult *Culicoides* at low temperatures and link BTV surveillance and entomological data (Carpenter et al. 2008b; Carpenter et al. 2009; EFSA 2007b; EFSA 2007c; EFSA 2008b; Meiswinkel et al. 2008a). Limitations in current entomological surveillance for zoning and trade in Europe include:

- Insect samples collected by light traps, widely used across Europe, did not accurately reflect populations of *Culicoides* which are significant vectors of BTV, and underestimated the importance of new species like *C. chiopterus* (Carpenter et al. 2008c).
- Limited value of stabling. Although the peak biting activity of the *C. obsoletus* group was at dawn and dusk, vectors fed on ruminant hosts throughout the day and stabling was of little value in minimising vector attack (Carpenter et al. 2008c).
- Differences in the relative abundance indoors and outdoors (endophily/exophily) between *Culicoides* species. This behaviour varied at different times of year. For example, *C. obsoletus* group midges were more likely to enter housing in autumn

[13] K. Nomikou in oral presentation Bluetongue Symposium, Brescia, Italy, 7 June 2008

than spring (Baldet et al. 2008). Thus housing, if midge-proof, may only be useful in regions where an exophilic vector predominates.
- A shortage of entomological expertise in identification and surveillance of BTV vectors (Carpenter et al. 2009).

Overwintering

The first report that demonstrated that BTV8 had survived winter in Europe was in June 2007 from Westphalia, Germany (Hoffmann et al. 2008).

Widespread reappearance of BTV8 in 2007 and 2008 in mainland Europe is attributed principally to maintenance of virus in adult vectors that survived over winter, while the contribution of vertical transmission in cattle remains unclear (EFSA 2008b; Hoffmann et al. 2008; MacLachlan and Osburn 2008; Promed Mail 2008i). However, the following issues should be noted:

- Elsewhere in the world, vertical transmission is not required for survival between seasons of other strains of BTV (Promed Mail 2008e).
- Vector species vary in their tolerance of low temperatures and some can survive temperatures of −1.5 °C for 53 days (Nevill 1971). Small numbers of adult *C. obsoletus/scotius* remain active in animal housing during winter and may contribute to survival of BTV from one year to the next (Baldet et al. 2008; Hoffmann et al. 2008; Losson et al. 2007; Meiswinkel et al. 2008b).
- BTV transmission was documented in Germany during the winters of 2007 and 2008 (Hoffmann et al. 2008), but there was no evidence of BTV transmission over winter in 2007-08 in the United Kingdom (Holliman and Watkins 2008; Promed Mail 2008i; Veterinary Laboratories Agency 2008).
- Several factors operating concurrently may contribute to virus survival including: infected animals which are not detected by blood testing; venereal, transplacental and oral transmission in mammalian hosts; infection of wildlife (especially deer); transovarial transmission in vectors; survival of adult *Culicoides* in animal housing; novel vectors (e.g. ticks) and mechanical transmission by *Melophagus ovinus* (Bouwknegt et al. 2010; Meiswinkel et al. 2008b; Meiswinkel et al. 2008a; Napp et al. 2011b; Wilson et al. 2008).

Vector seasonal activity

Seasonal activity of BTV8 vectors is poorly understood because surveillance is at an early stage in northern Europe and uncoordinated across the EU (Meiswinkel et al. 2008a). Vector activity is monitored in several countries in an attempt to reduce the risk of exporting viraemic animals and the cost of livestock movement bans. The date of the start and end of the vector free period, as defined by EC 1266/2007, is published by EU Member States (SCFCAH 2009c). However, the effect of these dates on trade in livestock and genetic material is not clear.

Control

EU legislation for bluetongue zoning, livestock movement, vector control, surveillance, vaccination and germplasm production is described in the risk assessment and Appendices 1 and 2. Initial BTV control measures in the EU comprised movement restrictions on livestock and germplasm, and vaccination. These were based on Council Directive 2000/75/EC and Commission Decision

2005/393/EC. Bluetongue regulations were revised in October 2007 (EC 1266/2007) and updated several times since then.

The spread of BTV8 in Europe has occurred because of progressive infection of suitable vectors, movement of viraemic animals into areas with competent vectors and limitations to vector control.

Vector control

Measures such as housing livestock to protect them from vectors and control of vectors in the environment or on livestock are considered inefficient, impractical, uneconomic or unacceptable for cattle and sheep (Carpenter et al. 2008b; EFSA 2007b; EFSA 2008b; EFSA 2008d; Mellor and Wittmann 2002; Wilson and Mellor 2009). The failure of measures to protect animals from vector attack was acknowledged in EU legislation in December 2008 in amendments to EC 1266/2007 (EC 1304/2008).

Animal movement

BTV continued to spread in western and northern Europe despite restrictions on livestock movement (Wilson and Mellor 2009).

Italy's approach to BTV control, which combined livestock testing with surveillance data, was not implemented in northern Europe during the spread of BTV8. After the emergence of BTV8 in 2006, Belgium, the Netherlands and Germany initially banned the movement of ruminants, semen and embryos but later lifted these controls. With the increase in bluetongue notifications, there was a rapid expansion of 20 km restriction zones and animal movement was permitted between these zones (Appendix 2). In accordance with EC 1266/2007, free movement of susceptible livestock without pre-movement BTV testing was permitted between zones in which the same serotypes of BTV circulated. Despite this regulation, some countries, such as the United Kingdom, implemented post-movement testing on imported ruminants because of variation in BTV prevalence and inadequate knowledge of circulating serotypes in the country of origin. Movement of livestock between zones with different serotypes was prohibited unless animals were vaccinated against BTV or tested negative to BTV prior to movement. However, tests may not detect all infected animals (Appendix 6) and vaccination programs varied between European countries.

Investigations of movement controls for BTV in Europe found that they had limited effect on the spread of virus:

- There was no relationship between control of livestock movement and bluetongue incidence in an analysis of weekly data in the region where bluetongue was first reported in Belgium, Germany and the Netherlands (EFSA 2007c; Mintiens et al. 2008a). Local spread of BTV8 occurred at a rate of about 10 to 15 km per week (Gerbier et al. 2008), which was consistent with rates of local spread resulting from *Culicoides* dispersal in other bluetongue epidemics (Calistri et al. 2004; Mellor et al. 2000).
- Livestock movement did not play a significant role in the dispersal of BTV in the United Kingdom in 2007 (DEFRA 2008b). Tracing and surveillance[14] found no local spread of BTV after movement of PCR positive animals into BTV free areas.

[14] The UK does not have a national sentinel herd surveillance system and virological data is largely derived from pre-movement testing before animals move to bluetongue unrestricted zones (Simpson, 2009).

- A review of bluetongue zoning, surveillance and livestock movement in France found that once BTV8 had appeared, a single serological test for BTV, without a risk assessment based on serological and entomological surveillance data, probably contributed to the inadequate control of BTV8 and export of viraemic livestock (European Commission: Health & Consumer Protection Directorate-General 2007).

Bluetongue vaccination in Europe

Vaccines against BTV have been recently reviewed (Alpar et al. 2009). Bluetongue MLVs have been used in eastern Europe since 1999 and in the EU Member States of Italy, France, Spain and Portugal since 2002 (Appendix 1). MLVs against serotypes other than BTV8 are currently used in Italy, despite European Commission recommendations in 2005 to use only inactivated vaccines against BTV. European Commission regulations prohibit the use of MLVs against BTV8. Inactivated bluetongue vaccines have been used in the EU since 2005 (Savini et al. 2008) and completely replaced MLVs in Corsica (in 2005 to 2006), Spain and Portugal (in 2007) (Caporale 2008).

Vaccines must be assessed and approved for marketing by a national regulatory authority or the European Medicines Agency (EMEA). Initially, vaccination against BTV was only permitted in bluetongue protection zones against circulating virus of the same serotype (European Commission 2009b). After February 2009, vaccination against BTV in the absence of virus circulation was permitted in 'lower risk areas' within protection zones. Movement of vaccinated cattle and sheep from a bluetongue restricted zone is prohibited until 60 days after bluetongue vaccination (Appendix 2).

Vaccination in accordance with European Commission legislation limited production losses from BTV but did not prevent its spread, partly because of poor harmonisation of vaccination programs between EU Member States which have implemented different programs with a range of objectives, types of vaccines and different levels of vaccine coverage. For example, Italy used MLV against BTV1 in 2007, while Spain, Portugal and France used inactivated vaccines against ongoing outbreaks of this serotype (Caporale 2008; Rodriguez-Sanchez et al. 2008a; Savini et al. 2008; SCFCAH 2008i; SCFCAH 2008k). In France in 2008, BTV1 continued to spread, despite mass vaccination, across the south-west and to the north and east (Figure 4). As a result, the Netherlands considered vaccination against BTV1 to manage the risk of its introduction in imported livestock. However, at the time, this was not permitted under EU legislation in the absence of circulating BTV1. Subsequently, revisions which allowed pre-emptive vaccination in bluetongue restricted zones were proposed in late 2008 after two cattle infected with BTV1 were imported into the Netherlands from France (Promed Mail 2008x; SCFCAH 2008l). In 2009 Belgium did not vaccinate against BTV1 after modelling of vector dispersal by wind showed that vaccine coverage of 78% of cattle and 64% of sheep in France prevented the spread of this serotype within 350 km of the Belgian border (Ducheyne et al. 2011).

Development of coherent vaccination policy against BTV in the EU has been impeded by controversy surrounding the use of MLVs (Alpar et al. 2009) (Appendix 1), which include:

- teratogenicity and restrictions on their use in pregnant sheep
- decreased semen quality in rams
- decreased milk production

- poor attenuation and disease in vaccinated animals
- introduction of BTV topotypes from other regions
- viraemia from the vaccine virus and transmission by vectors
- genetic reassortment.

Vaccination against BTV8 in Europe

Problems experienced with MLVs against BTV in southern Europe precluded their use against BTV8 and only inactivated vaccines against BTV8 are currently used in Europe. However, inactivated vaccines against BTV8 were not developed and available until late April 2008. These vaccines were approved under emergency provisions in Directive 2001/82/EC which were invoked to enable vaccination of as many animals as possible before vectors became active in 2008 (European Medicines Agency 2008). As a result draft guidelines for emergency vaccine authorisation, released for consultation from 19 June to 30 September 2008, allowed incomplete efficacy testing and removed requirements for vaccine field trials and expert opinion.

Vaccination against BTV is only permitted under EU law within a bluetongue protection zone:

- As a result, areas that were not infected but required protection from vaccination were required to become protection zones. For this reason, the whole of England was declared a protection zone for BTV8 on 1 September 2008 and Scotland in November 2008.
- An undesirable consequence of designating an area a protection zone in order to vaccinate against BTV, was that trade in livestock between protected zones with the same serotype was then permitted across Europe. Trade with zones that had a much higher prevalence of BTV, or in which a new serotype was spreading, increased the risk of importing BTV.
- Changes to EU regulations in February 2009 permitted the use of inactivated vaccines in a bluetongue protection zone without circulating virus (European Commission 2009b; RSE 2008). This change was considered important for disease control. It also reduced the reliance of importers on BTV testing, which was unable to differentiate infected from vaccinated animals.

A range of BTV8 vaccines are available in different countries but there have been problems with vaccine supply. Vaccine manufactured by Merial in the United Kingdom was delayed by the escape of foot-and-mouth disease virus from Pirbright in late 2007. Spread of BTV8 into Scandinavia placed further demand on vaccines and, in September 2008, Denmark sought one million doses from Austria (SCFCAH 2008h).

BTV8 vaccination policies and their objectives vary widely between Member States:

- In countries where BTV8 is considered endemic, vaccination is intended to minimise losses from clinical disease, while in others, for example, the United Kingdom, the absence of circulating virus in 2008 presented an option to consider eradication (Oura 2008). This was supported by surveillance in 2009 which concluded that there was no circulating BTV within the United Kingdom at a prevalence of 2% (SCFCAH 2010a). The absence of clinical cases of BTV8 in 2008 and 2009 in the United Kingdom was attributed to successful vaccination (Burgin et al. 2009), although weather conditions for vectors may also have been unfavourable (Simpson 2009; Szmaragd et al. 2010).

- Elimination of BTV is thought to require vaccination for about three years, but funding these campaigns may be a problem (European Commission 2008b). Based on use of MLVs in Italy for other BTV serotypes, the EU recommended vaccine coverage of at least 80% of susceptible animals to prevent bluetongue spread, while lower rates reduce economic losses (EFSA 2007b; Patta et al. 2004).

In most countries, blanket vaccination of all domestic ruminants — cattle, sheep and goats — was undertaken. In countries with high rates of naturally-acquired immunity to BTV, vaccination of only non-immune livestock is not feasible.

- Vaccination against BTV8 is compulsory in most EU countries and Switzerland but voluntary in England and the Netherlands (Caporale 2008). Vaccination was initially voluntary in France but compulsory after November 2009 (Oura 2008; SCFCAH 2008i; SCFCAH 2009a).
- In France, Belgium and Germany, vaccination of sheep and cattle against bluetongue was undertaken by veterinarians but in the United Kingdom vaccine was administered by farmers under veterinary supervision (DEFRA 2008a; Promed Mail 2008h; Promed Mail 2008{; SCFCAH 2008b; SCFCAH 2008i). In Belgium, vaccination of deer and goats is voluntary. Emergency vaccination against BTV8 was required in the Czech Republic in 2008 (SCFCAH 2008e). In France, vaccination against BTV8 commenced in March 2008 (Promed Mail 2008b) and in Switzerland veterinarians started vaccination of all cattle, sheep and goats in June 2008 (Promed Mail 2008d). Scotland commenced compulsory vaccination against BTV8 of cattle and sheep during winter 2008–09 and voluntary vaccination of other susceptible species (SCFCAH 2008g) but planned to make vaccination voluntary in 2010 (Scottish Government 2009).
- When BTV vaccines were first used in the United Kingdom, the lack of compulsory bluetongue vaccination and the absence of a national surveillance program did not meet OIE recommendations for the use of vaccines in an emergency (Blayney 2008; Orpin 2008; Saegerman et al. 2007; Simpson 2009). The United Kingdom Department of Environment, Food and Rural Affairs (DEFRA) funded a research project to measure bluetongue vaccine coverage and its effect on disease occurrence (Wood et al. 2008). Vaccine sales data for 2008 suggested an overall coverage of 60%, with 80 to 90% of cattle and sheep vaccinated in regions of the United Kingdom affected by BTV in 2007, and 20 to 30% in northern England (Promed Mail 2008l). Vaccination in Germany since 20 May 2008 covered 70% of cattle (17 million doses) and 90% of sheep (3 million doses) and was considered the reason for reduced numbers of BTV cases in 2008 (Promed Mail 2008j).

In some European countries BTV8 vaccination programs were maintained in 2009-10 while surveillance demonstrated the absence of circulating virus or very low numbers of BT cases (SCFCAH 2011a). The role of BTV vaccines in Member States that lift BT restriction zones and regain ‘free status for BT disease’ from the EC is not clear (SCFCAH 2011b; SCFCAH 2011c).

Biological characteristics of the European strain of BTV8

This section describes the biological characteristics of BTV8 circulating in Europe which are used to evaluate potential contamination of ruminant semen and embryos in the risk assessment. The importance of venereal and congenital transmission and immunotolerance in the spread of BTV8 remain uncertain (EFSA 2008d). However, it is acknowledged that these features present challenges for international trade in ruminant germplasm (De Clercq et al. 2008a; MacLachlan and Osburn 2008; Promed Mail 2008b).

Prior to the emergence of BTV8 in Europe, earlier reviews of other serotypes of BTV rejected claims of immunotolerance and that natural infections of BTV caused clinical signs in cattle and crossed the placental barrier (Barratt-Boyes and MacLachlan 1995; Kirkland and Hawkes 2004; Parsonson 1993):

- Transplacental transmission and foetal abnormalities in cattle and sheep were only observed with strains of BTV which were propagated in embryonated eggs and/or cell culture. These 'laboratory-adapted' strains of BTV serotypes 10, 11, 13 and 17 were developed primarily for MLVs. In general, sporadic cases of BTV-induced hydranencephaly in cattle occurred only in countries in which MLVs against BTV were used (Barnard and Pienaar 1976; De Clercq et al. 2008a; MacLachlan et al. 2000; Roeder et al. 1991; Waldvogel et al. 1992).
- In sheep and goats, transplacental infection and abortion were reported after infection with other, unadapted BTV serotypes from the United States (MacLachlan et al. 2000) and Cyprus (Richardson et al. 1985). However transplacental infection was not conclusively demonstrated in other studies in Australia (Johnson et al. 1992) and the United States (Parsonson et al. 1994a). Overall, transplacental infection and abortion in sheep and goats following natural infection with 'wild strains' of BTV was considered very rare (Kirkland and Hawkes 2004).
- Experimental infection of sheep showed that laboratory-adapted strains of BTV crossed the placenta and caused lesions in the foetus, including hydranencephaly (Anderson and Jensen 1969).
- Vertical transmission of laboratory-adapted BTV continues to limit the use of MLVs in pregnant ewes (Dungu et al. 2004; EFSA 2007b; Scientific Committee on Animal Health and Animal Welfare (SCAHAW) 2000).
- There is only one report of transplacental transmission of BTV1 and this was in llamas in France in 2008 (Meyer et al. 2009).

Transplacental transmission

In contrast to other wild-type BTV serotypes, BTV8 can cause transplacental infection and immunotolerance in calves. Following the extensive spread of bluetongue in northern Europe, large numbers of pregnant cows were infected with BTV8 during the breeding season of 2007. Initial reports of abortion and hydranencephaly in cattle in late 2007 were followed by investigations which showed that 10 to 42% of healthy calves were infected with BTV8 before birth (Darpel et al. 2009; De Clercq et al. 2008b; De Clercq et al. 2008a; Desmecht et al. 2008; Promed Mail 2007a; Promed Mail 2008c; Santman-Berends et al. 2010a; Santman-Berends et al. 2010b; Vercauteren et al. 2008; Wouda et al. 2008).

- In Northern Ireland, pregnant heifers imported from the Netherlands gave birth to calves which were RT-PCR positive for BTV8 during the vector free season. One calf was viraemic (Menzies et al. 2008).
- In early 2008, there were unofficial reports from Germany of a 'massive increase' in abortions, stillborn or weak calves as well as fertility problems since autumn 2007 (Promed Mail 2008c). These were followed by a report of 18 cases of hydranencephaly between October and February 2008 in the Netherlands. These cases included calves up to 28 days old with congenital abnormalities consistent with BTV infection *in utero*. All calves born alive tested negative for BTV by PCR but six dams were seropositive and PCR negative for BTV. However, of the foetuses aborted at six to eight months gestation, six of 11 were positive for BTV by PCR (Wouda et al. 2008). Over the same period, increased rates of abortion, neonatal mortality and malformations and calves which were both BTV antigen and antibody positive were reported from Belgium (De Clercq et al. 2008b; De Clercq et al. 2008a; Desmecht et al. 2008; SCFCAH 2008c; Vercauteren et al. 2008).
- In April 2008, the Slovak Republic also reported BTV in calves born from pregnant heifers imported from the Netherlands. Two of 17 calves were born seronegative but RT-PCR positive for BTV8 from seropositive but RT-PCR negative dams (SCFCAH 2008f). One of the calves was also positive for BTV on virus isolation. The United Kingdom reported three calves born in Suffolk in October and December 2007 were PCR positive for BTV8. Infection of 33% of calves *in utero* was confirmed by positive PCR tests for BTV on the blood of 61 calves born during the season of low vector activity (Darpel et al. 2008; Darpel et al. 2009; European Commission: Health & Consumer Protection Directorate-General 2008).

These reports were followed by formal studies of transplacental transmission:

- A survey of 123 healthy calves born in Belgium between 15 December 2007 and 15 March 2008 and tested before ingesting colostrum, showed that the prevalence in newborn calves of transplacental transmission from natural infections of BTV8 was 9.8% (De Clercq et al. 2008a). Over the same period, these authors also found evidence of BTV infection in 11% of 109 calves that died at birth or showed clinical signs of BTV within a month of birth, compared with 2% of 733 calves one to four months old without suspicion of BTV.
- BTV RNA was present in 41% of 68 aborted foetuses from cows suspected of BTV infection during pregnancy compared with 19% of 232 aborted foetuses from cows without suspicion of BTV infection (De Clercq et al. 2008a).
- 37% of healthy calves were infected *in utero* with BTV8 on Belgian farms born between 22 November 2007 and 31 January 2008 (Desmecht et al. 2008), while 16% of healthy calves were infected with BTV8 *in utero* in the Netherlands in 2007 (Santman-Berends et al. 2010b; van Wuijckhuise et al. 2008) and 21% in 2008 (Santman-Berends et al. 2010a). In 2008 a French study of cows vaccinated against BTV8 and naturally exposed to the virus during pregnancy showed that 42% of calves born to unvaccinated cows were RT-PCR positive for BTV8 (Galleau et al. 2009).
- BTV8 was isolated from one calf which lived for two days, after experimental infection of seven cows in late gestation (Backx et al. 2009). The authors speculated on the role of concurrent infection with bovine viral diarrhoea virus to cause vascular damage and enable BTV to cross the placenta. However, in earlier

studies by Wouda *et al*. (2008) cases of *in utero* infection with BTV8 were negative for bovine viral diarrhoea virus.

- Initial investigation of 33 lambs found no evidence of transplacental infection with BTV8 in sheep (De Clercq et al. 2008a). However transplacental infection was later demonstrated experimentally and shown to occur in 69% of ewes inoculated with field strains of BTV8 in mid pregnancy (van der Sluijs et al. 2011; Worwa et al. 2009). A study of natural BTV8 infection in 300 ewes which were pregnant at different times of year showed that up to 25% of ewes aborted and flock fertility was reduced by 50% (Saegerman et al. 2011).

The spectrum of clinical signs and serological responses observed in newborn calves was used to estimate the stage of gestation at which BTV8 infection occurred. Earlier studies with other serotypes of BTV (laboratory-adapted strains) showed that calves with central nervous system lesions were infected in early gestation (70 to 130 days) when immunotolerance could develop in cattle (MacLachlan et al. 2000).

- Cases of hydranencephaly in calves were attributed to infection with BTV8 in early gestation (Vercauteren et al. 2008; Williamson et al. 2010), while healthy seropositive calves were probably infected in late gestation (De Clercq et al. 2008a).
- Seropositive and RT-PCR positive calves tested at birth before colostrum intake were thought to be infected with BTV8 between 90 to 150 days (Menzies et al. 2008) and 90 to170 days gestation (De Clercq et al. 2008b).

Responses to transplacental transmission by EU Member States followed:

- To manage the risk that importation of pregnant animals could introduce BTV8 to uninfected areas, Ireland prohibited the importation of female cattle over 12 months and sheep over six months from bluetongue affected regions on 22 February 2008. Regulations implemented from November 2007 had allowed trade in BTV seropositive but PCR negative animals (Republic of Ireland Department of Agriculture 2008).
- Soon afterwards, the United Kingdom announced research into transplacental infection by BTV8 (IAH 2008; Promed Mail 2008a).
- On 29 April 2008, the EU announced restrictions on the movement of pregnant animals unless they were immune to BTV prior to mating or artificial insemination (EC 384/2008).

Immunotolerance

Calves born with evidence of BTV infection (based on PCR or virus isolation) but without a serological response are considered immunotolerant. Earlier claims of immunotolerance in calves from BTV infection were largely discredited (Barratt-Boyes and MacLachlan 1995; Kirkland and Hawkes 2004; Parsonson 1993). In contrast, there is clear evidence of immunotolerance with BTV8 in about 2% of calves:

- Surveys of calves born in the vector free period in Belgium (between 15 December 2007 and 15 March 2008) showed that about 10% were infected *in utero* with BTV8 and a quarter of these (i.e. 2.4% of all newborn calves) were immunotolerant (De Clercq et al. 2008a). When the diagnostic sensitivity and specificity of the RT-PCR test was considered, the true prevalence of transplacental infection was estimated as 2.3% in 733 calves aged one to four months and born without clinical signs of bluetongue.

- A study of calves tested before ingestion of colostrum found that of 12 calves, three infected naturally with BTV8 *in utero* (25%), were 'apparently immunotolerant' (PCR positive for BTV). One of these, a 'dummy calf', was also positive on virus isolation for BTV8 (Desmecht et al. 2008).
- Rates of abortion and immunotolerance in cattle due to BTV8 appear similar to those for bovine viral diarrhoea virus. However, unlike bovine viral diarrhoea virus, BTV-infected immunotolerant calves probably have a minor role in the maintenance of BTV over winter. Earlier studies suggested that calves were either able to clear infection within one month or died (De Clercq et al. 2008a; Desmecht et al. 2008). Later work showed that although healthy calves born during the vector free period remained RT-PCR positive for up to five months, the presence of maternal antibodies from colostrum prevented virus isolation from these calves, making them unlikely to infect vectors (Dedet 2009; Promed Mail 2008f; Williamson et al. 2010).

Presence of BTV8 in semen

BTV8 was detected in the semen of bulls with a history of acute clinical BTV8 infection in an artificial breeding centre in Belgium in 2007 (Vanbinst et al. 2010). Although the prevalence of BTV8 infection of semen could not be estimated from this study, the authors noted that, in contrast with other BTV serotypes, virulent wild-type BTV8 was shed easily in semen from bulls of all ages and without contamination of samples with blood.

Unconfirmed observations in France suggested that BTV8 RNA was detected in the semen of about 10% of naturally-infected bulls.

Infection with BTV8 also caused a transient reduction of semen quality in rams and bulls (Kirschvink et al. 2009; Muller et al. 2010). Poor quality semen may be discarded for failing to meet quality standards.

It is not known if BTV8 in infected semen can cause infection of the foetus or viraemia in cows mated by natural service or artificial insemination.

The conclusions of earlier reviews about the low risk of BTV in semen may not be valid for the European strain of BTV8, which shares characteristics with laboratory-adapted strains of BTV (De Clercq et al. 2008a; MacLachlan and Osburn 2008; Promed Mail 2008b; Vanbinst et al. 2010).

BTV contamination of cattle embryos - collected *in vivo*

BTV is categorised by the IETS as a disease of cattle for which there is sufficient evidence that the risk of transmission through embryo transfer is negligible, provided that embryos were properly handled between collection and transfer (Category 1) (IETS 2010a; OIE 2010c).

- International standards for processing embryos were based on a review by the Research Subcommittee of the IETS Import/Export Committee in 1991 of published experiments which investigated the risk of transmission of infectious disease through embryo transfer.
- Safety of cattle embryo transfer procedures was upheld in subsequent reviews (Stringfellow and Givens 2000a; 2000b; Wrathall et al. 2006) and research updates by the IETS Health and Safety Advisory Committee (HASAC) in 2004 and 2007 (IETS 2004; 2007). However, all studies in these reviews were

published before 1995, prior to emergence of BTV8, and none of the BTV serotypes evaluated (10, 11, 13, 17 and 18) caused clinical signs or transplacental transmission in cattle.

- There are no reports of BTV transmission after commercial transfer of approximately 2.5 million cattle embryos between 1986 and 1997 or from earlier periods when washing procedures were less consistently implemented (Stringfellow and Givens 2000b).
- In January 2009, a review presented to the Research Subcommittee of HASAC at the IETS Conference concluded that there was negligible risk of BTV transmission when *in vivo* derived embryos were processed according to IETS guidelines (IETS 2009). Although the authors considered the recent evidence of transplacental transmission of BTV8, their conclusions were general and based on experiments undertaken with BTV before 1998. There were no references to published or unpublished experiments which investigated contamination of semen or embryos with BTV8. HASAC recommended that the IETS ask the European Commission to conduct research which would provide evidence that BTV8 should remain a Category 1 disease for cattle embryos (IETS 2009).
- Uncertainty about the effects of BTV8 on bovine embryos was acknowledged by the OIE General Session in May 2009. A provision in the Bluetongue Chapter to exempt products from BTV trade restrictions includes bovine embryos collected *in vivo* 'except for BTV8 (under study)' (Article 8.3.2) (OIE 2010a).
- Recent experiments with cattle embryos collected *in vitro* showed high rates of infection of hatched blastocysts exposed to BTV8 (Vandaele et al. 2011). Based on these results, the authors called for further research on the susceptibility to BTV8 of zona pellucida-intact *in vivo* derived embryos.

BTV contamination of sheep and goat embryos - collected *in vivo*

BTV in sheep is categorised by the IETS as a Category 2 disease, in that preliminary evidence indicates that the risk of transmission of BTV through embryo transfer is negligible but additional experimental data is required (IETS 2010a; OIE 2010c) (see page 57).

- BTV readily contaminated washed sheep embryos which were exposed to virus *in vitro* or collected from viraemic ewes (Singh et al. 1997; Terblanche and Gerdes 2005). Embryos which were inadequately washed after collection from viraemic ewes caused seroconversion of recipients (Gilbert et al. 1987). However, washing according to IETS protocols prevented seroconversion in recipients (Hare et al. 1988; Singh et al. 1997; Terblanche et al. 2006).
- The susceptibility of sheep embryos to BTV8 infection has not been reported.

Evidence to assess the risk of BTV transmission via embryo transfer in goats was considered preliminary (IETS Category 4) (IETS 2010a; OIE 2010c; Thibier and Guerin 2000) (see page 57).

- BTV8 was not removed by washing according to IETS procedures of zona pellucida intact goat embryos fertilized *in vivo* and exposed to BTV8 *in vitro* (Al Ahmad et al. 2011).

Transmission of BTV8 via embryo transfer in small ruminants has not been studied.

Oral transmission

Oral transmission in ruminants and wildlife appears to be limited but may contribute to circulation and transmission of BTV8 during periods of low vector activity. Previously, oral transmission of BTV was only hypothesised to occur in bluetongue-endemic countries when wild carnivores consumed BTV-infected carcasses (Alexander et al. 1994).

- Two captive Eurasian lynx showed clinical signs of bluetongue after being fed foetal and stillborn ruminants from farms in a region of Belgium with a high incidence of BTV8 infection (Jauniaux et al. 2008). No deaths in other animals, including ruminants, occurred at the zoo at the time.
- Transmission of BTV8 between penned parturient cattle in the absence of vectors in Northern Ireland is presumed to have occurred from ingestion of infected placenta (Menzies et al. 2008).
- Backx *et al*. (2008; 2009) demonstrated transmission of BTV8 to calves fed colostrum which was artificially spiked with BTV8. One of five calves became PCR positive to BTV8 at eight days post partum, seroconverted 25 days later and remained PCR positive for 70 days. It is not known if BTV8 is excreted in colostrum or milk, through viral contamination of blood cells (Backx et al. 2009; Saegerman et al. 2011).
- Transmission of BTV11 to calves that were fed BTV-contaminated colostrum was demonstrated recently in the United States (Mayo et al. 2010).

Potential for genetic reassortment

Reassortment of genome segments has contributed to the evolution of BTV (Carpi et al. 2010). New risks for ruminant semen and embryos may occur if novel strains of BTV8 emerge with altered biological characteristics (Dal Pozzo et al. 2009a). The use of MLVs against BTV has contributed to genetic reassortment of the virus (Batten et al. 2008b; Maan et al. 2010; MacLachlan and Osburn 2008), although this was only thought to occur in Europe between vaccine strains (Caporale 2008) (Appendix 1). However, recent analysis showed reassortment between field strains of BTV serotypes 6 and 9 from the eastern Mediterranean (Nomikou et al. 2009) and between the European field strain of BTV8 with a vaccine strain of BTV6 in the Netherlands (Maan et al. 2010). Other opportunities for reassortment of BTV8 in the field include:

- Overlaps in distributions of BTV8 and BTV1 in Spain and France (Figure 4). In October 2008, France reported concurrent infections of BTV1 and BTV8 in 40 animals (Bureau de Sante Animale 2008). Reassortment can also occur in the vector.
- Genetic instability of BTV. Genetic sequencing showed small numbers of nucleotide changes in strains of BTV8 as it has moved across the United Kingdom (Maan et al. 2008; Mertens et al. 2008).

New serotypes of BTV detected in Europe over 2008

New serotypes of BTV other than BTV8 emerged recently in northern Europe. These serotypes are of limited clinical significance and thought to be circulating at low levels. However, their detection and evidence of reassortment with BTV8 emphasises the dynamic bluetongue situation in Europe and the need for sensitive tests to determine the BTV status of animals (Batten et al. 2010; Hoffmann et al. 2009b) (Appendix 6).

BTV serotype 6

- On 17 October 2008, the Netherlands reported clinical signs of BTV in cattle in three herds in the same region. Two of these herds had been vaccinated against BTV8. An unidentified type of BTV was detected in a fourth herd by testing prior to movement (Promed Mail 2008s).
- On 25 October 2008, the BTV Community Central Reference Laboratory at Pirbright confirmed the presence of BTV6 (Promed Mail 2008u). Later sequencing of the entire genome of this isolate showed that it was derived from reassortment of two segments of the MLV strain of BTV6 with the MLV strain of BTV2 (both produced in South Africa) and segments of the field strain of BTV8 from Europe (Maan et al. 2010). Experimental inoculation of this isolate of BTV6 caused seroconversion without clinical signs (Dedet 2009; Promed Mail 2008v; Promed Mail 2008w; Promed Mail 2008y; SCFCAH 2009b).
- The Netherlands report to the OIE on 24 October 2008 nominated 10 September 2008 as the start of the BTV6 incursion (OIE 2008c). Four infected cattle were detected by PCR testing in Germany on 5 November 2008. Initial controls on livestock movement in the Netherlands and Germany affected three million cattle, 300 000 sheep and 27 000 goats (DEFRA 2008d; SCFCAH 2008a; SCFCAH 2008l; SCFCAH 2008m). Belgium reported two imported calves as positive to BTV6 by RT-PCR testing in late December 2008 but no evidence of local spread. After extensive surveillance, BTV6 was reported at very low prevalence from 21 farms in the Netherlands and 23 farms in Germany (Eschbaumer et al. 2010a; SCFCAH 2009b).
- The EU removed control measures for BTV6 (and BTV11) in March 2009 after assessing evidence of limited circulation, transmission studies and genome sequencing. The European Commission Standing Committee on the Food Chain and Animal Health (SCFCAH) concluded that BTV serotypes 6 and 11 were non-virulent vaccine strains which did not cause clinical signs but recommended increased surveillance over the next transmission season (SCFCAH 2009b).
- The route of introduction of this strain of BTV6 into northern Europe was not determined at the time of writing, and assumptions about illegal use of MLVs remain controversial (Eschbaumer et al. 2010a; Promed Mail 2008y; Promed Mail 2009b).

BTV serotype 11

- BTV11 was reported in one cow in Belgium on 5 February 2009 (Promed Mail 2009a). Tests were conducted on a sample submitted to the Bluetongue Reference Laboratory at Pirbright in November 2008 after tests at the Belgian National Reference Laboratory failed to identify the BTV serotype. The infected animal

showed no clinical signs and was detected by routine surveillance (De Clercq et al. 2009).

- Sampling of the affected herd in November 2008 and January 2009 found no evidence of spread and control measures were not implemented (Promed Mail 2009a). However, in February 2009, seven farms located in three provinces were found to be infected at very low prevalence (3.8% of Belgian dairy cattle herds and less than 0.2% of animals) (De Clercq et al. 2009; Promed Mail 2009c). Control measures were lifted in March 2009.
- As for BTV6, the source of the BTV11 infection was not identified but genetic analysis showed that the Belgian strain of BTV11 was closely related but not identical to the reference strain of BTV11 from South Africa, which is assumed to be very similar to the MLV strain produced there (Promed Mail 2009b).
- Previously, BTV11 has been found in the United States, the Caribbean (Dominican Republic) and Africa (Nigeria, South Africa and Zimbabwe), where it has been isolated from *C. imicola* (Mertens et al. 2009a).

Toggenburg Orbivirus

In October 2008, Toggenburg Orbivirus (TOV), an Orbivirus proposed to be a 25th serotype of BTV, was reported in goats from north-east Switzerland (Hofmann et al. 2008; Maan et al. 2010; Promed Mail 2008|). Later surveys showed that TOV was widespread in goats in Switzerland since 1998 and also present in Germany and northern Italy (Chaignat et al. 2010).

Routine surveillance for BTV in early 2008 identified goats that were clinically normal but seropositive to BTV-specific antibody cELISAs, and positive to a real time RT-PCR assay developed by Orru *et al*. (2006). Importantly, other RT-PCR assays used in Europe failed to confirm the presence of BTV RNA (Chaignat et al. 2009; Hofmann et al. 2008).

In different regions of Switzerland up to 60% of goat herds were seropositive to TOV. Although the prevalence of TOV in goats was high (50 to 100%), cattle and alpacas on the same farms were antibody negative for BTV. In sheep flocks that were in contact with infected goat herds, approximately 4% of sheep were seropositive for TOV. Studies confirmed that TOV produced no clinical signs in the Swiss breeds of goats tested but resulted in mild clinical signs in White Alpine sheep (Chaignat et al. 2009; Chaignat et al. 2010).

Initial evaluation of field infection in goats suggested transplacental transmission of TOV but this was not supported by experimental studies (Chaignat et al. 2009; Planzer et al. 2011).

Information about TOV is limited:

- Goats are a reservoir host for TOV and natural infection causes mild clinical signs in goats and low levels of virus in sheep (Hofmann et al. 2008).
- There was no evidence of infection in several hundred cattle tested in Switzerland (Chaignat et al. 2010). However, absence of TOV in this species may be a result of vector affinity, rather than resistance to infection (Promed Mail 2008|). Susceptibility of cattle to TOV may be clarified by experimental inoculation with virus.
- TOV has not been isolated in cell culture or embryonated chicken eggs (Chaignat et al. 2009; Hofmann et al. 2010; Planzer et al. 2011).

- Distribution of TOV in Europe and elsewhere in the world is to be clarified and the vector for TOV has not been identified.

The significance of TOV is yet to be determined:

- Transmission to sheep appears to be difficult. Experimental infection with TOV caused either low levels of infection or mild symptoms of bluetongue in sheep but no disease in goats. Cattle do not appear to be infected (Chaignat et al. 2009; Chaignat et al. 2010; Hofmann et al. 2008).
- Detection of TOV by cELISA for BTV antibody may prevent trade but RT-qPCR assays specific for TOV have been developed (Hofmann et al. 2010). Goats exported from Switzerland to Sweden were destroyed after notification of their infection with TOV (Chaignat et al. 2009).
- Potential for reassortment between TOV and other Orbiviruses is unknown (Chaignat et al. 2010).

5 Risk Assessment: evaluation of BTV8 contamination of semen and embryos

The previous section of this review established BTV8 as a new hazard for ruminant genetic material. This section evaluates available evidence to determine whether BTV8 is more likely to contaminate ruminant genetic material than other BTV serotypes. Each stage of ruminant semen and embryo production in Europe[15] is assessed, as well as potential consequences should BTV8 infect recipients and spread in Australia.

Semen

Release assessment

The following factors influence the likelihood of BTV8 entering via the importation of ruminant semen:

Country or zone of origin

The Member States of the EU, Switzerland and Norway are countries infected with BTV, as defined by the OIE Code.

The likelihood of infection of semen donors with BTV is influenced by EU bluetongue policies. Current EU control measures for ruminant germplasm are based on the designation of bluetongue restriction zones. Confidence in the status of these zones is affected by new incursions of BTV and changes to patterns of vector survival or competence through climatic effects, land use and animal management (such as housing). Since ruminant germplasm can be stored for long periods, the bluetongue status of the exporting country at the *time of collection* needs to be clear.

Large areas of the EU are now confluent BTV restriction zones with no requirement for pre-movement testing under EU legislation. Importation of BTV PCR positive animals into BTV free countries has been reported (Promed Mail 2008l). Donor animals for artificial breeding programs may be infected with BTV during transport to a semen collection centre.

The time of year that semen is collected may modify the risk of its contamination with BTV. A simulation study of an area of South America where BTV is endemic showed that the risk of transmitting BTV from *in vivo* derived, washed cattle embryos was reduced from one in 30 000 to one in a million when embryos were collected in the season of low vector activity rather than the vector season (Sutmoller and Wrathall 1997).

EU legislation requires data from three years of entomological surveillance to demonstrate seasonal periods of low vector activity. Limited data suggested that there was no risk of BTV transmission in the Netherlands between January and March 2007 (Meiswinkel et al. 2008a) and that a ‘vector free’ period in the United Kingdom was

[15] The risk of introducing BTV8 into northern Europe via infected germplasm was recognised as a possibility that would require an (IRA) by the EU (Mintiens et al. 2008a).

maintained from 20 December 2007 to 15 March 2008 (Promed Mail 2008i). The only country to declare a new case of BTV infection during the winter of 2007–8 was Germany. A single animal developed clinical signs of bluetongue and subsequently tested positive for virus during February 2008 (Promed Mail 2008i). Germany reported its first new case of BTV for 2008 on 21 July 2008 in an unvaccinated sheep (Promed Mail 2008h) and subsequently 1 678 new cases of BTV for the reporting period 1 May to 3 October 2008 (Promed Mail 2008q). In contrast, there was 'no evidence of BTV8 circulation in the United Kingdom' throughout the transmission season in 2008 (Promed Mail 2008r) and 2009 (Gibbens 2010). However, for the countries under consideration in this review, there is insufficient information at present to assess seasonally-free zones or countries, especially if livestock are housed over winter.

Norway

The status of Norway as a country which met OIE Code requirements for freedom from bluetongue changed over the course of this review. Norway lies at 57 to 72 °N, and is adjacent to Sweden and approximately 115 km from Denmark. Both of these EU Member States had bluetongue restriction zones.

- Prior to the detection of bluetongue in Sweden, a risk assessment for bluetongue disease completed in August 2008 by the Norwegian Scientific Committee for Food Safety concluded that the probability of BTV8 being introduced into Norway was 'small'. However, the report acknowledged that the risk of introducing BTV8 was uncertain and considered the need for vaccination should bluetongue spread from Sweden. It also recommended that animal owners and veterinarians maintain a high level of vigilance (Rimstad et al. 2008).
- BTV was first detected in Norway on 20 February 2009 through routine surveillance of milk samples (OIE 2009d). In two cattle herds, 17 animals were subsequently confirmed as infected by RT-PCR testing (Promed Mail 2009d). Surveillance detected a total of four herds which were deemed to have been infected with BTV8 in late 2008. No other outbreaks were detected by sampling of bulk milk, beef cattle and sheep in 2009 or 2010 (SCFCAH 2010b).
- Norway did not vaccinate against BTV8. 'Risk animals' (pregnant seropositive cows calving before 1 June 2009) were culled and imported livestock were tested for BTV (SCFCAH 2010b). Based on serological and entomological surveillance over three years, Norway proposed to lift their restricted zone and informed the EC of their intention to regain their free status for bluetongue in late 2010 (SCFCAH 2010e).
- BTV8 circulated in countries adjacent to Norway in 2008:

- Sweden first detected BTV8 in cattle through surveillance of bulk milk samples on 6 September 2008 and commenced a vaccination program. By 2 October 2008, 16 infected sheep and cattle holdings were identified but only one clinical case was reported (OIE 2009e; Promed Mail 2008q). Overall, BTV8 infection was confirmed in 30 cattle herds and three sheep flocks in a wide area of southern Sweden in 2008 and surveillance identified 79 infected herds in January 2009. Vaccination against BTV8 was undertaken in 2009 and 2010 (SCFCAH 2010d). Sampling of wild ruminants detected one moose seropositive for BTV (Sternberg Lewerin et al. 2010). Introduction of BTV8 into Sweden was considered to be via windborne infected *Culicoides* species from Denmark or Germany (Agren et al. 2010). Sweden was recognised by the EC as free of circulating BTV8 in December 2010 (SCFCAH 2010d; SCFCAH 2010e).

- Denmark had one case of BTV8 in October 2007 (a sheep on the south east island of Lolland) without further spread reported that year (OIE 2007). Cattle, sheep and goats in the protection zone in southern Denmark were vaccinated against BTV8 from July 2008. During late 2008, 13 outbreaks of BTV8 in Denmark were reported in cattle and two in sheep. BTV was detected through surveillance of bulk milk samples and observation of clinical signs by vaccination teams (Danish Veterinary and Food Administration 2009; Rasmussen et al. 2010).
- Information on surveillance for BTV vectors in Norway is limited. However, Nomikou[16] reported that *C. pulicaris* had been detected there. The potential spread of BTV8 northwards in Europe prompted Latvia, a country with similar latitude to southern Norway (57 °N), to initiate an entomological surveillance program for BTV vectors. In 2007, 99% of all midges trapped in Latvia were vector species known to transmit BTV8 (Gunita and Rodze 2008). Monitoring in Sweden detected 30 new *Culicoides* species and widespread distribution of *C. obsoletus* and *C. scotius*, and the presence of *C. pulicaris* (Nielsen et al. 2010).
- Norway has implemented EU legislation for BTV management in its legislation, including amendments to EC 1266/2007[17].

Herd of origin

Effectiveness of measures to control BTV8 is reflected in the prevalence of the virus in each country. Natural and acquired immunity to BTV8 in Europe limited its prevalence in infected countries after 2008. At the time of the epidemic, it was difficult to determine the proportion of herds and animals infected with BTV8 in different regions of Europe because of a lack of published data, variable spread of the virus and the range of vaccination programs. Presence of other BTV serotypes (1, 6 or 11) or TOV may also confound serosurveillance results for BTV8 (Appendix 6).

Between herd prevalence: During early stages of the BTV8 epidemic, between 10 and 100% of flocks and herds were infected with BTV8 (Durand et al. 2010; Elbers et al. 2007a; Méroc et al. 2008; SCFCAH 2007b; van Schaik et al. 2008; Vandenbussche et al. 2008b). Between herd prevalence varied between regions within a country and with animal species and breed, possibly due to the preference of vectors for cattle rather than sheep. In some countries, the number of new BTV8 cases decreased in 2008, probably due to immunity acquired from natural infection or vaccination, while in France more new cases were recorded in 2008 than in 2007 (Promed Mail 2008q; Promed Mail 2008r).

Within herd prevalence: After the first season of spread of BTV8, the proportion of seropositive sheep and cattle in BTV-infected flocks and herds ranged from 0.2% to 80%. In the Netherlands, a higher proportion of animals were infected with BTV in small and suckler cow herds than dairy herds. Clinical signs of bluetongue were a poor indicator of within herd prevalence because of low morbidity rates and high case fatality rates (Durand et al. 2010; Elbers et al. 2007a; 2008a; van Schaik et al. 2008;

[16] K. Nomikou, oral presentation Bluetongue Symposium, Brescia, Italy, 7 June 2008

[17] Royal Ministry of Trade and Industry, Oslo, Norway, Regulation of 9 July 2008 amending Regulation 30 April no 416 as regards the control, monitoring or bluetongue and restrictions on movements of certain animals of susceptible species in relation to bluetongue. http://www.eftasurv.int/fieldsofwork/fieldgoods/tbt/dtr/dtrlists/dtrlist2008/2008-9017-n/2008-9017-ndoc3.doc (Accessed 6 February 2009).

Williamson et al. 2008). Up to 40% of wild red deer in Belgium were seropositive to BTV (Linden et al. 2008).

Semen collection centre: There are no specific references to BTV in the conditions for approval of semen collection centres in the EU (Council Directives 88/407/EEC for cattle semen and 92/65/EEC for sheep, goat and deer semen) (See Appendix 2). However, animals showing clinical signs of disease may not enter a semen collection centre (88/407/EEC, Annex B.1.4) and sheep, goat and deer semen donors must be in good health on the day of collection. These requirements are of limited value for subclinical infections of BTV8, which are typical in cattle and common in other susceptible species.

At semen collection centres in bluetongue restricted zones, semen donors must be tested for BTV unless they are protected from vector attack (EC 1266/2007). The BTV serological tests used in the EU (usually cELISA) generally have a diagnostic sensitivity above 99% but this may be as low as 87.8% in practice during an epidemic (Appendix 6). Laboratory ring trials conducted in 2006 and 2007 showed that BTV8 was detected reliably by all types of cELISA tests against BTV antibody and all real time RT-PCR assays used by EU National Veterinary Laboratories. However, several cELISA and PCR assays are used and these need to be capable of detecting all serotypes of BTV, such as newly introduced serotypes (BTV6 and BTV11). A vaccine safety study showed that seroconversion in sheep after a single dose of inactivated vaccine against BTV8 may not be reliably detected by commercial cELISA tests (Oura et al. 2009).

Although BTV testing has been an option for ruminant semen donors under EU legislation for intra-Community trade since 23 May 2005 (2005/393/EC and EC 1266/2007), compliance is not known and there may be insufficient knowledge of the bluetongue status of the region in which a semen collection centre is located. In addition, donors may not have been tested because they were considered protected from vectors. There is considerable uncertainty about the effectiveness of protection from vector attack.

If serological testing for BTV is undertaken, EU legislation requires serological testing of donors every 60 days during collection and 21 to 60 days after semen collection. There is a small risk that a donor which is viraemic on the last day of semen collection may not have seroconverted by 21 days after infection (Appendix 6). The effectiveness of serological testing of donor bulls every 60 days during collection to control BTV transmission has been questioned (Napp et al. 2011a).

Presence of BTV8 in semen

Early studies which showed that BTV could contaminate cattle semen resulted in trade restrictions. However, further analysis indicated that the method of infection, strain of BTV and age of bulls affected the presence of virus in semen. Studies in Australia suggested that cattle semen was rarely contaminated with BTV after natural infection with wild strains of virus. In contrast, BTV was detected in semen after experimental inoculation with laboratory-adapted strains of virus or inoculation of mature bulls (5-15 years) with either ‘wild’ or laboratory-adapted strains (Kirkland et al. 2004; Melville et al. 1993).

Early experiments in North America showed that bulls infected with BTV shed virus in their semen during and shortly after the viraemic period (Breckon et al. 1980) and

that BTV-infected semen also caused viraemia and seroconversion in inseminated cows (Barratt-Boyes and MacLachlan 1995; Bowen and Howard 1984; Kahrs et al. 1980; Thomas et al. 1985). Further investigation showed that wild strains of BTV were not isolated from semen once viraemia ended and contamination with blood or inflammatory cells ceased. Nor did BTV persist in testicular tissue (Barratt-Boyes and MacLachlan 1995; Kirkland et al. 2004). In addition, young bulls (two to four years) with natural or experimental infections of BTV did not shed virus in their semen (Bowen et al. 1983c; Bowen et al. 1985; Breckon et al. 1980; Foster et al. 1980; Gard et al. 1989; Groocock et al. 1983; Kirkland et al. 2004; Melville et al. 1993; Melville and Gard 1992; Phillips et al. 1986). These conclusions were based on observations of natural infections with BTV serotypes 1, 3, 16, 20 and 21 and experimental infections with wild and laboratory-adapted BTV serotypes 1 and 23 from Australia and serotypes from the United States.

Limited evidence suggests that laboratory-adapted BTV may be found in the semen of mature rams during viraemia (Kirkland and Hawkes 2004).

Suggestions that BTV could also cause persistent infection of semen, transplacental infection in cows and immunotolerant calves (Luedke et al. 1977; Luedke et al. 1982; Luedke and Walton 1980) were later refuted (Bowen et al. 1983c; Gard et al. 1989; Kirkland et al. 2004; Parsonson 1993; Parsonson et al. 1994b; Phillips et al. 1986). Reviews upheld the consensus that contamination of semen with BTV was intermittent and only occurred with laboratory-adapted strains of virus in older bulls, possibly associated with inflammatory changes to the reproductive tract (Dejucq and Jegou 2001; Kirkland and Hawkes 2004; Roberts et al. 1992; Wentink et al. 2000).

In contrast to other BTV serotypes, virulent wild-type BTV8 was shed in semen from young as well as older bulls, and without contamination of samples with blood, in a study of bulls with a history of acute clinical BTV8 infection in an artificial breeding centre in Belgium in 2007 (Vanbinst et al. 2010). The 19 bulls ranged in age from 11 to 89 months, with a mean age of 42 months. Of the 89 batches of semen tested, 48 were positive using a new RT-qPCR assay for BTV RNA in semen and BTV8 virus was isolated from four of nine semen samples from three of the bulls.

Unconfirmed testing for BTV in France found that semen from seven of 50 clinically normal bulls with acceptable semen quality was positive for BTV8 with a PCR test.

The period over which the European strain of BTV8 is shed in semen has not been investigated and studies of the duration of viraemia from BTV8 are limited. An Italian isolate of BTV8 was detected by virus isolation in the blood of experimentally infected calves for 39 days (Di Gialleonardo et al. 2011). Although there is no evidence that the length of the viraemic period from BTV8 differs from that of other serotypes (EFSA 2007b; 2008b), it is acknowledged that BTV8 has different biological properties to field strains of other BTV serotypes (EFSA 2007d; 2008a; 2008c; 2008d). The presence of BTV8 in semen may be more likely because the European strain shares characteristics with laboratory-adapted strains.

Vaccination

Although only inactivated (killed) vaccines against BTV8 are currently available and authorised for use in restricted zones in the EU, MLV against several BTV serotypes are available and the vaccination status of individual semen donors may not be

known. Tests for BTV which distinguish vaccinated from infected animals are not available commercially.

Concerns with BTV8 vaccines include incomplete efficacy and safety testing in the field (Oura et al. 2009), and the effect of vaccination on semen. The only study of semen quality after BTV vaccination was conducted using MLV against BTV2 (Bréard et al. 2007). Although virus was not detected by RT-PCR in the semen of inoculated rams, semen quality was reduced temporarily. Decreased concentration and motility of spermatozoa and an increased proportion of abnormal and dead spermatozoa were observed for up to 69 days after the first vaccination.

Prevention of viraemia by inactivated vaccines

While inactivated vaccines do not result in a viraemia from vaccine virus, occasionally they do not prevent viraemia after viral challenge. Unless they completely prevent viraemia, inactivated vaccines may not prevent BTV contamination of semen. This particularly appears to be the case with BTV8.

- Prior to development of inactivated vaccines against BTV8, the European Food Safety Authority (EFSA) scientific panel on bluetongue vectors and vaccines concluded that inactivated vaccines against other serotypes 'were able to fully protect the animals from viraemia' (EFSA 2007b). However, the report acknowledged that relevant vaccine efficacy studies varied widely in their methodology.
- Information on prevention of viraemia by inactivated vaccines against serotypes other than BTV8 is limited:
  - Correctly administered inactivated monovalent and bivalent vaccines for sheep and cattle against serotypes 2 and 4 completely prevented viraemia (detected by virus isolation) and were used in Corsica, Italy, Spain and Portugal from 2005 (Savini et al. 2007; 2008; 2009).
  - Recently developed inactivated vaccines against BTV serotypes 1 and 4 prevented viraemia (detected by PCR) in sheep and cattle (Plana Duran et al. 2008; Puentes et al. 2008).
  - An inactivated vaccine against BTV16 gave complete protection against viraemia in sheep (Savini et al. 2007).
  - Prevention of viraemia was incomplete after inoculation of sheep with an inactivated vaccine against BTV18. However, the period of viraemia after experimental challenge was reduced from 12 to eight days (as detected by virus isolation) (Ramakrishnan et al. 2006).
- Field reports suggested that some animals vaccinated against BTV8 were RT-PCR positive (Promed Mail 2008q). However, there are few efficacy studies to demonstrate the ability of inactivated vaccines against BTV8 to prevent viraemia:
  - Zulvac 8® (Fort Dodge) prevented viraemia (detected by PCR) in 20 lambs and restricted (but did not prevent) viraemia and clinical signs in 12 calves (Paradell et al. 2008). Another study showed that viraemia was prevented in five sheep after administration of two doses of Zulvac® 8 Ovis (Eschbaumer et al. 2009).
  - BTVPUR AlSap8® (Merial) protected five vaccinated lambs and five cattle from viraemia (detected by RT-PCR) after challenge with BTV8 (Hamers et al. 2009). Similar results were obtained for five sheep after administration of a single dose of this vaccine in another trial (Eschbaumer et al. 2009).

- Product information accompanying Bovilis BTV8® (Intervet), which is the vaccine used in the United Kingdom vaccination program, claims to only reduce viraemia in sheep and cattle (Intervet 2009). A recent efficacy study showed that one of seven sheep failed to seroconvert after a single dose of this vaccine. After challenge with field virus, this animal was persistently positive on RT-PCR testing but did not develop clinical signs of bluetongue (Oura et al. 2009). The authors suggested that failure to detect seroconversion with cELISA tests after vaccination may explain reports of seronegative animals in two consignments of vaccinated sheep imported from the United Kingdom and France to Romania in September 2008 (Promed Mail 2008o). later studies confirmed that 'many' sheep in the United Kingdom did not seroconvert after a single dose of inactivated vaccine (Oura et al. 2009). Investigation of colostral immunity following vaccination of ewes during pregnancy showed that 23% of lambs challenged with BTV8 became viraemic (detected by RT-PCR and virus isolation) (Oura et al. 2010). None of these lambs showed clinical signs but the authors concluded that they may be able to transmit virus to vectors.
- In Germany, a pilot efficacy study of inactivated BTV8 vaccines showed that following a single dose of BLUEVAC® 8, one of six sheep failed to seroconvert and then developed viraemia after challenge (Eschbaumer et al. 2009). The authors attributed this to incomplete administration of the vaccine under field conditions.
- Few safety trial data for inactivated vaccines against BTV8 are publically available. The Scottish government announced its vaccination program against BTV8 in November 2008 and stated 'The Merial vaccine is safe with clinical trials, due to be published shortly, demonstrating that it does not adversely affect the animals inoculated.' (Scottish Government 2008). A recent safety study of three inactivated vaccines against BTV8 (BTVPUR® Alsap 8, Merial; Zulvac® 8 Ovis or Bovis, Fort Dodge; BLUEVAC® 8, CZ Veterinaria) in large numbers of sheep and cattle showed no adverse effects in pregnant cows but did not investigate challenge by field virus or effects on semen or embryos (Gethmann et al. 2009). A follow-up study showed that between 76 and 100% of the sheep and more than 90% of cattle in this study were seropositive to BTV one year after vaccination (Wackerlin et al. 2010). When small numbers of the animals with low serum antibody levels were challenged with virus, one sheep and one cow tested positive with RT-PCR for BTV8. Similarly, neutralising antibodies were detected in 66 to 97% of cattle in Italy after vaccination with Zulvac® 8 Bovis (Calistri et al. 2010). Viral challenge was not investigated.
- The possibility of interference of BTV RNA from inactivated vaccines with BTV tests was investigated. An Austrian experiment showed that small amounts of BTV RNA were detected in sheep for up to 61 days after administration of inactivated BTV8 vaccine (Steinrigl et al. 2010). However BTV RNA was not detected in vaccinated sheep in two other studies (Eschbaumer et al. 2010b; Oura et al. 2009).

Viraemia after vaccination with modified live vaccines

Attenuated bluetongue vaccine virus is laboratory-adapted, and therefore more likely to be present in the semen of bulls than field virus (Kirkland et al. 2004). Vaccine strains of BTV may contaminate ruminant germplasm during the viraemia which

follows vaccination with MLV or if vaccine virus is not adequately attenuated. A recent review by Alpar *et al*. (2009) documented titres after vaccination with MLV against BTV serotypes 2, 9 and 16 that were well above the threshold for transmission to vectors:

- After inoculation with MLV against BTV2 in Italy, viral titres of $10^{4.5}$–$10^{5.3}$ $TCID_{50}$/ml were recorded and virus was detected in vectors and blood between 45 and 133 days after the final dose (Ferrari et al. 2005).
- In a study of poll Dorset sheep inoculated with vaccine virus (BTV2 and 9), titres reached $10^{2.5}$–$10^{6.25}$ $TCID_{50}$/ml and viraemia lasted up to 19 days post-infection (as detected by virus isolation) (Veronesi et al. 2005). A later, related study reported a maximum viraemia in sheep of 27 days after vaccination against BTV16 (Alpar et al. 2009).
- Peak titres exceeding 10^5 $TCID_{50}$/ml occurred after inoculation of sheep with a South African MLV against BTV16 (Monaco et al. 2004). Unusually, viraemia was detected by RT-PCR for a longer period after vaccination (five to 26 days post-infection), than after inoculation with a field isolate of BTV16 (five to 14 days post-infection).

Knowledge of serological and antigenic responses in vaccinated animals is based on a limited number of bluetongue serotypes, and differences between serotypes in these responses occur (EFSA 2007b). For example, a study with laboratory-adapted strains of BTV showed differences in the maximum duration of viraemia in bulls inoculated with BTV1 (27 days) compared with BTV23 (38 days) (Kirkland et al. 2004). The opinion of EFSA on the maximum length of viraemia from MLVs against BTV was based on data from Italy for a bivalent MLV. This study suggested that 24 days (sheep) and 28 days (cattle) after vaccination, the probability of an animal being viraemic was less than 0.01%. However, this period was extended to 60 days to include one animal which remained viraemic for up to 78 days after vaccination with under-attenuated vaccine against BTV16 (EFSA 2007b; EFSA 2007d; Savini et al. 2008).

Based on this information EFSA concluded that:

- Viraemia from BTV lasted less than 35 days in both cattle and sheep (detected by virus isolation).
- Viral titres reached levels which were capable of infecting vectors for periods of two to four days.
- Serological responses after MLVs were protective from 28 days in sheep and 32 days in cattle. Inactivated vaccines, which may require two initial doses, were protective after a similar period (28 and 38 days after the completion of vaccination).

As a result, EFSA recommended that 60 days was required after BTV vaccination before movement of livestock to a BTV free zone (EFSA 2007b; EFSA 2007d; Savini et al. 2008).

Semen collection and processing

Natural infection with BTV8 in clinically normal bulls decreased sperm motility for nine months and increased the proportion of malformed sperm for three months (Muller et al. 2010). Natural infection with BTV8 in rams decreased semen concentration and motility and the percentage of living spermatozoa for up to 85 days after clinical disease (Kirschvink et al. 2009). The decrease in semen quality was

possibly more severe and of greater duration after natural infection than after vaccination with MLV against BTV2 (Bréard et al. 2007).

Poor quality semen is more likely to be discarded.

BTV is not susceptible to antibiotics included with processed semen.

The potential for BTV contamination of semen during further processing has not been investigated e.g. during sex-sorting of sperm. Chapter 4.6, Article 4.6.7 of the OIE Code (2010b) recommends that equipment used for sex-sorting sperm should be clean and disinfected between animals and 88/407/EEC requires semen to be processed and stored in approved centres without coming into contact with any other consignment of semen.

Tests for BTV on semen include virus isolation in embryonated chicken eggs or cell culture and PCR tests. Inoculation of semen into sheep or calves is highly sensitive but has been largely replaced by isolation in embryonated chicken eggs (Afshar 1994; Clavijo et al. 2000; Ianconescu and Kaufman 1992; Kirkland et al. 2004; Kirkland and Melville 2006; Melville et al. 1993; Sawyer et al. 1992; Schultz et al. 1982). Testing semen for BTV is hampered by intermittent shedding of the virus in semen around the viraemic period and undiluted spermatozoa and seminal fluid may be cytotoxic for cells in culture and contain PCR inhibitors (Breckon et al. 1980; Wilson 1999). However, recently developed RT-PCR tests on semen appear promising (Kirkland et al. 2010; Vanbinst et al. 2010).

Semen freezing, storage and transport

Orbiviruses may remain infectious for decades when kept chilled at less than 15 °C in samples of blood, serum or albumin and BTV in frozen semen remains infectious. Although the freezing process reduced virus infectivity by about 90%, infectivity remained stable when virus was held at -70 °C (Mertens et al. 2000). BTV titres of extended frozen semen did not change after thawing and correction for dilution (Bowen et al. 1983c).

Frozen-thawed extended semen stored for eight months after collection from a bull experimentally infected with BTV17 remained infectious, and four of nine inseminated heifers seroconverted (Bowen and Howard 1984). The viral titre of the thawed, extended semen was$10^{2.2}$ $CEIVLD_{50}$/0.5 mL[18] (i.e. per straw) and three of the heifers developed comparable titres in blood after intrauterine inoculation with BTV.

Release assessment - conclusion

Compared with other serotypes of BTV, it is considered that there is an increased likelihood of BTV8 entering Australia in cattle semen from the EU, Switzerland and Norway, and sheep, goat and deer semen from the EU. The likelihood of entry of BTV8 is estimated as ***very low***.

This conclusion takes into account the epidemiology of BTV8 in Europe, current control measures for BTV in semen collection centres and more general measures, such as vaccination and restrictions on animal movement. These controls have not prevented further spread of BTV8 but have reduced its prevalence in some areas.

[18] $CEIVLD_{50}$ (median chicken embryo infective viral lethal dose)

Exposure assessment

The following factors were taken into account in assessing the likelihood of exposure:

Semen thawing

Differences in viability between BTV8 and other serotypes after semen thawing have not been investigated. BTV infectivity was retained in frozen-thawed extended semen (Bowen et al. 1985; Bowen and Howard 1984). Although BTV infectivity remained stable when held at -70 °C (Mertens et al. 2000), repeated freezing and thawing inactivated African horse sickness virus, a closely related Orbivirus (Musser and Burham 2006).

Transmission of BTV to semen recipients

There is no published research on the transmission of BTV8 by artificial insemination using frozen-thawed semen. As discussed earlier, characteristics which BTV8 shares with laboratory-adapted virus make it more likely to be transmitted venereally than other strains or serotypes of BTV.

A limited number of studies with other serotypes of BTV showed that insemination with BTV-infected semen caused viraemia and seroconversion in recipient cows. Artificial insemination with frozen-thawed semen collected from a bull infected with BTV17 resulted in three of nine heifers becoming viraemic, as detected by virus isolation, while four of nine seroconverted (Bowen and Howard 1984). Experimental inoculation of semen with BTV17 resulted in two of four inseminated heifers becoming viraemic (Thomas et al. 1985). Two superovulated cows inseminated with semen artificially infected with BTV became infected (Schlafer et al. 1990). All cows became viraemic and seroconverted after direct inoculation into the uterus of semen infected with BTV20 (Parsonson et al. 1987).

Multiple direct exposure of recipients to BTV could occur at several locations because of the wide distribution of imported semen in Australia.

Exposure assessment -conclusion

There is a **high** likelihood that recipients exposed to imported semen contaminated with BTV8 will become infected due to the direct route of exposure.

Consequence assessment

Likelihood of an outbreak, establishment or spread

Despite very high rates of infection and seroconversion to BTV8 in cattle in Europe in 2007 and 2008, clinical signs were less obvious in cattle than in sheep (Elbers et al. 2007a; Elbers et al. 2008a; van Schaik et al. 2008; Williamson et al. 2008). As a result, BTV8 may escape early detection in Australia in areas where cattle predominate and are extensively managed. An outbreak of BTV8 and its subsequent spread in Australia would depend on the distribution of competent vectors and vector

activity around the time of infection via insemination or birth of viraemic offspring (if transplacental transmission occurred). The possibility of multiple initial exposures in multiple locations at different times as the semen was used would increase the likelihood of establishment or spread of the virus.

- Over the past 20 years some of the exotic serotypes and strains of BTV detected in northern Australia have been spread by local vectors (St George et al. 2001). BTV8 was able to infect new vector species in Europe but it is not known if Australian *Culicoides* species would be competent vectors for this strain and serotype of virus. If existing Australian bluetongue vectors were infected, the distribution of BTV8 would probably be in the area defined by Australia's National Arbovirus Monitoring Program (NAMP). However, if other Australian vector species transmitted an exotic serotype of BTV, the distribution of these species would need to be determined (as occurred with BTV8 in northern Europe). Introductions of new vector species to Australia are also possible. For example, *C. wadai* was probably blown to northern Australia from Indonesia and within a decade had spread over 2 000 km across northern and eastern Australia and into NSW (Doyle 1992).
- Most BTV transmission occurs in Australia in late summer and autumn. Transmission typically occurs in northern Australia during and after the wet season and on the NSW coast in summer and autumn (St George et al. 2001). In northern Australia, most cattle are mated after the wet season, between March and May, while in southern areas beef cattle are inseminated during late winter to early summer, when vector activity is likely to be low. However, dairy cattle which occur in reasonable numbers in the zone of possible BTV transmission in NSW, may be inseminated all year round. Vector activity and BTV transmission has occurred during mating periods that were unseasonally wet and in irrigation districts. Deer mate in autumn when vectors are likely to be active (Invasive Animals CRC 2009).
- Sheep in spring-lambing flocks are more likely than cattle to be mated during the period of vector transmission (late summer and autumn). However, in Australia at least, cattle are the preferred host for *Culicoides* vectors. The feeding behaviour and host preferences of BTV8 vectors in northern Europe are not known (Carpenter et al. 2008b).

Infectivity of BTV8 for non-ruminant species in Australia is unknown. Antibodies to other serotypes of BTV in Australia have not been found in horses, donkeys, pigs, dogs, marsupials or humans (St George et al. 2001).

- The prevalence of BTV in Australian populations of feral fallow, red, sambar, chital, rusa and hog deer has not been investigated. Over the last two decades, the number of feral deer in Australia increased to an estimated 200 000 animals (Moriarty 2004). Wild deer are mostly located in coastal and tableland regions of south-east Australia (DECC 2008; Moriarty 2004), but herds are also established in the range of known BTV vectors in coastal NSW and southern and south-east Queensland, the Torres Strait islands and the Charters Towers area of Queensland (Harrison and Congdon 2002; Queensland Primary Industries and Fisheries 2009; Wet Tropics Management Authority 2008).

Impacts of an outbreak, establishment or spread

In general, an outbreak of exotic BTV8 in Australia is likely to have a greater impact than other exotic serotypes of BTV. Animal life or health at the regional level would be affected and the effect on international trade at the national level is likely to be significant, given the sensitivity of Australia's export markets to serotypes of bluetongue already in Australia. It is also likely to have an impact on control and surveillance programs, domestic trade and industries, and a minor effect on the environment (due to disposal of infected animals and excessive use of macrolides) and rural communities.

Expected consequences of an outbreak of clinical bluetongue in Australia, especially in sheep or goats, are described in AUSVETPLAN (Animal Health Australia 2008). Bluetongue is a Category 3 disease (funded 50% by government and 50% by industry) under the Emergency Animal Disease Response Agreement. The AUSVETPLAN strategy for bluetongue is to minimise the economic impact and eliminate the disease if circumstances permit, rather than pursue a 'stamping out' policy. Measures include quarantine and movement controls to prevent further spread, zoning, surveillance (using existing NAMP surveillance) and possibly vaccination. Control may include widespread use of macrocyclic insecticides for control of vectors on livestock and in dung.

If BTV8 spread and became established in Australia, rates of infection, disease and mortality in European breeds of cattle and sheep are likely to be similar to those experienced in Europe, with up to 3% of cattle showing clinical signs and reduced productivity, and an increase in herd mortality of about 1%. In northern Australia, a high proportion of cattle herds are likely to be infected if *Bos indicus* are as susceptible to BTV8 as European breeds. The number of cattle affected may be greater if BTV transmission in tropical regions of northern Australia is more efficient and occurs for a longer period than in temperate regions of Europe. If vectors were located further south, BTV8 could be expected to infect cattle, sheep, goats, deer and other susceptible ruminants, such as camelids.

- European data discussed earlier suggest that BTV8 is likely to cause severe disease and loss of productivity in up to 5 to 10% of sheep (roughly double the number of cattle). About 20 to 50% of sheep that show clinical signs would die. On average, BTV8 decreased the net return for milk production by 2% in bluetongue infected dairy herds in the Netherlands (van Schaik et al. 2008) and reduced gross margins in France for dairy herds by 1 to 8%, beef herds by 6 to 18% and sheep flocks by 4 to 106% (Dedet 2009; Promed Mail 2008z). By the end of 2007, the direct cost of BTV in northern Europe was estimated to be Euros 150 million, with losses several times higher from restrictions on trade (Mellor et al. 2009). In a recent study, the net control costs and production losses to the farm sector due to BTV8 in the Netherlands from 2006 until July 2008 were estimated to be Euros 163-175 million (Velthuis et al. 2010).
- BTV8 would also infect feral and exotic ruminants. Existing serotypes of BTV in Australia are not thought to be spread by native wildlife (St George et al. 2001), but rates of infection, morbidity and mortality from BTV8 in Australian wildlife are unknown.
- Australian breeds of sheep and cattle are likely to be highly susceptible to BTV8. Morbidity rates in sheep could be much higher than those suggested above and approach 30% with mortality rates up to 15%, similar to those of BTV serotypes in southern Europe with severe clinical signs. Case fatality rates in Australian

Merino sheep from BTV8 are likely to be similar to those of the more pathogenic serotypes of BTV in Australia, such as BTV23. Mortality rates in adult Merino sheep experimentally infected with Australian strains of BTV ranged from 0 to 32%, depending on serotype: 0% (BTV9); 8% (BTV serotypes 3 and 15); 12.5% (BTV16) to 32% (BTV23) (Johnson et al. 1992). In six British breeds of sheep experimentally infected with a South African strain of BTV3, mortality rates varied from 0 to 83% in different breeds (Jeggo et al. 1986). BTV8 caused moderate to severe clinical signs in all experimentally infected poll Dorset sheep in two studies (Darpel et al. 2007; Worwa et al. 2010).

Consequences of BTV8

The likelihood of spread of BTV8 in cattle, sheep, goats and deer is likely to be similar to other BTV serotypes. However, BTV8 may cause clinical signs in cattle which may enhance detection and if new vectors were involved its spread could be greater.

The impact on trade and animal health (in cattle) would be greater for BTV8 than other serotypes.

Based on the considerations above, the impact of the establishment or spread of BTV8 in Australia after introduction by contaminated semen is assessed as **high**.

Overall risk for semen

Overall, it is concluded that the risk of introduction and establishment or spread of BTV8 via the importation of semen from the EU, Switzerland and Norway is **low** and exceeds Australia's ALOP (very low). As a result, risk management measures for cattle, sheep, goat and deer semen are required.

Embryos

Release assessment

Factors taken into account in assessing the likelihood of ruminant embryos contaminated with BTV8 include the following:

Country of origin

The between herd prevalence of BTV8 infection in the EU, Switzerland and Norway is similar for embryo and semen donors (above).

Herd of origin

The within herd prevalence of BTV8 infection in the EU, Switzerland and Norway is similar for embryo and semen donors (above).

Cattle embryos
EU legislation on bluetongue (EC 1266/2007 Annex III.C.1) requires health certificates set down in 89/566/EEC, 95/388/EC or 93/444/EE for both cattle and non-cattle embryos which are intended for intra-Community trade or export to a third country to identify the BTV management measures applied (EC 1266/2007 Annex III.C). For cattle embryos collected *in vivo*, the only requirement is that donors not show clinical signs of bluetongue on the day of collection. BTV testing is not required. However, only a small proportion of cattle infected with BTV8 show clinical signs.

Sheep, goat and deer embryos
In bluetongue restricted zones in the EU, BTV testing of non-cattle embryo donors is required unless animals are protected from vectors. Specific risk management measures for BTV must be recorded on EU health certificates for intra-Community trade or export to a third country set out in 95/388/EC, 93/444/EEC or 92/65/EEC, according to amendments in EC 1266/2007.

Vaccination
The effect of vaccination against BTV8 on the risk of viraemia in embryo donors is similar to that for semen donors.

Contamination of embryos with BTV

BTV can contaminate ruminant embryos with an intact zona pellucida which are collected *in vivo* from viraemic donors. It has previously been considered that this risk is managed by embryo washing protocols developed by the IETS and incorporated into EU legislation and OIE Code recommendations for embryo transfer of all ruminant species (OIE 2010c; Stringfellow 2010).

- The use of BTV-infected semen in embryo transfer programs was thought to contribute ‘negligible’ risk of infecting cattle embryos collected *in vivo* or their recipients, as long as semen collection and embryo processing protocols were observed (IETS 2005; Wrathall et al. 2006). However, a review of studies conducted up until 1991 found that few experiments were of sufficient scale to draw legitimate conclusions (Roberts et al. 1992). In support of negligible risk,

there are no reports of BTV transmission from thousands of commercial embryo transfers in cattle undertaken worldwide (Stringfellow and Givens 2000b; Wrathall et al. 2006).

- Testing of cattle embryo donors for BTV is not required by EU legislation, the OIE Code or IETS protocols. The IETS classify BTV as a Category 1 disease which means that there is sufficient evidence that the risk of transmission of BTV is negligible, provided that embryos are handled according to IETS recommendations between collection and transfer. To date, the IETS has not included in its disease categorisation the possible contamination of embryos with BTV8 or MLV against BTV, and it is not known whether research programs are being conducted to investigate these issues. From 2009, the OIE has recommended that exemptions from BTV requirements for *in vivo* derived bovine embryos should not include BTV8, which is 'under study' (Article 8.3.2.) (OIE 2010a).

Cattle embryos

The risk of contamination of ruminant embryos with BTV8 may be greater than that of other serotypes because it possesses features of laboratory-adapted virus. These characteristics, such as clinical disease in cattle, presence in semen and ability to cross the placenta may increase the likelihood of BTV contamination of cattle embryos in general (Kirkland and Hawkes 2004). It is not known whether IETS washing protocols remove BTV8 from cattle embryos collected from viraemic donors.

Earlier studies in cattle showed that despite strong adherence of BTV to bovine embryos compared with other viruses (Gillespie et al. 1990), IETS washing and handling procedures appeared to consistently remove the risk of infecting recipients with both laboratory-adapted and field strains of virus (Acree et al. 1991; Bowen et al. 1983a; 1983b; Singh et al. 1982; Thomas et al. 1983; 1985):

- In a small study, transfer of cattle embryos which were washed three times after *in vitro* exposure to BTV11 caused seroconversion of three recipient heifers (Schlafer et al. 1990).
- Washing ten times without trypsin treatment prevented infection in recipients of 28 cattle embryos collected from viraemic heifers that were inoculated with laboratory adapted BTV18 or a field isolate of BTV17 passaged through cattle (Thomas et al. 1983).
- None of nine embryos collected from two viraemic heifers and washed according to IETS protocols but without trypsin treatment caused BTV17 infection in recipients or calves (Thomas et al. 1985).
- A large study of 169 embryos collected from 59 viraemic heifers infected with a field isolate of BTV11 showed that washing according to IETS protocols but without trypsin treatment prevented BTV infection of embryos, recipients of 110 embryos and 36 resulting calves (Acree et al. 1991).

Sheep and goat embryos

The risk of BTV contamination of sheep embryos appears to be greater than that for cattle embryos. As discussed earlier, the IETS designates BTV as a Category 2 disease in sheep and as a Category 4 disease in goats.

- BTV10 was transmitted to susceptible sheep by transfer of inadequately washed embryos infected *in vitro* or *in vivo* with virus (Gilbert et al. 1987).
- All 22 sheep embryos infected with BTV11 *in vitro* remained infective after washing according to IETS protocols (Singh et al. 1997). The authors concluded

that the zona pellucida of sheep was 'stickier' and less resistant to pathogens than that of cattle.

- Terblanche *et al*. (2005) also found that sheep embryos infected *in vitro* with a strain of BTV4 remained infected after processing according to IETS protocols. However, although this South African strain of BTV4 was highly virulent (MacLachlan et al. 2008), it was not transmitted after transfer from viraemic donors of embryos which were washed according to IETS protocols (Terblanche et al. 2006).
- Washing prevented infection of recipients after transfer of embryos collected from viraemic ewes infected with BTV11 (Hare et al. 1988).
- Washing according to IETS protocols also prevented transfer of BTV10 and 11 from 107 sheep embryos collected from viraemic donors (Singh et al. 1997).
- Goat embryos which were washed ten times before freezing did not transfer BTV to recipients despite being collected from does which were seropositive to Caribbean strains of BTV serotypes 6 and 14 (Chemineau et al. 1986). However, donors may not have been viraemic at the time of embryo collection.
- BTV8 infected *in vivo* derived goat embryos that were exposed to virus *in vitro* and then washed according to IETS protocols (Al Ahmad et al. 2011).

Collection, freezing and storage

Effectiveness of IETS washing procedures to remove BTV8 from contaminated *in vivo* derived cattle embryos has not been investigated. However IETS washing procedures did not prevent *in vitro* infection with BTV8 of *in vitro* derived cattle blastocysts (Vandaele et al. 2011) or *in vivo* derived goat embryos (Al Ahmad et al. 2011). In the latter experiment BTV RNA was found in all ten successive washing fluids.

- Other serotypes of BTV are considered very unlikely to contaminate cattle embryos which have been collected and processed according to IETS protocols, as long as the zona pellucida remains intact before and after washing.
- The effectiveness of washing procedures in removing BTV from sheep, goat and deer embryos may vary with BTV serotype (Terblanche et al. 2006). Evidence of *in vitro* contamination of sheep embryos that was not removed by IETS washing procedures shows that strict observance of surgical sterility in embryo transfer procedures is required (Hare et al. 1988; Singh et al. 1997; Terblanche et al. 2006). Viral contamination of washing fluids and instruments used in embryo collection were considered a source of infection of recipients (Terblanche et al. 2006).
- Infectivity of BTV after freezing embryos is probably similar to that for freezing semen.

Release assessment -conclusion

There is insufficient research to investigate potential BTV8 contamination of semen and embryos. The likelihood of BTV8 contamination of ruminant embryos collected *in vivo* is considered to be very low, based on the available evidence, including the resemblance of BTV8 to laboratory adapted strains of BTV which:

- cause disease in cattle as well as sheep, goats and deer
- can cross the placenta and cause foetal abnormalities in cattle
- are present in semen.

Cattle embryos:

Based on the evidence of transplacental transmission of BTV8 and the other considerations above, there is an increased likelihood of BTV8 contaminating cattle embryos compared with other serotypes of BTV. As a result, the likelihood of BTV8 entering Australia via contaminated cattle embryos from the EU, Switzerland and Norway is estimated as **very low**.

Sheep, goat and deer embryos:

Based on the evidence above, the likelihood of contamination of sheep and goat embryos with other serotypes of BTV is greater than for cattle embryos. There is insufficient information on BTV contamination of deer embryos. However, testing of sheep, goat and deer embryo donors is required in the EU. As a result, the likelihood of BTV8 entering Australia via contaminated sheep, goat and deer embryos from the EU is estimated as **very low**.

These assessments take into account embryo washing and processing protocols recommended by the IETS and OIE, the epidemiology of BTV8 in Europe, current control measures for BTV in artificial breeding centres and more general measures, such as vaccination and restrictions on animal movement. These controls have not prevented further spread of BTV8 but have reduced its prevalence in some areas.

Exposure assessment

The following factors were taken into account in assessing the likelihood of exposure:

Embryo thawing

Investigations of BTV infection via embryo transfer with serotypes other than BTV8 were conducted with fresh, rather than frozen embryos. Infectivity of BTV and BTV8 after thawing of contaminated embryos is likely to be retained, as for thawed semen.

Transmission of BTV to embryo recipients

There is no published research on the transmission of the European strain of BTV8 by transfer of *in vivo* derived embryos. As discussed earlier, inadequately washed embryos contaminated with other BTV serotypes caused high rates of seroconversion in recipients. Characteristics of BTV8 which resemble those of a laboratory-adapted virus may make it more likely to resist washing procedures and infect recipients than other strains or serotypes of BTV.

- Studies with serotypes of BTV other than BTV8 showed that transmission of virus to recipient cows was prevented by washing of cattle embryos (Acree et al. 1991; Bowen et al. 1983a; Singh et al. 1982; Thomas et al. 1983; Thomas et al. 1985).
- Adequate washing of embryos collected *in vivo* from donors also prevented transmission from viraemic sheep (Hare et al. 1988; Singh et al. 1997; Terblanche et al. 2006) and seropositive goats (Chemineau et al. 1986).

Multiple direct exposure of recipients in several locations may occur if BTV8 contaminated embryos are imported. Compared with semen, fewer recipients are

likely to be exposed because smaller numbers of embryos are imported and these are probably less widely distributed.

Exposure assessment -conclusion

It is considered that the likelihood that sheep, cattle, goat and deer recipients exposed to imported embryos contaminated with BTV8 will become infected is **high** due to the direct route of exposure.

Consequence assessment

Likelihood of an outbreak, establishment or spread

The risk of viraemic embryo recipients spreading BTV8 to insect vectors is the same as that for viraemic semen recipients, and depends on the presence of competent vectors at the site of embryo transfer. Data are not available on the location of embryo transfer programs in Australia but they are likely to be undertaken in more intensive management systems and, therefore, in southern regions. Dairy farms which transfer imported embryos may be located in the seasonal bluetongue transmission zone in NSW.

Impacts of an outbreak, establishment or spread

The impact of an outbreak, establishment or spread of BTV8 in Australia after introduction by contaminated embryos would be similar to that for semen.

Consequences of BTV8

The impact of the establishment or spread of BTV8 in Australia after introduction by contaminated cattle, sheep, goat and deer embryos is **high**.

Overall risk for embryos

The evaluation included IETS procedures for collecting and washing ruminant embryos, and control measures for BTV in Europe.

Cattle embryos:

Overall, it is concluded that the risk of introduction and establishment or spread of BTV8 via the importation of cattle embryos from the EU, Switzerland and Norway is **low** and exceeds Australia's ALOP (very low). As a result, risk management measures for cattle embryos are required.

Sheep, goat and deer embryos:

It is concluded that the risk of introduction and establishment or spread of BTV8 via the importation of sheep, goat and deer embryos from the EU is **low**. As a result, risk management measures for sheep, goat and deer embryos are required.

6 Risk management

The previous section of this review established that the risk of BTV8 contamination of semen and embryos exceeded Australia's ALOP and required risk management. Options to manage the risk of introducing BTV from imported cattle, sheep, goat and deer semen and embryos from the EU, and cattle semen and embryos from Switzerland and Norway include consideration of:

- BTV status of the country or zone of origin of the animal
- seasonality of vector activity
- protection from vectors
- vaccination
- testing of donors
- semen and embryo collection and processing protocols.

OIE Code

Australia's import requirements for ruminant semen and embryos are based on recommendations for BTV risk management in OIE Code Chapters 8.3, 4.6 and 4.7.

Country or zone free status for BTV

- Country or zone free status for BTV would be an acceptable risk management measure subject to recognition by the Australian Government. This recognition would be based on surveillance data submitted to Australia as part of a dossier of evidence according to the OIE Code (Chapter 8.3) and the consensus document between Australia and the EU, 'Principles of Zoning and Regionalisation' (SANCO/10157/2005) dated 23 May 2005 (see Appendix 5).
- Norway and Switzerland are countries infected with BTV, as defined by the OIE Code.
- Any exemptions from BTV testing for EU Member States in the Mediterranean basin should take into account the date of first notification of BTV in each country after 2000.

Season of low vector activity

The risk of transmission of BTV is reduced when vectors are less active (EFSA 2008b; 2008c) but it is difficult to accurately define these seasons in different regions in Europe for reasons outlined earlier.

- Recognition of a BTV seasonally free zone or season of low vector activity in an EU Member State would be based on the 'Principles of Zoning and Regionalisation' referred to above.
- Australia's recognition of such a zone in Switzerland or Norway should be by agreement with the exporting country, using similar evidence to that required for the EU and based on OIE recommendations.

Protection from vectors

Protection of donor animals from vector attack may reduce BTV infection rates but is considered insufficient as a sole risk management measure (Bauer et al. 2009; Baylis

et al. 2011; EFSA 2007b; EFSA 2008b; Mellor and Wittmann 2002; Papadopoulos et al. 2009).

EU legislation

Australian import conditions currently require semen or embryos imported from the EU to be collected and processed in accordance with the following EU legislation and amendments:

- Cattle semen: 88/407/EEC, or
- Cattle embryos: 89/556/EEC, or
- Sheep, goat and deer semen and embryos: 92/65/EEC.

In addition:

- All processing of semen, including sperm sorting for sex (semen sexing), must be undertaken at premises approved by the competent authority of the exporting country for the processing of semen.
- All embryos must be fertilised *in vivo* and not subjected to micromanipulation involving breaching of the zona pellucida. Embryos must have an intact zona pellucida at the time of storage.

Vaccination

Exclusion of semen and embryo donors which have been vaccinated against BTV may not be desirable or possible:

- Vaccination against BTV is mandatory or widely practiced in most European countries which export semen and embryos to Australia.
- Differentiation of vaccinated from BTV-infected animals is not available commercially at present. A further problem is that reassortment between vaccine and wild BTV strains has occurred and these reassortant viruses are circulating in Europe. It is not commercially feasible to distinguish the genotype of BTV in infected donors.
- Inactivated vaccines or MLVs against bluetongue may limit or prevent viraemia and therefore reduce the likelihood of contamination of ruminant semen and embryos with BTV.
- Effectiveness of different inactivated vaccines and MLVs used in each European country may vary. Absence of vaccine strains of BTV in semen has not been determined for all MLVs.

In view of these factors, recommended risk management for semen and embryo donors from Europe vaccinated against BTV is as follows:

- Vaccines against BTV must be inactivated and approved by the competent authority and should be administered to cattle, sheep, cattle or deer donors of semen or embryos at least 60 days before the commencement of semen or embryo collection.

Tests for semen and embryo donors

Emergence of new BTV serotypes and reassortants with BTV8 in Europe described earlier means that Australian import conditions should be revised to ensure that all serotypes of BTV are detected by serological and PCR tests.

- Cattle, sheep, goat and deer semen donors from countries deemed by Australia to be infected with any serotype of BTV must be tested for BTV infection.
- Sheep, goat and deer embryo donors from countries deemed by Australia to be infected with any serotype of BTV must be tested for BTV infection.
- Cattle embryo donors should be tested for infection with BTV8, unless the absence of this strain can be demonstrated in the exporting country to the satisfaction of Australian authorities. However, in practice it is difficult to specify testing for infection with BTV8 alone because:
  - BTV serological and agent identification tests typically detect more than one serotype, and
  - distribution of BTV8 in several European countries overlaps that of other serotypes and these distributions change.

  Thus the recommendation for BTV testing of cattle embryo donors does not specify BTV8.
- All tests for BTV should be validated according to the current OIE Terrestrial Manual, calibrated to a diagnostic sensitivity of at least 98.0%, and carried out in a laboratory approved by the competent authority for the exporting country.
- The laboratory conducting the test must participate in regular inter-laboratory tests (ring trials) to validate tests and be officially approved by the competent authority.
- Samples for testing for BTV should not be diluted or pooled.
- Tests should be applied, irrespective of the vaccination history of the donor animal. A donor whose blood has a positive result to the cELISA for bluetongue antibodies may still be able to meet import requirements for BTV providing agent identification tests on blood are negative.

Serological ***or*** agent identification tests which comply with the current OIE Terrestrial Manual may be used with the following conditions:

Serological tests

- Serological tests for BTV antibody should be restricted to the cELISA. The AGID test for BTV should not be used.
- Semen donors should be tested at least every 60 days throughout the collection period and between 28 and 60 days after the final collection for the consignment.
- Embryo donors should be tested between 28 and 60 days after embryo collection.

Serological testing of donors from 28 rather than 21 days after semen or embryo collection (as specified by the OIE) takes into account a longer period to seroconversion under some circumstances, coexistence of antibodies and virus in the early stages of BTV infection and the limited validation of cELISA tests in situations when animals are seroconverting from natural infection or vaccination.

Agent identification tests

OIE prescribed tests for international trade are virus isolation or real time RT-PCR to detect BTV antigen in blood.

- Virus isolation for BTV must be conducted on blood samples collected at commencement and conclusion of, and at least every seven days during semen collection, or on the day of embryo collection.
- Real time RT-PCR tests must be approved[19] and conducted on blood samples collected at commencement and conclusion of, and at least every 28 days during semen collection, or on the day of embryo collection.

Conclusion and recommendations

To achieve Australia's ALOP with respect to BTV risks identified in this review, it is recommended that all imported ruminant semen and *in vivo* derived embryos from the EU, Switzerland and Norway, including bovine embryos, are:

- collected and processed in accordance with OIE recommendations and EU legislation,

and

- collected from donors which are negative to serological or agent identification tests on blood samples for BTV, using tests capable of detecting all 24 known BTV serotypes,

and

- collected from donors which are not vaccinated with attenuated (modified live virus) vaccines against BTV. Donors must not be vaccinated with inactivated (killed) vaccines against BTV at less than 60 days before commencement of, or during, semen or embryo collection.

The diagnostic tests used must be appropriate for the purpose and sensitive. Emergence of several serotypes in Europe and their potential for reassortment means that the serological and agent identification tests must be able to detect all known BTV serotypes.

Equivalent approaches to managing identified risks may be accepted. Parties seeking to use alternative equivalent risk management measures to those identified above would need to provide a submission for consideration. Such proposals should include supporting scientific data that clearly demonstrate equivalence of the proposed alternative measures.

[19] Real time RT-PCR tests must be approved by the competent authority and be able to detect all known 24 BTV serotypes and be conducted in an officially approved laboratory. These tests must use primer sequences directed against highly conserved segments of the BTV genome which code for BTV serogroup (not serotype). An example of an approved test is the TaqMan real time RT-PCR test according to the method of Shaw *et al.* (2007), which uses two primers directed against segment 1 of BTV RNA.

7 Revised quarantine requirements

REVISED ANIMAL QUARANTINE REQUIREMENTS FOR BLUETONGUE VIRUS FOR CATTLE, SHEEP, GOAT AND DEER SEMEN AND EMBRYOS IMPORTED FROM THE EUROPEAN UNION, AND CATTLE SEMEN AND EMBRYOS IMPORTED FROM SWITZERLAND AND NORWAY

The following requirements for bluetongue apply to:

(a) All member countries of the EU at the time of this review, namely: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Republic of Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, United Kingdom, and

(b) Switzerland and Norway.

Semen

Vaccines (if used) against bluetongue must be inactivated and approved by the competent authority and should be administered to donors at least 60 days before the commencement of semen collection for this consignment.

Cattle, sheep, goat and deer semen donors were subjected to either:

- a competitive ELISA for antibody to the bluetongue virus group, with negative results, at least every 60 days throughout the collection period and between 28 and 60 days after the final collection for this consignment,

or

- an agent identification test for bluetongue virus on blood samples collected at commencement and conclusion of, and at least every seven days (virus isolation test) or at least every 28 days (approved PCR test*) during semen collection for this consignment, with negative results.

Note:

All tests for bluetongue virus should be validated according to the current *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, calibrated to a diagnostic sensitivity of at least 98.0% and carried out in a laboratory approved by the competent authority of the exporting country.

* Real time RT-PCR tests must be approved by the competent authority and be able to detect all known 24 BTV serotypes. These tests must use primer sequences directed against highly conserved segments of the BTV genome which code for BTV serogroup (not serotype). An example of an appropriate test is the TaqMan real time RT-PCR test according to the method of Shaw *et al.* (2007)**, which uses two primers directed against segment 1 of BTV RNA.

**Reference: Shaw A.E., Monaghan P., Alpar H.O., Anthony S., Darpel K.E., Batten C.A., Guercio A., Alimena G., Vitale M., Bankowska K., Carpenter S., Jones H., Oura C.A., King D.P., Elliott H., Mellor P.S., Mertens P.P. (2007). Development and initial evaluation of a real time RT-PCR assay to detect bluetongue virus genome segment 1. *Journal of Virological Methods*, 145, 2, 115-26.

Embryos
Vaccines against bluetongue must be inactivated and approved by the competent authority and should be administered to donors at least 60 days before the commencement of embryo collection for this consignment.

Cattle, sheep, goat and deer embryo donors were subjected to either:

- a competitive ELISA for antibody to the bluetongue virus group, with negative results, between 28 and 60 days after the collection of embryos for this consignment,

or

- a bluetongue virus isolation test or an approved PCR test* on a blood sample taken on the day of collection of embryos for this consignment, with negative results.

or, for cattle embryos only:

- The cattle embryos were collected prior to 1 May 2006.

Note:
All tests for bluetongue virus should be validated according to the current *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, calibrated to a diagnostic sensitivity of at least 98.0% and carried out in a laboratory approved by the competent authority of the exporting country.

* Real time RT-PCR tests must be approved by the competent authority and be able to detect all known 24 BTV serotypes. These tests must use primer sequences directed against highly conserved segments of the BTV genome which code for BTV serogroup (not serotype). An example of an appropriate test is the TaqMan real time RT-PCR test according to the method of Shaw *et al.* (2007)**, which uses two primers directed against segment 1 of BTV RNA.

**Reference: Shaw A.E., Monaghan P., Alpar H.O., Anthony S., Darpel K.E., Batten C.A., Guercio A., Alimena G., Vitale M., Bankowska K., Carpenter S., Jones H., Oura C.A., King D.P., Elliott H., Mellor P.S., Mertens P.P. (2007). Development and initial evaluation of a real time RT-PCR assay to detect bluetongue virus genome segment 1. *Journal of Virological Methods*, 145, 2, 115-26.

Appendix 1

Control measures for BTV in Europe

Some EU Member States, such as Greece, managed past BTV outbreaks by surveillance and slaughter without use of vaccines. Greece declared itself free of BTV in March 2005 (EFSA 2007b) but a new incursion occurred in 2008. Italy, continually infected with BTV since 2000, contained the distribution of disease through control of animal movement and extensive vaccination.

Movement bans and tests

In 2000, EU Member States applied import bans on livestock from Italy and the Balearic Islands of Spain. Italy imposed internal restrictions on livestock movement between BTV-infected and non-infected areas which required knowledge of vector activity and serological testing prior to movement. To support livestock trading, Italy commenced a national entomological and virological surveillance system based on a 20 km^2 grid, with serological testing of 58 sentinel cattle every 15 days during March and December (the spread period) and every 30 days during January and February (SCFCAH 2008j).

Italy maintains that serological testing prior to movement without intensive surveillance of animals and vectors presents an unacceptable risk of introducing BTV[20], and questioned the effectiveness of EU legislation which specifies larger bluetongue restriction zones and free livestock movement between these zones (Caporale 2008).

BTV vaccination

The use of vaccines against bluetongue in Europe to reduce livestock losses, and problems associated with the use of MLVs in the Mediterranean region prior to 2006, have been reviewed in the Proceedings of the Third International Bluetongue Symposium in Taorima, Italy in 2003, and by others (Alpar et al. 2009; Caporale 2008; EFSA 2007d; Mellor and Wittmann 2002; Savini et al. 2008).

Prior to the emergence of BTV8, vaccination policies in Mediterranean countries varied widely.

- Vaccination is compulsory in Italy, Portugal and Spain, but only Italy requires vaccination of cattle as well as sheep and goats for serotypes other than BTV8.
- Coverage of less than 80% of the susceptible population reduced control of bluetongue during outbreaks in the Balearic Islands and Corsica, where only sheep and goats were vaccinated (Caporale 2008). In Italy, the percentage of animals vaccinated in bluetongue restricted zones decreased to 40% in 2005 from prior levels of 85 to 98% in 2002–2004 (European Commission: Health & Consumer Protection Directorate-General 2006).

[20] V. Caporale, plenary address, EPIZONE Bluetongue Symposium, 7 June 2008

Modified live virus vaccines

Most MLV against bluetongue are multivalent vaccines (i.e. effective against two or more specific serotypes). These vaccines were imported from South Africa and were used in Bulgaria (1999–2000), Italy (2002–08), the Balearic Islands of Spain (2003) and France (Corsica) (2003–04) (European Commission 2007; Savini et al. 2008).

- Four monovalent MLVs from South Africa (against BTV serotypes 2, 4, and 9) (Onderstepoort Biological Products) and from Pakistan (against BTV16) and four multivalent vaccines were used at various times over the last decade in Europe and neighbouring regions.
- A South African pentavalent MLV against BTV serotypes 3, 8, 9, 10 and 11 was used in Bulgaria in 1999-2000 against an outbreak of BTV9.
- Locally-produced MLV against BTV4 was used in Turkey during bluetongue outbreaks in 2000–2003.
- Monovalent MLV against BTV2 was used in Tunisia in 2000–2002.
- In Italy, compulsory vaccination of all cattle, sheep and goats commenced in January 2002 with appropriate monovalent MLV produced in South Africa.
- Bivalent MLV against BTV2 and BTV9 was also used in Italy until 2004 when combinations of MLV against BTV serotypes 2, 4 and 16 and BTV serotypes 2, 4, 9 and 16 were used until the present.
- Israel continues to use pentavalent MLV against BTV serotypes 2, 4, 6, 10 and 16 against regular outbreaks of BTV, although only one outbreak of BTV16 was reported (in 2003) prior to its reappearance in 2008 (EFSA 2007d; Mellor et al. 2008; OIE 2009b; Savini et al. 2008).

MLVs contributed to the emergence of new strains of BTV serotypes in Europe, through inadequate attenuation, blanket administration and genetic reassortment. Reassortment makes the future behaviour of BTV uncertain and may also lead to unreliable results from RT-PCR tests (Batten et al. 2008b).

- Inadequate attenuation of a monovalent MLV for BTV16 resulted in its discontinuation in Italy and Corsica after observations of clinical signs in sheep and abortion in less than 0.5% of vaccinated animals (Savini et al. 2008).
- Insufficient attenuation of vaccines against BTV serotypes 16 and 2 also resulted in their transmission by vectors in southern Europe (Ferrari et al. 2005; Nomikou et al. 2008).
- An RT-PCR test was developed to differentiate the eight field and vaccine strains of BTV serotypes 2, 4, 9, 16 circulating in Italy (Polci et al. 2007). Other RT-PCR tests were developed to differentiate field from vaccine strains of BTV serotypes 2 and 9 ‘to avoid unjustified economic losses and legal disputes’ (Elia et al. 2008).
- Genetic analysis of BTV RNA segment 10 from serotypes other than BTV8 supported the view that the use of MLV strains had ‘facilitated’ reassortment between BTV strains in the Mediterranean basin (Balasuriya et al. 2008).
- Evidence of genetic reassortment between bluetongue vaccine strains was obtained in 2004 from an Italian isolate of BTV2 from a sheep vaccinated with MLV against BTV2 and BTV4 (Monaco et al. 2008).
- Reassortment was also demonstrated in a field isolate of BTV16 from Italy in 2002 which contained segments of vaccine strains of BTV2 (used in Italy in 2002) and vaccine strains of BTV16 (used in Israel annually since 1995) (Batten et al. 2008b). Interestingly, this reassortant strain was isolated before MLV

against BTV16 had been used in Italy (Caporale 2008) and probably entered Italy via North Africa (Batten et al. 2008b).

Reassortment of BTV was demonstrated in the field and experimentally in Australia and North America (McColl et al. 1994; Oberst et al. 1987; Stott et al. 1987).

- In the United States, genetic reassortment was demonstrated between two strains of BTV11 during a mixed infection with both strains in a heifer (Oberst et al. 1987).
- Reassortment of BTV can produce strains with different biological characteristics. In northern Australia, increased infectivity of BTV and longer viraemia were observed in reassortants of BTV20 and 21 with the Java C strain from Indonesia (Melville et al. 2005c). The length of viraemia with BTV21 increased from three to six weeks and with BTV20 from one to four weeks. In contrast, reassortment of BTV1 with the Java C strain did not change these characteristics.

MLVs caused transplacental transmission, abortion and stillbirths:

- Vaccination with MLVs against serotype 2 (alone or combined with serotype 9) in Italy and the Balearic Islands of Spain in 2000-2002 resulted in abortions or stillbirths in up to 0.53% of sheep and 0.18% of cattle. BTV was detected in a small proportion of foetuses (Savini et al. 2008).
- Similar problems were reported after use of MLV against BTV16 in Corsica (De Clercq et al. 2008a).

Inactivated vaccines

Inactivated vaccines, in monovalent and bivalent formulations, were developed for BTV1, 2 and 4 and these are used in France (Corsica), Italy, Spain and Portugal. An inactivated vaccine against BTV16 is under development (Savini et al. 2007).

Inactivated (killed) vaccines have fewer potential side effects than MLVs and do not contribute to reassortment, as long as quality assurance is adequate. However, they are more costly to manufacture and administer as two doses are needed to protect cattle, and annual boosters are required. Inactivated vaccines need to provide complete protection against viraemia after challenge with field virus (see page 48).

Vaccination against BTV1 in France commenced in March 2008 with inactivated vaccine (Promed Mail 2008b). Despite vaccination using 3.5 million doses in cattle and 4 million doses in small ruminants, BTV1 continued to spread across south-west France to the north and east to affect a total of 1 217 farms by 15 September 2008. At the same time, the number of farms in France infected with BTV8 was 13 856 and there was considerable overlap in the distribution of these serotypes (Promed Mail 2008n).

Differentiating infected from vaccinated animals

RT-PCR tests were developed to distinguish field from vaccine strains of BTV (Bréard et al. 2003; Elia et al. 2008; Monaco et al. 2006; Orru et al. 2004); although serological tests to differentiate infected from vaccinated animals (DIVA) are not yet available:

- Differences in levels of antibody to a BTV non-structural protein (NS3) in vaccinated and infected animals were detected by an ELISA test (Barros et al. 2009).

- Innovative Diagnostics (ID-VET)[21] was developing an ELISA based on different distributions of monoclonal antibodies to variable and conserved viral BTV proteins found in field infections but not in animals given inactivated vaccine (Pourquier and Lesceu 2008). Validation of the test will occur during 2008 after mass vaccination across Europe.
- Differences in the 'avidity' of serum antibody from BTV vaccinated and naturally infected animals may be the basis of another DIVA strategy (Fernandez-Pacheco et al. 2008b).

Future vaccines

Developments in BTV vaccines were recently reviewed by Noad and Roy (2009). Disabled infectious single cycle vaccines (DISC) may be protective after a single dose and enable infected and vaccinated animals to be distinguished. Recombinant vaccinia, capripoxvirus and canarypox virus-vectored for BTV4 vaccines are available (Alpar et al. 2009; Calvo et al. 2008; EFSA 2007d). Future vaccines for bluetongue including non-replicating virus like particles and recombinant vaccines (Boone et al. 2007; Schwartz-Cornil et al. 2008; Stewart et al. 2009), will need to be assessed as they become available.

[21] ID-VET. 167 rue Mehdi Ben Barka, 34 070 Montpellier, France. http://www.id-vet.com/index_gb3.htm

Appendix 2

EU legislation

This section examines EU legislation or control measures, including any requirements for mandatory testing of semen and embryo donors for BTV.

Legislation in the EU for Animal health requirements for ruminant germplasm and the control of bluetongue is summarised in Table 6. This review refers to consolidated versions of legislation available at http://eur-lex.europa.eu/.

- Animal health requirements for cattle semen are set down in Council Directive 88/407/EEC. This directive includes health conditions for intra-Community trade or importation from third countries and approval of semen collection centres.
- Requirements for intra community trade in and importation from third countries of cattle embryos in Council Directive 89/556/EEC include conditions for the collection, processing and storage of embryos but do not refer directly to BTV.
- Requirements for trade in and import into the EU of sheep, goat and deer semen and embryos are set out in Council Directive 92/65/EEC. This directive, which required animals to be free of clinical signs of bluetongue and not subject to official restrictions, was amended by EC 1266/2007 (see below).

Table 6. Current EU legislative documents which specify animal health requirements for ruminant germplasm and control measures for bluetongue.

Issue	Legislation
Cattle semen	Council Directive 88/407/EEC
Cattle embryos	Council Directive 89/556/EEC
Sheep/goat semen and embryos	Council Directive 92/65/EEC
Deer semen and embryos	Council Directive 92/65/EEC
Bluetongue	Council Directive 2000/75/EC
	Commission Regulation EC 1266/2007 of 26 October 2007 Amendments: EC 289/2008 of 31 March 2008 EC 384/2008 of 29 April 2008 EC 394/2008 of 30 April 2008 EC 708/2008 of 24 July 2008 EC 1108/2008 of 7 November 2008 EC 1304/2008 of 19 December 2008 EC 123/2009 of 10 February 2009 EC 789/2009 of 28 August 2009 EC 1156/2009 of 27 November 2009 EC 1142/2010 of 7 December 2010

- Provisions for the control bluetongue in the EU are set out in Council Directive 2000/75/EC of 20 November 2000, which includes establishment of restriction zones, bans on the movement of livestock and products and requirements for

monitoring and surveillance. Rules for this directive are implemented in EC 1266/2007 of 26 October 2007, which includes bluetongue requirements for semen and embryos. (This regulation replaced Commission Decision 2005/393/EC of 23 May 2005, after BTV8 emerged in northern Europe).

- An amendment to EC 1266/2007 on 31 March 2008 (EC 289/2008) required health certificates in EC 1266/2007, Annex III, sections B and C to specify BTV testing of donors of cattle, sheep, goat and deer semen and non-bovine embryos if, for the 60 days prior to and during collection, they had not been:
  - kept outside a restricted zone, or
  - protected from attack by vectors, or
  - kept during the seasonally vector free period in a bluetongue seasonally free zone

The BTV test requirements are the same as those in the OIE Code (see below).

As in the OIE Code, EC 1266/2007 does not require bluetongue testing of donors of cattle embryos collected *in vivo* but states that they should not show clinical signs of bluetongue.

The regulation also revoked, for bluetongue, requirement in EC 89/556 for cattle embryo donors to be kept in a holding not subject to quarantine measures.

Animal health certification for semen and embryos do not refer to requirements for vaccination specified in EC 1266/2007, which covers inactivated and modified live vaccines.

EU legislation was updated in response to emerging issues with BTV8. Problems with transplacental transmission of BTV and effectiveness of protection from attack by vectors have been recognised in EC 394/2008. This regulation was incorporated into EC 1266/2007 as an update on 20 April 2008. Animals that are moved from BTV-infected to free zones must be either vaccinated or shown to be naturally immune to BTV. Movement of calves less than 90 days old, which are too young to be vaccinated, is allowed if they are confined for protection from vectors. These were transitional measures until 31 December 2008 pending further scientific assessment (European Commission 2009a).

Prior to May 2006, requirements for semen and embryos collected in EU states infected with BTV (in southern Europe) are set out in EC 2005/393 Annex II, sections B and C. These requirements do not refer to a seasonally vector free period in a bluetongue seasonally free zone but otherwise were the same as those for EC 1266/2007.

EU zoning for BTV

- EU Member States from which Australia principally imports ruminant germplasm are currently designated bluetongue restricted zones under EU legislation.
- Movement of livestock between confluent restricted zones in the EU can occur subject to conditions determined by the importing country.
- Donor animals in artificial breeding centres in restricted zones are probably tested for BTV but there are exemptions. Exemptions also exist for donors in unrestricted or bluetongue seasonally-free zones.

- Bluetongue test requirements for vaccinated animals rely on knowledge of all the BTV serotypes circulating in a region. This information may be difficult to obtain when serotypes are spreading rapidly, e.g. BTV1 in France in 2008.

Zoning for BTV is the basis for controlling the movement of animals, semen and embryos in the EU. Restricted zones for BTV are defined in 2000/75/EC and consist of both protection[22] and surveillance[23] zones. Criteria for monitoring and surveillance programs are set out in EC 1266/2007. A bluetongue restricted zone is defined as a part of European Commission territory and may cross borders of Member States. Lists of bluetongue restricted zones must be published by SCFCAH. Each Member State must submit information on BTV serotypes circulating in each of their restricted zones and notify the Commission within 24 hours of any changes to these zones. An area cannot be removed from a restricted zone without demonstrating absence of BTV circulation for two years, following implementation of a bluetongue monitoring program (EC 1266/2007, Chapter 3, Article 6, point 2). Surveillance to demonstrate the absence of circulating virus must detect a prevalence of BTV infection of 10% with 95% confidence in areas where mass vaccination was implemented (EC 1266/2007 Annex I, point 1.3). Currently, bluetongue infected zones encompass most of the EU Member States from which Australia imports ruminant germplasm.

Under EU legislation (EC 1266/2007), the 'bluetongue *seasonally*-free zone' is defined as 'an epidemiological relevant geographical area of a Member State for which, for a part of the year, surveillance demonstrates no evidence of bluetongue virus transmission or of adult *Culicoides* likely to be competent bluetongue vectors'. The seasonally vector free period must be substantiated with data from a monitoring program over at least three years (EC 1266/2007, Annex III.A.1 and B.c). Confidence in the seasonally-free zone in Member States is limited by differences in surveillance methods between Member States and the need for more accurate vector trapping techniques to reflect distribution and activity of vectors (Carpenter et al. 2008c; Meiswinkel et al. 2008a). As more information on vector behaviour emerged, definitions of a 'vector free period' were modified (Meiswinkel et al. 2008a). However, EU legislation still refers to a seasonally vector free period for the purpose of determining a bluetongue seasonally-free zone (Annex V of EC 1266/2007). Criteria for this period include, 'in the absence of sound evidence supporting the determination of the maximum threshold, total absence of *Culicoides imicola* specimens and less than five parous *Culicoides* per trap.'

Animals can be moved within the same restricted zone *where the same bluetongue virus serotype or serotypes are circulating* (EC 1266/2007 Article 7 (1)). To move animals, semen and embryos from a holding, semen collection or storage centre in a BTV restricted zone, conditions in Annex III of EC 1266/2007 must be met. The competent authority at the destination may also set its own animal health conditions for movement (Article 8.1(b)). Thus movement of animals from BTV8 restriction zones in Germany to the same zone in the United Kingdom is permitted under EU law, but the United Kingdom tests imported animals on arrival and detected several cases of bluetongue PCR positive animals in this way (DEFRA 2008c). In contrast, movement of animals infected with BTV1 from France to Belgium and the

[22] Protection zone: a part of the Community territory having a radius of at least 100 km around the infected holding (2000/75/EC Article 8.2a).

[23] Surveillance zone: a part of the Community territory with a depth of at least 50 km extending beyond the limits of the protection zone and in which no vaccination has been carried out during the previous 12 months (2000/75/EC Article 8.2b).

Netherlands in November 2008 was not permitted, but occurred because BTV1 was not detected in the restriction zone of origin at the time of movement.

European Commission regulations controlling ruminant germplasm and BTV vaccination are discussed below.

Cattle semen

EU requirements to test cattle semen donors for BTV depend on EU bluetongue zoning, with exemptions if protected from vectors. However, EU legislation recently acknowledged that measures employed in the EU to protect against vector attack were not effective.

Council Directive 88/407/EEC sets out health certification for cattle semen for intra-Community trade or export to a third country. Amendments in EC 1266/2007 require health certificates to identify the BTV risk management options applied to the donor. These options include alternatives to BTV testing.

BTV testing of ruminant semen donors in bluetongue restricted zones was an option under EU legislation after 23 May 2005 (2005/393/EC). This decision was amended on 30 November 2006 to include zones for BTV8 and then replaced in 26 October 2007 by EC 1266/2007. Under Annex III of this regulation donors are exempt from testing for BTV if, for the 60 days preceding and during semen collection, they were:

- kept outside a restricted zone, or
- protected against attacks by vectors, or
- kept during the seasonally vector free period in a bluetongue seasonally-free zone.

Apart from issues with bluetongue zoning outlined above, exemptions from BTV testing of semen and embryo donors on the basis of protection from vector attack remain in EU legislation, despite acknowledgement by the EFSA scientific panel that protection from vectors is difficult (EFSA 2008c). In addition, ‘protection from attack’ is not defined and there is no method to substantiate protection or any requirement to inspect livestock or housing for vectors. In late 2008 an amendment to EC 1266/2007 acknowledged the ineffectiveness of protection of animals against vector attack (EC 1304/2008 dated 19 December 2008, points 3 and 5). Later amendments permitted semen and embryos to be collected from donors ‘protected against attacks by vectors in a vector proof establishment’ (EC 1266/2007, Annex III, sections B and C). The amendment also continued temporary provisions which enable EU destination countries to require BTV testing of animals, semen and embryos before movement (EC 1266/2007 Article 9a).

Test requirements for BTV under EC 1266/2007 are identical to those in the OIE Code (OIE 2010a) for ruminant semen donors.

88/407/EEC does not refer to vaccination against BTV, nor are there specific provisions for vaccination of semen and embryo donors in EC 1266/2007.

Cattle embryos

There are no EU requirements to test cattle embryo donors for BTV. Animal health conditions governing intra-Community trade in and importation from third countries of embryos of domestic animals of the cattle species (89/556/EEC) do not refer to bluetongue or bluetongue vaccination.

Cattle embryos can be collected from donors located in bluetongue restriction zones. EC 1266/2007 Annex III.C point 3 revokes, specifically for BT, a requirement for a donor to be kept in a holding not subject to veterinary prohibition or quarantine measures (89/556/EEC Annex B.2.a). However, some risk of BTV contamination of cattle embryos is acknowledged in EU legislation by the requirement for donor cows to show no clinical signs of bluetongue on the day of embryo collection (EC 1266/2007 Annex III.C.1). This contrasts with OIE Code Article 4.7.4, which requires embryo donors to be free of clinical signs of diseases not included in IETS Category 1. Since bluetongue is a Category 1 disease for cattle, the IETS and OIE recommendations mean that embryos may be collected from cattle donors clinically affected by bluetongue. This categorisation was developed assuming that BTV rarely caused disease in cattle, prior to the emergence of BTV8 and was since noted in the OIE Code as an issue 'under study'.

Conditions for the collection, processing, storage and transport of cattle embryos by approved embryo collection teams are set out in 89/556/EEC Annex A, Chapter II., and include a requirement that embryos must be washed be ten times and treated with trypsin in accordance with internationally recognised procedures.

Sheep, goat and deer semen and embryos

- EU requirements to test sheep, goat and deer semen and embryo donors for BTV depend on EU bluetongue zoning, with exemptions if protected from vector attack.

Trade in and imports into the EU of sheep, goat and deer semen and embryos are governed by 92/65/EEC[24]. Approved centres must be free of notifiable diseases, which include bluetongue (92/65/EEC Annex C). Health certificates require animals to be free of clinical signs of these diseases. Requirements that animals are not subject to official restrictions (Annex E of 92/65/EEC Part 3) were revoked for bluetongue by EC 2007/1266.

EC 1266/2007, Annex III.C requires sheep, goat and deer embryo donors in bluetongue restricted zones and seasonally free zones to be tested for BTV, unless protected from vectors. These requirements must also be met before movement of animals, their semen, ova and embryos from a holding or semen collection or storage centre located in a restricted zone (EC 1266/2007 Article 8.1.a).

Vaccination against BTV is permitted under conditions governing approval of artificial breeding centres, which require vaccination of susceptible animals against infectious diseases in conformity with European Commission legislation (92/65/EEC Annex C.1.g.ii).

Embryos must be processed in accordance with 'approved methods', which includes washing in accordance with 'international standards' and the sterilisation and handling of media (92/65/EEC Chapter III). The zona pellucida of sheep, goat and deer embryos must remain intact before and after washing but this chapter does not explicitly refer to the IETS Manual or the OIE Code, or specify the number of times embryos must be washed.

[24] 92/65/EEC sets animal health requirements for 'animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC' http://eur-lex.europa.eu (Accessed 25 August 2008)

EC 1266/2007 requires the donors of embryos of non-cattle species to be tested for BTV, or to reside outside a bluetongue restricted zone or be protected from vector attack for 60 days prior to embryo collection. (EC 1266/2007 Annex 3.C.2.a–d) as amended by EC 289/2008, dated 31 March 2008). Deer are not included in IETS disease categories.

Vaccination

- The bluetongue vaccination status of ruminant semen and embryo donors in the EU is uncertain because of the circulation of multiple serotypes of BTV in different European regions and variation in vaccination policies between Member States.

EU legislation for BTV vaccination:

- Does not require or prohibit vaccination against BTV of semen and embryos donors. Any vaccination against BTV must be 'according to a vaccination program adopted by the competent authority' (EC 1266/2007 Annex III.A.5).
- Requires health certificates for movement of animals out of bluetongue restricted zones to identify whether inactivated or MLVs have been used and against which BTV serotypes (EC 1266/2007, Annex III.A.5). Individual Member States determine the type of vaccines that they will approve in their vaccination program.
- Requires 60 days to elapse between vaccination and movement of an animal from a bluetongue restriction zone to a free zone.
- Requires semen donors and non-bovine embryo donors in bluetongue infected zones to test negative to bluetongue (unless protected from vector attack), whether they are vaccinated or not. Difficulties in interpreting bluetongue test results from vaccinates may discourage vaccination of donors against bluetongue (EC 1266/2007, Annex III.C).
- Does not require testing of cattle embryo donors in bluetongue infected zones, or EU health certificates for cattle embryos to record whether donors were vaccinated.

Regulations governing vaccination against BTV in EC 1266/2007 and amendments only permit vaccination of animals in restricted zones. There are no specific provisions for vaccination of semen and embryo donors, other than general requirements for vaccination against BTV before animal movement out of a restricted zone (Annex III.A.5). Under this provision, donors from restricted zones entering an artificial breeding centre in a free zone may need to be vaccinated.

In bluetongue restricted zones, semen and embryo donors may be vaccinated against any BTV serotype present in the region with an inactivated or MLV vaccine (or combination of these). Under EC 1266/2007, donors in restricted zones must also meet BTV test requirements or be protected from vector attack.

Council Directives 88/407/EEC (cattle semen) and 89/556/EEC (cattle embryos) do not require or prohibit vaccination of donors against BTV. There is a general requirement in 92/65/EEC for vaccination of susceptible animals in artificial breeding centres against infectious diseases. In Member States with compulsory vaccination programs against BTV, this condition may require sheep, goat and deer donors residing in artificial breeding centres to be vaccinated. However, this may not apply to donors of these species which have embryos collected on farms.

The use of live or inactivated vaccines in semen or embryo donors is not considered a risk under EU legislation. EC 708/2008 of 24 July 2008 amending regulation EC 1266/2007 states:

> ‘Animals that were immune to bluetongue infection before artificial insemination or mating, due to vaccination with a modified life vaccine or an inactivated vaccine, are not considered to pose any significant risk as regards that disease provided that sufficient time has elapsed between vaccination and insemination or mating. Regulation (EC) No 1266/2007, as amended by Regulation (EC) No 384/2008, only covers animals vaccinated by inactivated vaccines.’

Further,

> ‘As preliminary scientific information recently obtained does not indicate that there is an additional risk associated with pregnant animals vaccinated with live modified vaccines at least 60 days prior to insemination or mating, it should be possible to exempt all immunised animals vaccinated with either inactivated or modified live vaccines from the exit ban provided that sufficient time has elapsed between vaccination and insemination or mating.’

In February 2009, a new ‘lower risk’ bluetongue restricted zone was proposed to allow vaccination in the absence of circulating BTV virus, with conditions for movement in and out of this zone. This proposal was adopted in August 2009 (Regulation EC 789/2009 amending EC 1266/2007). Substantial amendments were also made to regulations for BTV surveillance and monitoring (Regulation EC 1108/2008 amending EC 1266/2007) and increased EU funding for BTV vaccination was made available. In 2008 and 2009 EU funding to Member States' BT eradication and monitoring programmes amounted to Euros 150 and 160 million. This decreased to Euros 66 million in 2010 and 16 million for 2011, as the number of BTV outbreaks declined (European Commission 2010).

Appendix 3

Interim Australian import policy for BTV

In July 2008, Australia imposed interim conditions for imported cattle embryos from the EU and semen and embryos from Switzerland with regard to BTV. In the case of Norway, as there were no imports of cattle semen and embryos at the time, any application to import was to be assessed on a case-by-case basis (Table 7). This also applied to imports of small ruminant semen and embryos from the EU. The interim conditions are detailed below.

Table 7. The status of import conditions after 8 July 2008 for ruminant semen and embryos from the EU, Switzerland and Norway considered in this review.

Commodity	Status
Cattle semen from EU	unchanged
Cattle semen from Switzerland	interim
Cattle semen from Norway	case-by-case basis
Cattle embryos from EU	interim
Cattle embryos from Switzerland	interim
Cattle embryos from Norway	case-by-case basis
Goat semen from EU	case-by-case basis
Sheep semen from EU	case-by-case basis
Deer semen from EU	case-by-case basis
Goat embryos from EU	case-by-case basis
Sheep embryos from EU	case-by-case basis
Deer embryos from EU	case-by-case basis

Embryos

Cattle embryos from the EU

Prior to the export of this consignment each embryo donor must be certified as follows for Bluetongue:

- A competitive ELISA (cELISA) test for antibodies (according to the World Organisation for Animal Health - OIE Terrestrial Manual) to the BTV group between 21 and 60 days after collection of embryos in this consignment, with negative results

or

- An approved BTV isolation test or PCR test (according to the World Organisation for Animal Health - OIE Terrestrial Manual) on a blood sample taken on the day of collection of embryos of this consignment, with negative results

or

- The embryos were collected prior to 1 May 2006.

Cattle embryos from Switzerland

Prior to the export of this consignment each embryo donor must be certified as follows for Bluetongue:

- A competitive ELISA (cELISA) test for antibodies (according to the World Organisation for Animal Health - OIE Terrestrial Manual) to the BTV group between 21 and 60 days after collection of embryos in this consignment, with negative results

or

- An approved BTV isolation test or PCR test (according to the World Organisation for Animal Health - OIE Terrestrial Manual) on a blood sample taken on the day of collection of embryos of this consignment, with negative results

or

- The embryos were collected prior to 1 May 2006.

Semen

Cattle semen from Switzerland

Switzerland is not a member of the EU. Prior to 17 June 2008, Australian policy required cattle semen from Switzerland to be collected from donors resident in a bluetongue free country or zone. However, after 8 July 2008, interim conditions for the importation of cattle semen from Switzerland required BTV testing of donor bulls. These conditions include the following requirements:

The semen was collected, processed and stored in accordance with Council Directive 88/407/EEC.

Bluetongue

- Blood samples drawn from each donor between 28 and 60 days after final semen collection for this consignment, gave negative results to the competition ELISA for BT antibodies.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 7 days during semen collection and gave negative results to a virus isolation test for BT.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 28 days during semen collection and gave negative results to a polymerase chain reaction test for BT.

[The veterinary certificate must indicate the option that applies. The attached table must include dates of sampling for test, type of tests used, test results.]

Appendix 4

Australian import conditions

The following import conditions for bovine semen and embryos from the EU are set out in full and include interim conditions for bovine embryos for BTV.

Veterinary certification for the importation of bovine semen from Member States of the European Union

1. During the period between the first and last semen collection for this consignment, the donor lived in a country or zone recognised by the OIE as free from foot and mouth disease (FMD) where vaccination is not practised and met the OIE Code Article definitions of country freedom from:

- rinderpest
- vesicular stomatitis
- contagious bovine pleuropneumonia
- lumpy skin disease
- Rift Valley fever.

2. Foot and mouth disease (FMD)
The semen was not collected:

France: between 5 February 2001 and 23 June 2001 (inclusive of these dates).

Netherlands: between 12 February 2001 and 25 August 2001 (inclusive of these dates).

Republic of Ireland: between 1 February 2001 and 22 June 2001 (inclusive of these dates).

United Kingdom: between 1 January 2001 and 15 January 2002 and between 1 July 2007 and 18 February 2008 (inclusive of these dates).

Cyprus: after 24 September 2007 (inclusive of this date).

Bulgaria: after 2 December 2010 (inclusive of this date).

[These declarations need only be made if the semen is collected in one of the above countries]

3. The semen in this consignment was collected, processed and stored under conditions that comply with the standards laid down in Council Directive 88/407/EEC and updating legislation.

4. Johne's disease (M. paratuberculosis)
Each donor showed no clinical signs of Johne’s disease during the collection period.

5. Bluetongue

- Blood samples drawn from each donor between 28 and 60 days after final semen collection for this consignment, gave negative results to the competition ELISA for bluetongue antibodies.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 7 days during semen collection and gave negative results to a virus isolation test for bluetongue.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 28 days during semen collection and gave negative results to a polymerase chain reaction test for bluetongue.

[The veterinary certificate must indicate the option that applies. The attached table must include dates of sampling for test, type of tests used, test results.]

6. Epizootic haemorrhagic disease of deer (EHD)

- The semen was collected from donors resident in an EHD free country or zone for at least 60 days prior to, and during, semen collection.

or

- Blood samples drawn from each donor between 28 and 60 days after final semen collection for this consignment, gave negative results to either an agar gel immunodiffusion (AGID) test or a virus neutralisation test for EHD antibodies.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 7 days during semen collection and gave negative results to a virus isolation test for EHD.

or

- Blood samples were drawn from each donor at the commencement and conclusion of semen collection and at least every 28 days during semen collection and gave negative results to a polymerase chain reaction test for EHD.

[The veterinary certificate must indicate the option that applies. The attached table must include dates of sampling for test, type of tests used, test results.]

7. Infectious bovine rhinotracheitis/ Infectious pustularvulvovaginitis (IBR/IPV)

- The semen in this consignment complies with requirements for IBR/IPV laid down in Council Directive 88/407/EEC and updating legislation,

or

- The semen was collected from donors whose serological status is unknown or positive for IBR/IPV, and from which an aliquot of each semen collection for export was subjected to a virus isolation test (by cell culture inoculation and a minimum of 2 passages if no cytopathic effect observed on first passage) or real-time polymerase chain reaction (RT-PCR) assay, with negative results. Only collections that have been tested as described above are eligible for importation to Australia. Semen from bulls collected in periods between tests is not eligible.

[The veterinary certificate must indicate the option that applies. The attached table must include dates of sampling for test, type of tests used, test results.]

NOTE: Diagnostic tests and interpretation of test results for IBR/IPV must comply with the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter on Infectious bovine rhinotracheitis / Infectious pustularvulvovaginitis.

8. All blood tests for disease were carried out at a laboratory approved by the Veterinary Administration of the exporting Member State to perform the test required for that disease.

9. An Approved Veterinarian:

- ensured the isolation of the donors from all other ruminants not of equivalent health status prior to semen collection;
- supervised the isolation period;
- supervised the collection of specimens for testing;
- supervised the collection and processing of the semen in accordance with the standards laid down in Council Directive 88/407/EEC and updating legislation;
- ensured that suitable antibiotics were added to the diluent and that diluents were prepared in accordance with the standards laid down in Council Directive 88/407/EEC and updating legislation, and
- verified the permanent identification of the semen straws with the identification details of the donor and date of collection or a code from which this information could be determined.

10. For sex sorted semen, either:

- Sex sorted semen is NOT included in this shipment,

or

- Sex sorted semen IS included in this shipment, and:

- equipment used for sex-sorting sperm was cleaned and disinfected between animals according the sex semen licensor's recommendations; and

- where seminal plasma, or components thereof, was added to sorted semen prior to cryopreservation and storage, it was derived from animals of same or better health status.

[The veterinary certificate must indicate the option that applies.]

11. Shipping containers

- The shipping container was new.

or

- Prior to loading, the shipper was emptied and inspected and any loose straws removed. The shipper, including all surfaces contacting the straws, was disinfected with one of the following disinfectants: 2% available chlorine, Virkon at manufacturers recommended rate or irradiated at 50kGy.

Only new liquid nitrogen was added to the tank

[The veterinary certificate must indicate the option that applies. For used shippers, the date of disinfection, the disinfectant used and its active chemical must be recorded on the health certificate.]

12. Reproductive material for export to Australia was identified in a legible and non-erasable manner, and was stored since the end of the collection period until export under the supervision of the Approved Veterinarian(s) in containers in which no biological material other than semen, embryos or ova of equivalent health status as specified in this Veterinary Certificate was held.

13. For this reproductive material, either:

- Reproductive material was NOT removed from containers for further processing or aggregation with other reproductive material.

or

- Reproductive material was removed from containers for further processing or aggregation with other reproductive material at an approved centre or laboratory. The dates of transfer, reason for transfer (e.g. for sex sorting), name of the approved centre or laboratory and the Approved Veterinarian ARE listed against the containers. The unique serial (SEAL) number of each shipping container IS included in this documentation.

Date of transfer.....

Reason for transfer
.....

Name of approved
laboratory.....

Approved
veterinarian/s.....

Container serial
number/s.....

[The veterinary certificate must indicate the option that applies.]

Reproductive material was not removed from containers for further processing, or aggregation with other reproductive material unless under the supervision of the Approved Veterinarian at an approved centre or laboratory.

14. An Official Veterinarian sealed the semen transport container with an official government seal prior to shipment and the number or mark on the seal was recorded on the certificate prior to export.

Veterinary certification for the importation of bovine embryos from Member States of the European Union

These conditions allow the import of embryos derived from domestic cattle (*Bos taurus* and *Bos indicus*), and breeds derived from these species only.

1. Foot and mouth disease (FMD)
The embryos were not collected:

France: between 5 February 2001 and 23 June 2001 (inclusive of these dates).

Netherlands: between 12 February 2001 and 25 August 2001 (inclusive of these dates).

Republic of Ireland: between 1 February 2001 and 22 June 2001 (inclusive of these dates).

United Kingdom: between 1 January 2001 and 15 January 2002 and between 1 July 2007 and 18 February 2008 (inclusive of these dates).

Cyprus: after 24 September 2007 (inclusive of this date).

Bulgaria: after 2 December 2010 (inclusive of this date).

2. Each donor (both male and female) has been continually resident and free from any quarantine restriction for the 90 days immediately prior to collection in part of the territory of a Member State or States recognised by the OIE as a foot and mouth (FMD) free zone where vaccination is not practised according to the OIE Code Article definitions.

3. Each donor (both male and female) has been continually resident and free from any quarantine restriction for the 90 days immediately prior to collection in part of the territory of a Member State or States which meets the OIE Code Article definitions for country freedom from the following diseases:

- Rinderpest
- contagious bovine pleuropneumonia
- lumpy skin disease
- Rift Valley fever
- vesicular stomatitis

4. Bluetongue
Prior to the export of this consignment each embryo donor must be certified as follows for bluetongue:

- A competitive ELISA (cELISA) test for antibodies (according to the World Organisation for Animal Health - OIE Terrestrial Manual) to the BTV group between 21 and 60 days after collection of embryos in this consignment, with negative results

or

- An approved BTV isolation test or PCR test (according to the World Organisation for Animal Health - OIE Terrestrial Manual) on a blood sample

taken on the day of collection of embryos of this consignment, with negative results

or

- The embryos were collected prior to 1 May 2006.

[The veterinary certificate must indicate the option that applies. The attached table must include dates of sampling for test, type of tests used, test results.]

5. Bovine pestivirus

Prior to the export of this consignment of embryos each female donor gave a negative result to one of the following tests for bovine pestivirus:

- an antigen-capture enzyme-linked immunosorbent assay (ELISA) on peripheral blood leucocytes

or

- a virus isolation test on blood or serum.

6. The embryos must be fertilised in vivo, collected and processed in accordance with Council Directive 89/556/EEC and meet the requirements in Section 2 of this document. This must be certified in the Veterinary Certificate.

7. The embryos in this consignment were not subjected to micromanipulation involving breaching of the zona pellucida and all had intact zona pellucida at the time of storage.

8. The embryos in this consignment have been stored:

- only with other embryos or semen collected for export to Australia;
- in sealed containers
- since the end of the collection period until export in an approved secure place.

9. Reproductive material suitable for import into Australia was identified and placed under the supervision of the official veterinarian in:

- a new shipper

or

- prior to loading, the shipper was emptied and inspected and any loose straws removed. The shipper, including all surfaces contacting the straws, was disinfected*

[*The date of disinfection, the disinfectant used and its active chemical must be recorded on the health certificate.]

[The following disinfectants will be accepted by AQIS: 2% available chlorine, 35% formaldehyde, Virkon, or irradiated at 50kGy.]

10. Only new liquid nitrogen was added to the tank. The container was sealed with an official government seal prior to export and the seal number was recorded on the veterinary certificate.

Appendix 5

Consensus document between Australia and the European Union: Principles of Zoning and Regionalisation (SANCO/10157/2005)

Dated: 23 May 2005

I. OIE

Article 1.3.5.1 of the Office International des Epizooties (OIE) Terrestrial Animal Health Code (2004) describes zoning or regionalisation (for the purposes of the Code these have the same meaning) as a procedure implemented by a country to define geographical areas of subpopulations of different animal health status within its territory for the purpose of international trade in accordance with the relevant chapters of the Code.

Article 1.3.5.2 states: "The requirements necessary to preserve the distinct health status of a zone must be appropriate to the particular disease and will depend on the epidemiology of the disease, environmental factors, control measures and surveillance.

The extent of a zone and its limits should be established by the Veterinary Administration on the basis of natural, artificial or legal boundaries and made public through official channels.

Animals and herds belonging to subpopulations need to be clearly recognisable as such. The Veterinary Administration must document in detail the measures taken to ensure the identification of the subpopulation and the recognition and maintenance of its health status.

Thus defined, the zones constitute the relevant subpopulations for the application of the recommendations in Part 2 of the Terrestrial Code."

II. Relevant principles

Certain principles should be used as criteria for applying and assessing zoning and regionalisation (for simplicity the term zone is used henceforth). In terms of their level of relevance these principles are interdependent and variable, and depend on the epidemiology of the disease in the area in which zoning is applied. Their application and assessment depends on such factors as:

- OIE classification of the disease
- basic scientific knowledge of the epidemiology of the disease, in particular as regards animals and commodities causing spread of disease.
- the specificity of the zones: - Geographical factors,
  - (Micro) climatological factors,
  - Infrastructural factors,

- Environmental factors.

The application of these principles for trade in no way compromises the rights and obligations of both importing and exporting Parties under the Agreement on the Application of Sanitary and Phytosanitary Measures (WTO SPS Agreement).

III. The Principles

1) Zones of different health status:

a) - Zones may be established in the course of eradication measures to control an outbreak of a disease including zoonoses or,
- Zones refer to the presence or absence of the disease / pathogen in a zone, different prevalence of the disease in zones or the control measures (including vaccination) in place in the zones.

b) Zones of different health status are separate and distinct. The following zones can be distinguished: infected zone, free zone, buffer zone, control program/surveillance zone, zones with a certain prevalence and vaccinated zones.

2) Borders of the zone:

- The function of the borders is to protect and/or define the free/buffer/control zone;
- The borders of the zones may be legal, natural or artificial (geographical/ physical) barriers;
- Legal borders are legally determined for the competence of an administration such as countries, states, provinces, communes, other administrative entities such as Shires, Divisions, etc;
- Natural borders include mountains, rivers, seas, lakes, etc;
- Artificial borders include physical features such as roads, canals, railways, and intangible lines such as geographic information system coordinates;
- Regardless of the type of border used, the zone status of each animal and each farm or management unit should be clear. The choice of the type of borders should always take into account the best available option or combination of options.

3) Legislation:

- Effective legislation must be in force and available to enable the establishment, maintenance and control of the zones and their borders;
- Effective legislation must be available for movement controls and movement restrictions for a period of time under conditions to be determined by the Competent Authority (CA) for all susceptible animals and animal products and risk material (where relevant);
- Effective legislation must be available for imposing actions to control the disease in the zone and to manage the zones (surveillance, sampling etc);
- The criteria of this legislation are that:
- it must allow establishment or lifting of zones without delay
- it must not be hindered by procedural/competence/budget problems
- it must be risk based and flexible, reflecting the different levels of risk.

4) Powers and performance of the CA

- The CA is in most cases the official veterinary service but may be any service that has been given this responsibility. The CA must be able to count on an effective co-operation with the police, army and any other services necessary for the enforcement of the measures;
- The CA should be a central service with central power and in case of decentralised power (such as federal states and territories or autonomous regions) structured provisions and legislation should be available to ensure an appropriate interregional co-operation.

5) Disease reporting

The disease for which zoning is carried out must be notifiable or reportable. Quality of disease reporting/notification depends on:

- disease surveillance, early investigation and reporting
- legal provisions for the disease being notifiable/reportable to the CA
- public awareness
- history of disease occurrence
- compensation provisions in case of obligatory eradication measures
- legal penalties in case of non-compliance.

6) Epidemiological investigations

- Investigations should take into account the epidemiology of the disease under consideration. For diseases that can be transmitted by contagious means, investigations should focus on tracing forwards and backwards from positive disease findings. For non-contagious diseases the investigations should take into account relevant information related to possible vectors.
- The effectiveness of these investigations depends on:
  - Epidemiological knowledge of a given disease in the zone under consideration
  - Experience, performance and power of the CA
  - Performance of the laboratories
  - Knowledge of trade structures and pattern
  - Knowledge of degree of risk posed by feral animal reservoirs
  - Quality of record keeping systems
  - Stability of pathogen or vector systems
  - Traceability of animals and animal products (where appropriate) depends on good identification and/or registration systems.

7) Reliability of laboratory procedures

- Reliability of laboratory procedures is crucial for confirmation of diagnosis, epidemiological investigations, surveillance, and movement controls
- Reliability must be judged in qualitative and quantitative terms. Laboratory capacity and speed of reporting may be crucial in certain circumstances.

8) Movement control and trade restrictions

- Movement control concerns movements within and between zones
- For diseases that can be spread by contagious means, the stability of a certain disease status in a zone depends on an effective movement control, which depends on:

- Performance and power of the CA and its co-operation with other services
- Traceability of animals and animal products via identification and/or registration systems
- Quality of record keeping systems.

- For diseases that cannot be transmitted by contagious means, the value of movement controls on animals or animal products depends on the epidemiology of the disease under consideration.

9) Level of surveillance

- To effectively manage the zones surveillance must be carried out inside and outside the different zones
- Surveillance programs should consider the epidemiology of the disease, and may include active and passive surveillance, as appropriate according to scientific standards
- Confirmed and suspected cases should be followed by epidemiological investigations and surveillance
- Surveillance programs should be designed according to:

  - the disease agent as regards:
    - surveys for evidence of the agent
    - routine sampling on farms, markets and abattoirs
    - sentinel animal and vector trapping programs
    - banking of samples for retrospective surveys
    - analysis of laboratory records.
  - the host population as regards:
    - demographics
    - movement and trade patterns
    - interaction between domesticated and wild animals
    - animal identification and registration systems
    - management factors.
  - environmental factors as regards:
    - air and quality
    - vector distribution and competence
    - topography
    - meteorology
    - degree of uniformity of the above.
  - infrastructure as regards:
    - feed distribution
    - marketing, distribution and slaughter of animals
    - pharmaceutical and other relevant industries
    - veterinary and practice
  - measures taken in the zone – see (10) below.

10) Measures in the zones

If disease is detected in a free/buffer/control zone, the status of that zone must be reassessed. Scientifically supportable measures may be taken to protect or re-establish the status of the zone, including:

- stamping out
- movement control
- stand still
- vaccination (including safety of vaccines used).

11) Control of entry

Zones of higher health status should be protected from disease incursions by measures that consider the epidemiology of the disease and are consistent with international guidelines. These measures may include controls on the importation of animals, genetic material, animal products, fomites, animal feeds including swill, biologics and border audit (as appropriate). These controls are intended to apply (where appropriate) to the boundary of a free zone, which may or may not be a national border.

12) Notification to the OIE

Where applicable the party involved notifies the occurrence of the disease to the OIE in accordance with the OIE rules.

IV. Procedures

The CA with the responsibility for implementing the zoning policy (the exporting Party) is in the best position to define and maintain the zone. Providing the zone is defined and maintained according to the requirements of the importing Party in agreement with the criteria laid down in this document, the decision of this CA shall be the basis for trade.

In order to maintain confidence in the authority of the exporting Party, the exporting Party shall inform the importing Party on an ongoing basis and without delay of any evolution in the disease situation and any measure taken.

In determining whether trade in animals and animal products can occur the importing party may decide to carry out an inspection in the territory of the exporting party concerning the implementation and enforcement of the zoning provisions. Such an inspection shall be carried out without delay and shall be carried out on the basis of an audit, including an assessment of the performance of the CA. The past history of the results of previous checks and controls on importation should also be taken into account.

The final decision whether trade in animals and animal products on the basis of zoning can occur lays with the importing Party. In consultation with the exporting Party, the importing Party may decide on additional guarantees or risk mitigating factors, such things as deboning/maturation, treatment, quarantine, time delays and tests. Decisions on zoning and risk mitigation/management requirements will be made in a manner that ensures that the rights and obligations of both importing and exporting Parties under the WTO SPS Agreement.

Appendix 6

Diagnostic tests for BTV

Diagnostic tests prescribed for international trade in the OIE Terrestrial Manual (2010) are:

1. agent identification tests by virus isolation or reverse-transcription polymerase chain reaction (RT-PCR) assay, and
2. serological tests, namely the competitive enzyme linked immunosorbent assay (cELISA). The agar gel immunodiffusion (AGID) test was a prescribed test for international trade until 2008 but is no longer recommended for this purpose because of problems with cross reactions with other Orbiviruses, especially epizootic haemorrhagic disease virus (OIE 2010e).

The ability of these tests to detect all BTV serotypes and strains circulating or likely to emerge in a region has been reviewed recently (Mertens et al. 2009b) and is discussed below.

Agent tests for detection of BTV

In the early stages of BTV infection, infectious virus and antibodies typically coexist in the blood of an infected animal for up to three to four weeks in sheep and four to eight weeks in cattle (Kirkland and St George 1996). However, as virus is cleared from the animal, small persistent amounts of viral RNA can be detected by RT-PCR tests for long periods (sometimes over 200 days). The presence of small amounts of RNA does not necessarily mean that animals are capable of infecting a vector, and the infectious period is more accurately indicated by virus isolation (see below). Much of the antigen testing for BTV8 in Europe for trade were undertaken with RT-PCR tests but this hampered knowledge of the period for which livestock were infectious. International standards for trade recommend negative results from either PCR or virus isolation tests on the blood of donors where testing is required.

Virus isolation

The OIE Terrestrial Manual recommends isolation of BTV in embryonated chicken embryos, as the success rate in cultured mammalian cells *in vitro* is often less (Melville et al. 2005c; OIE 2010e; Worwa et al. 2010). Similarly, virulence of BTV8 was reduced after growth on mammalian cells (Oura et al. 2009). Although virus isolation is known to have lower diagnostic sensitivity than RT-PCR, this might be overcome by repeated sampling of donors during the collection period.

RT-PCR

RT-PCR tests for BTV generally have a very high analytical sensitivity, meaning that extremely small amounts of viral antigen can be detected in a sample. False positive results can occur from contamination of samples if laboratory procedures are not carefully observed and only validated RT-PCR tests with positive controls should be

used from laboratories which have undergone proficiency testing[25,26, 27]. Real time RT-PCR tests are typically more accurate (i.e. more sensitive and specific) than conventional RT-PCR tests in high throughput diagnostic systems (Rodriguez-Sanchez et al. 2008b; Shaw et al. 2007).

The diagnostic sensitivity of an RT-PCR test may be reduced, resulting in an unacceptable proportion of false negative results, if test primers are not appropriate for all topotypes and strains of BTV in a region. It is important that the specified PCR test for BTV can detect all BTV serotypes and strains/topotypes and is kept up to date. To specify such a test requires some understanding of the structure and nucleotide variability of the BTV genome and recent developments in PCR tests for BTV.

The 2009 OIE Terrestrial Manual Chapter 2.1.3 on Bluetongue outlines which of the ten viral RNA segments that comprise the BTV genome are most conserved between serotypes and topotypes of BTV. The BTV virus consists of the ten RNA segments (Seg) surrounded by seven structural viral proteins (VP) arranged in layers (Figure 7 and Table 8). Proteins in the core of the virus are more highly conserved (VP1, VP3, VP4, VP6 and VP7, coded by Segs 1, 3, 4, 9, and 7 respectively). The VP7 protein forms the outer core and, despite being the major serogroup-specific antigen, its structure can vary between different strains or topotypes of BTV. The proteins of the outer capsid, VP2 and VP5 (coded by Segs 2 and 6 respectively), are highly variable and determine antibody responses (serotype). The other three segments of the BTV genome code for 'non-structural' proteins (NS), which are found in BTV-infected cells. NS1 and NS2 (coded by Seg 5 and Seg 8) are conserved, while NS3/N3a (coded by Seg 10) is more variable (Maan et al. 2008; Verwoerd and Erasmus 2004). Some of the more conserved genes also show limited variation between topotypes (Maan et al. 2008).

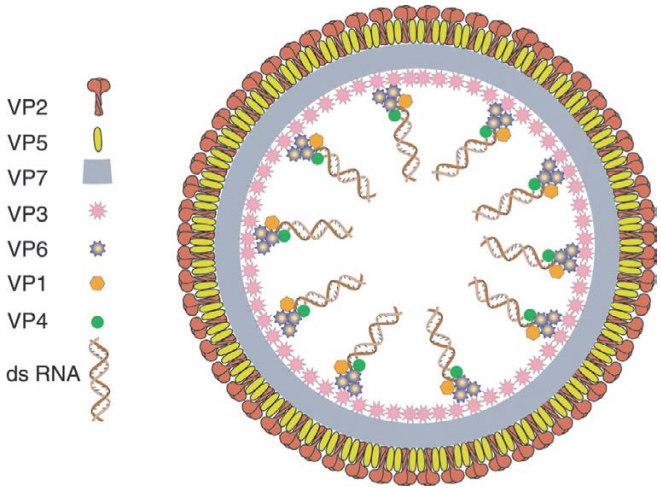

Figure 7. Representative arrangement of BTV structural proteins (VP) and double stranded RNA segments (dsRNA). Source: Schwartz-Cornil (2008) (http://dx.doi.org/10.1051/vetres:2008023)

[25] K. De Clercq, in plenary session of EPIZONE Conference, Brescia, Italy, 7 June 2008.
[26] S. Zientara (*personal communication* 22 September 2008).
[27] P. Mertens (*personal communication* 24 September 2008).

Table 8. The nucleotide variability of the ten RNA segments which code for structural viral proteins (VP) comprising the core and capsid of BTV and non-structural proteins (NS).

Protein type	Viral Protein	RNA segment	Nucleotide variability
Core	VP1	1	Conserved
	VP3	3	Conserved
	VP4	4	Conserved
	VP6	9	Conserved
	VP7	7	Conserved but some variation (not associated with topotype). Major serogroup specific antigen.
Capsid	VP2	2	Highly variable. Determines BTV serotype. Also varies with topotype.
	VP5	6	Highly variable. Determines BTV serotype. Also varies with topotype.
Non-structural	NS3/N3a	10	Conserved but varies with topotype
	NS1	5	Conserved
	NS2	8	Conserved

Primers derived from genes which are highly conserved in all 24 serotypes (Segs 3, 5, 7 and 9) may be used for serogrouping (i.e. will react with all serotypes of BTV) (Mertens et al. 2009b; OIE 2010e; Shaw et al. 2007). These tests are referred to as serogroup specific or pan-reactive tests.

RT-PCR tests using primers for nucleotide sequences in Seg 2 can be used to identify virus serotype. Several RT-PCR assays targeting Seg 2 have been developed for rapid identification of each of the 24 serotypes of BTV (Mertens et al. 2007a), while other RT-PCR assays targeting Seg 2 detect only subsets of BTV serotypes, e.g. only those circulating in the United States (Johnson et al. 2000).

Description of different RT-PCR tests

Primers used in a RT-PCR test for BTV are of critical importance to ensure that new and emerging strains of BTV are detected, as well as differentiation of field and vaccine isolates (Hoffmann et al. 2009a; Mertens et al. 2007b; Zientara et al. 2004). RT-PCR tests for highly conserved BTV genome segments can detect BTV regardless of serotype, while other tests can determine different serotypes and topotypes within each serotype. Tests for health certification of donors of ruminant germplasm need to detect BTV infection, rather than determine BTV serotype.

RT-PCR tests were developed for detecting all 24 serotypes of BTV with similar cycle threshold (Ct) values. These tests do not differentiate serotypes and use highly conserved regions of RNA. While Seg 10 (Elia et al. 2008; Gaunitz et al. 2008) and Seg 5 (Jiménez-Clavero et al. 2006; Polci et al. 2007) were used in this way, recent findings from full genome sequencing of BTV raised doubts about whether these, and several other gene sequences that were previously considered to be highly conserved,

are consistently preserved between serotypes and topotypes[28]. RT-PCR tests need to detect segments conserved in both 'eastern' and 'western' BTV groups and topotypes and reliably detect all serotypes and topotypes of BTV (Hoffmann et al. 2009a; Maan et al. 2007b; Maan et al. 2008). These problems may be overcome by real time RT-PCR assays based on primer-probe energy transfer (PriProET) which tolerate mutations and have the potential to detect variants of BTV (LeBlanc et al. 2010). The RT-PCR assay of Orru *et al*. (2006) targets Seg 10 but was only validated for BTV serotypes 1–9 and 16.

Published pan-reactive RT-PCR tests validated for all 24 serotypes of BTV are presented in Table 9. These tests were not validated for TOV. In general, real time RT-PCR assays, such as those of Shaw *et al*. (2007) and Toussaint *et al*. (2007), are preferable to conventional gel-based methods because they have the advantage of being less vulnerable to contamination and are suited to high-throughput diagnostic systems. A pan-reactive test targeting Seg 9 is under development (Mertens et al. 2009b). Further testing of the assay developed by LeBlanc *et al.* (2010) was required to determine its capacity to detect different strains of BTV.

Table 9. RT-PCR tests validated for all 24 BTV serotypes.

Group	Primer	Test type	Reference
Toussaint *et al.*	Two primers for Seg 1 and Seg 5.	Two real time RT-PCR assays using TaqMan® probes[29].	Toussaint *et al.* (2007)
Shaw *et al.*	Two primers for 'eastern' and 'western' sequences of Seg 1.	Real time RT-PCR assay using TaqMan® probes for two primers combined in a single tube reaction.	Shaw *et al.* (2007)
Anthony *et al.*	Two primers for different sections of Seg 7.	Duplex one-step gel-based RT-PCR.	Anthony *et al.* (2007)
LeBlanc *et al.*	Conserved region of Seg 10	Real time RT-PCR assay based on primer-probe energy transfer (PriProET)	LeBlanc *et al.* (2010)

RT-PCR can also be used to distinguish vaccination from infection, and infection in an individual with multiple BTV serotypes. Infection with more than one serotype can make serum neutralisation assays for BTV unreliable (Johnson et al. 2000; Mertens et al. 2007b). However, as discussed below, the tests of Shaw *et al*. (2007) and Toussaint *et al*. (2007) failed to detect TOV because of mismatches between primer sequences (Hofmann et al. 2008).

Validity of several RT-PCR tests for BTV8

Both tests developed by Shaw *et al*. (2007) and Anthony *et al.* (2007) are used in the EU Community Central Reference Laboratory for BTV at the Institute for Animal

[28] S. Maan (*personal communication* Bluetongue satellite symposium, Brescia, Italy, 7 June 2008)
[29] see Glossary

Health, Pirbright, United Kingdom. In ring trials of PCR assays used by 14 National Reference Laboratories in the EU conducted in June and September 2006, BTV8 was detected in sera by all six different assays (Batten et al. 2008a). Four laboratories used the real time RT-PCR tests methods of Shaw *et al*. (2007), two used methods of Toussaint *et al*. (2007), two used methods of Jiménez-Clavero *et al*. (2006), one used the method of Polci *et al*. (2007) and three laboratories in Holland and two in Germany used unpublished tests.

In these trials, all real time RT-PCR tests detected BTV8 by three days post infection in sheep and five days post-infection in cattle, except the test using the method of Jiménez-Clavero *et al*. (2006) (Batten et al. 2008a). The latter test, developed for detection of BTV in the Mediterranean basin, did not reliably detect BTV8 at 27 days post-infection in sheep or cattle although other tests were positive. Conventional (gel-based) RT-PCR tests used by nine of the National Reference Laboratories detected BTV8 at five days post-infection in sheep and ten days post-infection in cattle but were unreliable at 27 days post-infection. The gel-based RT-PCR test described by the OIE (2004) reliably detected BTV8 in cattle earlier (at five days post-infection) than the method described by Anthony *et al.* (2007).

In a later ring trial conducted in September 2007, some laboratories used more than one BTV assay and nine of 26 laboratories using RT-PCR used unpublished assays (Batten et al. 2008c). Ten laboratories used the method of Shaw *et al*. (2007) compared with four in the ring trial of the previous year; five used Toussaint *et al.* (2007); one used Jiménez-Clavero *et al.* (2006); one used Polci *et al*. (2007); five used an unpublished assay by Hoffmann *et al*. and four used other unpublished assays. The ability to detect new serotypes of BTV was limited in two laboratories using the method of Shaw *et al*. (2007) because they failed to use both primers required to detect both ‘eastern’ and ‘western’ topotypes of BTV.

This trial also indicated that some of the ten laboratories had difficulties in running particular gel-based PCR assays. BTV8 was detected in sheep and cattle samples in only one of six laboratories and two of six laboratories using the method of Anthony *et al.* (2007), respectively. In contrast, all four laboratories using the OIE method detected BTV8 in both species (Batten et al. 2008c). A second part of the study investigated whether laboratory throughput could be increased by pooling blood samples. The sensitivity of the assay by Shaw *et al*. (2007) to detect BTV8 on the first day of viraemia was compromised in diluted blood.

Based on results of seven RT-PCR and two gel-based PCR assays used in European National Reference Laboratories in 2007, Batten *et al.* (2008c) recommended that:

1. all National Reference Laboratories develop real time RT-PCR assays for BTV rather than using conventional methods,
2. laboratories need to constantly update PCR primers as new BTV topotypes emerge and regularly check for new serotypes,
3. laboratories need to regularly check for new serotypes, and
4. pooling samples leads to an unacceptable reduction in test accuracy.

By 2008, 22 of 25 National Reference Laboratories were using real time RT-PCR assays for BTV (Batten et al. 2010).

Validity of several RT-PCR tests for other serotypes of BTV and TOV

The need for tests which can detect new variants and serotypes of BTV in a region was highlighted by the spread of BTV1 in northern Europe and the emergence of

BTV6, BTV11 and TOV. Because the RT-PCR tests used in the Netherlands in October 2008 were not able to identify all serotypes of BTV, there were substantial delays in detecting an MLV vaccine strain of BTV6 (Promed Mail 2008u). This also led to misleading preliminary conclusions being made that this virus was a variant strain of BTV8 (Promed Mail 2008s; Promed Mail 2008t). TOV was identified using a modified version of the RT-PCR test of Orru *et al.* (2006) but it was not detected by pan-reactive assays developed by Shaw *et al*. (2007), Toussaint *et al.* (2007) and Hofmann *et al.* (2008). BTV11 was first detected using the pan-reactive assay of Toussaint *et al.* (2007) and confirmed using serogroup- and serotype-specific RT-PCR assays (Promed Mail 2009b).

As mentioned earlier, several research groups developed PCR tests to detect all established 24 BTV serotypes (Gaunitz et al. 2008; Maan et al. 2007a). The real time RT-PCR assay of Shaw *et al*. (2007) was able to detect 130 of 132 isolates from European BTV outbreaks from 1998 to 2007. The gel-based RT-PCR of Anthony *et al.* (2007) was able to reliably detect isolates of all 24 serotypes of BTV from a range of geographic locations derived from tissue culture, vectors and mammalian blood and tissues. Pan-reactive PCR assays were used in Great Britain and Denmark's national BTV surveillance programs in 2010 (SCFCAH 2010c; SCFCAH 2011a). RT-PCR tests were also developed for specific serotypes e.g. BTV1 (Aguero et al. 2008; Hoffmann et al. 2009b; Vandenbussche et al. 2009).

Validation of some other real time RT-PCR tests used in EU laboratories is limited:

- The assay based on the description of Toussaint *et al.* (2007) used in Belgium in 2006 had a diagnostic sensitivity of 99.5% (95% CI: 99.0–100.0%) and specificity of 98.5% (95% CI: 97.1–100.0%) (Vandenbussche et al. 2008b).
- The assay developed by Toussaint *et al.* (2007) detected field and vaccine strains of BTV 2, 4, 9 and 16. The limit of detection varied with virus serotype and strain from 0.005 to 0.05 $TCID_{50}$/ml.
- The effect of dilution on the assay of Toussaint *et al.* (2007) showed that ten-fold dilutions of samples reduced the sensitivity to 60% at 0.06 $TCID_{50}$/ml (95%CI: 42.2–75.5%), and 10% at 0.006 $TCID_{50}$/ml (95%CI: 3.6–25.7%). Specificity determined from 40 negative sheep and goat sera was 100% (95%CI: 92.5–100%).
- The assay of Jiménez-Clavero *et al.* (2006) detected all serotypes and strains circulating in the Mediterranean basin to viral concentrations in samples of 0.0054 $TCID_{50}$/ml but only 15 of 24 reference strains of BTV serotypes (Batten et al. 2008a).

A BTV RT-PCR assay developed by the Institute for Animal Health, Pirbright, United Kingdom which can detect 24 BTV serotypes, was recently licensed as a kit for commercial release (Qiagen 2008). Rapid development in some laboratories of a real time RT-PCR for BTV may mean that improved tests are being used but are unpublished.

RT-PCR testing of semen

Detection of BTV by isolation of virus from semen is impeded by toxicity of the spermatozoa and seminal plasma for cell culture (Howard et al. 1985). PCR testing of semen for BTV was suggested in the 1990s but there were problems with validating diluted semen because of inhibition by egg yolk in semen extenders (Akita et al. 1993). Test validation on ten samples of semen collected commercially from

seronegative and seropositive bulls was also hampered by the intermittent presence of BTV in semen (Wilson 1999).

A PCR assay to detect BTV RNA in bovine semen has been partly validated (Vanbinst et al. 2010) and another is under development in Australia in a joint project between Berrimah Veterinary Laboratory and NSW Agriculture Elizabeth Macarthur Agriculture Institute (EMAI) (Kirkland et al. 2010).

RT-PCR tests and vaccines

In a safety study of MLV against BTV2 in Corsica in 14 sheep, RT-PCR was able to detect vaccine virus in blood at titres as low as 1 $TCID_{50}$/ml over four to 32 days post-infection, while viraemia was not detected by virus isolation (tested at four, seven and 11 days post-infection) (Hammoumi et al. 2003).

Safety studies of inactivated BTV8 vaccines used three RT-PCR assays: the pan-reactive assays of Shaw *et al*. (2007) and Toussaint *et al*. (2007) and a specific assay for BTV8 developed by Hoffmann *et al*. (unpublished) (Eschbaumer et al. 2009; Oura et al. 2009).

Serological tests for BTV

As stated in the revised chapter on bluetongue in the OIE Terrestrial Manual (2009), AGID and indirect ELISA (iELISA) tests for BTV are not recommended because they are prone to cross reactions and require specific antisera to be developed for target species. These tests were superceded by cELISA tests, which can detect antibody to the BTV group as early as seven to eight days post-infection. This is much sooner than AGID tests (Kirkland and St George 1996; Shad et al. 1997).

Persistence of BTV8 antibody in naturally acquired infections is yet to be thoroughly investigated. Antibody to Australian BTV serotypes was reliably detected in naturally infected cattle for four years by cELISA but AGID testing showed a decline in antibody levels after one year, with 90% of cattle demonstrating detectable antibody at four years (Melville et al. 2005a).

cELISA

In two ring trials conducted to compare the performance of 23 EU National Reference Laboratories in 2006, six commercial cELISA kits tested against all 24 serotypes of BTV were able to detect all BTV serotypes circulating in Europe (Batten et al. 2008a). All cELISA kits detected BTV8 antibodies in cattle and sheep by 21 days post-infection and most by nine days post-infection.

A second ring trial of 27 National Reference Laboratories from EU Member States and three non-EU countries was conducted in September 2007 (Batten et al. 2008c). These laboratories tested a panel of 11 BTV serotypes (BTV1, 2, 3, 4, 8, 9, 10, 11, 15, 16, 23), including BTV8 from experimental animals collected at 21 days post-infection, with six commercial cELISA kits. All six assays were considered ‘fit for purpose’ in that they detected antibodies to the BTV serotypes circulating in Europe (BTV1, 2, 4, 8, 9, and 16) and BTV8 at 21 days post-infection. All kits correctly identified two negative sera but one kit failed to detect BTV10 and two had reduced sensitivity to BTV10 and 16.

However the diagnostic sensitivity of commercial kits used to test field sera when BTV is spreading or re-enters the population is poor. The OIE Code (2008)

recommends that serological testing to detect antibody to BTV is performed between 21 and 60 days after semen collection and 28 days prior to movement of susceptible species from a BTV-infected zone.

Testing during the BTV8 epidemic in Europe resulted in a low sensitivity for cELISA (87.8% with 95%CI: 85.1–91.1) compared to RT-PCR (99.5% with 95%CI: 99.0–100.0), while specificity was comparable (98.2% with 95%CI: 96.3–99.6 for cELISA, and 98.5% with 95%CI: 99.0–100.0 for RT-PCR) (Vandenbussche et al. 2008b). Similarly, during the 2001 BTV2 outbreak in Corsica, diagnosis of bluetongue infection using a commercial cELISA kit (VMRD Inc, Pullman, WA, USA) was unacceptably low (sensitivity: 78%, specificity: 85%) compared with RT-PCR results in field samples collected from 113 sheep (Biteau-Coroller et al. 2006). The test also had unacceptable accuracy (sensitivity of 70.8% and specificity of 87.2%) in 2 366 cattle sera collected during the vaccination campaign with MLV on Corsica during 2002. The specificity of this test was 100% when determined from 4 593 sera from cattle in mainland France from 2001 to 2004 (which was uninfected with BTV at that time). Several other references in the scientific literature report variable test sensitivity and specificity in relation to the commercial cELISA tests used (Afshar et al. 1989; 1993; Anderson et al. 1993; Cagienard et al. 2006; Gustafson et al. 1992; Jeggo et al. 1992; Lelli et al. 2003; 2004; Reddington and Reddington 1992; van Donkersgoed et al. 2004; Velic et al. 2004).

The poor sensitivity of the cELISA tests may be partially due to sampling animals yet to develop sufficient antibody responses, because BTV antibodies can only be detected from six to ten days post-infection (Koumbati et al. 1999). However, it is unlikely that such high proportions of BTV positive animals would be in early stages of infection at the time of sampling (between 12.2 and 29.2%). One possible explanation for the reduced sensitivity of cELISA testing in the field is that the infective dose of virus can also affect the sensitivity and timeliness of serological detection. Intermediate levels of infection with BTV8 delayed the appearance of antibodies to 22 days post-infection compared to ten to 14 days post-infection in the high dose group in studies of different infective doses of low passaged virus injected subcutaneously into groups of four lambs (Domingo et al. 2008).

Receiver operating characteristic analysis of cELISA test results obtained during an epidemic of BTV in Belgium showed that changing the test cut-off point could improve test accuracy (Vandenbussche et al. 2008b). However, this is not practical for large-scale screening as it requires interpretation of results against RT-PCR results to recalibrate each commercial kit. Most diagnostic laboratories processing large numbers of samples are only in a position to use the manufacturer's cut-off point.

In Germany in 2008, about 30 regional laboratories in 16 federal states performed tests for BTV using four commercial cELISA kits and a 'validated' but unpublished real time RT-PCR (Hoffmann et al. 2008). Positive results are confirmed by the National Reference Laboratory at the Friedrich-Loeffler–Institut, Insel Reims. Inoculation of three cattle with blood from a BTV8 infected cow resulted in a positive result by real time RT-PCR at 2 days post-infection in all animals. Seroconversion by cELISA occurred at 16 days post-infection in one animal but was not detected until day 30 in another and was not evident at day 30 in a cow given the highest dose of virus (Hoffmann et al. 2008).

Tests that allow early detection of antibodies to BTV after infection have clear advantages for screening semen and embryo donors. Inoculation of three lambs and

five calves with laboratory adapted BTV serotypes 2, 10, 11, 13 and 17 showed that all lambs were seropositive by AGID at two weeks and all the calves at four weeks (Reddington et al. 1991). Seroconversion after inoculation with BTV10 was not detected by cELISA until 21 days post-infection in all animals (eight sheep) (Shad et al. 1997). In a comparison of a commercial cELISA (BPS) kit with cELISA-I (conventional) tested against 24 serotypes of BTV[30] inoculated into 24 calves, detection at ten days post-infection was variable (BPS detected 19 of 24, while I-ELISA detected 23 of 24 serotypes). At 20 days post-infection, BPS detected 22 of 24 serotypes, I-ELISA detected all 24 serotypes but by 40 days post-infection both tests had detected all serotypes (Afshar et al. 1992; 1993). All 35 cattle inoculated with BTV11 were positive by 19 days post-infection using a cELISA test (Gustafson et al. 1992).

ELISA testing of bulk milk for BTV using blocking ELISA in Australia (Kirkland 2008) and iELISA in the Netherlands (Kramps et al. 2008) was validated and used as a surveillance tool in Switzerland (Hadorn et al. 2008) and Sweden (Promed Mail 2008l). Very early detection of all 24 serotypes of BTV (at five to seven days post infection) with an ELISA to detect antibodies specific to BTV VP7 was validated for use in serum or milk (Ranz et al. 2008).

Virus neutralisation tests (used to identify BTV serotype) in experimentally infected sheep indicated that low levels of neutralising antibodies may be a feature of some field strains of BTV8 (Worwa et al. 2009).

Persistence of BTV8 antibody in naturally acquired infections is not yet thoroughly investigated.

Detection of BTV6 and BTV11 in Europe

The isolate of BTV6 which appeared in October 2008 in the Netherlands was first detected with a gel-based RT-PCR using primers from VP-2 developed at the BTV Community Central Reference Laboratory by P. Mertens and S. Maan (Promed Mail 2008t). This test can identify all 24 serotypes of BTV. This test was used in an earlier study of virus isolates present in Europe up until 2007 (Mertens et al. 2007b) which did not, therefore, attempt to identify BTV6. It is not clear whether this test was used to detect exotic isolates of BTV in the United States, which included BTV6 (Johnson et al. 2007).

Samples containing BTV6 were not included in earlier ring trials of RT-PCR assays for BTV in EU National Reference Laboratories (Batten et al. 2008a; Batten et al. 2008c). Validation studies of various RT-PCR tests used in EU National Reference Laboratories to detect BTV show that tests published by the following authors detected BTV6 (based on a single sample of an isolate of BTV6 from South Africa): Shaw *et al.* (2007); Anthony *et al.* (2007); Toussaint *et al*. (2007) and Jimenez-Clavero *et al*. (2006). Detection of BTV6 by the following RT-PCR tests was not known: Polci *et al*. (2007); Hoffmann (unpublished) and other unpublished ('in house') tests, including those following OIE protocols. In late 2008 a limited ring trial of 13 National Reference Laboratories showed that BTV6 was detected by all BTV serogroup and BTV6 serotype-specific RT-PCR assays (Batten et al. 2010).

[30] BTV serotypes 1 to 20 from South Africa and BTV serotypes 10, 11, 13 and 17 from the United States

cELISA tests used in the EU should detect donors which are seropositive to BTV6 as well as BTV8. Antisera from a South African isolate of BTV6 were included in ring trials to test cELISA kits used in EU National Reference Laboratories in 2006 (Batten et al. 2008a) but not in 2007 (Batten et al. 2008c). In the 2006 trials, all 27 cELISA tests in 23 participating EU National Reference Laboratories detected BTV6 (Batten et al. 2008a). Further evaluation is required of cELISA kits to detect the BTV6 strain which emerged in the Netherlands in October 2008.

In late 2008, tests at the National Reference Laboratory in Belgium established that a new serotype of BTV had been detected using the RT-PCR test of Toussaint *et al*. (2007), which was confirmed with serogroup and serotype-specific RT-PCR assays . However, results for serum neutralisation tests were equivocal and final confirmation of BTV11 was undertaken at the BTV Community Central Reference Laboratory, Pirbright (Promed Mail 2009a; Promed Mail 2009b; Vandenbussche et al. 2009). Ring trials of EC National Reference Laboratories included samples for BTV11 and there was no report of failure to detect this serotype with commercial cELISA kits (Batten et al. 2008c). Four serotype-specific RT-qPCR assays for BTV serotypes 1, 6, 8 and 11 were developed in Belgium and used to detect these serotypes in imported animals in late 2008 (Vandenbussche et al. 2009). Similar assays for BTV serotypes 1, 6 and 8 were developed in Germany and used in regional veterinary laboratories (Hoffmann et al. 2009b).

Detection of Toggenburg Orbivirus

TOV was first investigated in clinically healthy goats in Switzerland in early 2008 which were positive at low levels to cELISA and a real time RT-PCR assay (Hofmann et al. 2008). The RT-PCR assay used was a modification of an assay developed by Orru *et al*. (2006) but this test does not identify all 24 established serotypes of BTV. Other RT-PCR tests were negative, including those developed to detect all known 24 BTV serotypes by Shaw *et al*. (2007) and Toussaint *et al*. (2007) because of mismatches between primer sequences. Sequencing of seven of ten viral RNA segments showed that the gene encoding VP2 placed TOV in the BTV serogroup. However, TOV was considered a completely new serotype of BTV because the greatest similarity to sequences of known BTV serotypes was 63% (for Seg 2) and 79% for (Seg 7 and Seg 10). A recently developed RT-PCR assay from Switzerland appears to detect all 24 serotypes of BTV as well as TOV[31] and a TOV specific RT-qPCR assay is available (Hofmann et al. 2010).

The virus was transmissible to sheep and goats by inoculation from infected animals but inoculated goats failed to show signs of disease (Hofmann et al. 2008). Inoculation of cattle was not attempted. At the time of writing, TOV could not be isolated from embryonated chicken eggs or mammalian or insect cell lines (Chaignat et al. 2009; Hofmann et al. 2010; Planzer et al. 2011)[32].

Not all commercial cELISA tests can detect TOV antibodies. In samples from field infections of goats and experimental infections of sheep, TOV antibodies were detected by BDSL, VMRD and INGENASA cELISAs, but BDSL appeared to be much more sensitive. TOV antibodies were not detected by IDVET and Pourquier cELISA tests (Chaignat et al. 2009).

[31] P. Kirkland, NSW DPI (*personal communication* 5 March 2009)
[32] P. Kirkland, NSW DPI, (*personal communication* 9 December 2008)

Test standardisation and validation

The rapid spread of BTV8 resulted in the rapid development of diagnostic tests for this strain, particularly RT- PCR tests. However, as mentioned earlier, some of these were not yet validated in the field[33]. The OIE Terrestrial Manual (2010e) recommends that diagnostic RT-PCR tests for BTV are accredited to an international standard such as ISO/ISEC 17025 and that laboratories participate in proficiency testing.

The European Commission regulates tests for certain diseases, mainly those on former OIE list A (including BTV) and major public health infections[34]. For EU regulated diseases, one national laboratory is appointed as a Central Community Reference Laboratory which is contracted to coordinate and provide quality assurance on the 27 National Reference Laboratories. The Institute for Animal Health, Pirbright in the United Kingdom is the Central Community Reference Laboratory for BTV and conducts ring testing of National Laboratories for BTV. The Central Community Reference Laboratory in many cases prepares a diagnostic manual which sets out procedures for laboratories to use within the EU. Where regulated tests are in place, it is the task of the National Reference Laboratory of each Member State to ensure that other laboratories in the country are following appropriate methods. Quality controls are also important for individual tests. For example, internal controls in RT-PCR assays, which ensure availability of sufficient target RNA, and checks that the reverse transcription and PCR amplification steps have occurred, were estimated to prevent 0.6% of 1 985 field samples being diagnosed incorrectly as negative (Vandenbussche et al. 2008a).

[33] K. De Clercq, in plenary session at EPIZONE Bluetongue Symposium, Brescia, Italy, 7 June , 2008
[34] P. Kirkland, NSW DPI, (*personal communication* 18 July 2008)

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