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Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep

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- FIRST PART –
BLUETONGUE DESEASE

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I. - INTRODUCTION

Bluetongue (BT) is a non-contagious, insect-transmitted disease of certain species of domestic and wild ruminants that is caused by BT virus (BTV), (MacLachlan & Pearson, 2004; Verwoerd & Erasmus, 2004). BTV infection of ruminants occurs throughout much of the temperate and tropical regions of the world, coincident with the distribution of specific species of Culicoides biting midges that are biological vectors of the virus (Gibbs & Greiner, 1994; Tabachnick, 2004). BT typically occurs when susceptible sheep are introduced into areas where virulent strains of BTV circulate, or when virulent strains of BTV extend their range into previously unexposed populations of ruminants. The global distribution of BTV has historically been between latitudes of approximately 40-50°N and 35°S but the virus recently (since 1999) has spread northward in parts of the Mediterranean Basin and appeared in North-Western Europe in 2006 (far beyond its prior known upper northern limits anywhere in the world). Palearctic region of Europe is the only continent other than Antarctica currently free of BTV infection.

From 1998 the incursion of BTV into the Mediterranean Basin is causing great economic losses, partly due to the disease itself, but mostly linked to the total ban of ruminant trade between the infected and non infected areas.

During a BTV-2 epidemic in Italy in 2000-2001, approximately 263.000 diseased sheep and goats were reported and 48.000 sheep and goats died. During a second epidemic in 2001-2002, approximately 251.000 diseased sheep and goats were reported and 73.000 sheep and goats died. In 2007 BTV-8 outbreaks occurred on over 20.000 farms in Germany with disease in ca, 35.000 cattle, sheep or goats.

In Sardinia BT was first reported on 18 August 2000 in a flock located in the south-western part of the island in the Cagliari Province. Serological confirmation of BT was obtained on 24 August and BTV-2 was isolated by the National Reference Centre for Exotic Diseases (CESME - Centro Studi Malattie Esotiche) on 1 September and identified subsequently by the Onderstepoort Veterinary Institute. In September 2003 circulation of BTV-4 was detected in Sardinia. In January 2004 circulation of BTV-16 was found by virus neutralisation test in 7 sentinel animals in the north-eastern part of Sardinia. Serotype 1 was first notified on 15 October 2006 in the south-western part of the island.

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The indirect costs associated with bluetongue can be much greater than the direct costs. Bluetongue is a "List A" disease of the Office of International Epizootics (OIE). List A diseases are those diseases which can spread rapidly and that have a considerable impact on the health of livestock. List A diseases are notifiable to the OIE, and often used as non-tariff trade barriers to prevent livestock from moving and potentially spreading disease. Livestock from bluetongue endemic areas, or regions where there are outbreaks, can be prevented through regulatory policy from moving to regions considered bluetongue-free. The economic impact in reducing livestock movement and trade can be considerable.

In an attempt to reduce direct losses due to disease and indirect losses due to the trade embargo caused by virus circulation, the Italian government has, since February 2002, been carrying out a compulsory BT vaccination campaign of all domestic ruminants using modified-live virus vaccines (MLV) produced by Onderstepoort Biological Products (OBP), South Africa. Based on the serotype/s present in a given area, various MLV monovalent serotype formulations were used.

Aim of this work was to evaluate the safety and the efficacy of BTV serotype 1 MLV vaccine, used in BT vaccination campaign of 2007.

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II. - HISTORY

The disease of BT was first described as 'malarial catarrhal fever' and 'epizootic catarrh of sheep' in the original written descriptions by investigators in South Africa. The name of "bluetongue" was later used to describe the distinctive cyanotic tongue of some severely affected sheep. The first descriptions of BT, an arboviral disease of ruminants, were published in the late 19th and early 20th centuries, although farmers in South Africa recognised the disease soon after the introduction of fine-wooled European breeds of sheep to that region of the world (Erasmus, 1975; Spreull, 1905). Prior to the 1940s, BT was thought to be confined to southern Africa. There is some evidence for the occurrence of bluetongue in Cyprus in the 1920s but the disease was not officially recognized outside of Africa until 1943, when a significant epidemic occurred in Cyprus and Palestine (Gambles, 1949). Bluetongue was soon recognized in several countries of the Middle East. In 1952 there was an extensive epidemic of blue tongue in California but this was eclipsed by an epidemic in Portugal and Spain in 1956/57 that killed about 180.000 sheep. The rapid spread of this potentially devastating disease prompted the publication by the Food and Agriculture Organization of the United Nations (FAO) of a book on Emerging Diseases of Animals in 1963, and the disease was added to List A of the World Organisation for Animal Health (OIE), thereby restricting the trade in animals from affected countries. Bluetongue viruses continued to spread, being found in Australia in 1977 and tropical America in the 1980s, but the worldwide absence of any significant losses to clinical bluetongue led in the 1990s to a reassessment of its significance. By the end of 2001 at least 10 Mediterranean countries have been affected by bluetongue, including six (France, Italy, Bulgaria, Macedonia, Yugoslavia and Tunisia) that had never previously experienced the disease and two within which hit her to unaffected areas (mainland Greece and the Balearic islands of Spain) have now been affected. The epidemic, which may not yet be over, has already claimed more than 250.000 sheep and this is, therefore, the largest epidemic of bluetongue yet seen. Despite this statistic, however, the real significance of the epidemic lies elsewhere.

In the Old World, bluetongue viruses are primarily transmitted by a single species of biting midge, Culicoides Imicola (Mellor *et al.*, 2000). This species also transmits the closely related African horse sickness virus. Between the late 1960s and 1998, there were several outbreaks of bluetongue and African horse sickness in the

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Mediterranean region and all were in countries that had also been affected in previous years (The Veterinary Journal 2002, available online at http://www.idealibrary.com).

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III. - AETIOLOGY

III.1 - BT VIRUS, TAXONOMY AND STRUCTURAL BIOLOGY

BTV belongs to the genus Orbivirus of the family Reoviridae, wich currently contains twelve genera of multi-segmented dsRNA viruses, including pathogens of a wide range of insects, reptiles, fish, crustaceans, mammals (including humans), plants and fungi, many of which are of economic, veterinary or medical importance, (Mertens, 2000). These viruses can be distinguished and identified by a number of different characteristic features, including capsid structure, number and size distribution of genome segments, host range, serological properties, protein composition, disease symptoms and most recently by sequence analyses and comparisons of individual genome segments (Tables I and II).

The orbiviruses (which are classified as members of the genus Orbivirus, within the family Reoviridae) characteristically have a ten-segmented dsRNA genome that is packaged as one copy of each segment within an icosahedral protein capsid (~85 nm diameter). The viruses are resistant to lipid solvents, which is typical of nonenveloped viruses. The viruses are relatively acid-labile, and slow freezing at -10 to -20 °C is deleterious to the virus. BTV is the prototype species of twenty-one different Orbivirus species now recognised by the International Committee for the Taxonomy of Viruses (Mertens, 2004).

Table I. Virus genera of the family Reoviridae
Genera of viruses with genomes composed of 10-12 segments of dsRNA (Mertens, 2004).

Genus	No. of genome segments	No. of species	No. of types (serotypes)	Tentative or unassigned isolates
1. *Orthoreovirus*	10	4	6	-
2. ***Orbivirus***	**10**	**21**	**160**	**11**
3. *Cypovirus*	10	16	16	3
4. *Aquareovirus*	11	6	unknown	5
5. *Rotavirus*	11	5	>23	2
6. *Coltivirus*	12	2	5	1
7. *Seadornavirus*	12	3	7	15
8. *Fijivirus*	10	8	8	-
9. *Phytoreovirus*	12	3	3	1
10. *Oryzavirus*	10	2	4	-
11. *Mycoreovirus*	11 or 12	3	3	-
12. *Idnoreovirus**	10	5	6	1

* the creation of the three new genera *Seadornavirus*, *Mycoreovirus* and *Idnoreovirus* has been approved by the International Committee for the Taxonomy of Viruses. The name *Idnoreovirus* is derived from 'Insect derived non-occluded **reovirus**'

Table II. The Orbivirus species (Mertens, 2004).

Orbivirus species currently recognised	No. of serotypes/strains	Tentative species/ unassigned viruses
1. African horse sickness virus (AHSV)	9 serotypes	
2. Bluetongue virus (BTV)	24 serotypes	Andasibe virus (ANDV)
3. Changuinola virus (CGLV)	12 serotypes	Ife virus (IFEV)
4. Chenuda virus (CNUV)	7 serotypes	Itupiranga virus (ITUV)
5. Chobar Gorge virus (CGV)	2 serotypes	Japanaut virus (JAPV)
6. Corriparta virus (CORV)	6 serotypes/strains[a]	Kammavanpettai virus (KMPV)
7. Epizootic haemorrhagic disease virus (EHDV)	10 serotypes/strains[a]	Lake Clarendon virus (LCV)
8. Equine encephalosis virus (EEV)	7 serotypes	Matucare virus (MATV)
9. Eubenangee virus (EUBV)	4 serotypes	Tembe virus (TMEV)
10. Ieri virus (IERIV)	3 serotypes	Codajas virus (COV)
11. Great Island virus (GIV)	36 serotypes/strains[a]	Tracambe virus (TRV)
12. Lebombo virus (LEBV)	1 serotypes	Yunnan orbivirus (YOV)
13. Orungo virus (ORUV)	4 serotypes	
14. Palyam virus (PALV)	13 serotypes/strains[a]	
15. Peruvian horse sickness virus (PHSV)[b]	1 serotype	
16. St Croix River virus (SCRV)[b]	1 serotype	
17. Umatilla virus (UMAV)	4 serotypes	
18. Wad Medani virus (WMV)	2 serotypes	
19. Wallal virus (WALV)	3 serotypes/strains[a]	
20. Warrego virus (WARV)	3 serotypes/strains[a]	
21. Wongorr virus (WGRV)	8 serotypes/strains[a]	
Total 21 virus species	160 serotypes/strains[a]	11 unassigned viruses

a) in some species the serological relationships between strains has not been fully determined

b) two new species of *Orbivirus* (SCRV and PHSV) have recently been recognised by the International Committee for the Taxonomy of Viruses, based primarily on genome segment sequence analyses and comparison (3, 33); Ndelle virus (previously classified as an *Orbivirus*) was also reclassified as an *Orthoreovirus* on this basis (2)

BTV virions are architecturally complex structures composed of 7 discrete proteins that are organised into two concentric shells, the outer and inner capsids, and a genome of 10 dsRNA segments (Fig. 1). The inner capsid consists of the structural protein VP3 which forms the sub-core upon which the VP7 containing "rings" or capsomers are located (Huismans *et al*., 1987*a*; Hyatt *et al*., 1988). There are 780 molecules of VP7 which form 260 triangular spikes and protrude 5 nm from the base of the inner capsid (Prasad *et al*., 1992). The arrangement of the trimers are such that there are 132 channels on the surface of the inner shell and are termed Types I, II and III. Type I channels are located along the icosahedral 5-fold axes and terminate inside the inner shell where they and the Type II channels, which surround the 5-fold axes, form indentations on the sub-core. Type III channels are located around the icosahedral 3-fold axes and traverse the inner-shell and into the sub-core where the minor structural proteins VP1, VP4 and VP6 are located (Fig. 2). A possible function

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of these Type III channels may be associated with the passage of metabolites and RNA into and from the core. Such a function would be consistent with the report by Huismans *et al.* (1987*b*), that uncoated BTV is transcriptionally active.
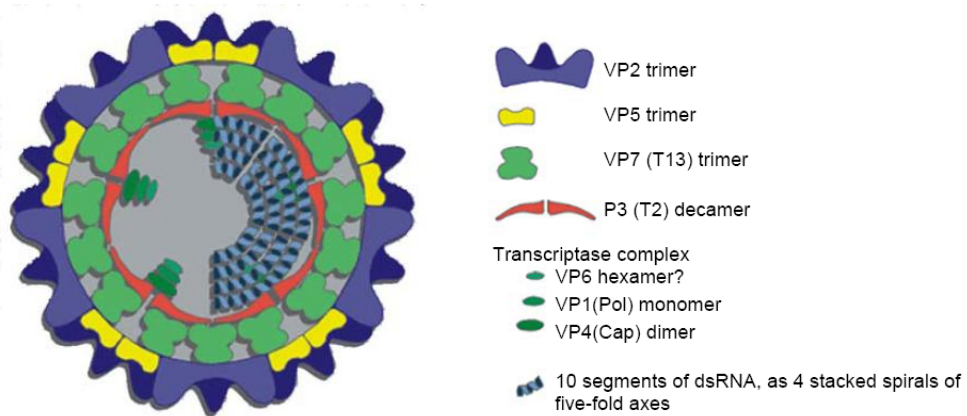
Fig. 1. Schematic diagram illustrating the structure of the bluetongue virus particle derived from biochemical, X-ray crystallography and cryo-electron microscopy (Mertens, 2004).
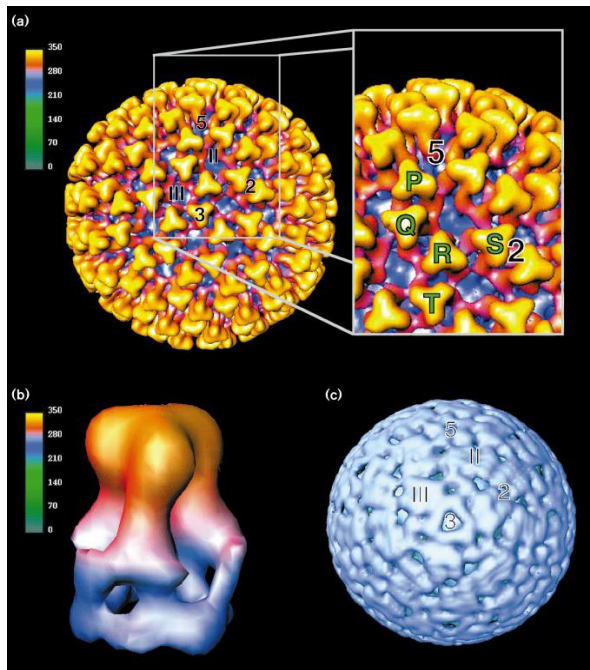
Fig. 2. The overall cryoEM 3-D reconstruction of the core of BTV-10. (a) Surface represent-tation of the core showing the trimers of VP7 in yellow, with an enlarged portion highlighting the protomeric unit of the viral capsid. The icosahedral axes are marked as are channels II and III in the layer of VP7. In the enlarged section, the five quasi-equivalent trimers are marked P (closest to the fivefold) to S (closest to the twofold axis) through to T (the trimer on the icosahedral threefold axis). (b) Close-up of a transverse view of a trimer of VP7 sitting on the inner layer of VP3, with the density coloured radially inward from yellow through to blue. (c) Figure of the cryoEM reconstruction of he core of BTV (radially cut at 260 Å), showing the smooth featureless inner layer of VP3 coloured in blue. The viral icosahedral axes and the positions of the channels II and III in the layer of VP7 are marked (Grimes, 1997).

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The outer capsid of BTV and other orbiviruses has been described as fibrillar. The outer-shell is composed of the major structural proteins VP2 and VP5. The outermost layer is composed of 180 copies, 'sail-shaped' VP2 protein, arranged as trimeric 'triskellion' structures, together with 360 copies of an inter-dispersed and underlying VP5 protein, which also appears to be arranged as 120 trimers. The outer capsid, is involved in cell attachment and virus penetration during the initial stages of infection. In addition to the seven structural proteins, three non-structural (NS) proteins, NS1, NS2, NS3 (and a related NS3A) are synthesised in BTV-infected cells. Of these, NS3/NS3A is involved in the egress of the progeny virus. The two remaining non-structural proteins, NS1 and NS2, are produced at high levels in the cytoplasm and are believed to be involved in virus replication, assembly and morphogenesis.

The components of the BTV outer capsid, proteins VP2 and VP5, are the most variable of the viral proteins. VP2, in particular, contains neutralising epitopes and by controlling the specificity of virus particle interactions with neutralising antibodies, determines the identity of the 24 BTV serotypes that are currently recognised using serum neutralisation (SN) assays. Sequence analyses of genome segment 2 (Moss *et al.,* 1992; Hewat *et al.,* 1992; Huismans *et al*., 1987*a*) and segment 6 (Eaton *et al.,* 1992) from representative isolates of all 24 BTV serotypes have recently been completed. Phylogenetic comparisons have demonstrated that variations in the nucleotide sequences of segment 2 and 6 and in the amino acid sequences of VP2 and VP5 show a high correlation with virus type as determined by SN assays (Moss *et al.,* 1992; Hewat *et al.,* 1992; Huismans *et al*., 1987*a*; Eaton *et al.,* 1992).

Importantly, there is considerable variation amongst field strains of BTV, even those of the same serotype, which reflects differences in the nucleotide sequence of each of the 10 distinct dsRNA segments of the BTV genome (Bonneau *et al.*, 1999; Pritchard *et al.*, 2004; Bréard *et al.*, 2007). Genetic heterogeneity of field strains of BTV occurs as a consequence both of genetic drift and genetic shift, the latter as a result of reassortment of viral genes during mixed infections of either the vertebrate or invertebrate hosts of the virus (Bonneau & MacLachlan, 2004; Pierce *et al.*, 1998; Samal *et al.*, 1987a, 1987b). Variation in the nucleotide sequence of individual genes occurs through a complex process of genetic drift and founder effect during alternating passage of BTV in its ruminant and insect hosts (Bonneau *et al*., 2001).

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III.2 - BT VIRUS REPLICATION

BTV interacts with the target cell surface via VP2 trimers binding to cell surface glycoproteins and possibly to other receptors. BTV core particles can also bind to cells (particularly insect cells) via VP7 trimers. The BTV particle is then internalized in endosomes via a clathrin-dependent endocytosis pathway (Hyatt *et al*., 1988). VP2 dissociates from the outer capsid layer in early endosomes. Acidification induces VP5 fusion with the endosomal membrane (Huismans *et al*., 1987), delivering the transcriptionally active core into the cell cytoplasm. Like other members of the Reoviridae family, BTV replicates within the cytoplasm of infected cells. Within the BTV core, the VP1 molecules transcribe positive sense ssRNA copies from each of the ten BTV genome segments (Studdert *et al*., 1966). These mRNA molecules are capped by the guanylyl-transferase and transmethylase activities of VP4 (De Mattos *et al*., 1994) and leave the particles via channels situated at the five fold axes of the core particle (Della Porta *et al.,* 1979). The viral mRNA serve as templates for translation in viral proteins, starting within two hours post infection (Murphey *et al.,* 1971). Viral positive RNA are directed to viral inclusion bodies (VIB) where the correct encapsidation of the different segments (nature and numbers) within the VP3 shell may involve interactions with the helicase VP6 (Pedley *et al*., 1988), the ssRNA binding NS2 protein (Mertens *et al*., 1985), and the VP1 and VP4 proteins. VP1 then synthesizes the negative strand RNA to produce dsRNA (Studdert *et al*., 1966; Fig 3). It has been proposed that each dsRNA segment independently associates with a different transcription complex (VP1, VP4 and VP6) located at the inner side of VP3 along a five fold axis, making a 'flower shape' in cryoelectromicroscopy (Della Porta *et al*., 1985).

Exchanges of dsRNA segments can occur when two different BTV (serotypes or strains) infect the same cells, contributing to the evolution of BTV through the process of reassortment. The process of reassortment may involve fusion of VIB formed with different viral particles. For unknown reasons, some segments are more often exchanged than others (Browne *et al*., 1967).

The VP3 subcores are relatively fragile and unstable structures (Gould, 1987) that serve as a scaffold for the addition of VP7 trimers, giving rise to more rigid and stable cores. The outer capsid proteins VP2 and VP5 appear to be added to the progeny core particle surface at the periphery of the VIB as they enter the host cell

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cytoplasm. Mature progeny virus particles are transported within the cytoplasm on microtubules involving VP2/vimentin interactions (Wade-Evans *et al*., 1992). Release of virions from the infected cell occurs via cell membrane destabilization mediated by the NS3 viroporin activity (Lecatsus, 1968), in some cases via budding, or as a result of cell death and lysis. Production of mature particles is exponential during the 8th and 24th hours post infection.
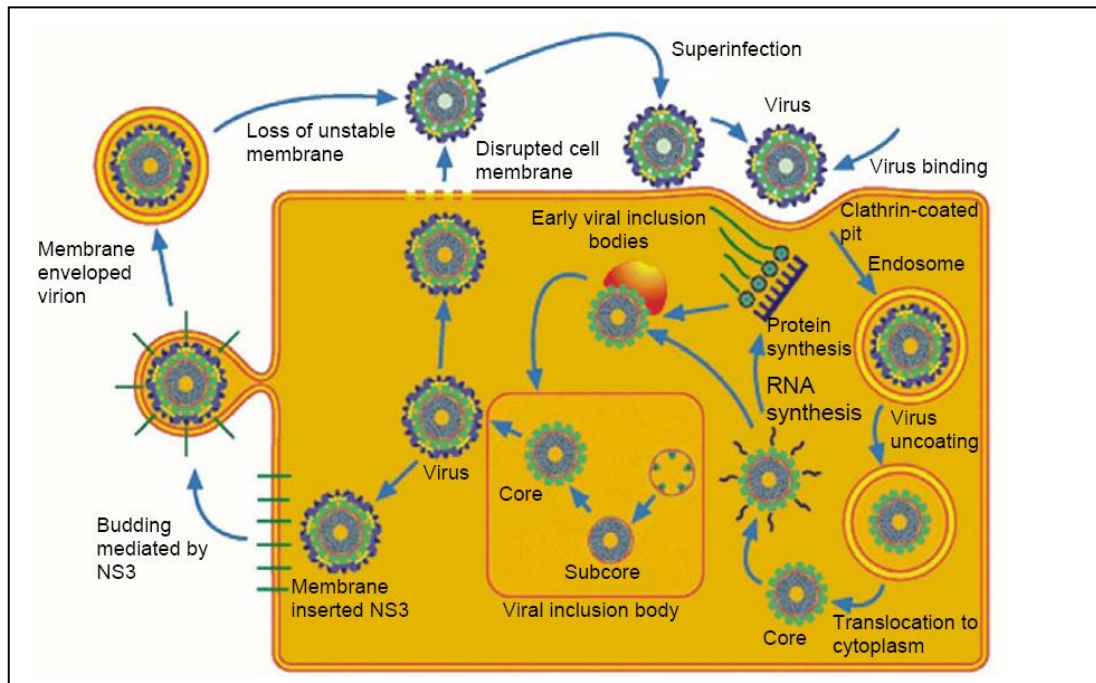
Fig. 3. Schematic diagram representing the lytic replication cycle of bluetongue virus (Mertens, 2004).

III.3 - SUSCEPTIBLE SPECIES

The generally accepted premise is that BTV is able to infect and replicate in all species of ruminant, domestic and wild, and there is a significant amount of information to support this assertion (Jessup 1985; House *et al.,* 1982; Stallnecht & Howerth 2004; MacLachlan, 2004).

Of the domestic species, sheep are the most severely affected. Indigenous South African breeds are less susceptible than the Merino, while most exotic breeds such as the European mutton breeds are more susceptible than the Merino. Susceptibility may also vary among individuals of a breed. Selected breeds as European fine wooled Merinos are highly susceptible to the virus while, Asian and African breeds are the most resistant. Sheep breeds from the temperate countries of the world are

more susceptible to the development of clinical disease than breeds adapted to the tropics. Older sheep are considered to show more severe disease than younger sheep. Goats are susceptible to infection although clinical disease is rarely encountered. Cattle are frequently infected with BT virus (BTV), but clinical disease is rare (Erasmus, 1975). The absence of BT in sheep does not necessary imply the absence of BTV or viral activity in a particular region or country at a time. Sheep could therefore be regarded as merely an indicator of the presence of the disease.

In the case of wild ruminants, clinical disease seems to be common only in North America where mortality and morbidity have been documented in white-tailed deer (Odocoileus virginianus), mule deer (O. hemionus), pronghorn antelope, (Antilocapra americana), Elk (Cervus elaphus), mountain goat (Oreamnos americanus) and bighorn sheep (Ovis canadensis) (Stallknecht & Howerth 2004).

Antibodies have been detected in wild carnivores in Africa, and cross-contamination between bluetongue and canine vaccines during vaccine manufacture has resulted in the death of some vaccinated dogs in the United States.

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IV. - EPIDEMIOLOGY

Bluetongue is biologically transmitted by Culicoides midges, but only a limited number of Culicoides species are efficient vectors. Cattle are the main amplifying hosts and are probably also important maintenance hosts. The competent Culicoides vector species feed more abundantly on cattle. The incidence and geographical distribution of bluetongue infections are determined largely by the distribution of insect vectors. The central role of the insect in BT epidemiology ensures that prevalence of the disease is governed by ecological factors and climatic, temperature, humidity and soil characteristics, which favour insect survival. In many parts of the world therefore, the disease has a seasonal occurrence, generally in late summer and early autumn.

IV.1 - DISTRIBUTION OF BTV

Although BTV infection occurs throughout extensive portions of the tropical and temperate regions of the world (Fig. 4), there are marked regional differences in the occurrence of virulent BT influenced by several factors, most importantly, geographical location, climate and vaccination history. There also are profound differences in the virus serotypes and/or principal species of *Culicoides* vector that occur within specific regions (Gibbs & Greiner, 1994; Tabachnick, 2004; MacLachlan & Osburn, 2006; Fig. 5; Table III). Both vector insects and ruminant animals are essential to the lifecycle of btv.

IV.1.1 - BTV GEOGRAPHICAL DISTRIBUTION IN EUROPE AND MEDITERRANEAN BASIN

An unknown disease of sheep was first reported from Cyprus in 1924. This disease was confirmed as being BT in 1943 (Gambles, 1949; Mellor & Pitzolis, 1979). Subsequently in Israel BT was first reported in 1943/44 and then with increasing frequency since that time (Shimshony, 2004).

Between 1956 and 1960, a large outbreak in Spain and Portugal caused the deaths of approximately 179.000 sheep (Manso-Ribeiro *et al.,* 1957; Lopez & Botija, 1958). More recently an outbreak of serotype BTV-4 has involved the Greek islands of Rhodes and Lesbos in 1979 and 1980 (Vassalos, 1980; Dragonas, 1981). Greece was then declared BTV free in 1991, but in the October of 1998 a new outbreak involved

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the islands of Rhodes, Kos, Samos and Léros (serotype BTV-9) (Mellor & Wittmann, 2002; Calistri et al., 2004; OIE, 1998).

From 1999 BTV infection spread progressively across most of the Mediterranean Basin, Balkan areas and more recently in north west Europe. Until now, 7 serotypes have been detected in the current outbreak in the Mediterranean Basin: BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, BTV-15 and BTV-16 (Mellor & Wittmann, 2002; www.reoviridae.org /dsRNA_virus_proteins/outbreaks.htm).

Table III. Recent global distribution of BTV serotypes and vectors (MacLachlan & Osburn, 2006).

Geographical distribution	Serotypes	Vectors	References
Africa	• North Africa - serotypes 1, 2, 4 recently isolated • Sub-Saharan Africa – endemic for serotypes 1 to 20, 22 and 24 • BTV 3 recently was isolated on la Réunion island.	*Culicoides imicola* and, *C. bolitinos* are the major vectors of all BTV serotypes. Other *Culicoides* species may be of lesser or local importance	Gerdes, 2004 Bréard et al, 2005
Australia	• only serotypes 1 and 21 are widely distributed across northern and eastern coastal regions of Australia • serotypes 3, 9, 15, 16, 20 and 23 occur only in the northern region of Australia but do not spread beyond	*Culicoides actoni, C. brevitarsis, C. fulvus, C. wadai*	Kirkland, 2004; Pritchard et al., 2004.
China and Taiwan	• BTV serotypes 1-4, 9, 11, 12, 15, 16, 21 and 23 have been identified, and others are also likely to be present • BTV infection recently was described in Taiwan	*Culicoides actoni, C. imicola* and *C. fulvus* probably are the major vectors. Other *Culicoides* species may also be involved but data have not been presented to confirm this.	Bonneau et al., 1999 Kirkland et al., 2002; Zhang et al., 2004. Ting et al., 2005.
Europe	• Serotype 8 is restricted to Northern Western Europe (Belgium, France, Germany, Luxembourg, the Netherlands) • Spain and Portugal recently have experienced serotypes 2 and 4 • Italy serotypes 1, 2, 4, 9 (excluding Sardinia) and 16 • France - serotype 2, 4, 16 in Corsica and serotype 8 in the northern mainland • BTV serotypes 1, 4, 9 and 16 in the eastern European countries	*Culicoides imicola, C. obsoletus, C. scoticus, C. pulicaris* and, most recently, *C. dewulfi* are proven vectors or have been implicated in the transmission of one or more BTV serotypes	See chapter on epidemiology
Indian subcontinent and adjacent areas	• BTV serotypes 1-4, 8, 9, 12, 16-18 and 23 have been isolated and antibodies to serotypes 6, 7, 11-15, 19, 20, have been reported but without virus isolation Serological evidence of BTV infection in Pakistan (serotypes 3, 9, 15, 16 and 18), Bangladesh and Sri Lanka has also been reported.	*Culicoides imicola, actoni* and *fulvus* are present and are major BTV vectors elsewhere. Other *Culicoides* species may also be involved but data have not so far been presented to confirm this.	Sreenivasulu et al., 2004.
Japan	• BTV serotypes 4, 11, 13, 20 and 21 have been isolated in Japan and antibodies to serotypes 1 and 12 have been reported but without virus isolation	*Culicoides brevitarsis*, a vector in Australia has been reported from Japan. Three other Orbiviruses, including the BT-related Ibaraki virus have been isolated from blood-free *C. oxystoma* suggesting that this species might also be a potential vector of BTV	Goto et al., 2004 Yanase et al 2005
Middle East	• Serotypes 2, 4, 6, 10 and 16 have historically been present in Israel and serotype 15 very recently was identified Other serotypes that have been identified in the region (Turkey, Syria etc) include 3, 9 and 13		Mellor, P; unpublished data. Shimshony, 2004.
North America	• Serotypes 10, 11, 13 and 17 in endemic regions (south of latitude 50°N in the west) other than the Gulf Region where other serotypes (1, 2 and perhaps others) also occur but these serotypes have not spread to areas where only *C sonorensis* occurs.	*C. sonorensis* is the main vector in continental North America and *C. insignis* is also present in the Gulf of Mexico region (Florida and coastal Mississippi, Louisiana etc).	Ostlund et al., 2004 Gibbs and Greiner, 1994
South and Central America, Caribbean Basin	• BTV infection common, but disease is rare) • BTV serotypes 1, 2, 3, 4, 6, 8, 10, 12 and 17 have been isolated, and antibodies to serotypes 14, 19 and 20 have been identified but without virus isolation. In South America the presence of BTV has been reported from Brazil, Argentina, Colombia, Guyana and Surinam, with isolation of BTV serotypes 4, 12 and antibodies have been identified to serotypes 4, 6, 12, 14, 17, 19 and 20. In Central America the virus has been reported from 11 countries.	*Culicoides insignis* and probably *C. pusillus* are the major vectors.	Mo et al., 1994; Gibbs and Greiner, 1994 Lager, 2004
Southeast Asia (Malaysia and Indonesia)	• BTV serotypes 1-3, 7, 9, 12, 16, 21, and 23 have been isolated and antibodies to serotypes 5, 6, 15 and 20 have been identified but without virus isolation.	The vectors are likely the same as those in Australia, and probably others of important.	Daniels et al., 2004; Pritchard et al., 2004

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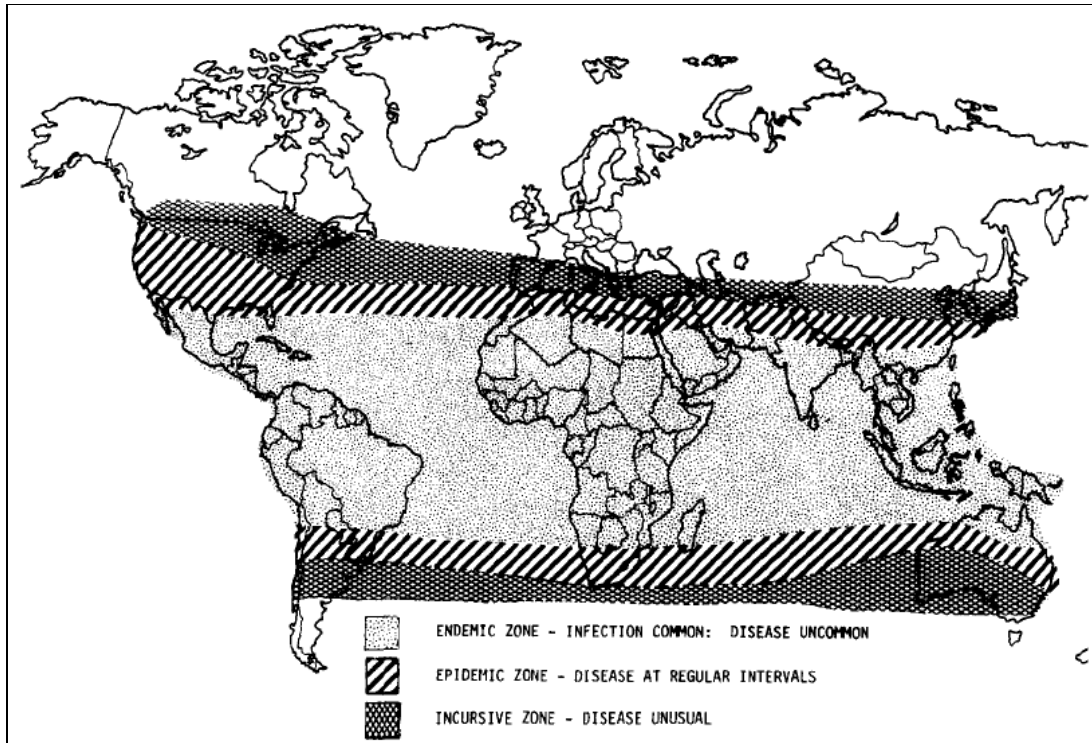
Fig. 4. Geographical distribution of bluetongue virus and clinical disease. The three zones are depicted to simplify analysis of the epidemiology; they are dynamic and represent parts of a spectrum of virus activity dependent upon climate, altitude and other factors. They should not be considered geographically accurate; e.g., no clinical disease has been recorded in Australia or South America. One would normally expect that in the endemic zone, most ruminants have antibody to the virus; in the epidemic zone, the percentage of animals with antibody to the virus varies and is focal, although herds/flocks with antibody can be found; and in the incursive zone, no ruminants with antibody can be found (Gibbs and Ellis, 1994).
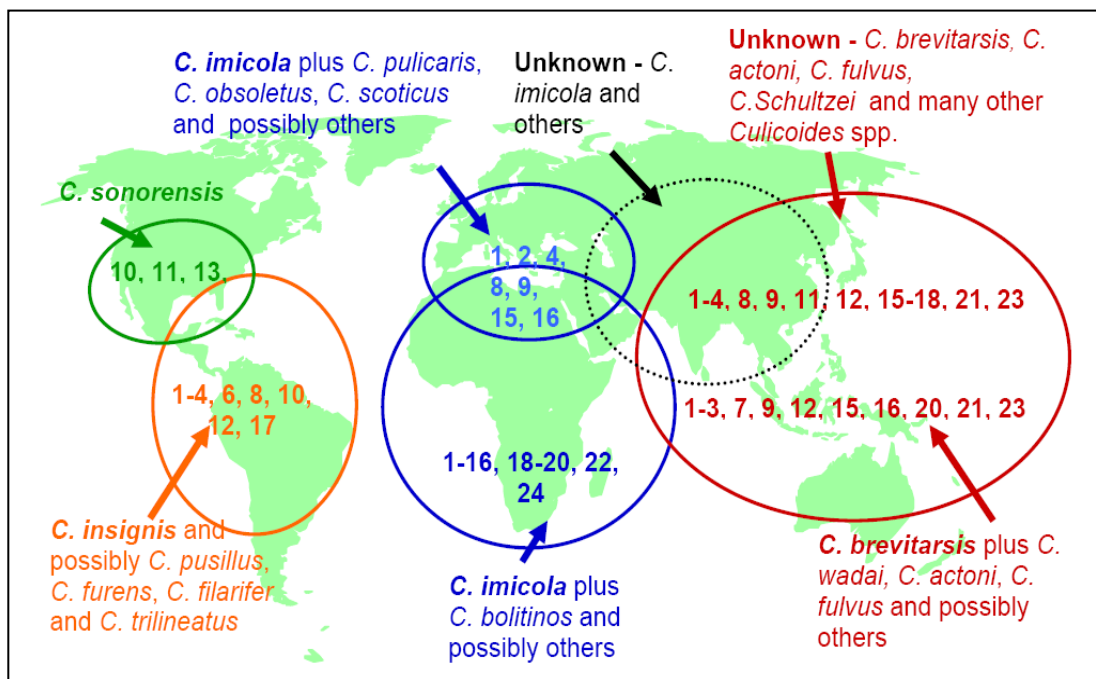
Fig. 5. Global distribution of BTV serotypes and vector species. The Culicoides species identified in bold are considered the principal vector of BTV in each region (MacLachlan & Osburn, 2006).

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IV.1.1.1 - ROUTES OF INTRODUCTION OF BTV SEROTYPES

Routes of introduction of such vector-borne diseases into EU are often not clear. There is a need for a multi-disciplinary approach to identify the source, ranging from tracing of animal/product movements to more sophisticated knowledge of virus and vector biology. Knowledge of vector biology and ecology is fundamental to the understanding of how these diseases behave and may become established in the EU. Genetic analysis of BT viruses isolated in Europe has shown that six serotypes of the virus (1, 2, 4, 8, 9 and 16) have entered the region since 1998. There are four apparently distinct routes by which these viruses have arrived: from the east via Turkey/Cyprus; from the eastern part of north Africa (Algeria, Tunisia) into Italy and the western Mediterranean Islands; from Morocco into southern Spain and Portugal and via an unknown route into North Western Europe BTV-15 was identified in Israel during 2006 (Fig. 6).
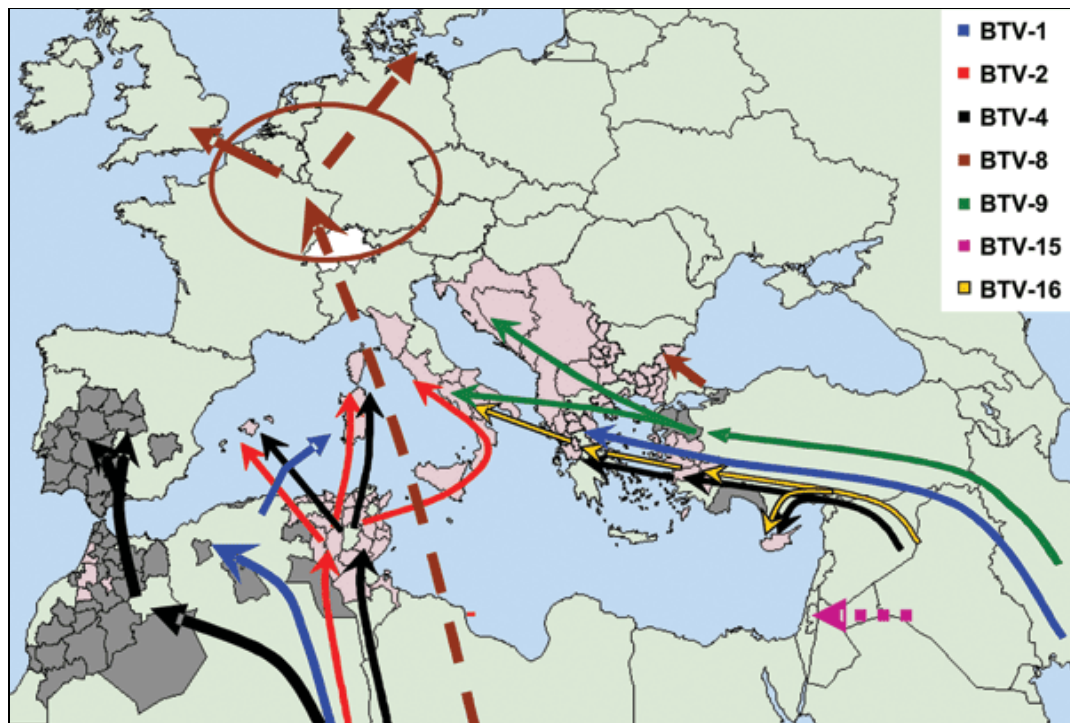
Fig. 6. Map of the possible routes of introduction of the different BTV serotypes isolated in Europe since 1998 (Saegerman *et al.,* 2008).

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IV.1.1.2 - BTV SEROTYPES IN EUROPE AND MEDITERRANEAN BASIN

IV.1.1.2.1 BTV-1 GEOGRAPHICAL DISTRIBUTION

Serotype 1 was first notified in the north-western part of mainland Greece (Panagiotatos, 2004) and in the island of Lesbos and Rhodes in 2001. For a long period of time BTV-1 was not detected in other countries in the region, until 2006 when in October it was recorded in Sardinia (OIE, 2006*a*). It has also been reported in 2006 in Algeria, in Morocco, in 2007 in Spain, in France, in Tunisia, in Portugal, in Libya and in Gibraltar (OIE 2006 and 2007 various Disease information Reports).

Phylogenetic analyses of segment 2 sequences of BTV serotype 1 Greek isolate and Algerian and Moroccan isolates showed that they are distinct, belonging to different 'eastern' and 'western' lineages respectively (www.iah.bbsrc.ac.uk/dsRNA_virus _proteins /BTV1-segment2-tree.htm).

The initial strain of BTV-1 in Greece is related to eastern viruses from India and Malaysia and is thought to have entered Europe from the east (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ ReoID/btv-1.htm).

This strain only persisted for a relatively short period in Greece and apparently did not spread to other European Countries. In 2006 BTV was detected in northern Africa and was shown by RT-PCR and sequence analysis to be caused by BTV type 1 (www.iah.bbsrc.ac.uk/ dsRNA_virus_proteins/ReoID/btv-1.htm).

The strain involved is most closely related to other western type 1 isolates from sub-Saharan Africa, but is clearly distinct from the South African vaccine strain. It is also distinct from the earlier Greek isolates of BTV-1 (www.iah.bbsrc.ac.uk/dsRNA_ virus_ proteins/ReoID/btv-1.htm).

IV.1.1.2.2 BTV-2 GEOGRAPHICAL DISTRIBUTION

In December 1999 BTV-2 was identified in Tunisia, along the east coast of the country (OIE, 2000*a*). During the summer of 2000 new outbreaks of the same serotype appeared in the whole northern part of Tunisia and in Algeria (OIE, 2000*b*). During the summer and autumn of 2000, Italy experienced the largest BT epidemics in Europe (OIE, 2000*c*; Calistri *et al*., 2004). Three regions were involved (Sardinia, Sicily and Calabria), inhabited by 54.7% of the entire Italian sheep and goats population.

Phylogenetic analyses of Seg-2 sequences of Tunisia isolate identified the virus as BTV serotype 2, belonging to a western lineage (www.iah.bbsrc.ac.uk/dsRNA_

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virus_proteins/BTV2-segment-2- tree.htm). The virus is related to other BTV-2 strains from Nigeria and South Africa, although it is distinct from the South African BTV-2 vaccine strain. The initial Tunisian isolates of BTV-2 are also almost identical to subsequent isolates made in Corsica and Sardinia indicating that BTV-2 managed to spread northwards, from north Africa into Italy and the western Mediterranean islands (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-2.htm).

In the early autumn of 2000 the disease was notified in the Spanish Balearic islands (OIE, 2000*d*) and in the French island of Corsica (OIE, 2000*e*). In the following years BTV-2 infection spread through the southern and central regions of Italy (Calistri *et al*., 2004).

IV.1.1.2.3 BTV-4 GEOGRAPHICAL DISTRIBUTION

In 1999 BTV-4 spread in several islands of the Greek. Later the same year the serotype spread also into the northern and eastern mainland areas of Greece. Western regions of mainland Greece were also involved in 2000, and western and central areas in 2001 (Panagiotatos, 2004; Nomikou, 2006 personal communication), and, almost at the same time, it appeared in Calabria region, the most southern region of mainland Italy (Calistri *et al*., 2004).

Phylogenetic analyses demonstrated that the Greek virus is similar to earlier isolates from Cyprus and Turkey (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv4-segment-2-tree.htm). It is also closely related to the Reference strain of BTV-4, which is also believed to have been originally derived from Cyprus (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-4.htm and http://www.iah. bbsrc.ac.uk/dsRNA_virus-proteins/ReoID/BTV-mol-epidem.htm). This suggests that the BTV-4 strain which invaded Greece and the Eastern Mediterranean region since 1999, has been circulating in the region, on the borders of Europe, for some time. Indeed several isolates of BTV-4 have subsequently been made in Israel. Phylogenetic analyses show that these European BTV-4 strains belong to a western lineage but are distinct from the South African vaccine strain (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-4.htm and http://www.iah. bbsrc.ac.uk /dsRNA_virus-proteins/ReoID/BTV-mol-epidem.htm).

In September 2003 circulation of BTV-4 was detected in Sardinia (OIE, 2003a). The virus spread also to the province of Nuoro, Sassari and Oristano (April, 2004).

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Further to the notification of an outbreak of BT in Cognocoli-Monticchi, by the south Corsica Department dated 31 October 2003, BTV-4 was identified at the laboratory of the French Agency for Food Safety (AFSSA) (OIE, 2003b). In the same year (2003) BTV-4 was also identified from the Spanish Balearic islands.

A vast epidemic of BTV-4 in 2004 involved also the northern part of Morocco (OIE, 2004*a*), spreading from there into the southern part of the Iberian Peninsula (OIE, 2004*b*; OIE, 2004*c*). The phylogenetic analyses of BTV detected in the Spanish island of Menorca showed that this was also a western strain of BTV-4, although it is clearly distinct from strains that had previously caused outbreaks in the eastern Mediterranean region (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ ReoID/btv-4.htm and http://www.iah.bbsrc.ac.uk/dsRNA_virus-proteins/ReoID/BTV-mol-epidem.htm). These conclusions were confirmed by Zientara *et al.,* (2006) working with Corsican isolates at Maisons Alfort in France. This virus is believed to have entered Europe from North Africa. The same strain of BTV-4 subsequently caused outbreaks and was isolated in Morocco and then spread to the Iberian peninsula in 2004, where it persisted through into 2005.

IV.1.1.2.4 BTV-8 GEOGRAPHICAL DISTRIBUTION

Serotype 8 had not been detected in Europe and the Mediterranean Basin before 2006, when it was the cause of a large epidemic involving five Northern Western European countries: Belgium (OIE, 2006*b*), The Netherlands (OIE, 2006*c*), Germany (OIE, 2006*d*), France (OIE, 2006*e*), Luxembourg (OIE, 2006*f*), United Kingdom, Denmark, Switzerland, Czech Republicc, Spain and Portugal (www.reoviridae.org /dsRNA_virus_proteins/outbreaks.htm). Multiple isolates of the virus were made from isolates across this region. Sequence analyses of Seg-2 from Dutch isolate demonstrated that the virus is from a western lineage from sub Saharan Africa, but is distinct from the BTV-8 vaccine strain (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/BTV-8-Seg-2-tree.htm). It is uncertain exactly how BTV-8 arrived in Northern Western Europe, but the absence of BTV-8 outbreaks in southern Europe suggests that it did not involve simple linear extension of earlier outbreaks and is likely to reflect a distinct entry route and mechanism.

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IV.1.1.2.5 BTV-9 GEOGRAPHICAL DISTRIBUTION

In the autumn of 1998, clinical and serological evidence of BTV-9 was recorded on 3 islands of Greek Dodecanese archipelago (Leros, Rhodes, Kos) and Samos, adjacent to the Anatolian Turkish coast (Mellor & Wittmann, 2002). During 1999 the virus was also recorded in south eastern Bulgaria (OIE, 1999), in European Turkey, western Anatolian Turkey, North-East and eastern mainland Greece (Mellor & Wittmann, 2002). In 2000 and 2001, BTV-9 was again detected in mainland Greece, initially in the northern areas and then in the NW near the Albanian and Former Yugoslav Republic of Macedonia (FYROM) borders (Panagiotatos, 2004).

New outbreaks of BTV-9 also occurred in southern Bulgaria in September 2001, and in the same month BTV-9 appeared for the first time in Serbia (OIE, 2001*a*) and in FYROM (OIE, 2001*b*). In October the infection was detected also in Kosovo (FRY) (OIE, 2001*c*) and, in December, in southern Croatia, Dubrovnik district (OIE, 2001*d*).

In September 2002 the National Veterinary Office of Bosnia and Herzegovina reported BTV-9 outbreaks in the Republic of Srpska and subsequently ruminants have been found seropositive in the Federation of Bosnia and Herzegovina (OIE, 2002). In the same period of time outbreaks again occurred in Bulgaria. In December 2002, Albania experienced for the first time one outbreak of BT in Librazhd, in the eastern part of the country (OIE, 2003*c*). After years of absence, in October 2006 BTV-9 reappeared in south eastern regions of Bulgaria (OIE, 2006*g*).

Sequence data for genome segment 2 of the BTV isolated in 1998 in Greece was compared to other isolates of BTV held in the reference collection at IAH Pirbright from around the world (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9.htm).

The resulting analysis shows that this is an 'eastern' virus, related to strains from Indonesia and Australia (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv9-segment-2-tree.htm).

IV.1.1.2.6 BTV-15 GEOGRAPHICAL DISTRIBUTION

A sample of a virus-isolate that was made in Israel from outbreaks of disease in 2006 was typed as BTV-15, by RT-PCR assays using serotype specific primers targeting genome segment 2. Subsequent phylogenetic analyses confirm the virus serotype and indicated that it belongs to a western lineage (www.reoviridae.org/

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dsRNA_virus_proteins/BTV15-Seg2-tree.htm). The number of BTV-15 isolates that are available is very limited. So it is difficult to be more precise about the origins of this virus strain. However, the existing data suggest that it is new to the region and may therefore represent a further threat to Europe in the future.

IV.1.1.2.7 BTV-16 GEOGRAPHICAL DISTRIBUTION

BTV-16 was first detected in 1999 in the Greek Dodecanese islands and in northern mainland Greece and in 2000 it was also detected in western Anatolian Turkey (Mellor & Wittmann, 2002) and on the Greek Island of Lesbos (Nomikou unpublished results). Since September 2003, a surveillance programme has been in place in Cyprus, and BTV-16 seroconverted sentinel animals were found in October 2003 (OIE, 2004*d*). The affected area was in Famagusta (Ammochostos) district, in the eastern part of the country. Moreover, in February 2004 a new outbreak of BT occurred in Larnaca district, also in the eastern part of the country, about 20 km south-west from the first outbreak.

In January 2004 circulation of BTV-16 in Sardinia was detected by virus neutralisation test in 7 sentinel animals (Calistri *et al*., 2004). In the summer of the same year BTV-16 was detected also in Corsica (OIE, 2004*e*).

The sequence analysis of Seg-2 and phylogenetic analysis (www.iah.bbsrc.ac.uk/dsRNA_virus_ proteins/btv16-segment2-tree.htm) of the initial European strain of BTV-16 isolated in Greece during 1999, show that this virus is from an eastern lineage and is very similar to strains of BTV-16 from Turkey and the South African reference strain of BTV-16 (www.iah.bbsrc.ac.uk/ dsRNA_virus_proteins/ReoID/btv-16.htm and http://www.iah.bbsrc.ac.uk/dsRNA_ virus-proteins /ReoID/BTV-mol-epidem.htm). The similarity between the Greek and Turkish strains and the South African reference strain of BTV-9 may seem surprising but the reference strain was originally derived from an outbreak in Pakistan. This suggests that the virus arrived in Europe from the east. Phylogenetic analyses have shown that the original European field strain of BTV-16 is closely related, although distinct from the BTV-16 vaccine strain. BTV-16 also appeared in Cyprus during 2004, and was a strain of this serotype which is similar to the earlier eastern Mediterranean isolates.

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IV.2 - MODES OF TRANSMISSION

Bluetongue virus is transmitted by biting midges in the genus Culicoides. Other Culicoides species can also transmit the virus and may be important locally. Ticks or sheep keds can be mechanical vectors but are probably of minor importance in disease transmission. Cattle are the major amplifying host due to their prolonged viremia and the feeding preferences of many Culicoides species. The virus is not transmitted by direct contact, or by indirect means, between animals in the absence of insect vectors. Animals can be infected experimentally by inoculation with infected blood. Therefore, iatrogenic transmission by needle transfer is considered possible but unlikely. Rarely, virus may be excreted in the semen when males are viraemic. Excretion is more likely if there is inflammation of the genital tract and if the animal is aged. Contaminated semen may infect recipient females, but this will not initiate a cycle of transmission unless competent insect vectors are abundant.

Introduction of BTV from an area into another can occur in 4 ways: through animal movement (domestic and wild ruminants) or animal product transport (semen, embryos); by infected vector Culicoides spp. carried by various living (plants, animals) or inanimate (airplanes, ships) means; through the active flight of infected vector Culicoides spp. (local propagation); and through passive flight of infected vector Culicoides spp. on the wind (responsible for long-distance dissemination).

IV.3 - THE CULICOIDES

Culicoides (Diptera: Ceratopogonidae) are small biting flies, 1-3 mm in size; 96% of the more than 1.300 species known world-wide are obligate bloodsuckers attacking mammals (including humans), birds, reptiles and even other insects. In some parts their attacks are legion, causing nuisance to humans or an acute allergic dermatitis in Horse, and have earned them names such as damnosus, diabolicus and irritans. They are distributed from the tropics to the tundra and from sea level to 4.200 m (in Tibet). Approximately 120 species of Culicoides occur in Europe; this diversity declines markedly towards the Arctic with only about 25 species living beyond the 60th latitude.

Fortunately, only a very small fraction (2.5%) of the Culicoides described world-wide have a proven involvement in the transmission of viral pathogens injurious to livestock; these pathogens include BTV, African horse sickness virus (AHSV),

epizootic haemorrhagic disease of deer virus (EHDV), Akabane virus (AKAV) and equine encephalosis virus (EEV). Furthermore, adults Culicoides also transmit several parasites of veterinary importance, as for example hemoparasites (Haemoproteus sp. in birds; Hepatocystis kochi in monkeys) and filariasis (Onchocerca sp. in horses and cattle).

Around the world approximately 32 species of Culicoides are considered to be involved in the transmission of BTV; these are listed in Table IV (adapted and updated from Meiswinkel *et al.,* 2004) according to subgenus and species complex. In sub-Saharan Africa C. Imicola and C. Bolitinos transmit BTV and also other orbiviruses including equine encephalosis virus (EEV) and African horse sickness virus (AHSV). In South Africa at least 14 serotypes of BTV have been isolated from C. Imicola alone (Nevill *et al.,* 1992) one of these being BTV-8, the serotype affecting Northern Western Europe in 2006. In the Mediterranean Basin C. Imicola (Fig. 7) is the principal vector of BTV; however, within southern Europe an additional four species are also implicated: C. Pulicaris, C. Scoticus, C. Obsoletus and C. Dewulfi. The latter three species are probably the most important vectors in northern Europe, where C. Imicola does not occur. In western North America the major vector of BTV is C. Sonorensis, which is replaced by C. Insignis in the south-eastern USA; in Central and South America C. Insignis and C. Pusillus are the known vectors. In south-east Asia and Australia the major vector is C. Brevitarsis complemented by the less widely prevalent C. Wadai, C. Fulvus and C. Actoni.

Fig. 7. A gravid female Culicoides Imicola collected from a location near bluetongue outbreaks in Sardinia in 2007.

Table IV. The 32 species of the genus *Culicoides* (Latreille, 1809), that play a greater or lesser role in the transmission of bluetongue disease across the world. These are assigned to their correct subgenus and species complex (where known); the species given in bold are those more clearly implicated in the field transmission of BTV.

Subgenus	Species Complex	Species
Avaritia Fox, 1955	Imicola	**C. imicola**
		C. brevitarsis
		C. bolitinos
	Obsoletus	**C. obsoletus**
		C. scoticus
	Dewulfi	*C. dewulfi*
	Orientalis	*C. fulvus*
		C. dumdumi
		C. orientalis
	Grahamii	*C. actoni*
	Pusillus	*C. pusillus*
	Suzukii	*C. wadai*
	Gulbenkiani	*C. brevipalpis*
		C. gulbenkiani
		C. tororoensis
Culicoides Latreille, 1809	Pulicaris	**C. pulicaris**
		C. magnus
Silvicola Mirzaeva and Isaev, 1990	Cockerellii	Species unknown
Monoculicoides Khalaf, 1954	Variipennis	**C. sonorensis**
	Nubeculosus	*C. nubeculosus*
		C. puncticollis
Remmia Glukhova, 1977	Schultzei	*C. oxystoma*
		C. nevilli
		Species unknown
Hoffmania Fox, 1948	Guttatus	**C. insignis**
		C. filarifer
	Peregrinus	*C. peregrinus*
	Milnei	*C. milnei*
Haematomyidium Goeldi, 1905	Complex unknown	*C. stellifer*
Oecacta Poey, 1853	Furens	*C. furens*
Meijerehelea Wirth and Hubert, 1961	Complex unknown	*C. pycnostictus*
Subgenus unknown	Complex unknown	*C. trilineatus*

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IV.3.1 - THE CULICOIDES LIFE CYCLE

Adult female Culicoides use a protein-rich blood meal to mature the ovaries and to develop between 30-450 eggs (measuring 350-500 μm in length and 65-80 μm in breadth) which are laid (oviposited) in batches. One batch of eggs is matured from each blood meal. Midges need to survive only five days to lay the first egg mass, but they must survive 10 days to transmit a virus during which time they may lay two to three egg masses. In general Culicoides eggs usually hatch in 4- 5 days at 25 ºC but in one northern European species (C. Grisescens) hatching was reported to take 7-8 months (Parker, 1949). For the tropical C. Imicola at 30 °C the eggs hatch within 2-3 days with the egg to adult cycle being rapid (9-10 days). However, low temperatures affect egg viability and in the case of C. Imicola below 6.5 °C viability decreases after 7 days with none hatching after 37 days (Nevill, 1967).

Culicoides have four larval instars. All 4 larval instars are slender and almost "hairless", which facilitates their characteristic "serpentine" movement through the semi-solid media they inhabit. The length of the larval development is regulated by temperature and nutritive richness of the breeding sites varying from a week in tropical species like C. loxodontis (Meiswinkel, 1992) to nearly two years in some Arctic species (Downes, 1962). The lengthening of the larval cycle across latitudes appears related to falling average annual temperatures and to the number of daylight hours; at 8 hours of light or less the larvae of many temperate species go into diapause and development is resumed the following spring when warmer, longer light, conditions return. Water content determines the suitability of a breeding habitat because the larvae and pupae will die when the habitat dessicates unless they are able to migrate to a joining areas that are still moist. The pupa is a short-lived, non-feeding stage from which the adult midge hatches 2 days to 4 weeks later (Fig. 8).

The longevity of adult Culicoides has not been measured precisely but it is likely that it lasts from a few weeks or less (usually) to several months (rarely). Nevill found also that at temperatures as low as -1.5 °C only 15% of C. Imicola adults lived beyond 15 days. From field data it has been estimated that the optimal temperatures for C. Imicola range between 18 and 38 ºC (Ortega *et al.,* 1998); for C. Obsoletus these have been calculated to be between 11-27.5 °C (Dzhafarov, 1964). Before Culicoides are able to mate both sexes require several days to reach sexual maturity. Mating occurs mainly around sunset but has been described for only a few species during which swarms of males are detected by single females, which then enter the

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swarms to be mated. Males feed exclusively on sugar sources such as plant nectar; while females feed principally on blood they have been reported to also visit flowers to obtain nectar.

It is widely assumed that Culicoides are generally crepuscular and may continue to be active throughout the night; however, many species are also (in particular those found at more northerly latitudes) troublesome in the day displaying two biting peaks: one after sunrise the other close to sunset. In general Culicoides disperse only short distances from their breeding sites. It is postulated that in the Mediterranean region C. Imicola can be passively carried for distances of over 100 km on winds (Sellers, 1975; Sellers *et al.,* 1977, 1978, 1979; Sellers & Pedgley, 1985; Sellers & Maarouf, 1989, 1991; Braverman & Chechik, 1996) and recent outbreaks of BT in the Spanish Balearics and in absence of animal movements onto the islands have been highlighted as an example of this (Alba *et al.,* 2004), in Sardina and in Corsica Island (Gerbier *et al.,* 2004).
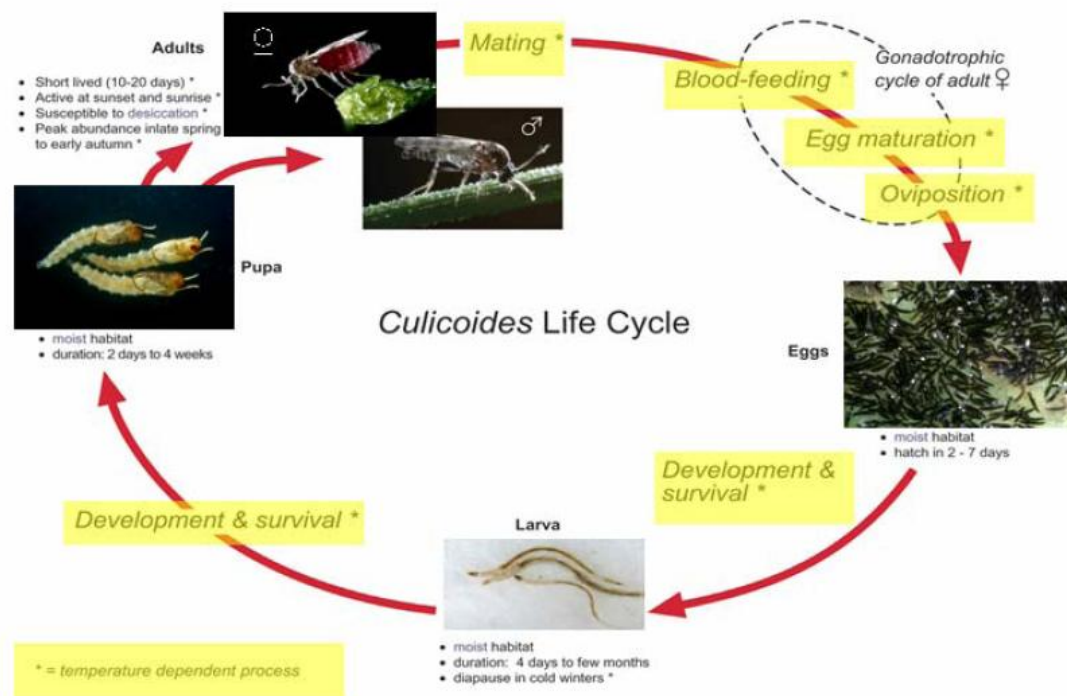

Fig. 8. The *Culicoides* life cycle (Purse *et al*., 2005).

IV.3.2 - VIRUS-VECTOR INTERACTIONS

Vector Culicoides become persistently infected with BTV for their entire lifespan after acquiring infection through feeding on an infected ruminant, whereas infection of the ruminant host is transient and the virus does not persist in a geographic region in the absence of competent insect vectors (Gibbs & Greiner, 1994).

BTV is ingested by adult female Culicoides during the course of blood feeding on a viraemic host and is deposited in the hind part of the insect's mid-gut along with the ingested blood. If the individual that has ingested the virus is a competent vector then the virus particles attach to the luminal surface of the mid-gut cells, penetrate into the cytoplasm of these cells by a process of endocytosis, and replicate in them. Progeny virus particles exit into the haemocoel of the vector through the abluminal surface of the gut cells, probably by a process of budding and disseminate through the body cavity suspended in the haemolymph. Secondary target tissues that include fat body, neural cells and the salivary glands may be infected during the course of this dissemination. In the case of the salivary gland cells, these are infected and a second cycle of replication ensues with fresh progeny virus particles exiting into the salivary ducts where they collect, sometimes in para-crystalline arrays and so are available for transmission during subsequent biting activity. The time interval between ingestion of virus and transmission is called the extrinsic incubation period and is temperature-controlled decreasing in duration as temperature rises. The high concentration of virus in the salivary ducts of infectious vectors means that BTV transmission is extremely efficient and in most cases the bite of a single midge will result in the infection of a susceptible host (O'Connell, 2002). However, transmission from an infected host to the vector is much less efficient and experimental work indicates that even at peak viraemia <2% of feeding individuals become infected (O'Connell, 2002). The blood of an infected animal is more infectious when virus titres are high, soon after infection (before antibodies develop). Nevertheless, experimental evidence suggests that even at peak viraemia with titres in excess of >5 $\log_{10}$ TCID^{50} of virus/ml, it can be difficult to persistently infect more than 1% of feeding vectors (O'Connell, 2002; Baylis *et al.,* 2004). As the titre of viraemia drops there is less chance of a biting midge imbibing an infectious dose of virus. However, midges can sometimes be infected even when the virus titre is too low to be recorded by conventional tests (i.e. <0.5 $\log_{10}$ TCID^{50} of virus/ml), (Bonneau *et al.,* 2002). This

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suggests that any titre of viraemia may be infectious for some vector midges should their competence rate and biting rate be sufficiently high.

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V. - PATHOGENESIS

The pathogenesis of BTV infection is similar in sheep and cattle, and most probably, all species of ruminants (Barratt-Boyes & MacLachlan, 1995; MacLachlan, 1994; Mahrt & Osburn, 1986; Pini, 1976). There are marked differences in the severity of disease that occurs in different ruminant species after BTV infection, however, with cattle being especially resistant to expression of BT disease. After initial replication in the lymph nodes draining the sites of inoculation, BTV disseminates to secondary sites, principally the lungs and spleen, where it replicates in endothelium and mononuclear phagocytes (Barratt-Boyes & MacLachlan, 1994). BTV can disseminate via lymph (Barratt-Boyes & MacLachlan, 1994) or/and via blood (Brodie *et al.*,1998). BTV infects monocytes both in vivo and in vitro. In vivo, infectious BTV can be retrieved transiently (<1 week) from monocytes (minimum 10^5 cells) (Brodie *et al.*,1998; Whetter *et al.*,1989).

Monocytes also express BTV antigens in vivo at low frequency (four NS2 antigen-positive monocytes per 2×10^5 peripheral blood mononuclear cells (Whetter *et al.*,1989). In vitro, around 15% of monocytes express BTV VP7 after 36 h in culture (Barratt-Boyes & MacLachlan, 1995) and they produce low amounts of infectious BTV in vitro (Whetter *et al.*,1989). Conversely, resting T lymphocytes are not efficient at supporting BTV replication (Whetter *et al.*,1989) unless they are activated by mitogens (Barratt-Boyes & MacLachlan, 1995). Interestingly, γδ T cell lines can be productively infected in vitro (Takamatsu *et al.*, 2003) and blood γδ T cells from infected sheep (3–13 days post infection) have been induced to produce infectious BTV when cocultivated with skin fibroblasts (Takamatsu *et al.*,2003). However, it is unclear how monocytes and possibly blast T cells are involved in vivo in the pathogenesis of BTV. Last but not least, infectious BTV can also be detected in the intracellular vesicles of erythrocytes, in which it does not replicate but persists in invaginations of cell membrane (MacLachlan *et al.*, 2004; Whetter *et al.*, 1989). The association of infectious BTV with erythrocytes is detected very early after infection (24 h) (Brodie *et al.*, 1998) and persists throughout viraemia (MacLachlan *et al.*, 2004; Shad *et al.*, 1997).

In sheep, the incubation period is usually 5 to 10 days, while in cattle can become viremic starting at 4 days post-infection. BTV infection in ruminants is characterized by a prolonged cell-associated viraemia that can persist in the presence of high titres

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of neutralizing antibody, although recovered animals are immune to re-infection with the homologous serotype of BTV. In sheep and cattle, infectious BTV can be detected in the blood for 35 to 60 days (Barratt-Boyes & MacLachlan, 1994) and viral structures for up to 160 days (Katz *et al.,*1994). It has been proposed that particles associated with erythrocytes are protected from early immune clearance. Furthermore, detection of BTV RNA up to 145 days after infection is remarkably similar to the lifespan of the ruminant erythrocytes, suggesting that erythrocytes are likely to be the critical mechanism that allows cattle to serve as natural reservoir hosts of BTV (Brewer *et al.,* 1994). Some other work indicated that BTV RNA can be detected even up to 222 days (Bonneau *et al.,* 2002).

In enzootic areas, BT usually appears in late autumn after long periods of quiescence (8-12 months), a phenomenon called overwintering (Takamatsu *et al.,* 2003). However, conventional models for the transmission of BTV suggest that if adverse winter conditions last for more than 100 days, the virus should be unable to survive from one year to the next. Persistence of BTV in the larvae of vector Culicoides is considered to be highly improbable (Mellor, 1990) although it cannot be ruled out (White *et al.,* 2005). Some authors have postulated that persistently infected γδ T cells, recruited by midge biting, could facilitate the transmission of BTV for periods as long as nine weeks post infection in Dorset sheep (Fig. 9) (Takamatsu *et al.,* 2003). However, this finding was not confirmed in another study using a different sheep breed (Merino sheep) (Lunt *et al.,* 2006).

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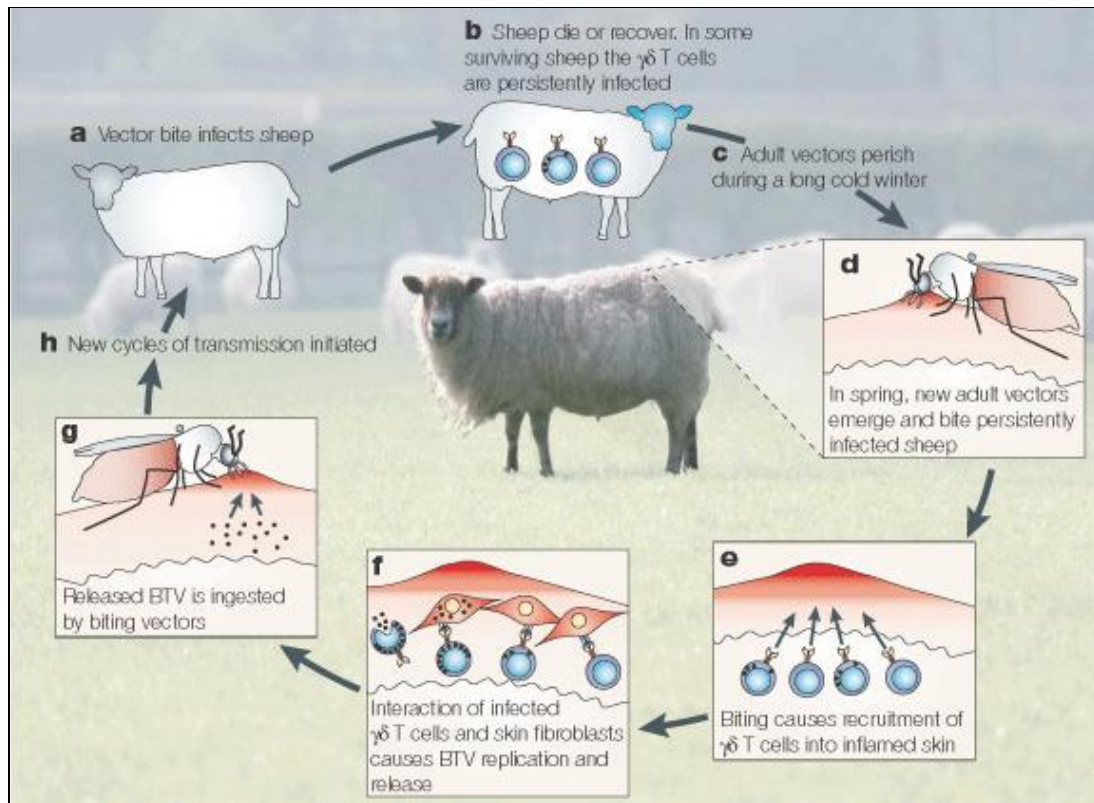
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Fig. 9. The figure shows how, on being bitten by an infected adult Culicoides vector, ruminant hosts become infected with BTV (a). In some infected hosts, the virus establishes a persistent infection of T cells. Surviving animals will be seropositive and aviraemic, but will still carry BTV in their T cells (b). In such apparently recovered animals the virus persists, covertly, over the cold vector-free winter months (c). In the following spring, new vectors emerge and commence biting ruminants, some of which are persistently infected (d). Vector biting causes localized skin inflammation, which initiates the recruitment of inflammatory cells, including infected T cells, into the inflamed areas (e). In the skin, interactions between the skin fibroblasts and the T-cell-specific surface molecule WC-1, causes growth arrest in the infected T cells and, through a mechanism that is not fully understood, converts the BTV persistent infection to a productive, lytic infection. This results in BTV replication, cell death and release of the virus into the skin at locations where the vectors are biting (f). The released virus is ingested by the vector Culicoides, infecting the vector (g) and initiating a new transmission cycle (h) (Adapted from Takamatsu *et al.*, 2003).

V.1 - CELLULAR RESPONSE TO BTV

Viral haemorrhagic diseases are not only a consequence of direct viral damage to the cells but also the result of intense and sometimes deregulated inflammatory processes. BTV reduces endothelial electrical resistance in vitro associated to p38 MAP kinase-dependent cytoskeletal rearrangements (Chiang *et al.,* 2006). In addition, BTV infections induce cell death in many cell types and an important inflammatory cell response.

In mammalian cells, BTV induces cell death (apoptosis and/or necrosis) in cell lines (Mortola *et al.,* 2004), microvascular ovine and bovine endothelial cells (De Maula *et*

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al., 2001), monocytes (Barratt-Boyes *et al.,* 1992) and in WC1-activated γδ T cells (Takamatsu *et al.,* 2003). In mammalian cell lines, uncoating of BTV, but not BTV replication, is required to trigger apoptosis (Mortola *et al.,* 2004). Extracellular treatment with a combination of the viral outer capsid proteins VP2 and VP5, and the cell penetration protein VP5 is sufficient to trigger apoptosis, involving activation of NF-κB (Mortola *et al.,* 2004). However, some cell types that support the replication of the virus do not demonstrate a cytopathic effect to BTV, such as insect cells (Mortola *et al.,* 2004), γδ T cell lines (Takamatsu *et al.,* 2003) and activated blood lymphocytes (Barratt-Boyes *et al.,* 1992). The budding versus the viroporin-mediated viral exit mechanism might partially explain this difference (Wirblich *et al.,* 2006).

Infection of bovine and ovine microvascular endothelial cells induces the transcription of interleukin 1 (IL-1), IL-8, IL-6, cyclooxygenase-2, and inducible nitric oxide synthase (De Maula *et al.,* 2001). These mediators have been involved in the pathogenesis of severe viral haemorragic fevers. Infection of sheep and cattle with BTV induces a plasmatic increase of prostacyclin and thromboxane (De Maula *et al.,* 2001). Thromboxane is a strong pro-coagulant factor whereas prostacyclin is a potent vasodilatator and inhibitor of platelet aggregation. There is a much higher prostacyclin/thromboxane ratio in cattle, which may explain the lower sensitivity of cattle to BTV induced microvascular injury and thrombosis (De Maula *et al.,* 2001).

BTV is also a strong inducer of type I IFN in vivo, in sheep (Foster *et al.,* 1991), cattle (MacLachlan *et al.,* 1985*a*) and mice (Jameson *et al.,* 1978). A strain of BTV serotype 8 was shown to be an extremely potent inducer in vivo in mice, even when UV-irradiated. Strains of many BTV serotypes have been reported as IFN inducers but they may differ in their ability to induce IFN depending on the cell context (Fulton *et al.,* 1982). A wide variety of cells produce type I IFN after BTV stimulation in vitro, including mouse embryo cells (Huismans, 1969), human leucocytes (Jameson *et al.,* 1978), leucocytes from adult sheep (Rinaldo *et al.,* 1975). Double stranded RNA from BTV also induced IFN in mice (Eksteen *et al.,* 1972). However, the exact viral component involved in the induction is unknown.

V.2 - IMMUNE RESPONSES AGAINST BTV

Ruminants infected with BT virus develop a variety of protective antiviral responses including production of interferon as well as virus-specific humoral and cellular

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immune responses. Such capabilities are acquired during gestation so that ruminants are born immunologically competent to BT virus. Antiviral responses of infected ruminants clear the virus from infected animals and prevent reinfection with homologous serotypes of the virus.

V.2.1 - INTERFERON PRODUCTION

BT virus is a very efficient inducer of interferon in vitro, and interferon is present in serum soon after infection of both cattle and sheep. While interferon likely limits the dissemination of BT virus throughout the body it is unlikely that it influences virus clearance as viremia continues long after interferon no longer is detected in serum (MacLachlan *et al., 1985a*).

V.2.2 - HUMORAL IMMUNITY AGAINST BTV

BT virus infected cattle and sheep develop a prompt humoral immune response to a variety of different viral proteins (Jeggo *et al.,* 1983; MacLachlan *et al.,* 1987; Reddington *et al.,* 1991; Richards *et al.,* 1988). Neutralizing antibodies in serum prevent reinfection in a serotype specific manner and virus neutralization is dependent upon the presence of antibodies directed at the outer capsid protein VP2 (Richards *et al.,* 1988; DeMaula *et al.,* 1993). Recent studies indicate that a least some neutralizing epitopes of BT viruses are conformationally dependent (DeMaula *et al.,* 1993), and the significance of individual neutralizing epitopes may vary considerably amongst field strains of the same serotype of BT virus (MacLachlan *et al.,* 1992; Rossitto *et al.,* 1992). Thus the same epitope may function as a major or minor neutralization determinant on different strains of the virus. Furthermore, epitopes which are responsible for neutralization of one strain of BT virus may exist in a non-neutralizing conformation on other strains or serotypes. Neutralizing epitopes may be shared between viruses of different serotypes of BT virus, which is consistent with the fact that animals infected with 1 or more serotypes of BT virus may develop neutralizing antibodies to serotypes of the virus to which they were never exposed. Despite the considerable variation of VP2 amongst different strains and serotypes of BT virus, similar regions of the protein apparently are critical to the neutralization of genetically and antigenically distinct viruses (DeMaula *et al.,* 1993). This suggests that these regions are evolutionarily conserved because of their critical roles in viral replication. Although neutralizing antibodes clearly prevent reinfection

of ruminants with a homologous virus (Jeggo *et al.,* 1983), neutralizing antibodies do not immediately clear virus from the circulation (MacLachlan *et al.,* 1991, 1987; Richards *et al.,* 1988). Thus BT virus may co-circulate with specific neutralizing antibodies for several weeks after infection of both cattle and sheep (Richards *et al.,* 1988). The prolonged viremia which occurs in BT virus infected ruminants likely reflects association of virus with blood cells, especially erythrocytes. The erythrocyte-associated viremia appears to protect the virus from rapid clearance by neutralizing antibody (MacLachlan *et al.,* 1991, 1990; Barratt-Boyes & MacLachlan, 1994; Leudke *et al.,* 1969; Brewer and MacLachlan, 1992).

Ruminants also develop antibodies to a variety of other viral proteins (Jeggo *et al.,* 1983; Richards *et al.,* 1988). These responses form the basis of diagnostic tests as core proteins such as VP7 and nonstructural proteins such and NSI have epitopes which apparently are common to all strains and serotypes of BT virus. Whether antibodies to proteins other than VP2 contribute to the protective antiviral response by mechanisms such as antibody-dependent cellular cytotoxicity currently is unknown. Recent studies indicate that the lymph node draining sites of virus inoculation develops a humoral immune response prior to the systemic response (Barratt-Boyes *et al.,* 1992). Thus antibody can be detected in efferent lymph from a regional node several days prior to its detection in serum. Antibodies to BT virus usually are detected in the serum of BT virus infected ruminants by 7-28 days after inoculation, depending upon the type of assay and route of animal inoculation used.

V.2.3 - CELLULAR IMMUNITY AGAINST BTV

Cell mediated immunity likely limits viral spread during the initial stages of BT virus infection of ruminants, although such responses do not lead to rapid elimination of the virus. Increased numbers of CD8+ T cells are present in efferent lymph from lymph nodes draining the site of BTV inoculation in cattle at 6-8 days after infection (Barratt-Boyes *et al.,* 1992), and peak numbers of CD8+ T cells were detected in blood at 14 days (Ellis *et al.,* 1990). CD8+ T cells possess cytotoxic activity and could lyse BT virus-infected cells and so reduce virus production. In vitro studies suggest that some resistance to BT virus infection can be adoptively transferred with lymphocytes, and that such resistance is not serotype-specific (Jeggo *et al.,* 1983). Attempts to quantitate cellular immunity by in vitro correlates such as the lymphocyte stimulation assay have produced variable results. This is hardly

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surprising given that BTV frequently is present in the peripheral blood mononuclear cells from infected cattle, and that the virus is rapidly cytolytic to dividing lymphocytes (Barratt-Boyes *et al.,* 1992). The induction of cytotoxic T lymphocytes in mice inoculated with BT virus has been documented (Jeggo *et al.,* 1982*a*, 1982*b*, 1982*c*), and similar cells presumably are induced in BT virus infected ruminants. Takamatsu and Jeggo (1989) propagated BTV-specific T lymphocyte cell lines from sheep previously inoculated with BT virus. The cultured cells proliferated after exposure to BT virus, with variable responses to heterologous serotypes (those to which the sheep were not exposed) and some lines exhibited cytotoxic activity, T cell lines which did not react with heterologous serotypes of BT virus were shown to specifically react with outer capsid protein VP2, the protein which also expresses the neutralization determinants of BT virus (Takamatsu *et al.,* 1990). Uncloned lines also reacted with core proteins as well as VP5, the other component of the viral capsid. Although poorly characterized at present, it is likely that cellular immune responses limit virus replication in the initial stages of BT virus infection of ruminants but it is most unlikely that they would exert any effect in clearing virus during prolonged, cell-associated viremia.

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VI. - DISEASE CHARACTERISTICS

The disease is characterised by inflammation of the mucous membranes, congestion, swelling and haemorrhages. Sheep are generally the worst affected, while cattle and goats do not usually show any clinical signs of disease and can carry the virus for a certain period of time and transmit it to other ruminants (Maclachlan, 2004). Severity of illness is influenced by the BTV serotype involved, animal breed and environmental factors like the direct and prolonged exposure to sun light. In fact, photosensitization plays a crucial role in developing serious forms; sheep sheltered from direct sun light exposure show less severe signs of congestion.

VI.1 - CLINICAL SIGNS

The vast majority of infections with bluetongue are clinically inapparent. In a percentage of infected sheep and occasionally other ruminants, more severe disease can occur.

In sheep, the clinical signs may include fever, excessive salivation, depression, dyspnea and panting. Initially, animals have a clear nasal discharge; later, the discharge becomes mucopurulent and dries to a crust around the nostrils (Fig. 10). The muzzle, lips and ears are hyperemic, and the lips and tongue may be very swollen. The tongue is occasionally cyanotic and protrudes from the mouth. The head and ears may also be edematous (Fig. 11). Erosions and ulcerations are often found in the mouth; these lesions may become extensive and the mucous membranes may become necrotic and slough. The coronary bands on the hooves are often hyperemic and the hooves painful (Fig. 12); lameness is common and animals may slough their hooves if they are driven. Pregnant ewes may abort their fetuses, or give birth to "dummy" lambs. Additional clinical signs can include torticollis, vomiting, pneumonia or conjunctivitis (Erasmus, 1975; Pini, 1976; Gard, 1984; Mahrt & Osburn, 1986; MacLachlan, 1994). The death rate varies with the strain of virus. Three or four weeks after recovery, some surviving sheep can lose some or all of their wool.

Infections in cattle are usually subclinical; often, the only signs of disease are changes in the leukocyte count and a fluctuation in rectal temperature. Rarely, cattle have mild hyperemia, vesicles or ulcers in the mouth; hyperemia around the coronary band; hyperesthesia; or a vesicular and ulcerative dermatitis. The skin may develop

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thick folds, particularly in the cervical region. The external nares may contain erosions and a crusty exudate. Temporary sterility may be seen in bulls. Infected cows can give birth to calves with hydranencephaly or cerebral cysts. Cattle that have clinically apparent disease may develop severe breaks in the hooves several weeks after infection; such breaks are usually followed by foot rot. Infections in goats are usually subclinical, and similar to disease in cattle (Leudke *et al.,* 1969; 1970). Although many infections in wild ruminants are inapparent, severe disease can occur in some species. In pronghorn antelope and whitetail deer, the most common symptoms are hemorrhages and sudden death.

VI.2 - POST MORTEM LESIONS

In sheep, the face and ears are often edematous. A dry, crusty exudate may be seen on the nostrils. The coronary bands of the hooves are often hyperemic; petechial or ecchymotic hemorrhages may be present and extend down the horn. Petechiae, ulcers and erosions are common in the oral cavity (Fig. 13), particularly on the tongue and dental pad, and the oral mucous membranes may be necrotic or cyanotic (Mahrt & Osburn, 1986; Pini, 1976). The nasal mucosa and pharynx may be edematous or cyanotic, and the trachea hyperemic and congested. Froth is sometimes seen in the trachea, and fluid may be found in the thoracic cavity. Hyperemia and occasional erosions may be seen in the reticulum and omasum (Fig. 14). Petechiae, ecchymoses and necrotic foci may be found in the heart (Fig. 15). In some cases, hyperemia, hemorrhages and edema are found throughout the internal organs. Hemorrhage at the base of the pulmonary artery is particularly characteristic of this disease (De Maula *et al.,* 2001). In addition, the skeletal muscles may have focal hemorrhages or necrosis, and the intermuscular fascial planes may be expanded by edema fluid.

In deer, the most prominent lesions are widespread petechial to ecchymotic hemorrhages. More chronically infected deer may have ulcers and necrotic debris in the oral cavity. They may also have lesions on the hooves, including severe fissures or sloughing.

VI.3 - MORBIDITY AND MORTALITY

In sheep, the severity of disease varies with the breed of sheep, virus strain and environmental stresses. The morbidity rate can be as high as 100% in this species.

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The mortality rate is usually 0-30%, but can be up to 70% in highly susceptible sheep. Similar morbidity and mortality rates are seen in bighorn sheep. Bluetongue is usually severe in whitetail deer and pronghorn antelope, with a morbidity rate as high as 100% and a mortality rate of 80-90%.

Most infections in cattle, goats and North American elk are asymptomatic. In cattle, up to 5% of the animals may become ill, but deaths are rare. In some animals, lameness and poor condition can persist for some time.

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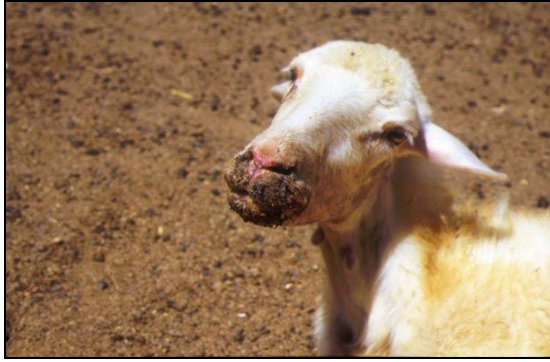Fig. 10.   Fig. 11

Fig. 12   Fig. 13

Fig. 14   Fig. 15

Fig. 10-14. Source: IZSAM – Sistema inforamativo della Blutongue; Fig. 15. Source: www.thecattlesite.com/

VII. - DIAGNOSIS

Bluetongue should be suspected when typical clinical signs are seen during seasons when insects are active. A recent history of wasting and foot rot in the herd supports the diagnosis.

VII.1 - DIFFERENTIAL DIAGNOSIS

The differential diagnosis includes foot-and-mouth disease, vesicular stomatitis, peste des petits ruminants, plant photosensitization, malignant catarrhal fever, bovine virus diarrhea, infectious bovine rhinotracheitis, parainfluenza-3 infection, coenurosis, contagious ecthyma (contagious pustular dermatitis), sheep pox, foot rot and Oestrus ovis infestation. In cattle and deer, EHD can also result in similar symptoms.

VII.2 - LABORATORY TESTS

Bluetongue can be diagnosed by isolating the virus in embryonated chicken eggs or cell cultures. Appropriate cell cultures include mouse L, baby hamster kidney (BHK)-21, African green monkey kidney (Vero), and Aedes Albopictus (AA) cells. Isolation in embryonated eggs is more sensitive than isolation in cell culture.

Bluetongue virus can also be isolated by inoculation into sheep, and sometimes suckling mice or hamsters. Animal inoculation is more sensitive than virus isolation in cell culture, and may be particularly valuable when the virus titer is very low. Bluetongue viruses can be identified to the serogroup level by immunofluorescence, antigen-capture enzyme-linked immunosorbent assay (ELISA) or the Immunospot test, as well as other techniques. These viruses can be serotyped with virus neutralization tests.

Polymerase chain reaction (PCR) techniques are widely used to identify the bluetongue virus in clinical samples. These techniques allow for rapid diagnosis and can identify the serogroup and serotype.

Serology is sometimes used for diagnosis. Antibodies appear 7 to 14 days after infection and are usually persistent. Available serologic tests include agar gel immunodiffusion (AGID), competitive ELISA, and virus neutralization. The AGID and indirect ELISA tests can identify serogroup-specific antibodies. A newer

monoclonal antibody-based competitive ELISA can also distinguish antibodies to viruses in the bluetongue serogroup from antibodies to the EHD serogroup. Virus neutralization tests can determine the serotype specificity of antibodies, but are cumbersome. Complement fixation has largely been replaced by other tests, but is still used to detect antibodies to bluetongue virus in some countries.

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VIII. - BLUETONGUE CONTROL STRATEGY

Bluetongue is an OIE-listed disease that has the potential for rapid spread with significant production losses and is of major importance to the international trade in ruminant livestock (including sheep, goats, cattle and deer).

The policy with regard to an outbreak of bluetongue is to minimise the economic impact and to eliminate clinical disease if circumstances permit. This may be feasible if the disease is detected early in isolated animals and infected vectors are absent, if the disease occurs in a vector-free area, or if frosts are imminent in vector areas. If the disease occurs in areas with competent vectors early in the vector season, control will be difficult.

VIII.1 - EUROPEAN UNION LEGISLATION

To begin on 1999, BTV infection spread progressively across most of Europa and the Mediterranean Basin. At the time, control of BT was covered by European Union (EU) Directive 92/119/EEC (European Council, 1992*a*), together with some other Office International des Épizooties (OIE) List A diseases (such as foot and mouth disease, rinderpest, sheep pox, swine vesicular disease etc.), and EU Directive 92/35/EEC (European Council, 1992*b*) had already defined specific measures against African horse sickness.

Directive 92/119/EEC outlined direct control measures and the demarcation of a 3 km radius protection zone and a 10 km radius surveillance zone, around infected farms. Direct control measures included the slaughter of all susceptible animals on farms and the possible extension of such measures to neighbouring farms suspected of being exposed. In November 2000, the EU, after reviewing the provisions of Directive 92/35/EEC (European Council, 1992*b*), issued EU Directive 2000/75/EC (European Council, 2000) defining specific rules for the control and eradication of BT.

Directive 2000/75/EC establish a combination of strategies to use to limit or control the BT, which included:

1. Quarantine and movement controls;
2. Slaughter of infected / suspected animals;
3. Surveillance and monitoring;
4. Husbandry modification;

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5. Vector control;

6. Vaccination.

The application of Directive 2000/75/EC, through the adoption of Decision 2001/138/EC (European Commission, 2001), has disrupted animal trade in at least a third of Italy. All existing EU legislation regarding the compensation of farmers was developed in relation to contagious diseases of OIE List A, mainly foot and mouth disease and hog cholera (classical swine fever). The control strategy used in Europe for outbreaks of these diseases resorts to stamping-out of infected and in-contact animals and, since 1990, vaccination is only an ancillary measure. In this context, the principal economic losses are direct, due to the slaughter of infected and in-contact animals. According to European legislation, any compensation for indirect losses would perturb the market. Moreover, losses suffered by farmers whose livestock is subjected to movement restrictions also affect the earnings of farmers living in free areas. This has been recognised only recently (July 2003) by the European Commission that enacted the Decision C(2003)2519fin (European Commission, 2003), authorising the region of Sardinia to compensate cattle farmers for indirect losses due to movement restrictions imposed from 6 September 2000 to 31 December 2001.

VIII.1.1 - QUARANTINE AND MOVEMENT CONTROLS

When a clinical outbreak of bluetongue occurs, the initial strategy is a quarantine of domestic ruminants on affected properties, while an epidemiological investigation is carried out. All environmental factors, including ruminant stocking densities, recent movements of ruminants onto and off the property, and recent rain and wind patterns, are recorded. The premises, on which the infected animals are detected, are subjected to quarantine and movement controls and are officially declared as Infected Premises (IP). After confirmation that BTV is circulating, a Restricted Zone (RZ) will then be imposed, consisting of an inner Protection Zone (PZ) - at least 100 km around an IP, and an outer Surveillance Zone (SZ) - at least 50 km in radius beyond that (A. Giovannini *et al.,* 2004*a*). The area outside of the Bluetongue Restricted Zone is referred to as Bluetongue-free. The movement of cattle or sheep are controlled within and from the control zones and exports of live animals may be subject to restrictions.

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VIII.1.2 - SLAUGHTER OF INFECTED / SUSPECTED ANIMALS

Slaughter of susceptible animals infected with bluetongue might, in some circumstances, be considered as a control measure, for example, in the context of single infected imported animal where no further disease is detected (as a precautionary measure to try to stop disease establishing). In some cases, it may be necessary to slaughter infected animals for animal welfare reasons or for trade purposes.

VIII.1.3 - SURVEILLANCE AND MONITORING

A surveillance and monitoring program for virus and competent vectors in affected or threatened areas start immediately when disease is detected. The survey will attempt to determine the extent of the virus and vectors, the serotype involved and its virulence. The survey will also help to define the limits of the bluetongue-free area. If vaccination is used, it will be necessary to distinguish between natural infections and vaccination responses. The epidemiological investigation should include:

• examination of the time and location of the outbreak and the location of the susceptible population;

• recording of recent movements of ruminants onto and off IP;

• identification of the species of vectors and virus serotypes present;

• collection of meteorological data;

• a serum survey of affected animals and contacts.

The size of the area may be very large (100 km radius), depending on meteorological and other factors assessed by epidemiologists.

VIII.1.4 - HUSBANDRY MODIFICATION

This measure is aimed at denying or reducing vector access to susceptible animals. Most vector species of Culicoides including C. Imicola are strongly exophilic (Meiswinkel *et al.,* 2000), therefore, housing susceptible stock during times of maximum vector activity (i.e. the crepuscular periods and during the night) will significantly reduce biting rates and hence the likelihood of infection. In addition, if obvious ports of access to such housing such as windows and doors are screened with material of fine mesh (e.g. sand-fly netting) or with coarser material impregnated with insecticide (e.g. a synthetic pyrethroid) this will reduce biting even further (Braverman, 1989). As BTV vector species of Culicoides seem to be more

attracted to cattle than to sheep some authorities have advocated running the two ruminant species together in order to divert vector-attention from the disease-susceptible sheep (Nevill, 1978).

However, this measure will not break the virus transmission cycle which will continue, covertly, through the cattle population and so is not recommended if virus eradication is the overall objective (Mellor, 1994).

VIII.1.5 - VECTOR CONTROL

It is rarely possible to completely eliminate populations of vector Culicoides. The main aim, therefore, is to reduce the number of potentially infecting bites that animals of the target species receive, to levels where maintenance of an epizootic becomes unsustainable (Anderson & May, 1991). Vector control can be tackled in a number of ways but it is important to remember that a combination of approaches is likely to yield the best results.

VIII.1.5.1 - HABITAT ALTERATION

This control method is dependent upon an ability to be able to recognize and then destroy the breeding sites of the vector species of Culicoides. Culicoides Imicola usually breeds in organically enriched (mainly animal dung), moist but not waterlogged soils. Such areas may be bare or covered with short grass (e.g. irrigated pastures) and need to remain moist for sufficient time to complete the developmental part of the vector's life cycle (i.e. at least 7-10 days). Consequently, slow draining or clay soils are better for C. Imicola than free-draining, nutrient-poor, sandy soils. High populations of C. Imicola usually occur only on livestock farms and seem to be a phenomenon of this type of activity. In such locations humans inadvertently `manufacture' an ideal Imicola habitat by providing ample water, soil contaminated with animal dung and a sedentary `blood bank' of domestic animals. Culicoides Imicola breeding sites can vary in size from a few metres in diameter (irrigation pipe leaks, cattle trough overflows, leaking taps) to whole pastures. Breeding site destruction may be easily achieved through habitat modification, when the sites are few in number and are small (e.g. by turning off taps, mending leaks and filling in or draining damp areas) but may be impossible or not economically feasible in other situations (Braverman, 1989).

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VIII.1.5.2 - ADULTICIDING

Older recommendations for broadscale, aerial application of insecticides with all of the attendant risks to non-target organisms are not likely to find favour in these more environmentally conscious times. However, targeted application of insecticides of known low mammalian toxicity (e.g. the synthetic pyrethroids) in and around animal housing and directly to the target animals themselves can be efficacious against Culicoides species and may be environmentally acceptable (Braverman, 1989; Braverman *et al.,* 1995). Intradermal or subcutaneous inoculation of systemic insecticides such as Ivermectin may also be effective at killing biting Culicoides (Standfast *et al.,* 1985). An additional advantage with this system and with such insecticidal food additives as tetrachlorvinphos, is that these drugs are eliminated in the faeces which, should they be deposited on breeding sites, are toxic to the immature stages of Culicoides (Jackson, 1989; Standfast *et al.,* 1985).

VIII.1.5.3 - LARVICIDING

Application of a larvicide such as "Abate" (American Cyanamid) (5% temephos granulated with gypsum) to Culicoides breeding sites, provides a slow but sustained release of the insecticide and may be effective for periods as long as 30 days (Holbrook, 1985). Such preparations are effective even when used on breeding sites that are rich in organic matter which makes them particularly suitable in Culicoides control (Braverman, 1989). Biological control of larval Culicoides by agents such as Bacillus thuringensis has apparently not proved successful (Lacey & Kline, 1983).

VIII.1.5.4 - REPELLENTS

There are several candidate and established repellents that have been tested at deterring Culicoides attack. However, none is completely effective and the deterrent effect, even of the best, rarely persists for more than a few hours (Schreck *et al.,* 1979; Braverman & Chizov-Ginzburg, 1997). Di-ethyl toluamide (DEET) seems to be the only commercially available repellent that has been shown to have a significant deterrent effect against Culicoides for periods of up to 4 h (Braverman & Chizov-Ginzburg, 1997). Since C. Imicola attacks apparently peak during the first 4 h of the night, if applied nightly to target animals, DEET may have a significant but temporary effect in reducing the biting rate of this species.

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IX. - VACCINES AGAINST BLUETONGUE IN EUROPE

The incursion of BTV into Mediterranean Europe is having a considerable negative economic impact, partly due to direct losses from mortality and reduced production in affected livestock but, more importantly, because of the total ban of ruminant trade between BTV-infected and non-infected areas (Calistri *et al.,* 2004). To limit direct losses and in an effort to minimize the circulation of BTV, as well as to allow the safe movement of animals, the Italian, French, Portuguese and Spanish authorities all undertook vaccination of livestock according to their individual national policies, the geographic distribution of the incurring BTV serotype(s), and the availability of appropriate vaccines. In France, only sheep were vaccinated whereas in Italy, all susceptible domestic ruminant species were vaccinated, i.e. sheep, goats, cattle and water buffalos (Patta *et al.,* 2004). In Spain, initially only sheep were vaccinated (2001/2003 in Balearic Islands) but in later outbreaks (southwest Spain) both cattle and sheep were subjected to vaccination (MAPA 2006). The Italian vaccination campaign was based on a risk assessment that demonstrated that such a vaccination strategy would prevent direct economic losses, significantly reduce virus circulation (Giovannini *et al.,* 2003) and minimize risks linked to the movement of animals from infected to free areas.

Different vaccines against BT have been used and these include inactivated whole [killed] virus preparations, virus-like particles (VLPs) produced from recombinant baculoviruses, live attenuated vaccines (modified live viruses, MLVs) and live recombinant vaccinia or canarypox virus-vectored vaccines (Murray *et al.,* 1996; Boone *et al.,* 2007). All have inherent potential advantages and disadvantages, but only MLVs and some inactivated vaccines are currently available under European Community approved national disease control programs. VLPs are also safe and have been shown to be efficacious in laboratory trials (Roy *et al.,* 1990, 1992, 1994; Roy, 2004), but their efficacy in the field is still under evaluation.

IX.1 - INACTIVATED VACCINES

The first inactivated vaccine that was developed and used in the field after the emergence of BT in Europe was the vaccine against BTV-2. Subsequently, a monovalent BTV-4 and a bivalent BTV-2 and -4 vaccines have been developed and

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used in Corsica, Spain, Portugal and Italy. Other inactivated vaccines have recently been developed or are under development.

Inactivated whole virus vaccines are very safe if properly produced. They can be highly efficacious (Stott *et al.,* 1985; Di Emidio *et al.,* 2004) and although not yet available, strategies for differentiating infected from vaccinated animals (DIVA) are theoretically possible with these type of vaccines. Their inherent potential disadvantages include their high costs of production, as vaccination requires large amounts of antigen; and the need for booster immunizations, as inactivated vaccines generally induce a relatively transient immunity.

IX.1.1 - QUALITY CONTROL

For quality control testing, no data were given regarding the control tests performed during the different stages of production of the inactivated vaccine; however, all companies must follow the current guidelines described in the EU legislation concerning the manufacture of veterinary vaccines.

IX.1.2 - SAFETY

Several studies have been conducted on sheep to evaluate the safety of the subcutaneous injection of inactivated prototype vaccines against BTV-2, BTV-4 and BTV-2&4 in either simple, repeated or overdose trials. In all conditions, the inactivated BTV prototype vaccines were very well tolerated as demonstrated by the absence of systemic reaction (fever, weight loss, reproductive dysfunction, etc.) related to vaccination. Some vaccines induced transient local reactions of variable severity (mild to moderate) with different frequency (unusual to common). These usually disappeared within 3 days but, in a single case, a moderate local reaction persisted for 2 weeks (Hamers *et al.,* 2006*a*). Anaphylactic shock was also reported in 0.02% sheep following vaccination with a BTV-4 inactivated vaccine. This event was observed only in areas where BTV-4 MLV had previously been used.

BTV-4 and BTV-2&4 inactivated vaccines have also been tested for safety in cattle (MAPA, 2006; Savini *et al.,* 2006*a*, 2006*b*). The vaccines were very well tolerated, and no side effects or local reactions were observed even when five doses of the BTV-4 inactivated vaccine were administered to the same animal (MAPA, 2006).

No data documenting systemic or local reactions in vaccinated animals are available from the field use of these vaccines, as no complaints were reported from farmers. In

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addition, as no significant antigenic variation was yet documented amongst the BTV-2 and -4 strains currently circulating in Europe, the strains used to produce the inactivated vaccine are still suitable for the production of effective vaccines against these serotypes.

IX.1.3 - EFFICACY

Most companies producing BTV inactivated vaccines follow the guidelines of the European Pharmacopea and Committee for Veterinary Medicines Products for quality and safety control and efficacy. Assessment of efficacy is based on clinical and virological data as well as on immunogenicity. Immunogenicity is assessed by the analysis of the antibody response induced by each immunization, as measured by ELISA and by titration in a VNT against the same serotype (BTV-2 or BTV-4). The efficacy of the vaccine is evaluated in vaccinated animals by inoculation of an infective dose of live virulent BTV. The level of viremia after virus challenge is considered the most objective way to assess the efficacy of the vaccine-induced immunity. The level of viremia is analyzed by either a BTV-specific quantitative real-time RT-PCR assay (Jiménez-Clavero *et al.,* 2006) or by virus isolation. In addition, clinical signs (fever, general congestion of the skin, edema and lameness) are evaluated after challenge.

The inactivated prototype BTV vaccines induced significant titer of neutralizing antibodies after either one or two injections in sheep. A booster effect was observed after the second immunization (Hamers *et al.,* 2006*a*, 2006*b*). In cattle, one dose of BTV-4 or BTV-2&4 inactivated vaccines induced a weak humoral response which rapidly declined to be undetectable 21 days following vaccination. However, the second dose of vaccine elicited high and stable titers of neutralizing antibodies (MAPA, 2006; Savini *et al.,* 2006*a*).

The number, age and breed of animals, as well as the challenge dose and virus strain vary for the different vaccines. A preliminary study was carried out to establish and standardize the optimal conditions for BTV vaccine trials on sheep and cattle. The effect of factors like the number of cell culture passages of the virus inoculum, the virus dose, type of inoculum and route of inoculation have all been evaluated. The infectivity of the field isolate was confirmed after four passages in cell culture, and the route of inoculation (subcutaneous or intravenous) did not affect either the occurrence of clinical signs or the duration and titer of viremia. Appropriate titers of

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viremia were also obtained with different inocula (infected blood or cell culture-propagated virus) at different doses [$2x10^7$ and $2x10^6$ $TCID_{50}$ (50% tissue culture infective dose)/dose or threshold cycle (TC): 25–27 and TC: 30–33]. The inoculation of cell culture-propagated virus-induced detectable viremia 3 days earlier (day 4) than that of blood from highly viremic sheep.

One or two doses of inactivated BTV-2, BTV-4 and BTV-2&4 vaccine at 3–4 week intervals gave full and significant protection against clinical signs and viremia in sheep that were intradermally challenged with virulent BTV-2 and/or BTV-4 a week or a month after last vaccination. For BTV-2, it was also shown that a single dose protected sheep against both clinical signs and viremia for at least 6 months (Hamers *et al.,* 2006*a*, 2006*b*). For BTV-2&4, two doses fully protect sheep for up to 12 months.

In cattle, efficacy studies have been performed on BTV-4 and BTV-2&4 vaccines. Two doses of the inactivated BTV-4 vaccine administered at a 24-day interval prevented viremia in vaccinated animals challenged with the homologous virulent serotype. Similarly, none of the animals vaccinated with two doses of BTV-2&4 inactivated vaccine developed detectable viremia following challenge with virulent field strains of BTV-2 and/or BTV-4 that were performed up to 1 month after the second vaccination (Savini *et al.,* 2006*a*). However, although a single dose of BTV-4 inactivated vaccine prevented viremia in vaccinated animals challenged 2 weeks after vaccination, a single vaccination did not fully prevent viremia in animals challenged 7 months after vaccination (MAPA, 2006).

The efficacy of the inactivated BTV vaccine was indirectly confirmed in the field when all but 2 of more than 40.000 seasonally migrating vaccinated Spanish cattle remained negative for BTV by RT-PCR after staying in a restricted area in the presence of BTV circulation (Jiménez-Clavero *et al.,* 2006).

IX.2 - MODIFIED LIVE VIRUS (MLV) VACCINES

Outside of the EU, including the USA, Turkey, Republic of South Africa and India, BTV MLVs are available for many BTV serotypes. MLVs are produced by adapting BTV field isolates to growth in vitro through serial passages in tissue culture or in embryonated chicken eggs. Stimulation of a strong antibody response by these vaccines directly is correlated with their ability to replicate in the vaccinated host.

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MLVs are cheap to produce in large quantities, they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used (Patta *et al.,* 2004; Caporale *et al.,* 2004; Dungu *et al.,* 2004). However, BTV MLVs suffer from a variety of documented or potential drawbacks including under-attenuation, whose impact may vary with sheep of different breeds. Potential adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring when used on pregnant females (MacLachlan *et al.,* 1985*b*; Venter *et al.,* 2004; Savini, *et al.,* 2004*a*; Monaco *et al.,* 2004*a*; Monaco *et al.,* 2004*b*; Ferrari, *et al.* 2005; Gerbier *et al.,* 2003). Another risk associated with the use of MLVs is that of their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of MLV genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but natural and local dissemination of BTV-2 and -16 MLV vaccine strains has already been documented in Europe. Natural dissemination of MLV strains of BTV likely also is responsible for the sporadic incidence of teratogenic defects in unvaccinated cattle in South Africa and North America. Finally, the intrinsic inability to serologically distinguish naturally infected from MLV vaccinated animals precludes the possibility of developing a DIVA strategy with the MLV vaccines.

After the incursion of BTV into Mediterranean Europe, the Spanish, French, Italian and Portuguese authorities have all carried out compulsory vaccination campaigns since 2000 using MLVs produced by Onderstepoort Biological Products in an attempt to reduce direct losses due to disease and indirect losses due to trade embargoes caused by the presence of BTV. At that time, these were the only commercially available BTV vaccines. Based on the serotype(s) present in a given country/area, various MLV monovalent serotype formulations have been used.

IX.2.1 - QUALITY CONTROL

Four monovalent MLV vaccines have been imported from South Africa and used in the EU (Table V). Before use, these vaccines were confirmed by the various EU National Laboratories to be free of bacterial, fungal and viral contaminants. Titer and serotype of the MLV batches also have been verified with no major discrepancies with information provided by the manufacturer. For the BTV-2 MLV, it was observed that the titer strongly decreased at temperatures above 35 °C (Hammoumi,

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et al., 2003), but that it was retained for at least 36–48 h if sterility conditions were ensured during rehydration and the reconstituted vaccine was stored at a temperature below 19 °C (Ronchi *et al.,* 2003).

Table V. Characteristics of the bluetongue virus vaccine strains (Savini *et al.*, 2007).

Modified live vaccine	Strain	Date, place of isolation	History
BTV-2	Vryheid/ 5036	1958, Republic of South Africa	Virus passaged 50 times in embryonated chicken eggs and plaque selected three times and passaged twice in BHK_{21} cells.
BTV-4	Theiler/ 79043	1900, Republic of South Africa	Virus passaged 60 times in embryonated chicken eggs, followed by three small plaque selections and nine passages in BHK_{21} cells.
BTV-9		1900, Republic of South Africa	Virus passaged 70 times in embryonated chicken eggs, followed by three small plaque selections and six further passages in BHK_{21} cells.
BTV-16	Pakistan/ 7766	Pakistan	Virus passaged 37 times in eggs, followed by three large plaque selections, two passages in BHK_{21} cells and one in VERO cells.

IX.2.2 - SAFETY

Modified live viruses have different potential adverse impacts according to the specific formulation used, the specific serotypes and the number of serotypes included in the vaccine.

IX.2.2.1 - FEVER AND SICKNESS

Apart from a study performed by the Institute of Animal Health at Pirbright using either BTV-2 or BTV-9 and Polled Dorset sheep, in which moderate to severe clinical signs of BT, albeit short lived, were observed following vaccination (Veronesi *et al.* 2005), only mild symptoms were observed in most experimental MLV vaccination studies. These were characterized by transient fever starting from the fifth day after vaccination (p.v.) and mild hyperemia of the oral cavity during the second week.

Reports of adverse events in the field greatly vary with the strain of BT MLV used for vaccination of the animals. The monovalent BTV-2 MLV vaccine was used in Corsica (from 2001 to 2004) and Italy (from 2002) on approximately 130.000 and 4.000.000 sheep and goats, respectively. In both locations, there were no or negligible adverse reactions reported after vaccination (Bréard *et al.,* 2004; Gerbier *et al.,* 2003; IZSAM, 2001; IZSAM, 2004). However, when the same vaccine was

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used in 2000/01 in Menorca and Mallorca on 320.000 sheep, adverse events were observed in 0.13% and abortion in 0.16% of the vaccinated animals (MAPA, 2006). The BTV-2 MLV vaccine was also used on more than 400.000 cattle during the 2002 Italian vaccination campaign with no adverse reactions reported.

The monovalent BTV-4 MLV vaccine has been only used in sheep in Corsica in 2004. No adverse reactions were reported (Bréard *et al.,* 2004). In 2003 a new outbreak of BT occurred in Menorca that was due to BTV-4. A combined BTV-2 and -4 MLV vaccine was used to vaccinate sheep and goats, and no side effects were observed. The same vaccine combination was used in 2004 in Tuscany, and the following year in Tuscany and Sardinia, involving some 4.000.000 sheep and goats. Again, no or negligible adverse reactions were reported. Similarly, no adverse side effects were recorded among approximately 400.000 cattle vaccinated with the combined BTV-2 and -4 MLVs in 2004 and among a similar number of animals in 2005.

A bivalent BTV-2 and -9 MLV vaccine was used to vaccinate sheep and goats in some regions of Italy since 2002. Of the more than 1.700.000 animals vaccinated, only a very small percentage ($<0.1\%$) developed fever and facial edema at 7–14 days p.v. (IZSAM, 2004). In the same vaccination campaign, BTV-2/BTV-9 MLVs were also administered to more than 600.000 cattle and no adverse reactions were reported. Similarly, a trivalent MLV vaccine containing BTV serotypes 2, 4 and 9 was used since 2005 on more than 1.000.000 sheep, goat and cattle, and no or negligible adverse reaction were reported.

In 2004, BTV-16 was isolated in Corsica by the AFSSA Maisons-Alfort laboratory (Zientara *et al.,* 2006). Vaccination of sheep with BTV-16 MLV was performed in the southern part of the island. Typical signs of BT were reported in vaccinated sheep several days after vaccination. The vaccination program was therefore immediately terminated. The nucleotide sequence of segments 2, 7, 8, 9 and 10 from the virus isolated from ill animals was determined and found to be exactly identical to that of the vaccine MLV strain (Zientara, unpublished).

Also in 2004, a trivalent MLV vaccine containing serotypes 2, 4 and 16 was used in Sardinia. However, a few weeks after vaccination, many vaccinated and unvaccinated sheep and goats became ill because of infection with the BTV-16 vaccine strain (Monaco *et al.,* 2006). These incidents were attributed to inadequate attenuation of the BTV-16 MLV that was in the vaccine, and for this reason the use

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of monovalent BTV-16 MLV vaccine was discontinued (Italian Ministry of Health, 2005). In contrast to what occurred in sheep and goats; however, the BTV-2, -4 and -16 MLV combination did not cause significant adverse reactions in cattle (approximately 600.000 vaccinated animals).

A polyvalent MLV containing BTV serotypes 2, 4, 9 and 16 was used in southern regions of Italy in 2004, and approximately 1.700.000 sheep and goats were vaccinated. No adverse reactions were reported. The same BTV-2, -4, -9 and -16 MLV combination did not cause adverse reactions in cattle (approximately 600.000 vaccinated animals).

Finally, the safety of a pentavalent BTV serotypes 1, 2, 4, 9 and 16 MLV vaccine was evaluated in sheep maintained in isolation facilities at the Afssa Sophia Antipolis laboratory. Significant fever (41-42 °C) was reported in many animals (unpublished data). Based on these observations, it was decided that this vaccine should not be used in the field.

IX.2.2.2 - EFFECT ON PREGNANCY

Experimental infection studies using MLV strains of BTV-2 or BTV-2 and -9 were conducted on cattle to evaluate potentially deleterious effects on reproduction. In none of these studies was any adverse effect on pregnancy observed (Monaco *et al.,* 2004*b*, 2004*c*; Lucifora *et al.,* 2004).

Abortions and/or stillbirth have however been reported in the various vaccination campaigns that used BTV MLVs. During the 2000/01 Balearic BTV-2 campaign, approximately 0.16% of the 320.000 vaccinated sheep aborted. Similarly, during the Italian BTV-2 vaccination campaign, abortion was reported on 0.42% and 0.18% of vaccinated sheep and cattle, respectively. However the virus was detected in only 0.06% and 0.01% of their respective aborted fetuses. In 2002, the combined BTV-2 and -9 MLV resulted in abortion of 0.53% and 0.14% of vaccinated sheep and cattle, respectively, although BTV was detected only in a small percentage of abortions (0.09% and 0.01% of the sheep and cattle fetuses, respectively) (Monaco *et al.,* 2004*c*).

IX.2.2.3 - EFFECT ON SEMEN

The effects of the BTV-2 MLV on the quality of semen were investigated (Bréard *et al.,* 2007) in 23 rams vaccinated at a 47-day interval. Although BTV was not detected in any of the semen samples, a decrease of the semen quality (volume,

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sperm concentration, motility, abnormal and dead spermatozoa) was demonstrated after the first vaccination. A decrease in semen quality was also observed after the second vaccination; however, at day 69, the semen quality of the vaccinated animals was not significantly different from those of the 23 controls.

IX.2.2.4 - MILK PRODUCTION

Given the economic importance of milk production, numerous studies have been conducted to determine the effects of several MLV combinations on milk production in both sheep and cattle.

Vaccination of sheep with either BTV-2 or BTV-2 &4 MLVs did not affect the quantity and quality of milk produced by the vaccinated animals (Zientara, unpublished; and (Caporale *et al.,* 2003; Cannas *et al.,* 2005; Giovannini *et al.,* 2004*b*).

Quite different were the observations made with BTV-2 and -9, BTV-2 and -16, BTV-2, -4 and -9 or BTV-2, -4, -9 and -16 MLVs. Vaccination with each of these combinations had a marked negative impact on total milk production with production decreases of 20–30% as compared to normal production levels. The decrease, evident in the second week following vaccination, was transient and not accompanied by significant changes in milk quality (cell count, pH, fat, protein and lactose) (Savini *et al.,* 2004*a*; Cannas *et al.,* 2005). It was suggested that the effects of vaccination on milk production were primarily due to the transient perturbation of health induced by the vaccine and not to a direct virus effect on the mammary tissue (Savini *et al.,* 2004*a*).

In contrast, vaccination of 30 cows with combined BTV-2 and -9 MLV vaccines had no effect on the production and quality (somatic cell count, pH, milk fat, protein and lactose content) of their milk (Monaco *et al.,* 2004*b*).

IX.2.2.5 - DURATION AND TITER OF VIREMIA

After immunization with MLVs, the attenuated virus circulates in the blood stream and so potentially can infect competent vectors and be transmitted to other susceptible hosts. Therefore, MLV vaccination should be performed in the cooler months when the Culicoides population and its activity typically are at the lowest level. This will limit the possibility of transmission of the vaccine strains by biting midges while immunizing susceptible animal populations before the next epidemic season.

Transmission of MLV strains of BTV to insects most likely would occur from viremic animals that are introduced into infection-free areas where competent Culicoides species are present and highly active. In this scenario, the magnitude and duration of viremia in vaccinated animals would be clearly important in determining whether or not MLV strains of BTV could be acquired and transmitted by local vectors. Although virus titers in blood less than 10^3 TCID$_{50}$/ml have traditionally been considered a ''safe'' threshold, authentic instances of insects acquiring BTV from animals with viremic titers less than 10^3 TCID^{50}/ml have been reported. Given the complex interaction of BTV, Culicoides vectors and animal hosts in the life cycle of infection, virus titers induced by MLV should be kept to an absolute minimum specially if field transmission of MLV strains is a concern.

Studies on the duration and titers of viremia have been performed on sheep and cattle following vaccination with different MLV combinations. Viremia following vaccination with BTV-2, -4, -9 and -16 MLV strains (including multivalent combinations) was found to persist for up to 24 days in sheep and 78 days in cattle (Monaco *et al., 2004a, 2004*b, 2006; Savini *et al.,* 2004*b*, 2004*c*, 2004d, 2005). Information pertaining to the MLV strains used in Europe is however limited; available data suggests that cattle vaccinated with BTV-2 and BTV-9 MLVs can be moved safely 32 days after vaccination (Monaco *et al.,* 2004*a*), whereas sheep vaccinated with the same strains can be moved 28 days following immunization (Savini *et al.,* 2004*a*). From the viremia data obtained in cattle following BTV-2, -4, -9 and -16 MLV vaccination, it was determined that cattle could be moved safely (risk of infection <0.01%) at 60 days after vaccination (Savini *et al.,* 2006*b*). The latter result, however, is most likely related to the inadequate attenuation of the BTV-16 MLV strain and cannot be extrapolated to MLV vaccines that do not include this serotype.

Apart from some BTV-2 MLV vaccination studies on sheep and cows where virus titers were never found to be higher than 10^3 TCID$_{50}$/ml (Hammoumi *et al.,* 2003; Monaco *et al.,* 2004*c*), all other MLV combinations which have been studied in sheep (BTV-2, BTV-9, BTV-16, BTV-2 and -9, BTV-2 and -4, BTV-2, -4 and -16, BTV-2, -4, -9 and -16) and cattle (BTV-2 and -9, BTV-2, -4, -9 and -16) gave rise, for a brief period of 2–4 days, to viremic titers above the infecting threshold at least in some of the vaccinated animals (Savini *et al.,* 2006*a*, 2006*b*, 2005*a*, 2004*a*; Monaco *et al.,* 2004*a*, 2006; Veronesi *et al.,* 2005;).

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No data have been reported, however, on the duration and titers of viremia in animals vaccinated with these MLV in the field, but local transmission of BTV-2 and BTV-16 vaccine strains in the field has been demonstrated (Ferrari *et al.,* 2005; Monaco *et al.,* 2006).

IX.2.3 - EFFICACY

An important factor in confirming the efficacy of MLV vaccines is their ability to elicit neutralizing antibodies in vaccinated animals. Neutralizing antibodies play a key role in protecting animals from disease and viremia following infection with the homologous wild-type BTV. Knowing the duration of the immune status derived from vaccination is of paramount importance for both planning the frequency of vaccine booster immunizations to adequately protect the animals against disease, and to facilitate the safe movement of vaccinated animals (OIE, 2005).

Experimental challenge studies have demonstrated that vaccination with the BTV-2 MLV strain prevented viremia in at least 90.5% of vaccinated cattle that were challenged at 7 months after vaccination with a dose of $2\times10^{5.8}$ TCID$_{50}$ of virulent homologous field isolate (Savini *et al.,* 2004*b*). Serological studies performed on cattle and sheep that were vaccinated with several MLV combinations have shown that more than 80% of the vaccinated animals had specific BTV antibodies (Hammoumi *et al.,* 2003; Gerbier *et al.,* 2003; Savini *et al.,* 2004*c*, 2004*e*). Colostral antibodies were found in calves born from vaccinated dams until 39 days of age (Savini *et al.,* 2004*c*).

The efficacy of MLV vaccination has widely been demonstrated in the field. Following the 2000/01 and 2003 BT vaccination campaigns in the Balearic Islands, no outbreaks have been detected since December 2003 in the area.

With regard to the vaccination strategy in Italy, several points warrant attention. First, on the basis of a risk assessment (Giovannini *et al.,* 2003) and considering the encouraging results of preliminary studies, the Italian Authorities decided to vaccinate all susceptible domestic ruminant species (i.e. sheep, goats, cattle and water buffalo) in the infected and at risk areas, with the aim of limiting direct losses and reducing virus BTV circulation (Patta *et al.,* 2004). Mass vaccination of susceptible populations started in January 2002, although the starting dates and the percentages of vaccinated population achieved varied greatly among regions (Patta *et al.,* 2004). In those areas where more than 80% of the target population was properly

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vaccinated before the new epidemic peak, clinical disease in sheep disappeared almost completely and virus circulation was significantly reduced (Patta *et al., 2004*), with substantial benefit to internal animal trade/movement. The results obtained in some Italian regions with mass vaccination of all susceptible domestic ruminants and the experience gained during the vaccination campaigns contributed to the modifications of BT international standards. Specifically, risk analysis can be used as an alternative to individual testing to assess immunity level in the population of origin and determine the risk of spreading infection to free areas by movement of vaccinated animals from infected territories (Giovannini *et al., 2003*). In particular, the analysis performed by Giovannini (Giovannini *et al., 2003*) indicates that when more than 80% of the susceptible population in the territory of origin was vaccinated, the risk associated with the movement of vaccinated animals to free areas appeared acceptable and could further be mitigated by ancillary control measures.

In the absence of effective inactivated vaccines and in an emergency, MLVs still represent a valid option for vaccination, provided that the quality, safety and efficacy of the MLV strains match EU standards. These vaccines can be an alternative also in a non-emergency situation when local conditions (e.g. in case a large amount of animal must be immunized in a very short period of time) indicate their use.

IX.3 - RECOMBINANT VACCINES

Several experimental recombinant vaccines have been described and they clearly have numerous inherent potential benefits, including rapid onset of immunity, lack of transmissibility and even a polyvalent strategy.

A recombinant vaccinia virus that expressed both VP2 and VP5 of Australian BTV serotype 1 induced variable titers of neutralizing antibody in sheep and afforded protection against homologous challenge (Lobato *et al., 1997*), but this approach has not been pursued further.

A recombinant capripoxvirus expressing VP7 was shown to provide partial protection against heterologous BTV challenge (Wade-Evans *et al.*, 1996), but like the recombinant vaccinia BTV vaccine, its development was not continued.

Finally, a recombinant canarypox virus-VP2/VP5 vaccine was recently described that induced highly effective protective immunity in sheep (Boone *et al., 2007*). This vaccine has a major inherent advantage in that the existing VP7 competitive ELISA

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assay would distinguish vaccinated from naturally infected animals (DIVA), and it utilizes an expression vector that is incorporated in several vaccines already in use in the EU and elsewhere. The vaccine still is at a development stage.

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X. - FLOW CYTOMETRY

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus.

The first practical applications of flow cytometry, beginning in the 1940's, were to counting blood cells in liquid suspension, on the one hand, and bacteria and other small particles in aerosol, on the other, based on measurements of light scattering; these signals were also used to provide estimates of cell size. In the early 1960's, light absorption measurements were used for quantitative flow cytometric analyses of cellular nucleic acid and protein. Flow cytometers in modern clinical haematology laboratories perform counts of red cells, white cells, and platelets in blood, as well as differential leukocyte counts, using combinations of electrical impedance, light scattering and light absorption measurements (Shapiro, 1995). As regards the veterinary sector the flow cytometry is lately obtaining greater consensus thanks to greater demand for specialist examinations in clinical diagnostic process of small animals.

X.1 - PRINCIPLES

One of the fundamentals of flow cytometry is the ability to measure the properties of individual particles. When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system. Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters
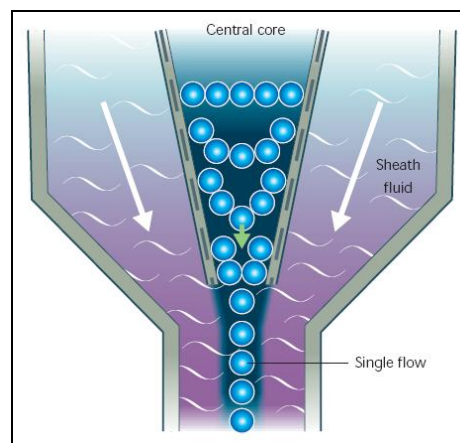

Fig. 14. Hydrodynamic focusing produces a single stream of particles

the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its center and zero velocity at the wall (Fig. 14).

The effect creates a single file of particles and is called hydrodynamic focusing. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid. Without hydrodynamic focusing the nozzle of the instrument (typically 70 μM) would become blocked, and it would not be possible to analyze one cell at a time.

After hydrodynamic focusing, each particle passes through one or more beams of light. Light scattering or fluorescence emission (if the particle is labeled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry.

Lasers produce a single wavelength of light (a laser line) at one or more discreet frequencies (coherent light). Arc lamps tend to be less expensive than lasers and exploit the color emissions of an ignited gas within a sealed tube. However, this produces unstable incoherent light of a mixture of wavelengths, which needs subsequent optical filtering.

Light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis, is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells.

Light measured approximately at a 90° angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample.

Fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorochrome-labeled cell surface receptors or intracellular molecules such as DNA and cytokines.

Flow cytometers use separate fluorescence (FL-) channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings.

The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types. 'Long pass' filters allow through light above a cut-off wavelength, 'short pass' permit light below a cut-off wavelength and 'band pass' transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption (Fig. 15).

When a filter is placed at a 45° angle to the oncoming light it becomes a dichroic filter/mirror. As the name suggests, this type of filter performs two functions, first, to pass specified wavelengths in the forward direction and, second, to deflect blocked light at a 90° angle (Fig. 16).
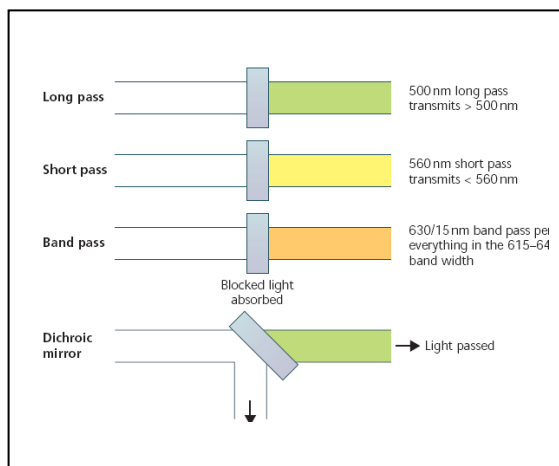

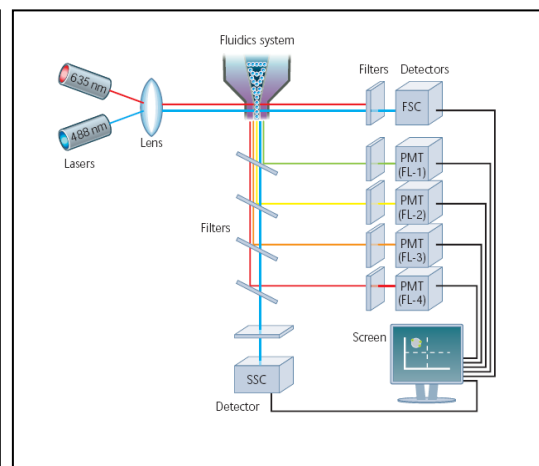Fig. 15. Different types of optical filters.

Fig. 16: Schematic overview of a typical flow cytometer setup.

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis.

The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each parameter are known

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as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

X.2 - IMMUNOPHENOTYPING

Immunophenotyping is a technique used to study the protein expressed by cells. This technique is commonly used in basic science research and laboratory diagnostic purpose. This can be done on cell suspension, tissue section (fresh or fixed tissue), etc. It involves the labelling of white blood cells with antibodies directed against surface proteins on their membrane. By choosing appropriate antibodies, the differentiation of subpopulations of normal o abnormal lymphocytes cells can be accurately determined. The labelled cells are processed in a flow cytometer, a laser-based instrument capable of analyzing thousands of cells per second. The whole procedure can be performed on cells from the blood, bone marrow or spinal fluid in a matter of a few hours.

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- SECOND PART –
Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep

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XI. - AIM

The aim of this study was to determine the safety of a newly produced BTV serotype 1 modified-live virus (MLV) vaccine and its efficacy in protecting sheep against bluetongue infection. For this purpose the protocol reported on the "Manual of diagnostic tests and vaccines for terrestrial animals" of the "Office International des Épizooties" (OIE, 2004*f*) was used, except for some additional laboratory tests useful to evaluate health status. In this way it was possible to establish the real vaccine's ability to prevent clinical insurgence of BT disease. Furthermore, in the challenge trial of efficacy test, white blood cell subsets of immunised and non immunised sheep following BTV-1 experimental infection were identified by flow cytometry. Finally a group of sheep were challenged with virulent strain of BTV2 in order to verify the protection of animals vaccinated with BTV1 vaccine against serotype 2, assuming serotype 2 to be antigenically correlated with serotype1.

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XII. - MATERIALS AND METHODS

Our experimental trial consists of different tests to ensure innocuity (safety tests) and efficacy (efficacy tests) of BTV-1 modified-live virus vaccine as described in the "Manual of diagnostic tests and vaccines for terrestrial animals" by OIE (OIE, 2004*f*). The MLV vaccine against BT serotype 1 used was manufactured and supplied by "Centro Studi Malattie Esotiche" (CESME) of "Istituto Zooprofilattico dell'Abruzzo e Molise - G. Caporale" in Teramo.

XII.1 - SAFETY TESTS

This test is conducted on sheep to evaluate the safety of inactivated vaccines in either simple or overdose trials as demonstrated by the absence of systemic (fever, reproductive dysfunction) or local reaction related to vaccination. Safety test is important also in determining whether or not attenuated virus strains of BTV could be acquired and transmitted by local vectors and could possibly lose some of its characteristic of attenuation to acquire other more pathogenic.

XII.1.1 - ANIMALS

Thirty-one Sarda sheep (Table VI) of various ages, healthy and BTV seronegative were selected for this trial. Animals were kept at the "Istituto Zooprofilattico Sperimentale della Sardegna" facilities in a Culicoides - free area. Animals were treated in accordance with national and European Union animal welfare regulations.

Clinical examination and laboratory analysis were executed in order to ensure sheep health condition. Blood samples were collected to verify eventual seropositivity against common ruminant infectious diseases (visna-maedi, pestivirus, paratubercolosis) and to determine haematological and biochemical parameters. Furthermore, echography examinations to diagnose pregnancy and identify its stage were performed.

XII.1.2 - VACCINE INOCULATION

A group of 19 sheep were vaccinated subcutaneously with 1 ml of 10^3 TCID$_{50}$ of BTV-1 modified-live virus vaccine; 7 sheep were vaccinated with 1 ml of decuple dose (10^4 TCID$_{50}$); the remaining 5 sheep were inoculated with 1 ml of sterile saline

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solution and were used as mock-vaccinated control animals to detect possible local circulation of wild BTV in the experimental group.

Table VI. Sheep used in the experimentation of the strain BTV-1 attenuated

Progressive	Identification number	Vaccine dose	Age	Sex	Birth Date
1	N19	10^3 TCID$_{50}$	2 years	Female	January
2	903	10^3 TCID$_{50}$	1 year	Female	End of February-March
3	907	10^3 TCID$_{50}$	1 year	Female	End of February-March
4	915	10^3 TCID$_{50}$	1 year	Female	End of February-March
5	912	10^3 TCID$_{50}$	1 year	Female	End of February-March
6	N06	10^3 TCID$_{50}$	2 year	Female	End of February-March
7	897	10^3 TCID$_{50}$	--------	Female	End of February-March
8	909	10^3 TCID$_{50}$	1 year	Female	End of February-March
9	917	10^3 TCID$_{50}$	1 year	Female	End of February-March
10	905	10^3 TCID$_{50}$	1 year	Female	End of February-March
11	908	10^3 TCID$_{50}$	1 year	Female	End of February-March
12	N08	10^3 TCID$_{50}$	2 years	Female	January
13	F3	10^3 TCID$_{50}$	4 years	Female	January
14	F4	10^3 TCID$_{50}$	4 years	Female	January
15	N14	10^3 TCID$_{50}$	2 years	Female	not pregnant
16	921	10^3 TCID$_{50}$	1 year	Female	not pregnant
17	895	10^3 TCID$_{50}$	7 years	Female	not pregnant
18	911	10^3 TCID$_{50}$	1 year	Female	not pregnant
19	899	10^3 TCID$_{50}$	7 years	Female	not pregnant
20	910	Placebo	1 year	Female	January
21	898	Placebo	7 years	Female	not pregnant
22	N16	Placebo	2 years	Female	January
23	F5	Placebo	4 years	Female	January
24	N09	Placebo	2 years	Female	January
25	916	10^4 TCID$_{50}$	1 year	Male	-------------
26	914	10^4 TCID$_{50}$	1 year	Male	-------------
27	919	10^4 TCID$_{50}$	1 year	Female	not pregnant
28	902	10^4 TCID$_{50}$	1 year	Female	not pregnant
29	918	10^4 TCID$_{50}$	1 year	Female	not pregnant
30	913	10^4 TCID$_{50}$	1 year	Female	not pregnant
31	900	10^4 TCID$_{50}$	1 year	Female	not pregnant

XII.1.3 - FOLLOW UP: HEALTH STATUS AND VIRAEMIA

After inoculation animals were followed to check their health status (clinical examination and laboratory analysis) and viraemia (isolation and titres).

XII.1.3.1 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

Recording of rectal temperatures and clinical examination were conducted daily for 36 days following inoculation to reveal any clinical signs. Sheep were monitored for possible teratogenic effect.

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XII.1.3.2 - BIOCHEMICAL ANALYSIS AND COMPLETE BLOOD COUNT (CBC)

Blood samples were collected from the jugular vein before the vaccination (day 1) and weekly for the following 21 weeks to follow temporal modification occurred in biochemical analysis. The parameters measured by an automatic clinical chemistry analyzer with spectrophotometric method (Dimension RXL, Dade Behring) were: albumin (ALB), azotemia (Urea), Gamma glutamyl transferase (GGT), creatinine (Crea), Glucose (GLU), total bilirubin (TB), total serum protein (TP), alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Creatine kinase (CK).

CBC was conducted on blood supplemented with ethylenediamine tetra-acetic acid (EDTA) with the following determinations: white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets (PLT), lymphocytes (Li), monocytes (Mo), neutrophils (Gran). CBC were performed by an automatic blood cell counter (ADVIA 2120, Siemens)

XII.1.3.3 - VIRUS ISOLATION AND TITRES

On all animals EDTA blood samples were collected three times per week for 30 days beginning from the day of vaccination. In order to determine the presence of the virus and to define the titres, samples have been tested by CESME. Blood samples collected from animals were washed three times with sterile phosphate-buffered saline (PBS) containing antibiotics. The washed blood cells were re-suspended in lactose peptone buffer and sonicated to disrupt the red cells. After sonication, 0.1 ml of the sonication product was inoculated intravenously into 12 days old embryonated chickens eggs, which were incubated in a humidity chamber at 34°C and candled daily. Any embryo deaths within the first 24 hours were regarded as non-specific. Embryos that died after between two and seven days were kept at 4°C and embryos that were still alive after seven days were euthanased. The infected embryos were dissected and the brains, lungs, hearts, livers and kidneys were collected and homogenised with sterile quarts powder. The tissue debris was removed by centrifugation, and the supernatant was inoculated into flasks containing confluent monolayers into Vero cells maintained in an antibiotic medium and incubated at

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37°C in a humid atmosphere containing 5% carbon dioxide (CO_2). The flasks were monitored daily for seven days for signs of cytopathic effects (cpe). If no cpe appeared, a second passage was made in cell culture, and incubated and monitored as before. The presence and identity of BTV in the culture medium of the cells showing cpe, and in those at the end of the second passage, were determined by immunofluorescence, using monoclonal antibody to BTV core protein VP7 (VMRD) and fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G. Virus characterised as BTV was typed by virus microneutralisation assays, using type-specific antibodies.

Virus titres were determined in the blood of viraemic animals as follows: the blood cells were washed three times in phosphate-buffered saline (PBS) containing antibiotics. After the last washing, the sample was resuspended in MEM with antibiotics (1/10 v/v) and sonicated. Four tenfold dilutions of each sample suspension (from 1:10 to 1:10000) were inoculated into 96 flat-bottomed microtitre plate wells, following the method described in the OIE Manual of standards for diagnostic tests and vaccines (2004). Four replicates were made for each dilution. Approximately 10^4 cells, in a volume of 100 μl of MEM plus antibiotics and 3% FCS, were added per well and the plates incubated at 37°C under 5% CO_2. The plates were examined after six days and the $TCID_{50}$ calculated.

XII.1.4 - REVERSION TO VIRULENCE

This test was executed to confirm that attenuated viruses do not revert to virulence in vaccinated sheep and it was conceived in three steps.

1° STEP

Blood samples were collected in EDTA by vaccinated animals belonging to the safety test group (19 sheep inoculate with a single dose vaccine) in correspondence with hyperthermia (7° - 10° days after vaccination). Samples were used to form two different pools that were titrated by CESME following the protocol previously described. The blood pool with higher virus titre was chosen to inoculate three seronegative sheep (40 ml/each). After inoculation animals were followed as previously described to evaluate their health status and viraemia for almost 3 weeks.

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2°STEP

After two weeks three seronegative sheep were inoculated (40 ml/each) with a blood pool collected from the first group during the hyperthermia phase. Health status and viraemia were valued as the previous point.

3°STEP

After other two weeks, three seronegative sheep were inoculated (40 ml/each) with a blood pool collected from the second group during the hyperthermia phase. Health status and viraemia were valued as the previous point.

XII.2 - EFFICACY TESTS

Assessment of efficacy is based on clinical and virological data as well as on immunogenity. Immunogenity is determined by the analysis of the antibody response induced by each immunization, as measured by ELISA and by titration in a serum neutralisation test (SN) against the same serotype. Vaccine efficacy is evaluated in vaccinated animals by inoculation of an infective dose of live virulent BTV. The level of viraemia, analyzed by virus isolation, and clinical signs together with laboratory analysis are evaluated after challenge.

XII.2.1 - IMMUNOGENITY

This test was executed to confirm the efficacy of BTV-1 modified-live virus vaccine by evaluating its ability to elicit neutralizing antibodies in vaccinated animals.

Serum samples have been examined for BTV antibodies using a commercially available competitive Enzyme-Linked Immunosorbent Assay (c-ELISA) (Lelli *et al.* 2003) and serum neutralisation test (SN) (Savini *et al*. 2004*b*).

Vaccinated and control animals were bled once a week for 15 weeks form the day of vaccination. The c-ELISA was conducted using a kit developed by CESME (Lelli *et al.* 2003). The purified antigen was diluted in pH 9.6 carbonate/bicarbonate buffer solution and dispensed into microplate wells where it was left to adsorb overnight at 4°C. After washing, the sera and monoclonal antibodies, labelled with peroxidase, were added. The antigen-antibody reaction was revealed by the addition of a substrate. Samples presenting an optical density less than 35% of that found in the control wells were considered as positive.

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The SN revealed the serotype and antibody titre; 50 µl of diluted serum from 1:10 to 1:280 were added to each test well of flat-bottomed microtitre plates and mixes with 100 $TCID_{50}$ of previously titrated BTV serotypes 1. They were incubated at 37°C in 5% CO_2, and after one hour approximately 10^4 Vero (African green monkey kidney) cells were added to each well in 100 µl of minimum essential medium (MEM) (Eurobio, France) containing antibiotics: penicillin 100 IU/ml (Sigma, Germany), streptomycin 100 µg/ml (Sigma), gentamicin 5 µg/ml (Sigma), nystatin 50 IU/ml (Sigma) and 10% foetal calf serum (FCS) (Sigma), were added to each well. After 3 days, the cytopathic effect (CPE) in the wells was evaluated and the antibody titre was defined as the highest serum dilution able to inhibit at least 50% of the virus CPE. The positive and negative reference sera, cell and virus control were included in each plate. Positive and negative control sera were kindly supplied by the OIE reference laboratory of the Onderstepoort Veterinary Institute (OVI) in South Africa.

XII.2.2 - CHALLENGE

The challenge study was performed about 7- 8 months after the inoculation of BTV1 modified-live virus vaccine. The sheep previously vaccinated were divided into two groups: the first group was inoculated with virulent strain of BTV1, to test the efficacy of the vaccine, the second group with BTV-2, in order to verify the protection of animals vaccinated with BTV-1 vaccine against serotype 2. Viraemia level, clinical examination, serological analysis and leukocytes subsets were carried out on challenged animals.

XII.2.2.1 - CHALLENGE WITH VIRULENT STRAIN BTV-1

XII.2.2.1.1 - ANIMALS

Twenty-four healthy Sarda sheep of various ages were used for this trial. Animals were kept at the "Istituto Zooprofilattico Sperimentale della Sardegna" facilities in a *Culicoides* - free area. Animals were treated in accordance with national and European Union animal welfare regulations. Seven months before the challenge started 12 sheep were vaccinated with 10^3 $TCID_{50}$/ml of BTV-1 modified-live virus vaccine and the remaining twelve were left unvaccinated.

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XII.2.2.1.2 - BTV STRAIN PREPARATION

A wild BTV strain isolated from the spleen of a sheep which died due to BTV-1 infection during 2006 epidemic in Sardinia was used for the challenge test.

The inoculum was prepared according to the method described by Savini *et al.* (2005*b*): the spleen was fragmented using sterile quartz powder, suspended in a lactose peptone buffer containing antibiotics, sonicated and centrifuged. After centrifugation, 100 µl of the suspension was inoculated intravenously into embryonated chicken eggs and then passaged on a confluent monolayer of Vero cells. At maximum cpe, the material was collected, divided into aliquots and stored at 80°C. The strain was then titrated and typed by the virus neutralisation test.

Virus titre

Eight ten-fold dilutions (from 10^{-1} to 10^{-8}) were tested for each positive sample. Six replicates for each dilution were dispensed into 96 flat bottomed microtitre plate wells; Vero cells at a concentration of approximately 10^4 cells/ml in MEM with antibiotics and 10% FCS were added to each well as the detection system. The test was read after six days' incubation at 37°C with 5% CO_2. The content, calculated using the Reed and Muench formula, is defined as the highest virus dilution producing a cpe in 50% of the inoculated Vero cells ($TCID_{50}$).

Serological Typing

Starting from 1:10, four ten-fold viral dilutions were placed in contact with each of 24 serum-specific antisera diluted 1:20, as described by OIE. The viral serotype was identified on the basis of the specific antiserum able to neutralise viral growth on a cell monolayer, detectable through 50% inhibition of the CPE.

XII.2.2.1.3 - ANIMAL INOCULATION

Nineteen sheep, 12 vaccinated and 7 unvaccinated, were intramuscularly injected with 1 ml containing 10^6 $TCID_{50}$/ml of wild strain BTV1. Five seronegative sheep were not infected and used as negative control to possibly evidence wild BT virus circulation.

XII.2.2.1.4 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

Recording of rectal temperatures and clinical examination were conducted daily for 4 weeks following inoculation to reveal any clinical signs.

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XII.2.2.1.5 - BIOCHEMICAL ANALYSIS AND CBC

Blood samples, with and without EDTA, were collected weekly for 6 weeks post infection (p.i.) for biochemical analysis and complete blood count (CBC). Laboratory analysis were executed from the Clinical Diagnostic Laboratory of the "Istituto Zooprofilattico Sperimentale della Sardegna" as previously described.

XII.2.2.1.6 - VIRUS ISOLATION AND TITRE

In order to determine the presence of BTV1 and eventually to define its titre, EDTA blood samples, collected thrice a week for 4 weeks p.i., have been analysed by CESME, using the methods described above.

XII.2.2.1.7 - SEROLOGICAL TESTS

To evidence eventual seroconversions due to the inoculated virus or others viral serotypes, serum samples were collected once a week for 7 weeks p.i. and examined for antibodies presence of varied serotypes 1, 2, 4, 8, 9 and 16 of the virus of the bluetongue using the competitive enzyme-linked immunosorbent assay (c-ELISA) and serum neutralisation test (SN), as previously described.

XII.2.2.1.8 LEUKOCYTES SUBSETS

Blood samples were collected at 0, +5, +7, +10, +14, +17, +21, +26, +33 to study leukocyte subset alterations following experimental infection.
Cells were directly stained with monoclonal antibodies (Table VII) conjugated with different fluorochromes in a three-colour "Lyse & Wash" staining procedure described below.

Table VII. the panel of monoclonal antibodies used in the study

Antigen	Clone	Label	Specificity	Supplier
CD4	44.38	FITC	sheep	Serotec
CD8	38.65	PE	sheep	Serotec
CD14	TUK4	Alexa 647	human	Serotec

An aliquot of 50 µl of EDTA blood samples were transferred into 12x75 mm tubes (BD Falcon, Franklin Lakes, NJ, USA), added with 5 µl of each monoclonal antibody (0.05 µg/µl) and gently vortexed before ice-incubation for 20 min in dark.

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Then, 450 µL of FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) were added to each tube and samples were incubated for 10 min at room temperature in dark. FACS Lysing Solution essentially lyses the red blood cells, maintaining cellular integrity without distortion, minimizing debris or platelet adhesion to leucocytes preparation. Following red blood cell lysis, samples were washed twice (200 x g for 5 min at 4°C) and after resuspending in sheet fluid (Facs Flow, BD Falcon, Franklin Lakes, NJ, USA) they were acquired in a FacsCalibur flow cytometer (BD, Franklin Lakes, NJ, USA). Data analysis were performed using CellQuest™ software (BD, Franklin Lakes, NJ, USA).

XII.2.2.2 - CHALLENGE WITH VIRULENT STRAIN BTV-2

XII.2.2.2.1 - ANIMALS

Eleven Sarda sheep of various ages were used for this trial. Animals were kept at the "Istituto Zooprofilattico Sperimentale della Sardegna" facilities in a *Culicoides* - free area. Animals were treated in accordance with national and European Union animal welfare regulations. Eight months before the challenge started 6 sheep were vaccinated with 10^3 $TCID_{50}$/ml of BTV-1 modified-live virus vaccine and the remaining 5 were left unvaccinated. Animal health status was tested as previously described before the challenge started.

XII.2.2.2.2 - BTV STRAIN PREPARATION

A wild BTV strain isolated from the spleen of a sheep which died due to BTV-2 infection during 2000 epidemic in Sardinia was used for the challenge test. The virus was isolated and titrated in the same way of the BTV-1. Sheep were infected with 1 ml containing 10^6 $TCID_{50}$/ml of wild strain BTV-2.

XII.2.2.2.3 - ANIMAL INOCULATION

Eight months after vaccination, 6 vaccinated and 5 unvaccinated Sarda sheep, were infected with the wild strain of BTV-2.

XII.2.2.2.4 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

Recording of rectal temperatures and clinical examination were conducted daily for 3 weeks following inoculation to reveal any clinical signs.

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XII.2.2.2.5 - BIOCHEMICAL ANALYSIS AND CBC

Blood samples, with and without EDTA, were collected weekly for 7 weeks post infection (p.i.) for biochemical analysis and CBC. Laboratory analysis were executed from the Clinical Diagnostic Laboratory of the "Istituto Zooprofilattico Sperimentale della Sardegna" as previously described.

XII.2.2.2.6 - VIRUS ISOLATION AND TITRE

In order to determine the presence of BTV-2 and eventually to define its titre, EDTA blood samples, collected thrice a week for 4 weeks p.i., have been analysed by CESME, using the methods described above.

XII.2.2.2.7 - SEROLOGICAL TEST

To evidence eventual seroconversions due to the inoculated virus, serum samples were collected once a week for 5 weeks p.i. and examined for antibodies presence serotypes 2 and 1 of the virus of the bluetongue using the competitive enzyme-linked immunosorbent assay (c-ELISA) and serum neutralisation test (SN), as previously described.

XII.3 - STATISTICAL ANALYSIS

Differences between the viraemic titres and antibody titres of the vaccinated and unvaccinated groups of sheep before and after challenge were analysed using the non-parametric Mann–Whitney test for independent. Statistical significance of the tests was based on P values equal or lower than 0.05.

Statistical analysis of laboratory data (biochemical analysis and CBC) was performed with analysis of variance (ANOVA) between groups and within groups (single dose, decuple dose and control); Tukey's Test was used to perform pairwise comparisons between different sampling times.

Statistical analysis of WBC subsets were performed by ANOVA. P values ≤ 0.05 was considered significant.

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XIII. - RESULTS

XIII.1 - SAFETY TESTS

XIII.1.1 - FOLLOW UP: HEALTH STATUS AND VIRAEMIA

XIII.1.1.1 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

Vaccinated animals did not developed any clinical signs suggestive of bluetongue as well as local reactions at the injection site. Inoculation of the vaccine appeared not to cause reproductive failures including abortion, stillbirth, newborn mortality or congenital anomalies. Control animals did not show any signs of clinical disease. Two vaccinated ewes, one with single dose and the other with decuple dose, showed fever ($>40°$ C) from day 6 to day 11 p.v. It could be evidenced short-term hyperthermia in sheep belonging to the three groups (single dose, decuple dose and control) during the whole trial period (Fig. 17).

It has to be recorded the death of a sheep (F4) that was vaccinated with single dose. To identify the cause of death, autopsy, bacteriological and virological examination were conducted. Autopsy established injury attributable to enterotoxemia. Virological examination showed the presence of BTV-1 in the spleen. In a blood sample taken by the animal four days before death were observed changes in some liver parameters in presence of normal CBC.

XIII.1.1.2 - BIOCHEMICAL ANALYSIS AND CBC

Statistical analysis performed for biochemical and CBC parameters showed significant differences between groups (single dose, decuple dose, control) in some sampling times. Data were all included in normal reference intervals typical of ovine specie except for AST, GGT and monocytes (see Appendix 1).

XIII.1.1.3 - VIRUS ISOLATION AND TITRES

Vaccinated animals (single and decuple dose) have shown virus titres for 14 days, starting from day 5 p.v. (Fig. 18). No significant differences were found between viraemia levels of the two groups. Eight animals (42.1%) of the group vaccinated with 10^3 TCID$_{50}$ and 4 (57.1%) of the group with 10^4 TCID$_{50}$ showed viraemia. In both groups the viraemia peak was found 7 days p.v. (Fig. 19). Three (15.8%) sheep of the group vaccinated with single dose and 2 (28.6%) of the group vaccinated with decuple dose showed, between the 5th and the 7th day p.v. levels of viraemia higher

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than those necessary to infect the vectors (10^3 TCID$_{50}$). It was not possible to isolate BTV from control group sheep blood samples.
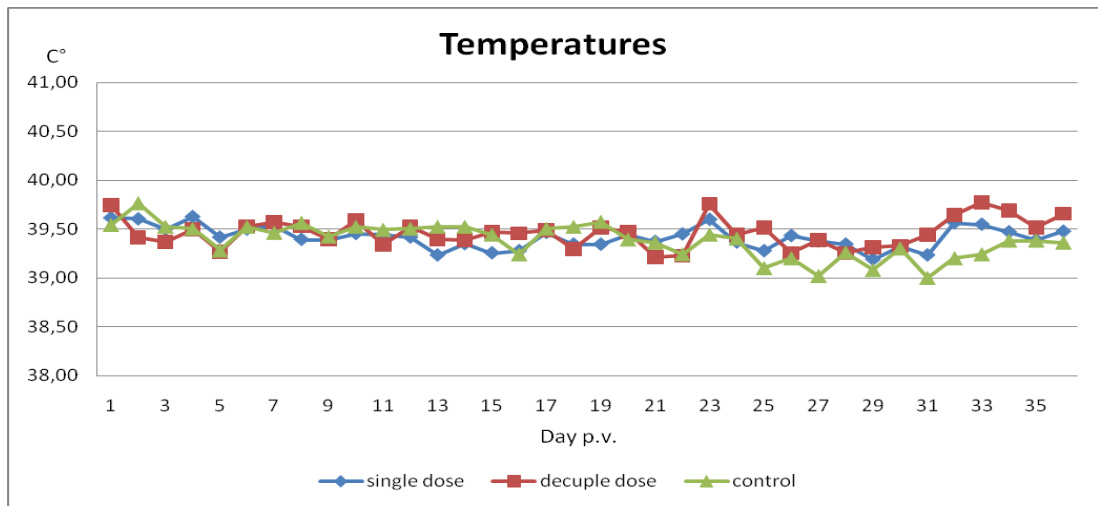
Fig. 17. Average evolution of rectal temperatures recorded in sheep vaccinated with single and decuple dose of BTV-1 virus vaccine and unvaccinated sheep (control).
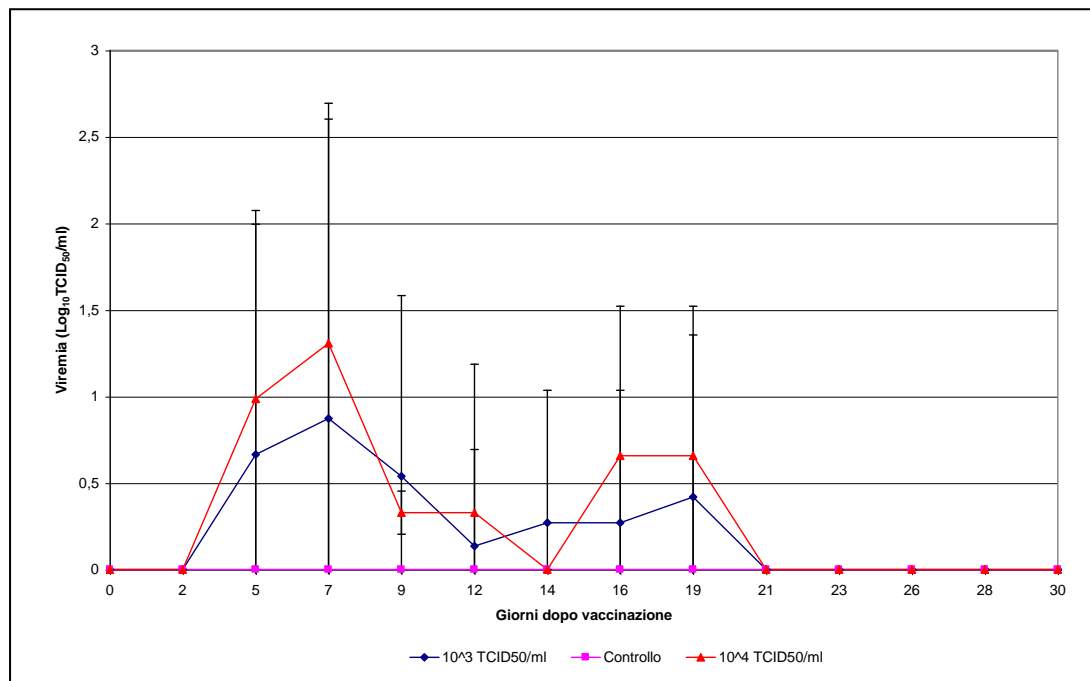
Fig. 18. Virus titres (mean ± SD) in sheep vaccinated with single (10^3 TCID$_{50}$/ml) and decuple dose (10^4 TCID$_{50}$/ml) of BTV-1 virus vaccine and unvaccinated sheep.

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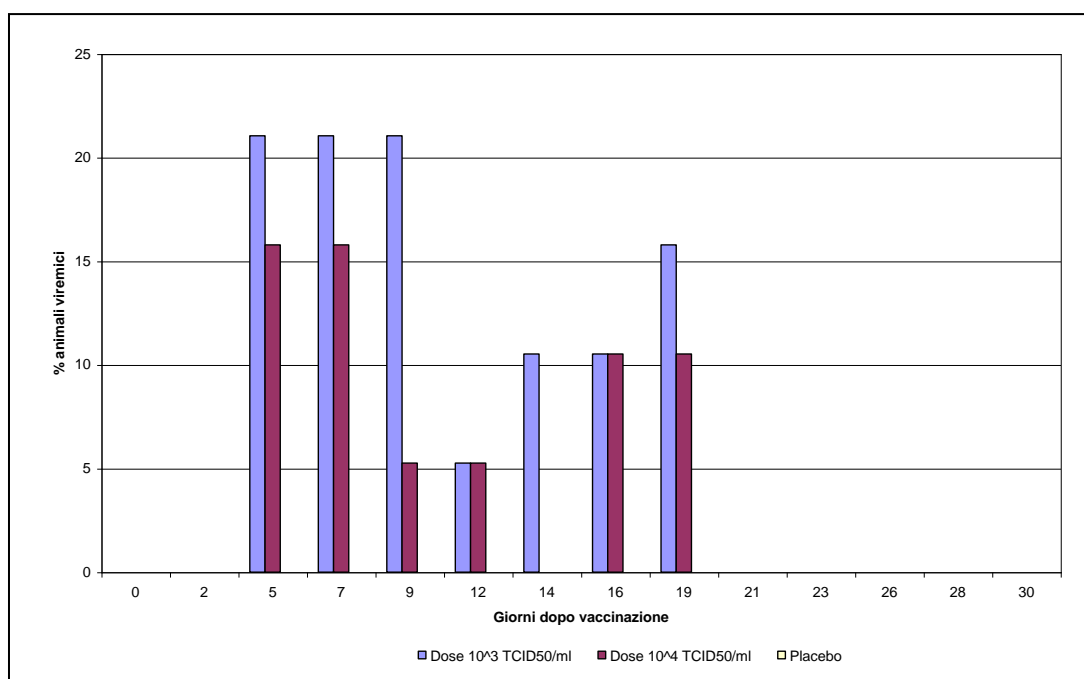
Fig. 19. Percentages of viraemic animals vaccinated with single and decuple dose of BTV-1 modified-live virus vaccine and unvaccinated sheep.

XIII.1.2 - REVERSION TO VIRULENCE

As reported in Table VIII, IX and X the blood pools collected seven days after each inoculation (Step 1, 2 and 3) showed higher virus titres than those collected ten days after inoculation. The virus titre of blood pool used for subsequent inoculation was $10^{3,63}$ $TCID_{50}$/ml in the first step, $10^{2,53}$ $TCID_{50}$/ml in the second step and $10^{4,3}$ $TCID_{50}$/ml in the third step.

Table VIII. Virus titres recorded in the pools of blood taken from vaccinated animals

Sample date	16/01/2007	18/01/2007
Days after vaccination	7	9
Virus titre ($TCID_{50}$/ml)	$10^{3,63}$	$10^{2,55}$

Table IX. Virus titres recorded in the pools of blood taken from animals after the first step.

Sample date	02/02/2007	05/02/2007
Days after infection	7	10
Virus titre ($TCID_{50}$/ml)	$10^{2,53}$	$10^{2,3}$

Table X. Virus titres recorded in the pools of blood taken from animals after the second step.

Sample date	16/02/2007	19/02/2007
Days after infection	7	10
Virus titre (TCID$_{50}$/ml)	$10^{4,3}$	$10^{2,3}$

Animals showed no clinical signs suggestive of BT disease during this trial. Fever (>40°C) was recorded in sheep of the second and third step (Fig. 20), which respectively showed hyperthermia between the 4th and the 9th day p.i. and between 8th and the 10th p.i.

Statistical analysys performed for CBC showed significant differences for limphocytes and neutrophils in the three groups even if data were all included in normal reference intervals typical of ovine specie. Biochemical analysis showed significant differences for ALT in the second step and Crea in the third step. Urea differed significantly in the first and second step, TB in the second step and CK in the first step, being these data all included in normal reference intervals typical of ovine specie (see Appendix 2).

No significant differences were found between viraemia levels of the three steps groups respect to the vaccinated aniamals. Higher virus titre occurred in animals belonging to the second step group (Fig. 21) while in the third step group more lasting levels of viraemia were found (14 days). All animals in the second and third step showed viraemia, while in the group of the first step BTV was found only in one blood sample. Levels of viraemia necessary to infect the vectors (10^3 TCID$_{50}$) were found in:

- 1 sheep of the first step, between the 5° and 10° day p.i.;

- 2 sheep of the second step, between the 5° and 10° day p.i.;

- 3 sheep of the third step, between the 5° and 12° day p.i.;

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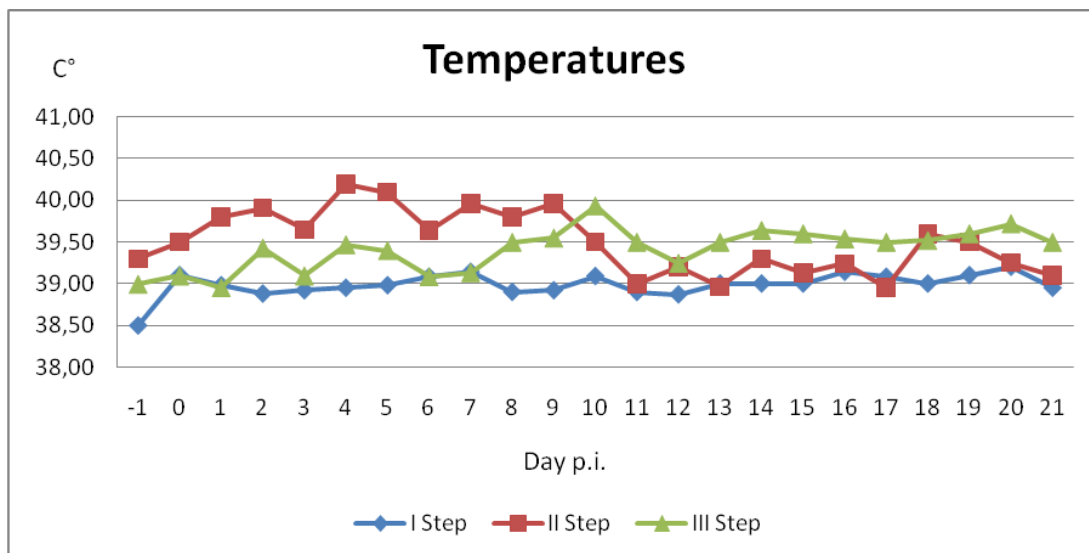
Fig. 20. Average evolution of rectal temperatures recorded in sheep in the three steps.
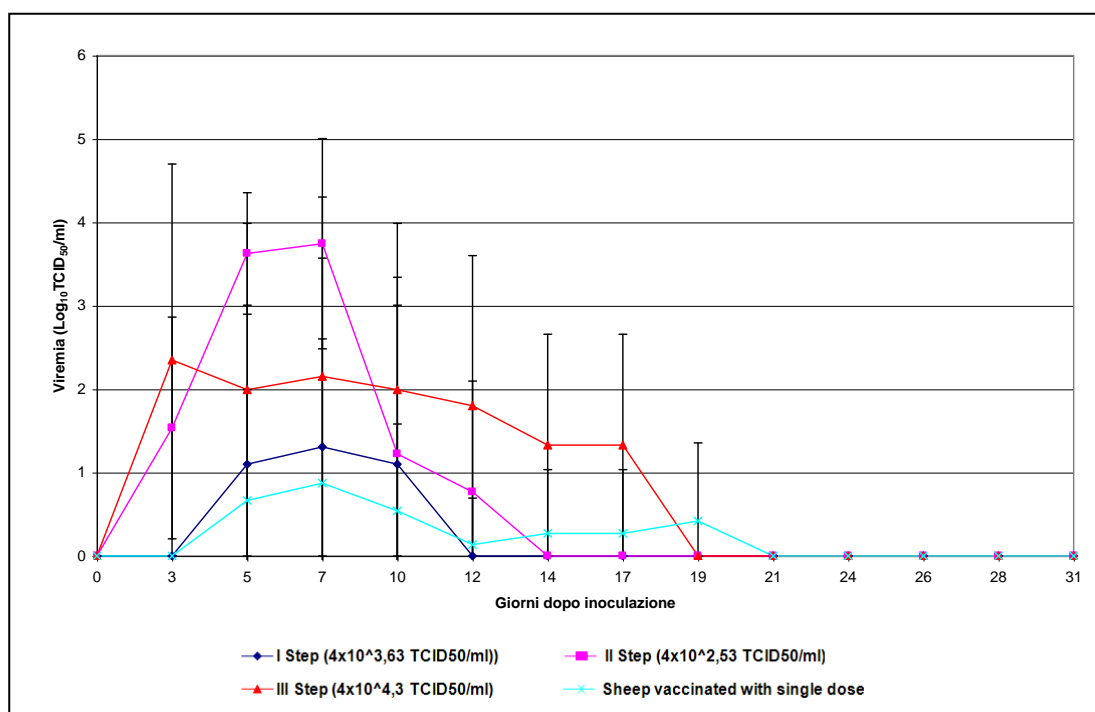
Fig. 21. Virus titres (mean ± SD) in sheep of the three steps and vaccinated with single dose of BTV-1 modified-live virus vaccine and unvaccinated sheep.

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XIII.2 - EFFICACY TESTS

XIII.2.1 - IMMUNOGENITY

From day 14 p.v. an antibody titres increase was observed both in fourteen animals (77.8%) of single dose vaccinated group and in 6 (85.7%) of decuple dose vaccinated group (Fig. 22). All animals, except one, seroconverted at 21 days p.v. with neutralising antibody titres peaking on day 35 p.v. There were no antibody titres for BTV-2, BTV-4, BTV-8, BTV-9, BTV-16, so as no BTV antibody titres have been found in control group.). No significant differences were found between neutralising antibody titres levels of the single and decuple dose groups. On the contrary the two groups differed significantly (P≤ 0,05) respect to the control group.
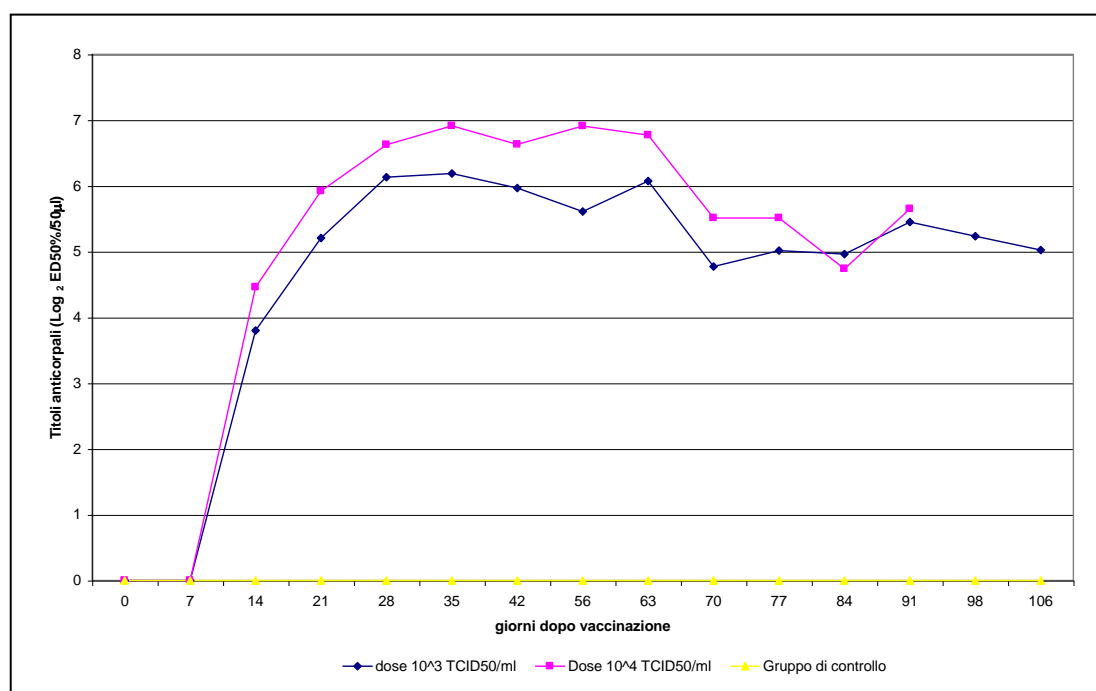
Fig. 22. Neutralising antibody titres in sheep after vaccination with BTV-1 modified-live virus vaccine.

XIII.2.2 - CHALLENGE

XIII.2.2.1 - CHALLENGE WITH VIRULENT STRAIN BTV-1

XIII.2.2.1.1 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

Control group animals developed hyperthermia from day 5 p.i. to day 9 p.i. peaking at day 7 p.i. (41.9°C) within the first 2 weeks of challenge. Only a sheep of vaccinated group, which did not produced neutralising antibodies, showed fever

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starting from day 4 p.i and lasting the following 4 days. Challenged vaccinated animals did not show any clinical signs and hyperthermia (Fig. 23).

XIII.2.2.1.2 - BIOCHEMICAL ANALYSIS AND CBC

Statistical analysis performed for biochemical and CBC parameters showed significant differences between and in the groups (vaccinated animals and control) in some sampling times. Data were all included in normal reference intervals typical of ovine specie except for ALT, CK, GGT, RBC and PLT in the control group and AST, MCH, MCHC and Mo in both groups (see Appendix 3).

XIII.2.2.1.3 - VIRUS ISOLATION AND TITRE

BTV-1 virus was isolated in the blood of all unvaccinated control animals from days 3 to 13 p.i., with peak titres observed on day 5 p.i. Control animals virus titres differed significantly (P≤ 0,05) from those of vaccinated animals on day 3, 5, 7 and 10 p.i. In vaccinated group virus isolation was not evidenced, except for one sheep which did not shown seroconvertion after vaccination performed seven months before (Fig. 24).

XIII.2.2.1.4 - SEROLOGICAL TESTS

In vaccinated animals an increase of antibody titres against BTV-1 was evidenced starting from 5 days p.i. and peaking after 19 days when it appeared 4 logarithms-fold higher than day 0. Control group animals showed antibody titres against BTV-1 from day 12 (Fig. 25). Statistical analisys performed between the two groups showed significantly different (P≤ 0,05) antibody titres on day 0 and 5.

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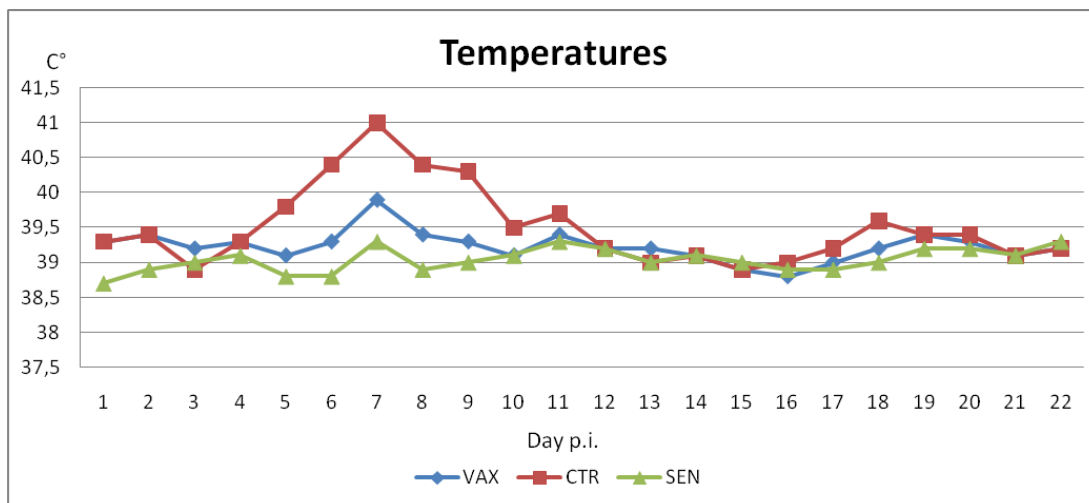
Fig. 23. Average evolution of rectal temperatures of sheep vaccinated (VAX), unvaccinated (CTR) and sentinel (SEN), after challenge with wild BTV1.
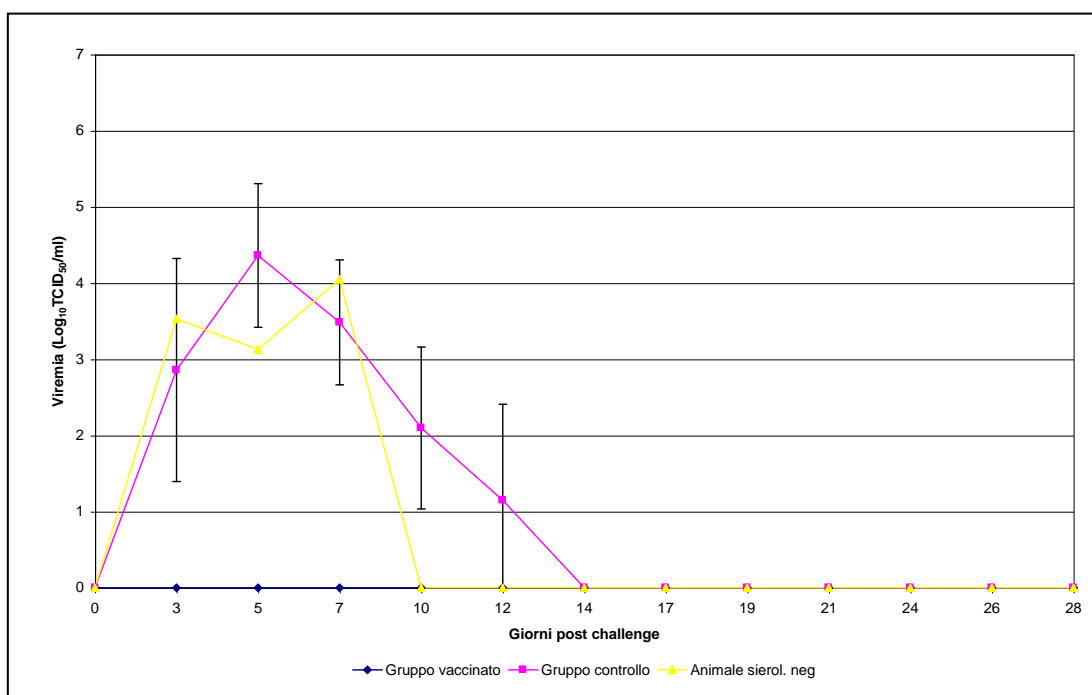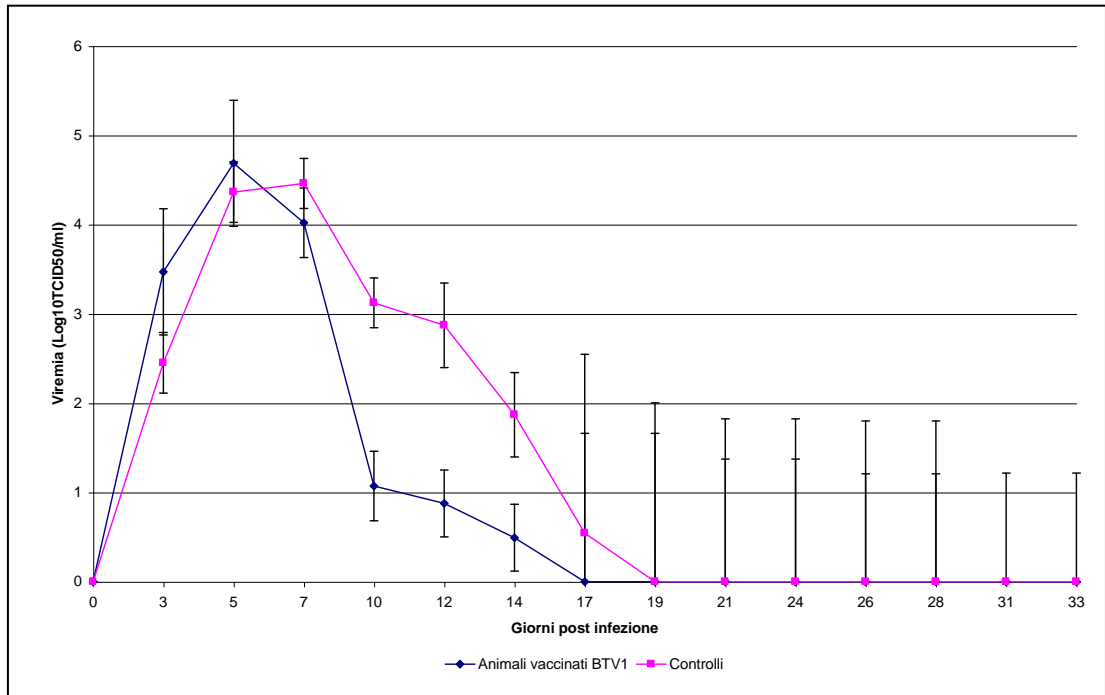

Fig. 24. Virus titres (± SD) in sheep vaccinated with BTV-1 modified-live virus vaccine and unvaccinated after challenge with homologous strain.
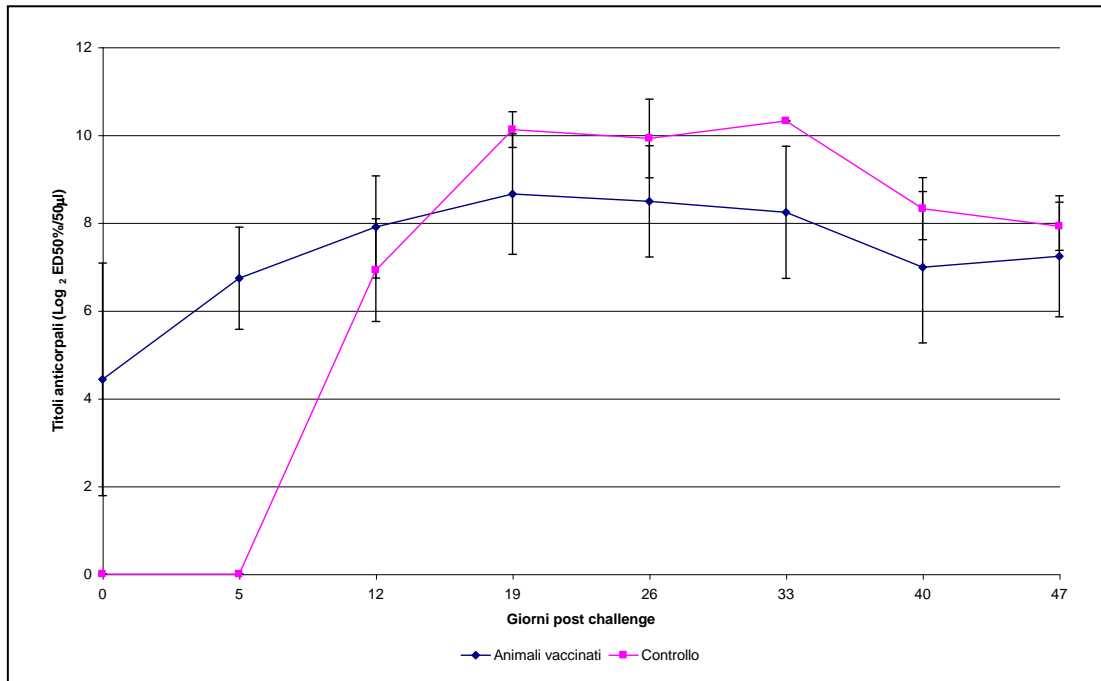
Fig. 25. Neutralising antibody titres in sheep vaccinated with BTV-1 modified-live virus vaccine after challenge with homologous strain.

XIII.2.2.1.5 - LEUKOCYTES SUBSETS

In ewes of the unvaccinated group the percentage of granulocytes markedly ($P \leq 0.05$) increased while the lymphocyte number significantly ($P \leq 0.05$) decreased compared to the values observed in the vaccinated animals. The latter change was the consequence of a CD4+ T-cells depletion. On day 5 and from day 10 till day 17 the unvaccinated group exhibited CD+4 T-cell lower percentage respect to vaccinated ewes ($P \leq 0.05$). As a consequence, the CD4/CD8 ratio also resulted significantly lower ($P \leq 0.05$) in the unvaccinated animals compared to the value observed in the vaccinated group on day 10 and 22. No significant differences were found in CD8 and CD14 percentages between two groups (Fig. 26).

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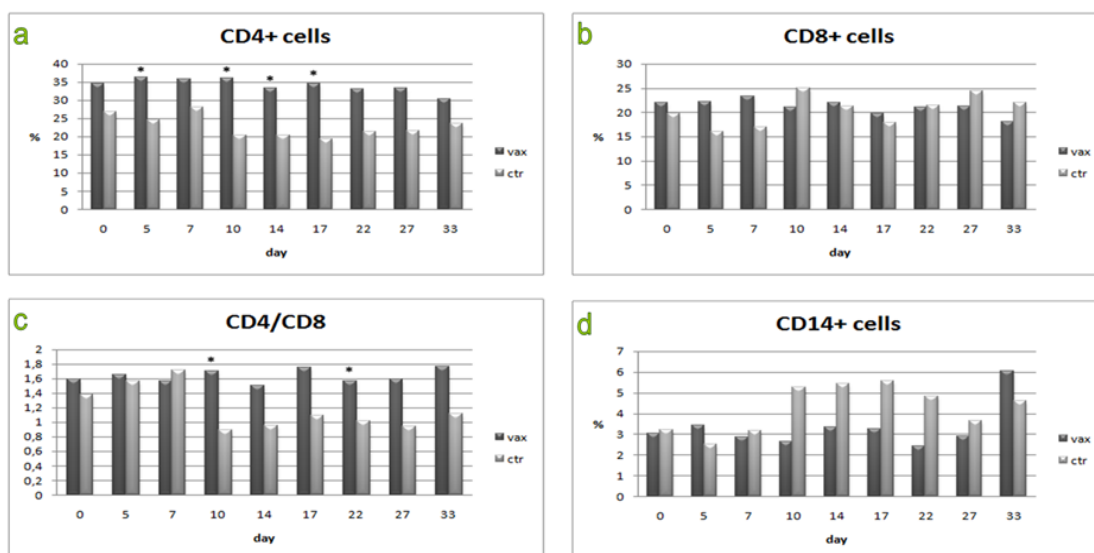
Fig. 26. Leukocytes subsets in vaccinated (VAX) and unvaccinated sheep (CTR). CD4 (a), CD8 (b), CD4/CD8 ratio (c), CD14 (d). CD4+ cells= lymphocytes T-helper; CD8+ cells= lymphocytes T cell suppressor/cytotoxic; CD14+ cells= monocytes. Day 0= BTV1 inoculation.

XIII.2.2.2 - CHALLENGE WITH VIRULENT STRAIN BTV-2

XIII.2.2.2.1 - CLINICAL EXAMINATION AND RECTAL TEMPERATURES

After challenge with virulent strain BTV-2, both groups of animals, vaccinated and not, have shown hyperthermia during the first week (Fig. 27).

XIII.2.2.2.2 - BIOCHEMICAL ANALYSIS AND CBC

Statistical analysis performed for biochemical and CBC parameters showed significant differences between and in the groups (vaccinated animals and control) in some sampling times. Data were all included in normal reference intervals typical of ovine specie except for RBC and MCV in the vaccinated group and MCH and MCHC in both groups (see Appendix 4).

XIII.2.2.2.3 - VIRUS ISOLATION AND TITRE

BTV-2 virus was isolated in the blood of all animals from day 3 to 14 p.i. in vaccinated group and from day 3 to 17 p.i. in control group, with peak titres observed on day 5 p.i. in both groups. Viraemia titres of vaccinated animals were lower respect to unvaccinated animals (P≤0.05) on day 10, 12 and 14 p.i. Animals vaccinated with BTV-1 showed viraemia for 11 days, while the control group for 14 days p.i. (Fig.28).

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XIII.2.2.2.4 - SEROLOGICAL TESTS

After challenge with virulent strain BTV-2, all animals showed neutralising antibody titres against BTV2 (Fig. 29). No significant differences were found between antibody titres of vaccinated and unvaccinated animals, except for day 12 when antibody titres were higher (P≤0.05) in vaccinated animals.

After inoculation with virulent strain BTV-2, in vaccinated animals an increase of antibody titres against BTV-1 was evidenced starting from 5 days p.i. and peaking after 14 days when it appeared 4 logarithms-fold higher than day 0. Antibodies increase was similar to that in sheep vaccinated with the vaccine BTV-1 infected with the homologous serotype (Fig. 30).
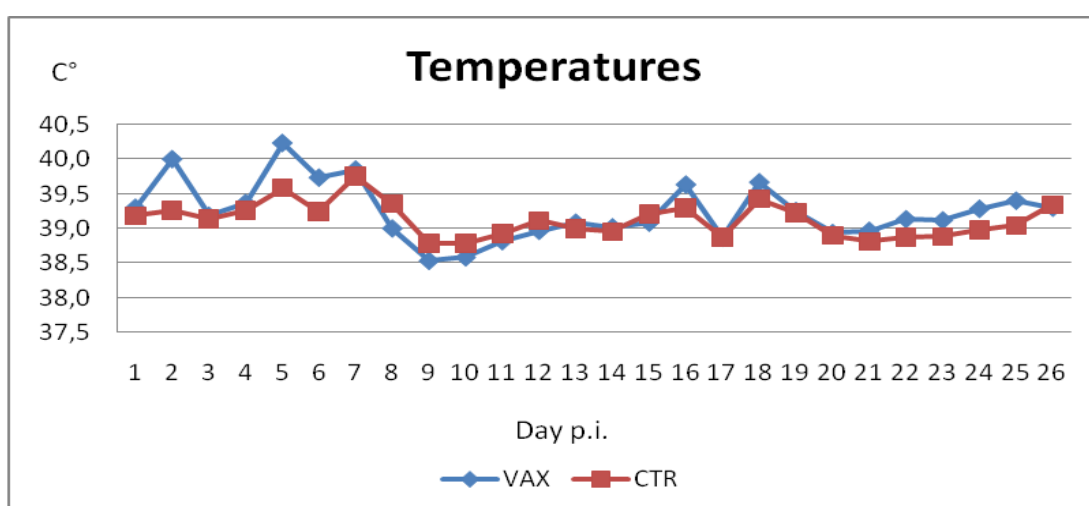
Fig. 27. Average evolution of rectal temperatures of sheep vaccinated (VAX) and unvaccinated (CTR), after challenge with virulent strain BTV-2.

Fig. 28. Virus titres (± SD) in sheep vaccinated with BTV-1 modified-live virus vaccine and unvaccinated after challenge with virulent strain BTV-2.
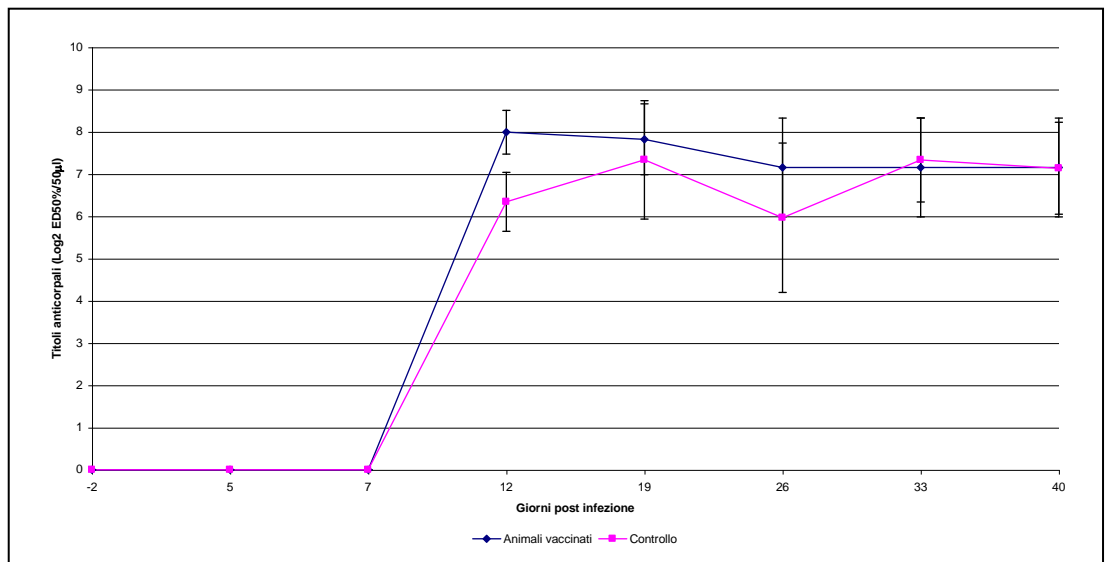
Fig. 29. Neutralising antibody titres against the serotype 2 in sheep vaccinated with BTV-1 modified-live virus vaccine after challenge with virulent strain BTV-2.
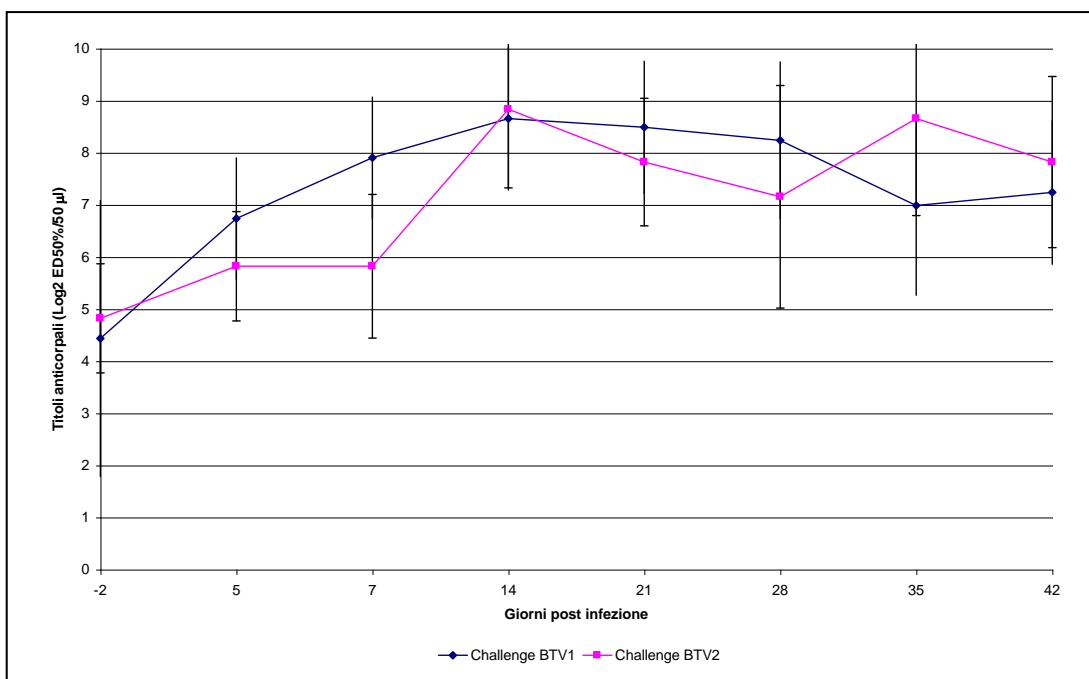
Fig. 30. Average of antibody titres against the serotype 1 of BTV found in groups of sheep vaccinated with live attenuated vaccine BTV-1 after challenge with virulent strains of BTV-1 and BTV-2.

XIV. - DISCUSSION

Vaccination against BTV is an essential tool, not only for the control of the disease but more importantly, for "safe" trade of live ruminants in accordance to OIE standards and EU legislation. To prevent BTV infection of ruminants, different types of vaccines, including modified live virus (MLV), inactivated vaccines, virus-like particles (VLP) produced from recombinant baculoviruses, and recombinant vaccinia virus vectored vaccines have been manufactured (Roy et al., 1992; Boone et al., 2007). MLV vaccines tested in this study, when administered in adult Sarda sheep, resulted safe and effective in complete prevention of clinical signs and detectable viraemia after challenge using virulent homologous virus. However, the challenge with virulent strain BTV-2 showed that the vaccine is not able to cross-protect vaccinated sheep, confirming that protective immunity against BT is associated with the presence of type-specific neutralising antibodies.

At the moment, only inactivated and MLV vaccines against BT are commercially available and used in the official vaccination campaigns. Although VLPs are safe and neat, their inconsistent efficacy when used in field trials (Roy et al., 1990, 1992, 1994; Roy, 2004) and difficulties with commercial production, cost, and long-term stability make them ineligible for field use (Savini et al., 2007a). Similarly, recombinant vector vaccines expressing both VP2 and VP5, even though revealing some potential in terms of safety and protection, still require further development before being ready for field use (Lobato et al., 1997).

Inactivated vaccines have been recently developed and marketed. The efficacy of an inactivated vaccine is fully dependent on the dose of virus, resulting in significantly higher virus mass than that of MLV. Two doses, in the presence of adjuvant, may often be required for inactivated vaccines considerably increasing the cost of vaccination. Inactivated vaccines for BTV-2 and/or BTV-4 have been developed, commercialized and successfully employed in the 2005–2006 BTV vaccination campaigns. In infected areas, when inactivated virus vaccines are used, it is recommended to wait for 60 days before moving the animals to take into consideration the risk that animals are infected at the moment of the vaccination.

Conversely, live attenuated vaccines are cheap to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used (Patta et al., 2004;

Dungu et al., 2004). Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. However, live attenuated BTV vaccines suffer from a variety of documented or potential drawbacks, including under-attenuation, the impact of which may vary with different breeds of sheep. Potential adverse consequences with the use of live attenuated vaccines are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring from pregnant females that are vaccinated during the first third of gestation. Another risk associated is their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of vaccine virus genes with those of wild-type virus strains (OIE, 2004f). The frequency and significance of these events remain poorly defined but natural and local dissemination of vaccine strains has already been documented in Europe (OIE, 2004f). When MLV are used, it is recommended to take into consideration, before movements of animals, the duration of viremia induced by the vaccinal strain. In consequence, the waiting period before movement should be 60 days considering that it was determined that cattle could be moved after that period.

When, in October 2006, BTV-1 was recorded in Sardinia, Ministry of Health has agreed that the use of vaccination is the only viable route to safeguard the livestock and to enable the movement of susceptible animals in accordance with national and European Union regulations. Since there was no inactivated BTV-1 vaccine yet available, it was decided to use a MLV vaccine as emergency measure (D.M. 12.03.2007). The only attenuated vaccine against BTV-1 available on the market was manufactured by "Ondersterpoort Biological Products Ltd" (OBP) in South Africa. However, the OBP was not able to provide the vaccine (for times of production and delivery) for the next vaccination campaign that it would be started in March 2007. Therefore Ministry of Health, with D.M. 12.03.2007, authorized the "IZS dell'Umbria e del Molise" to produce the modified live vaccine against BTV-1 for 2007 vaccination campaign. In attenuated vaccines the master or primary virus seed is prepared by BTV field isolates. Vaccine virus is attenuated by either passages in tissue culture, in embryonating chicken eggs or a combination of both. Vaccine virus manufactured by CESME has been obtained by cloning from South Africa tetravalent vaccine. and subsequently tested for the presence contaminant viruses of viable bacterial, fungal or mycoplasmal contamination.

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In this work we have evidenced the safety of BTV serotype 1 MLV vaccine and its efficacy in protecting sheep against bluetongue infection. For this purpose various trials were performed, as described in the "Manual of diagnostic tests and vaccines for terrestrial animals" by OIE (OIE, 2004*f*), to evaluate the innocuity and efficacy of this vaccine.

Animal health status assessment and viraemia detection after vaccine inoculation performed to evaluate its safety has given a positive outcome. In fact, vaccine, both in normal dose and decuple dose, did not cause significant clinical effects and long-term hyperthermia attributable to BT, proving that its use is safe. Analysis of laboratory data showed no changes in tested blood chemical parameters referable to the vaccine activity. Indeed, analysis of red cell parameters showed significant differences in some sampling times, but this were all included in normal reference intervals typical of ovine specie. As reported by OIE, MLV vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryonal death. As shown by our results, BTV-1 vaccine did not show any adverse effects in pregnant ewes if administered in the second half of gestation. Virus titre was carried out to determine if attenuated virus can be transmitted by insects feeding on vaccinated animals. Our data indicated that laboratory-adapted viruses can be transmitted by insect vectors, as already reported by other authors (Standfast *et al.*, 1985). A suitable procedure to determine attenuated virus transmissibility should require that sheep are vaccinated and, during viraemia, that they are exposed to competent, uninfected Culicoides, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. Due to the fact that the titre of attenuated virus in the blood of vaccinated sheep is low, very large numbers of Culicoides would be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. However, in South Africa it is estimated that the minimum titre of virus circulating in the bloodstream of an animal must be at least 10^3 TCID$_{50}$ before feeding Culicoides become infected, although it has also been suggested that a lower titre may sometimes be infective. Only attenuated viruses that generate titres under 10^3 TCID$_{50}$ are deemed to be acceptable as vaccines (OIE, 2004*f*). In this work the administration of MLV vaccine caused a

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transient viraemia in 8 animals (42.1%) of the group vaccinated with normal dose and in 4 (57.1%) of the group vaccinated with decuple dose. Measured viraemia was relatively modest in intensity and duration. However, 15,8% of animals vaccinated with single dose showed short-therm viraemia levels ($>10^3$ TCID$_{50}$) which are considered the threshold for possible Culicoides infection. Although for short time (48 hours), the possibility that insects could acquire vaccine virus by feeding on vaccinated animals and transmit it to other uninfected sheep or cattle cannot be prevented.

Reversion to virulence test was executed to evaluate the possibility of vaccine virus to loose some of its characteristic of attenuation to acquire other more pathogenic. Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep (OIE, 2004*f*). However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence during a number of sheep-insect replication cycles becomes a distinct prospect. In this trial vaccine virus, after three passages, caused a low increase of viraemia intensity and duration comparing to animals vaccinated with normal dose. Furthermore, clinical examinations together with biochemical analisys and CBC evidenced no severe health status alteration in inoculated animals. Our data suggest that virus vaccine after 3 passages on 3 groups of sheep has not changed its original features.

The administration of BTV-1 MLV vaccine resulted to be safe, not causing in vaccinated animals significant clinical effects, not showing any adverse effects in pregnant ewes if administered in the second half of gestation and not changing its original features after 3 passages. However, titres and duration of viraemia recommend an awaiting period before shipment of vaccinated animals equal to 60 days to prevent the spread of the virus.

The efficacy test performed in this study evidenced how administration of BTV-1 MLV vaccine was able to elicit significant neutralizing antibodies titres in all vaccinated animals and did not determine seroconversion against other serotypes.

This vaccine has an appropriate balance between attenuation of virulence and ability to replicate in sheep. The antigenic stimulus provided by its replication elicits complete protection against challenge using a virulent homologous virus and does not cause any detectable viraemia in serologically positive vaccinated animals. These results show that at least 91.6% of vaccinated sheep will not develop a BT viraemia when challenged with virulent homologous virus seven months after immunisation.

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Analysis of laboratory data showed in control group some changes in tested blood chemical parameters presumably referable to the virus activity. Indeed, analysis of red cell parameters showed significant differences in some sampling times, probably due to viral replication. Furthermore, it would appear that the risk of spreading BTV-1 infection through the movement of vaccinated sheep will be very low.

Furthermore, in the challenge test was performed the study of white blood cell subsets of immunised and non immunised sheep following BTV-1 experimental infection. Leukocytes subsets analisys showed no differences in CTL lymphocytes subsets between vaccinated and unvaccinated animals, although other authors reported how BTV infection caused CD8+ cells decrease. Jeggo *et al.* (1984) using cellular adoptive transfer demonstrated that CD8+ T cells could give a short-term heterotypic response able to protect sheep from different field strain BTV infection within a season. In our results we evidenced a CD4+ T cells depletion in unvaccinated sheep following virus inoculation. Being involved both in humoral and cell mediated immunity, CD4+ T-cells play a fundamental role in eliciting a protective antiviral response. Our findings suggest that live modified BTV-1 vaccine used in this trial does protect animals against clinical signs and haematological modifications. Nevertheless, the absence of any CD4+ cells decrease in vaccinated group confirm BTV-1 vaccine stimulation of cell mediated response immunity. Further studies are needed in order to better understand cell mediated immune response mechanisms involved in host defense response to BTV infection.

Finally, in this study we performed a challenge trial with virulent strain BTV-2 in vaccinated sheep in order to verify evidence of crossprotection. The hypothesis on a correlation between the serotypes 1 and 2 of BT was born from the observation of the facts occurring in Sardinia when the disease appeared. In fact it is known that BTV-1 and BTV-2 have a high pathogenic index and high epidemic potential (Saegerman *et al.,* 2008). During the first two epidemic season of BT in Sardinia, BTV-2 caused the death about 120.000 sheep (www.izs.it). After, several vaccination campaigns were carried out to immunize the livestock. When in 2006 BTV-1 was identified in Sardinia, it was expected the death of numerous susceptible animals, while was recorded the death of only 4.500 sheep. These data suggested that sheep, previously immunized for serotype 2 of BT, were even protected for the serotype 1. However, after challenge with wild strain BTV-2, sheep have not shown cross-protection for serotype 2. Furthermore, vaccine has been able to reduce the duration of viraemia in

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sheep infected with high titres of virulent strain of BTV-2. Moreover, infection with BTV-2 has produced an increase of antibody titres against serotype 1 similar to that obtained after challenge with homologous virus. It seems that infection with BTV-2 exercises a booster effect in respect of serotype 1 of BTV.

In conclusion, the administration of BTV-1 MLV vaccine was safe, not causing particular drawbacks, and effective, stimulating an adequate protection of animals in case of infection with wild strain of homologous serotype.

Results obtained in this study were confirmed from the compaign vaccine 2007, from which were not found reports of negative effects due to inoculation of the vaccine and the reduction of BTV-1 outbreaks, passed by 238 in 2006, to 15 in 2007.

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REFERENCES

- Alba A., Casal J., Domingo M., (2004). Possible introduction of bluetongue into the Balearic Islands, Spain, in 2000, via air streams. The Veterinary Record, 155: 460-461.

- Anderson, R.M. & May, R.M., (1991). Infectious Diseases of Humans: Dynamics and Control, Oxford: Oxford University Press., 757.

- Barratt-Boyes S.M. & MacLachlan N.J., (1995). Pathogenesis of bluetongue virus infection of cattle. J. Am. Vet. Med. Assoc., 206: 1322-1329.

- Barratt-Boyes S.M. & MacLachlan N.J., (1994). Dynamics of viral spread in bluetongue virus infected calves, Vet. Microbiol., 40: 361-371.

- Barratt-Boyes S.M., Rossitto P.V., Stott J.L., MacLachlan N.J., (1992). Flow cytometric analysis of in vitro bluetongue virus infection of bovine blood mononuclear cells, J. Gen. Virol., 73: 1953-1960.

- Baylis M., O'Connell L., Purse B.V., (2004). Modelling the distribution of bluetongue vectors. In Bluetongue, Part I (N. J. MacLachlan & J. E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. Veterinaria. Italiana, 40: 176-181.

- Bonneau K.R., MacLachlan N.J., (2004). Genetic diversification of field strains of bluetongue virus. Veterinaria Italiana, 40: 446-447.

- Bonneau K.R., DeMaula C.D., Mullens B.A., MacLachlan N.J., (2002). Duration of viraemia infectious to Culicoides sonorensis in bluetongue virus-infected cattle and sheep. Veterinary Microbiology, 88: 115-125.

- Bonneau K.R., Mullens B.A., MacLachlan N.J., (2001). Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/3A genes of bluetongue virus upon passage between sheep and cattle. Journal of Virolology, 75: 8298-8305.

- Bonneau K.R., Zhang N., Zhu J., Zhang F., Li Z., Zhang K., Xiao L., Xing W., MacLachlan N.J., (1999). Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. Virus Research, 61: 153-160.

- Boone J.D., Balasuriya U.B., Karaca K., Audonnet J.C., Yao J., He L., et al., (2007). Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. Vaccine; 25: 672-678.

- Braverman Y. & Chizov-Ginsburg A., (1997). Repellency of synthetic and plant-derived preparations for Culicoides imicola. Medical and Veterinary Entomology, 11: 355-360.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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- Braverman Y., Chechik, F., (1996). Air streams and the introduction of animal diseases borne on Culicoides (Diptera, Ceratopogonidae) into Israel. Revue Scientifique et Technique – Office International des Epizooties, 15: 1037-1052.

- Braverman Y., Wilamowsky A. & Chizov-Ginzburg A., (1995). Susceptibility of Culicoides imicola to cyhalothrin. Medical and Veterinary Entomology, 9: 443-4.

- Braverman, Y. (1989). Control of biting midges Culicoides (Diptera: Ceratopogonidae), vectors of bluetongue and inducers of sweet itch: a review. Israel Journal of Veterinary Medicine, 45: 124-9.

- Bréard E., Sailleau C., Nomikou K., Hamblin C., Mertens P., Mellor P.S., El Harrack M., Zientara S., (2007). Molecular epidemiology of serotype 4 bluetongue viruses isolated in the Mediterranean Basin between 1979 and 2004. Virus Research, 125: 191-197.

- Bréard E., Pozzi N., Sailleau C., Catinot V., Durand B., Dumont P., et al., (2007). Transient effect of the attenuated bluetongue virus vaccine on the quality of the ram semen. Vet. Rec. 160(13): 431-435.

- Bréard E., Hamblin C., Hammoumi S., Sailleau C., Dauphin G., Zientara S., (2004). The epidemiology and diagnosis of bluetongue with particular reference to Corsica. Res. Vet. Sci., 77: 1-8.

- Brewer A.W., MacLachlan N.J., (1994). The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization, Arch. Virol., 136: 287-298.

- Brewer A.W. and MacLachlan N.J., (1992). Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. Vet. Pathol., 29: 356-359

- Brodie S.J., Wilson W.C., O'Hearn P.M., Muthui D., Diem K., Pearson L.D., (1998). The effects of pharmacological and lentivirus-induced immune suppression on orbivirus pathogenesis: assessment of virus burden in blood monocytes and tissues by reverse transcription in situ PCR. J. Virol., 72: 5599–5609.

- Browne J.G. and Jochim M.M., (1967). Cytopathologic changes and development of inclusion bodies in cultured cells infected with bluetongue virus. Am. J. Vet. Res., 28: 1091-1105.

- Caporale V., Giovannini A., Patta C., Calistri P., Nannini D., Santucci U., (2004). Vaccination in the control strategy of bluetongue in Italy. (Developments in biologicals, vol. 119). Control of infectious animal diseases by vaccination. Basel, Switzerland: S Karger AG, 113-127 [book chapter: Conference paper].

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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- Caporale V., Di Emidio B., Nicolussi P., Piredda G., Salaris S., De Palmas S., et al., (2003). Experimental vaccination of sheep using a bluetongue attenuated live vaccine: a study of the effects on lactation. Vet. Ital. 39(4): 5-10.

- Calistri P., Giovannini A., Conte A., Nannini D., Santucci U., Patta C., Rolesu S., Caporale V., (2004). Bluetongue in Italy: Part I. Veterinaria Italiana, 40: 243-251.

- Cannas E.A., Nicolussi P., Bonelli P., Dore S., Denti G., Marongiu E., Migliaccio P., Savini G., (2005). Live modified bluetongue vaccine viruses in Sarda sheep: effects of three different formulations on milk production. In: Proceedings of the 12th international symposium of the world association of veterinary laboratory diagnosticians, Montevideo, Uruguay, 16–19 November 2005.

- Chiang E.T., Persaud-Sawin D.A., Kulkarni S., Garcia J.G., Imani F., (2006). Bluetongue virus and doublestranded RNA increase human vascular permeability: role of p38 MAPK, J. Clin. Immunol., 26: 406-416.

- De Mattos C.A., De Mattos C.C.P., Osburn B.I. and MacLachland N.J. (1994). Heterogeneity of the L2 gene of field isolates of bluetongue virus serotype 17 from the San Joaquin Valley of California. Virus Res., 31: 67-87.

- De Maula C.D., Leutenegger C.M., Bonneau K.R., MacLachlan N.J., (2002). The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue, Virology, 296: 330-337.

- De Maula C.D., Jutila M.A., Wilson D.W., MacLachlan N.J., (2001). Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells, J. Gen. Virol., 82: 787-794.

- De Maula C.D., Heidner H.W., Rossitto P.V., Pierce C.M. and MacLachlan N.J., (1993). Neutralization determinants of United States bluetongue virus serotype 10. Virology, 195: 292-296.

- Della Porta A.J., Parsonson I.M. and McPhee D.A., (1985). Problems in the interpretation of diagnostic tests due to cross-reactions between orbiviruses and broad serological responses in animals. In Bluetongue and Related Orbiuiruses (Edited by Barber T. L. and Jochim M. M.), 445-453. Alan R. Liss, New York.

- Della Porta A.J., McPhee D.A. and Snowdon W.A., (1979). The serological relationships of orbiviruses. In Arbouirus Research in Australia, (Edited by St George T. D. and French E. L.), 64-71. Proceedings of the second symposium 17-19 July, Brisbane: CSIRO-QMIR.

- Di Emidio B., Nicolussi P., Patta C., Ronchi G.F., Monaco F., Savini G., et al., (2004). Efficacy and safety studies on an inactivated vaccine against bluetongue virus serotype 2. Vet. Ital., 40: 640-644.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

97

- Downes J. A., (1962). What is an arctic insect? Canadian Entomologist, 94: 143-162.

- Dragonas P., (1981). Fièvre catharrale au mouton. L'Office International des Epizooties Monthly Circular. 9:10.

- Dungu B., Gerdes T., Smit T., (2004). The use of vaccination in the control of bluetongue in southern Africa. Vet. Ital., 40: 616-622.

- Dzhafarov S. M., (1964). Blood-sucking midges (Diptera, Heleidae) of the Transcaucasus. Akademija Nauk Azerbaidzanskoi SSR, Instituta Zoologicheskiy, 414.

- Eaton B.T., Hyatt A.D. and White J.R., (1987). Association of bluetongue virus with the cytoskeleton. Virology, 157: 107-116.

- Eksteen P.A., Huismans H., (1972). Interferon induction by bluetongue virus and bluetongue virus ribonucleic acid, Onderstepoort J. Vet. Res., 39: 125-131.

- Ellis J.A., Luedke A.J., Davis W.C., Wechsler S.J., Mecham J.O., Pratt D.L. and Elliot J.D., (1990). T lymphocyte subset alterations following bluetongue virus infection in sheep and cattle. Vet. Immunol. Immunopathol., 24: 49-67.

- Erasmus B.J., (1975). Bluetongue in sheep and goats. Aust. Vet. J., 51: 65-170.

- European Commission, (2003). Aiuto di Stato No. 662/2001 – Italia (Sardegna). Aiuti a favore degli allevatori di bovini per i danni causati dall'epizoozia Blue Tongue, C(2003) 2519fin, 27 July. EC, Brussels, 8 pp. (europa.eu.int/comm/secretariat_general/sgb/state_aids /agriculture/n662-01.pdf accessed on 28 July 2004).

- European Commission, (2001). Decision of 9 February 2001 establishing protection and surveillance zones in the Community in relation with bluetongue (2001/138/EC). Off. J., L 050: 17-19.

- European Council, (2000). Council Directive 2000/75/EC of 20 November 2000 laying down specific provisions for the control and eradication of bluetongue. Off. J., L 327: 74-83.

- European Council, (1992*a*). Council Directive 92/119/EEC of 17 December 1992 introducing general Community measures for the control of certain animal diseases and specific measures relating to swine vesicular disease. Off. J., L 062: 69-85.

- European Council, (1992*b*). Council Directive 92/35/EEC of 29 April 1992 laying down control rules and measures to combat African horse sickness. Off. J., L 157: 19-27.

- Ferrari G., De Liberato C., Scavia G., Lorenzetti R., Zini M., Farina F., et al., (2005). Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. Prev. Vet. Med., 68: 10-13.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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- Foster N.M., Luedke A.J., Parsonson I.M.,Walton T.E., (1991). Temporal relationships of viremia, interferon activity, and antibody responses of sheep infected with several bluetongue virus strains, Am. J. Vet. Res., 52: 192-196.

- Fulton R.W., Pearson N.J., (1982). Interferon induction in bovine and feline monolayer cultures by four bluetongue virus serotypes, Can. J. Comp.Med., 46: 100-102.

- Gambles R.M., (1949). Bluetongue of sheep in Cyprus. Journal of Comparative Pathology, 59: 176- 190.

- Gard G.P., (1984). Studies of bluetongue virulence and pathogenesis in sheep. Technical Bulletin No. 103, Department of Industries and Development, Darwin, Australia.

- Gerbier G, Hendrikx P., Roger F., Zientara S., Biteau-Coroller F., Grillet C., Baldet T., Albina E., (2004). Bluetongue control using vaccines: an experience from the mediterranean islands in Europe. Veterinaria Italiana, 40: 611-615.

- Gerbier G., Hendrikx, P., Roger F., Zientara S., Biteau-Coroller F., Grillet C., Baldet T., Albina E., (2003). Bluetongue control using vaccines: an experience from the mediterranean islands in Europe. In: Proceedings of the third OIE bluetongue international symposium, Taormina, Sicily, 26-29 October 2003.

- Gibbs E.P. and Ellis C.G., (1994). The epidemiology of bluetongue. Comp. Immun. Microbiol. infect. Dis., 17: 207-220.

- Gibbs E.P., Greiner E.C., (1994). The epidemiology of bluetongue. Comparative Immunology, Microbiology and Infectious Diseases, 17: 197-206.

- Giovannini A., Calistri P., Conte A., Savini L., Nannini D., Patta C., Santucci U. & Caporale V., (2004*a*). Bluetongue virus surveillance in a newly infected area. Vet. Ital., 40 (3): 188-197.

- Giovannini A., Conte A., Panichi G., Calistri P., Dessı̀ M., Foddis F., et al., (2004*b*). Effects of vaccination against bluetongue on milk production and quality in cattle vaccinated with live-attenuated monovalent type 2 vaccine. Vet. Ital. 40(4): 648-653.

- Giovannini A., Conte A., Calistri P., Di Francesco C., Caporale V., (2004*c*). Risk analysis on the introduction into free territories of vaccinated animals from restricted zones. Vet. Ital. 40(4): 697-702.

- Giovannini A., MacDiarmid S., Calistri P., Conte A., Savini L., Nannini D., et al., (2003). The use of risk assessment to decide the control strategy for bluetongue in Italian ruminant populations. J. Risk Anal., 24(6): 1737-1753.

- Gould A.R., (1987). The complete nucleotide sequence of bluetongue virus serotype 1 RNA3 and a comparison with other geographic serotypes from Australia, South Africa and the United States of America, and with other orbivirus isolates. Virus Res., 7: 169-183.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

99

- Hamers C., Werle-Lapostolle B., Rehbein S., Blanchet M., Mure-Ravaud K., Schumacher C., Hudelet P., (2006*a*). Six month efficacy of an inactivated BTV-2 industrial vaccine against a virulent BTV-2 challenge in sheep. In: Proceedings of the ninth international symposium on double-stranded RNA viruses, Cape Town, South Africa, 21–26 October 2006.

- Hamers C., Hudelet P., Blanchet M., Werle-Lapostolle B., Sailleau C., Breard E., Zientara S., (2006*b*). Efficacy of an inactivated bivalent BTV-2/BTV-4 industrial vaccine against a BTV-2 or BTV-4 virulent challenge in sheep. In: Proceedings of the ninth international symposium on double-stranded RNA viruses, Cape Town, South Africa, 21–26 October 2006.

- Hammoumi S., Breard E., Sailleau C., Russo P., Grillet C., Cetre-Sossah C., et al., (2003). Innocuity and activity of the monovalent bluetongue serotype 2 vaccine. Specific detection of the genome of the vaccine strain by RT-PCR. J. Vet. Med., B50: 316-321.

- Hewat E.A., Booth T.F. and Roy P., (1992). Structure of bluetongue virus particles by cryoelectron microscopy. J. Struc. Biol., 109: 61-69.

- Holbrook, F.R., (1985). Research on the control of bluetongue in livestock by vector suppression. In Bluetongue and Related Orbiviruses, ed. Barber, T. L. & Jochim, M. M., New York: Alan R. Liss Inc., 617-20.

- House J.A., Groocock C.M6., Campbell C.H., (1982). Antibodies to bluetongue viruses in animals imported into United States zoological gardens. Canadian Journal of Comparative Medicine, 46: 154-159.

- Huismans H., Van Dijk A.A. and Els H.J., (1987*a*). Uncoating of parental bluetongue virus to core and subcore particles in infected L cells. Virology, 157: 180-188.

- Huismans H., Van der Walt N.T., Cloete M. and Erasmus B.J., (1987*b*). Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. Virology, 157: 172-179.

- Huismans H., (1969). Bluetongue virus-induced interferon synthesis, Onderstepoort J. Vet. Res. 36: 181-185.

- Hyatt A.D. and Eaton B.T., (1988). Ultrastructural distribution of the major capsid proteins within bluetongue virus and infected cells. J. gen. Virol., 69: 805-815.

- Italian Ministry of Health, (2005). Nota del 19 gennaio. Bluetongue— provvedimenti ed impiego del vaccine sierotipo 16 nella IV campagna di vaccinazione 2004–2005, n.1720.

- IZSAM, (2004). Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'. Vaccino e danni vaccinali, /http://gis2.izs.it:7777/bluetongue/doc_tec/danni_vacc/vaccini.pdfS.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

100

- IZSAM, (2001). Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'. Safety and potency testing of bluetongue vaccines. Testing of monovalent bluetongue vaccine (serotype 2) for innocuity, potency and reversion to virulence. Contract DG Sanco 00/0127. Final report. Teramo, pp. 19. /http://www.east-btnet.izs.it/document_r/research/final_report.pdfS.

- Jackson H.C., (1989). Ivermectin as a systemic insecticide. Parasitology Today, 5: 146-56.

- Jameson P., Schoenherr C.K., Grossberg S.E., (1978). Bluetongue virus, an exceptionally potent interferon inducer in mice, Infect. Immun., 20: 321-323.

- Jeggo M.H., Wardley R.C., Brownlie J. (1984). A study of the role of cell-mediated Immunity in bluetongue virus infection in sheep, using cellular adoptive transfer techniques. Immunology, 52: 403-410.

- Jeggo M.H., Gumm I.D. and Taylor W. P., (1983). Clinical and serological response of sheep to serial challenge with different bluetongue virus serotypes. Res. Vet. Sci., 34: 205-211.

- Jeggo M.H. and Wardley R.C., (1982a). The induction of murine cytotoxic T lymphocytes by bluetongue virus. Archiv. Virol. 71: 197-206.

- Jeggo M.H. and Wardley R.C., (1982b). Generation of cross-reactive cytotoxic T lymphocytes following immunization of mice with various bluetongue virus types. Immunology, 45: 629-635.

- Jeggo M.H. and Wardley R.C., (1982c). Production of murine cytotoxic T lymphocytes by bluetongue virus following various immunization procedures. Res. Vet. Sci., 33: 212-215.

- Jessup D.A., (1985). Epidemiology of two orbiviruses in California's native wild ruminants: preliminary report. In: Bluetongue and Related Orbiviruses, (eds Barber T.L. and Jochim M.M.), AR Liss, New York, 53-56.

- Jiménez-Clavero M.A., Aguero M., San Miguel E., Mayoral T., Cruz Lopez M., Ruano M.J., et al., (2006). High throughput detection of bluetongue virus by a new real-time on clinical samples from current mediterranean outbreaks. J. Vet. Diagn. Invest., 18: 7-17.

- Katz J., Alstad D., Gustafson G., Evermann J., (1994). Diagnostic analysis of the prolonged bluetongue virus RNA presence found in the blood of naturally infected cattle and experimentally infected sheep, J. Vet. Diagn. Invest., 6: 139-142.

- Lacey L.A. & Kline D.L., (1983). Laboratory bioassay of Bacillus thuringensis (H-14) against Culicoides spp. And Leptoconops spp. (Ceratopogonidae). Mosquito News, 43: 502-503.

- Lecatsus G., (1968). Electron microscopic study of the formation of bluetonguc virus. Onderstepoort J. Vet. Res., 35: 139 150.

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- Lelli R., Portanti O., Langella V., Luciani M., Di Emidio B., Conte A., (2003). Produzione di un Kit ELISA competitiva per la diagnosi sierologica della Bluetongue. Vet. Ital., 47: 5-13.

- Luedke A.J., Jochim M.M., Bowne J.G. and Jones R.H., (1970). Observations on latent bluetongue virus infection in cattle. J. Am. Vet. Med. Assoc., 15: 1871-1879

- Leudke A.J., Jochim M.M. and Jones R.H., (1969). Bluetongue in cattle: viremia. Am. J. Vet Res. 30: 511-516.

- Lobato Z.I., Coupar B.E., Gray C.P., Lunt R., Andrew M.E., (1997). Antibody responses and protective immunity to recombinant vaccinia expressed-expressed bluetongue virus antigens. Vet. Immunol. Immunopathol., 59: 293-309.

- Lopez A.C., Botija C.S., (1958). Epizootie de fièvre catarrhale ovine en Espagne (blue tongue). Bull. Off. Int. Epiz., 50: 65-93.

- Lucifora G., Rossi P., Calistri P., Giovannini A., (2004). Effects of bivalent bluetongue virus serotypes 2 and 9 vaccine on reproductive performance of cattle: a case study in Calabria, Italy. Vet. Ital., 40: 654-656.

- Lunt R.A., Melville L., Hunt N., Davis S., Rootes C.L., Newberry K.M., et al., (2006). Cultured skin fibroblast cells derived from bluetongue virus-inoculated sheep and field-infected cattle are not a source of late and protracted recoverable virus, J. Gen. Virol., 87: 3661–3666.

- MacLachlan N.J., Osburn B.I., (2006). Impact of bluetongue virus infection on the international movement and trade of ruminants. Journal of the American Veterinary Medical Association, 226: 1346-1349.

- Maclachlan N.J., (2004). Bluetongue: pathogenesis and duration of viraemia. Vet. Ital., 40 (4): 462-467.

- Maclachlan N.J., Pearson J.E. (2004). Bluetongue: Proceedings of the Third International Symposium. MacLachlan, N.J. and Pearson, J.E. (Eds.). Veterinaria Italiana, 40: 1-730.

- MacLachlan N.J., (1994). The pathogenesis and immunology of bluetongue virus infection of ruminants. Comp. Immunol. Microbiol. Infect. Dis., 17: 197-206.

- MacLachlan N.J., Nunamaker R.A., Katz J.B., Sawyer M.M., Akita G.Y., Osburn B.I., Tabachnick W.J., (1994). Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and in vitro feeding of Culicoides variipennis, Arch. Virol., 136: 1–8.

- MacLachlan N.J., Rossitto P.V., Heidner H.W., Iezzi L.G., Yilma T., DeMaula C.D. and Osburn B.I., (1992). Variation amongst the neutralizing epitopes of bluetongue viruses isolated in the United States in 1979-1981. Vet Microbiol., 31: 303-316.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

102

- MacLachlan N.J., Barratt-Boyes S.M., Brewer A.W, and Stott J.G., (1991). Bluetongue virus infection of cattle. In Bluetongue, African Horse Sickness and Related Orbiviruses (Edited by Walton T. E. and Osburn B. l.) CRC Press, Boca Raton. 275-736.

- MacLachlan N.J., Jagels G., Rossitto P.V., Moore P.F. and Heidner H.W., (1990). The pathogenesis of experimental bluetongue virus infection of calves. Vet. Pathol., 27: 223-229.

- MacLachlan N.J., Heidner H.W. and Fuller F.J., (1987). Humoral immune response of calves to bluetongue virus infection. Am. J. Vet. Res., 48: 1031-1035.

- MacLachlan N.J., Thompson J., (1985*a*). Bluetongue virus-induced interferon in cattle, Am. J. Vet. Res., 46: 1238-1241.

- MacLachlan N.J., Osburn B.I., Stott J.L., Ghalib H.W., (1985*b*). Orbivirus infection of the bovine fetus. Prog. Clin. Biol. Res., 178: 79-84.

- Mahrt C.R. & Osburn B.I., (1986). Experimental bluetongue virus infection of sheep; effect of vaccination: pathologic, immunofluorescent, and ultrastructural studies. Am. J. Vet. Res., 47: 1198-1203.

- Manso-Ribeiro J., Rosa-Azevedo J.A., Noronha F.O., Braco-Forte M.C., Grave-Pereira C. & Vasco-Fernandez M., (1957). Fièvre catarrhale du mouton (bluetongue). Bullettin de l'Office International des Epizooties, 48: 350-367.

- MAPA, (2006). Report of the efficacy test performed in Spain with different commercially available vaccine against BTV4 in cattle and sheep. MAPA report.

- Meiswinkel R., Gomulski L.M., Delécolle J.-C., Goffredo M., Gasperi G. (2004). The taxonomy of Culicoides vector Complexes – unfinished business. Veterinaria. Italiana, 40: 151-159.

- Meiswinkel R., Baylis M. & Labuschagne K., (2000). Stabling and the protection of horses from Culicoides bolitinos (Diptera: Ceratopogonidae), a recently identified vector of African horse sickness. Bulletin of Entomological Research, 90: 509-15.

- Meiswinkel R., (1992). Afrotropical Culicoides: C. (Avaritia) loxodontis sp. nov. a member of the Imicola group (Diptera: Ceratopogonidae) associated with the African elephant in the Kruger National Park, South Africa. Onderstepoort Journal of Veterinary Research, 59: 145-160.

- Mellor P.S., Wittmann E.J., (2002). Bluetongue virus in the Mediterranean basin, 1998-2001. The Veterinary Journal, 164: 20-37.

- Mellor P.S., Leake C.J., (2000). Climatic and geographic influences on arboviral infections and vectors. Revue Scientifique et Technique de l'Office International des Epizooties, 19: 41-54.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

103

- Mellor P.S., (1994). Bluetongue. State Veterinary Journal, 4: 7-10.

- Mellor P.S., (1990). The replication of bluetongue virus in Culicoides vectors, Curr. Top. Microbiol. Immunol., 162: 143-161.

- Mellor P.S., Pitzolis G., (1979). Observations on breeding sites and light-trap collections of Culicoides during an outbreak of bluetongue in Cyprus. Bullettin of. Entomological. Reseach., 69: 229-234.

- Mertens P.P., Diprose J., Maan S., Singh K.P., Attoui H., Samuel A.R., (2004). Bluetongue virus replication, molecular and structural biology. Veterinaria Italiana, 40: 426-437.

- Mertens P.P.C., Arella M., Attoui H., Belloncik S., Bergoin M., Boccardo G., Booth T.F., Chiu W., Diprose J.M., Duncan R., Estes M.K., Gorziglia M., Gouet P., Gould A.R., Grimes J.M., Hewat E., Hill C., Holmes I.H., Hoshino Y., Joklik W.K., Knowles N., López Ferber M.L., Malby R., Marzachi C., McCrae M.A., Milne R.G., Nibert M., Nunn M., Omura T., Prasad B.V.V., Pritchard I., Samal S.K., Schoehn G., Shikata E., Stoltz D.B., Stuart D.I., Suzuki N., Upadhyaya N., Uyeda I., Waterhouse P., Williams C.F., Winton J.R. & Zhou H.Z., (2000). Reoviridae. In Virus taxonomy. Seventh Report of the International Committee for the Taxonomy of Viruses (M.H.V. Van Regenmortel, C.M. Fauquet, D.H.L. Bishop, C.H. Calisher, E.B. Carsten, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle & R.B. Wickner, eds). Academic Press, London, 395-480.

- Mertens P.P.C. & Sangar D.V., (1985). Analysis of the terminal sequences of the genome segments of four orbiviruses. Virology, 140: 55-67.

- Monaco F., Cammà C., Serini S., Savini G., (2006). Differentiation between field and vaccine strain of bluetongue virus serotype 16. Vet. Microbiol., 116: 45-52.

- Monaco F., De Luca N., Spina P., Morelli D., Liberatore I., Citarella R., et al., (2004*a*). Virological and serological response of cattle following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9. Vet. Ital., 40(4): 657-660.

- Monaco F., De Luca N., Morelli D., Pisciella M., Palmarini S., Di Giandomenico M., et al., (2004*b*). Field vaccination of cattle using a bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9: effect on milk production. Vet. Ital., 40(4): 661-663.

- Monaco F., Bonfini B., Zaghini M., Antonucci D., Pini A. & Savini G., (2004*c*). Vaccination of cattle using monovalent modified-live vaccine against bluetongue virus serotype 2: innocuity, immunogenicity and effect on pregnancy. Vet. Ital., 40(4):671-675.

- Mortola E., Noad R., Roy P., (2004). Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells, J. Virol., 78: 2875-2883.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

104

- Moss S.R., Jones L.D., Nuttal P.A., (1992). Comparison of the major structural core proteins of tick-borne and Culicoides - borne orbiviruses. J. gen. Virol., 73: 2585-2590.

- Murphey F.A., Borden E.C., Shope R.E., Harrison A., (1971). Physiochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus--a new taxonomic group: electron microscopic studies. J. gen. Virol., 13: 273-288.

- Murray P.K., Eaton B.T., (1996). Vaccine for bluetongue. Aust. Vet. J., 73: 207-210.

- Nevill E.M., Erasmus B.J., Venter G.J., (1992). A six-year survey of viruses associated with Culicoides biting midges throughout South Africa (Diptera: Ceratopogonidae). In: Bluetongue, African horse sickness, and related orbiviruses: (eds. Walton TE and Osburn BI), Proceedings of the Second International Symposium, Paris, France. 314-319.

- Nevill E.M., (1978). The use of cattle to protect sheep from bluetongue infection. Journal of the South African Veterinary Association 49: 129-30.

- O'Connell L., (2002). Entomological aspects of the transmission of arboviral diseases by Culicoides biting midges. PhD, Thesis, University of Bristol.

- OIE. World Organisation for Animal Health (2006*a*). Bluetongue in Italy. Disease Information, 9 November, 19: 775-776.

- OIE. World Organisation for Animal Health (2006*b*). Bluetongue in Belgium. Disease Information, 24 August, 19: 616-617.

- OIE. World Organisation for Animal Health (2006*c*). Bluetongue in The Netherlands. Disease Information, 24 August, 19: 612-613.

- OIE. World Organisation for Animal Health (2006*d*). Bluetongue in Germany. Disease Information, 24 August, 19: 618.

- OIE. World Organisation for Animal Health (2006*e*). Bluetongue in France. Disease Information, 31 August, 19: 636-637.

- OIE. World Organisation for Animal Health (2006*f*). Bluetongue in Luxembourg. Disease Information, 7 December, 19: 852.

- OIE. World Organisation for Animal Health (2006*g*). Bluetongue in Bulgaria. Disease Information, 19 October, 19: 736.

- OIE. Terrestrial animal health code, (2005). World Animal Health Organization.

- OIE. World Organisation for Animal Health (2004*a*). Bluetongue in Morocco. Disease Information, 24 September, 17: 273.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

105

- OIE. World Organisation for Animal Health (2004*b*). Bluetongue in Spain: serological findings in the peninsular territory, in sentinel animals. Disease Information, 15 October, 17: 302.

- OIE. World Organisation for Animal Health (2004*c*). Bluetongue in Portugal. Disease Information, 26 November, 17: 353.

- OIE. World Organisation for Animal Health (2004*d*). Bluetongue in Cyprus. Disease Information, 19 March, 17: 83-84.

- OIE. World Organisation for Animal Health (2004*e*). Bluetongue in France, in the island of Corsica. Disease Information, 17 September, 17: 266-267.

- OIE. World Organisation for Animal Health (2004*f*). Bluetongue, Chapter 2.1.9. In Manual of diagnostic tests and vaccines for terrestrial animals, Paris.

- OIE. World Organisation for Animal Health (2003*a*). Bluetongue in Italy. Circulation of virus serotype 4 in Sardinia. Disease Information, 26 September, 16: 209.

- OIE. World Organisation for Animal Health (2003*b*). Bluetongue in France, in the island of Corsica. Disease Information, 31 October, 16: 242-243.

- OIE. World Organisation for Animal Health (2003*c*). Bluetongue in Albania. Disease Information, 14 February, 16: 47.

- OIE. World Organisation for Animal Health (2002). Bluetongue in Bosnia and Herzegovina. Disease Information, 6 September, 15: 171.

- OIE. World Organisation for Animal Health (2001*a*). Bluetongue in Yugoslavia. Disease Information, 2 November, 14: 252.

- OIE. World Organisation for Animal Health (2001*b*). Bluetongue in Former Yugoslav Republic of Macedonia. Disease Information, 12 October, 14: 234.

- OIE. World Organisation for Animal Health (2001*c*). Bluetongue in Kosovo (FRY), territory under United Nations interim administration. Disease Information, 12 October, 14: 242.

- OIE. World Organisation for Animal Health (2001*d*). Bluetongue in Croatia. Disease Information, 21 December, 14: 291.
- OIE. World Organisation for Animal Health (2000*a*). Bluetongue in Tunisia. Disease Information, 7 January, 13: 1.

- OIE. World Organisation for Animal Health (2000*b*). Bluetongue in Algeria. Disease Information, 28 July, 13: 109.

- OIE. World Organisation for Animal Health (2000*c*). Bluetongue in Italy. Disease Information, 1 September, 13: 150.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

106

- OIE. World Organisation for Animal Health (2000*d*). Bluetongue in Spain: in the Balearic Islands. Disease Information, 13 October, 13: 181.

- OIE. World Organisation for Animal Health (2000*e*). Bluetongue in France: in the island of Corsica. Disease Information, 3 November, 13: 195.

- OIE. World Organisation for Animal Health (1998). Bluetongue in Greece. Disease Information, 27 November, 11: 166.

- Ortega M.D., Mellor P.S., Rawlings Pro M.J., (1998). Seasonal and geographical distribution of Culicoides imicola, C. pulicaris group and C. obsoletus group biting midges in central and southern Spain. Archives of Virology [Suppl.], 14: 85-91.

- Panagiotatos D.E., (2004). Regional overview of bluetongue viruses, vectors, surveillance and unique features in Eastern Europe between 1998 and 2003. Veterinaria Italiana, 40: 61-72.

- Parker A.H., (1949). Seasonal and daily incidence of certain biting midges (Culicoides Latreille: Diptera, Ceratopogonidae). Transactions ofthe Royal Entomological Society London (A).

- Patta C., Giovannini A., Rolesu S., Nannini D., Savini G., Calistri P., et al., (2004). Bluetongue vaccination in Europe: the Italian experience. Vet Ital, 40(4): 601-610.

- Pedley S., Mohamed M.E.H., Mertens P.P.C., (1988). Analysis of genome segments from six different isolates ofbluetongue virus using RNA RNA hybridization: a generalized coding assignment for bluetongue viruses. Virus Res., 10: 381-390.

- Pierce C.M., Balasurya B.R., MacLachlan J., (1998). Phylogenetic analysis of the S10 gene of field and laboratory strains of bluetongue virus from the United States. Virus research, 55: 15-27.

- Pini A., (1976). Study on the pathogenesis of bluetongue: replication of the virus in the organs of infected sheep. Onderstepoort J. Vet. Res., 43: 159-164.

- Prasad B.V.V., Yamaguchi S. Roy P., (1992). Three-dimensional structure of single-shelled bluetongue virus. J. Virol., 66: 2135-2142.

- Pritchard L.I., Sendow I., Lunt R., Hassan S.H., Kattenbelt J., Gould A.R., Daniels P.W., Eaton B.T., (2004). Genetic diversity of bluetongue viruses in south East Asia. Virus Research, 101: 193- 201.

- Reddington J.J., Reddington G.M. and MacLachlan N.J., (1991). A competitive ELISA for detection of antibodies to the group antigen of bluetongue virus. J. Vet. Diagn. Invest., 3: 144-147.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

107

- Richards R.G., MacLachlan N.J., Heidner H.W. and Fuller F.J., (1988). Comparison of virologic and serologic responses of lambs and calves infected with bluetongue virus serotype 10. Vet. Microbiol., 18: 233-242

- Rinaldo C.R. Jr., Overall J.C. Jr., Glasgow L.A., (1975). Viral replication and interferon production in fetal and adult ovine leukocytes and spleen cells, Infect. Immun., 12: 1070-1077.

- Ronchi G.F., Franchi P., Battistini L., Di Emidio B., (2003). Stability study of bluetongue attenuated live vaccine serotype 2 after rehydration. Vet. Ital., 39(2): 11-14.

- Rossitto P.V. & MacLachlan N.J., (1992). Neutralizing epitopes of the serotypes of bluetongue virus present in the United States. J. Gen. Virol., 73: 1947-1952.

- Roy P., (2004). Genetically engineered structure-based vaccine for bluetongue disease. Vet. Ital., 40: 594-600.

- Roy P., Bishop D.H., LeBlois H., Erasmus B.J., (1994). Long-lasting protection of sheep against bluetongue challenge after vaccination with like-like particles: evidence for homologous and partial heterologous protection. Vaccine,12: 805-811.

- Roy P., French T., Erasmus B.J., (1992). Protective efficacy of virus-like particles for bluetongue disease. Vaccine, 10: 28-32.

- Roy P., Urakawa T., Van Dijk A.A., Erasmus B.J., (1990). Recombinant virus vaccine for bluetongue disease in sheep. J. Virol., 64: 1998-2003.

- Saegerman C., Berkvens D., Philip S.M., (2008). Bluetongue epidemiology in the european union. Emerging Infectious Diseases, 40: 539-544.

- Samal S.K., Lviingstone C.W., McConnell S., Ramig R.F., (1987a). Analysis of mixed infection of sheep with bluetongue virus serotypes 10 and 17; evidence of genetic reassortment in the vertebrate host. Journal of Virology, 61:1086-1091.

- Samal S.K., El-Hussein A., Holbrook F.R., Beaty B.J., Ramig, F., (1987b). Mixed infections of Culicoides variipennis with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. Journal of General Virology, 68: 2319-2329.

- Savini G., MacLachlan N.J., Sanchez-Vizcaino J., Zientara S., (2007). Vaccines against bluetongue in Europe. Comparat. Immunol. Microbiol. Infect Dis., doi:10.1016/j.cimid.2007.07.006.

- Savini G., Hamers C., Migliaccio P., Leone A., Hudelet P., Schumacher C., Caporale V., (2006*a*). Assessment of efficacy of a bivalent inactivated vaccine against bluetongue virus serotypes 2 and 4 in cattle. In: Proceedings of the 24th world buiatrics congress, Nice, France, 15-19 October 2006.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

108

- Savini G., Conte A., Di Gennaro A., Leone A., Migliaccio P., Bonfini B., Di Ventura M., Monaco F., (2006*b*). Virological and serological responses in cattle following vaccination with modified-live vaccine against bluetongue virus serotypes 2, 4, 9 and 16. In: Proceedings of the ninth international symposium on ds-RNA viruses, Cape Town, South Africa, 21-26 October 2006.

- Savini G., Cannas E.A., Bonelli P., Petruzzi M., Fresi S., Dimauro C., Di Gennaro A., Nicolussi P., (2005*a*). Live modified bluetongue vaccine viruses in sarda sheep: clinical signs, haematology and chemistry. In: Proceedings of the 12th international symposium of the world association of veterinary laboratory diagnosticians, Montevideo, Uruguay, 16–19 November 2005.

- Savini G., Goffredo M., MonacoF., Di Gennaro A., Cafiero M.A., Baldi L., De Santis P., Meiswinkel R., Caporale V., (2005*b*). Bluetongue virus (BTV) isolations from the Obsoletus Complex (Culicoides, Diptera: Ceratopogonidae) in Italy. Vet Rec., 157: 133-139.

- Savini G., Monaco F., Facchinei A., Pinoni C., Salucci S., Cofini F., et al., (2004*a*). Field vaccination of sheep with bivalent modified-live vaccine against bluetongue virus serotypes 2 and: effect on milk production. Vet. Ital., 40(4): 627-630.

- Savini G., Monaco F., Citarella R., Calzetta G., Panichi G., Ruiu A., et al., (2004*b*) Monovalent modified-live vaccine against bluetongue virus serotype 2: immunity studies in cows. Vet. Ital., 40(4): 664-667.

- Savini G., Monaco F., Calistri P., Panichi G., Ruiu A., Leone A., et al., (2004*c*). Neutralising antibody response in cattle after vaccination with monovalent modified-live vaccine against bluetongue virus serotype 2. Vet. Ital., 40(4): 668-670.

- Savini G., Tittarelli M., Bonfini B., Zaghini M., Di Ventura M., Monaco F., (2004*d*). Serological response in cattle and sheep following infection or vaccination with bluetongue virus. Vet. Ital., 40(4): 645-647.

- Savini G., Monaco F., Conte A., Migliaccio P., Casaccia C., Salucci S., et al., (2004*e*). Virological and serological response of sheep following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9. Vet Ital 2004;40(4):631–4.

- Schreck C.E., Smith N. & McGovern T.P., (1979). Repellency of selected compounds against two species of biting midges (Diptera: Ceratopogonidae: Culicoides). Journal of Medical Entomology, 16: 524-527.

- Sellers R.F., Maarouf A.R., (1991). Possible introduction of epizootic hemorrhagic disease of deer virus (serotype 2) and bluetongue virus (serotype 11) into British Columbia in 1987 and 1988 by infected Culicoides carried on the wind. Canadian Journal of Veterinary Research, 55: 367-370.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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- Sellers R.F., Maarouf A.R., (1989). Trajectory analysis and bluetongue virus serotype 2 in Florida 1982. Canadian Journal of Veterinary Research 53: 100-102.

- Sellers R.F., Pedgley D.E., (1985). Possible windborne spread to western Turkey of bluetongue virus in 1977 and of Akabane virus in 1979. Journal of Hygiene (London) 95: 149-158.

- Sellers R.F., Gibbs E.P.J., Herniman K.A.J., Pedgley D.E., Tucker M.R. (1979). Possible origin of the bluetongue epidemic in Cyprus, August 1977. Journal of Hygiene (Cambridge) 83: 547.

- Sellers R.F., Pedgley D.E., Tucker M.R., (1978). Possible windborne spread of bluetongue to Portugal, June-July 1956. Journal of Hygiene, Cambridge 81: 189.

- Sellers R.F., Pedgley D.E., Tucker M.R., (1977). Possible spread of African horse sickness on the wind. Journal of Hygiene, Cambridge 79: 279-297.

- Sellers R.F., (1975) Bluetongue in Cyprus. Australian Veterinary Journal 51: 198-203.

- Shad G., Wilson W.C., Mecham J.O., Evermann J.F., (1997). Bluetongue virus detection: a safer reversetranscriptase polymerase chain reaction for prediction of viremia in sheep, J. Vet. Diagn. Invest., 9: 118–124.

- Shapiro H.M., (1995). Practical flow cytometry: Third Edition. Wiley-Liss, pp. 542.

- Shimshony A., (2004). Bluetongue in Israel – a brief historical overview. Veterinaria Italiana, 40: 116-118.

- Spreull J., (1905). Malarial catarrhal fever (bluetongue) of sheep in South Africa. J. Comp. Pathol. Therapy, 18: 321-337.

- Stallnecht D.E., Howerth E.W., (2004). Epidemiology of bluetongue and epizootic disease in wildlife: surveillance methods. Veterinaria Italiana, 40: 203-207.

- Standfast, H.A., Muller, M.J. & Wilson, D.D., (1985). Mortality of Culicoides brevitarsis fed on cattle treated with Ivermectin. In Bluetongue and Related Orbiviruses, ed. T. L. Barber & M. M. Jochim. New York: Alan R. Liss Inc., 611-616.

- Stott J.L., Barber T.L., Osburn B.I., (1985). Immunologic response of sheep to inactivated and virulent bluetongue virus. Am. J. Vet. Res., 46:.1043-1049.

- Studdert M.J., Pangborn J. and Addison R.B., (1966). Bluetongue virus structure. Virology, 29: 509 511.

- Tabachnick W.J., (2004). Culicoides and the global epidemiology of bluetongue virus infection. Veterinaria Italiana, 40: 145-150.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

110

- Takamatsu H., Mellor P.S., Mertens P.P., Kirkham P.A., Burroughs J.N., Parkhouse R.M., (2003). A possible overwintering mechanism for bluetongue virus in the absence of the insect vector, J. Gen. Virol., 84: 227–235.

- Takamatsu H., Burroughs J.N., Wade-Evans A.M., Mertens P.P., (1992). Analysis of bluetongue virus serotype-specific and cross reactive ovine T-cell determinants in virus structural proteins, In: Walton T.E., Osburn B.I. (Eds.), Bluetongue, African horse sickness and related orbiviruses, CRC Press, Boca Raton, FL, USA, 491–497.

- Takamatsu H., Burroughs J.N., Wade-Evans A.M. and Mertens P.P.C., (1990). Identification of a bluetongue virus serotype l-specific ovine helper T-cell determinant in outer capsid protein VP2. Virology, 177: 396-400.

- Takamatsu H. and Jeggo M.H., (1989). Cultivation of bluetongue virus-specific ovine T cells and their crossreactivity with different serotype viruses. Immunology, 66: 258-263.

- Vassalos M., (1980). Cas de fièvre catharrale du mouton dans l'ile de Lesbos (Grece). Bullettin de l'Office International des Epizooties, 92: 547-555.

- Venter G.J., Gerdes G.H., Mellor P.S., Paweska J.T., (2004). Transmission potential of South African Culicoides species for live-attenuated bluetongue virus. Vet. Ital., 40: 198-202.

- Veronesi E., Hamblin C., Mellor P.S., (2005). Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). Vaccine, 23(48/49): 5501-5509.

- Verwoerd D., Erasmus B.J., (2004). Bluetongue. In: Coetzer J.A., Tustin R.C. (Eds). Infectious Diseases of Livestock, 2nd ed. Oxford University Press, Cape Town, 1201-1220.

- Wade-Evans A.M., Mertens P.P.C. and Belsham G.J., (1992). Sequence of genome segment 9 of bluetongue virus (serotype 1, South Africa) and expression analysis demonstrating that different forms of VP6 are derived from initiation of protein synthesis at two different sites. J. Gen. Virol., 73: 3023-3026.

- Whetter L.E., Maclachlan N.J., Gebhard D.H., Heidner H.W., Moore P.F., (1989). Bluetongue virus infection of bovine monocytes, J. Gen. Virol., 70: 1663-1676.

- White D.M., Wilson W.C., Blair C.D., Beaty B.J., (2005). Studies on overwintering of bluetongue viruses in insects, J. Gen. Virol., 86: 453-462.

- Wirblich C., Bhattacharya B., Roy P., (2006). Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101, J. Virol., 80: 460-473.

- Zientara S., Bréard E., Sailleau C. (2006). Bluetongue: Characterization of Virus Types by Reverse Transcription-Polymerase Chain Reaction. Vannier P,

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Espeseth D (eds): New Diagnostic Technology : Applications in Animal Health and Biologics Controls, Basel, IABs/Karger series, Developments in Biologicals, 126: 187-196.

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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- Doctor Giovanni Savini of "Centro Studi Malattie Esotiche" (CESME), "Istituto Zooprofilattico dell'Abruzzo e Molise - G. Caporale" in Teramo.

Legend:

Analysis of variance (ANOVA) between groups: different superscripts (a, b, c) denote statistically significant differences $P \le 0.05$.

Analysis of variance (ANOVA) within groups: different superscripts (single dose= *, decuple dose= + and control= #) denote statistically significant differences $P \le 0.05$ respect to prevaccination value.
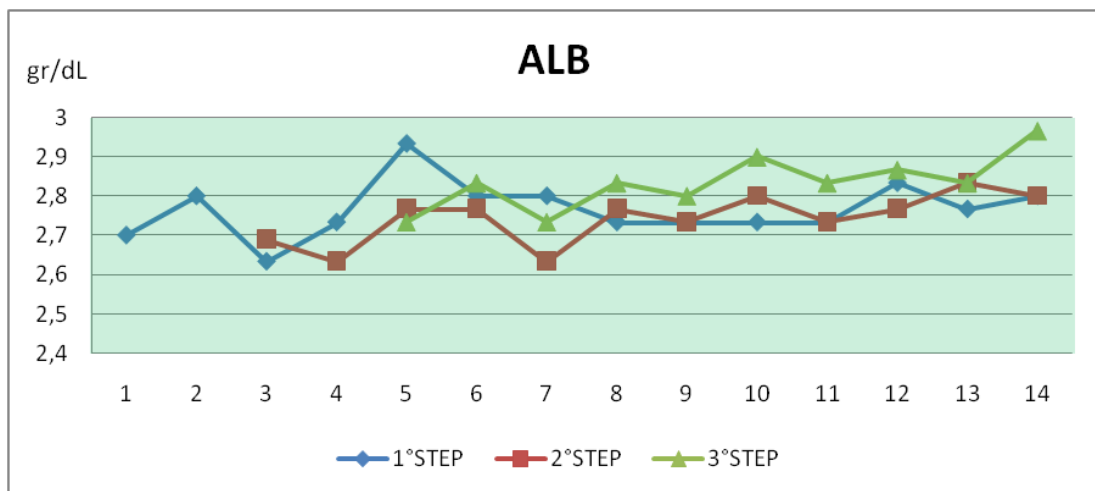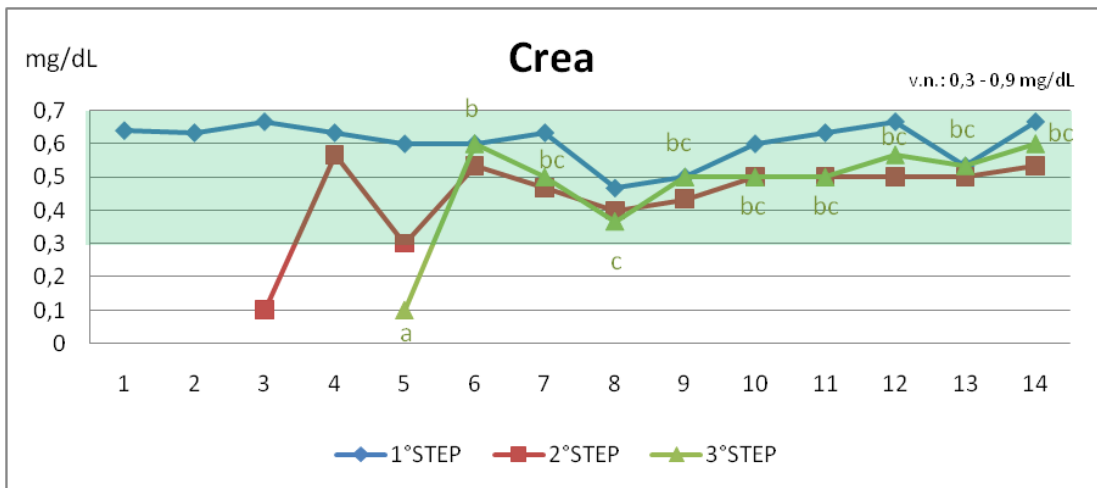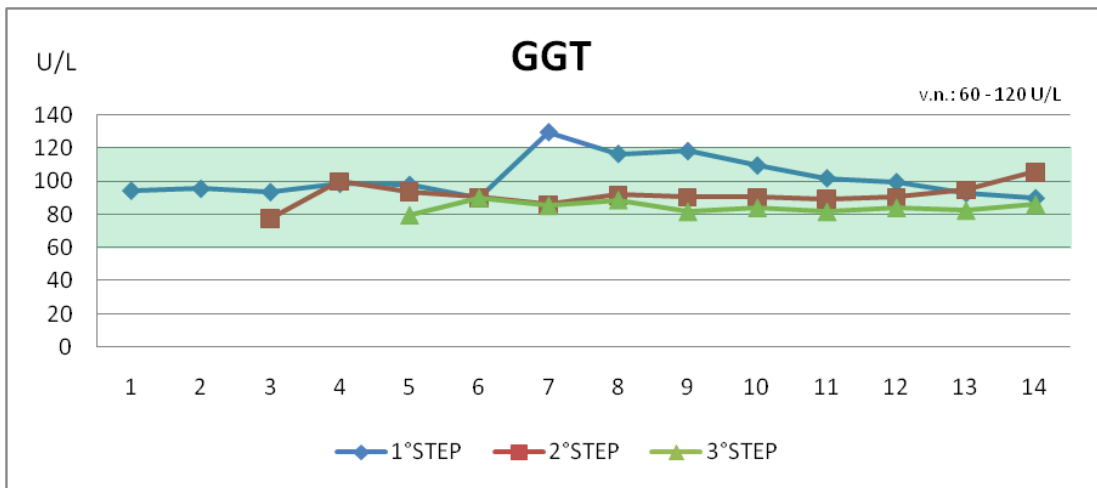
v.n.: normal value

■ : normal reference range

Blood sampling:

Number	Data	Number	Data
1	05/01/2007	12	11/04/2007
2	17/01/2007	13	18/04/2007
3	24/01/2007	14	26/04/2007
4	31/01/2007	15	02/05/2007
5	07/02/2007	16	09/05/2007
6	14/02/2007	17	16/05/2007
7	07/03/2007	18	23/05/2007
8	14/03/2007	19	30/05/2007
9	21/03/2007	20	07/06/2007
10	28/03/2007	21	13/06/2007
11	04/04/2007		

BIOCHEMICAL ANALYSIS

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GLC

v.n.: 40 - 80 mg/dL

TB

v.n.: 0 - 0,5 mg/dL

TP

v.n.: 6 - 8,5 g/dL

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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ALP

v.n.: 45 - 250 U/L

ALT

v.n.: 15 - 45 U/L

AST

v.n.: 70 - 200 U/L

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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COMPLETE BLOOD COUNT

WBC

10³/µL

v.n.: 4,0 - 12,0 10³/µL

singola — decupla — ctr

RBC

10⁶/µL

v.n.: 9,0 - 15,0 10⁶/µL

singola — decupla — ctr

Hb

g/dL

v.n.: 9,0 - 15,0 g/dL

singola — decupla — ctr

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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MCHC

g/dL

v.n.: 29,0 - 40,0 g/dL

PLT

$10^3/\mu L$

v.n.: 200 - 600 $10^3/\mu L$

Li

%

v.n.: 40,0 - 78,0 %

Mo

v.n.: 1,0 - 6,0 %

Gran

v.n.: 10,0 - 60,0 %

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Legend:
Analysis of variance (ANOVA) within groups: different superscripts (a, b, c) denote statistically significant differences $P \le 0.05$.

v.n.: normal value
: normal reference range

Blood sampling:

Number	Data	Number	Data
1	26/01/2007	8	14/03/2007
2	31/01/2007	9	21/03/2007
3	07/02/2007	10	28/03/2007
4	14/02/2007	11	04/04/2007
5	21/02/2007	12	11/04/2007
6	28/02/2007	13	18/04/2007
7	07/03/2007	14	26/04/2007

BIOCHEMICAL ANALYSIS

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Urea — mg/dL — v.n.: 25 - 60 mg/dL

GGT — U/L — v.n.: 60 - 120 U/L

Crea — mg/dL — v.n.: 0,3 - 0,9 mg/dL

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

GLC

TB

TP

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Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

CK
v.n.: 80 - 260 U/L

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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COMPLETE BLOOD COUNT

WBC

10³/µL

v.n.: 4,0 - 12,0 10³/µL

Legend: 1°STEP, 2°STEP, 3°STEP

RBC

10⁶/µL

v.n.: 9,0 - 15,0 10⁶/µL

Legend: 1°STEP, 2°STEP, 3°STEP

Hb

g/dL

v.n.: 9,0 - 15,0 g/dL

Legend: 1°STEP, 2°STEP, 3°STEP

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

MCHC

g/dL

v.n.: 29,0 - 40,0 g/dL

1°STEP   2°STEP   3°STEP

PLT

$10^3/\mu L$

v.n.: 200 - 600 $10^3/\mu L$

1°STEP   2°STEP   3°STEP

Li

%

v.n.: 40,0 - 78,0 %

1°STEP   2°STEP   3°STEP

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Mo

v.n.: **1,0 - 6,0** %

Gran

v.n.: **10,0 - 60,0** %

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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Legend:

Analysis of variance (ANOVA) between groups: superscript (*) denote statistically significant differences $P \leq 0.05$.
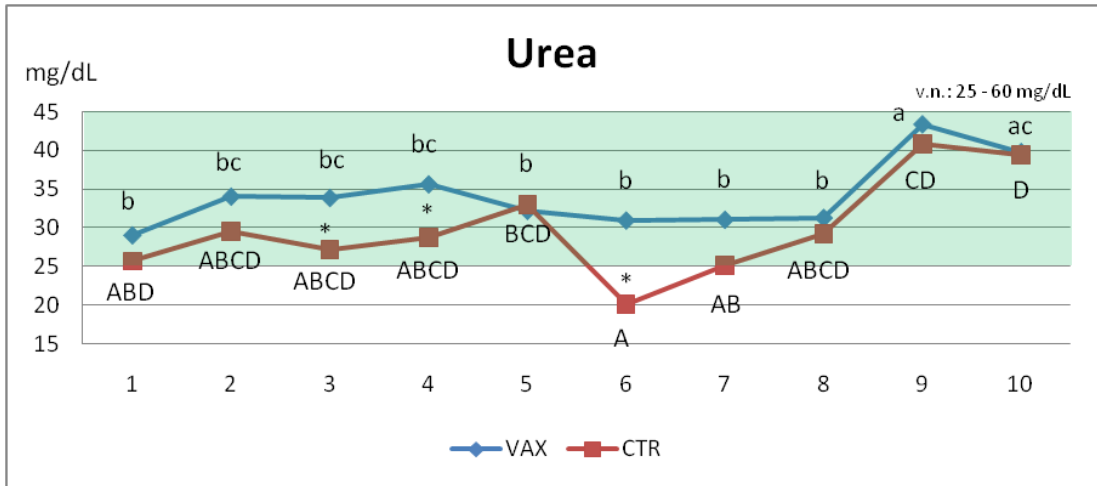
Analysis of variance (ANOVA) within groups: different superscripts (letters) denote statistically significant differences $P \leq 0.05$.
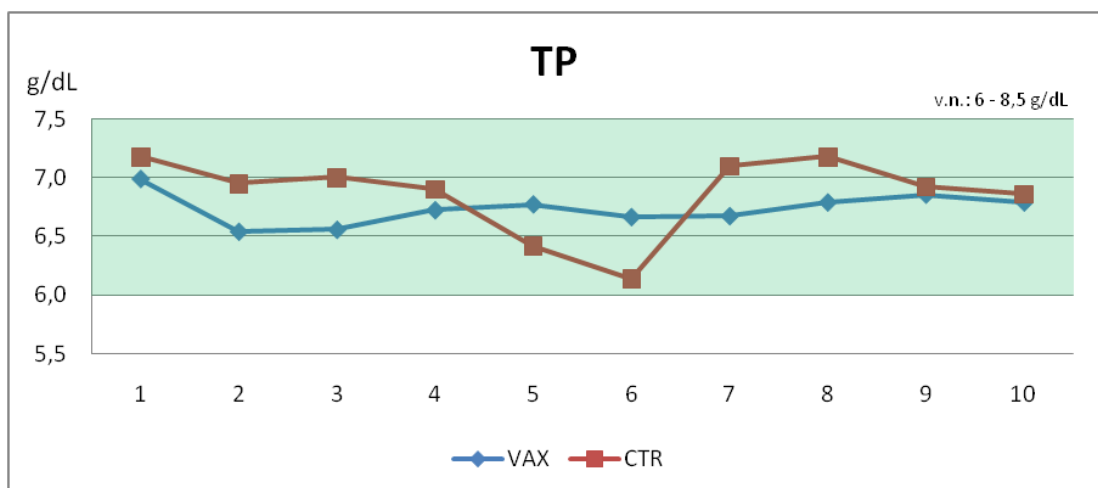
v.n.: normal value

▭ : normal reference range

Blood sampling:

Number	Data	Number	Data
1	01/08/2007	6	05/09/2007
2	08/08/2007	7	12/09/2007
3	17/08/2007	8	19/09/2007
4	22/08/2007	9	26/09/2007
5	29/08/2007	10	03/10/2007

BIOCHEMICAL ANALYSIS

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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133

GLC

mg/dL

v.n.: 40 - 80 mg/dL

VAX — CTR

TB

mg/dL

v.n.: 0 - 0,5 mg/dL

VAX — CTR

TP

g/dL

v.n.: 6 - 8,5 g/dL

VAX — CTR

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

134

ALP

U/L

v.n.: 45 - 250 U/L

200

ab   a   ab   ab   ab   b   ab   ab   ab   ab

150

100

50

*   *

0

1   2   3   4   5   6   7   8   9   10

VAX   CTR

ALT

U/L

v.n.: 15 - 45 U/L

120

*   *

100

80

60

40

20

0

1   2   3   4   5   6   7   8   9   10

VAX   CTR

AST

U/L

v.n.: 70 - 200 U/L

800

600

400

200

0

1   2   3   4   5   6   7   8   9   10

VAX   CTR

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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COMPLETE BLOOD COUNT

WBC

10³/μL

v.n.: 4,0 - 12,0 10³/μL

RBC

10⁶/μL

v.n.: 9,0 - 15,0 10⁶/μL

Hb

g/dL

v.n.: 9,0 - 15,0 g/dL

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

138

MCHC

v.n.: 29,0 - 40,0 g/dL

PLT

v.n.: 200 - 600 10³/µL

Li

v.n.: 40,0 - 78,0 %

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari
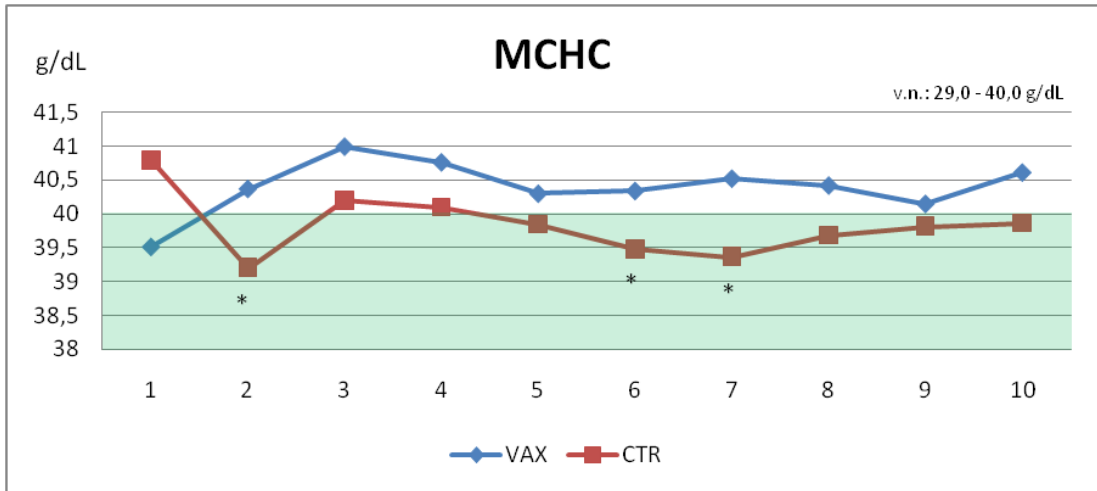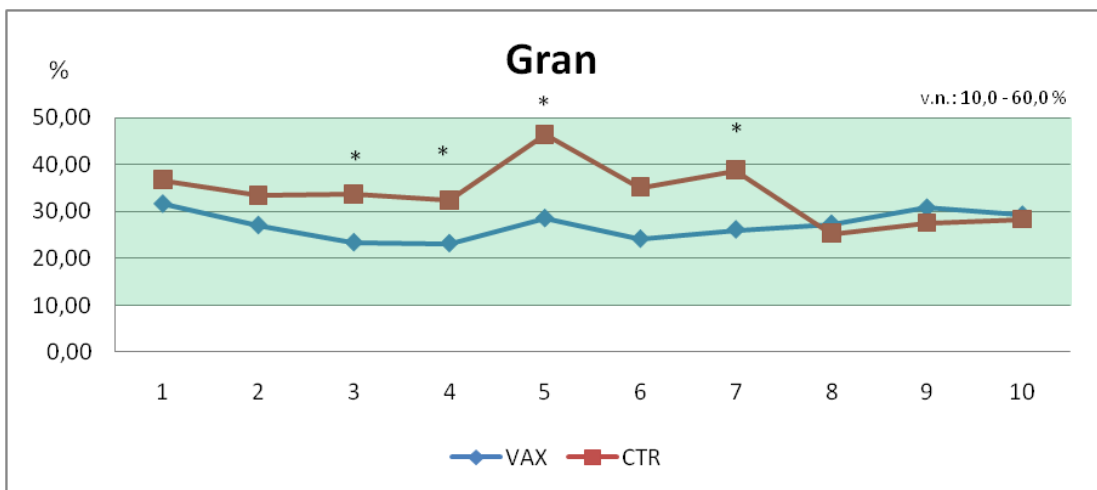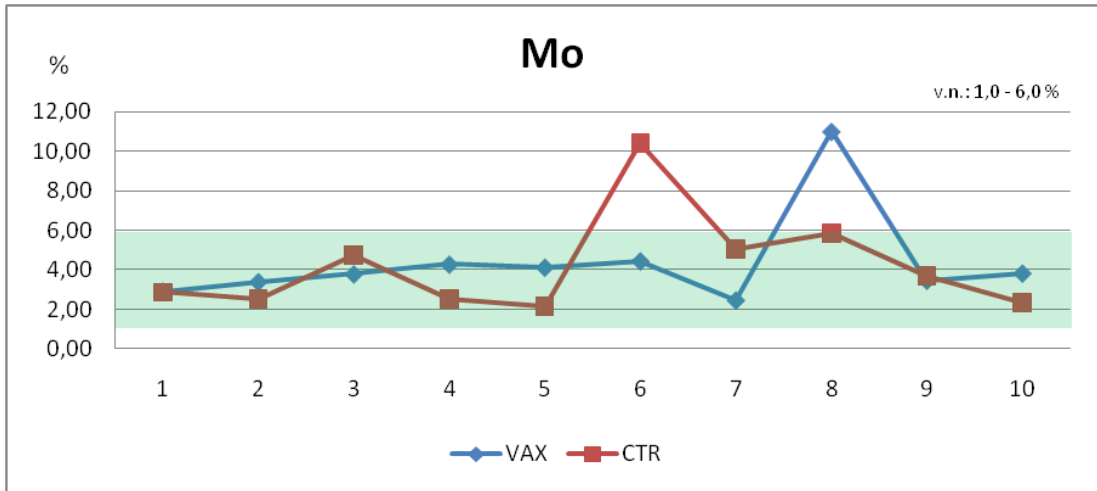
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Legend:

Analysis of variance (ANOVA) between groups: superscripts (*) denote statistically significant differences $P \leq 0.05$.

Analysis of variance (ANOVA) within groups: different superscripts (letters) denote statistically significant differences $P \leq 0.05$.

v.n.: normal value

▭ : normal reference range

Blood sampling:

Number	Data	Number	Data
1	12/10/2007	5	07/11/2007
2	17/10/2007	6	14/11/2007
3	24/10/2007	7	21/11/2007
4	31/10/2007		

BIOCHEMICAL ANALYSIS

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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142

GLU

v.n.: 40 - 80 mg/dL

mg/dL

*

BD   BCD   BD   D

AC

A
*

ACB

SINGOLA   CTR

TB

v.n.: 0 - 0,5 mg/dL

mg/dL

*

SINGOLA   CTR

TP

v.n.: 6 - 8,5 g/dL

g/dL

a

ab

b   b   b   b   b

SINGOLA   CTR

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

143

ALP

U/L

v.n.: 45 - 250 U/L

— SINGOLA — CTR

ALT

U/L

v.n.: 15 - 45 U/L

*

— SINGOLA — CTR

AST

U/L

v.n.: 70 - 200 U/L

— SINGOLA — CTR

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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CK

v.n.: 80 - 260 U/L

U/L

SINGOLA   CTR

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

145

COMPLETE BLOOD COUNT

WBC

$10^3/\mu L$

v.n.: 4,0 - 12,0 $10^3/\mu L$

SINGOLA   CTR

RBC

$10^6/\mu L$

v.n.: 9,0 - 15,0 $10^6/\mu L$

SINGOLA   CTR

Hb

g/dL

v.n.: 9,0 - 15,0 g/dL

a   b   ab   ab   ab   ab   ab

SINGOLA   CTR

Ht

%

v.n.: 27,0 - 45,0 %

MCV

fL

v.n.: 28,0 - 40,0 fL

MCH

pg

v.n.: 8,0 - 12,0 pg

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Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

Mo

v.n.: 1,0 - 6,0 %

Gran

v.n.: 10,0 - 60,0 %

Dott. Marco Canalis, *"Monovalent modified-live vaccine against Bluetongue serotype 1: Safety & Efficacy studies in sheep"*, Tesi di Dottorato in "Scienze e Tecnologie Zootecniche" – Università degli Studi di Sassari

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