Monitoring and surveillance of emerging arboviroses in livestock: what did we learn from Bluetongue and Schmallenberg virus epidemics?

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List of abbreviations

ABV arthropod-borne virus or arbovirus

ADNS Animal Disease Notification System

AIC Akaike Information Criteria

ANOVA Analysis of variance

ARSIA Association Régionale de Santé et d'Identification Animales

BT Bluetongue

BTV Bluetongue virus

BTV-8 Bluetongue virus serotype 8

c-ELISA competitive enzyme-linked immunosorbent assay

CI confidence interval

CODA-CERVA Veterinary and Agrochemical Research Centre

Cp-values Crossing Point values

DGZ Dierengezondheidszorg Vlaanderen

DIVA Differentiating Infected from Vaccinated Animals

DSe diagnostic sensitivity

DSp diagnostic specificity

EC European Commission

ECDC European Center for Disease Prevention and Control

EDTA Ethylenediaminetetraacetic acid (uncoagulated blood samples)

EFSA European Food Safety Authority

EID emerging infectious disease

ELISA Enzyme-linked immunosorbent assay

EU European Union

FAO Food and Agricultural Organization

FASFC Belgian Federal Agency for the Safety of the Food Chain

FLI Friedrich Loeffler Institute

GEE Generalized Estimating Equations

ICC Intraclass Correlation Coefficient

IDW Inverse Distance Weighting

ITG Institute of Tropical Medicine

KMI-IRM Royal Meteorological Institute of Belgium

LCP Laboratory Contingency Plan

MCC Milk Control Centers

MS European Member States

NRL National Reference Laboratory

OD optical densities

OIE Office International des Epizooties-World Organization for Animal Health

Pan-BTV/S5 RT-qPCR non-serotype specific RT-qPCR targeting segment 5

PN percentage negativity

ROC Receiver Operating Characteristic

RT-qPCR real-time reverse transcription polymerase chain reaction

SANITEL Belgian animal identification and registration system

SBV Schmallenberg virus

VFP vector-free period

WS winter screening

CHAPTER I

GENERAL INTRODUCTION

PREFACE

In 1962, Nobel Price virologist Mac Burnet wrote the following: "One can think of the middle of the twentieth century as the end of one of the most important social revolutions in history, the virtual elimination of the infectious diseases as a significant factor in social life" (Burnet, 1962). Unfortunately, the last decades have proven he was wrong; as described by Jones et al. (2008), the number of human emerging infectious diseases (EID) has continued to increase after 1960, reaching a peak in the 1980s, and still representing a significant burden on global economies and public health.

The concept of EID started to be used in the late 1980s, when major epidemics occurred around the globe which surprised many scientists who considered such diseases to be of the past or limited to the less developed parts of the world (Chomel, 1998). It has primarily been applied to diseases affecting humans (Cleaveland et al., 2001). The basic definition of an EID is an infection that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range (Morse, 1995; WHO, 2013). If the disease was present in the region in the past and at one point considered controlled and eradicated, the disease is considered to be re-emerging.

Recent EIDs have predominantly originated from animal reservoirs. More than 60% of the roughly 400 human EIDs that have been identified since 1940 are zoonotic (Jones et al., 2008).

The emergence of an infection can be seen as a two-step process, with: 1. the introduction of the pathogen into a new host population and 2. the establishment and spread within the new host population (Morse, 1995). Most EIDs appear to be caused by pathogens already present in the environment given an opportunity to infect new host populations. Introduction and spread of infectious diseases are known to be driven by a wide range of factors. The emergence can result from the modification of the pathogen characteristics (e.g., by mutation or recombination) and/or the host(s) characteristics, which themselves are thought to be driven largely by socio-economic, environmental and ecological factors. The increase of world-wide exchanges and consumerism, among others, are factors known to be precipitating disease emergence, by placing naïve hosts at increased contact with a previously unfamiliar pathogen or its natural host (Cleaveland et al., 2001; Dufour et al., 2008; Morse, 1995). The obvious increase of those factors suggests that EIDs will continue to occur in the future.

Indeed, even when adjusting for the improvement of global surveillance, it is observed that the frequency with which new pathogens emerge is clearly increasing in time (Morse et al., 2012).

Influenza, Malaria and West Nile virus are only a few examples among many others of recent emerging or re-emerging threats. The potential pandemic spread of such diseases, as it was observed for instance during the H1N1 Influenza epidemic in 2009 (Mexican Flue), and the negative economic and social consequences this will bring, clearly emphasizes the paramount need for improvement of the anticipation, surveillance and control of EIDs (Morse et al., 2012).

I.I. Emerging arboviral infections

One fourth of the EID events which occurred between 1940 and 2004 in the global human population (25.4%) were caused by viral and prion pathogens. Indeed, because of their poor mutation error-correction capacity and often high rates of nucleotides substitution, viruses are prone to adapt easily to new hosts. In addition, viral diseases are less easily controlled compared to other types of infectious diseases because of their difficulty to be treated, and are thus more likely to spread within the host populations (Morse, 1995; Jones et al., 2008).

Arthropod-borne viruses or arboviruses (ABV) are viral pathogens transmitted among vertebrate hosts by hematophagous arthropod vectors including mosquitoes, sandflies, biting midges and ticks. The last International Catalogue of ABV listed more than 550 ABV and related viruses. They are found in diverse viral families, including *Togaviridae* (genus *Alphavirus*); *Bunyaviridae* (genera *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus*); *Flaviviridae* (genus *Flavivirus*); *Rhabdoviridae* (genus *Vesiculovirus*); *Orthomyxoviridae* (genus *Thogotovirus*); *Reoviridae* (genus *Orbivirus*); and *Asfarviridae* (genus *Asfarvirus*). ABV are all RNA viruses except for African swine fever virus which is the only known DNA virus in this category (Weaver and Reisen, 2010). RNA viruses in general are characterised by higher mutation rates compared to DNA viruses (Cleaveland, 2001). Consequently, ABV are especially prone to emerge in new host populations. More than 150 of the ABV are known to cause human and/or animal diseases. Table 1 summarizes

information on important ABV which infect domestic and wild ruminants. Almost half of these ruminant viruses also have a zoonotic potential.

During the last decade, several human and animal pathogenic ABV, including West Nile virus (Monaco et al., 2010), Japanese encephalitis (Mackenzie et al., 2004) and Bluetongue virus (BTV) (Toussaint et al., 2006), have emerged and caused epidemics in countries where they had never before. Because of the requirement for certain vectors, ABV infections mostly occur in tropical and subtropical regions. Indeed, the cooler climate experienced in Northern latitudes means that there are fewer species of arthropod compared to tropical regions. Because ABV are totally dependent on their vectors, a good knowledge of the distribution of the latter is absolutely necessary in order to ensure a comprehensive understanding of the risk for introduction of a given virus. For example, after the emergence of the Chikungunya virus in the Indian Ocean islands in 2005 (Flahaut, 2007), and in Italy in 2007, the European Center for Disease Prevention and Control (ECDC) worked together with entomologists to develop *Aedes albopictus* distribution maps for Europe (ECDC, 2009).

Nevertheless, ABV infections often persist at very low levels in a population until some changes in viral/vector genetics, host/vector population composition/dynamics, or environmental features occurs and make them amplify to epidemics levels (Weaver and Reisen, 2010). Human behavior seems to play a non-negligible role in the abovementioned factors. Vectors may be introduced to new geographic areas inter alia by human travel, international trade and animal/plant movement (Mintiens et al., 2008a; EFSA, 2012a). The increased level of international flux has greatly facilitated the spread of viruses and vectors, which means that the geographical barriers faced by infectious diseases in the past have largely been removed. Also, environmental factors, including urban expansion and population growth have facilitated contacts between hosts and vectors. In addition, climate change is thought to play a significant role in the emergence of ABV (Hollidge, 2010). Receptivity of a given population to viral establishment seems to be closely related to climatic conditions (e.g, seasonal duration) conducive to viral replication. Global warming will create altered environmental conditions leading to changes in vector range, vertebrate host and dynamics (Maclachlan and Guthrie, 2010; Weaver and Reisen, 2010). BTV started to spread in Europe because climate warming allowed the main vector (*Culicoides imicola*) to expand its geographical distribution northward to include parts of Southern Europe (Purse et al., 2005). Following the heat wave in 2003, a French group of experts identified six priority diseases

that were likely to evolve in response to global warming. Five out of the six diseases were vector-borne and four out of five were ABV (ANSES, 2005).

Table 1: List of important arboviruses affecting domestic and wild ruminants; Viruses in italic are zoonotic (sources: Karabatsos, 1985; Johnson et al., 2012, and Powers, 2009)

Name	Genus	Vector	Affected hosts	Established	OIE listed disease
Akabane virus	Orthobunyavirus	Culicoides, Aedes, Culex and Anopheles	Cattle, sheep, goat	Japan, Sout-East Asia, Australia, Turkey, South Africa	no
Aino virus	Orthobunyavirus	Culicoides, Aedes, Culex and Anopheles	Cattle, sheep	Japan, Australia	
Bluetongue virus	Orbivirus	Culicoides	Several domestic and wild ruminant species	America, Africa, Asia, Australia, Middle East	yes
Bovine ephemeral fever virus	Ephemerovirus	Culicoides, Anopheles	Cattle and water buffalo	Africa, Asia, Australia, Middle East	no
Dugbe virus	*Nairovirus*	*Tick*	*Human, cattle*	*Africa*	*no*
Epizootic haemorrhagic disease virus	Orbivirus	Culicoides	Deer, cattle	Americas, Africa, Asia, Australia	yes
Inkoo virus	Orthobunyavirus	Aedes	Cattle	Finland	no
Louping ill virus	Flavivirus	Ixodes ricinus	Sheep, cattle	British Isles	no
Murray Valley encephalitis virus	*Flavivirus*	*Culex annulirostris*	*Human, horse, cattle*	*Australia, Indonesia*	*no*
Nairobi sheep disease virus	Nairovirus	Ripicephalus appendiculatus	Sheep, goat	East Africa	yes
Palyam virus	Orbivirus	Culicoides, Culicine moquitoe	Cattle, sheep	Africa, Asia, Australia	no
Rift Valley fever virus	*Phlebovirus*	*Aedes*	*Human, sheep, goat, camel*	*Africa*	*yes*
Russian spring-summer encephalitis virus	*Flavivirus*	*Ixodes persulcatus*	*Human, cattle, goat*	*Eurasia*	*no*
Thogoto virus	Thogotovirus	Tick	Sheep	Africa	no
Vesicular stomatitis virus	*Vesiculovirus*	*Phlebotomine sandfly, Culicoides, Simulium blackfly*	*Cattle, horse, pig, (human)*	*America*	*yes*
Wesselsbron virus	*Flavivirus*	*Aedes*	*Human, sheep, cattle*	*Africa, Asia*	*no*
West Nile virus	*Flavivirus*	*Culex*	*Human, cattle, horse, avian*	*Africa, Eurasia, America*	*yes*
Western equine encephalitis virus	*Alphavirus*	*Culex*	*Human, cattle, horse*	*America*	*yes*

I.II. Surveillance of emerging arboviral infections

I.II.1. Objectives of surveillance

*S*urveillance of animal infectious diseases is essential to improve animal health, protect public health, support economic growth, and enhance access to international markets for trade in animals. The Food and Agricultural Organization (FAO) wrote that 'In an increasingly globalized world, veterinary surveillance systems and services are vital to detect these diseases early enough and to prepare contingency plans to contain those outbreaks' (FAO, 2000; Mariner et al., 2011). Surveillance aims at producing health data designed to guide decision-making.

The two terms 'monitoring' and 'surveillance' are often used interchangeably in animal health programs. Stricto sensu, *disease monitoring* is defined as the ongoing efforts directed at assessing the disease status of a given population; The efforts consisting in the routine recording, analyses and distribution of information related to the disease. The term *disease surveillance* on the other hand is used to describe a more active system and implies that some form of directed action will be taken if the data indicate a disease level above a certain threshold (Christensen, 2001; Salman et al., 2003; Dufour and Hendrickx, 2009). In order to facilitate the reading of the text, we will from here on simplify the term 'monitoring and surveillance' by using instead the word 'surveillance'.

When designing an appropriate surveillance system for an EID, the initial step will be identifying and evaluating the risk of introduction and establishment of specific diseases in free host-populations. At the European level, the European Food Safety Authority (EFSA) is responsible for assessing and communicating on all risks associated with the food chain. Under the framework of Council Regulation (EC) No 178/2002 and as a consequence of the emergence of BTV in North-Western Europe in 2006 (Toussaint et al., 2006), a call for proposals was published on the EFSA website in August 2007 (CFP/EFSA/AHAW/2007/02) to provide an evaluation of the distribution of arthropod vectors and their potential for transmitting emerging vector-borne animal diseases and zoonoses. In this context, potential tick and mosquito vectors and associated ABV were listed for risk anticipation (EFSA, 2009a,b). Repeated assessments of the risk of emergence of specific pathogens present in the

animal population of neighboring regions and trading partners need to be performed at a regular basis (Doherr and Audigé, 2001).

Figure 1 displays the example of an emerging ABV epidemic curve with its different phases. The objectives of the surveillance process applied to an EID usually follow the evolution of the epidemic curve and are adapted to the epidemiological stage (Thulke et al., 2009; Randolph and Rogers, 2010; EFSA, 2011a).

The objective of surveillance during the initial phase of the epidemic, at t_0 (figure 1), will be to detect the introduction of the infection. At this stage, the host population which is being closely watched over is still naïve (i.e., without any history of the exotic/unknown infection) and is therefore fully susceptible to the infection. Should the introduction occur, the objective will be to detect it as soon as possible (early detection).

In the progress of any epidemic, following an exponential rise, the number of new cases eventually reaches a maximum and then starts to decrease. In the context of an ABV, in our latitudes, the transmission process fades out naturally as the vector activity decreases when the winter season begins. At this point of the epidemic (at t_{1a} and t_{1b}), it is usually time to take stock of the situation. Thus, the main surveillance objective will now be to assess the extent of the infection. The result of this assessment will also constitute the basis for future sampling, for predicting future spread, and will also be one of the aspects to consider before deciding on possible intervention measures such as vaccination.

Control measures such as mass vaccination or slaughter are implemented in the infected area to contain the epidemic. At t_2, the surveillance process will consist in evaluating the impact of the control measure. To do so, an option can be to monitor the targeted decrease of the prevalence indicator, thus, by assessing the extent of the infection as it is done at t_1.

At t_3, the epidemic has ceased following a natural fade-out of transmission or effective control actions. No more outbreaks are reported in the country. At this stage, it is important to provide sufficient proof that the animals are not infected anymore, especially for lifting trade restrictions for OIE-listed diseases (World Organization for Animal Health). The objective of surveillance at this point will be to investigate if a given population is truly free from the disease. Once this has been successfully accomplished, the focus will move again to the first

stage of surveillance, but, at this point, taking into account the fact that the population is not naïve anymore.

Figure 1: Example of emerging arboviral infection epidemic curve (*t0*: before epidemic starts, *t1a* and *t1b*: plateau after epidemic episode, *t2*: after control measures and *t3:* when epidemic has stopped)

Next to general recommendations for animal health surveillance, the OIE *Terrestrial Animal Health Code* sets out specific guidelines for improving surveillance of arthropod-vectors of animal diseases (OIE, 2013a). Even though the objectives of the programs as described in figure 1 will be similar to that of any other EID, arthropod-borne diseases pose an additional challenge to animal health surveillance systems due the complexity of their transmission cycle. A sound surveillance program will require a thorough understanding of the biology, ecology and interactions of the vertebrate and arthropod hosts and a multidisciplinary approach involving both animal health specialists and entomologists will always be needed.

The main objectives of the vector surveillance will be determining the areas and seasons of risk for emergence/re-emergence of the disease and implementing an early-detection system for vectors and/or vector-borne pathogens in those risk units during the vector season. Therefore, it is necessary to establish: 1. whether the vector(s) is (are) present in a region or not, and in the case it is, 2. the distribution of the vector(s) in the region, 3. the abundance of the vector(s) and its (their) spatial and temporal variability (OIE, 2013a; OIE, 2013b). In case the vector is not (yet) present in an area, a risk assessment will be useful for identifying potential pathways for introduction and subsequently watch them closely. Next to this, the detection of the pathogen itself in arthropod populations is a key element of the entomological surveillance.

I.II.2. Strategies for surveillance

There are various ways of classifying surveillance strategies available to meet the objectives described in previous section. Dufour and Audigé (1997) and Doherr and Audigé (2001) propose several classification criteria including the type/source and the method of data collection. As shown in figure 2, most surveillance programs of ABV are based on two methods of data collection: 1. the reporting of clinical suspicions (so–called 'passive surveillance') and 2. the sample-based surveys (so-called 'active surveillance').

Passive surveillance is a pivotal source of information for detecting EID, since it theoretically covers the whole susceptible animal population which is under farmer and/or veterinarian surveillance, continuously in time. The population is expected to express the clinical form of the disease, when infected (Doherr and Audigé, 2001). The veterinarians decide to submit samples for further diagnosis to the veterinary laboratories. Notification of suspect cases of OIE listed ABVs (Table 1) to the competent authorities is usually mandatory within national disease surveillance legislation. The detection and subsequent investigation of disease suspicions obviously relies on a chain of events including the capacity of animal-health professionals to recognize the clinical signs. Passive surveillance is not only important in the initial stage of the epidemic (at *t0*), but remains also very useful during the outbreaks. Indeed, the early detection of new cases helps controlling the impact of the epidemic.

Active surveillance is defined as the periodic collection of samples or case reports from veterinary authorities (Doherr and Audigé, 2001; Hadorn and Stark, 2008). The main difference between active and passive surveillance is that the former follows a prescribed sampling protocol. A very common example of active surveillance is the cross-sectional survey which will give a snapshot in time and space and will allow estimating the prevalence of the infection, the clinical disease, or the immune status of a population. In most cases, this type of surveys will provide a more valid picture of the impact of the EID than when relying on passive surveillance only. Many data sources are usually available, such as samples collected at the farm or animal health data records. The main drawback of active surveillance will be its cost, especially when a large sample is required.

Sentinel animals are a form of targeted surveillance with a prospective study design. They consist in cohorts of unexposed animals (initially seronegative) managed at fixed locations and monitored regularly to detect new infections (seroconversions) (OIE, 2013b). Risk-based approaches can help improve sentinel systems and give the possibility to concentrate resources on specific areas and periods which have been identified to be at-risk for the emergence. Hadorn et al. (2002) illustrated how taking account of risk assessments regarding disease introduction when designing surveys reduce considerably the sample size needed. In the context of an ABV, high-risk patterns can be identified based on several risk criteria (or a combination of criteria): 1.presence of the vector 2.vector ecological requirements (climatic and geographic factors) and 3.exposure of the hosts. Some ABV are transmitted by different species of arthropods and thus different risk criteria have to be dealt with simultaneously when building the sampling design. The elaboration of risk-based sentinel networks totally relies on the sufficient epidemiological knowledge of the disease which often requires among other things a preliminary entomological surveillance system to be in place. This kind of approach thus obviously supposes that the risk of emergence is well known by the veterinary authorities.

In the context of an emerging ABV, a substantial part of the active surveillance system concerns the surveillance of the vector itself (figure 3). Many scientific sampling techniques have been developed for capturing vector arthropods. Depending on the targeted ABV and on the purpose of the surveillance linked to the stage of the epidemic (figure 2), the vector surveillance protocol will vary in terms of: 1. the type of traps used, 2. the number/density of traps, 3. the location sites of traps and 4. the frequency of sampling. Once the objective of the

surveillance program has been clearly defined, historical data on the vector and the ABV should be collected. The choice of the type and the number of traps as well as their frequency of use should be based on this data, taking the following into account: 1.biology of the arthropod, 2.ecological criteria relevant to the arthropod, and 3.host population characteristics. Still depending on the objective of the entomological surveillance, the collection protocols should be able to obtain information about different life stages of the vector. For instance, if an arthropod is absent from an area, the trapping should target the developmental stages that are most likely to be introduced (OIE, 2013a). The detection of the ABV itself in the vector will usually be by performing identification at species level and analyzing pools of arthropods.

As pointed out by figure 2, the role of diagnostic tests is central as they will contribute to both passive and active surveillance. The diagnosis usually consists in identifying the presence of the virus, viral genetic material or of antibodies in potential samples, but can also take the form of a vector's morphological identification. The selection of the method to use will depend again on the purpose of the surveillance. The effectiveness of the surveillance programs will rely largely on the performance of the diagnostic tests that is used (sensitivity (DSe) and specificity (DSp) of the tests), which is closely linked to the status of the targeted population and to the dynamics of the infection (EFSA, 2011a).

Syndromic surveillance is a method for early identification of the impact of potential health threats which can complement the traditional surveillance systems. It consists in the real-time screening of non-specific health indicators for atypical syndromes. There are many sources of information which can be suitable for that purpose, such as data from veterinary practitioners, milk recording, fertility/abortion recording, rendering plant, etc. The data are usually initially collected for other reasons than surveillance; therefore, this approach is economically interesting since it would only rely on data routinely collected in many countries. The principal advantage of syndromic over clinical surveillance is that the former reduces under-reporting as well as the time lag linked to passive surveillance by monitoring continuously animal populations before laboratory confirmation. This is a relatively emerging field which has already encountered limitations principally regarding the availability of animal-health data, but the costs of overcoming these barriers are justified by their utility (Shephard, 2006; Dorea et al., 2011).

Figure 2: The different components of the monitoring and surveillance program of an arbovirus

I.III. Bluetongue virus

I.III.1. General

As shown in table 1, Bluetongue (BT) is an ABV holds a very important place in the history of EIDs, as it was added in the sixties to the former List A of the OIE International Zoosanitary Code as one of the 15 diseases that "are highly contagious and pose particularly serious threats to national and regional economies" (Gibbs and Greiner, 1994). Indeed, BT can cause mild to spectacular outbreaks in ruminant populations and has in addition an adverse impact on worldwide trade due to restrictions on the source of animals (MacLachlan and Osburn, 2006).

BT is caused by BTV, the type species of the genus *Orbivirus*. There are several antigenic strains of BTV. Recently, it was suggested to add a 25th and 26th member to the 24 serologically distinct serotypes of the virus already internationally recognized (Hofmann et al., 2008; Maan et al., 2011). Furthermore, there are variations in the virulence characteristics of individual strains of the same serotype (MacLachlan, 1994).

Adult females of haematophagous midges of the genus *Culicoides* (Diptera: Ceratopogonidae) currently are the only known vectors for transmitting BTV between animals (Du Toit, 1944; Osburn, 1994). The geographical distribution of Bluetongue around the world is a reflection of the Culicoides' distribution and the climatic conditions favorable to the transmission cycle of the virus. Therefore, BT was traditionally understood as occurring around the world between latitudes of approximately 40°N and 35°S (figure 3), although in certain areas (i.e. western North America, China and Kazakhstan) it may extend up to around 50°N (Dulac et al., 1989; Zhang et al., 1999, 2004; Lundervold et al., 2003; Mellor et al., 2008; Wilson and Mellor., 2009). The *Culicoides* species that serve as the principal vectors of the virus differ between regions. Historically, *C. imicola* is believed to be the main vector in Africa and around the Mediterranean Basin, *C.variipennis* in North America, *C. insignis* in Central and South America and *C.fulvus* and *C.wadai* in Australasia (MacLachlan, 1994). So far, six proven or potential *Culicoides* vectors have been recognized in Western Europe: C.*imicola* and five potential vectors of the Obsoletus and Pulicaris species complexes (EFSA, 2011b). Several episystems have been recognized around the world as relatively stable relationships between the different species of *Culicoides*, distinct strains of BTV and susceptible host species, despite extensive and ongoing trade and movement of ruminants between individual episystems (Caporale et al., 2004).

In Europe, the only areas known to have experienced the disease before the 1990s were parts of Portugal, Spain and Greek islands (Manso-Ribeiro and Noronha, 1958; Sellers, 1975; Vassalos, 1980). These epidemics however have never persisted. From 1998 onwards, the situation changed for BT emerged in several areas from Southern and Eastern Europe that had so far never recorded the virus (Anon, 1999a,b; 2000a,b,c, d; 2001a,b,c; Di Ventura et al., 2004; Boinas et al., 2005). At least five serotypes (BTV-1, -2, -4, -9, and -16) of BTV were involved in the BTV incursions between 1998 and 2005 (Mellor et al., 2008). It was shown that BTV was probably spread to those countries by the introduction of infected *Culicoides* by the wind or by moving infected animals from the Eastern and Southern boundaries of Europe (Gomez-Tejedor, 2004; Wilson and Mellor, 2009).

As described in figure 4, both *Culicoides* and ruminants are essential for the life cycle of BTV. Following the introduction of BTV through the skin of the host via the bite of an infected vector, the virus reaches the regional lymph nodes where initial replication occurs. BTV is then transported via the efferent lymphatic vessels throughout the body to secondary

sites of replication, mainly the lymph nodes, the lungs and the spleen. The virus replicates principally in the vascular endothelium and mononuclear phagocytes and is then released massively into the blood stream (Whetter et al., 1989; MacLachlan, 1994). The viraemic ruminant host becomes a source of virus for the *Culicoides* and act as a transient reservoir for maintenance of BTV. The maximal duration of viraemia is known to be around 8 and 9 weeks for sheep and cattle, respectively (Richards et al., 1988; Singer et al., 2001). The probability of a susceptible biting midge to become infected is dependent on the level of viremia of the host and the competence of the vector. When infected, the vector becomes persistently infectious for its entire lifespan which lasts more or less a few weeks (Gibbs and Greiner, 1994).

In addition, it was shown that BTV excretion in semen might occur sporadically (Howard et al., 1985). More recent findings have demonstrated that the transmission of BTV from ruminant to ruminant was possible via the transplacentary and presumably oral routes (De Clercq et al., 2008; Menzies et al., 2008; Backx et al., 2009; Mayo et al., 2010). Cases of contamination of a canine vaccine with BTV showed that dogs are also susceptible to BTV infection (Akita et al., 1994).

Common clinical signs of BT are pyrexia, inflammation of the oral mucosa, tongue and coronary band, excessive salivation, oedema of the head, anorexia, muscle degeneration and lameness (Breard et al., 2004; OIE, 2013c). Death may occur in 8-10 days, but the main impact of BTV infection comes from the indirect losses due to abortion and delayed convalescence (McKercher et al., 1953). BTV can infect most species of domesticated and wild ruminants although the clinical form of the infection is mostly seen in sheep and some species of deer (Erasmus, 1975; Osburn, 1994). The incidence of clinical disease is influenced by several other factors such as the breed and age of the host and the virus strain involved (Gibbs and Greiner, 1994). In cattle, BT is usually described as sub-clinical, but during the early stages of an epidemic in a naïve population, clinical cases are often reported (MacLachlan, 1994).

Figure 3: Map of the estimated global range of bluetongue virus prior to 1998 (from Wilson and Mellor, 2009)

Figure 4: Bluetongue virus transmission cycle

I.III.2. Emergence of Bluetongue virus in North-Western Europe

BTV is considered an 'emerging virus' since it has recently expanded its range towards North-Western Europe, outside its previous environment. Starting August 2006, a first epidemic of BTV was diagnosed in The Netherlands, Belgium and Germany. As early as June that year, Belgian veterinarians started to observe unusual clinical signs in cattle which were primarily attributed to photosensitization or exposure to mycotoxins. In August 2006, several different practitioners in different sheep flocks in the Netherlands reported similar clinical signs and, for this reason, a contagious disease was suspected. The first outbreak was confirmed in the southern part of the Netherlands (van Schaik et al., 2007). At the end of the year, outbreaks were also reported in Luxemburg and France (OIE Animal Health Department, 2006; Toussaint et al., 2006). The virus was first isolated at the Veterinary and Agrochemical Research Centre (CODA-CERVA, Belgium) and identified as BTV-serotype 8 (BTV-8) by scientists of the European Commission (EC) Reference Laboratory for BTV (Pirbright, United Kingdom) (CRL, 2006; Toussaint et al., 2007). The introduction of this serotype came as a surprise, since it was not circulating in Europe at that time. Prior to this epidemic, BTV-8 had only occurred in Africa, Central America, Malaysia, and India/Pakistan (Herniman et al., 1980; Hassan, 1992; Mo et al., 1994; Daniels et al., 2004; Gerdes, 2004). Even though several possibilities have been hypothesized, the route of introduction of BTV-8 still remains undiscovered (Mintiens et al., 2008a). Serotype 8 was shown to differ from other serotypes in: 1.its ability for transplacental transmission and contamination of semen, 2.its extensive spread in North-Western Europe, and 3.its capacity to induce important clinical signs in cattle (EFSA, 2007b; De Clercq et al., 2010; Vercauteren et al., 2008; Vanbinst et al., 2010; EFSA, 2011b).

In May 2007, a sentinel cow seroconverted in Germany and it became evident that BTV-8 somehow overwintered from 2006 and re-emerged within the original affected countries. In addition, the virus emerged in the United Kingdom, Denmark, Switzerland and the Czech Republic (OIE, 2007). It is still not clear how BTV-8 managed to survive winter 2006-2007. Some hypotheses refer to the vector and others to the host (Garigliany et al., 2011). During the second wave of the BTV-8 epidemic in the Netherlands, Elbers et al. (2009) found a median morbidity rate of 5.6% and 4.1% in sheep and cattle, respectively. The scale of the epidemic at that time became so important that the European Union (EU) decided to launch a massive vaccination campaign in 2008. In Belgium, mandatory vaccination of cattle and

sheep and voluntary vaccination of veal calves, goats and cervids with inactivated vaccine against BTV-8 took place between May 2008 and December 2010. From January 2011 onwards, vaccination of ruminants against BTV-8 was still possible but no more compulsory. Ever since the start of the vaccination campaign, no more clinical cases of BT occurred. Subsequently, active surveillance demonstrated that BTV-8 was no longer present and, in consequence, Belgium regained its free status in 2012. In countries adjacent to Belgium and in most European member states (MS), BTV-8 has now been eradicated after almost six years of struggle. In total, between 1st July 2006 and 30 April 2008, 58156 outbreaks of BTV-8 were confirmed throughout Europe (Figure 5).

Furthermore, two more BTV serotypes were detected in 2008 in North-Western Europe: serotype 11 in Belgium and serotype 6 in Germany and the Netherlands but the epidemics were rapidly jugulated (De Clercq et al., 2009). Data on the genetic sequence available indicates a high similarity with the BTV strains that were used to produce the South African modified live vaccines for the two serotypes (EC, 2009).

Figure 5: Temporal distribution of the number of outbreaks due to BTV-8 (source: Surveillance Network for Bluetongue, 2013)

I.III.3. Control of Bluetongue virus

Culicoides control using chemical (insecticides, larvicides and/or repellents) or physical methods (removal of larval breeding sites on farm holdings) can be used to limit the transmission and spread of BTV. There are currently no chemical products authorized in the EU specifically for use against *Culicoides*, but some compounds used to control other types of parasites have also the potential to impact upon *Culicoides.* The treatment of breeding sites remains difficult as habitats are poorly defined for most species. (EFSA, 2008). Globally, the use of control measures against *Culicoides* will only have temporary effects (Bréard et al., 2004) and in any case can surely not be considered by itself to control an epidemic of BTV.

Slaughter of BTV infected ruminants (stamping-out) may, in some specific situations, be considered as a control measure. In general, this type of policy is applied in the context of a disease for which no vaccine is available (or is not wanted to be used for specific reasons) or when the epidemic has reached very low incidence (Horst et al., 1999). A stamping-out strategy may be used as control measure against BTV, for instance, in the context of single infected imported animal where no further disease is detected (DEFRA, 2008).

Traditional control measures include the restriction of animal movements from infected areas. Most European countries require testing ruminants and animal products in provenance of a country which is not free of BTV. Since the year 2000 through Council Directive 2000/75/EC, European legislation provides directives on control measures implemented on animals, live animal products, or vectors, aiming at preventing the dispersion or eliminating BTV (CEC, 2000, 2005; Mintiens et al., 2008b). MS are to integrate these directives in their national BTV control programs. These measures include the establishment of three levels of zones in which movement restrictions should be applied in case of outbreak (*Restricted zones: 20 km-radius, the protection and the surveillance zones*) and a ban on susceptible animal species and on their biological products (semen, ova and embryos) leaving those zones. Nevertheless, even though these types of measures are essential in controlling BTV, they will never be totally efficient in preventing the virus from spreading. The reason for this is that no movement restriction can possibly be applied to the main actor of BTV's transmission, namely the *Culicoides* vector.

Vaccination of susceptible hosts is known to be the most efficient way of controlling BT and is also used to allow safe trade in live animals in accordance to the OIE standards and

European legislation. Live attenuated vaccines have been used for ages and are cheap to produce and effective in controlling clinical BT in areas where the disease is endemic like in South Africa (Hammoumi et al., 2003). However, those vaccines are known to encounter safety issues. Indeed, cases of reversions to virulence, reassortments and teratogen effects have been reported in the past (Dungu et al., 2004; Saegerman et al., 2007; Savini et al., 2008). Inactivated vaccines have been developed in the recent years principally to avoid these issues, but, are more expensive to produce and the induced immune response is generally short and requires repeated doses. However, they are effective when administrated in two doses and protect fully against both the clinical signs and the viraemia (EFSA, 2007a). Vaccination programs for the control of BT in ruminants have limitations due to difficulties in differentiating infected from vaccinated animals based on serology (DIVA). Recombinant DNA vaccines together with a suitable diagnostic test would allow that distinction to be made (Barros et al., 2009; Anderson et al., 2013).

In an emergency situation, such as the BTV-8 epidemic in 2007, MS are allowed to use vaccine without a marketing authorization, after consultation of the EC (Saegerman et al., 2007).

I.III.4. Surveillance of Bluetongue virus

Guidelines for BT surveillance are proposed in Chapter 8.3 of the OIE *Terrestrial Animal Health Code*. Because the impact and epidemiology of BT may differ in different regions of the world, these guidelines should be adapted by each country to its local conditions (OIE, 2013). Commission Regulation (EC) No 1266/2007, last amended on 30 May 2012 (CEC, 2007), introduces the obligation for MS to implement bluetongue monitoring and surveillance programs, which outcomes will guide veterinary authorities in the decision-making process. The minimal requirements have been summarized in table 2 and should always include clinical, active laboratory-based and entomological components. The requirements have been classified according to the surveillance aim: 1.to detect any possible incursion of BTV and 2.to demonstrate the absence of certain serotypes of BTV in a MS or epidemiological relevant geographical area (during a period of at least two years).

BT appears on the list of diseases 'notifiable' to the OIE, which means that an affected country has always to report a new outbreak to the OIE. According to EC No 1266/2007, MS should always notify outbreaks (i.e. holdings where at least one case has been confirmed) of BTV through the Animal Disease Notification System (ADNS). In Belgium, next to the traditional clinical surveillance described previously as well as in table 2, a compulsory reporting system of cattle abortions is implemented in the framework of Brucellosis surveillance (Abortus Protocol). All dead foeti samples presenting typical lesions of BT are tested by Real time Reverse-transcription polymerase chain reaction (RT-qPCR) to exclude the diagnosis of BTV (DGZ, 2013).

The 'design prevalence' (table 2) is a critical epidemiological notion in the active laboratory-based surveillance component, which will influence sample size. Indeed, if the purpose of the survey is to demonstrate freedom from infection at a given confidence level, sample size will increase as the expected prevalence decreases. The value of design prevalence can be derived from past observations or from disease modeling and will depend on the epidemiological context. In a recent Scientific Opinion pertaining to BT monitoring and surveillance, EFSA recommended to decrease the prevalence level prescribed at that time in the EU Regulation for the purpose of demonstrating freedom from disease (Cannon, 2002; EFSA, 2011a). In Regulation (EC) No 1266/2007, it is stated that in the absence of relevant information on the design prevalence for the target population, a prevalence of 20% should be used to detect an incursion of BTV in the susceptible population of a relevant geographical area and a design prevalence of 5% must be considered to demonstrate freedom of BTV.

In contrast to surveillance programs for non-vector diseases in which the epidemiological reference unit is usually the holding, the unit of reference for BTV is a geographical area ('geographical unit of reference' in table 2). It should be taken into consideration in the sampling protocol of the BTV sentinel system. This is due to the central role of *Culicoides* in the BTV transmission cycle and to the fact that midges are never confined to a specific holding. This unit of reference was defined based on past experience with BT surveillance as a grid of around 45 x 45 km (EFSA, 2011a).

The objective of the entomological component as described in table 2, is to determine the 'vector-free period' which is an important concept in the surveillance of BTV. Linked to climatic parameter conditions, vector densities and activity fluctuates greatly seasonally. For

instance, BTV replication is not possible in *Culicoides* under a certain temperature threshold (11-13°C) (Carpenter et al., 2011). In Belgium, the main activity of *Culicoides* was shown to occur between August and November (De Deken et al., 2008). In accordance to the European Regulation, the following criteria based on the surveillance activities should be taken into account to establish the vector-free period (VFP): 1.no evidence of BTV circulation, 2.cessation of vector activity, 3.captures below a maximum threshold and, 4.maximum temperature threshold. This concept is important in terms of legislation as it is a period during which restrictions of animal movements are loosened (EPIZONE, 2011).

In addition, EC No 1266/2007 states that an integrated approach at European level is required in order to be able to analyze seamlessly the epidemiological data provided by the surveillance programs of the different MS. In this context, a web-based application has been developed by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale to gather BTV surveillance data at the EU level (BT-Net system). The system collects, stores, analyses and disseminates relevant epidemiological data on BTV between the different MS (EC, 2013).

Several virological assays are available to detect BTV, antigen or viral RNA. RT-qPCR is a very sensitive and fast technique which is able to detect low concentrations of viral RNA. It is a valid method for the purpose of surveillance. However, viral RNA can be detected by RT-qPCR in the blood of the host longer than the infectious virus itself (MacLachlan, et al., 1994). The only method able to detect the presence of infectious BTV in a sample is viral isolation from eggs/mammal or insect cells (EFSA, 2011a). The most commonly used techniques for revealing the presence of BTV-antibodies in animal samples are the competitive enzyme-linked immunosorbent assay (c-ELISA), the indirect ELISA for bulk milk samples, the agar gel immunodiffusion and the serum neutralization method. The c-ELISA is able to detect the presence of antibodies against all BTV serotypes. Because of its high DSe and DSp and low cost, the c-ELISA is often the method of choice to monitor BTV circulation. However, this test cannot be used for identifying BTV infections in a vaccinated population since it can't make any distinction between infected and vaccinated animals. Furthermore, ELISA tests are not able to discriminate BTV serotypes from one and another. The seroneutralisation is the only serotype-specific serological assay existing up to now (EFSA, 2011a).

Table 2: Summary of Commission Regulation (EC) No 1266/2007 (last amended 30 May 2012) minimum requirements for BT monitoring and surveillance programs

Surveillance component	Activities
Passive clinical	-ongoing system of early detection and reporting of suspicions.
Active laboratory-based	-annual program consisting of at least one of the following: serological/virological monitoring with sentinel animals, surveys or risk-based surveillance. -at least once a year. -sampled animals should be non-vaccinated, exposed to the vector and representative of susceptible population. -sample size for appropriate design prevalence prevalence with 95% confidence in each geographical unit of reference. -positive screening tests should be followed by specific serotype tests.
Entomological	-permanently sites traps to determine the population dynamics of the vector. -aspiration traps equipped with ultraviolet light operated at least 1 night/week at least during the month before the expected end of the VFP and 1 night/month in each geographical unit of reference during the VFP. -a proportion of the collected midges sent to specialized laboratory for counting and identifying species.

I.IV. Schmallenberg virus

I.IV.1. General

During summer 2011, an unidentified disease appeared in adult cattle in several farms located in Germany and the Netherlands (19 November 2011, Promed). The animals showed non-specific acute symptoms (milk drop, watery diarrhea and high hyperthermia (>40°C)) and recovered after a few days. In December that year, researchers from the Friedrich Loeffler Institute (FLI) (Greifswald, Germany) isolated and identified a new emerging virus which was shown to be responsible for the abovementioned symptoms and was provisionally named after the municipality in Germany where it was first identified: the Schmallenberg virus (SBV). Metagenomic analysis indicated that the virus was closely related to viruses from the Simbu serogroup, part of the family *Bunyaviridae*, genus *Orthobunyavirus*. (Hoffmann et al., 2012; Muskens et al., 2012).

Several viruses from the Simbu serogroup such as Shamonda, Akabane (Table 1) and Aino viruses are known to be teratogenic for ruminants when infected during a vulnerable period during gestation and may lead to abortion or congenital malformation (Yanase et al., 2012; OIE, 2013). Starting from December 2011, domestic ruminants in Germany and the Netherlands suffered from abortions and stillborn malformed offspring with signs including the hydranencephaly-arthrogryposis syndrome (Garigliany et al., 2012a; Herder et al., 2012; Van den Brom et al., 2012).

RT-qPCR made available by the FLI was used to confirm SBV suspected cases (Hoffmann et al., 2012). The presence of SBV was demonstrated in Germany, the Netherlands, Belgium, the United Kingdom and France and to a lesser extent in Italy, Luxembourg, Spain, Switzerland, Denmark, Austria, Ireland, Sweden, Norway, Finland, Poland and Estonia (EFSA, 2012b; OIE, 2013; Figure 6). In Belgium, the first reports of malformation in newborn lambs occurred mid-December 2011 and were confirmed by RT-qPCR in a farm in the North of the country located near the Dutch border (23 December 2011, Promed). At the end of August 2012, SBV had been detected in 408 cattle holdings, 167 sheep holdings and 2 goat holdings in Belgium.

Figure 6: Schmallenberg virus confirmed herds by week of first report and country all species until April 2013 (source: EFSA, 2013)

I.IV.2. Epidemiology of Schmallenberg virus

Scientific research on the epidemiology of SBV is ongoing in the different affected countries with the financial support of the EC (EC, 2012). The OIE summarizes and updates regularly the scientific knowledge which has been accumulated on the virus in its SBV Technical Factsheet (OIE, 2013).

SBV is now known to affect several species of ruminants. Thus far, the virus was detected by RT-qPCR or virus isolation in cattle, sheep, goat, bison and roe deer. In addition, SBV was confirmed by serology in various wildlife species: red deer, fallow deer, sika deer, alpaca and mouflon (Jack et al., 2012; Beer et al., 2013; OIE, 2013; 30 May 2012, ProMed).

The identification of SBV in biting midges (*Culicoides spp.*) collected during summer and autumn of 2011 suggests the central role of the vectors in transmitting the virus (De Regge et al., 2012; Rasmussen et al., 2012; Elbers et al., 2013). Further information is required to exclude other arthropod species from the range of putative SBV vectors. Direct transmission of the virus from animal to ruminant has so far not been detected and has never been reported in other Simbu serogroup viruses either (Beer et al., 2013). SBV-genome was detected in bull semen. However, virus transmission by insemination remains to be proven; further research is ongoing to determine the importance of semen and embryos in the transmission of SBV. As a precautionary measure, several countries have already applied restrictions on imports of bovine semen and embryos collected after 1 June 2011 from all EU countries (20 December 2012, ProMed).

SBV is able to cross the placenta and infect the foetus, which can result in viraemic offspring (Garigliany, 2012; Van den Brom et al., 2012). The temporal patterns of the epidemics differ depending on the host species and the production type. This is to put in relation with the different timings for calving and lambing and to the time interval in pregnancy during which infection can lead to disease in the foetus. Linked to the gestational stage of the animal at infection time, SBV causes more or less severe damage to the foetus (Conraths et al., 2013).

The incubation period observed in an experimental challenge trial on cattle ranged between 2 and 5 days post-inoculation and the viraemia was very short with duration of maximum 6 days (Hoffmann et al., 2012). An antibody response to SBV can be detected in infected animals within 12 to 14 days. In analogy to infections with other viruses of the Simbu serogroup (Taylor and Mellor, 1994) and based on the results of a recent experimental study showing that neither oral exposure nor contact infection could induce SBV-infection in re-infected cattle (Wernike et al., 2013), it seems likely that infection with SBV induces protective immunity. It is not yet known how long protection might last and be detectable (Conraths et al., 2013).

I.IV.3. Control and surveillance of Schmallenberg virus

Acting on the control of potential vectors or rescheduling breeding outside the vector season were during the 2011-2012 epidemic the only available preventive measures that could be

implemented at the farm level. Since summer 2013, a vaccine is available in the United Kingdom (Merck Animal Health, 2012). In addition, based on the current scientific knowledge, the OIE has proposed test procedures that may help countries free of SBV to protect themselves from a potential introduction (OIE, 2013). As described previously, the SBV-genome can be detected by RT-qPCR (Hoffmann et al., 2012) and antibodies against the virus can be demonstrated by virus neutralization test, indirect immunofluorescent antibody test and ELISA (Conraths et al., 2013). The latter is the best diagnostic procedure for large-scale screening.

Unlike BTV for which specific surveillance requirements are foreseen, nothing is compulsory for SBV, since the virus, as other Simbu serogroup viruses, is not classified as notifiable to the OIE. However, affected countries have notified the OIE of the presence of SBV under required reporting procedures for emerging diseases. In the Netherlands, immediately after the emergence of the virus was recognized, SBV has been declared officially a reportable disease to the Dutch veterinary authorities. Later on during the epidemic, other countries including Germany and France, made the reporting mandatory as well (Beer et al., 2013).

Next to this, the Belgian compulsory Abortus protocol (as described in the BTV section) has now been extended to test also samples from foetuses with malformations typical of SBV with RT-qPCR.

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CHAPTER II

OBJECTIVES OF THE THESIS

The introduction of BTV-8 into the North-Western European ruminant population in 2006 was a major and unexpected animal health event. At that time, the virus was known to be an important pathogen of ruminants, but was still completely exotic to North-Western Europe. A few years later, history seemed to be repeating itself with the emergence of another ABV affecting also ruminants in the same geographical area. This time, the guilty party was a novel virus closely related to viruses from the Simbu serogroup, also totally unknown in Europe before summer 2011. The two viruses emerged in the exact same region at a five-year interval, meaning that this area probably includes an unidentified route of introduction.

At the moment there are very few endemic ABV in ruminant populations in North-Western Europe. This means that there is room for many other emergences as these populations are still naïve to many viruses (Table 1) (Johnson et al., 2011). In light of the increased risk of ABV emergence in Europe linked to globalization and climate change, it has become imperative for animal-health professionals to proactively improve the surveillance process. The occurrence of BTV and SBV in Belgium are two recent and unprecedented illustrations of ABV emergences which can be used as models to identify key elements to enhance surveillance in the future.

The main objective of this thesis is to analyze the surveillance activities which were implemented in Belgium in the context of the BTV-8 and SBV epidemics in order to be able to provide recommendations to anticipate and prepare adequately the potential next ABV emergence. This will be done by answering the following questions:

- How well were we prepared in Belgium for the BTV-8 epidemic?
- Did the BTV-8 epidemic experience helped to be better prepared for the SBV epidemic?
- What is the reliability of the early case detection for BTV-8 based on clinical surveillance?
- What are the other alternatives for the early detection of an ABV?
- Why and how to evaluate the spread of an ABV epidemic?
- How to plan and conduct the evaluation of a vaccination campaign against an ABV?
- How to demonstrate freedom from disease in a population immunized against an ABV?

CHAPTER III

EMERGENCE OF BLUETONGUE VIRUS IN BELGIUM

Establishing the spread of bluetongue virus at the end of the 2006 epidemic in Belgium

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Abstract

Bluetongue (BT) was notified for the first time in several Northern European countries in August 2006. The first reported outbreaks of BT were confirmed in herds located near the place where Belgium, The Netherlands and Germany share borders. The disease was rapidly and widely disseminated throughout Belgium in both sheep and cattle herds. During the epidemic, case-reporting by the Veterinary Authorities relied almost exclusively on the identification of herds with confirmed clinical infected ruminants. A cross-sectional serological survey targeting all Belgian ruminants was then undertaken during the vector-free season. The first objective of this study was to provide unbiased estimates of BT-seroprevalence for different regions of Belgium. Since under-reporting was suspected during the epidemic, a second goal was to compare the final dispersion of the virus based on the seroprevalence estimates to the dispersion of the confirmed clinical cases which were notified in Belgium, in order to estimate the accuracy of the case-detection based on clinical suspicion. True within-herd seroprevalence was estimated based on a logistic-normal regression model with prior specification on the diagnostic test's sensitivity and specificity. The model was fitted in a Bayesian framework. Herd seroprevalence was estimated using a logistic regression model. To study the linear correlation between the BT winter screening data and the case-herds data, the linear predicted values for the herd prevalence were compared and the Pearson correlation coefficient was estimated. The overall herd and true within-herd seroprevalences were estimated at 83.3 (79.2-87.0) and 23.8 (20.1-28.1) per cent, respectively. BT seropositivity was shown to be widely but unevenly distributed throughout Belgium, with a gradient decreasing towards the south and the west of the country. The analysis has shown there was a strong correlation between the outbreak data and the data from the survey (r=0.73, p<0.0001). The case detection system based on clinical suspicion underestimated the real impact of the epidemic, but indicated an accurate spatial distribution of the virus at the end of the epidemic.

Introduction

Bluetongue (BT), a vector-borne viral disease, is transmitted in ruminant populations almost exclusively by several species of biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) (Côtre-Sossah et al., 2004; Pili et al., 2006). BT virus (BTV) is a species of the genus *Orbivirus* within the *Reoviridae* family. To date, 24 distinct BTV-serotypes have been identified (Gorman, 1990; Takamatsu et al., 2003). BT can cause spectacular outbreaks and has an adverse impact on worldwide trade due to restrictions on the source of animals (Green et al., 2005; FAO, 2006). It thus appears on the list of diseases notifiable to the World Organisation for Animal Health (OIE). Influenced by several factors such as geographical location, the incidence of clinical disease is highly variable. BT disease is uncommon in many areas where BTV is endemic (MacLachlan, 2004). The virus is traditionally known to be distributed around the world in countries lying in the tropics and subtropics, although it may extend further north like in parts of Western North America and Xinjiang, China (Dulac et al., 1989; Gibbs et al., 1994; Qin et al., 1996). The virus has been documented as far as 45°N in Southern Europe (Caporale et al., 2004).

In August 2006, very unexpectedly, BT was for the first time notified in The Netherlands, Belgium and Germany. (OIE Animal Health Department, 2006; Toussaint et al., 2006). Later on during the epidemic, related cases were also reported in France and Luxembourg. The virus incriminated was identified as BTV-serotype 8 (CRL, 2006; Toussaint et al., 2007a), which prior to this epidemic had only occurred in Africa, Central America, Malaysia, and India/Pakistan (Herniman et al., 1980; Hassan, 1992; Mo et al., 1994; Daniels et al., 2004; Gerdes, 2004). Although the possible routes of introduction were investigated, the exact origin remains unknown (Mintiens et al., 2008). Based on the data from the early stages of the epidemic, the rate of the local spread was estimated to be around 15 km/week, partially reflecting the rapid extension of BTV in Northern Europe (Gerbier et al., 2008).

In Belgium, the first 11 ever reported BT outbreaks were confirmed in the near East-border part of the country on the 19th of August 2006, in both sheep and cattle herds (Toussaint et al., 2007b). Despite the measures implemented banning animal movement, the disease was rapidly and widely disseminated throughout the Belgian territory. By December 2006, a total of 695 herds or flocks were declared "case herds" of which 297 were cattle herds. During the epidemic, case-reporting by the Belgian Veterinary Authorities relied almost exclusively on

the identification of herds with confirmed clinical infected ruminants. Laboratory diagnoses were mostly used for confirmation of BTV infections in ruminants reported with BT-like clinical signs. Therefore under-reporting was suspected.

During the winter of 2006/2007, it was assumed that climatic conditions were unfavourable for further propagation of BTV. The last cases of the epidemic in Belgium were reported by the Veterinary Authorities on 15 December 2006. A serological and virological cross-sectional survey (BT winter screening) targeting all Belgian ruminants was undertaken in January-February 2007 in order to establish the true final dispersion of the virus across the country. The first objective of the study was to provide unbiased estimates of BT-seroprevalence for different regions of Belgium. A second objective was to compare the final dispersion of the virus based on the seroprevalence estimates to the dispersion of the confirmed clinical cases which were notified in Belgium, in order to estimate the accuracy of the case-detection based on clinical surveillance. This paper presents the descriptive epidemiology of the BT winter screening 2007.

Material and Methods

Sampling design for the BT winter screening

The study population of the winter screening consisted of dairy cattle of more than two years old which were housed in dairy farms with on-farm delivery of dairy products. Cattle were sampled because of expected higher prevalence in this species compared to sheep (Ward et al., 1994). Only dairy cattle were considered for sampling since serologically negative animals that were to be identified by the BT winter screening would participate afterwards in a longitudinal BT sentinel animals monitoring programme (logistically dairy animals are sampled more easily). The sampling frame was provided by the list of 1245 diary herds with on-farm delivery of dairy product previously identified for the official Belgian Leucois-Brucellosis winter screening. In this programme, all animals of more than two years were sampled.

Since no prior information on the herd prevalence was available, the number of herds to be sampled was based on an expected prevalence of 50 per cent (maximal variance), a desired

absolute precision of 5 per cent and 95 per cent confidence level. Since at the time of sample size's selection, no information was available on the diagnostic test's sensitivity and specificity, these were assumed to be perfect. A sample of 384 herds was set to be selected (Cannon and Roe, 1982). A one-stage cluster sampling design was performed with stratification of the herds by province and proportional allocation according to province surface.

Diagnostic methods

Samples were collected by the official farm veterinarians and conditioned to serum samples at the regional laboratories of 'Dierengezondheidszorg Vlaanderen' and the 'Association Régionale de Santé et d'Identification Animales'. The serum samples were assayed using a commercially available competitive ELISA (c-ELISA) kit (ID Screen® Blue Tongue Competition for detection of anti-VP7 antibodies; ID.VET, Montpellier, France) which was carried out according to the OIE Manual of Standards (OIE, 2004) and to the procedure described by the manufacturer. Results were expressed as percentage negativity (PN) compared to the negative kit control and cut-off settings considered were those provided by the manufacturer. Samples which presented a PN less or equal to 35 per cent, between 35 and 45 per cent, and greater than 45 per cent were considered as positive, doubtful and negative, respectively. Doubtful results were classified positive in the data analysis. Using RT-qPCR as reference test during the epidemic, the diagnostic sensitivity and specificity of the c-ELISA was estimated at 87.4 per cent (95%CI: 83.5-90.4) and 99.0 per cent (95%CI: 97.2-99.6), respectively (Vandenbussche et al., 2007).

Case herds

Case herds were mostly herds (cattle or ovine) for which the veterinary practitioner, who has been consulted by the animal owner, identified suspicious clinical cases and where at least one of those animals was subsequently confirmed positive using a laboratory test (c-ELISA and/or real-time PCR) and then notified to the veterinary authorities (EFSA, 2007). A maximum of three animals were sampled per herd. In addition, herds without clinical signs but with seropositive animals which were then confirmed positive with real-time PCR (Toussaint et al., 2007a) were also included. For example, animals could be detected when

tested serologically for certification prior to trade between zones with different BTV-8 status within the country or prior to export. EDTA blood and serum samples were tested at the Belgian National Reference Laboratory (VAR).

Statistical methods

The estimation of within-herd seroprevalence was based on a logistic-normal regression model. For the BT winter screening data, let Z_i be the number of positive tested animals out of N_i tested animals from herd *i*. It was assumed that the number of positive animals followed a binomial distribution:

$$Z_i \sim Bin(N_i, p_i^a),\qquad(1)$$

with p_i^a the apparent prevalence. The true prevalence p_i^t, reflecting the true serological status of the animals, was derived from the following equation (Rogan and Gladen, 1978) taking the sensitivity and specificity of the c-ELISA test into account:

$$p_i^a = Se \times p_i^t + (1-Sp) \times (1-p_i^t),\qquad(2)$$

or,

$$p_i^t = \frac{p_i^a + Sp - 1}{Se + Sp - 1},$$

where *Se* is the test sensitivity and *Sp* is the test specificity.

To account for possible correlation among the animals from the same herd, the seroprevalence in herd *i* was modelled as

$$logit(p_i^t) = \beta + u_i,\qquad(3)$$

with $u_i \sim Normal(0, \sigma^2)$ being the normally distributed random intercepts for each herd. This model is a special form of a generalized linear mixed model as described by Molenberghs and Verbeke (2005). The Intraclass Correlation Coefficient (ICC) given by

$$ICC = \frac{\sigma^2}{\sigma^2 + \pi^2/3},$$

was estimated to establish the correlation between the infection status of two animals within a herd. Since the sensitivity and specificity were no fixed or known values, a prior distribution for the sensitivity and specificity was assumed. Thus, model specification was further extended by assuming a beta-distribution for the S*e* and *Sp* parameters:

$$Se \sim beta(a_1, b_1), \qquad\qquad\qquad (4)$$

$$Sp \sim beta(a_2, b_2), \qquad\qquad\qquad (5)$$

where a_1, b_1, a_2, b_2 were chosen based on literature material (Vandenbussche et al., 2007). In summary, the within-herd seroprevalence estimation was based on a logistic-normal regression model, accounting for the test sensitivity and specificity of the test. The model is given by equations (1) to (5). Because of its hierarchical structure, it was fitted in a Bayesian framework, using WinBUGS software (http://www.winbugs-development.org.uk). Non-informative priors were used for all model parameters. Posterior seroprevalence distributions and 95 per cent central credibility intervals were generated. A density plot of the predicted within-herd seroprevalence estimates, was produced based on the logistic-normal regression model (1)-(5).

The herd seroprevalence (probability that a herd was infected) was estimated using a logistic regression model:

$$Y_i \sim Bernouilli(p^h), \qquad\qquad\qquad (6)$$

$$Logit(p^h) = \beta,$$

where p^h is the apparent herd prevalence. This model was extended by allowing different β 's for the different provinces, in order to estimate the province-specific herd seroprevalences. For the purpose of this study, a herd was considered as positive if at least one of the sampled animals had a positive ELISA test result, otherwise it was considered negative.

In both models described above, the design-effect was taken into account by weighting each observation by the inverse of the sampling probability. Provinces which were under-

represented in the sample were attributed a higher weight, whereas the over-represented received smaller weight. Herd density data was extracted from the Belgian animal identification and registration system (SANITEL) to provide estimates of the population at risk. A map showing herd density for cattle at municipality level was produced using ArcView GIS 3.2. (ESRI).

A map showing the distribution of within-herd seroprevalence in the country was produced. The true within-herd prevalence estimates of the farms which were sampled were interpolated by Inverse Distance Weighting (IDW) (Shepard, 1968; ESRI, 1996).

In order to estimate the accuracy of the case-detection based on clinical surveillance, the linear correlation between the BT winter screening data and the case data was estimated. For both data sets, herd prevalence per municipality was estimated based on logistic regression models, as in equation (6). In order to account for spatial differences, a flexible smoothing method was used to estimate the spatial trend. It is assumed that

$$Logit(p^h) = f(x, y),$$

where $f(x, y)$ is some unspecified smooth function of the *x*- and *y*-coordinates. The method used penalized splines with radial basis function, fitted as a generalized linear mixed model (Eilers et al., 1996; Ruppert, Wand and Carroll, 2003). This method was implemented in the SAS procedure GLIMMIX (9.1.3. SAS, Inc.).

To study the linear correlation between the two datasets, the linear predicted values $f(x, y)$ for herd prevalence, resulting from the logistic regression models applied to the two datasets, were compared and the Pearson correlation coefficient was estimated. For the outbreak data to be comparable with the winter screening data, solely cattle results were used for this part of the analysis. Maps showing the distribution of herd prevalence estimates at municipality level were produced.

Daily meteorological data on the mean temperature were collected at 247 weather stations in The Netherlands, Belgium, Germany and France. The raw temperature values were interpolated between the locations of the weather stations using IDW. A model of the German meteorological service for large scale maps was used to adjust the interpolated temperature values for the correlation between temperature and altitude (Müller-Westermeier, 1995;

Ahrens, 2006). In a first step, the raw temperature data were reduced to sea level and interpolated on a grid cell size of 250 m using the IDW algorithm. Finally, the interpolated values were adjusted for the height in the actual topography using altitude data with a resolution of 90 m (NASA SRTM data). Maps of the spatially interpolated mean daily temperature were produced for six dates at monthly intervals.

Results

Bluetongue winter screening

A total of 25,846 cattle from 344 herds were sampled between the first and the 31st of January 2007. An average of 75 animals (standard deviation, 51), ranging from 1 to 370, were sampled per herd. Among those samples, 5008 gave positive results. The overall herd seroprevalence was estimated at 83.3 per cent (95%CI 79.2-87.0). Province-specific herd seroprevalence estimates are shown in Figure 1. The highest estimates were found in provinces located in the north-east of Belgium, near the place where the epidemic started.

Figure 1: Bluetongue herd seroprevalence (%) and associated CI95% at the provincial level in Belgian dairy cattle based on the winter screening data, January 2007

The true overall within-herd prevalence was 23.8 per cent (95%CI 20.1-28.1). The spatial distribution of the within-herd prevalence is presented in Figure 2. The highest provincial within-herd prevalence estimates were found in Limburg and Liege provinces. There was a second focus around the city of Ghent in East Flanders province. On the other hand, the lowest within-herd seroprevalence was found in Hainaut province.

Figure 2: Distribution of within-herd seroprevalence (%) in Belgian dairy cattle based on the winter screening data, January 2007

Figure 3 shows the density plot of within-herd seroprevalence estimates. A large variability in within-herd seroprevalence was observed. However, in most herds, within-herd seroprevalence was between 0 and 20 per cent. The within herd ICC was estimated at 0.41 (95%CI 0.36-0.47). This shows that correlation between the infectious statuses of two animals within a herd was high.

Figure 3: Density plot of the farm-specific within-herd seroprevalence estimates based on the winter screening data

Figure 4 presents herd density at municipality level. High herd densities in 2006 were mainly observed in the western part of the country. Figure 5 shows maps of the spatially interpolated temperature for the 30 June, 30 July, 30 August, 30 September, 30 October and 30 November 2006. Around the possible time of introduction (June-July), the temperature was high in Belgium. The global temperature trend during the BT epidemic consisted of two warm time periods (June-July and End-September) separated by a cooler period. Local differences in mean temperature were also suggested by the maps.

Figure 4: Cattle herd density at the municipality level in Belgium, 2006

Figure 5: Maps of the spatially interpolated temperature in Belgium on the (a.) 30 June (b.) 30 July (c.) 30 August (d.) 30 September (e.) 30 October and (f.) 30 November 2006

Case herds

Between the 18th of August and the 31st of December 2006, a total of 1445 cattle and 893 sheep samples were analysed. The overall BT herd prevalence was estimated at 0.7 per cent (95%CI 0.7-0.8) for cattle herds and 1.3 per cent (95%CI 1.2-1.4) for sheep flocks. Herd prevalence estimates at the provincial level for cattle and sheep are shown in Table 1. Estimates were found to be more or less twice higher for sheep than for cattle. However, the distributions of herd prevalence estimates at the provincial level were found to be similar in the two species.

Table 1: Bluetongue herd prevalence (%) and associated 95%CI in Belgian cattle and sheep population based on the 2006 case herd data

Province	Herd-prevalence (%) (95%CI)	
	cattle	sheep
Antwerp	0.7 (0.5-1.0)	1.5 (1.1-2.0)
East Flanders	1.5 (1.3-1.8)	2.2 (1.9-2.5)
Flemish Brabant	0.8 (0.5-1.2)	1.2 (0.9-1.6)
Hainaut	0.1 (0.0-0.3)	0.1 (0.0-0.3)
Liege	1.6 (1.3-2.1)	1.5 (1.1-2.2)
Limburg	1 (0.7-1.4)	3.2 (2.5-4.0)
Luxembourg	0.2 (0.1-0.4)	0.1 (0.0-0.5)
Namur	0.3 (0.2-0.7)	0.5 (0.3-0.9)
Walloon Brabant	1 (0.5-2.1)	0.3 (0.1-1.2)
West Flanders	0.1 (0.1-0.2)	0.4 (0.3-0.7)

Comparison of winter screening and cattle case herd results

Based on the linear predicted values for herd prevalence of the winter screening and the cattle case herd data, the Pearson correlation coefficient was 0.73 and significant (p-value<0.0001). Figure 6 consists of two maps showing the distribution of BT herd prevalence estimates at municipality level based on cattle case-herd and winter screening data. The patterns indicated by the two maps seemed to be similar to each other.

Figure 6: Bluetongue herd prevalence at the municipality level in Belgian dairy cattle based on (a.) the 2006 case herd data and on (b.) the winter screening data.

Discussion

Starting from the original focus in the area where Belgium, The Netherlands and Germany share borders, the epidemic gradually disseminated throughout the Northern European countries. The epidemic predominantly spread horizontally along an East-West axis (EFSA, 2007). In Belgium, until October 2006, case herds were mainly limited to an area situated in the Eastern part of the country. Early September 2006, the area of chief concern appeared to

be the infectious status of the "still free" provinces; therefore, a serological screening was conducted and demonstrated freedom of BTV infection for all the provinces in which no case herd was notified at that time (Vandenbussche et al., 2007). The first case in East Flanders was notified on September 18 and the infection then further continued its spread to the west. At the end of the epidemic, BTV-seropositivity in dairy cattle herds was shown to be widely but unevenly distributed throughout Belgium. Seroprevalence was found to be the highest near the area of first infection with a gradient decreasing towards the south and the west of the country. In The Netherlands, the same distribution was observed, with, in this case, a gradient decreasing towards the Northern part of the country (Elbers et al., 2007). Based on case herd data, Gerbier et al. (2007) identified two spatial clusters of cases in Belgium which centered around the cities of Maastricht (the Netherlands) and Ghent. The authors stated that a gap between the two clusters remained by the end of the epidemic. The results of the present study on the other hand demonstrated a herd seroprevalence gradually decreasing towards the west with no higher level around the Ghent area. The within-herd seroprevalence map (Figure 2) revealed areas around Maastricht and Ghent where the within-herd seroprevalence was high. The highest within-herd seroprevalences were found on farms situated in Liege, Limburg, Flemish and Walloon Brabant provinces most certainly due to the fact that those regions were affected at the beginning of the epidemic. However, further study of specific risk factors such as local temperature, farm management system, and abundance of vector, is needed to better understand the spatial variation in the occurrence of BT and to allow a more efficient control of the infection in the future. The second focus around the city of Ghent could be explained for instance by the high cattle farm density in this area (Figure 4) which could be a risk factor for within-herd propagation of BT. Visual examination of the temperature maps (Figure 5) suggested that hilly areas were always cooler compared to areas of lower elevation. Those lower temperatures may have had an influence on the life cycle of *Culicoides* and the replication of BTV in the vectors. The high ICC reflected the important correlation between two animals within a herd with respect to the presence/absence of BTV. A study conducted in Kazakhstan demonstrated also significant clustering at farm level (Lundervold et al., 2003). The authors pinpointed the fact that this effect could be related to local variations in the vector's distribution.

Clinical signs of BT appear as soon as five days post-infection. Therefore, in the early stages of an epidemic, infected animals are more quickly detected by clinical examination than by serology. In Italy, during the 2000-2001 BT-outbreak, sero-surveillance only debuted in the

decreasing phase of the epidemic curve (Giovannini et al., 2004). In a reporting system such as the one implemented during the course of the outbreak in Belgium, a succession of events has to occur before a case is detected. Theoretically, the reporting of suspect cases allows for a view of the situation for the entire susceptible population which is under owner and veterinary observation. This first relies on the assumption that the infection will produce clinical signs; hence, subclinical cases will go unnoticed (Doherr et al., 2001). BTV has in the past been isolated in several countries without clinical disease being recognised (Gibbs et al., 1994; Mulhern, 1985). Based on the sparse data from whole-herd-sampling during the Northern European epidemic, it has been shown that a high proportion of cattle within a herd could be PCR or seropositive, while not showing any BT-clinical signs. Moreover, owners and veterinarians in Belgium had never previously experienced this exotic disease; therefore clinical signs were unfamiliar to them (Elbers et al., 2008). Also, owners may have been reluctant to report cases for fear of consequent loss of trade. The winter screening revealed indeed a higher prevalence than demonstrated by the reporting of clinical cases. Results demonstrated a high level of exposure to BTV in the dairy herds. The results confirm the fact that BTV spreads very quickly in an immunologically naïve ruminant population. The first Italian epidemic of BT in 2000 in Sardinia has demonstrated a rate of spread of 30 km per week and 80% of the island had been infected. Both in Sardinia and Sicilia, serological surveillance detected virus circulation to be wider than shown by clinical surveillance (Calistri et al., 2004; Giovannini et al., 2004). Serological screening demonstrated a BT animal-prevalence levels ranging from 3.23 to 61.11% in Albania after recent first infection (Di Ventura , 2004). In the present study, the obtained Pearson correlation coefficient showed that the spatial distributions of the virus indicated by the two datasets (Figure 6) were very similar. However, there was a large scale-difference in estimated prevalences. The case detection system based on clinical suspicion underestimated the real impact of the epidemic, but indicated an accurate spatial distribution of the virus at the end of the epidemic.

In theory, each individual within the target population, namely the Belgian ruminant population, should have had an equal chance of being selected for sampling. For accessibility matters, only dairy herds with on-farm delivery of dairy product were considered in the sampling frame. Moreover, solely animals older than 24 months were sampled. Sub sampling presents an opportunity for selection bias which must be accounted for when willing to extrapolate the results to the target population. From the outbreak data, BT herd prevalence level in sheep was higher than in cattle. Ovine BTV infection cases might have been easier to

detect since this species is commonly known to be more prone to develop the clinical form of the disease (Gibbs et al., 1994). However, the particularity of this BTV-8 epidemic was that the virus was able to induce severe clinical signs in cattle (Thiry et al., 2006). Moreover, the results of the confirmation analyses showed that clinical signs observed in cattle were more specific than those observed in sheep (Toussaint et al., 2007b). In general, seroprevalence is known to be higher in cattle than in small ruminant populations (Ward et al., 1994; Di Ventura et al., 2004). On the other hand, a study conducted on the Indian sub-continent, demonstrated a higher seroprevalence in sheep than in cattle, with 45.71% and 33.4%, respectively (Sreenivasulu et al., 2004). Those findings demonstrate differences which can occur when sampling a particular species instead of another. In the same way, many studies have concluded that older cattle were more likely to be positive to BTV antibodies than younger cattle, related to a greater opportunity for repeated exposure to the virus (Uhaa et al., 1990; Ward et al., 1994; Lundervold et al., 2003). Factors such as breed specific genetics or management methods differ a lot between a beef and a dairy cattle herd; hence, the level of prevalence may not follow identical patterns. Moreover, the "on-farm delivery of diary products" characteristic of selected herds may be associated for instance to a more artisanal agriculture which may indirectly have consequences on disease control. An analysis performed by Green et al. in the United States (2005), has not proven herd type to be a significant risk factor. However, this conclusion may depend on local conditions and consequently differ for Belgian cattle.

The only indication a positive serological result gives is that the tested animal was at one point infected with the virus. Due to resistance in host population, future outbreaks would probably occur more silently in herds which were already infected during the 2006 epidemic. In this case, the genuine dissemination of the virus would certainly be much more extensive than the distribution of suspected cases (Purse et al., 2006).

Conclusion

These findings currently provide the best information available on the unprecedented occurrence of BT in Belgium and emphasized the rapid and non-confined spread of the virus in a susceptible ruminant population. Local variations in estimated prevalence should be further investigated to help identify particular risk factors and be able to better control future

outbreaks. The results of the winter screening were also used to set up a sentinel program in the country. This study showed that the case detection system based on clinical suspicion underestimated the real impact of the epidemic, but provided an accurate indication of the spatial distribution of the virus at the end of the epidemic

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CHAPTER IV

SECOND EPISODE OF BLUETONGUE

Bluetongue in Belgium: Episode II

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Abstract

Bluetongue (BT) is an arthropod-borne viral disease of ruminants. In August 2006, domestic ruminant populations in Northern Europe became infected with BT virus serotype 8 (BTV-8). The first BTV-8-case of the year 2007 in Belgium was notified in July. This case was the starting point of a second wave of BT outbreaks. The main objective of this study was to describe the evolution and the clinical impact of the second episode of BT in Belgium. In addition, the main differences with the previous episode (August-December 2006) are reported. Both outbreak and rendering plant data were analysed. Overall cumulative incidence at herd level was estimated at 11.5 (11.2-11.8) and 7.5 (7.3-7.8) % in cattle and sheep populations, respectively. The findings went in favour of a negative association between within-herd prevalence in 2006 and the risk of showing clinical signs of BT in 2007 (via protective immunity). A high level of correlation was demonstrated between BT incidence and small ruminant mortality data when shifting the latter of one week backwards. This result supports the hypothesis that the high increase in small ruminant mortality observed in 2007 was the consequence of the presence of BT. For cattle, the correlation was not as high. An increase in cattle foetal mortality was also observed during the year 2007 and a fair correlation was found between BT incidence and foetal mortality.

Keywords

Introduction

Bluetongue (BT) is an arthropod-borne viral disease of both wild and domestic ruminants. BT virus (BTV) is a species of the genus *Orbivirus* within the *Reoviridae* family which is transmitted in ruminant populations almost exclusively by several species of biting midges of the genus *Culicoides* (*Diptera: Ceratopogonidae*). Of more than 1400 *Culicoides* species worldwide, fewer than 20 are actual or possible vectors of BTV (Cêtre-Sossah et al., 2004). To date, 24 distinct BTV-serotypes have been identified. The virus is traditionally known to be distributed around the world in countries lying in the tropics and subtropics, although it may extend further north like in parts of western North America and Xinjiang, China (Dulac et al., 1989; Gibbs and Greiner, 1994; Qin et al., 1996). Until recently, the virus has been documented as far as 45°N in southern Europe. (Gibbs and Greiner., 1994; Caporale, 2004). The vast majority of BT infections are clinically unapparent. Cattle can act as a reservoir for disease to keep the infection circulating. Sheep are more prone to show clinical signs. When the disease does occur, common clinical signs are pyrexia, inflammation of the oral mucosa, excessive salivation, oedema of the head (OIE, 2008a). Reproductive disorders such as infertility, congenital abnormalities, and abortion are also reported in infected sheep and cattle. BTV has been shown to cross the placenta barrier and may be arbortigenic and teratogenic (Luedke, 1985).

In August 2006, domestic ruminant populations in Northern Europe became infected with BTV. Very unexpectedly, BT was notified in the Netherlands, Belgium and Germany. (OIE, 2006; Toussaint et al., 2006). The index cases were reported in the area where the three countries share borders. Later on during the epidemic, related cases were also declared in France and Luxembourg. The virus incriminated was identified as BTV-serotype 8 (CRL, 2006; Toussaint et al., 2007). By the end of the first epidemic in December 2006, a total of 695 outbreaks were notified in Belgium. One of the particularities of this BTV-8 epidemic was the virus' capability of inducing severe clinical signs in cattle (Thiry et al., 2007; Elbers et al., 2008). At the end of January 2007, a cross-sectional serological study was performed ('winter screening 2007') in the Belgian cattle population in order to establish the spread of BTV after the 2006 episode (Méroc et al., 2008). The findings of the study emphasized the rapid and non-confined spread of the virus. Indeed, overall herd seroprevalence in January 2007 attained 83% and all Belgian provinces were concerned. Overall within-herd seroprevalence was estimated at 24%. The question whether BTV would over-winter was of

major concern at that time. In June 2007, a sentinel cow was confirmed positive in North-Rhein Westphalia (Germany). The first Belgian case of BT in 2007 was suspected in a flock located in Antwerp (Oelegem), subsequently confirmed at the National reference laboratory (CODA-CERVA) and notified on the 17 July 2007. This case was the starting point of a new wave of BT outbreaks in Belgium. In 2007, BTV-8 re-emerged in the Netherlands, Belgium, Germany, Luxembourg and northern France, and was also reported in the United Kingdom, Switzerland, Denmark and the Czech Republic (OIE, 2008b). Farmers suggested that BT induced more severe clinical signs in 2007 than it did the previous year.

The main objective of this study was to describe the evolution and the clinical impact of the 2007 BTV episode in Belgium. In addition, the main differences with the previous epidemic (19 August to 15 December 2006) are reported.

Materials and Methods

Temporal evolution of the incidence

Outbreak data (case data at farm level) was obtained from the Belgian Federal Agency for the Safety of the Food Chain (FASFC) for the 2006 and 2007 episodes. Outbreaks are mostly herds for which the veterinary practitioner, who has been consulted by the animal owner, identified suspicious clinical cases and where at least one of those animals was subsequently confirmed positive using a laboratory test (c-ELISA and/or real-time RT-PCR) and then notified to the veterinary authorities (EFSA, 2007). A maximum of three animals were sampled per suspected herd. Weekly incidence counts of BT outbreak herds were calculated for each species separately based on the dates when cases were reported to the FASFC. Temperature data was obtained from the Royal Meteorological Institute of Belgium (KMI-IRM), and *Culicoides* data from the Institute of Tropical Medicine (ITG) who was coordinating the entomological surveillance. The temporal distribution of the epidemic (incidence curve), the mean weekly temperature data, and the weekly numbers of *Culicoides* trapped during 2007 were analysed.

Spatial evolution of the incidence

Livestock density data was extracted from the Belgian animal identification and registration system (SANITEL) to provide estimates of the population at risk at the start of the 2007 episode. The cumulative incidence (probability that a herd at risk developed BT during the year 2007; based on outbreak data) and 95% confidence intervals were estimated.

Outbreaks' characteristics

The characteristics of the outbreaks were defined by analysing the distributions of the species and the herd types involved. In order to establish the influence of the herd immune status at the start of the episode on the incidence of BT during the episode (i.e. risk of becoming an outbreak), 286 dairy cattle which had been sampled during the 'winter screening 2007' and were found to be BT-seropositive at that time (Méroc et al, 2008), were investigated. A multiple logistic regression model was used to model the probability of a herd to become an outbreak according to its seroprevalence level at the end of the first BT episode, controlling for two other independent variables (herd province and herd size) (Agresti, 2002).The level of significance used was fixed to 0.05.

Rendering plant

In order to obtain information on BT mortality in Belgium in 2007, rendering plant data (Rendac) from 2002 onwards were acquired. Homogeneity in numbers of carcasses for years 2002 to 2005 (i.e. historical data, before emergence of BTV-8 in Northern Europe) was verified using a one-way Analysis of variance (ANOVA; Neter et al., 1996). The cumulative differences (starting from week 28, which is the date of onset of the 2007 episode) of number of carcasses collected by the rendering plant between 2007 and average (2002-2005) for cattle and for sheep and goat were calculated. The average number of carcasses that were collected per week from 2002 to 2005 was calculated and compared to the weekly numbers that were collected in 2006 and 2007, respectively. Multiple pair-wise comparisons between 2007 and average (2002-2005), 2006 and average (2002-2005), and between 2006 and 2007 were performed using t-tests with Bonferroni correction (Bonferroni, 1937).

To study the impact of BT on ruminant mortality, the BT weekly incidence curve for 2007 was compared to rendering plant data for the same time period. The correlation between BT incidence curve and the curve of weekly differences (2007 minus average (2002-2005)) was evaluated using the Pearson correlation coefficient. Statistical significance of the test was based on a p-value equal or lower than 0.05. The date of sampling was chosen in the BT dataset as reference to mimic the true date of disease onset. The analyses were performed for small ruminants and cattle separately. In addition, per ruminant species, different age/weight categories available in the Rendac dataset were considered (for sheep/goats: lambs and adults; for cattle: ≤50 kg calves and adults). The same comparison process was followed for dead cattle foeti, but, in this case, on a wider time scale (i.e. over both 2006 and 2007 calendar years).

All statistical analyses were computed using SAS software, Version 9.1.3.

Results

Temporal evolution of the incidence

Weekly BT incidence counts for each species concerned are shown in figure 1. The first outbreak was reported during week 28 and a peak was reached at week 36-38. We notice that, after having steadily diminished following week 38, the number of new cattle outbreaks has increased again at week 45 and this tendency continued until week 51. Pertaining to sheep population, the incidence curve in 2007 indicated first an exponential trend which reached a peak of 452 new outbreaks notified during week 36. Following this week, the number of new outbreaks reported decreased continually. At the end of the year 2007, a total of 6870 outbreaks were reported throughout the country. Temperature (figure 1) started to decrease slowly from week 35 until week 44 when it increased again a little and then decreased again. The weekly numbers of *Culicoides* (figure 2) which were trapped increased from week 16 until week 40 when the numbers started to sharply diminish.

Figure 1: Weekly incidence counts of reported Bluetongue outbreaks and weekly mean temperatures in Belgium in 2007 (week 23-52).

Figure 2: Weekly incidence counts of reported Bluetongue outbreaks and weekly total numbers of *Culicoides* trapped in Belgium in 2007 (week 14-52)

Spatial evolution of the incidence

Cumulative spatial distribution of confirmed cases with monthly intervals are presented along with farm density data in figure 3a (cattle) and figure 3b (sheep). The first outbreaks reported in 2007 were mainly located in East Flanders, and, until September, the vast majority of cases were reported in this province (for location, see figures 4a and b). The epidemic progressively spread to all provinces and especially towards West Flanders and Hainaut. At the end of the epidemic, BT was widely but unevenly distributed across all Belgian provinces. The disease, in both species, was mainly notified in regions where farm density was high. The vast majority of the outbreaks were located in the western half of Belgium. Indeed, 26.1 and 21.2% of the outbreaks were situated in East and West Flanders respectively, while only 1.4%, 4.9 and 3.7% of the cases in 2007 were located in Walloon Brabant, Liege, and Limburg provinces, where many of the outbreaks were reported in 2006.

Figure 3a: Distribution of Bluetongue 2007 cattle outbreaks and cattle farm density data in Belgium at date a. 1st September 2007, b. 1st October 2007, c. 1st November, d. 1st December 2007

Figure 3b: Distribution of Bluetongue 2007 sheep outbreaks and sheep farm density data in Belgium at date a. 1st September 2007, b. 1st October 2007, c. 1st November, d. 1st December 2007

Overall cumulative incidence at herd level based on confirmed outbreaks was estimated at 11.5 (11.2-11.8) and 7.5 (7.3-7.8) % in cattle and sheep populations, respectively. Province-specific cumulative incidence estimates and their 95% confidence intervals are presented for cattle in figure 4a and for sheep in figure 4b. The incidence in cattle was the highest in the southern part of Belgium. It was found to be the highest in Luxembourg and the lowest in Liege, Limburg and Flemish Brabant. For sheep, the highest estimate was found for East Flanders.

Figure 4: Province-specific BT cumulative incidence estimates (in %) and 95%CI for the year 2007. a. in Belgian cattle population. b. in Belgian sheep population. (based on confirmed cases)

Outbreaks' characteristics

In 2007, cattle outbreaks represented 64.4%, while sheep herds represented 34.9% of the total outbreaks (1% left: goat, bison, lama and deer). Leaving herds with non-identifiable herd-type aside, 55% of the cattle outbreaks were of dairy type. Two hundred and one of the dairy herds that were seropositive during the 'winter screening 2007' suffered clinical outbreaks during the 2007 episode. Controlling for herd size and province, the results of the logistic regression model indicated that a unit increase of within-herd seroprevalence is a significant protecting factor for BT in 2007 (OR=0.96; 95%CI 0.95-0.98; p<0.0001).

Rendering plant

The results of the one-way ANOVA showed no statistical significant difference between the mean numbers of carcasses across years 2002 to 2005 for the two categories of ruminants (p-values >0.05). Thus, homogeneity was assumed in the historical data and the average numbers (2002-2005) were further considered in the analyses as reference number. At the end of the year 2007, cumulative differences in number of carcasses between 2007 and reference value had reached 30693 carcasses in small ruminant population and 17517 carcasses in cattle population. Table 1 shows the results of the analysis made in order to compare mean weekly differences in mortality. The most important difference was observed for adult sheep and goats when comparing 2007 to average (2002-2005), with 554 carcasses per week more in 2007 (p<0.05). An important difference was also seen for adult cattle with 122 carcasses more in 2006 than on average (2002-2005) (p<0.05). Figure 5a and 5b show the differences of number of carcasses between 2007 and average (2002-2005) for different sub-categories for cattle and small ruminant, respectively. For cattle, differences in all sub-categories are positive from week 35 onwards. For small ruminants, the difference curve clearly increased between week 29 and 43. This difference seemed to be attributed almost totally to adult carcasses. However, a small increase for lamb carcasses was also noticed.

Table 1: Differences between mean weekly numbers of carcasses collected yearly by the rendering plant and 95% CI Bonferroni (* indicates statistical significance)

	sheep and goats			cattle	
Year comparison	**lambs**	**adults**	**dead foeti**	**calves(≤50kg)**	**adults**
2007-average(2002-2005)	120 (-1;241)	554 (260;840)*	80 (54;103)*	99 (-135;33)	120 (18;222)*
2006-average(2002-2005)	114 (-6;235)	142 (-144;428)	23 (-3;47)	96 (-138;330)	122 (20;223)*
2007-2006	6 (-114;126)	412 (126;697)*	57 (31;81)*	3 (-232;238)	-1.54 (-104;101)

Figure 5: Weekly differences in number of carcasses collected by the rendering plant between 2007 and average (2002-2005). **a.** cattle, **b.** sheep and goats

Figure 6a and 6b show the BT incidence curves along with the rendering plant data curve (differences between 2007 and average (2002-2005)) for both species. The visual analysis of the cattle curves (Figure 6a) shows mortality differences were positive from week 35 onwards. This positive mortality pattern seemed to follow the increase of the BT incidence curve for cattle. For sheep (Figure 6b), the two curves clearly follow the same trend with a delay of more or less one week between them. Table 2 presents Pearson correlation coefficient estimates and associated p-values showing the correlation between BT incidence and rendering plant data for each species and sub-category within species. The Pearson correlation coefficient was shown to be the highest for adult sheep and goats when shifting back the rendering plant data of one week with r=0.98 (p<0.001). Correlation between BT and mortality was not as good for cattle as it was for sheep and goats. Figure 6c shows the cattle BT incidence curve along with the rendering plant data for dead foeti. For dead foeti, correlation was found to be the highest when shifting back the data of 3 weeks (r=0.57; p<0.0001).

Figure 6: Bluetongue weekly incidence curve and difference in number of carcasses collected by the rendering plant per week between 2007 and average (2002-2005) for cattle in Belgium in 2007.**a.** cattle**. b.** sheep and goats

Table 2: Pearson correlation coefficient estimates and associated p-values showing correlation between Bluetongue incidence and number of carcasses collected by the rendering plant in 2007

time lag (nb week)	sheep and goats		cattle	
	lambs	adults	calves (≤50kg)	adults
-1	0.50(<0.001)	0.57(<0.001)	0.56(<0.001)	0.47(<0.01)
0	0.63(<0.001)	0.83(<0.001)	0.52(<0.001)	0.45(<0.001)
1	0.73(<0.001)	0.98(<0.001)	0.62(<0.001)	0.54(<0.001)
2	0.67(<0.001)	0.83(<0.001)	0.64(<0.001)	0.53(<0.001)

Figure 6c: Bluetongue weekly incidence curve and difference in number of dead cattle foeti collected by the rendering plant per week between 2006/2007 and average (2002-2005) in Belgium, from week 1 2006 to week 52 2007

Discussion

The epidemic in 2007 expanded in such a way that within the two first months, four times more outbreaks were identified than during the entire 2006 episode. The higher overall cumulative incidence estimates in 2007 compared to 2006 may be due to many causes or to a combination of these causes:

- a higher level of infection in the population (linked to the presence of a considerable virus reservoir at the onset of the epidemic and/or to a change in *Culicoides* abundance (Elbers et al., 2007)).
- a more severe disease within individual animals infected (which could be a consequence of a variation in the virus strain and is still to be examined (Maan et al., 2008)).
- a better awareness of the clinical signs by the owners.

The observed provincial differences in cumulative incidence (figure 4a and 4b) could be explained by various risk factors such as the within-herd prevalence level at the end of the 2006-epidemic (via protective immunity). The cumulative incidence in the cattle population (figure 4a) was the highest in the province of Luxembourg where seroprevalence at the end of the 2006-epidemic was the lowest. The lowest estimates were found in Liege, Limburg and Flemish Brabant, where seroprevalence was the highest at the end of 2006 (Méroc, 2008). These results and those of the limited analysis using the results of the 'winter screening 2007' seem to go in favour of a negative association between within-herd prevalence in 2006 and the risk of showing clinical signs of BT in 2007. The preliminary results from a longitudinal study in the Netherlands indicated that animals infected during the 2006-epidemic did not become infected (no PCR positives) again during the 2007-epidemic (Elbers et al., 2007). Another risk factor could be for instance the average size of herds in the province. Luxembourg leads with a mean of 130 cattle /herd, followed by Namur with 105 cattle/herd. We notice that the highest BT incidences in cattle were observed in the provinces of Luxembourg and Namur (figure 4a).

As it was already the case during the first epidemic, BTV-8 appears to cause clinical disease both in sheep and cattle, whereas the clinical form of the disease is commonly known to be

restricted to sheep. The ratio of large to small ruminant farms affected was larger in 2007 compared to 2006, since at the end of the 2006-epidemic, the distribution was 57.3% of sheep outbreaks and 42.7% of cattle outbreaks. From figure 1, it can be observed that the sheep incidence started to diminish two weeks before that of the cattle (week 36 vs. week 38). This apparent delay between incidence curves could be the consequence of cattle not producing many clinical signs except for a loss in milk production, therefore owners often delayed the moment when they would call the veterinarian (De Deken, personal communication). This important impact of BT on milk production in cattle may explain the predominance of dairy type (55%) within reports of BT cattle herds. The latter may also be related to the fact that dairy cattle are more frequently and closely observed by the farmers compared to beef cattle. One notable difference between the two episodes of BT was also the confirmation of goat outbreaks in 2007, whereas no caprine case had been recorded in 2006. This particularity was also reported in the other countries concerned by BTV-8 in 2007 (Dercksen et al., 2007).

Figure 1 shows that the BT incidence curves seem to have globally followed the same trend as the temperature curve, with a delay of 2-4 weeks. The results of a study pertaining to association between temperature and BT incidence during the 2006-BT-epidemic, found the strongest correlation between the 2 parameters when shifting back the BT incidence data of 4 weeks (EFSA, 2007). Nevertheless, even if temperature was clearly demonstrated to influence *Culicoides'* life cycle, BTV replication in the vector and the biting rate of the midges, it is difficult to consider these associations by themselves. Indeed other parameters such as humidity are known to have confounding effects on the association (Mellor, et al., 2000). During the 2006-epidemic, two peaks of notifications were observed end-August and mid-October. However, BT epidemics are commonly known to follow a unimodal temporal distribution (Erasmus, 1985). Normally, as the cold weather intensifies towards the end of autumn, blood feeding of the midges declines. Baldet and Delécolle (2007) have partially explained the unusual pattern of last year's epidemic by unfavourable climatic conditions prevailing in August which could have led to a decrease in the vector population.

Many reproductive disorders in cattle have been reported during the year 2007 in Belgium. Those presumed consequences of BT included infertility, early embryonic deaths, abortions and stillbirths. It has well been demonstrated in the past that strains of BTV which have been modified by the passage in cell culture (e.g. modified live virus vaccine strains) are able to cross the ruminant placenta and consequently have various outcomes according to the timing

of foetal infection (MacLachlan, 2000). Recent laboratory analyses on aborted foeti, newborn calves and dam-newborn pairs provided evidence of the capacity of BTV serotype 8 to pass the placental barrier (De Clercq et al., 2008). The present study indicated an increase in foetal mortality in 2007, as well as a fair correlation between the incidence curve of BT and that of abortions when shifting back the mortality data of 3 weeks (Figure 6c). The association between BT and abortion is however difficult to objectify using this type of study design, since various impacts on embryo/foetus may have occurred according to the gestation stage the dam was infected.

Death may occur in 8-10 days in diseased animals. Infected ruminants either die from BT directly or from a secondary bacterial infection. Mortality usually ranges between 10 and 20 percent, but can reach 70 percent in individual flocks (Breard et al., 2004). The analysis demonstrated a strong and significant correlation between the incidence of BT and adult sheep and goat mortality since the coefficient was close to 1 (table 2). However, this high correlation does not necessarily induce a relation of causality between the two events. Some factors such as the breeds of the infected animals or the presence of concomitant diseases may here interact.

The objective of this paper was to describe the evolution and the clinical impact of the BT 2007 episode. Even though further validation is needed, some conclusions can be drawn:

- Incidence counts were related to the population density of the susceptible hosts. BT was mainly reported in regions where farm density was high.

- The findings went in favour of a negative association between the within-herd seroprevalence level at the end of 2006 and the risk of showing clinical signs in 2007.

- The impact of BTV-8 on mortality was more important in small ruminant than in cattle population.

- A high level of correlation was demonstrated between small ruminant mortality and BT incidence data when shifting back the mortality data of one week. This finding supports the hypothesis that the high increase in small ruminant mortality in 2007 was the consequence of the presence of BT. For cattle, the correlation was not as high.

- An increase in cattle foetal mortality was observed during the year 2007 and a fair correlation was found between BT incidence and foetal mortality.

Acknowledgements

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CHAPTER V

EVALUATION OF THE BLUETONGUE VACCINATION CAMPAIGN

Evaluation of the first cattle vaccination campaign against Bluetongue virus in Belgium

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Abstract

In order to control the devastating impact of Bluetongue in ruminant population, Belgium decided to start using an inactivated vaccine in 2008. The objective of the study was to evaluate the effect of this first vaccination campaign. Three cross-sectional screenings were undertaken in the cattle population during winters of 2007 and 2008 (before vaccination) and 2009 (after vaccination). The results showed that the target of 80% of vaccination coverage was attained but late in the season. The effect of several vaccination factors on the serology and RT-PCR response were analysed using linear and generalized mixed models. The interaction between time since vaccination and the booster injection's achievement was significantly associated to the change in serology after vaccination (β= -32.69; p=0.02). The study demonstrated the importance of respecting the vaccine's protocol in its entirety and in due time, for the level of antibodies depends on it.

Keywords

Bluetongue virus serotype 8; Cattle; c-ELISA; inactivated vaccine; real time RT-PCR

Introduction

Bluetongue (BT) is a vector-borne viral disease of ruminants. BT virus (BTV) is the type species of the genus *Orbivirus* within the *Reoviridae* family. Thus far, 25 distinct BTV-serotypes have been identified (Gorman, 1990; Hofman et al., 2008; Takamatsu et al., 2003). Midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are, until now, the only proven vectors in the transmission of BTV from ruminant to ruminant (Côtre-Sossah et al., 2004; Pili et al., 2006; Vanbinst, et al, 2009). BT can cause mild to spectacular outbreaks and therefore appears on the list of diseases notifiable to the World Organisation for Animal Health (OIE). It thus has an adverse impact on worldwide trade due to restrictions on the source of animals (Green et al., 2005). The vast majority of BT infections are clinically unapparent. Cattle act as a reservoir of BTV and keep the infection circulating. Cattle appear to be much more attractive to *Culicoides* spp. than sheep and this may enhance their importance as carriers (Ward et al., 1994). Sheep on the other hand are more prone to show clinical signs (Gibbs et al., 1994). When the disease does occur, common clinical signs are pyrexia, inflammation of the oral mucosa, excessive salivation, oedema of the head (OIE, 2008).

BTV is considered as an "emerging virus" since it has recently expanded its range towards Northern Europe, outside its previous environment (as far as 45°N). Starting in August 2006 from the area where Belgium, the Netherlands and Germany share borders, an epidemic of BTV serotype 8 (BTV-8) gradually disseminated throughout the North-Western European countries (Toussaint et al., 2007), causing the most severe outbreak of this disease ever recorded. In 2007, BTV-8 re-emerged in the same countries and extended also to the United Kingdom, Switzerland, Denmark and the Czech Republic (OIE, 2008). In order to control the devastating effect of BTV-8, several European Member States decided to start vaccination before the following summer season (Council Directive 2000/75/EC). In the past, BTV vaccination has been successfully used by a number of European countries which have been affected by the infection (Savini et al., 2008). The vaccination campaign intended to reach a target of at least 80% of coverage in order to stop the spread of the infection and ultimately to eradicate the disease. This target was fixed by the European Commission based on past mass vaccination experience for other diseases (P.Houdart, personal communication). In Belgium, compulsory vaccination of cattle and sheep with inactivated vaccine against BTV-8 began in May 2008, after a devastating epidemic starting during summer 2007 (Méroc, 2009). This means that livestock population was already highly immunized before the campaign took

place, making it impossible to distinguish infected from vaccinated animals based on serology. The objective of this study was to explore the effect of livestock vaccination against BTV-8 in Belgium.

Materials and methods

Cross-sectional serological data

In Belgium, since the first episode of BTV-8 (second half of 2006), three cross-sectional serological surveys were undertaken in the same cattle herds during the winter periods (Winter Screenings (WSs) were performed during winter 2006-2007 (=WS1), during winter 2007-2008 (=WS2) and during winter 2008-2009 (=WS3)) (Méroc et al., 2008). Table 1 summarizes the characteristics of the WSs. The main difference between the three samplings relates to the age of the sampled cattle: in WS1, only adult cattle (>24 months of age) were sampled, whereas in WS2 and WS3 all cattle were considered. In WS3, the same number of animals was sampled in each age category (balanced sample), which was not the case in WS2 (unbalanced sample).

Table 1: Summary of the three winter screening (WS) samples

	WS1	WS2	WS3
Sampling period	1st -31 January 2007	1st January- 20 May 2008	15 December 2008- 7 February 2009
Number of animals	25 846	21 498	7 175
Number of herds	344	235	203
Age characteristics	adults (>2 years)	unbalanced stratification, all ages	balanced stratification, all ages

The serum samples were analysed using the 'ID Screen1 Bluetongue Competition' assay (ID VET, Montpellier-FRANCE) according to the manufacturer's instructions but with the cut-off

determined by Vandenbussche et al. (2008) ($\leq$65 positive; >65 and $\leq$75 doubtful; >75 negative). Results were expressed as percentage negativity (PN%= (Optical Density sample/Optical Density negative kit control)*100). Bad quality sampling results were deleted from the final datasets.

The serological results from the WSs were evaluated by first estimating the three within-herd seroprevalences using logistic-normal regression models (SAS Inc, Version 9.2). In order to make these seroprevalences comparable from one to the other, the estimations were restricted to adult animals. The true prevalences, reflecting the real serological status of the animals, were derived by taking the sensitivity (87.8%) and the specificity (98.2%) of the c-elisa test into account (Rogan and Gladen, 1978; Vandenbussche et al., 2008).

Virological data

During WS3, uncoagulated blood samples (EDTA) were collected by the official farm veterinarians and were conditioned at the regional laboratories of 'Dierengezondheidszorg Vlaanderen' and the 'Association Régionale de Santé et d'Identification Animales'. The EDTA samples were analysed with a RT-qPCR as described by Vandenbussche et al. (2008). Based on a cut-off which was set at a Ct-value of 40, results were defined as 'positive' (<40) or 'negative' ($\geq$40).

Vaccination data

During the vaccination campaign, information on the vaccination progress of each livestock farm (date of primo- and booster-vaccination rounds and brand of vaccine used) was recorded by the Belgian Federal Agency for the Safety of the Food Chain (FASFC). These vaccination data concerning the herds sampled within the WSs were extracted and analyzed.

Change in individual serological profiles between WS2 and WS3

In order to evaluate the individual serological profiles between WS2 and WS3, the following variables were created first:
- 'QuantiWS2' and 'QuantiWS3' were defined as the percentage of negativity obtained by subtracting the PN% values at WS2 and WS3 (continuous variables) from 100 in

order to display an increased antibody response when there is truly an increase of antibodies.

- 'Change' (%), a continuous variable obtained by subtracting QuantiWS2 from QuantiWS3, which indicates for a given animal the change in serology between WS2 (before vaccination) and WS3 (during or after vaccination).

- 'StatusWS1', a dichotomous variable indicating whether the animal came from a herd where within-herd seroprevalence in WS1 was above the mean prevalence of 27% or not (Méroc et al., 2008).

- 'Time', a continuous variable reflecting the number of months between the date of first injection at herd level and the sampling date of the individual animal. After consideration of the variable's histogram which clearly showed a bimodal pattern, it was decided to create from it a dichotomous variable determining if 'time' was superior to 3 months or not.

- 'Two injections', a dichotomous variable indicating whether the animal had received its second vaccination (=1) or not (=0) at moment of sampling.

- 'Age' (months), a continuous variable indicating the age of the animal. This was extracted from SANITEL.

The profiles of cattle, strictly seropositive at WS2 (n=2360) were compared to that of animals strictly seronegative at WS2 (n=199). For the latest, the effects of vaccination variables ('Time', 'Two injections' and 'Brand'), 'StatusWS1' and 'Age' on the 'Change' were analysed at animal level. First, univariable associations between the response 'Change' and the different categorical independent variables ('Time', 'Two injections', 'Brand' and 'Status WS1') were explored using one-way ANOVAs. The clustering within the herds was not taken into account in this part of the analysis. Residuals plots were used to ensure that the assumptions underlying the ANOVAs were met. Then, a linear mixed model which takes into account the correlation among animals from a same herd by adding a random herd effect, was used for multivariable modelling (Dohoo, 2003). All pairwise correlations among predictor variables were examined using Pearson and Spearman correlation coefficient in order to identify pairs of variables containing essentially the same information. If the correlation between two variables was above 0.7, only the variable with the smallest p-value at the univariable analysis was kept in the multivariable model. The initial model considering all independent variables and two-way interactions was adjusted by looking for the most

parsimonious model as well as comparing the Akaike Information Criteria (AIC) estimates of all possible fits. The smaller the AIC value, the better a particular model fits (Akaike, 1974). A probability value of less than 0.05 indicated a statistically significant result. To assess the validity of the model, standardised residuals were computed and graphical methods were used; the homoscedasticity assumption was verified by plotting the standardised residuals against the predicted means and a normal probability plot for the residuals was used to examine the normality.

RT-PCR positives

The univariable association between the RT-PCR status and the age of the animal was explored by means of a marginal model, the generalized estimating equations (GEE), which takes into account the clustering within herds (Liang and Zeger, 1986). A dichotomous variable 'seroWS2' was created where 'seroWS2'=1 when on a herd at least one animal remained seronegative at WS2. The effects of the vaccination variables, status2007 and seroWS2 on the RT-PCR status of the herd were analysed using a multivariable logistic regression. The best model was looked for by comparing AIC estimates of all possible models with main effects and their two-way interactions. The Hosmer-Lemeshow goodness-of-fit test was used to assess the fit of the model (Dohoo, 2003). The results for the risk factors, from fitting the final model, were expressed as odds ratios along with their corresponding 95% confidence intervals and probability values. A probability value of less than 0.05 indicated a statistically significant result.

Results

Cross-sectional serological data

The true within-herd seroprevalence in adult cattle increased from 23.8% (95%CI: 20.1%-28.1%) at WS1, to 98.84% (95%CI: 97.63%-99.58%) at WS2 and, eventually, to 100% (95%CI: 99.5%-100%) at WS3.

Vaccination data

The herds sampled during WS3 were all completely vaccinated (two injections) between 6 May 2008 and 16 January 2009. The lag time between primo- and booster injection ranged between 11 and 161 days, with an average of 29 days and a standard deviation of 14. Figure 1 shows the progress in time of the vaccination campaign. At the end of November 2008, 80% of the Belgian cattle herds had started vaccination. Eighty percent of the herds had completed the vaccination process at the end of December 2008.

Figure 1: Cumulative proportion of number of herds vaccinated according to date of **a**. first injection **b**. last injection

Change in individual serological profiles between WS2 and WS3

The individual change in the 'Quanti' value between WS2 and WS3 is presented in figure 2. The serological patterns of animals strictly positive at WS2 (using a cut off level of quantiWS2>35%) is shown in figure 2a. Of those animals, it appears that 88% were already highly seropositive (quantiWS2>80%) at that time and stayed highly positive at WS3 (straight horizontal line). A portion of animals however, were highly positive at WS2 and became less positive at WS3 (quantiWS3<80%) (9% of animals) or even negative (quantiWS3<25%) (5% of animals). Figure 2b shows the same information for animals negative at WS2 (using a cut off level of quantiWS2<25%).

Figure 2: Individual quantitative serological response (quanti) at winterscreening 2008 (WS2) and winterscreening 2009 (WS3). **a.** animals BT-seropositive at WS2. **b.** animals BT-seronegative at WS2**.**

It is observable that quantiWS3 ranges from 0 to 100% with more values around 100%. The quantiWS3 distribution of animals seronegative at WS2 is presented in figure 3. Eighty percent of those animals became seropositive (quantiWS3>25%).

Figure 3: Distribution of quantitative serological response (quantiWS3) of animals BTV-seronegative at winterscreening 2008 (WS2)

From 199 animals we could extract data for which WS2 and WS3 serological responses were available and that were seronegative at quantiWS2 (using a cut off level of quantiWS2<25%). For those animals, quantiWS3 was significantly higher (mean=59; standard deviation=33.33) than quantiWS2 (mean=4.6; standard deviation=6.61) (p<0.0001). Out of these 199 animals, 185 (93%) demonstrated an increase in their serological response between WS2 and WS3. Table 2 shows the distribution of the variable 'Change' among the different levels of the categorical independent variables considered in the multivariable model analysis. The animals with a longer duration between first vaccine injection and sampling ('Time'=1) have a

superior 'Change' on average than the others. In the same way, subjects which had received two vaccine injections at sampling time ('Two injections'=1) had a significantly higher 'Change' than those that received only one vaccination.

Table 2: Description of the increase in serology between winterscreening 2008 (WS2) and winterscreening 2009 (WS3) ('Change') among categories of discrete variables and results of one-way ANOVAs (p-value)

Variable	Type	n	%mean	%sd	p-value
Time	0	72	45.54	36.96	0.03
	1	127	58.29	31.62	
Two injections	0	74	38.74	34.3	<0.0001
	1	125	63.67	30.26	
StatusWS1	0	148	53.27	32.27	0.42
	1	51	57.69	38.6	
Brand	both	52	54.75	30.96	0.83
	brand1	89	55.64	35.27	
	brand2	58	52.18	34.93	

The results of the multivariable model at the animal level are presented in table 3. It appears that after taking into account all independent covariates simultaneously and adjusting for herd effect, the only significant effect was the interaction between 'Time' and 'Two injections'.

Table 3: Results of the linear mixed model modelling the increase in serology between winterscreening 2008 (WS2) and winterscrening 2009 (WS3) ('Change')

Variable	Type	Coefficient	Standard Error	P-value
Age		0.34	0.31	0.27
Time		27.69	10.01	<0.01
Two injections		52.01	11.28	<.0001
StatusWS1		8.05	6.42	0.21
Brand	both	17.11	11.4	0,33
	brand1	25.67	9.11	
	brand2	15.04	10.17	
Time*Two Injections		-32.69	12.31	0.02
Injection*Age		-0.57	0.35	0.11

Table 4 describes the mean 'Change' for each of the patterns formed by combining 'Time' and 'Two Injections'. The results show that by themselves 'Time' (48.64-31.2=17.44%) and 'Two injections' (70.42-31.2=39.22%) make 'Change' increase. However, when those factors are both present, instead of adding the sum of the two main effects (17.44+39.22=57%) to the baseline level of mean 'Change', only 61.53-31.2=30.33% is added. Considering only animals with 'Two injections=1', time between this last injection and sampling was significantly shorter in the group with 'Time=0' (9.5 days) compared to the group with 'Time=1' (51.3 days) (p-value<0.001).

Table 4: Description of mean 'Change' (winterscreening 2008 (WS2) and winterscreening 2009 (WS3)) among different patterns formed by 'Two Injections' and 'Time'

	Two injections=0		Two injections=1		Total	
Time	n	%mean Change	n	%mean Change	n	%mean Change
0	42	31.2	30	70.42	72	47.54
1	32	48.64	95	61.53	127	58.29
Total	74	38.75	125	63.67	199	54.4

RT-PCR positives

Out of 5792 animals tested with RT-PCR, 146 (2.5%) turned out to be positive. In 56 out of 211 herds (26.5%), at least one animal was positive. Mean WS2 seroprevalence in those PCR positive herds (mean of 0.99 (s.d.= 0.02)) was found to be statistically similar to that of the other herds (mean= 0.97(s.d=0.09)) (p=0.17). The results of the GEE model at the animal level indicated that the effect of the animal's age on the RT-PCR response wasn't statistically significant (OR=0.98; p-value=0.46). Table 5 presents the estimated odds ratios from the multiple logistic regression model at the herd level. The results showed that 'StatusWS1', 'Time', 'Two injections' and 'SeroWS2' acted as protective factors on the risk of being RT-PCR positive (OR<1). However, none of these effects were found to be statistically significant, except for 'Time' that was borderline significant.

Table 5: Results of the multivariable logistic regression modelling the risk of a herd having a positive rt-PCR result: estimated odds ratios for independent variables (and 95% confidence interval limits (CL)) and p-values (comparison to reference category for each variable)

Variable	Type	OR	95%CI	P-value
Time		0.6	0.34-1.07	0.08
Two injections		0.66	0.36-1.18	0.16
StatusWS1		0.64	0.33-1.25	0.19
SeroWS2		0.7	0.4-1.24	0.22
Brand	both vs brand1	1.32	0.82-2.18	0.25
	brand2 vs brand1	0.83	0.53-1.29	0.41
	both vs brand2	1.59	0.71-3.55	0.26

Discussion

The results of the three cross sectional studies showed that adult cattle population in Belgium had reached a seroprevalence level of nearly 100% since the end of the second episode of BTV-8 (WS2). Antibodies typically appear within one or two weeks after infection and this response may last for several years (Afshar et al., 1989; Walton and Osburn, 1992). Ward and Carpenter (1996) suggested a duration of immunity of 33 months compatible with prevalence observed in the field. Therefore, in Belgium, we were obviously dealing before the onset of vaccination with a situation where at least some of the host sub-populations had already obtained natural immunity towards BTV-8. However, because of all newborn animals, part of the cattle population will continuously remain susceptible. Another factor which influences the change in within-herd seroprevalence from one WS to the other is cattle movement. For example, importation of animals from a BTV-8 free area into a herd will contribute to make its global serology level decrease.

The objective of vaccinating was four-fold: i) Reduce clinical disease, ii) Stop dissemination of BTV, iii) Facilitate live animal trade and iiii) lead to eradication in the future. An inactivated vaccine was used in Belgium against serotype 8. Compulsory vaccination of all domestic cattle and sheep began in May and had to be completed before 31 December 2008.

Cattle born later than 1 September 2008 and other species of ruminants could also take part to the campaign on a voluntary basis. It was decided to start vaccination before the summer period since the two previous episodes of BTV-8 started in August 2006 and July 2007 (Méroc et al., 2009). The serological profiles of cattle still seronegative before the vaccination campaign was interesting since it turned out that 80% of these animals had seroconverted at WS3 (Figure 3). Considering that only a small number of infections seems to have occurred during 2008 (2.5% of samples positive at RT-PCR), this would bring us to say that the vaccination's target of reaching 80% of coverage was correctly attained. However, pertaining to the first injection of cattle herds, only by end November was 80% coverage attained (figure 1). The whole process was not completed until January 2009. This discrepancy between planning and reality is related to organisation and delays in reception of vaccine stocks linked to the emergency context. We may ask ourselves what is the purpose of legislation allowing vaccination after the high risk period has already largely started and until December 31.

After a second episode of BTV-8 in 2007 devastating in terms of ruminant morbidity and mortality, the first reason for vaccinating massively (i.e. reduce the clinical impact) seems to be justified since only 50 outbreaks were reported in 2008. However, as aforementioned, cattle were obviously already largely immunized before the campaign was launched, so it is quite difficult to determine the role vaccination truly played here.

This study showed that a majority of the cattle was already highly seropositive at WS2 and was still seropositive at WS3. However, in a certain proportion of animals, a decrease in the serologic response was observed between the two years. This could be linked to several factors. First of all, we have to keep in mind that the sensitivity of the c-elisa test is not perfect and so we therefore must be careful in interpreting the quantitative serologic response. A possible explanation could also be the age of the animals at WS2. Young animals, still having colostral antibodies at the time of WS2 have lost them since then. A decrease of the immunity response occurring naturally with time could also be plausible. In either case, an animal becoming truly seronegative at WS3 would probably mean that there was a problem at one point in the vaccination process: either the vaccination wasn't done, or it was incorrectly done, or it didn't work on this particular animal for one reason or another.

Significant univariate associations were found between the individual change in serology on one hand and 'Time' and 'Two Injections' on the other hand. Indeed, the vaccine producers

indicated in their leaflet (European Medicines Agency, 2010) that the inactivated vaccines should be administrated in two separate doses and that the onset of immunity occurs more or less three weeks after administration of the booster injection. These findings pinpoint the importance of respecting the vaccination protocol in its entirety and of vaccinating in due time. Multivariable modelling showed a significant interaction between 'Time' and 'Two Injections'. For those animals that had received the booster injection at sampling time, a longer time since first injection ('Time'=1) was associated to a lower 'Change' (Table 4). This association seems to reflect the decrease of the serologic response after reaching a peak linked to the booster injection, since it was also shown that 'Time' was positively correlated to the time between second injection and sampling. The evaluation of vaccination could only be done on 199 animals as these animals were the only ones still seronegative just before vaccination, while all the others were already seropositive. Another drawback is that vaccination variables were solely available at herd level. Thus, individual variation within herds regarding the risk factors could not be accounted for in the analysis and this may have induced a bias.

Only 2.5% of the samples in this study were PCR positive. First of all, we have to consider the limits of the RT-PCR test itself. Indeed, Vandenbussche et al (2008) found that the specificity of the test used in the present study was of 98.5% (95%CI: 97.1-100.0). It could thus be possible that as much as 1.5% (with a maximum of 2.9%) of the samples were false positives, leaving us with 1% of true positives. Considering the situation where animals were truly positive and knowing that cattle stays RT-PCR positive for a maximum period of more or less 200 days after it has been infected, this would probably mean that the positive animals in this study were infected during the second half of 2008 (during or even after the vaccination campaign). It is important to notice that we have absolutely no information on the status of the animals between WS2 and WS3, and it may well be that they were RT-PCR positive at one point between the two screenings and then became negative before WS3 was undertaken. Thus we have to keep in mind that the number of positives could be underestimated. The use of sentinel herds for instance instead of annual cross-sectional surveys would allow a better estimation of the annual incidence rate of infection.

We could have expected a difference in age between cattle PCR positive and negative, with more animals being younger in the group of positives because less protected (lower seroprevalence and no vaccination in young calves). However, no age difference was detected

between the positive and negative group. The findings of the multivariable study showed that the vaccination and serology variables were protective factors but didn't indicate significant effect of any of these, except for the time since first injection that was borderline significant. Unfortunately, because of a lack of positive cases (2.5% of animals sampled) and no vaccination data at animal level, all risk factors except for the age could not be studied at animal level. Other extrinsic factors such as conservation of the doses, the way administration was performed, or animal health at time of injection were not considered at all in the multivariable analysis and obviously are critical points which should be accounted for.

Conclusion

In conclusion, at first sight the vaccination objectives of reducing clinical symptoms and stopping the dissemination of BTV-8 seem to have been fulfilled as few outbreaks and positive RT-PCR results were detected after the campaign took place in 2008. However, the intrinsic effect of vaccination was difficult to evaluate as there was already a high seroprevalence in cattle at moment of vaccination. The target of 80% of vaccination coverage was well attained but late in the year. This study demonstrated the importance of respecting the vaccination protocol in its entirety and of vaccinating in due time.

Acknowledgements

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CHAPTER VI

DEMONSTRATE FREEDOM OF BLUETONGUE VIRUS

Bluetongue sentinel surveillance program and cross-sectional serological survey in cattle in Belgium in 2010-2011

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Abstract

Bluetongue virus serotype 8 (BTV-8) emerged in Central Western Europe in 2006 causing a large scale epidemic in 2007 that involved several European Union (EU) countries including Belgium. As in several other EU member states, vaccination against BTV-8 with inactivated vaccines was initiated in Belgium in spring 2008 and appeared to be successful. Since 2009, no clinical cases of Bluetongue (BT) have been reported in Belgium and BTV-8 circulation seemed to have completely disappeared by spring 2010. Therefore, a series of repeated cross-sectional surveys, the BT sentinel surveillance program, based on virus detection in blood samples by means of real-time RT-PCR (RT-qPCR) were carried out in dairy cattle from the end of 2010 onwards with the aim to demonstrate the absence of BTV circulation in Belgium. This paper describes the results of the first two sampling rounds of this BT sentinel surveillance program carried out in October-November 2010 and January-February 2011. In addition, the level of BTV-specific maternal antibodies in young non-vaccinated animals was monitored and the level of herd immunity against BTV-8 after 3 consecutive years of compulsory BTV-8 vaccination was measured by ELISA.

During the 1st sampling round of the BT sentinel surveillance program, 15 animals tested positive and 2 animals tested doubtful for BTV RNA by RT-qPCR. During the 2nd round, 17 animals tested positive and 5 animals tested doubtful. The positive/doubtful animals in both rounds were re-sampled 2-4 weeks after the original sampling and then all tested negative by RT-qPCR. These results demonstrate the absence of BTV circulation in Belgium in 2010 at a minimum expected prevalence of 2% and 95% confidence level. The study of the maternal antibodies in non-vaccinated animals showed that by the age of 7 months maternal antibodies against BTV had disappeared in most animals. The BTV seroprevalence at herd level after 3 years of compulsory BTV-8 vaccination was very high (97.4% [95% CI: 96.2-98.2]). The overall true within-herd BTV seroprevalence in 6-24 month old Belgian cattle in early 2011 was estimated at 73.4% (95% CI: 71.3-75.4).

Keywords: Bluetongue virus; Sentinel surveillance; RT-PCR; Seroprevalence; Vaccination; Immunity

Introduction

Bluetongue (BT) is an infectious, non-contagious disease of ruminants caused by Bluetongue virus (BTV), an arbovirus of the genus *Orbivirus* within the *Reoviridae* family (Mertens et al., 2004). The virus can only be transmitted by certain species of *Culicoides* biting midges (Mellor et al., 2000; Tabachnick, 2004). In the past, BTV infection was limited to tropical and subtropical regions around the world between latitudes of approximately 40-50ºN and 35ºS but during the epidemic caused by BTV serotype 8 (BTV-8) in Western Europe in 2006-2007, the virus spread far beyond the previously known northern boundaries for BTV (Toussaint et al., 2006). Up to date, 24 distinct BTV serotypes have been identified, a 25th has been suggested in goats in Switzerland (Hofmann et al., 2008), and a novel BTV isolate from Kuwait has been proposed as BTV-26 (Maan et al., 2011). BTV outbreaks can cause substantial economic losses due to clinical disease especially in sheep (Calistri et al., 2004; Elbers et al., 2008b; Elbers et al., 2009) and, more importantly, due to the restrictions on trade of animals and animal products between BTV infected and non-infected areas (MacLachlan and Osburn, 2006).

In August 2006, BTV-8 unexpectedly emerged in Central Western Europe affecting Belgium, Luxembourg, the Netherlands, Germany and the North of France (Toussaint et al., 2007; Wilson et al., 2007; Elbers et al., 2008a; Méroc et al., 2008). The origin and the route of introduction of BTV-8 into Central Western Europe remain unknown (Mintiens et al., 2008). The virus survived the winter and a large-scale epidemic started in July 2007, this time also involving the United Kingdom, Switzerland, Denmark and the Czech Republic. Due to the size of the epidemic, a BTV-8 vaccination campaign using inactivated vaccines was implemented by the Belgian authorities in spring 2008 (FAFSC, 2008). A clear decline in the number of clinical cases and in virus circulation was observed in 2008, most likely as a result of natural immunity, vaccination and a number of climatic factors (Zientara et al., 2010).

In Belgium, a cross-sectional survey of cattle, the BT winter screening, has been carried out once a year during winter since 2007 in order to assess the spread of BTV-8 infection after each period of vector activity and to assess the level of herd immunity induced either by natural infection, by vaccination, or by a combination of both (Méroc et al., 2008). In addition, spleen samples from aborted bovine foetuses have been tested by BTV real-time RT-PCR (BTV RT-qPCR) since the start of the vaccination campaign in 2008 in order to monitor the level of trans placental infections (Garigliany et al., 2011). All these results

suggested that a low level of virus circulation was still present in 2009. From spring 2010 onwards there seemed to be a complete disappearance of BTV-8 infection (Garigliany et al., 2011). Based on this, it was decided to initiate repeated cross-sectional surveys every 3 months in non-vaccinated dairy cattle from the end of 2010 onwards with the aim to demonstrate the absence of BTV circulation in Belgium and, consequently, to re-gain Belgium's BTV free status in accordance with European Commission (EC) Regulation No 1266/2007 (CEC, 2007). For this purpose, a number of young non-vaccinated animals from selected herds are tested for the presence of BTV RNA by RT-qPCR performed on EDTA blood. The repeated cross-sectional studies can be considered as a sentinel system whereby the selected herds (and not the animals) function as sentinel units. This paper describes the results of the first two sampling rounds of the BT sentinel surveillance program carried out in October-November 2010 and January-February 2011 and the results of the BT winter screening 2011 to assess the serological status of animals and herds after 3 consecutive years of compulsory vaccination against BTV-8 in Belgium

Materials and methods

Repeated cross-sectional studies in non-vaccinated animals: Bluetongue (BT) sentinel surveillance program

- Sampling design

A total of 300 dairy herds, 30 herds in each Belgian province, were selected as sentinel herds for the BT sentinel surveillance program. These herds were randomly selected from the list of 6,600 active Belgian dairy herds that were expected to have a minimum of 15 animals present between 4 and 12 months of age at the start of the sentinel program. The first two sampling rounds in the sentinel surveillance program were carried out in October-November 2010 and January-February 2011. During each sampling round, a target number of 15 non-vaccinated animals between 4 and 12 months of age were sampled in each sentinel herd.

The number of animals to be sampled was calculated to detect an incidence of BTV infection of 2% with 95% confidence in each geographical unit of reference in accordance with EC Regulation No 1266/2007 (CEC, 2007). To facilitate the practical organisation of the

program, the surface area of each Belgian province was assumed to be equal to 2 geographical units of reference of 45 x 45 km or approximately 2,000 km^2 each. The obtained sample size of 150 animals per geographical unit was then multiplied by 1.5 to ensure that enough samples would be obtained. As such the final target sample size was estimated at 4,500 animals.

- Diagnostic methods: RT-qPCR and ELISA

EDTA blood samples were collected by the farm veterinarians and sent to the National Reference Laboratory (NRL) for BTV at the Veterinary and Agrochemical Research Centre (CODA-CERVA, Brussels, Belgium). There, samples were tested for the presence of BTV RNA by means of a non-serotype specific quantitative reverse-transcription PCR assay targeting BTV segment 5 (pan-BTV/S5 RT-qPCR) according to the method described by Vandenbussche et al. in 2010. Test results were classified as follows: Crossing Point values (Cp-values) <40.0 were classified as positive, Cp-values ≥40.0 but <45.0 were classified as doubtful, and Cp-values ≥45.0 were classified as negative. For the cut-off Cp-value of 40, the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of this RT-qPCR assay was estimated at 99.5% (95% Confidence Interval: 99.0-100.0) and 98.5% (95% CI: 97.1-100.0), respectively (Vandenbussche et al., 2008).

All samples that tested positive or doubtful by pan-BTV/S5 RT-qPCR were also tested by serotype-specific RT-qPCR assays for BTV-8 (BTV-8/S2), BTV-1 (BTV-1/S2), BTV-6 (BTV-6/S2) and BTV-11 (BTV-11/S2) since those were the serotypes that have been detected over the past years in Central Western Europe (De Clercq et al., 2009; Maan et al., 2010). The serotype-specific RT-qPCR assays were performed as described by Vandenbussche et al. in 2009. Cut-off Cp-values were identical to the pan-BTV/S5 RT-qPCR.

During both sampling rounds, with the exception of one animal, all animals that tested positive or doubtful by pan-BTV/S5 RT-qPCR were re-sampled 2-4 weeks (mean of 17 days) after the original sampling. These samples were also tested by pan-BTV/S5 RT-qPCR as described above.

In order to monitor the level of maternal antibodies against BTV in young non-vaccinated animals, plasma samples from a random selection of 25% of all sentinel herds sampled during the 1st sampling round were tested for the presence of BTV-specific antibodies by means of a

commercially available competitive ELISA (c-ELISA) (ID Screen[®] Blue Tongue Competition, ID VET, Montpellier, France) performed according to the OIE Manual of Diagnostic Tests (OIE, 2011) and the instructions provided by the manufacturer. Results were expressed as a percentage negativity (PN) compared to the negative kit control and were classified into positive (PN ≤65), doubtful (PN >65 but ≤75) and negative (PN >75) results based on the optimal cut-off point for diagnostic purposes of 65 PN determined by Vandenbussche et al. (2008). All doubtful results were classified as positive in the data analysis. The DSe and DSp of the c-ELISA corresponding with this approach were determined by means of a Receiver Operating Characteristic (ROC) analysis as 92.0% (95% CI: 88.8-94.6) and 95.7% (95% CI: 92.7-97.7), respectively (Vandenbussche et al., 2008).

- Statistical methods

For the study of the decline of maternal antibodies, estimations of the within-herd antibody prevalence (with 95% CI) were made for 3 different age categories: below 6 months of age, 6-8 months and 9-12 months of age. The estimation of the within-herd antibody prevalence was based on a Generalized Estimating Equation (GEE) using the GENMOD procedure in SAS[®] version 9.2 (SAS Institute Inc., Cary, NC, USA) taking into account the possible correlation among animals within the same herd. A logit link function and binomial distribution were assumed. An exchangeable working correlation was assumed. Apparent within-herd antibody prevalence estimates and their 95% CI's were converted into true antibody prevalence estimates by means of the Rogan and Gladen estimator (1978) assuming a sensitivity of 92.0% and a specificity of 95.7% for the c-ELISA.

BT winter screening 2011: herd immunity after 3 years of compulsory vaccination

- Sampling design

A total of 1,100 cattle herds were selected for this cross-sectional serological survey carried out between January and March 2011. The selection consisted of the 300 dairy herds that were already selected for the BT sentinel surveillance program and an additional 800 dairy, beef or mixed cattle herds. The additional herds were randomly selected, stratified by province and proportional to the number of herds present in each province. The sampling frame was provided by the list of all 22,609 active Belgian cattle herds that remained after the

exclusion of veal holdings, herds with less than 10 animals present at the time of selection, and the already selected 300 BT sentinel herds. Within each of the 1,100 herds, blood samples were collected by the farm veterinarians from a target number of 10 animals between 6 and 12 months of age and 10 animals between 12 and 24 months of age. In the BT sentinel herds, it was allowed to sample the same animals for the serological testing and for the virus detection by RT-qPCR.

The sample size for the BT winter screening 2011 was based on the sample size that had been calculated for the winter screening the year before. The screening carried out during the winter of 2009-2010 was primarily designed to demonstrate the absence of BTV circulation in Belgium (minimum expected prevalence of 2%) by means of testing by RT-qPCR. Despite the fact that the objective of the BT winter screening 2011, due to the introduction of the BT sentinel surveillance program, changed to becoming a seroprevalence survey, it was felt appropriate to keep the same sample size. A sample size of 1,100 herds is more than sufficient to make reliable seroprevalence estimations.

- Diagnostic methods

Blood samples were collected by the farm veterinarians and conditioned to serum samples at the regional laboratories of "Dierengezondheidszorg Vlaanderen (DGZ)" and the "Association Régionale de Santé et d'Identification Animales (ARSIA)". Serum samples were tested for the presence of BTV-specific antibodies using a commercially available competitive ELISA (c-ELISA) (ID Screen® Blue Tongue Competition, ID VET, Montpellier, France) performed according to the OIE Manual of Diagnostic Tests (OIE, 2011) and the instructions provided by the manufacturer.

- Statistical methods

The estimation of the within-herd seroprevalence was based on a GEE as described earlier. Estimations of the within-herd seroprevalence were made by Belgian province, by age category (6-12 months and 12-24 months) and by production type. For the latter, herds were classified as dairy herds if they were commercial milk suppliers as defined by their presence in the registers of the Belgian Milk Control Centers (MCC), and were classified as non-dairy herds if they were absent in those registers. Again, all obtained apparent within-herd

seroprevalence estimates were converted into true seroprevalence estimates by means of the Rogan and Gladen estimator (1978).

The estimation of the herd seroprevalence was obtained using a logistic regression (GENMOD procedure, SAS® version 9.2). A herd was considered seropositive if at least one of the sampled animals had a positive or doubtful c-ELISA test result.

Maps were produced using ArcMap® version 3.2.1 (ESRI, Redlands, CA, USA). An interpolation map showing the distribution of the true within-herd seroprevalence across Belgium was produced using the Inverse Distance Weighting (IDW) tool. This deterministic method determines cell values using a linear-weighted combination set of sampling points, in our case the 12 nearest herds. Assigned weights were an inverse function of the distance to these neighboring herds.

Results

BT sentinel surveillance program

- Results of RT-qPCR

During the 1st sampling round in October-November 2010, a total of 3,684 animals from 264 BT sentinel herds were sampled. An average of 14 animals (range between 3 and 15) were sampled in each herd. During the 2nd sampling round in January-February 2011, a total of 2,150 animals from 202 sentinel herds were sampled. An average of 11 animals (range between 1 and 15) were sampled per herd. Out of the total of 283 sentinel herds that have been sampled in the BT sentinel program up to now, 182 herds were sampled during both sampling rounds, 81 herds were sampled in October-November 2010 only and 20 herds were sampled in January-February 2011 only.

During the 1st sampling round, 15 animals tested positive and 2 animals tested doubtful by pan-BTV RT-qPCR (Table 1). These 17 animals originated from 5 different herds (Figure 1a) located in 4 different Belgian provinces. All RT-qPCR positive/doubtful animals were born in the herd in which they were sampled and their age ranged between 3 and 11 months with a mean of 8 months. Out of the 15 pan-BTV positive samples, 10 tested positive, 3 tested doubtful and 2 tested negative by BTV-8 specific RT-qPCR. Both samples that tested

doubtful by pan-BTV RT-qPCR tested negative by BTV-8 specific RT-qPCR (Table 1). Results of the BTV-1, BTV-6 and BTV-11 specific RT-qPCR tests were all negative. The serological status of the 17 animals that tested positive/doubtful by pan-BTV RT-qPCR was verified by means of the c-ELISA performed on plasma. All animals were seronegative for BTV. When re-sampled 2-4 weeks after the original sample collection, all 17 animals tested negative by pan-BTV RT-qPCR.

Table 1: Results of pan-BTV/S5 and BTV-8/S2 RT-qPCR during the 1st and 2nd sampling rounds of the BT sentinel surveillance program in Belgium (2010-2011)

	Pan-BTV/S5 RT-qPCR			BTV-8/S2 RT-qPCR		
	Number of animals tested	Number of animals positive	Number of animals doubtful	Number of animals tested [a]	Number of animals positive	Number of animals doubtful
1st sampling round (Oct-Nov 2010)	3,684	15	2	17	10	3
2nd sampling round (Jan-Feb 2011)	2,150	17	5	22	13	2

[a] Only samples that tested positive or doubtful by pan-BTV/S5 RT-qPCR were tested by BTV-8/S2 RT-qPCR.

Figure 1: Cp-values for all animals that tested positive or doubtful by pan-BTV/S5 RT-qPCR, organised by herd, during the 1st round (Figure 1a) and 2nd round (Figure 1b) of the Bluetongue sentinel surveillance program in Belgium in 2010-2011. Different animals within the same herd are displayed in different colors

During the 2nd sampling round, 17 animals tested positive and 5 animals tested doubtful by pan-BTV RT-qPCR (Table 1). These 22 animals originated from 12 different herds (Figure 1b) located in 7 different provinces. The 12 herds were different from the 5 herds with positive/doubtful results during the 1st round. During the 2nd round also all animals that tested positive or doubtful by pan-BTV RT-qPCR were born in the herd in which they were sampled. Their age ranged between 3 and 13 months with a mean of 7 months. Out of the 17 pan-BTV RT-qPCR positive samples, 11 were confirmed to be BTV-8 positive, 1 tested doubtful and 5 tested negative by BTV-8 serotype specific RT-qPCR. Out of the 5 pan-BTV RT-qPCR doubtful samples, 2 tested positive, 1 tested doubtful and 2 tested negative by BTV-8 serotype specific RT-qPCR (Table 1). Results of the BTV-1, BTV-6 and BTV-11 specific RT-qPCR assays were again all negative. A verification of the serological status was not possible for 8 out of the 22 animals with positive/doubtful pan-BTV RT-qPCR results as not enough plasma was left for performing the c-ELISA. One 4-month-old animal tested positive for antibodies against BTV. The remaining 13 animals were BTV seronegative. When re-sampled 2-4 weeks after the original sample collection, all animals, with the exception of 1 animal that was not re-sampled, tested negative by pan-BTV RT-qPCR.

- Maternal antibodies in young animals

In order to monitor the level of BTV-specific antibodies in young non-vaccinated animals during the sentinel program, a total of 1,008 plasma samples from 68 randomly selected herds in the 1st sampling round were tested by c-ELISA. True within-herd antibody prevalence estimates for animals below 6 months of age, 6-8 month old and 9-12 month old animals are shown in Table 2. Figure 2 shows the quantitative results of the c-ELISA in function of age. This figure clearly shows that by the age of 7 months maternal antibodies against BTV had disappeared in most animals.

Table 2: Bluetongue true within-herd antibody prevalence (%) in young non-vaccinated cattle during the 1st round of the Bluetongue sentinel surveillance program in Belgium in 2010

Age category (median age)	Number of animals tested	Within-herd antibody prevalence (%)	95% Confidence Interval
< 6 months (5 months)	193	21.8	13.3-32.8
6-8 months (7 months)	470	1.0	0.0-4.9
9-12 months (10 months)	274	3.3	0.0-9.1

Figure 2: Quantitative results of BTV antibody ELISA (c-ELISA) in function of age for a random selection of non-vaccinated animals during the 1st **526** round of the Bluetongue **527** sentinel surveillance program in Belgium in 2010-2011

BT winter screening 2011: herd immunity after 3 years of compulsory vaccination

A total of 16,616 animals from 1,030 herds (292 BT sentinel herds and 738 additional herds) were sampled for the yearly cross-sectional serological survey. An average of 16 animals (range between 1 and 25) were sampled per herd. Among all animals, 10,823 (65.1%) tested positive and 557 (3.4%) tested doubtful for antibodies against BTV. The overall true within-herd seroprevalence in 6-24 month old Belgian cattle and within-herd seroprevalence estimates per production type are shown in Table 3. Figure 3 presents within-herd seroprevalence estimates for each Belgian province. Higher seroprevalence estimates were found in the north than in the south of the country. The within-herd seroprevalence was highest in the provinces of West Flanders, East Flanders and Antwerp. The spatial distribution of the true within-herd seroprevalence in Belgium is presented in Figure 4. The within-herd seroprevalence in the sampled herds ranged between 0 and 100%. Approximately half the herds sampled (54.5%) had a true within-herd seroprevalence equal to or above 80% (Figure 5). The true within-herd seroprevalence was higher in the 12-24 month old animals (79.5%, 95% CI: 77.4-81.4) than in the 6-12 month old animals (66.3%, 95% CI: 63.4-69.1).

Table 3: Bluetongue overall true within-herd seroprevalence (%) and within-herd seroprevalence (%) per production type in 6-24 month old Belgian cattle during the winter screening 2011

Type of herd	Number of herds tested	Within-herd seroprevalence (%)	95% Confidence Interval
Dairy	612	73.2	70.5-75.8
Non-dairy	418	73.7	70.3-77.0
Total	1,030	73.4	71.3-75.4

Figure 3: Bluetongue within-herd seroprevalence (%) and associated 95% Confidence
Interval at the provincial level in Belgian cattle based on the winter screening 2011

Figure 4: Distribution of Bluetongue within-herd seroprevalence (%) in Belgian cattle based on the winter screening 2011

Figure 5: Histogram of Bluetongue within-herd seroprevalence (%) in Belgian cattle based on the winter screening 2011

The overall BTV seroprevalence at herd level in Belgium was estimated at 97.4% (95% CI: 96.2-98.2). The herd seroprevalence was very high in each province and ranged between 94.6% (95% CI: 88.5-97.6) in Liege and 99.4% (95% CI: 96.1-99.9%) in West Flanders.

Discussion

This paper describes the results of the first two sampling rounds of a series of repeated cross-sectional studies, the BT sentinel surveillance program, in Belgium carried out in October-November 2010 and January-February 2011, and the results of a large cross-sectional serological survey for BTV, the BT winter screening, performed between January and March 2011 in 6-24 month old Belgian cattle.

EC Regulation No 1266/2007 (CEC, 2007) last amended in July 2011 prescribes that a BT sentinel monitoring program has to be implemented by all EU member states in BTV restricted zones. Rather than providing fixed guidelines, this regulation is oriented towards setting the minimum requirements for BT monitoring and surveillance, leaving a certain degree of flexibility with each member state to design its own monitoring and surveillance system in order to meet the objectives. In Belgium, repeated cross-sectional studies were initiated from the end of 2010 onwards with the aim to demonstrate the absence of BTV circulation in Belgium. Due to the wide spread of the BTV-8 infection in Belgium during the epidemic in 2007 (Méroc et al., 2009) and due to the introduction of compulsory vaccination of cattle and sheep against BTV-8 in 2008, a serological testing was of no longer use to monitor the level of BTV infection. As BTV-8 RNA can be detected by RT-PCR in the blood of infected cattle for several months after infection (MacLachlan, 2004; Di Gialleonardo et al., 2011), RT-PCR was believed to be a valid method for the monitoring of BTV circulation. Because of its high diagnostic sensitivity (99.5%) and specificity (98.5%) (Vandenbussche et al., 2008) the pan-BTV/S5 RT-qPCR assay was selected as the diagnostic test to be used. It has been shown that vaccination, even with inactivated vaccines, may interfere with the results of RT-qPCR performed on blood (Steinrigl et al., 2010). It was therefore decided to only sample young non-vaccinated animals between 4 and 12 months of age in selected dairy herds due to the all-year round availability of young calves in those herds and because of the easier sampling logistics compared to beef herds. Through these repeated cross-sectional

surveys, a practical and relatively cheap "sentinel" system for BTV was installed in Belgium to meet the objectives of regulation No 1266/2007. Belgian farmers and veterinarians appreciated this way of monitoring more than the "classical" sentinel system for which the same animals need to be sampled at each sampling occasion. The latter causes serious practical problems and hampers trade.

During the 1st sampling round in October-November 2010, significantly more herds and animals (3,684 samples, 264 herds) were sampled than during the 2nd round in January-February 2011 (2,150 samples, 202 herds). This can be explained by the fact that the compulsory BTV-8 vaccination is often carried out during the winter months November and December while the animals are housed, resulting in few unvaccinated young animals being left early in the year compared to the months October-November. Still, the sample size obtained in both sampling rounds was sufficiently large to draw reliable conclusions with respect to freedom of disease. Considering that BTV-8 RNA can be detected in the blood of calves for at least 150 days following infection (Di Gialleonardo et al., 2011) and the main activity of the vector was demonstrated to occur in Belgium between August and November (De Deken et al., 2008), the samplings performed in October-November 2010 and January-February 2011 allow us to draw conclusions regarding the occurrence of any possible BTV circulation during the whole year 2010.

During the 1st sampling round, 15 animals tested positive and 2 animals tested doubtful by pan-BTV/S5 RT-qPCR. During the 2nd sampling round, 17 animals tested positive and 5 animals tested doubtful. It was remarkable that BTV RNA could not be detected anymore in any of these animals when they were re-sampled 2-4 weeks later. BTV RNA can be detected by RT-PCR in blood much beyond the period during which infectious virus can be isolated (MacLachlan, 2004; Di Gialleonardo et al., 2011). Therefore, we believe it is very unlikely that these animals would have been infected with BTV. Moreover, a verification of the serological status of several of the animals at the first sampling showed that they were BTV seronegative with the exception of one animal. Although it is possible for an infected animal to test seronegative if the animal is sampled shortly after infection before seroconversion has occurred, it is very unlikely that this would have been the case for all these animals. One 4-month-old animal tested positive for BTV antibodies but seen the young age of this animal, these antibodies may well be of maternal origin. Indeed, our random selection of animals tested for antibodies during the 1st sampling round of the sentinel program included 80 calves

at the age of 4 months. Thirty-six (44%) of these calves tested BTV antibody positive (Fig. 2). By the age of 7 months maternal antibodies had disappeared in almost all animals. To facilitate the interpretation of the RT-qPCR results in the next sampling round, the age of the animals to be sampled will be increased to 8-14 months and an additional clotted blood sample for verification of the serological status by means of the c-ELISA performed on serum will be collected.

When testing large numbers of negative animals (from an infection free population with or without a history of BTV infection) it is considering the characteristics of the used RT-qPCR assay completely acceptable to observe some positive test results. Therefore, it is recommended to re-sample these positive animals a few days or weeks later as we did in our study. A more strict recording and verification of the BTV-8 vaccination status at individual animal is also recommended and is crucial to allow a correct interpretation of the RT-qPCR results. Based on the results generated in the sentinel program so far, we conclude that there was no BTV circulation in Belgium in 2010 (minimum expected prevalence of 2%, 95% confidence level). This conclusion is further supported by the fact that no clinical cases of BT were found in 2010 and the fact that all spleen samples from aborted foetuses tested by RT-qPCR after mid-April 2010 were negative (Garigliany et al., 2011). The BT sentinel program will continue in 2011 and 2012 to demonstrate the absence of BTV circulation in 2011 and to consequently re-gain Belgium's BTV free status.

The cross-sectional serological survey performed between January and March 2011 showed a true BTV within-herd seroprevalence of 73.4% (95% CI: 71.3-75.4) in 6-24 month old animals. This is below the 80% vaccination coverage target that was set at the start of the vaccination campaign (CEC, 2008). In fact, only half of the herds sampled reached this 80% target. It has to be kept in mind though that our survey was carried out in 6-24 month old animals and that some of the youngest animals may not have been vaccinated yet or may have been vaccinated in the face of maternal antibodies, resulting in the absence of a humoral immune response post-vaccination (Vitour et al., 2011). Moreover, no animals above 24 months of age, which are all expected to be seropositive due to natural infection or the receipt of at least one booster vaccination, were included in our survey. Therefore, the true within-herd seroprevalence of the entire Belgian cattle population is expected to be higher than the 73.4% estimated in our study. It was noticed that the within-herd seroprevalence in 2011 decreased compared to the winter screening in 2009-2010 (true within-herd prevalence of

82.4%, 95% CI: 80.4-84.3; unpublished data). This may indicate that the motivation of farmers or veterinarians to vaccinate against BTV-8 has somewhat decreased. Indeed, a questionnaire performed in 2010 showed that only 48% of the farmers would continue to vaccinate their cattle against BTV-8 if vaccination was no longer compulsory (unpublished data). From the 1st of January 2011, the compulsory vaccination against BTV-8 is discontinued in Belgium. A cross-sectional serological survey carried out in 2012 or 2013 would be very useful to monitor the decline of the vaccine-induced population immunity against BTV-8.

Conclusion

The results of this study demonstrate the absence of BTV circulation in Belgium in 2010. The BTV seroprevalence at herd level after 3 years of compulsory BTV-8 vaccination was very high (97.4%). The overall true within-herd BTV seroprevalence in 6-24 month old cattle in Belgium in early 2011 was estimated at 73.4%. Maternal antibodies against BTV had disappeared by the age of 7 months.

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CHAPTER VII

EMERGENCE OF SCHMALLENBERG VIRUS IN BELGIUM

Distribution of Schmallenberg virus and seroprevalence in Belgian sheep and goats

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Abstract

A serological survey to detect Schmallenberg virus (SBV)-specific antibodies by ELISA was organised in the Belgian sheep population to study the seroprevalence at the end of the epidemic. One thousand eighty two sheep samples which were collected from 83 herds all over Belgium between November 2011 and April 2012 were tested. The overall within-herd seroprevalence and the intra-class correlation coefficient (ICC) were estimated at 84.31% (95%CI: 84.19-84.43) and 0.34, respectively. The overall between-herd seroprevalence was 98.03% (95%CI: 97.86-98.18). A spatial cluster analysis identified a cluster of six farms with significantly lower within-herd seroprevalence in the south of Belgium compared to the rest of the population (p=0.04). It was shown that seroprevalence was associated to flock density and that the latter explained the presence of the spatial cluster. Additionally, 142 goat samples from 8 different herds were tested for SBV-specific antibodies. The within-herd seroprevalence in goats was estimated at 40.68% (95%CI: 23.57-60.4%). The results of the current study provided evidence that almost every Belgian sheep herd has been in contact with SBV during 2011 and should be taken into consideration as part of comprehensive SBV surveillance and control strategies.

Keywords

Schmallenberg virus; Seroprevalence; Spatial cluster; ELISA; Sheep

Introduction

At the end of summer 2011, an unidentified disease appeared in adult cattle in several farms located in Germany and the Netherlands (19 November 2011, Promed). The animals showed non-specific acute symptoms (milk drop, watery diarrhea and hyperthermia (>40°C)) and recovered after a few days. The Friedrich Loeffler Institute (FLI, Germany) conducted a metagenomic analysis in cattle blood samples and identified a new virus phylogenetically closely related to viruses of the Simbu serogroup of the genus *Orthobunyavirus* of the family *Bunyaviridae* (Hoffmann et al., 2012). This virus was shown to be responsible for the abovementioned symptoms and was named after the place in Germany it was first identified: the Schmallenberg virus (SBV) (Hoffmann et al., 2012; Muskens et al., 2012). Several viruses from the Simbu serogroup such as Shamonda, Akabane and Aino viruses are known to be teratogenic for ruminants when infected during a vulnerable period during gestation and may lead to abortion or congenital malformation (Yanase et al., 2012). In line with this, it was observed that starting from December 2011, cattle, sheep and goats in Germany and the Netherlands suffered from abortions and stillborn malformed offspring with signs including hydranencephaly and arthrogryposis. Real-time reverse transcription PCR (RT-qPCR) was made available by the FLI and was used to analyze SBV suspected animals (Hoffmann et al., 2012). Thus far, most SBV cases have been confirmed in Belgium, France, Germany, the Netherlands and the United Kingdom, and more sporadic cases were confirmed in Italy, Luxembourg, Spain, Switzerland, Denmark and Sweden (EFSA, 2012). In Belgium, the first reports of malformation in newborn lambs occurred mid-December 2011 and were confirmed by RT-qPCR in a farm from the North of the country located near the Dutch border (23 December 2011, Promed). At the end of August 2012, SBV had been detected by RT-qPCR in 407 cattle holdings, 167 sheep holdings and 2 goat holdings in Belgium.

The viruses from the Simbu serogroup are all arthropod-borne viruses mainly transmitted between animals via *Culicoides* spp. and *Culex* mosquitoes (WHO, 1961; Takahashi et al., 1968; Yanase et al., 2005). The detection of SBV in pools of heads of *Culicoides obsoletus sensu stricto* and *Culicoides dewulfi* captured in Belgium during the period September-October 2011 (11 March 2012, Promed; De Regge et al., 2012) suggests a role of *Culicoides* in the transmission of SBV. So far, only transplacental and arthropod-borne transmissions have been described for SBV and there is no evidence that the virus could be transmitted horizontally (EFSA, 2012). Considering that SBV seems to be spread by *Culicoides*, it can be

assumed that in Northern Europe it will be seasonally dependent and the important question at the moment is to know if a new wave of viral circulation could be expected in 2012 during the period of activity of *Culicoides*. Knowledge about the immunological anti-SBV status in the host population that has been acquired during the first circulation period in 2011 is needed to address this question since it could largely influence the outcome of a possible new appearance of the virus. Seroprevalence surveys in affected countries are therefore essential to estimate the real impact of SBV infection in 2011 and help to predict the potential further spread of the disease. In this context, a serological survey was conducted in the Belgian small ruminant population to investigate the situation at the end of the first wave of the SBV epidemics.

Material and Methods

Sampling design

The SBV sampling frame consisted of serum samples initially collected from sheep and goats in the context of the Maedi-Visna and Caprine Arthritis and Encephalitis programme for trade certification (Royal Decree 24-03-1993). This programme works on a voluntary basis. After applying for the accreditation scheme, two samplings are carried out in the flock at a time interval of six to twelve months. All animals older than one year are sampled. All samples collected between 4 November 2011 and 4 April 2012 were included in the current study.

Diagnostic methods

Blood samples were collected by farm veterinarians. Blood samples were conditioned and serum was collected at the regional laboratories in Belgium: 'Dierengezondheidszorg Vlaanderen' and the 'Association Régionale de Santé et d'Identification Animales'. The serum samples were subsequently sent to the Veterinary and Agrochemical Research Centre (CODA-CERVA) where they were tested for the presence of SBV-specific antibodies using a commercially available ELISA kit (ID Screen® Schmallenberg virus Indirect ELISA kit, Montpellier, France). The validation of the test conducted by the manufacturer, using serum samples originating from France and Germany, demonstrated a relative sensitivity of 96.47% (95%CI: 93.43%-98.13%) and specificity of 99.75% (95%CI: 99.26%-99.92%) (IDVET,

2012). Results were expressed as S/P percentage using the optical densities (OD) from the ELISA reader (S/P % = OD$_{sample}$/OD$_{positive\ control}$ x 100). A cut-off prescribed by the manufacturer was used to assign the samples into a category (positive, negative, doubtful). Samples which presented an S/P lower or equal to 60%, between 60 and 70 per cent, and greater than 70 per cent were respectively considered as negative, doubtful and positive. In this study the doubtful results were considered as positive in the data analysis.

Seroprevalence

The sampling design of the study implied a hierarchical structure of the data, with animals typically clustered within flocks. Therefore, a marginal model, the generalized estimating equations (GEE) (Liang and Zeger, 1986), which takes into account the resulting correlation among animals, was used to estimate the within-herd seroprevalence with 95 per cent confidence intervals. In the current study, an exchangeable working correlation was assumed. The xtgee procedure in STATA® software version 10.0 was applied to fit the model. The within-herd seroprevalence estimates of the positive sheep flocks were plotted as a density-scale histogram.

A generalized linear mixed model with normally distributed random intercepts for each flock u_i~*Normal* $(0, \sigma^2)$ (Molenberghs and Verbeke, 2005) was then used to establish the correlation between the infection status of two animals within a flock. The gllamm procedure in STATA® was applied to estimate the Intraclass Correlation Coefficient (ICC):

$$ICC = \frac{\sigma^2}{\sigma^2 + \pi^2/3}$$

Between-herd seroprevalence (probability that a flock was infected) was estimated using a logistic regression model with the logit function in STATA®. For the purpose of this study, a flock was considered as positive if at least one of the animals sampled was positive, otherwise it was considered negative.

Design-effect was taken into account by weighting each observation by the inverse of the sampling probability (number of flocks sampled per province/number of flocks per province and number of animals sampled per flock/number of animals per flock).

Spatial analyses

In order to predict within-herd seroprevalence for unmeasured locations in Belgium, an interpolation from the data points was performed by the method of Inverse Distance Weighting based on the 6 nearest neighbours (Shepard, 1968; ESRI, 1996). A map showing the distribution of within-herd seroprevalence estimates was produced using ArcGIS, version 9.3.1 (ESRI).

A purely spatial weighted normal model was used to scan for clusters of sampled flocks with either high or low levels of within-herd seroprevalence. In short, this is done by using a variable circular window size and noting the number of observed and expected observations inside the window at each location. The model tests the null hypothesis that seroprevalence is homogeneously distributed among the flocks (no clusters of flocks with unusually high or low seroprevalence). The method uses a likelihood ratio test to identify clusters. To test the significance of this likelihood, 1000 Monte Carlo simulations were performed to obtain its distribution and clusters with p-value<0.05 were considered as statistically significant. Flock density by municipality was calculated using census data and farm X and Y coordinates extracted from the central identification and registration system of the Belgian Federal Agency for the Safety of the Food Chain (SANITEL). Subsequently we conducted the spatial analysis adjusting for flock density to investigate for the existence of clusters after taking into account this potentially explanatory variable. In order to do so, the residuals obtained after fitting a univariable linear regression model with flock density as covariate were used as observed values instead as the original within-herd prevalence estimates. In addition, the Spearman's rank correlation coefficient was estimated in order to investigate the correlation between within-herd seroprevalence and flock density. All calculations were done using SaTScan 8.2.1. (Kulldorff, 1997) and STATA® software version 10.0.

Results

Seroprevalence

A total of 1082 sheep from 83 flocks were sampled between 4 November 2011 and 4 April 2012. The number of sampled animals per flock ranged from 2 to 110 (median=8). The

geographical localisation of the sampled flocks is shown in figure 1. The within-herd seroprevalence and the ICC in sheep were estimated at 84.31% (95%CI: 84.19-84.43) and 0.34, respectively. In the seropositive flocks (at least one animal detected seropositive), the predicted value for within-herd seroprevalence ranged from 38.6% to 96.71% (median=89.28%) (figure 2). The between-herd seroprevalence in sheep was 98.03% (95%CI: 97.86-98.18).

In addition, 142 goats from 8 flocks were also sampled during the same period (figure 1). The within-herd seroprevalence was estimated at 40.68% (95%CI: 23.57-60.4%) for goats.

Figure 1: Sheep and goat flocks sampled for Schmallenberg virus (SBV) in Belgium between 4 November 2011 and 4 April 2012 and flock density (sheep and goat) at the municipality level

Figure 2: Density-scale histogram of within-herd seroprevalence estimates (%) in Belgian sheep flocks positive to Schmallenberg virus (SBV)

Spatial analyses

Figure 3 illustrates the geographical distribution of within-herd seroprevalence with interpolated predictions for unmeasured points. The prevalence is globally high (80-90%) and evenly distributed across the country. The regions where seroprevalence was the highest were those located in the North-West of Belgium. The levels of within-herd seroprevalence were low in the South-Eastern part of the country and a significant spatial cluster of six farms was identified in that region. The mean seroprevalence in this cluster was 59.5% and outside the cluster 84.87% (p=0.04) (figure 3). Another spatial cluster of 32 farms in the Western part of Belgium had on the other hand a higher mean seroprevalence observed (87.94%) than expected (79.86%), but was not significant (p=0.08). Ovine flock density at the municipality level ranges from 0 to 8 flocks/km². The spatial cluster scanning was conducted again, taking flock density into account, and no significant cluster was detected. A statistically significant

Spearman's rho of 0.25 (p=0.02) was obtained, indicating a moderate positive association between within-herd seroprevalence and flock density.

Figure 3: Geographical distribution of Schmallenberg virus (SBV) within-herd seroprevalence in Belgian sheep flocks and spatial cluster for low within-herd seroprevalence

Discussion

The results of this survey demonstrated that the SBV seroprevalence in sheep was extremely high in winter 2011-2012. SBV seems to have circulated all over Belgium during the 2011 vector season since the between-herd seroprevalence was almost 100%. The interpolation of within-herd seroprevalence estimates showed a high level of infection all over the territory. Within the positive flocks, seroprevalence ranged from 38.6% to 96.71%, but the great majority of the flocks (70%) had a seroprevalence above 80%. An ICC of 0.34 was found,

indicating that the correlation between two animals within a flock with respect to SBV result was high. The ICC of a livestock infectious disease is usually less than 0.2 and ranges from 0.04 to 0.42 (Otte et Gumm, 1997). From a screening among Dutch dairy cattle by virus neutralization, a seroprevalence on animal level of 72.5% was found (Elbers et al., 2012). The first preliminary results of studies from other affected countries also showed a high seroprevalence with results from Germany and France at animal level ranging from 61 to 100% in cattle (EFSA, 2012).

The regions where seroprevalence was the highest were those located in the North-East of Belgium corresponding to areas with high sheep density. Indeed, the results of this study demonstrated that the geographical pattern of seroprevalence was dependent on that factor. The existence of a spatial cluster with low seroprevalence seems to be linked to the fact that flock density in this part of the country is very low and may result in a smaller virus reservoir in the environment and consequently a weaker risk to disseminate the infectious disease by the vector, compared with high density regions.It should be noticed that the predicted seroprevalence in the South of Belgium result from an interpolation of sparser data compared to other areas, and this will eventually have an impact on the confidence intervals of the predictions.

The sampling was done on a voluntary basis and the interested owners were those who practise trade. Therefore, the flocks which were sampled may have had some particular features in their management, size, etc. This type of sampling obviously presents opportunity risk of selection bias which must be accounted for when considering the results of the study. It seems also important to mention that sample size in the screening was limited to available samples and that a lack in number of observations will inevitably lead to less precise estimations. However, pertaining to sheep, the number of animals sampled per flock seems reasonable given that the median size of Belgian flocks is 4 and that a median number of 8 animals were sampled. At the flock level, expecting a high between-herd seroprevalence (90-95%), a sample size of 73-139 flocks would be required to reach a desired confidence level of 95% and accepted error of 5%. Thus, the 1082 sheep from 83 flocks analysed in this study seem suitable to give reasonable estimates of seroprevalence in Belgium. Moreover, representativeness is obtained as the sampled flocks were well distributed within the 10 Belgian provinces and the sampling probability of each animal and farm was taken into account in the analysis by weighting observed values.

The current screening was not able to give very precise estimates for the goat population, as pointed out by the considerably large confidence interval, but gives an indication that the infection level in this species might be lower than it is in sheep and cattle. This would mean that a considerable portion of the goat population remains susceptible and may be sufficient to maintain the infection in Belgium during the following transmission season.

Our results show that there is a high discrepancy between the number of flocks in which the presence of SBV was confirmed by rRT-PCR (167 (0.6% of the total flock population in Belgium)) and the number of flocks that have been infected with the virus based on the serological results. The first factor to explain this difference could be underreporting. This can be expected when dealing with a new virus for which farmers and animal health professionals are completely unfamiliar. Moreover, SBV is not a notifiable disease in Belgium and there is no obligation to report infections to the authorities. The second factor is related to the absence of symptoms in adult sheep. Thus far, the disease has only been observed in lambs after vertical transmission. Indeed, if the ewe was contaminated before or after the vulnerable period during gestation, the infection would have passed unnoticed. The exact vulnerable period in sheep is currently being investigated but in reference to other viruses of the Simbu serogroup, should last more or less three weeks (Hashinguchi et al., 1979; Steukers et al., 2012). Finally, a third factor is the absence of virus in some lambs despite clinical suspicion indicating a possible clearance of the virus before birth.

It is interesting to note that despite a shorter viremia (Hoffmann et al., 2012), SBV has spread much faster than Bluetongue virus serotype 8 (BTV-8) did after its emergence in the same region five years ago (Elbers et al., 2008; Méroc et al., 2008). Several possibilities can be suggested to try and explain this difference of dissemination between the two *Culicoides*-transmitted viruses: 1. additional routes of transmission exist for SBV, 2. the host range of SBV is not limited to ruminants, 3. the infectious dose and the competence of *Culicoides* for transmitting the two viruses are not similar (Kirkland, 2012), 4. SBV was already present in Belgium prior to 2011 and 5. no legal measures such as live animal standstill were taken for SBV as it was the case for BTV-8. Although some of those hypotheses have already been investigated, more research is still necessary to elucidate clearly which of those should be retained.

In conclusion, the results of the current study provided evidence that almost every Belgian sheep herd has been at one point in contact with SBV. Thus, if we extrapolate from other viruses from the Simbu group like the Akabane virus which is the most well-known species of the serogroup, this means that the vast majority of sheep could by now have acquired natural protective immunity against SBV which may last for a few years (Anonymous, 2008; Steukers et al., 2012). Therefore, it seems probable that even if the virus overwinters, regions with high seroprevalence such as Belgium should not experience a similar epidemic in 2012-2013 and that only animals located in areas that are still free of SBV could in turn become infected, just as lambs after the waning of maternally derived antibody. Regions neighbouring affected areasare probably the most at risk for virus circulation, especially since *Culicoides* from the Obsoletus group, the identified vectors of SBV, are present in most European countries except Northern parts of Norway and Sweden (EFSA, 2012). Nevertheless, even in highly immunised livestock, because of all new-born animals, part of the host population will continuously remain susceptible. For these different reasons, the cluster identified in the South of Belgium with lower seroprevalence than the rest of the country should be prioritized in the context of future surveillance actions. In Australia, Akabane disease is controlled by acting on herd management, for instance by moving susceptible animals into endemic areas in time to develop immunity before they are first bred (Kirkland, 2012).

The findings presented in the current study should be taken into consideration as part of comprehensive SBV surveillance strategy. It is important to underline the paramount need to answer specific questions such as the duration of post infection protective immunity and the exact host range. Especially since, the emergence of exotic and newly identified arthropod-borne pathogens seems to happen more and more frequently in recent years.

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CHAPTER VIII

GENERAL DISCUSSION

The surveillance activities which were organized in Belgium during the BTV-8 and SBV epidemics in order to meet the different objectives evolving along with the epidemiological stages are summarized in table 1. These activities were presented and analyzed in this thesis:

- In Chapters III, IV and VII, the first introduction of BTV and SBV in Belgium was described, giving the opportunity to address several approaches to install early detection systems.
- The design and results of a study to evaluate BTV-8 vaccination was presented and criticized in Chapter V.
- Chapter VI summarized the results of the surveillance activities which were implemented in the cattle population for substantiating freedom of BTV disease.

The protocols, implementations and objective fulfillments of the different surveillance activities are reviewed and discussed in this section.

Table 1: Summary of the different surveillance activities which were carried out in Belgium during and after the BTV-8 (2006-2012) and the SBV epidemics (winter 2011-2012)

Surveillance stage	Strategy BTV-8	Strategy SBV
1. Early (re)detection	-Clinical reporting -*Culicoides* monitoring -Animal sentinel system in cattle based on c-ELISA (2007) -abortus protocol	-Clinical reporting -*Culicoides* monitoring -abortus protocol
2. Impact description	-Cross-sectional surveys based on c-ELISA in cattle and sheep (winter 2006-2007 and 2007-2008) -To further measure clinical impact: clinical reporting+ results of surveys in outbreak herds	-Cross-sectional serological surveys (winter 2011-2012 and 2012-2013) -To further measure clinical impact: clinical reporting+ results of surveys in outbreak herds
3. Control progress assessment	-Annual cross-sectional survey in cattle based on c-ELISA and RT-qPCR (winter 2008-2009) -Control clinical reporting and results of abortus protocol	/
4. Proof of freedom from infection	-Repeated cross-sectional surveys in cattle every 3 months based on RT-qPCR (2010-2012) -Control clinical reporting and results of abortus protocol.	/

VIII.I. How well were we prepared in Belgium for the Bluetongue virus epidemic?

VIII.I.1. Anticipation of the epidemic

Anticipating EID will contribute to facilitate timely recognition of outbreaks and their control. To be ready to face an epidemic, several aspects have to be considered beforehand:

- Laboratory preparedness

As a consequence of the emergence of BTV in Southern Europe, several diagnostic assays had already been implemented before 2006 in CODA-CERVA. In collaboration with French researchers, Toussaint et al. (2007a) had developed and partially validated two RT-qPCR able to detect strains from the 24 BTV serotypes. Next to this, two c-ELISA, virus isolation and a virus neutralization test had also been implemented in CODA-CERVA before the emergence of the virus in 2006. All this enabled prompt and fast confirmation of the first clinical cases of BT (Toussaint et al., 2007b). Besides the diagnostic tools available at that moment, a Laboratory Contingency Plan (LCP CODA-CERVA) was foreseen, designating the different responsibilities, procedures and supplies needed in case of an outbreak. BTV was already included in the LCP in order to have a crisis scenario ready in case the virus was introduced in Belgium. Diagnostic tests which meet the OIE standards were thus already available in Belgium before the emergence, even though the immediately available stock and expertise were relatively limited.

- Surveillance systems for early detection

For a long time, the presence of the main vector species of BTV, *Culicoides imicola*, has been considered the main risk factor for an emergence of BTV in Europe. Because *Culicoides imicola*, had been discovered in the Southern European countries following the 1998-2005 outbreaks, BTV surveillance such as sentinel networks was already implemented in most of these countries prior to 2006 (Guis et al., 2011). In contrast, *Culicoides imicola* was and still is absent in Northern Europe, and, for this reason, BT was not expected to occur in this region. In addition, since most introductions of BTV into Europe before 2006 occurred via Anatolian Turkey or the Maghreb, Southern Europe was thought at that time to be the most exposed area of the continent (Wilson and Mellor, 2009). In 2003, Elbers et al. (2003) had evaluated the risk of BTV introduction in Northern Europe. Mainly because of evolving climatic conditions and increased animal flux in the South-North direction in Europe, the

authors anticipated that *Culicoides imicola* might expand its range of distribution and, in consequence, that an introduction of BTV in Northern Europe was possible. As a consequence, the authors advocated an incremental response with regard to BTV by developing a crisis strategy for BTV control and deepening strongly knowledge on vector distribution and competence. None the less, in most northern European countries, with the exception of Switzerland and the Netherlands (Elbers et al., 2003; Racloz et al., 2006b; Takken et al., 2008), no surveillance activities for early detection were in place to try to detect an eventual incursion of BTV in this region.

- Clinical disease recognition

When BTV-8 emerged in 2006, veterinary practitioners in Belgium were at least 'theoretically' prepared to recognize the presence of the virus in hosts, since the virological, clinical and epidemiological aspects of this former-exotic virus were already addressed during the veterinary studies curriculum at that time. However, because of the abovementioned reasons, disease awareness among most veterinarians and farmers was quite inexistent. Moreover, because BTV clinical signs are usually restricted to sheep, disease descriptions mainly applied to sheep (Elbers et al., 2008). As we have seen both in Chapters III and IV, in contrast to other strains of BTV, the European strain of BTV-8 had also the capacity to induce important clinical signs in cattle, possibly explaining the misdiagnosis at the beginning of the epidemic in 2006. However, it was clear that the clinical signs in cattle were observed and recognized during the second wave of the epidemic in 2007. This illustrates that epidemics can help to prepare the veterinarians to make their differential diagnosis, based on clinical signs, more accurate than before.

- Vector surveillance

Next to the possibility that BTV could emerge in Northern Europe via the range expansion of *Culicoides imicola*, another possibility for the virus to spread towards this region was suspected. Indeed, BTV had also been isolated from two other species groups, *Culicoides obsoletus* and *Culicoides pulicaris* during the 2000 to 2003 Italian epidemic. (Caracappa et al., 2003; Miranda et al., 2003; Savini et al., 2003 ; De Liberato et al., 2005). Even though the potential of *Culicoides obsoletus* as BTV vector was thought to be quite low, the group species was known at that time to be present and abundant, especially during spring and autumn, in Europe from Italy to Great Britain (Rawlings and Mellor, 1994). As a consequence

of the findings mentioned previously, this means that we already knew before the BTV-8 epidemic that the presence of *Culicoides imicola* was not necessarily required to initiate a BTV epidemic in Europe. Funding for areas such as entomological surveillance was relatively neglected in Belgium before 2006. Expertise pertaining to *Culicoides* was virtually non-existing at that time, and had therefore to be organized in an emergency context. An entomological monitoring program has been subsequently implemented in 2007 by the FASFC and coordinated by IMT. In 2006, a cross-sectional study was organized to improve knowledge of the genus *Culicoides* and to identify plausible vectors during the 2006-epidemic (De Deken, 2008). The following years and until 2012, *Culicoides* captured with traps located all over Belgium were counted on a regular basis in order to monitor the activity period of the vectors.

- Identification and surveillance of introduction pathways

There are several potential entry paths of BTV into a new area: 1.via infected animals, 2.via infected *Culicoides* (via wind, plants, airplanes, etc) and 3.via infected biological products (Mintiens et al., 2008). Even though the likelihood of BTV emergence depends on several additional factors, including the occurrence of favorable climatic conditions, attempts to identify critical geographical points and periods at particular high risk of BTV introduction could have been initiated earlier, in order to instigate surveillance in these 'hotspots'. Indeed, detecting the newly introduced BTV-8 earlier by better surveillance could have reduced the impact of the epidemic.

VIII.I.2. Follow-up of epidemic surveillance

From the start of the BTV-8 epidemic, trans-boundary collaborations favored by European financing have enabled fast development of expertise, especially pertaining to the fields of entomology and epidemiology. A local epidemiology network was spontaneously created by epidemiologists from the first line countries (i.e., Belgium, Germany and the Netherlands) immediately after the emergence. Shortly after, EFSA was requested by the EC to form a network of experts from the different MS and from the Commission and to produce epidemiological reports. The final EFSA report described the results of analyses on factors associated with the introduction, establishment and spread of BTV-8 in North-Western

Europe (EFSA, 2007). Several other European projects have arisen from the epidemic (e.g. EPIZONE Internal Calls IC 6.6 BT-Epidemiology and IC 6.7, IC 6.9 BT-DYNVECT). In addition, Northern MS have beneficiated from the help of Southern European MS which shared actively their past experience in terms of BTV surveillance and control, in particular their expertise in the field of *Culicoides* identification. Individual MS have greatly benefited from the different outputs produced by all these European collaborations, since it helped improve globally the quality of the surveillance system. Nevertheless, sharing and analysis data at the European scale turned out not to always be simple tasks. For instance, in the framework of EPIZONE Internal Call BT-DYNVECT, the use of vector data for the development of models at the European level was hampered by differences in trapping protocols between MS (Guis et al., 2011).

The urgent need for an integrated approach and a harmonization of data from the different MS to be able to analyze correctly surveillance data became quickly obvious during the progress of the BTV-8 epidemic. It was more than one year after the onset of the epidemic, in November 2007, that Commission Regulation (EC) 1266/2007 implementing rules for Council Directive 2000/75/EC entered into force. The Annex I to Regulation introduced minimum requirements to harmonize BTV surveillance across the different countries. In light of the experience gained during the BTV-8 epidemic, the regulation has been amended twelve times following its initial publication. Recently, the EC was in need of scientific advice on two epidemiological parameters which are included in Commission Regulation (EC) 1266/2007: 1.design prevalence for different epidemiological circumstances and 2.the size of the geographic unit of reference. Based on the Scientific Opinion delivered by EFSA answering the EC's request (EFSA, 2011), and because the epidemiological situations as regards to BT may vary considerably between MS, the minimum requirements in Annex I were simplified in May 2012 to allow for more flexibility to design national surveillance programs.

Focusing now on Belgium, the particularity of this vector-born epidemic was well dealt with by establishing several multidisciplinary collaborations. For instance, a team of entomologists, virologists and epidemiologists worked together in the framework of a project conceived to optimize the surveillance of BT, by studying among others, the role of different *Culicoides* species as BTV vectors and the association between the spread of the virus and wind characteristics (Federal Governmental Services; RF-6187).

In brief, even though concerns concerning the possibility of BTV emergence had been expressed beforehand, only limited resources and expertise were available in Belgium prior to 2006. In consequence, nothing was done to try to prevent BTV from being introduced and established, and the country was not truly prepared to face the problems posed by the vector-borne epidemic.

VIII.II. Did the BTV experience helped to be better prepared for the Schmallenberg virus epidemic?

VIII.II.1. Anticipation of the epidemic

BTV-8 and SBV emerged in the exact same region. This means that this area is probably a 'hot-spot' for (re-)emergence (Beer et al., 2013). After the emergence of BTV-8 in 2006, it could have been interesting to study the characteristics of this geographical region to try to identify the route of introduction. This would have maybe helped to anticipate more a second emergence of ABV at a five-year interval. In contrast to BTV which was an exotic emerging disease, SBV was a totally new virus when it emerged and, in consequence, its emergence was totally unpredictable. Because of this, initially no diagnostic tools were available to detect it. However, metagenomic analysis has proven to be an effective tool to detect such unknown viruses (Blomström, 2011; Hoffmann et al., 2012). And as a consequence, diagnostic tools became available very early after the identification of the virus. It highlights the need to optimize this technology and to ensure that it is available at least in some reference laboratories in order to allow early recognition of potential other newly emerging pathogens.

For most Belgian farmers, the BTV-8 epidemic was still a relatively recent unpleasant memory at the time SBV emerged. We can therefore hypothesize that both farmers and veterinarians have gained experience from the BTV-8 crisis and that it must have helped in the recognition and reporting of clinical signs. On the other hand, as we have seen in Chapter VII, most SBV infections were asymptomatic, especially in adult animals. In this case, a good option to detect early enough the start of an epidemic, could have been to analyze beforehand different data sources such as fertility data in a structured syndromic surveillance system (Madouasse et al., 2011). Unfortunately, no such system was in place in a structured way in Belgium at that time.

VIII.II.2. Follow-up of epidemic surveillance

Both at national and supranational levels, several scientific networks, resulting directly from the BTV-8 epidemic, were very helpful for managing efficiently this new ABV emergence. This has contributed to allow a fast mobilization of the different research teams and available resources. Exchanges between European laboratories of protocols and diagnostic reagents essential to organize the diagnosis of SBV in the different affected countries were greatly facilitated by this past common experience.

Because SBV was a new virus, at the beginning of the epidemic, nothing much was known about its epidemiology and only extrapolations from related viruses such as Akabane virus could be made. The fact that these related viruses were known to be transmitted by mosquitoes and biting midges led to the hypothesis that SBV might be spread by the same vectors. At the onset of the SBV epidemic, fortunately, the entomological surveillance program implemented in the context of the BTV-8 epidemic was still in place in Belgium. *Culicoides* continued to be captured regularly with traps located all over the country until the end of 2012. These captures were used by scientists from CODA-CERVA to show that several *Culicoides* species, previously shown to be vectors of BTV, were most probably also involved in the transmission process of SBV (De Regge, 2012). In addition, the BTV-8 emergence inadvertently contributed to improve knowledge and scientific expertise in the field of entomology, which has contributed to facilitate the management of the SBV crisis.

Furthermore, because of the high similarities between the two diseases both in their clinical and transmission aspects, the transposition of surveillance activities such as the screenings (Chapter VII; Méroc et al., 2013) and the abortion protocol to SBV were straightforward. Thus, we may conclude that, even though the BTV-8 epidemic did not contribute to prevent SBV from emerging in the same region, it considerably contributed to aid in the follow-up of the crisis.

VIII.III. How to plan surveillance in the context of an emerging arboviral infection?

VIII.III.1. Early detection

The early detection of a new ABV in animal and/or vector populations is essential to provide prompt warning of the onset of an epidemic and to guaranty all control policies to be developed effectively. The detection of suspicious clinical cases (passive surveillance) is a critical component of the early recognition process and represents a very important source of information since it (theoretically) covers continuously in time the whole susceptible population as well as the whole range of potential emerging viruses. In Chapter III, by comparing the final dispersion of the BTV based on seroprevalence estimates to that of notified clinical cases, we demonstrated that the case detection system based on clinical suspicion provided an accurate indication of the spatial distribution of the virus. Because the first clinical signs appear as soon as five days after the animal is infected, whereas BTV antibodies can only be detected by c-ELISA from 7-10 days post-infection (OIE, 2009), this demonstrates the importance of the passive surveillance component for BTV early detection. Welby et al. (2013) showed indeed that in terms of early detection, this surveillance component was the most effective. However, the results of the first Belgian BTV serological survey demonstrated that the case detection system based on clinical suspicion underestimated greatly the impact of the epidemic (between-herd prevalence of 0.7% versus 83.3% based on the results from the cross-sectional serological study) (Chapter III). Exactly the same fact was observed for SBV (Chapter VII). Indeed, the main drawback of clinical surveillance is that it relies totally on a chain of events to occur first. As a start, clinical surveillance requires the infection to produce clinical signs, which is not always the case. During the BTV-8 epidemic in 2006, it has been demonstrated that a fair proportion of cattle within a herd could be infected (RT-qPCR/c-ELISA positive) without showing any clinical signs. This was also clearly the same situation during the SBV epidemic, since adult cattle rarely showed acute clinical signs of the infection and adult sheep did not present any acute sign of the disease at all. We have seen in Chapter III, that there was a difference in terms of disease occurrence between cattle and sheep; the BTV-8 between-herd prevalence after the first episode in 2006 was estimated at 0.7 per cent (95%CI 0.7-0.8) for cattle herds and 1.3 per cent (95%CI 1.2-1.4) for sheep flocks. We also saw in Chapter IV, that impact of BTV-8 on mortality was more important in small ruminant than in cattle population. In line with this, it seems advisable to reinforce furthermore clinical surveillance especially in groups of animals more

prone to manifest the disease if infected, such as sheep for the specific case of BT. Nevertheless, because of strain-specific characteristics with respect to the clinical expression of the infection (as it was the situation for BTV-8 with its capacity to induce important clinical signs in cattle), it is also fundamental to ensure a broad coverage of all susceptible domestic and wild animal populations.

Passive surveillance then also obviously relies totally on the recognition of pathognomonic signs of the disease ('awareness') by the farmers and veterinarians (animal-health professionals). This is not always warranted, especially in the context of an infection with an exotic virus such as BTV-8 or a new virus like SBV. Because of this, implementing this type of surveillance necessitates high vigilance of all involved animal-health professionals from farm to lab, maintained through regular contact, such as meetings with animations and simulation exercises designed to enhance disease awareness and facilitate access to differential diagnosis for the principal threats previously identified through a risk assessment. In case of reduced resources, it might already help to increase vigilance in a 'subpopulation' of animal-health professionals ('sentinel vets or sentinel group of veterinarians'). For most ABV, enhancing disease awareness is even more essential during the vector activity (approximately April-November in North-Western Europe) seasons. Indeed, past experience has shown that the first outbreaks of BTV epidemics are mostly diagnosed between mid-August and October (Gomez-Tejedor, 2004; Toussaint et al., 2007b). It is also essential to formulate a proper clinical case definition in order to increase the sensitivity of the passive surveillance system as well as its specificity and avoid unnecessary losses (expensive and time consuming laboratory confirmation procedures, trade impact, etc). Both the examples of BTV-8 and SBV have proven that this was not always an obvious task. Indeed, the clinical signs of BT may vary a lot between the host species and are never pathognomonic of the disease. It is so, that Toussaint et al. (2006) reported that at the beginning of the BTV-8 epidemic in Belgium, contagious echtyma was diagnosed for several sheep that showed BT signs. In addition, the range and severity of clinical signs can sometimes evolve over time and between different serotypes (Doherr and Audigé, 2001; Dungu et al.., 2004). This pinpoints the need to monitor the range of the clinical signs and to adapt the clinical case definition if necessary.

In situations for which the risk of emergence has been clearly identified, additional surveillance activities will increase the probability of detecting an emerging disease.

Introducing an active component in the surveillance process will allow identifying additional cases of disease. Moreover, this will also globally contribute to enhance disease awareness and thus improve the passive surveillance component (Doherr and Audigé, 2001). In any case, the active system should be adequate for long run application, that is as long as the ABV is absent from the population. One approach commonly used is sentinel surveillance which consists in cohorts of unexposed animals managed at fixed locations and monitored regularly to detect the emergence of new infections (OIE, 2013a). Sentinel systems designed to detect emergences of ABV have been developed in many countries (Giovannini et al., 2004; Melville et al., 2004; Biteau-Coreller, 2006; Racloz et al., 2006a). Saegerman et al. (2009) described the sentinel network which has been organized in Belgium during spring 2007 to detect the re-emergence of BTV-8 after the 2006-2007 VFP. Starting on 20 March 2007, a total of 4050 initially seronegative cattle (from 300 farms) were tested monthly by c-ELISA and confirmed by RT-qPCR in case of a positive result. The sampling design was prepared following guidelines for BTV surveillance proposed in Chapter 8.3 of the OIE *Terrestrial Animal Health Code* (OIE, 2013b). In line with these guidelines, cattle were selected as study population. Concerning *Culicoides*-borne viruses in general, the target animal species will often be cattle, since, there are known to be the preferred host species of the midges (Nevill, 1978). We decided to focus on dairy cattle because they are easier to monitor compared to other cattle types. Sentinel herds were selected based on the results of the cross-sectional serosurvey described in Chapter III and all herds with at least 20 animals seronegative were included in the sampling frame.

Sample size will depend on several criteria, among which the design prevalence. The latter may in some instances be defined by the legislation, as it is the case for BTV since November 2007 (cf. Commission Regulation (EC) No 1266/2007), or should be derived from field data if nothing is foreseen for this. Sample size for the 2007 BT-sentinel system was calculated based on OIE guidelines to allow the detection of a design prevalence of 2% with 95% CI in each geographical unit of reference. The latter corresponded to a conservative estimation of the expected monthly rate of seroconversion in an infected zone. Because of the central role of *Culicoides* in the BTV transmission cycle and to the fact that midges are never confined to a specific holding, the unit of reference for BTV is a geographical area: the geographical unit of reference. The latter was and is still defined as a grid of 45 x 45 km (approx. 2000 km²). This size was chosen because it is a compromise between the 20 km zone around infected

holdings as foreseen in EU legislation and the size of the administrative unit as defined in Directive 64/432/EEC (EFSA, 2011). In Belgium, a province (district) represents more or less two times this geographical unit in terms of surface, simplifying the organization of the sentinel network. It was chosen here to monitor seroconversion, but virus presence, or clinical signs of the disease may also be monitored. As abovementioned, the former is not always adapted to early detection, since antibodies need time before they become detectable. On the other hand, instigating active clinical surveillance performed by expert veterinarians may be more appropriate to meet this objective. For BTV, given, the presence of the virus may be detected in cattle blood for 150 days post-infection (Bonneau et al., 2002; Di Gialleonardo et al., 2011), the regular testing (e.g., every 4 months) of sentinel animals with RT-qPCR should ensure adequate early detection of BTV, even in an immunized population. In contrast, given the very short viraemia linked to SBV infection (with duration of maximum 6 days (Hoffmann et al., 2012)), this option would not be feasible in practice for this virus. In the case of SBV, a sentinel system based on serology (testing sentinel animals with c-ELISA for instance) would be more appropriate. At last, OIE guidelines recommended maximizing the chance of detecting BTV activity by selecting sentinel sites more at risk of infection (risk-based surveillance). This recommendation was not followed because no risk assessment had been performed at that time. Today, sampling designs could be enhanced by using outputs of analyses carried out since then, such as for example the results found by Faes et al. (2013) which identified, among others, land cover to be a risk factor for BT incidence.

Even though the Belgian sentinel network made possible the detection of BTV-8 re-emergence in July 2007, the first seroconversions were not detected before the first outbreaks occurred identified with clinical surveillance. Among the various key-parameters stated by the authors to help improve the quality of this type of system, is its earlier implementation during the year than the beginning of spring as it was done in 2007. Indeed, the exact starting point of the vector period is usually unknown. Moreover, it has been shown that *Culicoides* were able to overwinter inside cowsheds (Losson et al., 2007; Zimmer et al., 2010).

Sentinel systems may also focus on the vectors instead of the hosts. Monitoring vector populations at fixed sampling points may be useful to detect an introduction or an increase of known vector populations. This is important in order to identify areas and periods at risk (vector-active periods) for ABV introduction. Another possibility is to use these repeated collections of vectors to monitor the presence of the virus, as this was recently done in Italy to

investigate whether SBV had spread in a given area (Goffredo et al., 2013). Even though difficult to implement and labor-intensive, this type of sentinel surveillance is important to consider and maybe the only one truly helpful in some situations, since it is known that ABVs are capable of developing transmission strategies such as vertical transmission or overwintering that can ensure their survival in a region even in the absence of disease in host animals (Gould et al., 2006).

VIII.III.2. Impact description

At the point of the epidemic where the vector activity and the number of new outbreaks have decelerated and reached a steady state, the main surveillance objective will be to assess the extent and the impact of the infection.

During the first episode of the BTV-8 epidemic (2006), in the affected countries, a follow-up visit was executed by official veterinarians in the herds where a clinical suspicion had been reported and consisted of: 1. a clinical inspection of all animals in the herd, 2. sampling (EDTA blood and serum) of maximum three animals and testing with RT-qPCR and c-ELISA in case of a confirmed clinical suspicion, and 3. an epidemiological investigation using a standardized questionnaire. The questionnaire included questions concerning the date of first clinical suspicion, the number of animals that had clinical signs/died, as well as information on contact herds. In the context of the SBV epidemic, a similar epidemiological survey has been organized in a portion of selected Belgian outbreak herds and its analysis is currently in progress. This kind of clinical surveillance data provides the approximate timing of the infection. The presumed dates of infection occurrence can be used to build an epidemic curve. The information is of considerable value for modeling, for developing causal hypotheses and for predicting future spread of the infection (Martin et al. 1987). In addition, this gives indication on the origin of the epidemic (index case) and will help investigating on the route of introduction of the virus. Also, the numbers of animals that had clinical signs or died following the infection can be used to estimate key parameters such as the morbidity, mortality and case fatality rates (Elbers et al., 2008). This kind of clinical surveillance information provides valuable data to establish the impact of an epidemic. However, it has also several limitations mentioned previously like the fact that it will usually lead to an underestimation of the true picture. In addition, this type of surveillance principally works

only at the herd scale. In the case of BTV-8 and SBV in Belgium, a maximum of three animals were sampled in each suspicious herd to be tested with RT-qPCR and c-ELISA. This sample size is too small to estimate the proportion of animals infected (i.e, the within-herd prevalence); indeed, at the time sampling was done, we had no information on the expected prevalence, and, to estimate an unknown prevalence (50%) at a confidence level of 95% in a cattle herd of 90 animals (median size of Belgian cattle herds), a sample size of 73 is theoretically necessary and in a sheep flock of 4 animals (median size of Belgian sheep flocks), all sheep need to be sampled (Cannon and Roe, 1982). From the results presented in Chapter III we could now adjust slightly sample size; indeed, to estimate BTV-8 within-herd seroprevalence in an infected cattle herd after first transmission season, a sample size of 69 would be sufficient (with expected seroprevalence of 23.8%).

The results of passive surveillance may be used as a tool to determine between-herd prevalence (proportion of outbreaks in population). However, as shown in Chapter III and for the different reasons afore-mentioned, this kind of approach will often lead to an underestimation of the latter.

Next to this, as described in Chapter IV, a high level of correlation was observed between ruminant mortality/abortion rate and BT incidence data. In the context of the SBV epidemic in Belgium, bovine abortion and birth records from 2010-2012 have been analyzed and have shown an increase in the number of abortion reported and a decrease in the number of cattle births between September 2011 and March 2012 (Van der Stede, 2012). This kind of population-level data can be used to gain more reliable information on the impact of an epidemic as it was done for example by Perrin et al. (2010) to estimate indirectly the excess mortality during to the BTV-8 epidemic.

To have an unbiased estimation of the true impact of an epidemic, it will usually be essential to organize a screening of the population at risk. As described in Chapters III, V, VI and VII, cross-sectional surveys were organized in the Belgian domestic ruminant populations during the VFP following the different BTV-8 and SBV epidemics episodes. These surveys allowed estimating the geographical distribution of the infection, between-herd, within-herd seroprevalence and animal seroprevalence. Depending on the objective and the ABV, the samples can be tested either to identify the presence of the virus (virology) or of acquired specific antibodies (serology). A positive result in serology indicates that the tested animal

was at one point infected with the specific virus. As a whole (all sampled animals together), this gives an indication of the level of immunity of the population. Besides allowing a valid impact assessment to be made, the level of immunity is also an essential parameter to anticipate a potential overwintering of the ABV by defining on time the optimal national control strategy. An insufficient level of immunity will be the justification for implementing a vaccination campaign within an animal population. Moreover, identifying differences with respect to the level of immunity between categories of animals (e.g. age class, geographical localization of the farm, etc), will allow prioritizing control measures if necessary.

The results of the screening studies may also serve as basis for future surveillance; indeed, design prevalences to estimate sample sizes for future surveillance can be derived from observed prevalence estimates. However, EFSA performed a systematic literature review of prevalence data from MS and concluded that the plateau prevalence (after prevalence rise) of BTV-8 was markedly higher than that of other serotypes in Southern Europe (EFSA, 2011). This would be an important factor to take into account if a new serotype of BTV would emerge in the future.

From the results of our cross-sectional surveys we also estimated ICC of 0.41 and 0.34, respectively for BTV-8 in the Belgian cattle population (Chapter III) and for SBV in the Belgian sheep population (Chapter VII). This parameter indicates the level of correlation between two observations within a cluster (e.g., herd). Indeed, individuals within herds are often more similar than animals selected randomly from the target population. The ICC of a livestock infectious disease is usually less than 0.2 and ranges from 0.04 to 0.42 (Otte et Gumm, 1997; Dohoo et al., 2003). Sample size needs to be inflated to adjust for this clustering. The inflation will depend on both the ICC and the size of the clusters (Killip et al., 2004). As an illustration, let's calculate the effective sample size of the BTV-8 sentinel system described in previous section using the following formula:

$$\text{ESS} = \frac{m \times k}{1 + (m-1)\rho}$$

with ESS= effective sample size
m= number of units in the cluster (15 animals sampled/herd in our example)
k= number of clusters (=30 herds sampled/province in our example)
ρ= ICC (0.41 in our example)

Hence, in our example ESS= 67. This means that, although 450 animals (30 herds with 15 animals each) were sampled per province, from a statistical point of view, only 67 animals participated in the study (more or less 7 times less). Thus, to be able to detect seroconversion with 95% confidence, the sample should have been instead of 3033 animals (i.e. 202 herds with 15 animals each).

The prevalence results will contribute to evaluate indirectly the level of under-reporting and this will allow defining the sensitivity of the passive surveillance system. It can be also useful to instigate awareness campaigns. This may contribute to improve the surveillance system globally. The survey outcomes can also be used for subsequent modeling: to predict future spread (knowing the amount of susceptible animals), to estimate the cost of the epidemic by linking them with productivity data, to identify risk factors for infection which is important to instigate a risk-based early detection system, and to study the role of the vector by identifying links between entomological and serological data. The resulting epidemiological information will be crucial to improve globally surveillance and control.

The timing of the cross-sectional survey described in Chapter III, V and VI (January-March), initially selected for practical reasons in the context of the Brucellosis-Leucosis programme (WS), since the animals are in stables at that time of the year, fitted perfectly for a *Culicoides*-borne disease such as BT (and later on SBV). It coincides indeed with the VFP and thus to the end of a transmission season. During winter months, the incidence is very low and the epidemic curve has reached a plateau. In consequence, it is the right moment to take stock of the situation. In contrast, as it was described in Chapter VII, planning a cross-sectional survey in domestic small ruminants was more problematic. The first difficulty was to find available samples collected following a structured sampling design. Blood samples originally collected in the context of the Maedi-Visna and Caprine Arthritis and Encephalitis program for trade certification (Royal Decree 24-03-1993) were used for this purpose. This program works on a voluntary basis; interested owners are those who practice trade. Therefore, the flocks which were sampled may have some particular features in their size (median flock size in the study was 8 versus 4 in a random flock), management, etc. This kind of sampling design presents opportunity risk for selection bias. Indeed, estimating the extent of an epidemic necessitates the sample to be as representative of the target population as possible. Provided the sampling units are randomly selected, a serological screening will yield unbiased seroprevalence estimates (Martin et al., 1987). Each individual unit within the target population should have

an equal chance of being sampled, but this is rarely the case in practice. In the different cross-sectional studies described in this thesis, we saw indeed that the choice of the samples is often mainly dependent of practical and financial constraints (cf. dairy cattle older than 24 months in holdings with on-farm delivery of milk products in Chapter III). As a consequence, most samples are likely biased since they were seldom representative of all host species, breeds, herd types, ages, etc. This is also the reason why using data collected initially in the framework of surveillance for subsequent epidemiological research purposes should always be done with caution. Protocols designed for surveillance networks are not always adapted to study causal hypotheses (Dufour and Audigé, 1997). In order to ensure that adequate samples are always available to organize screenings, a collection of samples that can be kept in refrigerators (serum banks) representative of the different animal species is an interesting approach which has been developed in Belgian regional and national reference laboratories (ARSIA, DGZ and CODA-CERVA) since 2009. In addition, these samples constitute a sampling frame useful for potential retrospective monitoring, to date the introduction of a virus in a population (Martin et al., 1987).

The quality of the at-risk population data is also a critical parameter to allow correct design and analysis of surveys. In Belgium, all herds of ruminants are supposed to be registered in SANITEL. In addition, cattle are ear-tagged individually and recorded in this database. The identification of sheep and goat flocks is not as complete and no recording of the identification at the animal level is organized.

VIII.III.3. Control progress assessment

Several indicators can be used to assess the impact of intervention strategies designed to jugulate the epidemic such as the vaccination against BTV-8 which started in 2008 in Belgium (Chapter V).

A first line of approach can be to monitor the incidence of new outbreaks. As stated in Chapter V, only 50 outbreaks were reported in Belgium after the vaccination against BTV-8 was implemented in the cattle and sheep population, compared to 6870 the year before the onset of the campaign. In addition to monitoring the incidence of new outbreaks, it can be valuable to follow up ruminant rendering plant data, as it had a positive correlation with BT

incidence (Chapter IV). It is important to keep in mind that it is possible that virus transmission continues to occur at low levels in a highly immunized population and that it is useful to carry out a survey to demonstrate in a structured manner changes in infection prevalence and/or in seroprevalence compared to a starting point. In the context of vaccination, a decrease of infection is whished for, whereas an increase in immunity is expected. We have seen in Chapter V that one of the requests of the FASFC was to evaluate if the targeted antibody coverage of 80% had been correctly reached after the first vaccination campaign against BTV-8 which began in May 2008. However, vaccination was carried out in a population in which a high proportion of animals had already been infected during the previous BTV episodes (2006-2008). Antibodies against BTV appear in infected animals typically within one or two weeks post-infection and last for several years (Afshar et al., 1989; Walton and Osburn, 1992). Because of this and because no distinction between vaccinated and infected animals could be made based on serology (DIVA), the intrinsic effect of vaccination was hard to evaluate given the sampling design. Indeed, the survey (i.e., two cross-sectional serological surveys performed in the same cattle herds during consecutive VFP: one before and the other after the start of the vaccination) was initially designed to answer another objective (i.e, to describe seroprevalence after the consecutive transmission seasons, as described in previous section). The strategy for sampling and to avoid this issue consisted on focusing on individuals still seronegative before the vaccination's onset and on estimating the level of seroconversion in this portion of the host population. However, based on the results of those surveys, we had no information about the status of the animals between the two surveys, and it may well be that some animals were infected at one point between the screenings and became seropositive because of natural infection, and not as a consequence of vaccination. These are the reasons why in this survey animals were also tested by RT-qPCR. Nevertheless, given that BTV-8 RNA can be detected in cattle for at 150 days following infection, it is possible that some animals were RT-qPCR positive at one point between the two screenings and then became negative before the second survey was carried out. In conclusion, the sampling design which was used at that time was not adapted to reach its main goal. An alternative approach could have been to select a representative sample of seronegative cattle and to test them with RT-qPCR more frequently throughout the year (e.g. every 4 months) to exclude infection and to prove that seroconversion was due to vaccination. This kind of repeated surveys organized in the same herds over the years is an adaptive form

of sentinel network surveillance and might be useful for different objectives in certain epidemiological circumstances (o.a. prove freedom of disease).

VIII.III.4. Proof of freedom from infection

When the epidemic has ceased following effective control actions and/or a natural fade-out, for OIE listed diseases such as BT, it is essential to actively substantiate freedom from infection in order to lift trade restrictions. Therefore, the purpose of the surveillance actions is now the same as before the emergence, hence, to detect the potential presence of infected individuals in host and/or vector populations.

Guidelines to demonstrate freedom from infection after a BTV epidemic in the EU are informed in Commission Regulation (EC) No 1266/2007 and set conditions to prepare the sampling design of the surveillance survey. Cameron and Baldock (1998) proposed a method based on statistical formulae which may be used both to calculate sample size and to interpret accurately the results of freedom of infection surveys. This method takes into account the performance of the diagnostic test used (DSe and DSp) and provides the exact probability of observing the obtained results under the null hypothesis (that the disease is present in the population) and under the alternative hypothesis (that the population is free from the disease). Freedom of infection surveys imply that again a switch from a representative sampling design towards a more targeted sampling is done. The number of animals to be sampled was calculated to detect a minimal incidence of BTV infection of 2% with 95% confidence in each geographical unit of reference in accordance with EC Regulation No 1266/2007. This value has recently been reviewed in an EFSA Scientific Opinion and it was shown that it was slightly higher than the median observed value in an endemically infected area (EFSA, 2011).

We have already seen so far that repeated cross-sectional surveys were useful in order to organize sentinel systems (early detection) and to estimate the spread of an epidemic. From the end of 2010, repeated cross-sectional surveys have also been organized every three months in the same cattle farms with the objective of proving the absence from BTV infection (Chapter VI). This particular type of sentinel network usually causes less practical constraints and is thus often more appreciated by farmers and veterinarians than the traditional system for which the same animals need to be sampled at each sampling round.

However, the system has been confronted to several issues during the analysis of its outputs, related to the recent infectious history of the target population. When implementing this type of sentinel network, it is essential to keep in mind that, at this stage, the host populations have considerably changed since the emergence. Consequently, this has an impact on the choice of the diagnostic test used and on the individuals that are to be sampled. Indeed, a serological testing was of no longer use to monitor the infection in the highly immunized population. In consequence, RT-qPCR was selected as the diagnostic test to be used. However, because vaccination, which has been carried out compulsory during three consecutive year in Belgium (from 2008 to 2010), may interfere with RT-qPCR results (De Leeuw et al., 2013), the correct recording of the vaccination history of the animal sampled is a critical parameter to allow a correct interpretation of the results. Unfortunately, the vaccination data at animal level is not centralized in Belgium, leaving room for potential misrecordings and/or interpretation errors. Because the positive samples were to be confirmed using the c-ELISA, it was also essential to make sure that animals were free of maternal antibodies in order to avoid interference with the c-ELISA results. Therefore, after having evaluated the average age of maternal immunity decline, and having observed that by the age of 7 months maternal antibodies had disappeared in most animals, it was decided to focus on cattle between 8 and 14 months in non-vaccinated herds, keeping in mind that it is still possible for outliers to occur. Again, the interpretation of the results is totally dependent on the availability and validity of administrative data.

Concerning SBV, the duration of maternal-derived antibodies against the virus is not yet known, but Tsutsui et al. (2009) studied the duration of maternal immunity against Akabane virus in calves and found that 4-5 months is the estimated age when the maternal antibodies decay with a 90% probability. In a recent study, Elbers et al. (2013) found an average duration of 6 months between birth and first appearance of a seronegative status in young calves (min: 4 months; max: 8 months). If we were to organize a study to prove freedom from disease for SBV, we could thus also select young calves older than 8 months of age.

After Belgium re-gained its BTV free status, the repeated surveys were replaced by annual cross-sectional surveys which took place once a year during winter (January-February) 2012 and winter 2013 (unpublished data). As mentioned previously, if using only RT-qPCR to test the animals only once a year, it is possible to miss some animals infected during the previous year. Also, because of the aforementioned reasons, testing cattle only by c-ELISA, will not always constitute a sufficient proof of infection/non-infection either. In consequence, the best

option would be to use the two types of tests in parallel to gather enough information to decide on the real status of the samples.

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CHAPTER IX

RECOMMENDATIONS FOR THE SURVEILLANCE OF LIVESTOCK EMERGING ARBOVIROSES

Along this thesis, it has been clear that for an ABV surveillance system to be effective, an integrated approach of the three components - clinic, serology/virology and entomology - is fundamental. To anticipate a potential new ABV emergence, it is therefore advisable to organize regular simulation exercises with all concerned animal-health professionals (farmers, veterinarians, entomologists, laboratories, epidemiologists and the veterinary authorities). This thesis also demonstrated the importance of tailoring surveillance activities specifically to the objectives evolving with the epidemic stage. In addition, the surveillance system should be adapted continuously to the state of epidemiological knowledge (e.g. using adapted ICC and design prevalence). Table 2 summarizes the specific recommendations which were made in this thesis for BTV-8 and SBV surveillance.

To ensure early detection of an ABV epidemic, clinical surveillance is fundamental but depends highly on the level of morbidity of the disease and on disease awareness. To answer these two limitations it is therefore advisable to: 1) increase surveillance especially in animal categories more prone to show clinical signs and 2) increase globally disease awareness from farm to lab, for the principal threats identified, by defining and communicating (via education, internet, etc) updated case definitions to animal-health professionals facilitating access to differential diagnosis and communicating clearly information on reporting procedures. It is also essential (also for an unknown disease) to designate beforehand a Reference Laboratory responsible to follow the disease situation and its evolution across the world.

Next to this, when a risk of emergence has been assessed, it is also essential to implement an active component such as sentinel surveillance and follow this. The latter ideally should be organized both in vector and animal populations. The sentinel program should ensure that the virus is absent from the vector population and provide regular updates on vector counts to define periods at risk for introduction. The animal surveillance system should focus more specifically on: 1.host species more prone to be exposed to the virus and 2.risk zones/periods of introduction identified through risk assessment.

In order to describe the impact of an ABV epidemic, it is useful to gather and analyze population-level data and conduct individual epidemiological surveys in outbreak herds to estimate mortality/morbidity rates, timing of infection, etc.

To describe the extent of an ABV epidemic and prepare future potential control and surveillance strategies, it will be important to organize at the end of vector-active seasons, cross-sectional surveys representative of the main host population(s). These surveys greatly depend on the availability of unbiased samples which can be ensured through the development and management of serum banks. The quality of information and registration systems of these samples and animals is also a critical parameter to conduct correctly these surveys. A continuous investment of time and money for improvement of data quality and electronic systems (Laboratory information management system) to manage the data is recommended.

In order to assess the control progress, it is essential to design and carry out a survey tailored to answer the specific question (e.g. what is the vaccination coverage and its impact?) taking into account the characteristics of the infection and of the diagnostic test which will be used.

In order to demonstrate freedom of infection after an ABV epidemic, it is essential to organize targeted sampling taking into account the fact that the population is not naïve anymore. The implementation of repeated cross-sectional surveys is a convenient approach. Bad quality administrative data such as vaccination history may jeopardize the effectiveness of the system and lead to unwanted misinterpretation issues. It will be important to study and define the age of maternal immunity fade out.

Table 2: My recommendations for the surveillance activities which could have been carried out in Belgium during the BTV-8 (2006-2012) and the SBV epidemics (winter 2011-2012)

Surveillance stage	Strategy BTV-8	Strategy SBV
1. Early detection	-Improve disease awareness and reporting systems (especially for sheep population). -Risk-based cattle (dairy) sentinel system based on non-specific RT-qPCR testing every 4 months, covering (at least) vector active season. -*Culicoides* monitoring prior to 2006-emergence to define vector active season and places at risk. -abortus protocol : testing for BTV with RT-qPCR	-Improve reporting systems. -*Culicoides* monitoring prior to 2011 to define periods/places at risk of new *Culicoides*-ABV emergence. -Organize structured syndromic surveillance (e.g. using fertility data). -abortus protocol
2. Impact description	-Representative cross-sectional surveys at the end of vector active season based on c-ELISA in both cattle and sheep. -To further measure clinical impact: use clinical reporting+ results of surveys in outbreak herds+ mortality and abortus protocol data.	-Representative cross-sectional surveys at the end of vector active season based on ELISA testing in both cattle and sheep. -To further measure clinical impact: use clinical reporting+ results of surveys in outbreak herds+ mortality and abortus protocol data.
3. Control progress assessment	-To estimate vaccination coverage: representative cross-sectional survey based on c-ELISA + to prove that immunization is not due to infection: repeated cross-sectional surveys every 4 months (from start to end of vaccination campaign) survey in seronegative cattle (non-vaccinated and between 8-14 months) based on RT-qPCR -Control clinical reporting and results of abortus protocol	-To estimate vaccination coverage: representative cross-sectional survey based on ELISA + to prove that immunization is not due to infection: repeated cross-sectional survey every 4 months (from start to end of vaccination campaign) surveys in seronegative cattle (non-vaccinated and between 8-14 months) based on ELISA testing. -Control clinical reporting and results of abortus protocol
4. Proof of freedom from infection	-Risk-based repeated cross-sectional surveys in non-vaccinated cattle (verify status) between 8 and 14 months of age (verify identification) based on RT-qPCR and c-ELISA in parallel every 4 months (confirmation as soon as possible in case of positive result). -Control clinical reporting and results of abortus protocol.	-Risk-based sentinel system based on ELISA in seronegative cattle every 4 months (confirmation as soon as possible in case of positive result). -Control clinical reporting and results of abortus protocol.

Summary

Arthropod-borne viruses or arboviruses (ABV) are viral pathogens transmitted among vertebrate hosts by hematophagous arthropod vectors. More than 150 ABV are known to cause human and/or animal diseases. Bluetongue virus (BTV) is a virus which belongs to the genus *Orbivirus* and is transmitted to ruminants by midges of the genus *Culicoides*. Its emergence in 2006 in North-Western Europe was a major and unexpected animal health event. Five years later, history seemed to be repeating itself with the emergence of another virus affecting also ruminants in the exact same geographical area; research teams identified a novel ABV, the Schmallenberg virus (SBV), closely related to viruses from the Simbu serogroup part of the *Orthobunyavirus* genus and shown to be also transmitted to ruminants by *Culicoides*.

In light of the increased risk of ABV emergence in Europe linked to globalization and climate change, it has become imperative for animal-health professionals to proactively improve the surveillance process aimed at producing data designed to guide decision-making. The objectives of surveillance applied to an emerging ABV usually follow the evolution of the epidemic curve: 1.early detection, 2.impact description, 3.control progress assessment and 4.proof of freedom from infection. An overview of ABV emergence and its surveillance was described in **Chapter I**.

The main objective of this thesis (**Chapter II**) was to use the recent examples of BTV and SBV emergences to analyze the surveillance activities which have been implemented in Belgium in the context of the two epidemics in order to be able to identify key elements and provide recommendations to enhance ABV surveillance in the future.

In Chapter III, the emergence of BTV in Belgium was described. At the onset of the epidemic, case-reporting by the Veterinary Authorities relied almost exclusively on the identification of herds with confirmed clinical infected ruminants. A cross-sectional serological survey targeting Belgian cattle was then carried out during the vector-free season. The first objective of this survey was to obtain unbiased estimates of BTV-seroprevalence after the first transmission period. The overall between-herd and true within-herd seroprevalences were estimated respectively at 83.3% (79.2-87.0) and 23.8% (20.1-28.1). Since under-reporting was suspected during the epidemic, a second objective was to compare

the final dispersion of the virus based on the seroprevalence estimates to that of the confirmed clinical cases which were notified, in order to estimate the accuracy of the case-detection based on clinical suspicion. The analysis showed there was a strong correlation between the outbreak data and the data from the survey. The case detection system based on clinical suspicion underestimated the true impact of the epidemic, but indicated an accurate spatial distribution of the virus at the end of the epidemic.

In Chapter IV, the evolution and the clinical impact of the 2007 episode of Bluetongue (BT) in Belgium were analysed. Overall cumulative incidence based on confirmed clinical cases was estimated at 11.5% (11.2-11.8) and 7.5% (7.3-7.8) % respectively in the cattle and sheep populations and a negative association was found between within-herd seroprevalence in 2006 and the risk of showing clinical signs of BT in 2007. A very high level of correlation was demonstrated between BT incidence and small ruminant mortality data when shifting the latter of 1 week backwards supporting the hypothesis that the high increase in small ruminant mortality observed in 2007 was the consequence of the presence of BT. In contrast, for cattle, the correlation was not as high. An increase in cattle foetal mortality was also observed during the year 2007 and a fair correlation was found between BT incidence and foetal mortality.

In Chapter V, a study designed to evaluate the effect of the first vaccination campaign against BTV was presented and criticized. The results showed that the target of 80% of vaccination coverage was reached. However, vaccination was carried out in a population in which a high proportion of animals had already been infected. Because of this, and because no distinction between vaccinated and infected animals could be made based on serology, the intrinsic effect of vaccination was hard to evaluate given the sampling design.

In Chapter VI, the design and partial results of the repeated cross-sectional surveys carried out in cattle from the end of 2010 onwards with the aim to demonstrate the absence from BT in Belgium were described and discussed. The results demonstrated the absence of BTV circulation in 2010 at the design prevalence of 2% and 95% confidence level. In addition, the study of the maternal antibodies in non-vaccinated animals showed that, by the age of 7 months, BTV-specific maternal antibodies had disappeared in most animals.

In Chapter VII, we described the results of a cross-sectional serological survey organised in the sheep and goat population to study the SBV seroprevalence after the first transmission period of the virus in Belgium. The overall within-herd seroprevalence and the intra-class

correlation coefficient (ICC) were estimated at 84.31% (95%CI: 84.19-84.43) and 0.34, respectively. The overall between-herd seroprevalence was 98.03% (95%CI: 97.86-98.18). It was shown that seroprevalence was associated to flock density and that the latter explained the presence of a spatial cluster with lower seroprevalence. These results provided evidence that almost every Belgian sheep herd has been in contact with SBV during 2011.

In Chapters VIII and IX, we have discussed that although concerns concerning the possibility of BTV emergence had been expressed beforehand, only limited resources and expertise were available in Belgium before 2006. As a result, the country was not truly prepared to face the problems posed by the ABV epidemic. It was observed that even though the BTV experience did not contribute to prevent SBV from becoming established, it considerably facilitated the follow-up of the epidemic. In addition, the different surveillance activities carried out during the BTV and SBV epidemics were reviewed in details. In summary, this highlighted the need to integrate continuously the three surveillance components -clinic, serology/virology and entomology- and demonstrated the importance of tailoring surveillance activities specifically to the objectives evolving with the epidemic stage and the state of epidemiological knowledge. We proposed concrete recommendations for the different surveillance activities which could have been carried out in Belgium during the BTV and SBV epidemics.

Samenvatting

Arbovirussen (ABV) zijn virale pathogene micro-organismen die door hematofage arthropode vectoren overgedragen worden onder gewervelde gastheren. Meer dan 150 ABV die humane en/of dierziekten veroorzaken werden beschreven. Het blauwtongvirus (BTV), een virus dat tot het genus *Orbivirus* behoort, wordt naar herkauwers overgedragen door knutten van het genus *Culicoides.* Het opduiken van dit virus in Europa in 2006 was een belangrijke en onverwachte gebeurtenis in de dierengezondheid. Vijf jaar later, leek de geschiedenis zich te herhalen met het opduiken van een ander virus dat eveneens runderen aantastte op precies dezelfde geografische locatie; onderzoeksgroepen identificeerde een nieuw ABV, het Schmallenberg virus (SBV), dat nauw verwant bleek tot virussen van de Simbu serogroep van het *Orthobunyavirus* genus, en dat ook overgedragen bleek te worden naar herkauwers door *Culicoides.*

In het licht van het verhoogde risico tot opduiken van ABV in Europa, gelinkt aan de globalisatie en de klimaatsverandering, is het voor diergeneeskundige professionals noodzakelijk geworden om proactief het bewakingsproces, dat gegevens tracht te verzamelen om de besluitvorming te ondersteunen, te verbeteren. De objectieven van een bewakingsprogramma, toegepast op een opduikend ABV, volgen meestal de evolutie van een epidemische curve: 1. vroegtijdige detectie, 2. beschrijving van de impact, 3. beoordeling van de vooruitgang in de beheersing en 4. bewijs van vrijheid van infectie (**hoofdstuk I**).

Het belangrijkste doel van dit doctoraat (**hoofdstuk II**) was de recente voorbeelden van de BTV en SBV uitbraken te gebruiken om de bewakingsactiviteiten, die in België werden geïmplementeerd in het kader van de twee epidemieën, te analyseren zodat sleutelelementen kunnen gedefinieerd worden en aanbevelingen kunnen gemaakt worden om de ABV bewaking in de toekomst te verbeteren.

In hoofdstuk III werd het opduiken van BTV in België beschreven. Aan het begin van de epidemie steunde het rapporteren van casussen door de diergeneeskundige autoriteiten bijna enkel op de identificatie van beslagen met bevestigde klinisch geïnfecteerde herkauwers. Een cross-sectionele serologische studie, gericht op Belgisch rundvee, werd vervolgens gedurende het vector-vrije seizoen uitgevoerd. De voornaamste doelstelling van deze studie was onpartijdige schattingen van de BTV-seroprevalentie te verkrijgen na de eerste transmissie

periode. De algemene bedrijfsseroprevalentie en de ware binnen-bedrijfsseroprevalentie werden respectievelijk geschat op 83.3% (79.2-87.0) en 23.8% (20.1-28.1). Aangezien tijdens de epidemie onder-rapportering verwacht werd, werd bijkomend beoogd de accuraatheid in te schatten van de op klinische verdenking gebaseerde casus-detectie. Hiervoor werd de uiteindelijke spreiding van het virus gebaseerd op de seroprevalentie schattingen vergeleken met de uiteindelijke spreiding van de gemelde bevestigde klinische casussen. De analyse toonde een sterke correlatie aan tussen de uitbraakgegevens en de data van de cross-sectionele studie. De casus-detectie, gebaseerd op klinische verdenking, onderschatte de ware impact van de epidemie maar gaf echter wel een accurate spatiale distributie van het virus aan het einde van de epidemie aan.

In hoofdstuk IV werd de evolutie en de klinische impact van de '2007 episode' van BTV in België geanalyseerd. De algemene cumulatieve incidentie werd in de rundvee -en schapenpopulatie respectievelijk geschat op 11.5% (11.2-11.8) en 7.5% (7.3-7.8) %. Een negatieve associatie werd aangetoond tussen de binnen-bedrijfsseroprevalentie in 2006 en het risico tot vertonen van klinische symptomen van BTV in 2007. Een sterke correlatie werd aangetoond tussen de BTV incidentie en mortaliteitsgegevens van kleine herkauwers wanneer laatstgenoemde 1 week vroeger werd verplaatst, de hypothese ondersteunend dat de sterke mortaliteitsstijging bij kleine herkauwers die werd waargenomen in 2007 het gevolg was van de aanwezigheid van BTV. In tegenstelling tot de schapenpopulatie was deze correlatie bij het rundvee niet zo uitgesproken. Een stijging in de foetale rundvee-sterfte werd eveneens waargenomen tijdens het jaar 2007 en een matige correlatie werd beschreven tussen BTV incidentie en foetale sterfte.

In hoofdstuk V werd een studie, ontworpen om het effect van de eerste vaccinatiecampagne tegen BTV te evalueren, kritisch bekeken. De resultaten toonden dat het doel van 80% vaccinatiedekking werd gehaald. De vaccinatie werd echter uitgevoerd in een populatie waarin een groot deel van de dieren reeds geïnfecteerd was. Hierdoor, en omdat er voorlopig geen serologisch onderscheid gemaakt kan worden tussen gevaccineerde en geïnfecteerde dieren, was het intrinsieke effect van de vaccinatie moeilijk te evalueren in deze steekproefopstelling.

In hoofdstuk VI werden het ontwerp en de resultaten besproken van de herhaalde cross-sectionele studies, uitgevoerd in rundvee vanaf het eind van 2010 om de afwezigheid van

BTV in België te staven. De resultaten toonden de afwezigheid van BTV circulatie in 2010, aan een verwachte prevalentie van 2% en een 95% betrouwbaarheidsniveau. De bijkomende studie naar maternale antistoffen in niet-gevaccineerde dieren toonde aan dat, aan de leeftijd van 7 maanden, BTV-specifieke maternale antistoffen bij de meeste dieren verdwenen waren.

In hoofdstuk VII werden de resultaten beschreven van een cross-sectionele serologische studie die werd georganiseerd in de schapen en geitenpopulatie om de seroprevalentie van SBV na de eerste transmissieperiode van het virus in België te bestuderen. De algemene binnen-bedrijfsseroprevalentie en de ICC (Intra-class correlation coefficient) werden geschat op respectievelijk 84.31% (95%CI: 84.19-84.43) en 0.34. De algemene bedrijfsseroprevalentie was 98.03% (95%CI: 97.86-98.18). De seroprevalentie bleek geassocieerd te zijn met de kuddedensiteit, wat de aanwezigheid van een spatiale cluster met significant lagere binnen-bedrijfsseroprevalentie verklaarde. Deze resultaten leverden het bewijs dat bijna elk Belgische schapenbeslag in contact was gekomen met SBV in 2011.

In hoofdstukken VIII en IX werd aangehaald dat, ondanks dat de bezorgdheid omtrent het mogelijk opduiken van BTV op voorhand werd gedeeld, slechts beperkte middelen en expertise beschikbaar waren in België voor 2006. Hierdoor was het land niet echt voorbereid om de problemen betreffende een door vectoren overgedragen epidemie aan te pakken. Ondanks dat de expertise van de BTV epidemie het neerstrijken van SBV niet heeft kunnen voorkomen, vergemakkelijkte ze de opvolging van de SBV epidemie aanzienlijk.

Bijkomend werden de verschillende bewakingsactiviteiten, uitgevoerd tijdens de BTV en SBV epidemieën, bekeken. Dit onderstreepte de noodzaak om continu de drie surveillance componenten - klinische presentatie, serologie/virologie en entomologie - te integreren en toonde het belang aan van het aanpassen van bewakingsactiviteiten volgens de objectieven, evoluerend met de epidemiologische fase en het niveau van epidemiologische kennis. Concrete aanbevelingen werden voorgesteld voor de verschillende bewakingsactiviteiten die in België uitgevoerd hadden kunnen worden tijdens de BTV en SBV epidemieën.