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Benefits of PCR and decentralization of diagnosis in regional laboratories in the management of Bluetongue in France

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France,
National Reference Laboratory for BT.

Summary
Since 1998, Bluetongue virus (BTV) serotypes 1, 2, 4, 6, 8, 9, 11 and 16 have spread throughout Europe. In 2006, BTV serotype 8 (BTV-8) emerged unexpectedly in Northern Europe, in countries such as Belgium, France, Germany, Luxembourg, and the Netherlands, to spread rapidly in the following year throughout the rest of Europe. In 2007, BTV-1 spread in Southern Europe, in Spain and in South of France. In 2008, 2 more BTV serotypes were detected in Northern Europe: BTV-6 in the Netherlands and in Germany, and BTV-11 in Belgium. The European incursion of BTV has caused considerable economic losses, including direct losses from mortality and reduced production, as well as indirect losses generated by ensuing bans on trade of ruminants between infected and non-infected areas. Given the significance of the disease, all affected countries have established control and eradication measures that have evolved together with the availability of detection and prevention tools such as Polymerase Chain Reaction (PCR) tests and vaccines, respectively. This paper describes how the French National Reference Laboratory for BT has managed diagnosis during the fast and massive spread of BTV-1 and 8 in 2007 and 2008.

Vantaggi dell'impiego della PCR e della diagnostica decentralizzata in laboratori regionali per la gestione della Bluetongue in Francia

Parole chiave
Bluetongue,
BTV-1,
BTV-8,
Francia,
Laboratorio di Referenza Nazionale per BT.

Riassunto
Diversi sierotipi del virus della Bluetongue (1, 2, 4, 6, 8, 9, 11 e 16) hanno circolato in Europa dal 1998. Nel 2008, il sierotipo 8 (BTV-8) è inaspettatamente apparso in Nord Europa, Belgio, Francia, Germania, Lussemburgo e Olanda per diffondersi nel 2009 nel resto d'Europa. Il sierotipo 1 (BTV-1) è stato rilevato nel 2007 in Europa meridionale, Spagna e Sud della Francia. I sierotipi 6 e 11 si sono diffusi nel 2008 in Olanda e Germania (BTV-6) e in Belgio (BTV-11). La presenza di BTV in Europa ha causato ingenti perdite economiche riferibili a danni diretti (mortalità e riduzione della produzione) e danni indiretti (blocco della movimentazione di ruminanti tra aree infette e aree non infette da BTV). Vista l'importanza delle conseguenze, tutti i paesi affetti da BT hanno posto in atto misure per il controllo e l'eradicazione della malattia. Questo articolo descrive come il Laboratorio di Referenza Nazionale francese per la BT ha gestito la diagnosi della Bluetongue durante le epidemie da BTV-1 e BTV-8 registrate nel 2007 e 2008.

Introduction

Bluetongue virus (BTV) is the causative agent of Bluetongue (BT), an insect-transmitted disease of domestic and wild ruminants (Verwoerd and Erasmus 2004, MacLachlan *et al.* 2009). Bluetongue virus is the prototype member of the genus *Orbivirus*, family *Reoviridae* (Mertens *et al.* 2004). As a reovirus, it has a segmented genome of double-stranded RNA (dsRNA) and its virions have a characteristic morphology and structure. A total of 26 BTV serotypes have been described (before 2008, only 24 serotypes were identified) on the basis of serotype-specific virus neutralization assays and genome segment 2 sequences (Hofmann *et al.* 2008b, Maan *et al.* 2011).

BTV infection occurs in most temperate and tropical regions of the world, and its epidemiology coincides with the distribution of specific species of *Culicoides* biting midges that act as biological vectors of the virus (Gibbs and Greiner 1994, Tabachnick 2004). Bluetongue typically occurs when susceptible animal species are introduced into areas where virulent strains of BTV circulate, or when virulent strains of BTV extend their range into previously unexposed ruminant populations. Historically, BTV in Europe did not occur North of parallel 40°N, however the BTV-8 epidemic extended to 53°N in 2006 (Toussaint *et al.* 2006).

From 1998 to 2006, BTV serotypes 1, 2, 4, 9, and 16 have spread throughout the Mediterranean coast of European Region (Mellor *et al.* 2008, Rodríguez-Sánchez *et al.* 2008). In 2006, BTV-8 emerged and, in the following year, it spread rapidly throughout most of Europe. In 2008, 2 more BTV serotypes were detected in Northern Europe: BTV-6 in the Netherlands and Germany, and BTV-11 in Belgium (De Clercq *et al.* 2009). So far, the European Commission has spent a large amount of money on BTV vaccination in various Member States. Nonetheless, the European incursion of BTV has caused considerable economic losses, including direct losses from mortality and reduced production along to indirect losses generated by ensuing bans on the trade of ruminants between BTV-infected and non-infected areas.

Laboratory preparedness

The origin and the conditions of introduction of the highly pathogenic strain of BTV serotype 8 that has now spread throughout most of Europe remain unknown (MacLachlan and Guthrie 2010). Since its emergence in Northern Europe, this virus has spread to the Mediterranean basin, Scandinavia, and the Middle East. This strain of BTV serotype 8 exhibits several distinctive features that are unusual amongst other field strains of BTV, notably its ability to cross the ruminant placenta with high frequency (MacLachlan and Guthrie 2010). Vertical transmission has previously been considered to be largely or exclusively a property of BTV strains modified by growth in embryonated eggs or cell culture (MacLachlan and Guthrie 2010).

In outbreak situations, especially with a novel virus (or organism), it is important to understand the full spectrum of disease, as well as the way in which the new pathogen relates to infectivity, the routes it uses to spread, and the outcomes of laboratory tests. Therefore, the laboratory response during the early stages of an outbreak focuses on development of virological and immunological methods for diagnosis, for contact tracing, and for epidemiological studies into sources (differentiation between BTV-1 or BTV-8 in French area, where both serotype were detected), modes of transmission, identification of risk groups, and screening of potential animal reservoirs. These needs were raised for BTV-8 (and BTV-1) emergence in Europe but also, few years later, with Schmallenberg virus detection.

Molecular diagnosis of BT

Conventional BTV RT-PCRs

Before 2004, the molecular diagnosis of BTV was performed by conventional Real Time Polymerase Chain Reaction (RT-PCR). However, this method has been proved to have some weaknesses (Hoffmann *et al.* 2009).

When designing primers for molecular assays, it is important to select areas of the BTV genome that are sufficiently conserved to enable the detection of all 26 serotypes and related topotypes, and which are, at the same time, sufficiently divergent from sequences of the members of both other closely related Orbivirus species (other 'serogroups') and between serotypes in the same serogroup. The degree of variation detected within individual BTV genome segments (*e.g.,* between geographic groups) complicates the selection of appropriate targets.

However, several of the more conserved BTV genome segments have previously been used as targets for molecular diagnostic methods, including: genome segment 5, encoding the highly conserved non-structural 'tubule' protein NS1 [currently recommended as an RT-PCR target by the OIE (OIE 2014)]; genome segment 7 (despite its variability), encoding the BTV core surface protein, and major BTV virus-species/serogroup-specific antigen, VP7 (Gumm and Newman 1982, Huismans 1981, Mertens *et al.* 1987), genome segment 10

(encoding NS3/3a), in which nucleotide variation between gene sequences of up to 20% have been detected (Pierce *et al.* 1998, Van Niekerk *et al.* 2003), and genome segment 1 encoding VP1, the viral RNA-dependent RNA polymerase, one of the most highly conserved proteins of reoviruses (members of the family *Reoviridae*) (Huang *et al.* 1995, Mertens and Diprose 2004).

Many of the conventional RT-PCR assays that have been developed so far are able to detect BTV RNA in clinical samples (blood and spleen), including those targeting genome segments 2, 3, 6, 7, and 10 (reviewed in Zientara *et al.* 2004). However, these methods require agar gel electrophoresis to show the amplification of the target sequence, thus limiting both the speed of testing (Hoffmann *et al.* 2009) and the number of samples that can be tested at the same time.

Real-time RT-PCRs (rRT-PCR) for the detection of BTV

Prior to the BTV-8 outbreak in Northern Europe in the Summer of 2006, few random PCR (rPCR) assays had been published for the detection of BTV (Hoffmann *et al.* 2009). The first published rRT-PCR assay for the detection of BTV used primers designed from the NS1 gene (Seg-5) (Wilson *et al.* 2004). However, this assay detected only 11 out of the 19 serotypes tested (serotypes 20-24 were not tested). During the very same year, another rRT-PCR was published using FRET-probe technology targeting genome segment 2 (VP2) (Orru *et al.* 2004, De Santis *et al.* 2004). This assay was used in Italy to differentiate wild-type BTV-2 from the vaccine strain and was able to distinguish between vaccinated and infected animals. In 2006, a rRT-PCR assay was developed using a conserved region in RNA segment 5 of BTV-2 and BTV-4 (Jimenez-Clavero *et al.* 2006). This assay detected all the recent Mediterranean isolates that were tested (including isolates of serotypes 2, 4 and 16), BTV vaccine strains for serotypes 2 and 4, and also 15 out of the 24 BTV reference strains. The primers and probe were positioned in a part of segment 5 that had various mismatches, reducing the sensitivity of the assay. Moreover, the assay was only able to detect field isolates (but not the reference strain) of BTV-4 and BTV-16, and in the recent Northern European outbreak, BTV-8. This assay showed a reduced sensitivity for the detection of the field strain of BTV-8 compared to other assays (Batten *et al.* 2008a). Subsequent studies have indicated that the field strain of BTV-16 from Italy was a reassortant virus, containing genome segment 5 derived from a BTV-2 vaccine strain (Batten *et al.* 2008b), potentially explaining the differences in the sensitivity of the assay for field and reference strains of this serotype. In 2006, a quantitative rRT-PCR was also developed using a Molecular Beacon (MB) fluorescent probe designed within the NS3 conserved region of segment 10 (Orru *et al.* 2006). This assay successfully detected 10 serotypes (BTV-1, 2, 3, 4, 5, 6, 7, 8, 9, 16) (Hoffmann *et al.* 2009).

Since the start of the Northern European outbreak in August 2006, many rRT-PCR assays have been developed and are currently being used in many countries across Europe (all the rRT-PCR assays that have been published up to 2008 are described in Table I). Some assays remain unpublished and, at the time of writing, 3 published rRT-PCR assays are able to detect representative strains from all 24 serotypes of BTV, 2 of these assays were described in the same article published by Tussaint and colleagues

Table I. *Published real-time RT-PCR assays for the detection of BTV (from Hoffmann* et al. *2009).*

PCR target	Detection method	Reference	Strain identification
BTV Segment 1 (VP1)	Duplex RT-PCR	Shaw *et al*. (2007)	Representatives from all 24 serotypes, and multiple field strains/topotypes Able to differentiate between 'eastern' and 'western' strains of BTV
BTV Segment 1 (VP1)	One step RT-PCR	Toussaint *et al.* (2007)	Representatives from all 24 serotypes and field strains from Mediterranean basin
BTV Segment 5 (NS1)	Two step RT-PCR	Toussaint *et al.* (2007)	Representatives from all 24 serotypes and field strains from Mediterranean basin
BTV Segment 5 (NS1)	One step RT-PCR	Polci *et al.* (2007)	Representative from serotypes 2, 4, 9 and 16 isolated in Italy and their respective vaccine strains
BTV Segment 5 (NS1)	One step RT-PCR	Jimenez-Clavero *et al.* (2006)	Representatives from 17 serotypes (BTV-1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 14, 15, 16, 17, 18, 22, 23)
BTV Segment 5 (NS1)	One step RT-PCR	Wilson *et al.* (2004)	Representatives from 11 serotypes (BTV-1, 5, 6, 7, 8, 10, 11, 13, 17, 18, 19)
BTV Segment 10 (NS3)	MB fluorescent probe	Orru *et al.* (2006)	Representatives from 10 serotypes (BTV-1, 2, 3, 4, 5, 6, 7, 8, 9, 16)
BTV Segment 2 (VP2)	FRET RT-PCR	De Santis *et al.*, 2004 and Orru *et al.*, 2004	Differentiates field and vaccine strain of BTV-2

MB = molecular beacon; FRET = fluorescence resonance energy transfer.

(Toussaint *et al.* 2007). These 2 assays detect strains of the different BTV serotypes from the Mediterranean region, as well as prototype strains of 24 serotypes (BTV-25 and 26 were not discovered). Both assays have similar detection limits and detect 100 RNA copies. The primers were designed to target different genomic segments in a one-step procedure to amplify BTV genome segment 1, and a two-step procedure to amplify genome segment 5. These assays have the advantage of a quantitative output, which gives an estimate of viral load and both assays include an internal beta-actin control (Toussaint *et al.* 2007).

A further duplex rRT-PCR assay has been recently published by Shaw and colleagues (Shaw *et al.* 2007). The authors designed 2 sets of primers and probes to target segment 1 of Eastern and Western BTV. This combined 'duplex' assay was evaluated using a wide variety of test samples, including tissue culture-derived viruses, infected tissue, and blood samples from cattle and sheep and infected *Culicoides* midges. This assay showed no cross-reactions with closely related orbiviruses and gave positive results with all the viruses tested, including a wide range of 129 different BTV isolates derived from different geographical locations (different topotypes), reference strains of all 24 BTV serotypes, and multiple field strains of BTV serotypes 1, 2, 4, 8, 9, and 16 from European outbreaks that have occurred since 1998 (although the BTV-9 vaccine and a Sicilian isolate of BTV-9 gave weaker but still positive responses). This assay was able to detect less than 10 template copies per reaction. Differences in the sequences of Seg-1 also made it possible to use separately the 2 primer sets from the duplex assay to distinguish viruses of Eastern and Western origins (Hoffmann *et al.* 2009).

A one step rRT-PCR method has also been described (Polci *et al.* 2007). This method detects strains of BTV serotypes 2, 4, 9, and 16 isolated in Italy as well as their respective vaccine strains. Preliminary tests showed that this assay was able to identify serotype 1, which was introduced into Italy towards the end of 2006, and also serotypes 8, 13, 14, and 19 (Hoffmann *et al.* 2009).

Given the high variability of BTV and the fact that real-time probes are very sensitive to probe-target mismatches (Jimenez-Clavero *et al.* 2006), it is important to emphasise that these assays may not be able to recognise all BTV strains and that the sensitivity of the assays may not be the same for every strain. It would thus be wise, as emphasised by Toussaint and colleagues (Toussaint *et al.* 2007), to run 2 methods that amplify different genomic regions in parallel, as this would avoid the risk of missing a mutant, a recombinant or a reassortant strain. The OIE BT Reference Laboratories recommend to use rRT-PCR assays targetting BTV segment 10 (NS3). The method described in OIE manual is an adaption from Hofmann *et al.* (2008a) and is proven to be capable of detecting all known BTV serotypes and strains currently circulating. Results of PCR-based BTV diagnosis need to be interpreted with caution, as BTV RNA has been detected in blood from both infected cattle and sheep for at least 30 days, and sometimes up to 90 days, after virus isolation has ceased to be positive (Bonneau *et al.* 2002, Katz *et al.* 1993, MacLachlan 1994). Thus detection of virus-specific nucleic acid by these methods indicates a recent virus infection, but it does not necessarily indicate the presence of infectious virus in the animal (Hoffmann *et al.* 2009).

Role of the National Reference Laboratories

Following the discovery of BTV-8 in the North of Europe, molecular detection methods were developed by several groups and deployed internationally, through an international collaborative laboratory response. The generally preferred diagnostic method for detection of BTV [or other orbiviruses like Epizootic hemorrhagic disease virus (EHDV) or African Horse sickness virus (AHSV)] is rRT-PCR.

Before the emergence of BTV-8 in 2006 in Europe (Maan *et al.* 2008), rRT-PCR was (almost) not used for BTV detection. The conventional RT-PCR followed by sequencing was the methodology used by the majority of the National Reference Laboratories (NRLs). Since then, there are published and commercial rRT-PCR tests for BTV and published rRT-PCR tests for EHDV (Toussaint *et al.* 2007, Sailleau *et al.* 2012, Hoffmann *et al.* 2009, Wilson *et al.* 2009, Clavijo *et al.* 2010). There is also a PCR array for BTV serotype determination (Maan *et al.* 2012). Every year, a ring-test organized by the European Reference Laboratory for Bluetongue (at the Pirbright Institute, UK) assesses these different molecular tools used in the NRLs of many European countries. The conclusions of these ring-tests allow each NRL to assess and compare its molecular diagnostic method and to choose the best molecular tools adapted for detection of specific serotypes that are spreading or may emerge.

Network of regional laboratories

In Autumn 2007, BTV-8 spread from Belgium to France, while BTV-1 spread from Spain to France (Figure 1). The French Ministry of Agriculture asked veterinarians to send samples to the BT NRL (Animal health laboratory of Maisons-Alfort). During this period, we received 2 to 300 samples per day for

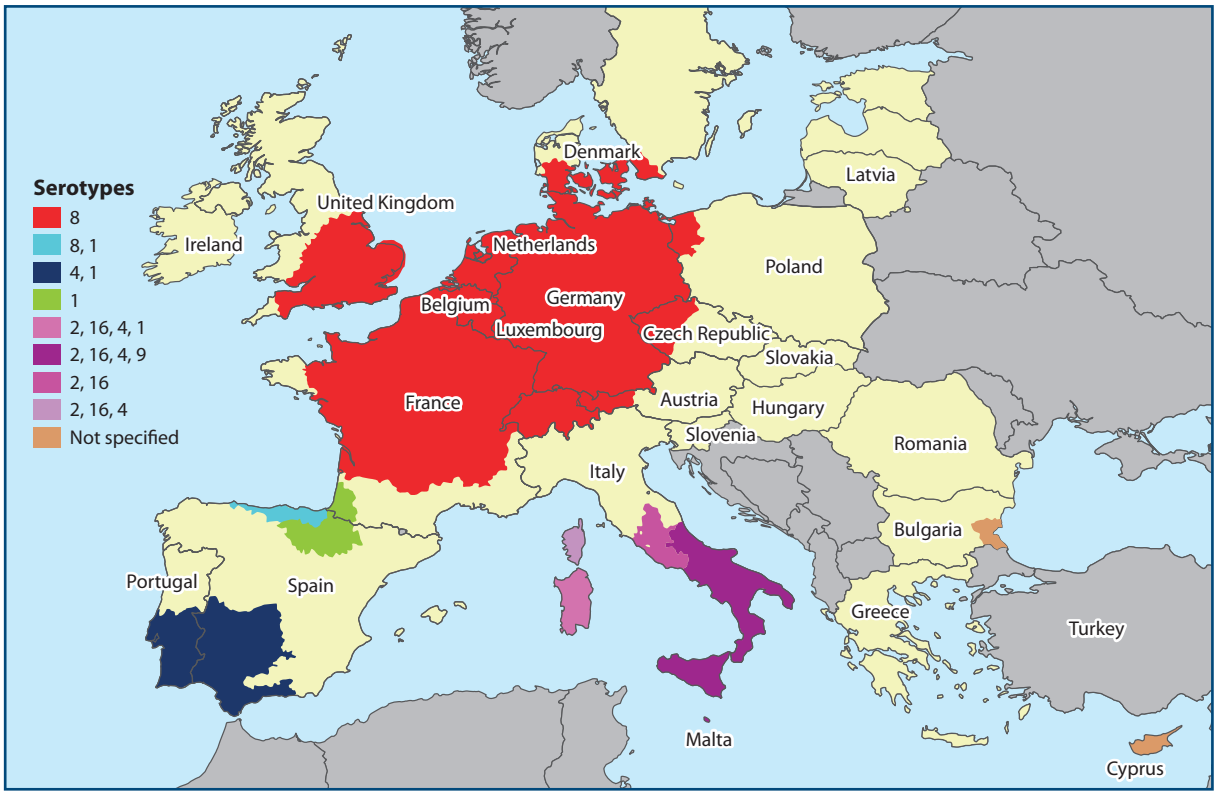


Figure 1. *Bluetongue virus distribution in Europe in 2007.*

testing by rRT-PCR, although the technical staff was only composed of 2 people.

Moreover, the spread of BTV-8 to the centre of France induced a ban on the trade from France to the North Italy. It is worth noticing that each Autumn more than 500,000 cattle are transported from France to Italy for fattening. Given the high economic and health consequences of the outbreak, the French Ministry of Agriculture asked the NRL to propose measures to rapidly manage this trade.

In 2007, we used the rRT-PCR (Toussaint *et al.* 2007) that we had developed in collaboration with the Centrum voor Onderzoek in Diergeneeskunde en Agrochemie (CODA-CERVA, Dr Kris de Clercq). We rapidly contacted some private companies involved at that time in selling PCR kits. LSI (Lyon, France, Dr Eric Sellal) and AES-ADIAGENE (Saint-Brieuc, France, Dr Béatrice Blanchard) answered positively to our request. For 2 months we collaborated with the 2 companies to transfer knowledge and reference materials (BTV RNAS, positive and negative samples, strains) and to validate the kits. As soon as the ANSES NRL laboratory validated the kits (based on the detection of a portion of the segment 5 of each 24 BTV serotypes), it contacted the French regional laboratories under the authority of the Ministry of Agriculture.

More than 60 regional laboratories agreed to be part of this BTV network. The French NRL organized in a few days a training session for the technical staff of the regional labs and a ring-test, focusing particularly on the ability of these kits to be automated.

Two months after the NRL was solicited by the veterinary authorities, a network of 67 labs was constituted and was able to test in good conditions hundred of thousands of blood samples per day. All the cattle that needed to be exported to Italy were tested by rRT-PCR. Moreover, many samples were tested for diagnosis, surveillance, or international trade.

With the introduction of BTV-1 in 2007 in the South of France, it was important to follow the spread of these 2 serotypes. Specific rRT-PCRs for BTV-8 and 1 were developed by LSI and AES-ADIAGENE companies and validated by the French NRL. These kits and methods were then decentralized in the regional labs.

Since 2007, and until 2011, a national ring-test organized by the French NRL allowed for controlling the performance of these labs for the detection of all BTV serotypes and the differentiation between BTV-1 and 8. This proved the efficacy of the existence of a wide network for the first time in France in 2007 for the detection and surveillance of a notifiable disease.

In conclusion is worthwhile stressing that the collaboration and networks (EPIZONE, European Union reference laboratory organized meetings)

developed between all the NRLs in Europe have really helped and facilitated the development of new methods to detect and characterize this new virus. This is fundamental to maintain and reinforce such networks.

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References

Batten C.A., Bachanek-Bankowska K., Bin-Tarif A., Kgosana L., Swain A.J., Corteyn M., Darpel K., Mellor P.S., Elliot, H.G. & Oura C.A. 2008a. Bluetongue virus: European Community inter-laboratory comparison tests to evaluate ELISA and RT-PCR detection methods. *Vet Microbiol*, **129** (1-2), 80-88.

Batten C.A., Maan S., Shaw A.E., Maan N.S. & Mertens P.P. 2008b. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Res*, **137**, 56-63.

Bonneau K.R., DeMaula C.D., Mullens B.A. & MacLachlan N.J., 2002. Duration of viraemia infectious to *Culicoides sonorensis* in Bluetongue virus-infected cattle and sheep. *Vet Microbiol*, **88**, 115-125.

Clavijo A., Sun F., Lester T. Jasperson D. C. & Wilson W. C. 2010. An improved real-time polymerase chain reaction for the simultaneous detection of all serotypes of Epizootic hemorrhagic disease virus. *J Vet Diagn Invest,* **22** (4), 588-593.

De Clercq K., Mertens P., De Leeuw I., Oura C., Houdart P., Potgieter A.C., Maan S., Hooyberghs J., Batten C., Vandemeulebroucke E., Wright I.M., Maan N., Riocreux F., Sanders A., Vanderstede Y., Nomikou K., Raemaekers M., Bin-Tarif A., Shaw A., Henstock M., Bréard E., Dubois E., Gastaldi-Thiéry C., Zientara S., Verheyden B. & Vandenbussche F. 2009. Emergence of bluetongue serotypes in Europe, part 2: the occurrence of a BTV-11 strain in Belgium. *Transbound Emerg Dis*, **56** (9-10), 355-361.

De Santis P., Orru G., Solinas F., Piras V., Savini G. & Caporale V. 2004. Molecular differentiation of field and vaccine strains of bluetongue virus serotype 2 using the real-time polymerase chain reaction and fluorescence resonance energy transfer hybridisation probe. *Vet Ital*, **40**, 572-576.

Gibbs E.P. & Greiner E.C. 1994. The epidemiology of bluetongue. *Comp Immunol Microbiol Infect Dis*, **17**, 197-206.

Gumm I.D. & Newman J.F. 1982. The preparation of purified bluetongue virus group antigen for use as a diagnostic reagent. *Arch Virol*, **72**, 83-93.

Hoffmann B., Beer M., Reid S.M., Mertens P., Oura C.A., van Rijn P.A., Slomka M.J., Banks J., Brown I.H., Alexander D.J. & King D.P. 2009. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Vet Microbiol*, **139** (1-2), 1-23.

Hofmann M., Griot C., Chaignat V., Perler L. & Thür B. 2008a. Blauzungenkrankheit erreicht die Schweiz. *Schweiz Arch Tierheilkd*, **150** (2), 49-56. doi: 10.1024/0036-7281.150.2.49.

Hofmann M.A., Renzullo S., Mader M., Chaignat V., Worva G. & Thuer B. 2008b. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg Infect Dis*, **14** (12), 1855-1861.

Huang I.J., Hwang G.Y., Yang Y.Y., Hayama E. & Li J.K. 1995. Sequence analyses and antigenic epitope mapping of the putative RNA-directed RNA polymerase of five U.S. bluetongue viruses. *Virology*, **214**, 280-288.

Huismans H. & Erasmus B.J. 1981. Identification of the seroype-specific and group specific antigens of Bluetongue virus. *Onderstepoort J Vet Res*, **48** (2), 51-58.

Jimenez-Clavero M.A., Aguero M., San Miguel E., Mayoral T., Lopez M.C., Ruano M.J., Romero E., Monaco F., Polci A., Savini G. & Gomez-Tejedor C. 2006. High throughput detection of bluetongue virus by a new real-time fluorogenic reverse transcription-polymerase chain reaction: application on clinical samples from current Mediterranean outbreaks. *J Vet Diagn Invest*, **18**, 7-17.

Katz J.B., Alstad A.D., Gustafson G.A. & Moser K.M. 1993. Sensitive identification of bluetongue virus serogroup by a colorimetric dual oligonucleotide sorbent assay of amplified viral nucleic acid. *J Clin Microbiol*, **31**, 3028-3030.

Maan S., Maan N.S., Nomikou K., Veronesi E., Bachanek-Bankowska K., Belaganahalli M.N., Attoui H. & Mertens P.P. 2011. Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait. *PLoS One*, **6** (10), e26147. doi: 10.1371/journal.pone.0026147.

Maan S., Maan N.S., Ross-smith N., Batten C.A., Shaw A.E., Anthony S.J., Samuel A.R., Darpel K.E., Veronesi E., Oura C.A., Singh K.P., Nomikou K., Potgieter A.C., Attoui H., van Rooij E., van Rijn P., De Clercq K., Vandenbussche F., Zientara S., Bréard E., Sailleau C., Beer M., Hoffman B., Mellor P.S. & Mertens PP. 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology*, **377** (2), 308-318. doi: 10.1016/j.virol.2008.04.028.

Maan N.S., Maan S., Belaganahalli M.N., Ostlund E.N., Johnson D.J., Nomikou K. & Mertens P.P. 2012. Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. *PLoS One*, **7** (2), e32601. doi: 10.1371/journal.pone.0032601.

MacLachlan N.J. & Guthrie A.J. 2010. Re-emergence of bluetongue, African horse sickness, and other Orbivirus diseases. *Vet Res*, **41** (6), 35. doi: 10.1051/vetres/2010007.

MacLachlan N.J., Drew C.P., Darpel K.E. & Worwa G. 2009. The pathology and pathogenesis of bluetongue. *J Comp Pathol*, **141**, 1-16.

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

Mellor P.S., Carpenter S., Harrup L., Baylis M. & Mertens P.P. 2008. Bluetongue in Europe and the Mediterranean basin: history of occurrence prior to 2006. *Prev Vet Med*, **87**, 4-20.

Mertens P.P., Diprose J., Maan S., Singh K.P., Attoui H. & Samuel A.R. 2004. Bluetongue virus replication, molecular and structural biology. *Vet Ital*, **40**, 426-437.

Mertens P.P. & Diprose J. 2004. The bluetongue virus core: a nano-scale transcription machine. *Virus Res*, **101** (1), 29-43.

Mertens P.P., Pedley S., Cowley J. & Burroughs J.N. 1987. A comparison of six different bluetongue virus isolates by cross-hybridization of the dsRNA genome segments. *Virology*, **161**, 438-447.

Orru G., Ferrando M.L., Meloni M., Liciardi M., Savini G. & De Santis P. 2006. Rapid detection and quantification of bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. *J Virol Methods*,**137** (1), 34-42.

Orru G., De Santis P., Solinas F., Savini G., Piras V. & Caporale V. 2004. Differentiation of Italian field and South African vaccine strains of bluetongue virus serotype 2 using real-time PCR. *J Virol Methods*, **122**, 37-43.

Pierce C.M., Balasuriya U.B. & MacLachlan N.J. 1998. Phylogenetic analysis of the S10 gene of field and laboratory strains of bluetongue virus from the United States. *Virus Res*, **55**, 15-27.

Polci A., Cammà C., Serini S., Di Gialleonardo L., Monaco F. & Savini G. 2007. Real-time polymerase chain reaction to detect bluetongue virus in blood samples. *Vet Ital*, **43** (1), 77-87.

Rodríguez-Sánchez B., Iglesias-Martín I., Martínez-Avilés M. & Sánchez-Vizcaíno J.M. 2008. Orbiviruses in the Mediterranean basin: updated epidemiological situation of bluetongue and new methods for the detection of BTV serotype 4. *Transbound Emerg Dis*, **55** (5-6), 205-214.

Sailleau C., Zanella G., Breard E., Viarouge C., Desprat A., Vitour D., Adam M., Lasne L., Martrenchar A., Bakkali-Kassimi L., Costes L. & Zientara S. 2012. Co-circulation of bluetongue and epizootic haemorrhagic disease viruses in cattle in Reunion Island. *Vet Microbiol*, **155**, 191-197.

Shaw A.E., Monaghan P., Alpar H.O., Anthony S., Darpel K.E., Batten C.A., Guercio A., Alimena G., Vitale M., Bankowska K., Carpenter S., Jones H., Oura C.A., King D.P., Elliott H., Mellor P.S. & Mertens P.P. 2007. Development and initial evaluation of a realtime RT-PCR assay to detect bluetongue virus genome segment 1. *J Virol Methods*, **145** (2), 115-126.

Tabachnick W.J. 2004. *Culicoides* and the global epidemiology of bluetongue virus infection. *Vet Ital*, **40**, 145-150.

Toussaint J.F., Vandenbussche F., Mast J., De Meester L., Goris N., Van Dessel W., Vanopdenbosche E., Kerkhofs P., De Clercq K., Zientara S., Sailleau C., Czaplicki G., Depoorter G. & Dochy J.M. 2006. Bluetongue in northern Europe. *Vet Rec*, **2**, 327.

Toussaint J.F., Sailleau C., Breard E., Zientara S. & de Clercq K. 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods*, **140** (1-2), 115-123.

Van Niekerk M., Freeman M., Paweska J.T., Howell P.G., Guthrie A.J., Potgieter A.C., van Staden V. & Huismans H. 2003. Variation in the NS3 gene and protein in South African isolates of bluetongue and equine encephalosis viruses. *J Gen Virol,* **84** (Pt 3), 581-590.

Verwoerd D. & Erasmus B.J. 2004. Bluetongue. *In* Infectious Diseases of Livestock 2nd Edition (Coetzer J.A. & Tustin R.C. eds). Oxford University Press, Cape Town, South Africa, 1201-1220.

Wilson W.C., Stallknecht D.E. & Mecham J.O. 2004. Field–deployable real time polymerase chain reaction detection of bluetongue and epizootic haemorrhagic disease viral ribonucleic acid. *Vet Ital*, **40** (4), 587-593.

World Organisation for Animal Health (OIE). 2014. Bluetongue *In* Manual of Diagnostic Tests and Vaccines for Terrestrial animals. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.03_BLUETONGUE.pdf

Wilson W.C., Hindson B.J., O'Hearn E.S., Hall S., Tellgren-Roth C., Torres C., Naraghi-Arani P., Mecham J.O. & Lenhoff R.J. 2009. A multiplex real-time reverse transcription polymerase chain reaction assay for detection and differentiation of Bluetongue virus and Epizootic hemorrhagic disease virus serogroups. *J Vet Diagn Invest*, **21** (6), 760-770.

Zientara S., Bréard E. & Sailleau C. 2004. Bluetongue diagnosis by reverse transcriptase-polymerase chain reaction. *Vet Ital*, **40** (4), 531-537.