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

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Riassunto
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).

Bluetongue virus 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...
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 fluorescence resonance energy transfer (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, BTV-2, BTV-3, BTV-4, BTV-5, BTV-6, BTV-7, BTV-8, BTV-9, BTV-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

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

MB = molecular beacon; FRET = fluorescence resonance energy transfer.
same article published by Tussaint and colleagues (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 BTV-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 haemorrhagic 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
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 BTV-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 BTV-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)
Management of BT in France  

Zientara et al.

References


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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