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Evaluating the most appropriate pooling ratio for EDTA blood samples to detect Bluetongue virus using real-time RT-PCR

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ABSTRACT

The control of Bluetongue virus (BTV) presents a significant challenge to European Union (EU) member states as trade restrictions are placed on animals imported from BTV-affected countries. BTV surveillance programs are costly to maintain, thus, pooling of EDTA blood samples is used to reduce costs and increase throughput. We investigated different pooling ratios (1:2, 1:5, 1:10 and 1:20) for EDTA blood samples to detect a single BTV positive animal. A published real-time RT-PCR assay (Hofmann et al., 2008) and a commercial assay (ThermoFisher VetMax™ BTV NS3 kit) were used to analyse BTV RNA extracted from pooled EDTA blood samples. The detection rate was low for the onset of infection sample (0–2 days post infection (dpi); C_T 36) irrespective of the pooling ratio. Both assays could reliably detect a single BTV-positive animal at early viraemia (3–6 dpi; C_T 33) when pooled, however, detection rate diminished with increasing pooling ratio. A statistical model indicated that pooling samples up to 1:20, is suitable to detect a single BTV positive animal at peak viraemia (7–12 dpi) or late infection (13–30 dpi) with a probability of detection of > 80% and > 94% using the Hofmann et al. (2008) and VetMAX assays, respectively. Using the assays highlighted in our study, pooling at ratios of 1:20 would be technically suitable in BTV-endemic countries for surveillance purposes. As peak viraemia occurs between 7–12 days post infection, a 1:10 pooling ratio is appropriate for post-import testing when animals are sampled within a similar time frame post-import.

1. Background

Bluetongue (BT) is an infectious haemorrhagic disease of ruminants, caused by bluetongue virus (BTV) of the genus Orbivirus within the family Reoviridae (Maclachlan et al., 2009). BTV is a serologically and genetically diverse virus that is transmitted between ruminant hosts via Culicoides biting midges (Carpenter et al., 2013). BT is a notifiable disease and trade restrictions are placed on countries which have active BTV infection within EU member states (Anonymous, 2000). Since 2007, a variety of BTV serotypes (BTV-1, BTV-2, BTV-4 BTV-8, BTV-9, and BTV-16 have circulated within the EU and have incurred animal movement restrictions (Belbis et al., 2017; Niedbalski, 2015). In addition to these "typical" BTV serotypes, BTV-25, BTV-26 and BTV-27 represent a novel clade of serotypes which circulate predominately in goats but which unlike other BTV serotypes, can be transmitted directly (Batten et al., 2014; Bréard et al., 2018). Although vaccines are available for BTV, only a limited number of inactivated BTV vaccines (BTV-1, BTV-2, BTV-4 and BTV-8) are approved for use within the EU (More et al., 2017). Therefore, control measures for BTV largely rely on animal movement restrictions and appropriate surveillance and testing programmes.

Real-time RT-PCR is the main diagnostic test for the detection of BTV in ruminants and allows competent authorities to control animal trade within the EU. The significant cost of BTV surveillance programmes represents a financial burden for member states and their respective diagnostic laboratories (Pinior et al., 2015). To reduce some of the costs associated with surveillance programmes, pooling of EDTA blood samples is practiced in a number of laboratories (Vandenbussche et al., 2008a). However, the most appropriate pooling ratio to allow for the detection of a single BTV-positive animal within a pool has not been fully determined. A previous report (Vandenbussche et al., 2008a) found that a large percentage of samples containing high C_T values (C_T > 35) would not be detected using a pooling ratio (defined as 1/ n where n is the number of samples constituting the pool) of 1:10 if animals were at an early stage of viraemia. Another study found that pooling of samples taken at peak viraemia would allow for detection of a BTV–positive animal but that pooling of samples taken at onset of infection would reduce the detection ability of the assay (Batten et al., 2009). The real-time RT-PCR assays investigated in both studies (Shaw et al., 2007; Toussaint et al., 2007) were designed to detect BTV

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serotypes 1–24, however, since that time a number of new serotypes have been identified (Hofmann et al., 2008; Schulz et al., 2016) which may present a detection challenge for those assays.

Recently, a number of real-time RT-PCR assays have been recognised as being broadly-reactive whilst achieving the greatest sensitivity and have thus been adopted by a large proportion of BT-testing laboratories. Within its remit as European Union Reference Laboratory for BT, The Pirbright Institute organises an annual inter-laboratory proficiency test (PT) to assess the diagnostic assays in use in EU member state national reference laboratories (NRLs) and representative laboratories of third countries. During the 2016 PT, the Hofmann et al. (2008) assay and the commercial assay ThermoFisher VetMAX™ BTV NS3 kit (hereafter: VetMAX assay) were identified as being the most-widely used whilst yielding the greatest analytical sensitivity.

This study describes an investigation into the most appropriate pooling ratio for EDTA blood samples to allow for the detection of a single BTV infected animal at four different stages of BTV infection. The C_T values for onset of infection C_T 36 (0–2 dpi), early viraemia C_T 33 (3 to 6 dpi), peak viraemia C_T 27 (7 to 12 dpi) and late infection C_T 30 (13–30 dpi) were ascribed based on a review of BTV animal infection studies. EDTA blood samples containing BTV levels representative of different stages of BTV infection were pooled at 1:2, 1:5, 1:10 and 1:20 ratios and tested using the Hofmann et al. (2008) and VetMAX assays. The results from our study would support a risk-based rationale for using various pooling ratios in different BTV epidemiological situations.

2. Methods

2.1. Review of BTV experimental infection studies

A review of data from BTV experimental infection studies was performed to yield a range of C_T values recorded over a 30-day BTV-infection period for bovines, ovines and caprines. Google scholar was used to source articles published since 2007 containing the following search parameters; “Bluetongue virus”, “infection”, “kinetics”, “experimental”, “pathological”, “real-time RT-PCR”. Articles were further screened for those reporting C_T values either in the article text or easily interpretable from figures. Eleven articles were chosen which represented a total of 12 experimental BTV infection studies in bovines (n = 4), ovines (n = 6) and caprines (n = 2). C_T values greater than 40 were ascribed as undetected (Undet.), therefore, the data compiled from the 12 experimental studies only considers C_T values lower than 40 (Table 1). The studies involved infection with BTV serotypes BTV-1 BTV-4, BTV-6, BTV-8 and BTV-26.

2.2. BTV isolates used for investigation and preparation of samples

Based on the current BTV situation in Europe, three recent European BTV serotypes (BTV-1, BTV-4 and BTV-8) were obtained from the Orbivirus Reference Collection held at The Pirbright Institute. Five-hundred microliters of the isolates: SPA2014/08 (BTV-1), ROM2014/06 (BTV-4) and FRA2015/01 (BTV-8) were diluted in BTV-negative bovine EDTA blood to yield a representative BTV C_T value for four stages of BTV infection: onset of infection, early viraemia, peak viraemia and late infection. To create the pools, 500 µl of the neat sample (the representative C_T value sample) was added to an appropriate volume of BTV-negative bovine EDTA blood to simulate pooling at different ratios of 1:2, 1:5, 1:10 and 1:20. A pooling ratio of 1:20 for the 0–2 dpi sample was not performed as it was assumed that the real-time RT-PCR assay would not detect BTV in these samples.

2.3. RNA extraction and real-time RT-PCR analysis

BTV RNA was extracted from 100 µl of EDTA blood using the KingFisher Flex automated extraction platform (ThermoFisher Scientific, Paisley, UK) and the MagVet Universal nucleic acid extraction kit (ThermoFisher) and RNA was eluted into 80 µl. Neat EDTA blood samples were extracted in triplicate while each of the pooled EDTA blood samples (1:2, 1:5, 1:10 and 1:20) were extracted and tested in ten replicates. Five microliters of BTV RNA was denatured at 95 °C for 5 min prior to analysis using two different real-time RT-PCR assays. The VetMAX assay was used according to the manufacturer’s instructions. The Hofmann et al., (2008) assay was performed using 15 µl of the reaction mix using the Express One-Step Superscript qRT-PCR kit (LifeTechnologies, Paisley, UK) containing 1 × reaction mix, 400 nM forward and reverse primers, 200 nM probe, 0.5 µl Rox, and 2 µl of enzyme mix in each well. Cycling conditions were as follows: reverse transcription at 50 °C for 15 min and 95 °C for 20 s min, and then 45 cycles of PCR, with each cycle consisting of 95 °C for 3 s, 56 °C for 30 s and 72 °C for 1 min. Real-time RT-PCR was performed on an Applied Biosystems 7500 Fast instrument (LifeTechnologies) using the fast ramp rates to provide results within 90 min for both assays.

2.4. Statistical analysis

Results of the pooling investigation were analysed using generalized linear models with binomial errors and a logit link function. The response variable was proportion of pools positive and explanatory variables were “assay” (Hofmann assay vs VetMAX assay), “C_T value of the positive sample”, “serotype” (BTV-1, BTV-4 or BTV-8) and “pooling ratio” (neat, 1:2, 1:5, 1:10, 1:20). The initial model was one including all explanatory variables as additive effects and two- and three-way interactions between “assay”, “C_T value” and “serotype”. Model selection proceeded by stepwise deletion of non-significant ($P < 0.05$) terms in the model, as judged by likelihood ratio tests. The final model

Table 1
Mean C_T values recorded during experimental BTV infection.
Data compiled from (Batten et al., 2014; Batten et al., 2013; Dal Pozzo et al., 2009; Darpel et al., 2016; Darpel et al., 2007; Eschbaumer et al., 2010; Hoffmann et al., 2008; Moulin et al., 2012; Schulz et al., 2017; Sluijs et al., 2013; Worwa et al., 2010).

Representative dpi	Bovine Mean C_T value ± SD	Ovine	Caprine
0	Undet. ± n.a.	Undet. ± n.a.	Undet. ± n.a.
1	34.66 ± 2.09	n.d. ± n.a.	n.d. ± n.a.
2	33.69 ± 1.57	34.55 ± 4.61	37.20 ± n.a.
3	34.42 ± 4.11	33.48 ± 3.13	n.d. ± n.a.
4	33.44 ± 4.07	26.86 ± 6.33	34.60 ± 1.53
5	28.97 ± 4.45	25.95 ± 4.10	n.d. ± n.a.
6	28.37 ± 3.55	23.35 ± 3.96	n.d. ± n.a.
7	25.36 ± 2.83	23.66 ± 3.13	23.90 ± 1.08
8	25.82 ± 3.87	23.92 ± 2.03	n.d. ± n.a.
9	26.49 ± 3.10	26.39 ± 1.94	22.30 ± 0.58
10	26.33 ± 3.66	25.69 ± 2.90	n.d. ± n.a.
11	28.00 ± n.a.	22.33 ± 2.69	21.27 ± 1.22
12	27.48 ± 3.63	26.46 ± 2.44	n.d. ± n.a.
13	26.13 ± 4.02	27.25 ± 1.77	n.d. ± n.a.
14	26.85 ± 3.29	25.28 ± 3.31	23.64 ± 0.62
15	n.d. ± n.a.	32.5 ± 2.12	n.d. ± n.a.
16	25.43 ± 1.80	26.94 ± 2.46	26.19 ± 2.89
17	28.26 ± 6.09	n.d. ± n.a.	n.d. ± n.a.
18	28.79 ± 3.96	27.83 ± 4.20	25.13 ± 2.18
19	n.d. ± n.a.	29.62 ± 3.58	n.d. ± n.a.
20	n.d. ± n.a.	26.75 ± 2.17	n.d. ± n.a.
21	28.91 ± 4.59	26.77 ± 3.15	27.54 ± 2.26
22	n.d. ± n.a.	29.72 ± 4.57	n.d. ± n.a.
23	27.83 ± 3.38	29.85 ± 2.53	28.71 ± 1.77
24	25.00 ± n.d.*	30.90 ± 3.59	n.d. ± n.a.
25	32.48 ± 5.19	30.51 ± 2.15	30.53 ± 0.32
26	n.d. ± n.a.	25.34 ± 1.66	n.d. ± n.a.
27	28.98 ± 0.59	30.90 ± 2.22	n.d. ± n.a.
28	n.d. ± n.a.	n.d. ± n.a.	29.23 ± 2.45
29	n.d. ± n.a.	25.17 ± 2.46	n.d. ± n.a.
30	27.46 ± 1.23	29.42 ± 1.30	30.00 ± 0.75

dpi. days post infection, Undet., undetected, n.d. no data available.
* based on a single C_T value, n.a. not applicable.