Investigating Superinfection Exclusion Between Two Important Bluetongue Virus Serotypes

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Introduction

Bluetongue virus (BTV) is a vector-borne virus transmitted between ruminant hosts by *Culicoides* biting midges. BTV causes the severe haemorrhagic disease Bluetongue. Its genome is divided into 10 segments of double stranded (ds)RNA and there are currently 28 serotypes of BTV. The virus can reassort these segments which has led to high diversity. The generation of re-assorted progeny has been shown *in vitro* using a simultaneous co-infection of 2 BTV serotypes (superinfection). However, in the field it is more likely that these infections would be asynchronous, and studies showed that *in vitro* this limited the ability of the virus to produce re-assorted progeny (superinfection exclusion). Two strains of BTV were studied in synchronous and asynchronous infections in BTV-relevant cell lines using a variety of molecular and bioimaging techniques. We hope to better understand the conditions needed for reassortment. The serotypes used are known to cocirculate and present a high risk to the UK.

Methods

BTV strain MOR2007/01 from serotype 1 (BTV-1) and BTV serotype 8 (BTV-8), strain UK2007/01, were obtained from the dsRNA virus collection at the Pirbright Institute. Virus working stocks for this study were generated by adapting each virus to either mammalian cells or *Culicoides* KC cells. These were then infected onto BFA (Bovine Foetal Aortal) or KC cells at an MOI of 0.1. For synchronous infections both serotypes were added at the same time. For asynchronous infections the second virus was added after increasing periods of time. Viral RNA was extracted using a Kingfisher and Ct values determined using a BTV Segment 2 duplex qRT-PCR.

Visualisation of serotype-specific viral mRNA was carried out using fluorescent *in situ* hybridisation (FISH) adapted from the Stellaris® RNA FISH Protocol for adherent cells with probes designed for segment 2 of each serotype. BTV-1 probes used FAM (blue colour) and BTV-8 probes used Cal Flour 590 (red colour). The images were taken on a laser scanning confocal microscope.

Results

Superinfection exclusion was shown to occur in BTV infections. Initially, both BTV-1 and BTV-8 were modelled in BTV relevant cell lines (Figure 1) to confirm both were capable of replicating together. Once a delay to the of the second virus was added, the CT of the virus started to drop (Figure 2A). Both viruses were capable of excluding each other (Figure 2B), however the time taken to completely exclude appears to be strain specific on a cell culture level.

Superinfection Exclusion was also observed in insect cells (Figure 3). With a 24 hour delay there was complete exclusion of the second virus. A loss of the second virus at zero hours addition further suggests that virus binding could be being inhibited. This was also noted in the BFA cells with a 24 hour delay.

FISH probes were designed which are serotype-specific and show no cross reactivity (Figure 4A and 4B). Two serotypes were shown to be replicating in the same cell at the same time indicated by the combined red and green labels (Figure 4C).

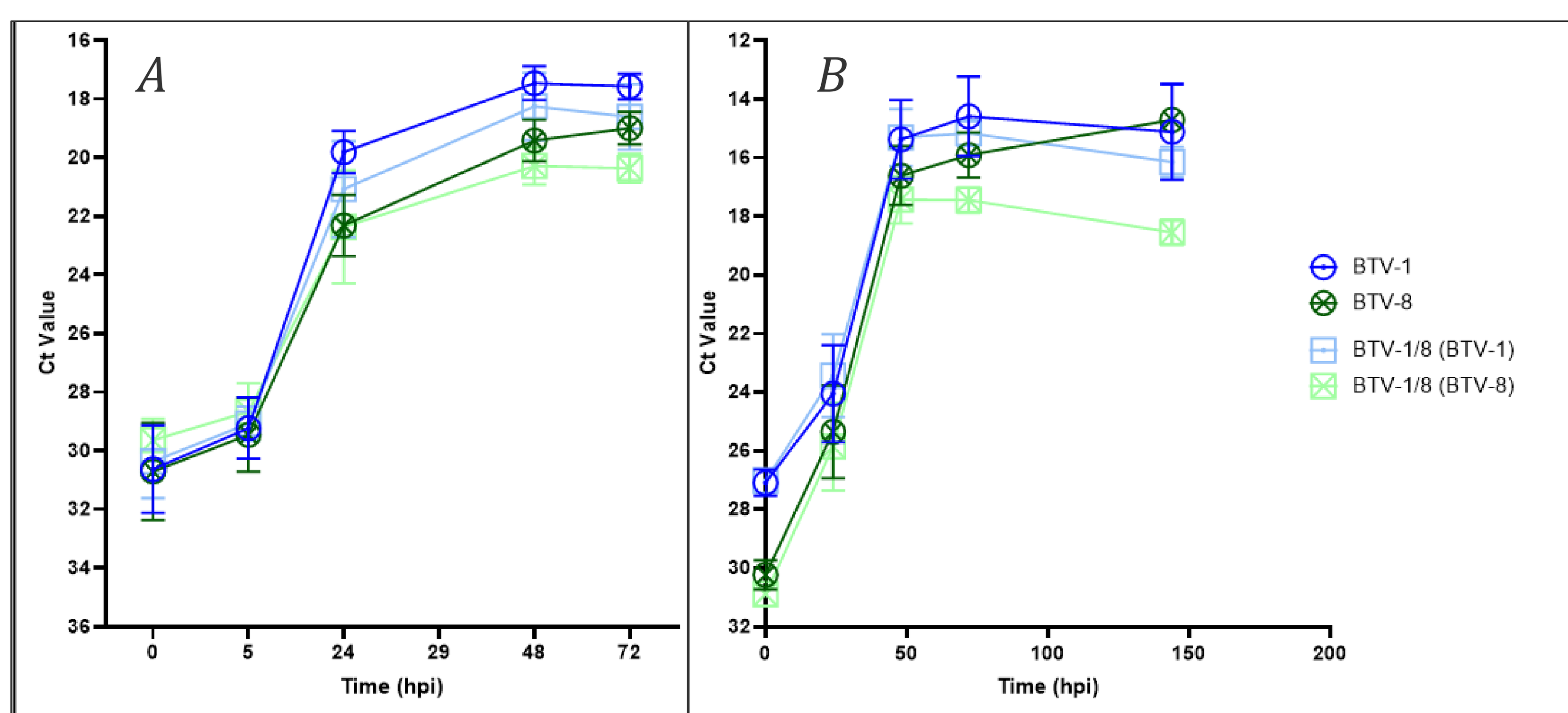
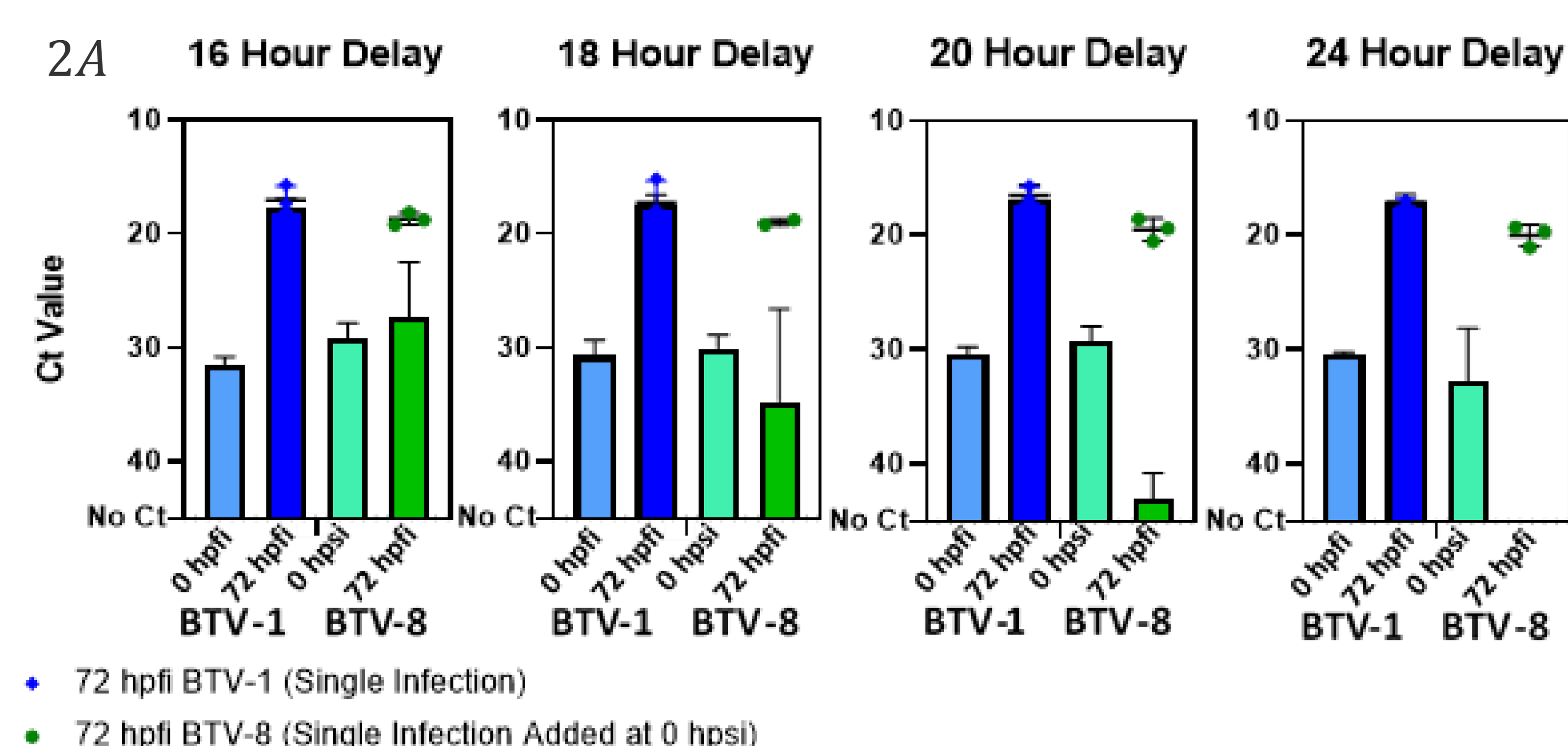


Figure 1. Single and dual-synchronous infections of BTV-1 (blue and light blue) and BTV-8 (green and light green). The lighter colours represent each virus in the dual infection and the darker their respective single infections. 1A) corresponds to BFA cells seeded at 2x10^5 in 24 well plates incubated 37 ºC. 1B) is in KC cells with a cell density of 1x10^6 and incubated at 28 ºC. The results are expressed as mean Ct values based on 3 independent experiments.



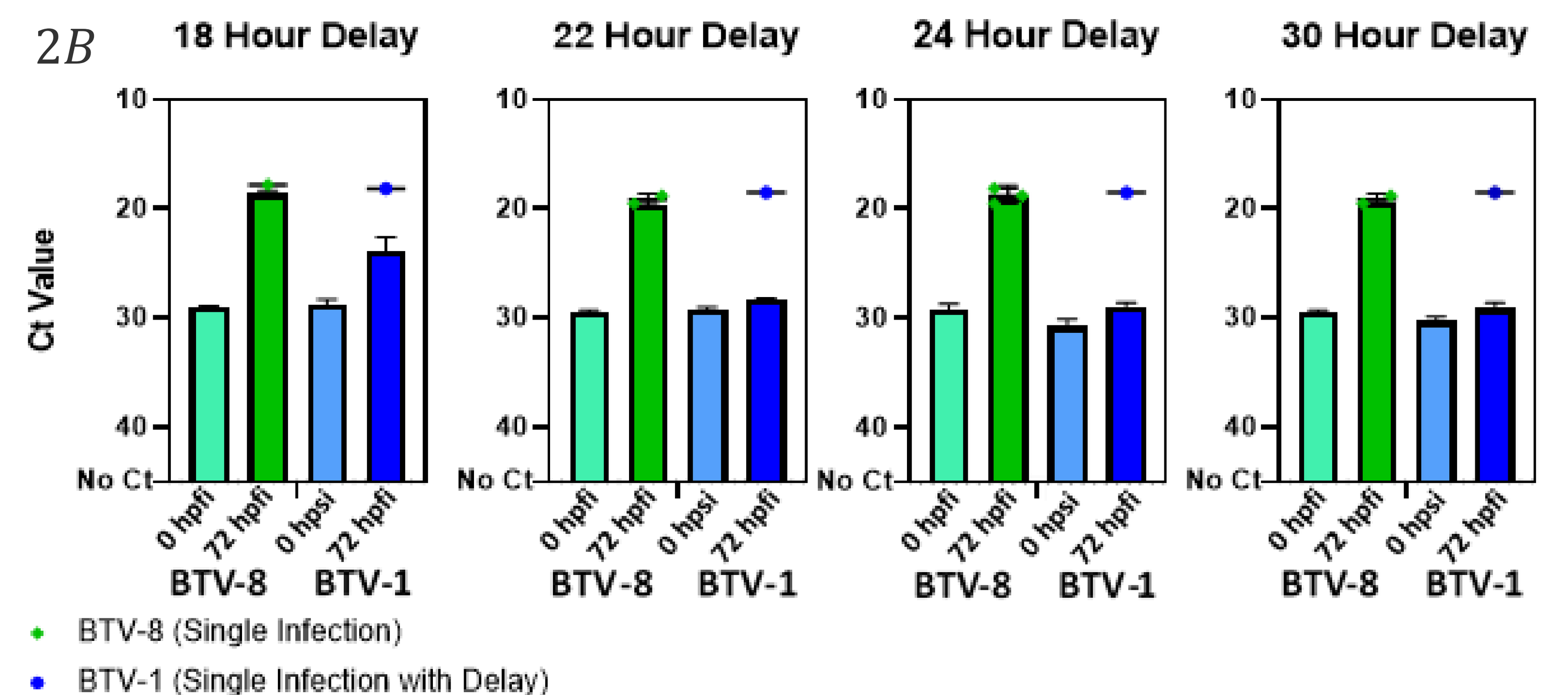


Figure 2. Asynchronous infections of BTV-1 (blue and light blue) and BTV-8 (green and light green) in BFA cells. Hpfi is hours post first infection and is the T0 for the first virus and the endpoint; hpsi is hours post second infection and represents the time zero for the second virus. The dots above the bars show the equivalent Ct values for single infections and therefore the maximum expected. 2A) corresponds to BFA cells seeded at 2x10^5 in 24 well plates incubated at 37 ºC. In these infections BTV-1 was the initial virus. 2B) in this instance BTV-8 was the initial virus. The results are expressed as mean Ct values based on 3 (A) or 1 (B) independent experiments.

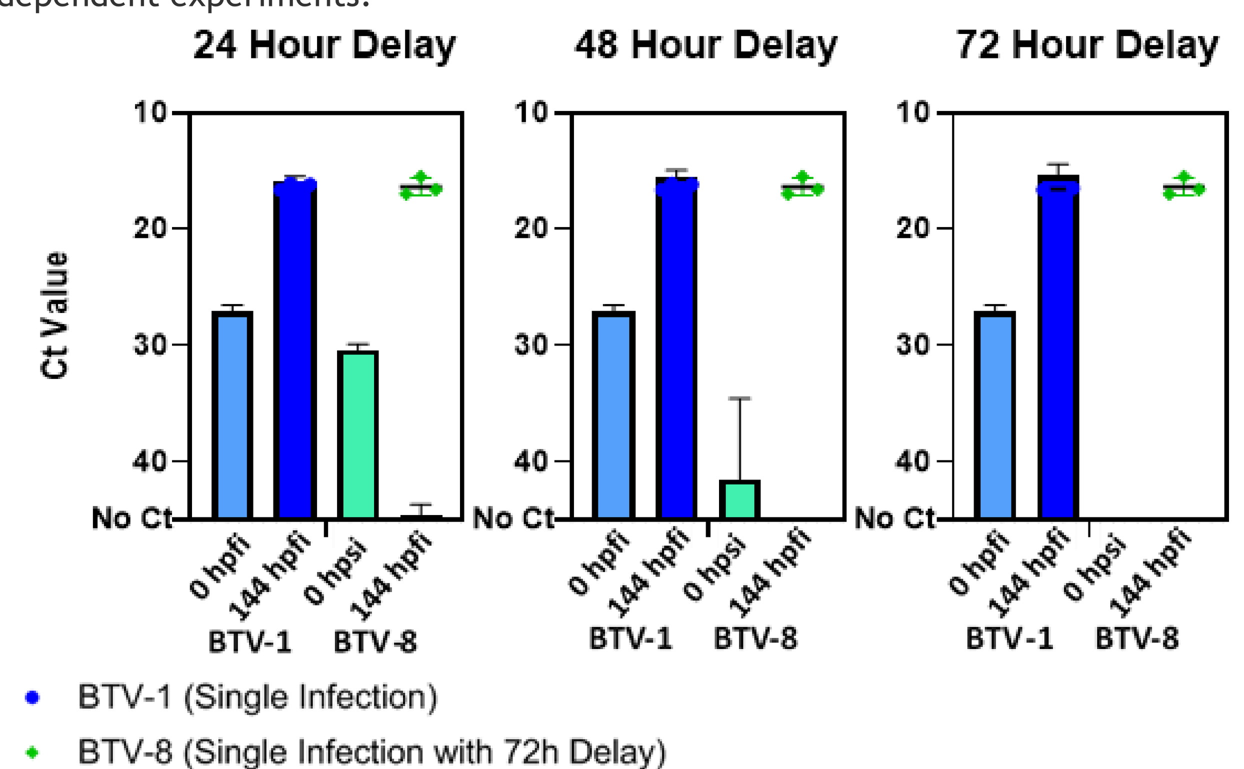


Figure 3. Asynchronous infections of BTV-1 (blue and light blue) and BTV-8 (green and light green) in KC cells. Hpfi is hours post first infection and is the T0 for the first virus and the endpoint; hpsi is hours post second infection and represents the time zero for the second virus. The dots above the bars show the equivalent Ct values for single infections and therefore the maximum expected. Here the cell density was 1x10^6 and incubated at 28 ºC. The results are expressed as mean Ct values based on 2 independent experiments.

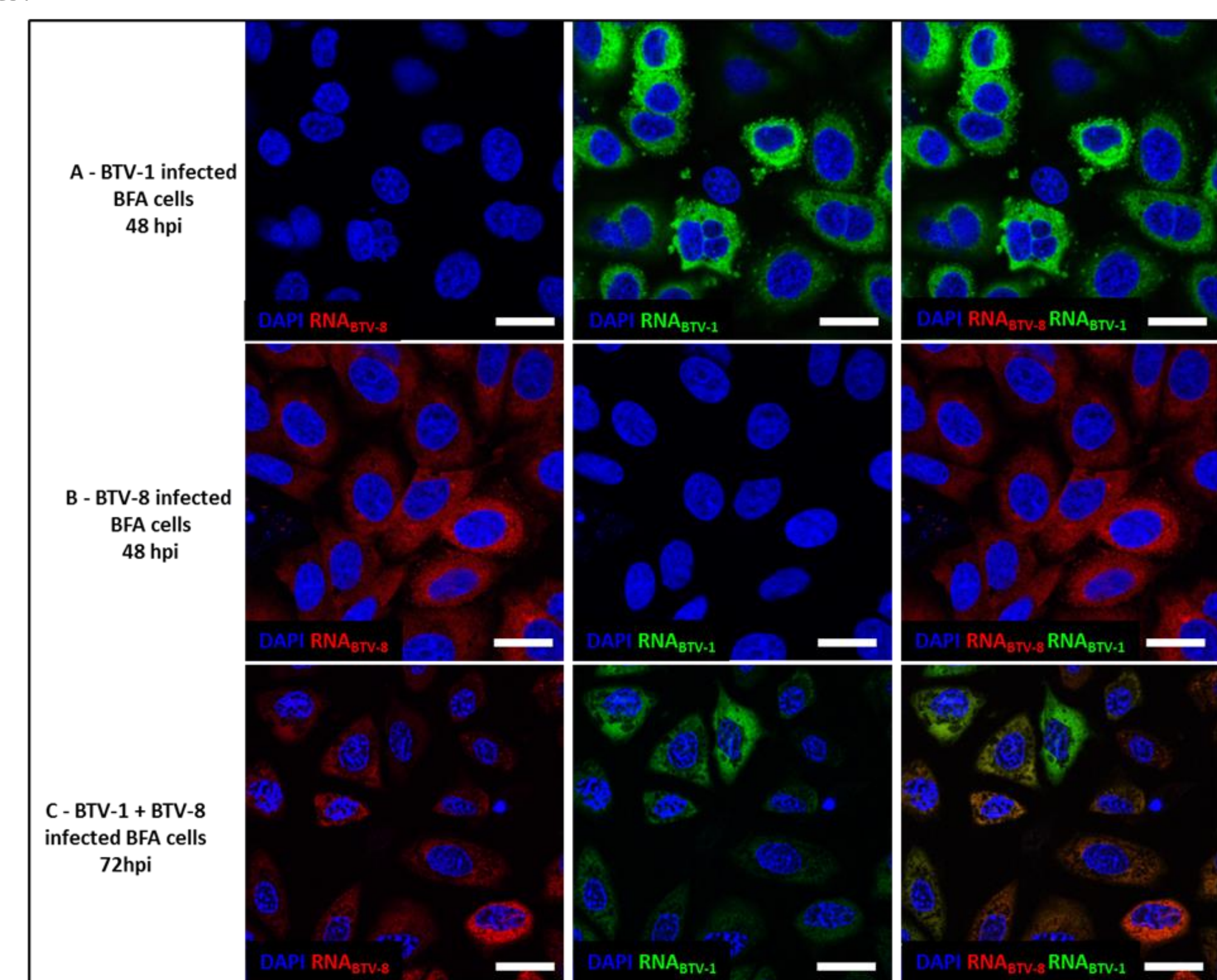


Figure 4. BFA cells were infected with BTV-1 (A), BTV-8 (B) or a mix of BTV1/8 (C) at a MOI of 0.1. At 48 hpi (A and B) or 72 hpi (C), cells in all panels were fixed with 4% PFA and labelled for DNA (blue), BTV-1 Seg-2 mRNA (green) and BTV-8 Seg-2 mRNA (red) following the Stellaris FISH protocol. Cells were visualized by confocal microscopy, and the scale bar represents 10 µm in all panels.

Conclusions

- Superinfection exclusion is demonstrated using BTV-1 and BTV-8 in both bovine and midge cell culture where the superinfecting virus is delayed. The time at which this occurs is strain specific.
- While the mechanism remains unknown, preliminary data points towards blocking the binding of the superinfecting virus.
- Novel serotype-specific probes have been designed and optimised and show no cross reactivity. These have been used to show for the first time that 2 serotypes can replicate in the same cell.
- Superinfection exclusion at a single cell level and the mechanisms behind it are currently being investigated. A FISH-Flow approach using these BTV-1 and BTV-8 probes is also being optimised.

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