

Viral replication kinetics and *in vitro* cytopathogenicity of parental and reassortant strains of bluetongue virus serotype 1, 6 and 8

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1. Abstract

Bluetongue virus (BTV), a segmented dsRNA virus, is the causative agent of bluetongue (BT), an economically important viral haemorrhagic disease of ruminants. Bluetongue virus can exchange its genome segments in mammalian or insect cells that have been co-infected with more than one strain of the virus. This process, may potentially give rise to the generation of novel reassortant strains that may differ from parental strains in regards to their phenotypic characteristics. To investigate the potential effects of reassortment on the virus' phenotype, parental as well as reassortant strains of BTV serotype 1, 6, 8, that were derived from attenuated, and wild type strains by reverse genetics, were studied *in vitro* for their virus replication kinetics and cytopathogenicity in mammalian (Vero) cell cultures. The results indicate that genetic reassortment can affect viral replication kinetics, the cytopathogenicity and extent/mechanism of cell death in infected cell cultures. In particular, some reassortants of non-virulent vaccine (BTV-1 and BTV-6) and virulent field origin (BTV-8) demonstrate more pronounced cytopathic effects compared to their parental strains. Some reassortant strains in addition replicated to high titres *in vitro* despite being composed of genome segments from slow and fast replicating parental strains. The latter result may have implications for the level of viraemia in the mammalian host and subsequent uptake and transmission of reassortant strains (and their genome segments) by *Culicoides* vectors. Increased rates of CPE induction could further suggest a higher virulence for reassortant strains *in vivo*. Overall, these findings raise questions in regards to the use of modified-live virus (MLV) vaccines and risk of reassortment in the field. To further address these questions, additional experimental infection studies using insects and/or animal models should be conducted, to determine whether these results have significant implications *in vivo*.

2. Keywords

Bluetongue virus, bluetongue, reassortment, cytopathogenicity, replication kinetics, apoptosis, necrosis, modified-live virus vaccine

3. Introduction

Bluetongue virus (BTV) is the prototype of the genus *Orbivirus* in the family *Reoviridae*. The virus (26 distinct serotypes) is the causative agent of bluetongue (BT), an economically important disease of wild and domestic ruminants, including sheep, cattle and goats (Spreull, 1905; Verwoerd & Erasmus, 2004). Bluetongue virus causes injury to the vascular endothelium, resulting in pathophysiological changes including vascular thrombosis, ischaemic necrosis, haemorrhage and vascular leakage (MacLachlan *et al.*, 2009). Acutely affected sheep may show clinical signs including fever, oral and nasal erosions/discharge, oedema (particularly of the face, submandibular region and ears), cyanosis of the tongue, coronitis, respiratory distress, anorexia and death (MacLachlan *et al.*, 2008; Verwoerd & Erasmus, 2004). Bluetongue virus evolves through a complex process of antigenic drift and shift, coupled with evolutionary selective pressures and founder effect. These evolutionary processes have resulted in substantial genetic and phenotypic diversity amongst BTV strains in the field (Bonneau & MacLachlan, 2004). For example, particular strains such as attenuated egg and/or cell-adapted modified-live virus vaccines (MLVs) and the current European strains of BTV-8 (wild type) can cross the placenta, resulting in the birth of offspring that may be virus positive and/or demonstrate severe developmental defects. On the other hand, some BTV strains are unable to do so (Desmecht *et al.*, 2008; MacLachlan *et al.*, 2000; Osburn, 1994; Savini *et al.*, 2012; Vercauteren *et al.*, 2008). The severity of clinical signs has further been suggested to be influenced by differences in the virulence of the virus, which varies in a strain specific manner, independently of virus serotype (Caporale *et al.*, 2011).

Bluetongue virus is transmitted through the bites of *Culicoides* midges (Du Toit, 1944) and has historically been considered endemic in temperate and tropical regions between the latitudes of 40-50 °N and 35° S (Gibbs & Greiner, 1994). However, during the last decade of the 20th century and first decade of the 21st century, BTV has spread to latitudes far north of its traditional boundaries (Purse *et al.*, 2005). Since 1998, several BTV serotypes (1, 2, 4, 6, 8, 9, 11 and 16) of both wild type and vaccine origin have been introduced into Europe, resulting in unprecedented genetic diversity amongst strains circulating on the continent (Saegerman *et al.*, 2008). Bluetongue virus contains a genome that consists of ten segments of linear dsRNA. Due to this segmentation, the virus can exchange its genome segments in mammalian or insect cells that have been co-infected with more than one strain of the virus

(Samal *et al.*, 1987a; Samal *et al.*, 1987b). Genetic reassortment can potentially generate reassortants that differ from parental strains in their phenotypic characteristics. Bluetongue virus reassortment has previously been associated with changes in serological properties (Cowley & Gorman, 1989; Maia & Osburn, 1993; Mertens *et al.*, 1989), changes in plaque morphology (Shaw *et al.*, 2013) and novel virulence properties in mice and ruminants (Waldvogel *et al.*, 1987; Waldvogel *et al.*, 1992a; Waldvogel *et al.*, 1992b). Reassortment may also potentially generate strains that differ in transmissibility by the insect vector (Riegler, 2002). Several BTV reassortants have since 2002, been isolated from the field in Europe and included both wild-type/wild-type and wild-type/vaccine strain reassortants (Batten *et al.*, 2008; Maan *et al.*, 2010; Shaw *et al.*, 2013). Multiple unique clinical manifestations of BT in sheep in Israel may also be associated with infections by reassortant strains, although this has not been confirmed under experimental conditions (Brenner *et al.*, 2011).

The BTV genome encodes seven viral proteins (VP1-VP7) and five non-structural proteins (NS1, NS2, NS3/NS3a, and NS4) (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011; Van Dijk & Huisman, 1988). The mature virion is non-enveloped and composed of a triple layered icosahedral protein capsid, consisting of a sub-core (VP3, encoded by Seg-3), a core surface layer (VP7, encoded by Seg-7), and an outer protein layer (VP2 and VP5, encoded by Seg-2 and Seg-6, respectively). The sub-core encloses the ten genome segments as well as multiple copies of three enzymatic proteins (Gouet *et al.*, 1999; Grimes *et al.*, 1998; Hewat *et al.*, 1992); VP1 (RNA-dependent RNA polymerase, encoded by Seg-1) (Boyce *et al.*, 2004), VP4 (capping and trans-methylase enzyme, encoded by Seg-4) (Ramadevi *et al.*, 1998) and VP6 (RNA-dependent ATPase and helicase, encoded by Seg-9) (Stauber *et al.*, 1997). Viral protein 7 is a ligand for the insect cell receptor and appears to be able to mediate the attachment and infection of insect cells by core particles (Basak *et al.*, 1997; Xu *et al.*, 1997). The outer capsid proteins are involved in the attachment (VP2) (Hassan & Roy, 1999) and release (VP5) (Forzan *et al.*, 2004; Hassan *et al.*, 2001) of the virus into the cytoplasm of mammalian cells. The non-structural proteins play a role in regulating viral protein expression (NS1, encoded by Seg-5) (Boyce *et al.*, 2012), morphogenesis (NS2 encoded by Seg-8) (Kar *et al.*, 2007) and intracellular trafficking/release (NS3/NS3a, encoded by Seg-10) (Hyatt *et al.*, 1993). The function of NS4 (encoded by an out-of-frame open reading frame within the VP6 frame) is currently unclear. This protein localises in the nucleolus of infected cells early in the infection cycle and appears to give a replication advantage to certain strains

of BTV in interferon treated mammalian cells. NS4 may therefore play a role in the evasion of host cell anti-viral defence mechanisms (Belhouchet *et al.*, 2011; Ratnien *et al.*, 2011).

Only limited studies have been conducted to investigate the effect of reassortment on BTV phenotype. The potential consequences of reassortment in regards to the course and epidemiology of the disease therefore remain uncertain. An incomplete understanding of the molecular determinants that influence BTV virulence and transmissibility further complicates this issue (reviewed by Coetzee *et al.*, 2012). The generation of reassortants of BTV by co-infection of different *in vitro* and *in vivo* systems (forward genetics) has been described (Oberst *et al.*, 1987; Ramig *et al.*, 1989; Samal *et al.*, 1987a; Samal *et al.*, 1987b; Shaw *et al.*, 2013). In addition, a reverse genetics system for BTV has recently been developed (Boyce *et al.*, 2008). The latter technology allows the *de novo* generation of desired reassortants that can be used to study the phenotype of BTV in a specific manner. In the case of reverse genetics, *in vivo* studies have indicated that some phenotypic characteristics of rescued strains remains preserved (i.e. virulence and ability to cross the placenta), highlighting the suitability of this approach to generate viruses that can be used as models for BTV virulence and pathogenicity studies (Rasmussen *et al.*, 2013; Van Gennip *et al.*, 2012b).

The objective of the present study was to compare different phenotypic properties of selected reverse genetics parental and reassortant strains of BTV serotype 1, 6 and 8 in mammalian cell culture, in order to examine whether reassortment can affect the *in vitro* phenotype of the virus. Selected *in vitro* parameters may reflect differences in virulence and transmissibility of BTV *in vivo*. In this study parental and reassortant strains were compared in regards to their virus replication kinetics, their rate of induction of CPE, the effects of viral infection on cell viability and the degree of induction of different mechanisms of cell death (apoptosis/necrosis).

4. Materials and methods

4.1. Viruses

The BTV reverse genetic strains/reassortants used in this study were previously described by van Gennip *et al.* (Van Gennip *et al.*, 2010; Van Gennip *et al.*, 2012). Briefly, BTVs and BTV reassortants were generated by transfecting BSR cells (a BHK-21 sub-clone) with

capped T7 RNA transcripts derived *in vitro* from cloned cDNA's of BTV serotype 1, 6 and 8 or by using a targeted single genome segment modification strategy (Van Gennip *et al.*, 2010; Van Gennip *et al.*, 2012) (**Table 1**). The consensus sequences of genome segments have been submitted to Genbank [BTV-1 (FJ9697191- FJ9697281), BTV-6 (QG506472–QG506481) and BTV-8 (GQ506451-GQ506460)] of the National Centre for Biotechnology (NCBI, (<http://www.ncbi.nlm.nih.gov/>)). The strains were selected for this study due to their availability from previous studies; the fact that the viruses were reassortants of attenuated and virulent strains, and that the parental strains had been evaluated for their virulence in ruminants.

The reverse genetics strain of BTV-1 (rgP1) is genetically closely related to the South African reference strain of BTV-1 (RSArrr/01) and to the MLV strain of serotype 1 produced by Onderstepoort Biological Products (OBP). Experimental infection of sheep with rgP1 has demonstrated that the virus is avirulent and only induces a few days of increased body temperature and a very low and transient viraemia in sheep (Dr. Piet van Rijn, personal communication). The rgP6 strain is derived from a BTV-6 field strain that was isolated in the Netherlands in 2008 (BTV6/net08). Segment-1 to Seg-9 shows 99.7-100% nucleotide sequence identity to the South African BTV-6 MLV strain. Segment-10 (NS3/NS3a) of BTV6/net08 is however highly similar (98.4%) to the South African BTV-2 MLV strain (Maan *et al.*, 2008; Van Gennip *et al.*, 2012b). Previous studies have demonstrated that BTV6/net08 and rgP6 are avirulent, and cause only mild/inapparent infections in sheep and cattle (Eschbaumer *et al.*, 2009; Van Gennip *et al.*, 2012b; Van Rijn *et al.*, 2012). The parental rgP8 strain is based on consensus sequences of BTV-8/net07/01 (collection nr of The Pirbright Institute), a cattle isolate of BTV-8 from 2007 in the Netherlands. Both the reverse genetics and ancestral virus are virulent in experimentally infected sheep and cattle and can cause significant morbidity and mortality (Van Gennip *et al.*, 2012b). Both rgP6 and rgP8 contain silent mutations that are spread across different genome segments with respect to the BTV6/net08 and BTV-8/net07/01 consensus sequences. These mutations were incorporated during construction of the rescue plasmids, and allow the viruses to be distinguished from the cognate field strains.

Two additional strains were included as controls. These included BTV8/net07/01 and the original BTV-6 MLV of Onderstepoort Biological Products (OBP, Pretoria, South Africa). The BTV-6 MLV strain was originally isolated from the field in South Africa and attenuated

by sequentially passaging the virus in embryonated chicken eggs and cell culture (60x embryonated chicken egg passages, 3x plaque purifications, 7x passages on BHK cells).

4.2. Virus characterization

Before commencing experimental work, whole genome sequencing of the selected viral strains was conducted using Ion Torrent sequencing (ABI Life Sciences) in order to confirm the identity and constellation of BTV genome segments. “FLAC amplification” (full-length amplification of cDNAs) of each of the viral genomes was conducted as previously described (Potgieter *et al.*, 2009). The preparation of DNA libraries, emulsion PCR and unidirectional sequencing on the Ion Torrent system (314/316 chip) was performed according to the manufacturer’s instructions. Fastq files containing the sequence data and quality values were imported into the CLC genomics software (V5.5, <http://www.clcbio.com/>) and quality trimmed using default settings. Sequence reads were assembled with the CLC software by either mapping reads to reference sequences of BTV that were downloaded from the National Centre for Biotechnology (NCBI, (<http://www.ncbi.nlm.nih.gov/>)) website or assembled using shotgun assembly (*de novo*) as appropriate, using default parameters. Consensus and/or contig sequences (>500 nt. in length) were subjected to a multi-nucleotide BLASTn (Basic Local Alignment Search Tool) at NCBI, in order to determine the sequence identities. In general sequencing yielded nearly full length sequences for each of the genome segments. All viruses further demonstrated 99-100% nucleotide sequence identity to the corresponding genome segments of the parental strains available on Genbank (results not shown).

4.3. Reassortant virus nomenclature

The nomenclature of the BTV strains in the present study (**Table 1**) is similar to that described by Meiring *et al.*, (2009). The names are preceded by the prefix “rgP” or “rgR” to indicate parental and reassortant strains derived by reverse genetics. Thus, rgR6-8(10) indicates a BTV reassortant based on rgP6 with Seg-10 of rgP8.

4.4. Cell culture

All experiments were carried out using Vero cells (African green monkey kidney cells, American Type Culture Collection, Manassa, USA). Vero cells were propagated in

Dulbecco's minimal essential medium (DMEM) containing 5% gamma-irradiated foetal calf serum (FCS, Highveld Biological, South Africa), 10% tryptose phosphate broth (v/v) and 1 mg/L gentamycin and incubated at 37°C in an atmosphere containing 5% CO₂ and 98% humidity until 100% confluent. In order to prepare virus stocks, virus was passaged 3x on Vero cells until complete CPE was observed. The virus was released by 1x freeze/thawing, clarified by centrifugation at 2,000 x g for 20 minutes and immediately titrated in triplicate on Vero cells (Kärber, 1931). Working stocks were diluted to 3 log₁₀ TCID₅₀/mL in supplemented DMEM prior to conducting each assay. Pilot studies were conducted using viruses of different titres (up to 3 log₁₀ TCID₅₀) (results not shown) and a dose of 100 TCID₅₀ (0.01 mL of stock virus) selected. Using this dose, CPE is normally observed within 3-4 days post infection (dpi) in cell cultures in 96 well cell culture plates. All assays were performed in confluent cell monolayers in either 16 (E-plates) or 96-well cell culture plates (total volume 0.28 mL). In order to prepare Vero monolayers, wells in cell culture plates were seeded with 38 400 cells and left to equilibrate for 24 hours prior to viral infection. The assays were done in triplicate and readings averaged between replicates for each virus. The assays were in addition repeated on different days using different stock viruses in order to confirm particular data trends (representative data presented in **Table 1** and **Figures 1-4**). Cell free medium and/or mock infected controls were included in the different assays as appropriate.

4.5. Virus replication kinetics

In order to generate virus growth curves, cell monolayers in 96-well plates were infected with virus (100 TCID₅₀), harvested at 24, 48, 72 and 96 hours post infection (hpi) and cell culture supernatant collected and titrated as previously described. Individual cell culture plates were frozen and thawed 3x times prior to titration, to facilitate virus release from infected cells into the cell culture medium.

4.6. Cytopathic effect

The onset and extent of CPEs were recorded in Vero cultures infected with each of the strains by continuously measuring the development of CPE using an electrical impedance assay (RTCA-DP system, xCELLigence, ACEA Biosciences) (Spiegel, 2009). Cell monolayers in 16-well E-plates were infected with virus at 24 hours post seeding and cell index (CI) values measured every 30 minutes over a 7-10 day period using the RTCA-DP system. Cell index

values were averaged across replicates and normalised to the approximate time of virus addition (23.3-24.0 hpi). The time to a 50% reduction in cell index value (CI₅₀) was subsequently determined. The CI₅₀ value or CPE induction rate was defined as the time point at which the CI value had decreased to 50% of the maximum for each of the normalized CPE curves.

4.7. Cell viability and mechanism of cell death

In order to measure the ability of the viruses to cause cell death, a cell viability/necrosis/apoptosis assay (ApoTox-Glo™ Triplex Assay, Promega, catalog nr. TM322) was conducted in Vero cultures. Confluent monolayers in 96-well (black) plates were infected at 24 hours post seeding (100 TCID₅₀) and cellular markers for cell viability, apoptosis and necrosis measured at 72 hpi, according to the manufacturers' instructions, using a luminometer (i.e. bioluminescence and/or fluorescence; Glomax, Promega).

4.8. Correlation between *in vitro* phenotypic properties

A Spearman's rank correlation (Rho) test was used to examine the correlation between the *in vitro* parameters that were measured in this study, using mean data values (Corder and Foreman, 2009). An online Rho calculator was utilized to calculate r_s and probability values (p) for pair-wise comparisons of the phenotypic parameters (<http://www.socscistatistics.com/tests/spearman/Default2.aspx>). The results were interpreted at the 95% significance level ($P < 0.05$). Calculated r_s and probability (p) values for each pair-wise comparison are presented in **Table 2**.

5. Results

5.1. Virus replication kinetics

An evaluation of the virus replication kinetics of parental, reassortant and control strains indicated an increase in viral titre between 24-96 hpi. Differences in virus titres were observed between 24-72 hpi, and maximum titres recorded at 96 hpi for most strains. Consequently, strains were compared at this sampling point (**Figure 1 and Table 1**). At 96 hpi, rgP6 yielded the lowest titre (3.9 log₁₀ TCID₅₀/mL, SD ± 0.1). The rgP1 and rgP8 strains

in contrast yielded higher titres of $6.3 \log_{10} \text{TCID}_{50}/\text{mL}$ ($\text{SD} \pm 0.1$) and $5.2 \log_{10} \text{TCID}_{50}/\text{mL}$ ($\text{SD} \pm 0.2$), respectively. The wild-type BTV-8 strain (BTV8/net07/01) replicated to a titre of $5.9 \log_{10} \text{TCID}_{50}/\text{mL}$ ($\text{SD} \pm 0.4$) whereas the BTV-6 MLV strain replicated to $4.8 \log_{10} \text{TCID}_{50}/\text{mL}$ ($\text{SD} \pm 0.3$).

Reassortants of rgP1 and rgP6 replicated to titres that appeared to correspond to either one or the other parental strain. The rgR1-6(1,3,4,8,9) and rgP1 strains demonstrated similar titres ($5.8 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$ and $6.3 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$, respectively), whereas rgP6 and rgR1-6(2,6,7,10) in comparison replicated to similar ($3.9 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$ and $4.0 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.4$, respectively) but lower titres. The rgP1/rgP8 reassortants, replicated to titres that appeared to be similar to both parental strains. Some variation was measured between rgP1/rgP8 reassortants, that ranged from 5.7 ($\text{SD} \pm 0.4$) to $6.8 \log_{10} \text{TCID}_{50}/\text{mL}$ ($\text{SD} \pm 0.4$). The reassortant rgR8-6(9,10) ($5.9 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$) demonstrated a similar titre as rgP8 ($5.2 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.3$), whereas rgR6-8(10) ($4.1 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.6$), replicated to a similar titre as rgP6 ($3.9 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$).

5.2. Cytopathic effects

The development of CPE was measured by using an electrical impedance assay (xCELLigence, Roche). The assay measures changes in trans-monolayer electrical resistance in response to cellular morphological changes in the whole cell culture monolayer. The method was therefore suitable to measure the course of CPE induction and replace subjective visual scoring by microscopy. The results of the electrical impedance assay are expressed as CI_{50} values in **Figure 2 (A to D)** and **Table 1**. In general, infected cells continued to grow for at least 10 to 15 hours similar to mock-infected cells, after which CI values started to decline. Eventually, all viruses caused complete CPE that was confirmed by a CI value of zero.

The rates of CPE induction of the different strains are expressed as CI_{50} values in hours post infection (hpi). Parental reverse genetics strains (**Figure 2A**) showed individual CPE induction rates. Virulent strain rgP8 caused CPE the quickest ($\text{CI}_{50} = 64.5$, $\text{SD} \pm 4.1$). Avirulent strain rgP6 was the slowest ($\text{CI}_{50} = 98.0$, $\text{SD} \pm 5.8$), whereas the other avirulent strain rgP1 had an intermediate CI_{50} value of 71 hpi ($\text{SD} \pm 4.7$). The South African BTV-6 MLV strain and the field isolate of BTV-8 (BTV8/net07/01) showed similar CPE induction

rates as the rgP6 and rgP8 strains, respectively (BTV-6 MLV, $CI_{50} = 91, SD \pm 5.8$; BTV8/net07/01, $CI_{50} = 61.0, SD \pm 3.5$).

CPE induction rates of rgR1-6(1,3,4,8,9) and rgR1-6(2,6,7,10) differed from the parental strains (**Figure 2B**). Strain rgR1-6(1,3,4,8,9) induced CPE faster ($CI_{50} = 68, SD \pm 1.3$) than rgP1 ($CI_{50} = 71.0, SD \pm 4.7$), whereas rgR1-6(2,6,7,10) was delayed ($CI_{50} = 142.5, SD \pm 17.9$) as compared to rgP6 ($CI_{50} = 98, SD \pm 5.8$).

For the rgP1 and rgP8 reassortant strains (**Figure 2C**), the CPE induction rates appeared to be similar to that of rgP8 ($CI_{50} = 64.5, SD \pm 4.1$) and distinct from that of rgP1 ($CI_{50} = 71.0, SD \pm 4.7$). Notably several reassortants [rgR1-8(5,8), rgR1-8(2,5,8), rgR1-8(5,6,8), rgR1-8(5,8,10), ($CI_{50} = 48.0, SD \pm 2.4$ to $62.0, SD \pm 7.0$)] showed faster CPE induction rates as compared to rgP8 ($CI_{50} = 64.5, SD \pm 4.1$).

The reassortants rgR8-6(9,10) and rgR6-8(10) demonstrated CPE induction rates that appeared distinct from both parental strains (**Figure 2D**). The strain rgR8-6(9,10) ($CI_{50} = 49.0, SD \pm 3.5$) induced CPE faster than rgP6 ($CI_{50} = 98.0, SD \pm 5.8$), which was closer to that demonstrated by rgP8 ($CI_{50} = 64.5, SD \pm 4.1$). The rgR6-8(10) strain in turn demonstrated delayed CPE induction ($CI_{50} = 112.0, SD \pm 8.1$) relative to rgP6 ($CI_{50} = 98.0, SD \pm 5.8$).

In summary, the CPE induction rates of the reassortant strains as measured by the electrical impedance assay and expressed as CI_{50} values, appeared to be different from that of the parental strains in several cases. Certain reassortants showed either increased or decreased CPE induction rates with respect to the parental strains, whereas some reassortants showed CI_{50} values close to that of one of the parental strains.

5.3. Cell viability and mechanism of cell death

The results of the viability assay indicated that most of the studied strains reduced the viability of infected cell cultures at 72 hpi with 50-80% compared to mock-infected controls (**Figure 3** and **Table 1**). The few exceptions were rgR1-6(1,3,4,8,9), rgR1-6(2,6,7,10) and rgR6-8(10), which demonstrated smaller decreases. The last two strains demonstrated an

approximate decrease of 20-30%, whereas rgR1-6(1,3,4,8,9) demonstrated a decrease of 40-50%.

The results from the necrosis/apoptosis assay indicated that visible CPE was associated both with necrotic and apoptotic cell death at 72 hpi (**Figure 4** and **Table 1**). The strains varied widely in the extent of induced necrotic and apoptotic cell death. For the cytotoxicity assay, this varied from a 2–12 times increase in cytotoxicity, whereas for apoptotic cell death this varied from 2–17 times increase, relative to mock-infected controls. In particular rgR1-6(2,6,7,10) demonstrated low levels of both apoptosis and necrosis.

5.4. Correlation between *in vitro* phenotypic properties

Calculated Spearman correlation coefficients (r_s) and probability (p) values (**Table 2**) for a pair-wise comparison of the *in vitro* phenotypic properties, indicated a statistically significant positive correlation between the mean titre of virus at 96 hpi and extent of apoptosis at 72 hpi [$r_s = 0.701$, p (0.011) < 0.05]; a statistically significant positive correlation between CPE induction rate (CI_{50}) and the percentage of cell viability remaining at 72 hpi [$r_s = 0.874$, p (0.005) < 0.0002]; a statistically significant negative correlation between CPE induction rate and extent of apoptosis at 72 hpi [$r_s = -0.748$, p (0.005) < 0.05] and a statistically significant negative correlation between the percentage of cell viability remaining and extent of apoptosis at 72 hpi [$r_s = -0.699$, p (0.005) < 0.05]. Statistically significant correlations could not be identified for the remaining comparisons.

6. Discussion

The objective of this study was to evaluate and compare selected *in vitro* properties of reverse genetics parental and reassortant (as well as control) strains of BTV-1, 6 and 8 in Vero cell cultures, in order to evaluate whether genetic reassortment could potentially affect the *in vitro* phenotype of the virus. In order to evaluate the effects of BTV reassortment, the test strains were compared in Vero cell culture in regards to selected phenotypic parameters. These parameters included an evaluation of viral replication kinetics, the rate with which the viruses induced CPE, the effect of viral infection on cell viability and the ability of the viruses to induce different mechanisms of cell death (apoptosis/necrosis). The choice of parameters was based on studies that have previously been documented with viruses from other genera, in

which similar parameters were investigated as potential indicators of potential changes in *in vivo* virulence (Baumgartner *et al.*, 1991; DeMaula *et al.*, 2001; Freistadt & Eberle, 1996; Slosaris *et al.*, 1989; Tscherne & Garcia-Sastre, 2011; Dortmans *et al.*, 2011). Differences in viral replication kinetics *in vitro* - and by implication possible differences in the ruminant host - was further considered to be of interest, due to its potential influence on the level of viraemia, and by implication the efficiency of the virus' uptake by the insect vector during blood feeding (Veronesi *et al.*, 2010).

The results from the *in vitro* study supported the notion that reassortment could potentially affect the phenotype of the virus, with reassortment appearing to be associated with changes in at least some of the phenotypic properties that were evaluated in this study. However, there was in general a lack of correlation between the measured parameters (with the exception of the rate of CPE induction) and previously documented differences in parental strain virulence in ruminants. For example, at 96 hpi the avirulent rgP1 was able to replicate to a higher titre ($6.3 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.2$) in cell culture than avirulent rgP6 [$3.9 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.1$], whereas the rgP1 titre was more similar to that of virulent rgP8 ($5.2 \log_{10} \text{TCID}_{50}/\text{mL}$, $\text{SD} \pm 0.2$). All three parental strains further demonstrated a similar reduction in infected cell viability at 72 hpi ($30.7\% \pm 5.2$ to $43.2\% \pm 3.5$). No overall pattern was discernable in regards to the documented virulence of the parental strains *in vivo* and extent of either cytotoxicity or necrosis at 72 hpi. Overall, this lack of correlation could be explained by general limitations in the applicability of *in vitro* models to the *in vivo* situation. The clinical manifestation of BT in the ruminant host, is a multi-factorial phenomenon that is thought to be influenced not only by intrinsic differences in the phenotype of BTV strains, but also by external host, vector and environmental factors that cannot be duplicated in cell culture (DeMaula *et al.*, 2001; Foster *et al.*, 1991; MacLachlan *et al.*, 2009). *In vitro* conditions further differ from *in vivo* conditions in that cells are clonally derived and some cell types such as for example Vero cells as was used in this study, lack accompanying antiviral defences such as the interferon response and/or even specific viral receptor sites.

Despite the limitation of *in vitro* models in regards to their applicability to the *in vivo* situation, it was of interest to speculate whether particular phenotypic changes observed in cell cultures for the reassortant strains, may be reflected in *in vivo* changes in either transmissibility and/or virulence. For example, it was observed that some wild-type/MLV reassortants replicated to high titres, [(e.g. rgR1-6(1,3,4,8,9) and rgR8-6(9,10) replicated to

>5 log₁₀ TCID₅₀/mL, SD ±0.1)], despite containing genome segments from the slow replicating rgP6 strain (3.9 log₁₀ TCID₅₀/mL, SD ±0.1). The replication of reassortant strains composed of genome segments from slow and fast replicating parental strains to high titres *in vitro*, as was observed in this study, may imply that these strains and therewith their genome segments may spread more efficiently in the field (e.g. either by influencing the peak level and/or duration of infectious viraemia). The possibility of MLV reassortment in this regard is particularly disconcerting as one specific goal of attenuation of BTV strains is to reduce the level of viraemia after vaccination of the ruminant host to a maximum titre of 3 log₁₀ TCID₅₀/mL in blood (OIE, 2008). The titre has been proposed as a threshold with respect to onward transmission of the virus by *Culicoides* species (Savini *et al.*, 2008). Additional *in vivo* studies should be conducted, to assess whether reassortment can alter the duration and level of viraemia in the ruminant host and whether such changes may be reflected in altered transmissibility of reassortant strains by *Culicoides* vectors.

An investigation of viral CPE induction rates indicated that the CI₅₀ values for the parental strains corresponded partially to documented differences in virulence of the parental strains in ruminants. Thus, it was observed that the virulent strain rgP8 and control strain BTV-8 demonstrated faster CPE induction rates than attenuated strains of rgP1, rgP6 and the BTV-6 MLV vaccine strains, respectively. Changes in CPE induction rates were further observed for nearly every reassortant type analysed. For rgP1/rgP6 reassortants, reassortment appeared to be associated both with increased [rgR1-6(1,3,4,8,9)] and decreased CPE induction rates [rgR1-6(2,6,7,10)] (**Figure 2B** and **Table 1**). The rgP1/rgP8 reassortants in turn which contained two or three genome segments from rgP8 [i.e. rgR1-8(5,8), rgR1-8(2,5,8), rgR1-8(5,6,8), rgR1-8(5,7,8), rgR1-8(5,8,10)] demonstrated increased CPE induction rates as compared to rgP1 (**Figure 2C** and **Table 1**). In the case of rgP6/rgP8 reassortants, rgR6-8(10) showed a delayed CPE induction compared to both parental strains, whereas the reassortant rgR8-6(9,10) showed a similar CPE induction rate as rgP8 (**Figure 2D**, **Table 1**). Thus genetic reassortment between parental strains with distinct CPE induction rates resulted in variable and somewhat unpredictable CPE induction rates for the reassortant strains. Altered CPE induction rates for some reassortant strains could potentially reflect changes in the rate with which these strains may cause tissue damage *in vivo*, and may thus potentially reflect altered virulence. Additional studies in either mice and/or ruminants should be conducted, in order to clarify whether there is a correlation between CPE induction rate and *in vivo* virulence of the virus.

Both necrosis and apoptosis have been demonstrated to play a role in BTV pathogenicity *in vivo* (DeMaula *et al.*, 2001; Umeshappa *et al.*, 2010). It was therefore of interest to evaluate whether the effect of viral infection on cell viability and/or induction of necrosis and apoptosis differed in cell cultures that were infected with the parental and reassortant strains. The Apotox Triplex assay results indicated that the effects of viral infection on cell viability, as well as the extent of necrosis and apoptosis induction varied widely between cell cultures that were infected with the different parental and reassortant strains. However in general a clear correlation and/or overall pattern between the parental and reassortant strains could not be observed. Nevertheless, the assay provided useful data in order to evaluate the relationship between the different *in vitro* phenotypic properties. The strong positive correlation [$r_s = 0.701$, $p (0.0111) < 0.05$] that was observed between viral yield at 96 hpi and extent of apoptosis induction at 72 hpi, was interpreted as reflecting widespread damage to the cell culture monolayer as a result of apoptosis, and associated virus release at 72-96 hpi. Secondly, the strong positive correlation [$r_s = 0.874$, $p (0.0002) < 0.05$] that was observed between the CPE induction rate and percentage of cell viability remaining at 72 hpi, was interpreted to reflect widespread morphological changes and associated cell death for highly cytopathic strains and vice versa for less cytopathic strains. This result suggests that the appearance of CPE is closely correlated to cell death. Thirdly, the strong negative correlation [$r_s = -0.748$, $p (0.0051) < 0.05$] that was observed between the CPE induction rate and extent of apoptosis induction at 72 hpi, suggests that increased rates of CPE induction was associated with increased levels of apoptosis induction for highly cytopathic strains and vice versa for less cytopathic strains. It therefore appears that the development of CPE is closely associated with cell death due to apoptosis. Finally, the strong negative correlation [$r_s = -0.699$, $p (0.8798) < 0.05$] that was observed between the percentage of cell viability remaining and extent of apoptosis induction at 72 hpi, suggests that apoptosis was the dominant mechanism of cell death in contrast to necrosis (cytotoxicity), for which a statistically significant correlation with cell viability could not be identified.

Bluetongue virus has been reported to be able to induce apoptosis in a wide range of mammalian cells cultures including Vero cells though uncoating of the VP2 and VP5 outer capsid proteins during the infection/fusion process (DeMaula *et al.*, 2001; Mortola *et al.*, 2004; Nagaleekar *et al.*, 2007). It has further been shown that the degree of apoptosis induction varies in endothelial cells from different anatomical regions from ovine and bovine, and that the dominant mechanism of cell death is influenced by the presence of pro-

inflammatory cytokines such as IL-1 and TNF- α (DeMaula *et al.*, 2001). In this study, evidence was obtained that apoptosis was the dominant mechanism of cell death instead of necrosis. This is in agreement with finding of DeMaula *et al.*, (2001), where endothelial cells that were infected with non-purified cell culture grown BTV (as was done in this study) primarily die due to apoptosis, due to the presence of pro-apoptotic mediators in the cell culture medium. A correlation was observed between the rate of CPE induction, the percentage of cell viability remaining at 72 hpi, and the extent of apoptosis induction in infected cell cultures in this study. Furthermore, these properties appear to vary between parental and reassortant strains. Concerning differences in *in vitro* phenotype that were identified between the parental and reassortant strains in this study, similar results have been reported by Shaw *et al.*, (2013) for selected *in vitro* phenotypic properties (i.e. replication kinetics and plaque morphology) for BTV-1 and BTV-8 reassortant strains. The results are also in accordance with studies that have been conducted with African horse sickness virus (AHSV) reassortants in Vero cell cultures, where reassortants demonstrate differences in their viral replication kinetics, the extent with which the strains are able to induce CPE, and the extent with which reassortants are able to permeabilize the cell membrane (cytotoxicity) (Meiring *et al.*, 2009).

The results from the study emphasise the potential, sometimes unpredictable consequences of reassortment between avirulent and virulent BTV strains. Apparently, reassortants with different constellations originating from parental strains with different phenotypic characteristics will not necessarily have intermediate *in vitro* characteristics. In general, it was not possible to correlate the exchange of any particular genome segment to phenotypic changes of parental strains *in vitro*, as the majority of reassortants were composed of multiple heterologous genome segments from both parental strains. Due to the difficulty with assigning phenotypic properties to particular genome segments when using multi-reassortants, it is recommended that future studies specifically focus on utilizing mono-reassortants. Nevertheless, an examination of genome segment constellations in this study suggested that variation in several of the genome segments and/or encoded proteins may have been associated with changes in BTV phenotype (e.g. VP2, VP5, NS1, NS2 and NS3/NS3a). These genome segments have previously been implicated as potentially being associated with changes in virulence of either BTV or the closely related AHSV *in vivo* (Caporale *et al.*, 2011; Huismans & Howell, 1973; Meiring *et al.*, 2009; O'Hara *et al.*, 1998; Owens *et al.*, 2004). In this study, only a small number of BTV reassortants that contained specific genome

constellations were studied. These viruses were generated using reverse genetics under *in vitro* (i.e. artificial) conditions. Such specific viral constellations are therefore unlikely to emerge in the field. Nevertheless the study of the phenotypic characteristics of reassortant strains generated either by forward and/or reverse genetics, represents a first step in order to begin to understand the potential consequences of BTV reassortment.

Overall, this work raises questions in regards to the potential risk of reassortment between circulating MLVs and/or wild-type viruses in the field. Bluetongue virus MLV strains could contain several unfavourable phenotypic characteristics, not necessarily encountered in wild-type strains (e.g. inadequate attenuation, ability to cross the ruminant placenta and/or increased transmissibility) (Kirkland *et al.*, 2004). These traits may be transferred from one strain to another during the reassortment process. Although evidence for the transfer of specific phenotypic traits due to reassortment is lacking, the potential of reassortment to transfer viral properties between different strains was considered important by animal health investigators in Europe during the 2006-2008 BTV-8 outbreak. Indeed, it was initially speculated that the ability of the European strain of BTV-8 to cross the placenta was acquired through reassortment with an MLV and/or unknown wildtype strain that demonstrated this ability (Brenner *et al.*, 2011). Reassortment also poses a risk in regards to the use of disabled infectious single cycle vaccines in the field. Disabled infectious single cycle (DISC) vaccines are virus strains that contain deletions in essential regions of the viral genome, preventing replication of the virus in the ruminant host. However, the use of these strains in the field may still result in reassortment. An approach that has been suggested to overcome this problem involves the alteration of the virus genome using a sub-optimal codon approach, thereby reducing the competitive advantage of DISC strains and/or their genome segments in the field (Roy *et al.*, 2009).

The advent of next generation sequencing technologies and genome characterisation of BTV field isolates will doubtless lead to the isolation of many more BTV reassortants from the field in future. This necessitates additional studies to identify the molecular determinants of BTV that influence viral phenotype, as well as studies to investigate the potential effect of reassortment of particular genome segments. The recent development of reverse genetics system through which mono-reassortants can be generated in a precise manner (Boyce *et al.*, 2008), will facilitate such studies in future. In summary the present study contributes to previous findings that genetic reassortment between different BTV parental strains, may

result in the generation of reassortant strains with altered phenotypic properties, at least in under *in vitro* conditions. Additional “parallel” *in vivo* and *in vitro* studies with the parental and reassortant strains should be conducted, in order to assess the implications of the results from this study *in vivo*.

7. Competing interests

The author(s) declare that they have no competing interests.

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10. Tables

Table 1: Summary of parental, control and reassortant strain phenotypic properties as measured using the various assays.

Virus name	Mean virus titre (log₁₀ TCID₅₀/mL) at 96 hpi	Time to CI₅₀^a	Mean percentage cell viability remaining at 72 hpi^b	Mean increase in cytotoxicity at 72 hpi^b	Mean increase in apoptosis at 72 hpi^b
rgP1	6.3 ± 0.1	71.0 ± 4.7	43.2 ± 3.5	5 ± 0.9	14.6 ± 0.9
rgP6	3.9 ± 0.1	98.0 ± 5.8	33.8 ± 8.9	9.8 ± 1.5	12.3 ± 0.7
rgP8	5.2 ± 0.2	64.5 ± 4.1	30.7 ± 5.2	8.9 ± 1.1	11.4 ± 1.2
BTV-8 (BTV8/net07/01)	5.9 ± 0.4	61.0 ± 3.5	28.2 ± 0.1	8.8 ± 0.2	9.6 ± 2.2
BTV-6 (MLV)	4.8 ± 0.3	91.5 ± 5.8	23.7 ± 1.4	8.6 ± 0.1	12.8 ± 2.0
rgR1-6(1,3,4,8,9)	5.8 ± 0.1	68.0 ± 1.3	54.7 ± 5.0	11.6 ± 0.3	11.6 ± 0.5
rgR1-6(2,6,7,10)	4.0 ± 0.4	142.5 ± 17.9	76.4 ± 2.3	1.2 ± 0.8	1.8 ± 0.1
rgR1-8(5,8)	6.3 ± 0.3	52.5 ± 2.9	27.1 ± 6.5	10 ± 1.8	17 ± 0.5
rgR1-8(2,5,8)	5.7 ± 0.4	48.0 ± 2.4	20.2 ± 1.2	7.6 ± 0.4	16.5 ± 0.1
rgR1-8(5,6,8)	6.3 ± 0.0	62.0 ± 7.0	21.2 ± 1.4	6.2 ± 0.2	16.2 ± 0.4
rgR1-8(5,7,8)	6.8 ± 0.3	64.0 ± 3.5	23.3 ± 5.2	5.9 ± 0.2	15.8 ± 0.3
rgR1-8(5,8,10)	6.3 ± 0.0	53.5 ± 0.8	20.0 ± 2.1	7.2 ± 0.2	13.4 ± 0.2
rgR6-8(10)	4.1 ± 0.6	105.0 ± 7.0	76.1 ± 2.6	8.4 ± 0.7	4.4 ± 0.3
rgR8-6(9,10)	5.9 ± 0.1	50.5 ± 4.1]	21.7 ± 1.2	8.1 ± 1.2	13 ± 1.2

^a Cell index values were normalised at 23.3 hpi

^b Cell viability, cytotoxicity and apoptosis data are presented as averaged values relative to mock infected controls.

Table 2: Calculated Spearman correlation (r_s) coefficients and probability (p) values that were generated for pair-wise comparisons of the *in vitro* properties/parameters (Table 1) in the present study. Mean values for each property were used to calculate the correlation coefficients. Probability values were interpreted at the 95% significance level ($p < 0.05$). Probability values are indicated in brackets next to the r_s values.

	CPE induction rate	Cell viability (72 hpi)	Cytotoxicity (72 hpi)	Apoptosis (72 hpi)
Median titre at 96 hpi	-0.523 (0.0810)	-0.537 (0.0700)	-0.278 (0.3816)	0.701 (0.0111)
CPE induction rate	N/A	0.874 (0.0002)	-0.133 (0.6802)	-0.748 (0.0051)
Cell viability (72 hpi)	N/A	N/A	0.084 (0.7952)	-0.699 (0.0051)
Cytotoxicity (72 hpi)	N/A	N/A	N/A	-0.049 (0.8798)

11. Figures

Figure 1: Replication kinetics of parental, reassortant and control strains in Vero cell culture. Cell cultures in 96 well plates were infected with 100 TCID₅₀ of each of the test strains and supernatants collected at 24, 48, 72 and 96 hpi. Supernatants were immediately titered in Vero cells by limiting dilution analysis. Error bars indicate the standard deviations for replicate readings.

Figure 2A

Figure 2B

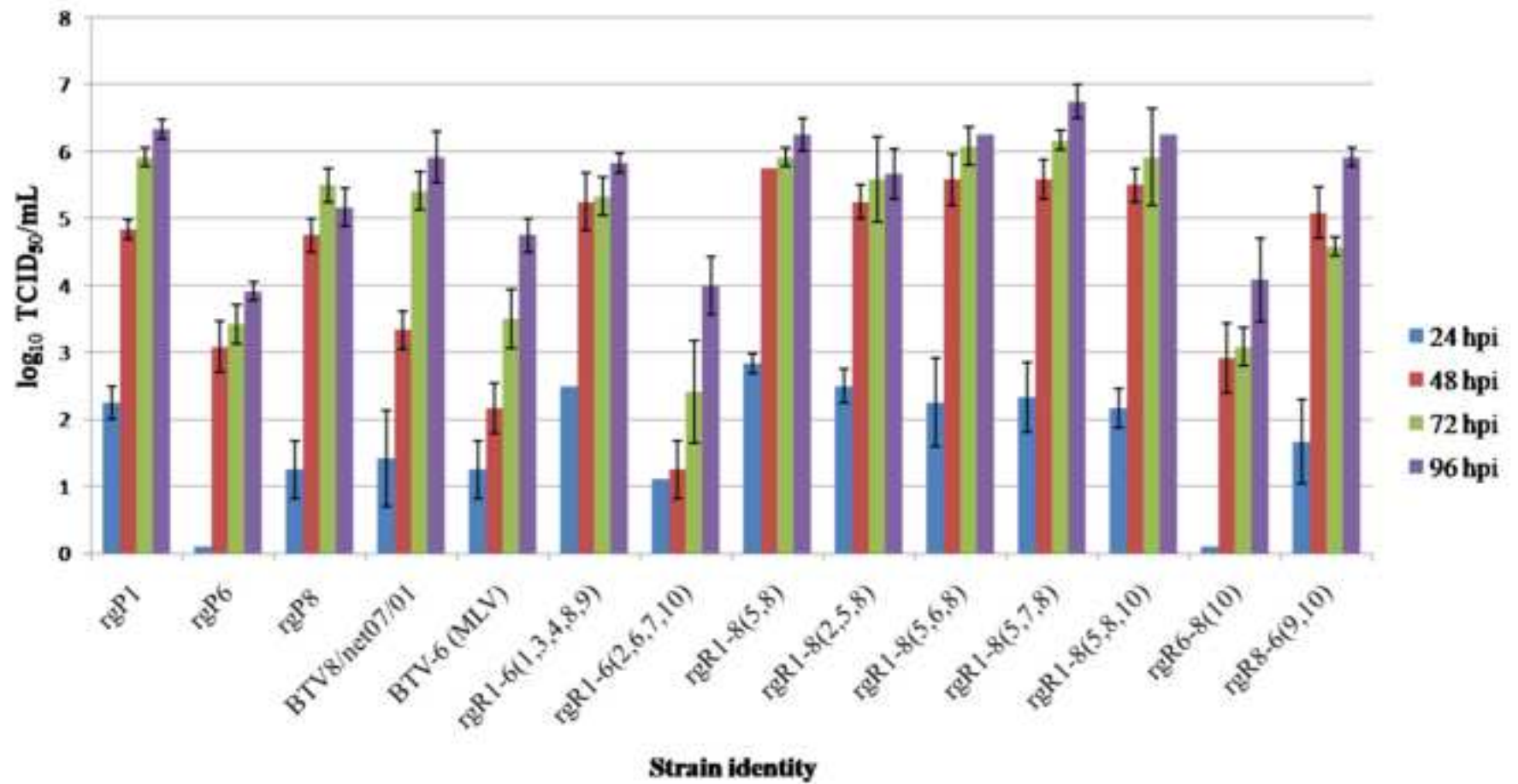
Figure 2C

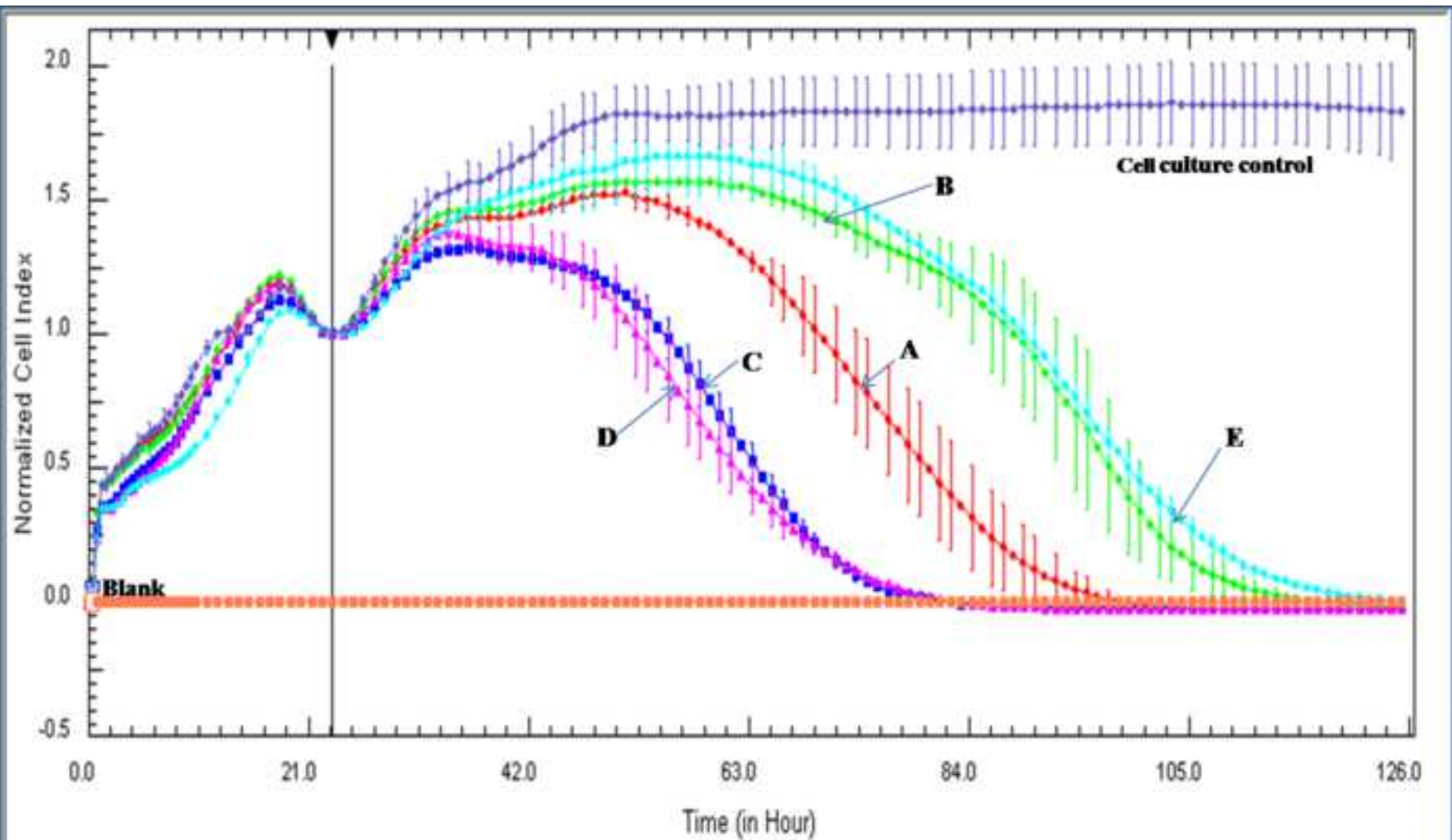
Figure 2D

Figure 2: Cytopathic effect profiles and induction rates of parental, control and reassortant strains in Vero cell cultures. Sixteen well E-plates were seeded with 38 400 Vero cells and left to equilibrate for 24 hours. Three wells each were subsequently infected with 100 TCID₅₀ of the test strains and CI values measured by using the RTCA-MP system every 30 minutes over a 7-10 day period. Cell index values were normalised at 23.28 hpi and CI₅₀ values calculated. Error bars on each composite curve indicate the standard deviations for individual CPE curves. Bluetongue virus strains are summarised in the different figures in relevant groupings (**Figure 2A**, parental and control strains; **Figure 2B**, BTV-1/6 strains/reassortants; **Figure 2C**, BTV-1/8 strains/reassortants; **Figure 2D**, BTV-6/8 strains/reassortants) and their corresponding CI₅₀ values (and standard deviations) indicated below the individual figures. Note that CI₅₀ values are given as averaged values for each composite curve.

Figure 3: Effect of test strain infection (100 TCID₅₀) on Vero cell culture viability at 72 hpi. Cell viability values were measured by using the Apotox Triplex assay at 72 hpi. The data is presented as the average percentage of viability remaining relative to that of mock-infected controls. Each bar is composed of data generated from three independent replicates. Error bars indicate standard deviations for replicate readings.

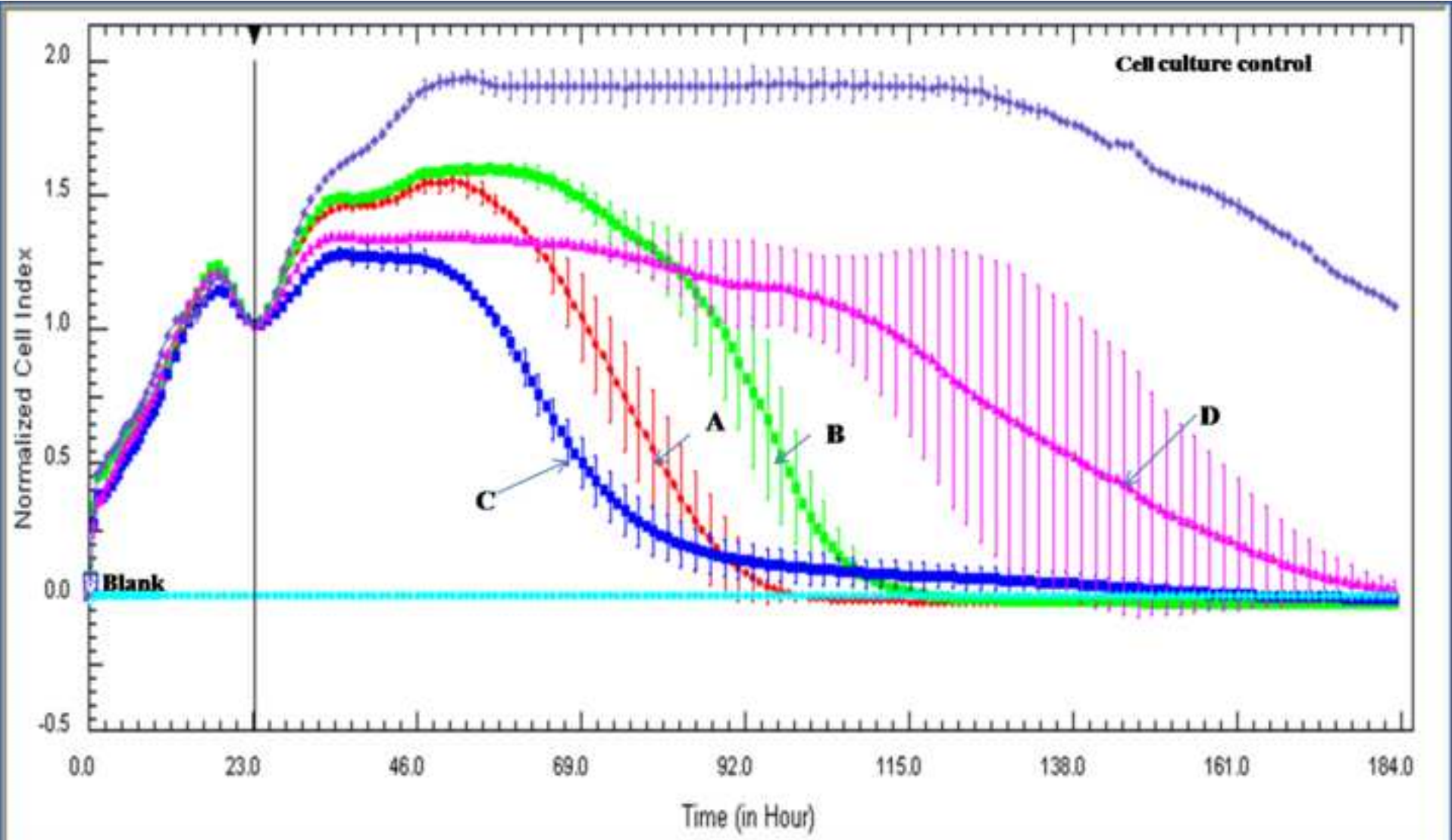
Figure 4: Evaluation of the mechanism and degree of cell death induced by test strain infection (100 TCID₅₀) in Vero cells at 72 hpi. Cellular markers for apoptosis and necrosis were measured by using an Apotox Triplex assay. The results are presented as the average increase in apoptosis/necrosis relative to that of mock-infected controls. Each bar is composed of data that was generated from three independent replicates. Error bars indicate the standard deviations for replicate readings. Cytotoxicity values are indicated in blue and apoptosis in maroon.





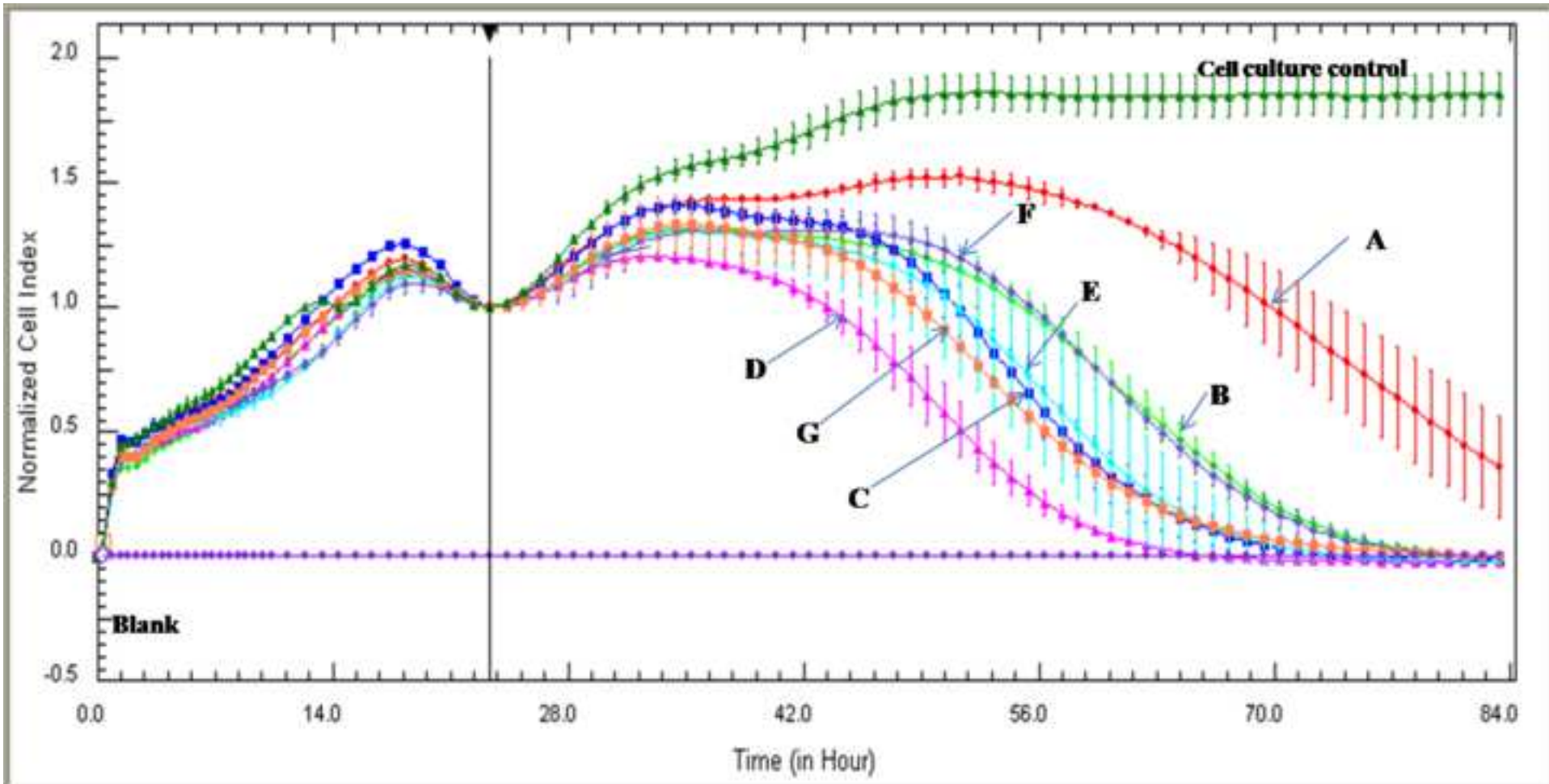
Curve identities and CI_{50} values for BTV parental and control strains

- A) rgP1 [71.0 ± 4.7]
- B) rgP6 [98.0 ± 5.8]
- C) rgP8 [64.5 ± 4.1]
- D) BTV-8 (BTV8/net07/01) [61.0 ± 3.5]
- E) BTV-6 (MLV) [91.5 ± 5.8]



Curve identities and CI_{50} values for BTV-1 and BTV-6 strains/reassortants

- A) rgP1 [71.0 ± 4.7]
- B) rgP6 [98.0 ± 5.8]
- C) rgR1-6(1,3,4,8,9) [68.0 ± 1.3]
- D) rgR1-6(2,6,7,10) [142.5 ± 17.9]



Curve identities and CI_{50} values for BTV-1 and BTV-8 strains/reassortants

A) rgP1 [71.0 ± 4.7]

B) rgP8 [64.5 ± 4.1]

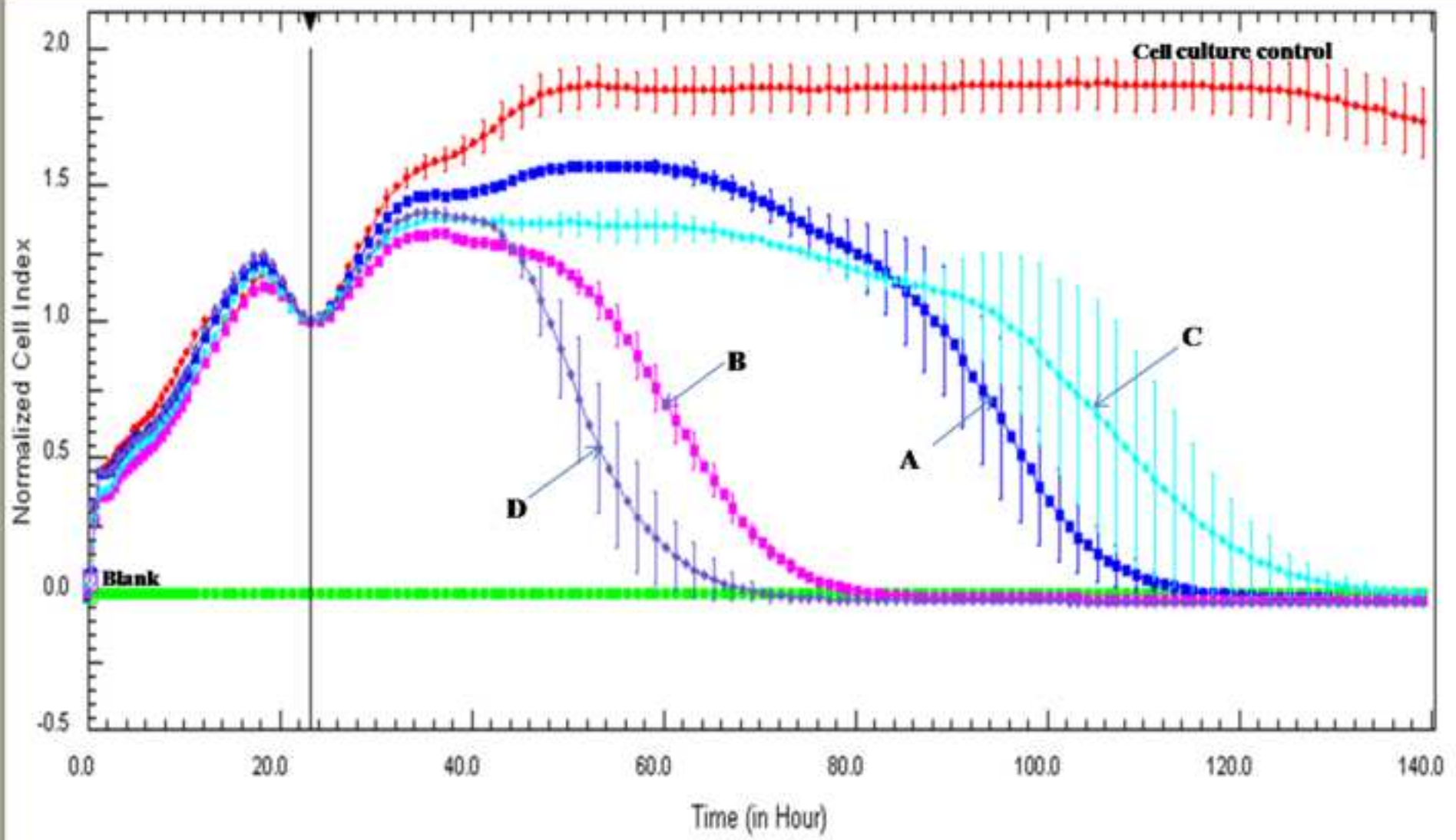
C) rgR1-8(5,8) [52.5 ± 2.9]

D) rgR1-8(2,5,8) [48.0 ± 2.4]

E) rgR1-8(5,6,8) [62.0 ± 7.0]

F) rgR1-8(5,7,8) [64.0 ± 3.5]

G) rgR1-8(5,8,10) [50.5 ± 4.1]



Curve identities and CI_{50} values for BTV-6 and BTV-8 strains/reassortants

- A) rgP6 [98.0 ± 5.8]
- B) rgP8 [64.5 ± 4.1]
- C) rgR6-8(10)[112 ± 8.1]
- D) rgR8-6(9,10)[49.0 ± 3.5]

