Experimental co-infections of calves with Bluetongue virus serotypes 1 and 8

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Abstract

The contemporarily circulation of multiple Bluetongue virus (BTV) serotypes or strains within the same territory, can imply the co-infection of the ruminant and/or the vector populations. As a consequence, the clinical and pathological outcomes of the co-infection as

well as the biological properties of the viral progenies could be influenced and suffer of relevant variations.

In this study, two independent experiments of co-infections were realized in calves using European strain of BTV serotype 1 and 8 (BTV-1 and BTV-8, respectively), with the objective to study their clinical and virological outcomes in comparison with BTV-1 and BTV-8 single infections.

Synchronous co-infections using the same titre for the two viral strains were performed and the clinical signs were quantified using a standardized clinical form. Serotype-specific real-time RT-PCRs and viral isolation were used to monitor the course of viraemia. Neutralizing antibody titres were measured along the experiments, and necropsy with viral detection in the affected organs was performed.

BTV-8 was detected in the EDTA-blood as well as in the organs of co-infected calves, while BTV-1 viraemia was inconstant. In parallel, the development of viraemia and high titres of anti-BTV-1 neutralizing antibodies in calves after BTV-1 single infection proved that the inoculum was infectious and the detection protocols were efficient.

Several hypotheses could explain the predominant detection of BTV-8 in the co-infected calves, such as the occurrence of a privileged BTV-8 segment 2 reassortment, like recently described during *in vitro* BTV-1/BTV-8 co-infections, the interference between the two viral strains or a higher BTV-8 tropism for the bovine species.

Keywords:

Bluetongue virus, serotype 1 and 8; calves, experimental infection; single infection; co-infection.

1. Introduction

Bluetongue disease in domestic and wild ruminants is caused by bluetongue virus (BTV) a member of the *Orbivirus* genus in the *Reoviridae* family (Mertens et al., 2005). BTV is a non-enveloped virus with a genome composed of 10 linear segments of dsRNA encoding for eleven distinct virus proteins (Mertens et al., 2005; Ratinier et al., 2011). BTV is characterised by great genetic and antigenic variability. At the time of writing, 26 distinct serotypes have been identified (Hofmann et al., 2008; Maan et al., 2007, 2011). BTV serotype 8 (BTV-8) emerged in Northern and Central Europe in August 2006 and it was characterized by a high virulence in sheep and, notably, in the bovine populations (Guyot et al., 2008). During its progressive radial distribution, its co-circulation with BTV serotype 1 (BTV-1) occurred in Spain and in the South of France. In numerous outbreaks, BTV-1 and BTV-8 were contemporarily detected, which implied the infection of the ruminant population with both viruses. Co-infection with two BTV serotypes or strains can lead to several biological consequences, such as variations of the clinical and pathological outcomes in the different susceptible species, or genetic rearrangements within the viral populations during the replication in the co-infected host. Reassortment and recombination are both phenomena which have been proved to occur for BTV in the field (He et al., 2010; Maan et al., 2012). Recently, a field reassortant strain between BTV-1 and BTV-8 has been identified, through full-genome sequencing, and its biological properties have been studied *in vitro* in comparison to its parental viruses (Shaw et al., 2012).

In this study, two independent experiments of co-infections were realized in calves using European isolates of BTV-1 and BTV-8, with the objective to study their clinical, virological and immunological outcomes in comparison with BTV-1 and BTV-8 single infections.

2. Materials and Methods

2.1 First in vivo co-infection

Four 12 months-old female Holstein calves have been used in this study. Before the beginning of the experiment, the calves were tested to confirm their naïve condition towards BTV, bovine viral diarrhea virus (BVDV) and Bovine herpesvirus 1 (BoHV-1). Furthermore, a veterinarian was attesting their healthy condition after a detailed clinical examination. The calves were confined in an insect-secure zone at the Experimental Infectiology Platform (PFIE) of the INRA center of Tours (Nouzilly, France). The local ethical committee approved the experimental protocol (dossier n. 2011-10-1).

The calves were divided in two groups of two animals, one being an environmental control group and another being infected. After one week of acclimatization, the infection was performed with 10^6 $TCID_{50}$ of BTV-1 and an equal dose of BTV-8. The infection was performed in the neck, subcutaneously (SC) and intravenously (IV). The BTV-8 strain BEL2006/01 was used, and the origin and the passage history of this strain have been published elsewhere (Martinelle et al., 2011; Toussaint et al., 2007b). The BTV-1 strain was kindly provided by Dr. Bernd Hoffmann from the Friedrich-Loeffler-Institut (FLI) (Eschbaumer et al., 2011). Only four days after the co-infection, Dr. Hoffmann found out that the BTV-1 inoculum was contaminated with BTV serotype 15 (BTV-15) (Eschbaumer et al., 2011).

The animals were monitored daily by a veterinarian for the duration of the experiment (21 days). A standardized clinical form was used during the examination of the animals (Saegerman et al., 2008) which was performed as previously described (Dal Pozzo et al., 2009a). A daily clinical score and a cumulative score at the end of the experiment were calculated for each animal. The average of the cumulative score was used to compare the two

groups. After 21 days post-infection (PI), animals were euthanized following an IV injection of pentobarbital. Spleen, prescapular and mesenteric lymph nodes, were collected and stored at -80°C for viral RNA detection.

2.2 In vitro kinetic growth of BTV serotypes

In order to compare the *in vitro* growth properties of the three BTV serotypes used in the first *in vivo* co-infection, their kinetic of replication was compared using VERO cell culture. First, plaque-purified BTV-1 and BTV-15 were obtained. Confluent 6-well microtiter plates (1×10^6 VERO cells/well) were infected with serial 10-fold dilutions (from 10^{-1} to 10^{-7}) of the inoculum (infected cell culture supernatant received from the FLI). Plaque assay was performed as described by Matsuo and Roy (2009). BTV isolates were obtained following a plaque-picking method. After five sequential passages of the two plaque-purified viruses on VERO cells, the absence of any residual contamination was verified by BTV-1 and BTV-15 specific real-time RT-PCR. The virus titres of the two stocks were determined by the end-point dilution assay (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml.

Plaque-purified and titrated BTV-1 and BTV-15, together with the original BTV-8 inoculum employed in this first experiment, were used in a one-step growth assay, with confluent VERO cells, a multiplicity of infection (m.o.i.) of 0.02 and following a protocol previously described (Matsuo et al., 2011). After 0, 4, 8, 12, 20, 28, 48 and 72 hours incubation, the supernatant was removed and stored at -80°C. For each time point PI the virus titre was determined in triplicate by the end-point dilution assay (Reed and Muench, 1938) and expressed as $TCID_{50}$/ml. For the three tested viruses the final titre was expressed as the median value of the three replications.

2.3 Second in vivo co-infection

Nine 6 months-old male Holstein calves have been used in this study and the same inclusion criteria used in the first experiment were verified. The calves were confined in an

insect-secure zone in the biosafety level 3 animal house of the CODA-CERVA (Machelen, Belgium). The experiments were approved by the local ethical committee (dossier n. 110228-01). The calves were divided in three groups of three animals: mock-infected calves, BTV-1 single infection, and BTV-1/BTV-8 co-infection. After two weeks of acclimatization, the infection was performed with 10^6 $TCID_{50}$ of virus (the group of co-infected calves received a dose of 2×10^6 $TCID_{50}$). The infection was performed in the neck, SC and IV. Mock-infected calves received 1 ml of uninfected cell culture supernatant SC and IV. Prior to the second experiment, BTV-1 and BTV-8 inocula were tested to exclude other serotype contaminations.

The animals were monitored for 35 days using the same protocol described for the first experiment. Similarly, euthanasia and necropsy were performed.

2.4 Laboratory analysis

2.4.1 BTV detection in biological samples

During the first and the second *in vivo* experiment, EDTA-blood samples were collected daily and stored at -80°C. These samples were used to detect the levels of viraemia by serotype-specific real-time RT-PCR, according to Vandenbussche and co-authors (2009) for BTV-1 and BTV-8, and Eschbaumer and co-authors (2011) for BTV-15. The construction of plasmid standard curves allowed the expression of the detected serotype-specific Segment 2 (Seg-2) RNA in copy number/µl, and within the same group of calves, the median value was calculated. Bovine beta-actin was contemporarily amplified as internal control (Toussaint et al., 2007a).

BTV RNA detection by the serotype-specific real-time RT-PCR was also performed from the organs collected during the two necropsies, as previously described (Martinelle et al., 2011). The detected serotype-specific Seg-2 RNA was expressed in copy number/µl.

In order to detect viable infectious virus in the bloodstream during the viraemic peak, EDTA-blood samples of the first experiment (3, 6, 8, 10, 12, 15 and 18 days PI) as well as of

the second experiment (9, 13 and 17 PI), were used for BTV isolation on ECEs (Bréard et al., 2003). In case of embryonic death between 24 hours and 5 days PI, the homogenized embryo tissues were used for RNA extraction and BTV-1, BTV-8 and BTV-15 specific real-time PCR were used to identify the isolated viral strain(s).

2.4.2 Serological analysis

For serological analysis, whole blood was collected daily. It was centrifuged at 3000 rpm for 10 minutes and the serum was stored subsequently at -20°C. Neutralizing anti-BTV-1, BTV-8 and BTV-15 VP2 antibodies were searched in the sera of the calves of the first experiment at several time-points (days 0, 6, 9, 13, 15, 18 and 21 PI). In the second experiment, only neutralizing anti-BTV-1 and BTV-8 VP2 antibodies were searched (at days 0, 7, 16, 29 PI). The method used to detect and measure the anti-BTV neutralizing antibodies has been previously described (Martinelle et al., 2011). The median titre of the anti-BTV neutralizing antibodies within the same group of calves was calculated.

3. Results and discussion

To the authors' knowledge, this is the first report of experimental *in vivo* co-infections of bovines with BTV-1 and BTV-8 European isolates.

The first *in vivo* experiment was characterized by the accidental triple co-infection of the calves. *A posteriori*, using a BTV-1 and a BTV-15 specific real-time RT-PCR (Eschbaumer et al., 2011; Vandenbussche et al., 2009), BTV-1 and BTV-15 Seg-2 RNA were quantified up to 4×10^6copies/µl and 1×10^5copies/µl, respectively in the original BTV-1 inoculum. The co-infected calves did not show hyperthermia, while other typical signs of bluetongue disease were present such as conjunctivitis, congestion of the dental pad with erosions and ulcers and congestion of the interdigital area (data not shown). The average of the cumulative score obtained by the group of co-infected calves was significantly higher compared to the control group (Fisher Exact Test $P<0.001$) (Fig. 1A). While control calves

were constantly negative, an increasing detection of BTV RNA started as soon as the first day PI in the blood of co-infected calves (Fig. 2A). While BTV-8 Seg-2 RNA was predominant, BTV-1 Seg-2 RNA was detected inconstantly, and always lower than 10 copies/µl, which is close to the limit of detection of the real-time (Vandenbussche et al., 2009). BTV-15 Seg-2 RNA was never detected in the bloodstream (Fig. 2A). In the course of viraemia, the significantly higher detection of Seg-2 BTV-8 RNA (comparison of the daily median values Seg-2 RNA number of copies/µl by two-factor ANOVA with repeated measures on one factor, P=0.001) was associated with the exclusive isolation of BTV serotype 8. BTV-8 isolation was obtained from the blood of both co-infected calves and the mean percentage of BTV-8 positive embryos/day is shown in Fig. 2A. The dominance of BTV-8 was further confirmed by the development of anti-BTV-8 neutralizing antibodies and the absence of measurable anti-BTV-1 and anti-BTV-15 neutralizing antibodies (data not shown). Furthermore, only Seg-2 BTV-8 RNA was detected in the organs of the co-infected calves (in average, 3.5×10^3 copies/µl in the spleen, 17 copies/µl and 5 copies/µl in the in the prescapular and mesenteric lymph-node, respectively). An interesting finding in one of the co-infected calves and confirmed also after histological examination, was the presence of a localized haemorrhage attacking 1/3 of the pulmonary artery wall.

The lack of evidence of BTV-1 and BTV-15 replication in the co-infected calves during the first *in vivo* experiment was surprising and a first hypothesis, i.e. different dynamic of replications of the three involved viruses, was formulated. The *in vitro* kinetic growth of the three BTV serotypes was evaluated in VERO cells. The median virus titres measured for each virus at the different time-points were compared using a two-factor ANOVA with repeated measures on one factor, showing a significantly different profile of the three kinetic growth curves (P=0.001) (Fig. 3). Unexpectedly, BTV-1 had an earlier and more abundant replication during the first 28 hours PI compared to the two other strains. BTV-8, however,

achieved equal high titres at 48 and 72 hours PI. Because of this efficient *in vitro* replication, other factors, than a minor kinetic growth, had to be considered to explain the lack of BTV-1 detection during the first *in vivo* co-infection. On the contrary, the *in vitro* assay pointed out a lower BTV-15 titre at all time-points tested (Fig. 3) and, together with the BTV-15 1 Log_{10} lower virus titre in the inoculum, it could represent a useful element of explanation of the lack of replication of this viral strain *in vivo*.

Because of the accidental BTV-15 contamination, a second *in vivo* co-infection was performed. The second experiment was characterized by the inclusion of a group of calves infected only with BTV-1. Previously, BTV-8 single infections had been performed using the same viral inoculum and the same experimental protocol (age and bovine breed, viral titre and inoculation route, clinical examination, BTV-8 RNA detection and isolation in the course of viraemia, anti-BTV-8 neutralizing antibodies) (Martinelle et al., 2011). The clinical signs shown by BTV-8 calves after single infection were previously described (Martinelle et al., 2011), while here we report the average of the cumulative clinical score (Fig. 1C). Calves after BTV-1 single infection had a one-two day hyperthermia (between 39.6 and 40.4°C), oral congestion (together with ptyalism in one case) and conjunctivitis (data not shown). Co-infected calves had a similar transient hyperthermia, oral congestion and erosions at the dental pad and internal lips, conjunctivitis (data not shown). The comparison of the average cumulative clinical scores revealed the absence of significant difference between the group of calves with BTV-1 single infection and BTV-1 and BTV-8 co-infection (Pearson's chi-squared test, P=0.813) (Fig. 1B). BTV-8 single infection attained the highest average cumulative clinical score compared to BTV-1 single infection and BTV-1 and BTV-8 co-infection. This finding is consistent with the more severe clinical outcomes observed during BTV-8 natural infections in bovines during the 2006-2008 epidemic in Europe, compared to other BTV strains and serotypes, and in particular to BTV-1 during its circulation in Spain

and in France since 2006 and 2007, respectively (Allepuz et al., 2010; Dal Pozzo et al., 2009b). In the course of both BTV-1 and BTV-8 single infections, calves developed viraemia, but the comparison of the median values of Seg-2 RNA (Log_{10} number of copies/µl) detected in the two groups, showed a significantly earlier detection of BTV-8 viraemia, which lasted until the end of the experiment, while BTV-1 viraemia was already over 16 days PI (Fig. 2B and 2C). In co-infected calves only BTV-8 viraemia was constantly detected and lasted until the end of the experiment, however, the median values of BTV-8 Seg-2 RNA (Log_{10} number of copies/µl), measured after the second week PI, were significantly lower compared to the group of calves with BTV-8 single infection (two-factor ANOVA with repeated measures on one factor, P=0.001) (Fig. 2B and 2C). Only in 2 out of the 3 co-infected calves and only at some sporadic days PI, BTV-1 Seg-2 RNA could be detected (at the limit of detection of the real-time RT-PCR) (data not shown). Interestingly and unlike previously observed during the first *in vivo* co-infection, the attempts to isolate BTV in ECE from the blood of co-infected calves, allowed the isolation of BTV-8 as well as BTV-1. Several ECE, used to isolate BTV from EDTA-blood samples collected at day 9 and 13 PI, were co-infected with both viruses, although BTV-8 confirmed to be predominant (Fig 2B).

Compared to the first experiment, in the second *in vivo* co-infection anti-BTV-1 neutralizing antibodies could be measured, although their titre was significantly lower than anti-BTV-8 neutralizing antibodies (data not shown). After BTV-1 single infection, Seg-2 BTV-1 RNA was detected only in the prescapular lymph-nodes of the three calves (at the limit of detection of the real-time RT-PCR), while after BTV-8 single infection, the viral RNA could be detected in the spleen and in the mesenteric lymph-nodes as well (Martinelle et al., 2011). These results highlight another interesting difference between BTV-1 and BTV-8 infection in bovine, such as a possible serotype-specific distribution of the two viruses in the organs. In the co-infected calves, Seg-2 BTV-8 RNA was detected in the spleen (at the limit

of detection), and in the prescapular lymph-nodes (20 copies/µl) together with Seg-2 BTV-1 RNA (at the limit of detection).

One of the main findings of both *in vivo* experiments was the disequilibrium in the detection of BTV-8 compared to BTV-1 (and BTV-15 in the first experiment). During the triple co-infection, BTV-8 was the only virus detected, on the contrary, in the absence of BTV-15, also BTV-1 was found (isolation during viraemia and development of anti-BTV-1 neutralizing antibodies). Viral interference can have an influence on the biological features of the future viral progeny in the course of co-infection. Nevertheless, while it has been described during synchronous co-infections between influenza virus strains (Wanitchang et al., 2012) and between influenza and Newcastle disease virus (Ge et al., 2012), viral interference occurred *in vitro* during asynchronous co-infections between BTV strains (Ramig et al., 1989). In this study, synchronous co-infections were performed, and concerning BTV-1 and BTV-8, equal viral titres were used. Recently, Shaw and co-authors (2012) presented the results of *in vitro* co-infections with BTV-1 and BTV-8 European strains. These authors showed that reassortant viruses could be generated *in vitro* and that the majority derived Seg-2 from their BTV-8 parental virus (Shaw et al., 2012). In our study, only serotype-specific real-time RT-PCRs have been used to detect BTV-1, BTV-8 and BTV-15, meaning that the detection of these strains was dependent to the frequency of the Seg-2. If, reassortment is a frequent event (Shaw et al., 2012; Ramig et al., 1989), we could hypothesize that the highest detection of BTV-8 Seg-2 during the first and the second *in vivo* co-infections was a consequence of a privileged reassortment of this segment within the viral population. This last hypothesis requires further investigations, but it represents a fascinating perspective allowing to confirm a previous *in vitro* observation. In the event that reassortment could not be proved and all the other segments would have the same BTV-8 origin, another possible explanation would be the highest efficiency of the BTV-8 strain used in this study (BTV-8 isolated during

the 2006 outbreak in Belgium) to infect bovines. The reasons underneath could be numerous, such as a difference in viral kinetic during *in vivo* replication, that could occur in various phases of the viral cycle such as a highest affinity of this BTV-8 strain for bovine receptors, or a faster *in vivo* replication cycle providing a dominance of BTV-8 over BTV-1 facilitated by an interference mechanism.

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Figure captions

Figure 1

The average cumulative scores, obtained after the cumulative sum of the clinical scores attributed to the calves of the same group, are presented for the first and the second *in vivo* experiment (respectively, A and B) and the BTV-8 single infection (C). The infected groups of calves are presented together with the correspondent control group. In A, calves were euthanized 21 days PI.

Figure 2

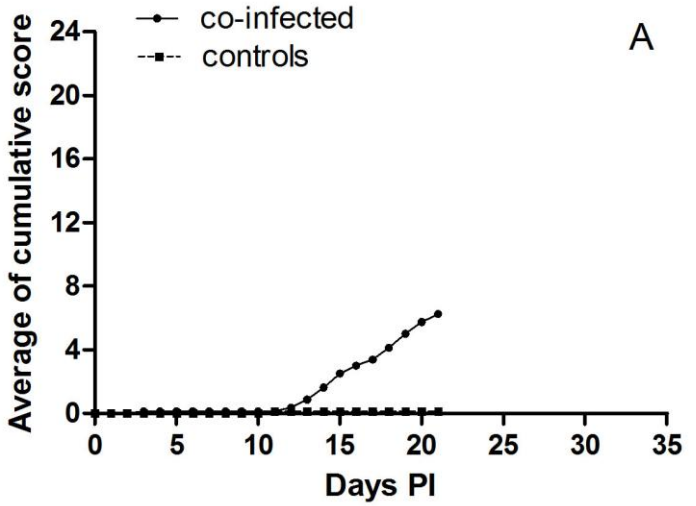
Viraemia was monitored during the first and the second *in vivo* experiment (respectively, A and B) and the BTV-8 single infection (C). In A, calves were euthanized 21 days PI. The median values of the number of copies of BTV-1, BTV-8 and BTV-15 Seg-2 RNA (in Log_{10}) detected in the blood at different days PI, are shown.

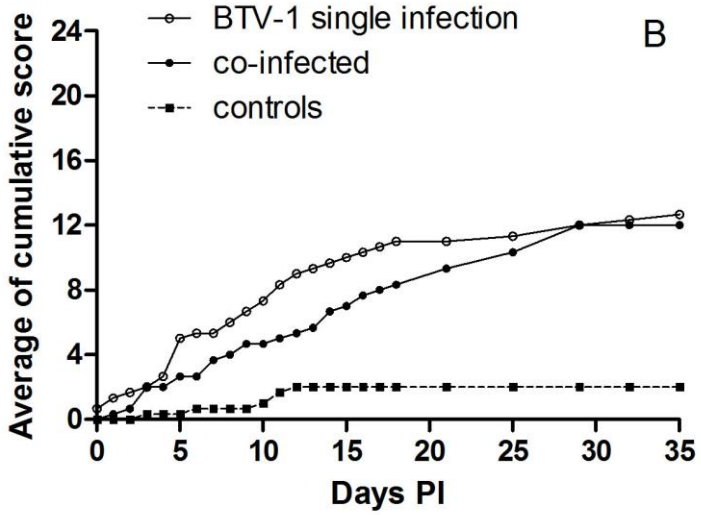
The numbers next to some points along the curves, indicate the mean percentage of ECE with positive BTV isolation starting from the blood collected the same day PI.

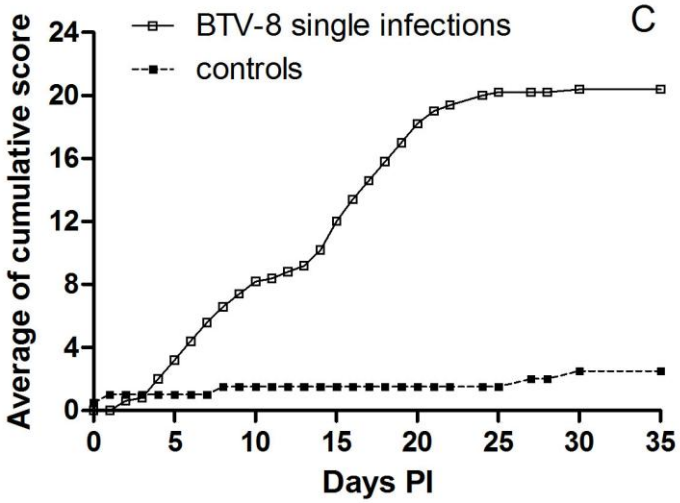
Figure 3

In vitro kinetic growth of BTV-1, BTV-8 and BTV-15. The virus titres (in Log_{10} of $TCID_{50}$/ml) are expressed as the median values of three results obtained independently.

Figure 1

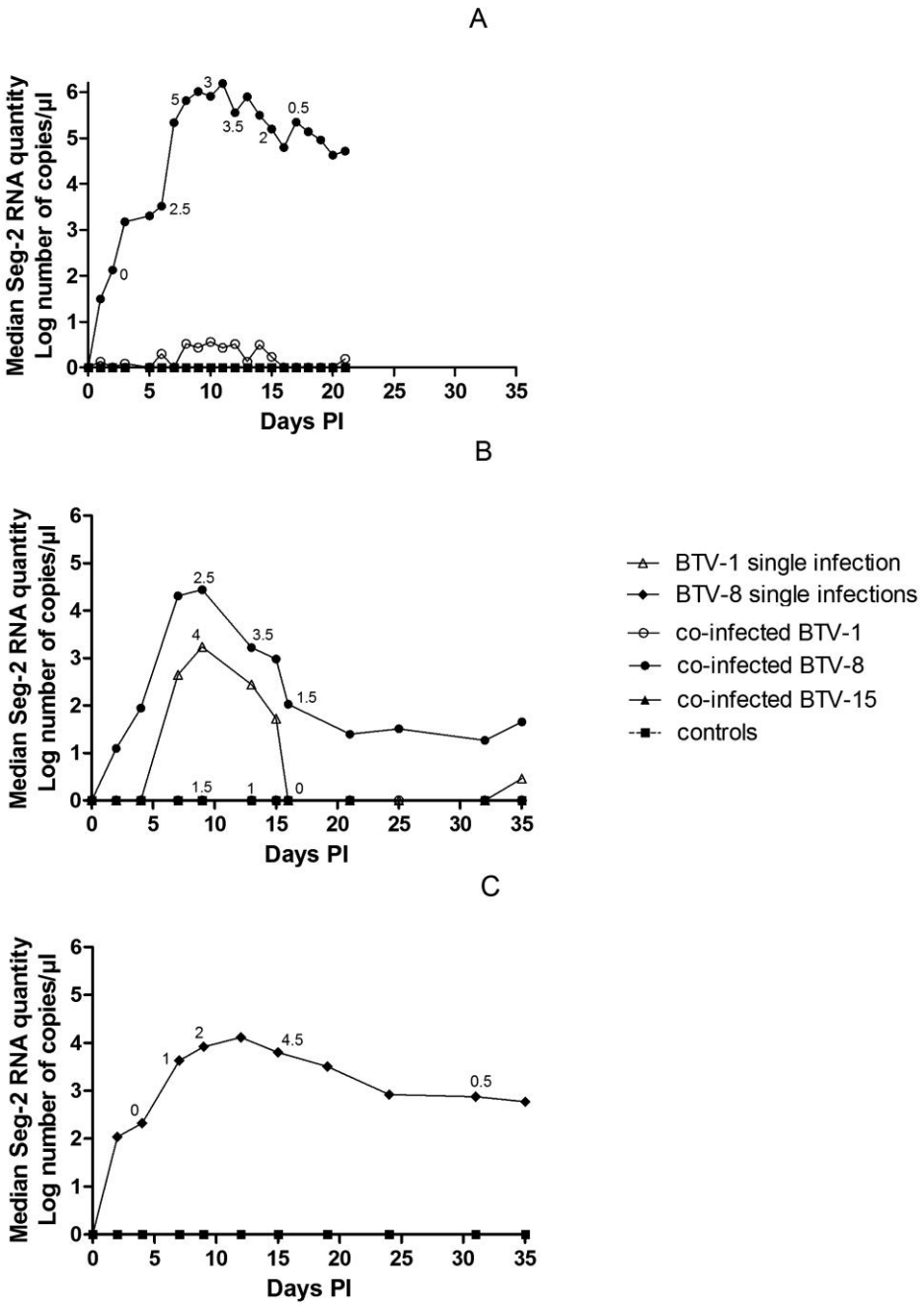

co-infected
controls
A
Average of cumulative score
Days PI


BTV-1 single infection
co-infected
controls
B
Average of cumulative score
Days PI


BTV-8 single infections
controls
C
Average of cumulative score
Days PI

392

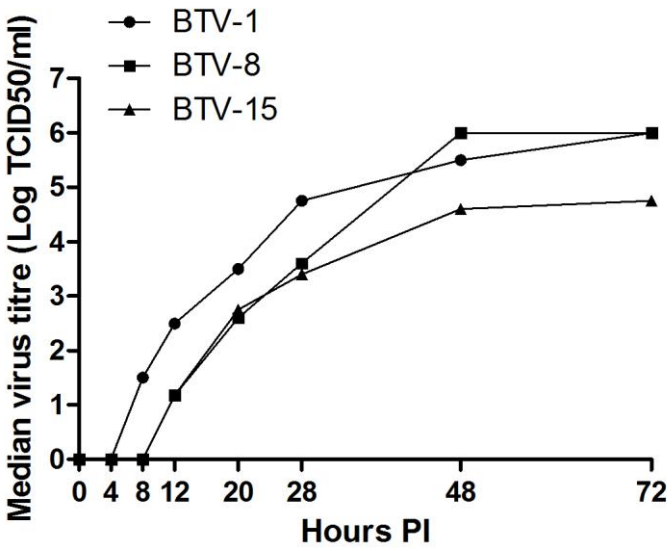
Figure 2



393

394

395 Fig. 3


BTV-1
BTV-8
BTV-15
Median virus titre (Log TCID50/ml)
0
1
2
3
4
5
6
7
0 4 8 12 20 28 48 72
Hours PI

396