Temperature affects reptarenavirus infection and inclusion body formation in a homotypic in vitro model.

Francesca Baggio[1,2], Iman Krijestorac Berbic[1,2], Eleni Michalopoulou[1], Jussi Hepojoki[1,3], Anja Kipar[1], Udo Hetzel[1]

The BIBD Group, [1]Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, 8057 Zurich, Switzerland; [2]Center for Clinical Studies, Vetsuisse Faculty, University of Zurich, 8057 Zurich, Switzerland; [3]Department of Virology, Medicum, Faculty of Medicine, University of Helsinki, 00290 Helsinki, Finland.
Contact: udo.hetzel@uzh.ch

Introduction

Reptarenaviruses cause Boid Inclusion Body Disease (BIBD), a lethal disease affecting in particular captive Boa constrictor populations. BIBD is characterized by cytoplasmic inclusion bodies (IBs), mainly composed of reptarenavirus nucleoprotein (NP), in many cell types[1-3]. Previous work by our group showed that reptarenavirus RNA, NP amount and IB formation decline when infected cells are kept at 37°C instead of 30°C[4] . The current study aimed to assess more in depth the effect of temperature on reptarenavirus growth and IB formation, a feature of potential relevance for snakes as poikilothermic animals. In addition, the effect of reptarenavirus infection on cell growth at different temperatures was also examined.

Material and Methods

Cells: *Boa constrictor* kidney-derived cell line (I/1Ki)

Virus: University of Giessen virus 1 (UGV-1) single reptarenavirus isolate

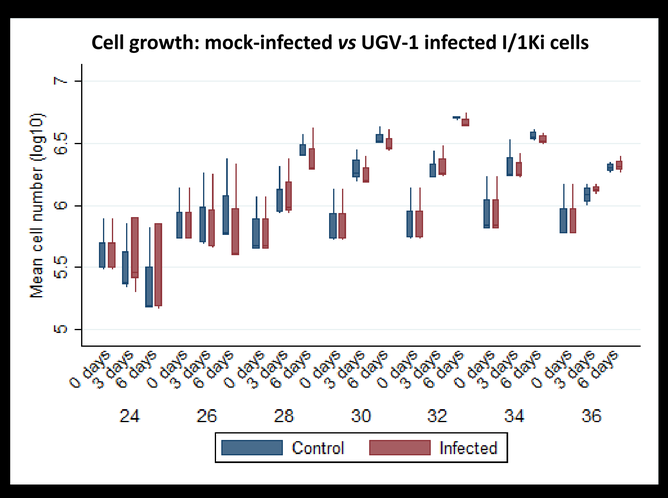
Methodologies:

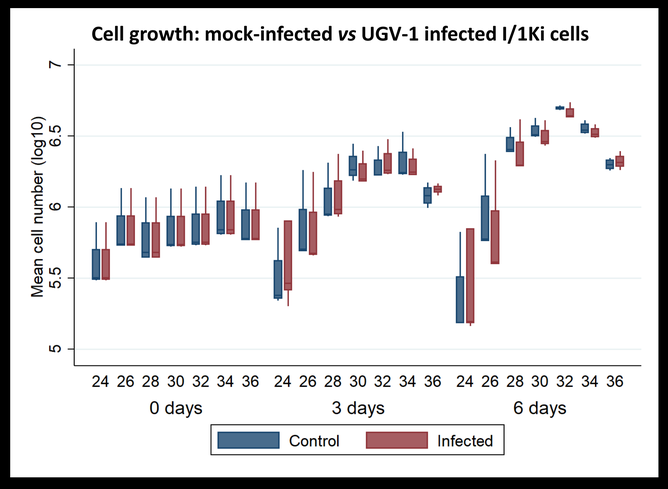
I/1Ki cells were inoculated with UGV-1 and incubated at different temperatures (24-36°C):

- Cell growth was assessed in infected *vs* mock-infected cells at 3 and 6 days post-inoculation (dpi)
- Reptanavirus RNA release was quantified through qRT-PCR analyses on cell culture supernatants
- IB formation was assessed and quantified in formalin-fixed, paraffin-embedded cell culture pellets by immunocytochemistry for viral NP and morphometry (Visiopharm)
- IB size/number and subcellular changes were determined via transmission electron microscopy (TEM) and immuno-EM

Results

1. Cell proliferation of I/1Ki is optimal at 30-34°C and not significantly affected by UGV-1 infection.

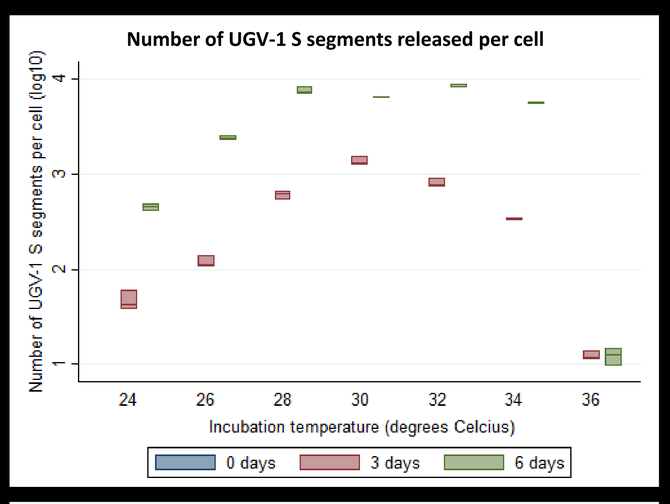


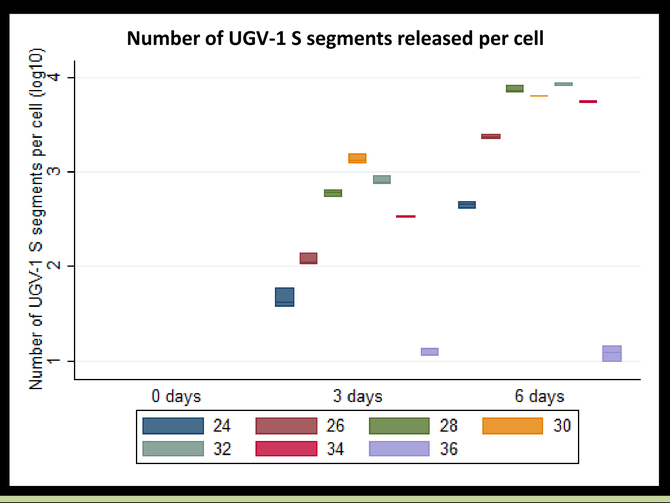


UGV-1 infected *vs* mock-infected I/1Ki cells were incubated at different temperatures between 24 and 36°C on 6-well plates. The same amount of cells ($7*10^5$) was initially seeded for all temperatures. Cell growth measurements were performed before virus inoculation, at 0 dpi, in order to exclude the non-adhering cells from the calculation of the proper multiplicity of infection (MOI=10), and at 3 and 6 dpi. In both infected and mock-infected cells, 3 replicates per time point were analyzed for each temperature. I/1Ki cells properly proliferated at 28-36°C whereas at 24-26°C cell counts were not significantly different over time. Statistical analyses were performed using Stat13® and pairwise mean comparisons were performed using the Bonferroni correction.

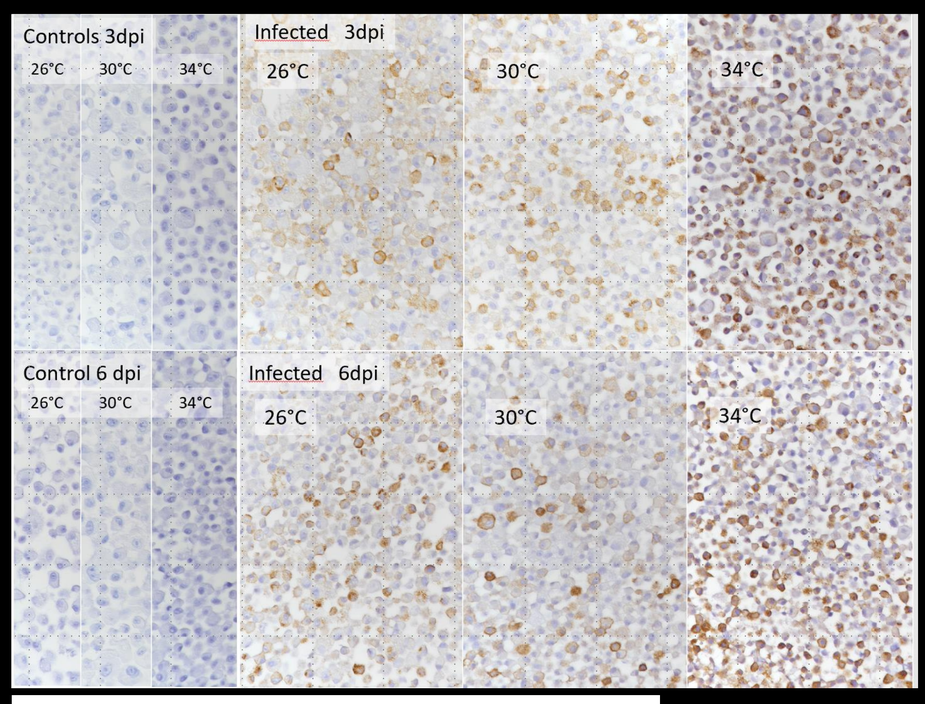
2. Viral RNA release per cell is highest at 28-32°C and does not differ significantly within this temperature range.

To determine virus release from infected cells, viral RNA was first isolated from cell culture supernatants of UGV-1-infected I/1Ki cells on 6-well plates. For each temperature, supernatants were collected at 3 and 6 dpi, in 3 replicates per time point. Afterwards, qRT-PCR analyses were performed to quantify the number of UGV-1 S segment copies released per cell in the cell culture supernatants. Except at 36°C, where UGV-1 S release per cell between 3 and 6 dpi was not statistically different, at all other temperatures UGV-1 S release per cell was higher at 6 *vs* 3 dpi indicating increasing replication of UGV-1 in I/1Ki cells over time.
For the statistical analyses pairwise mean comparisons were performed using the Bonferroni correction.

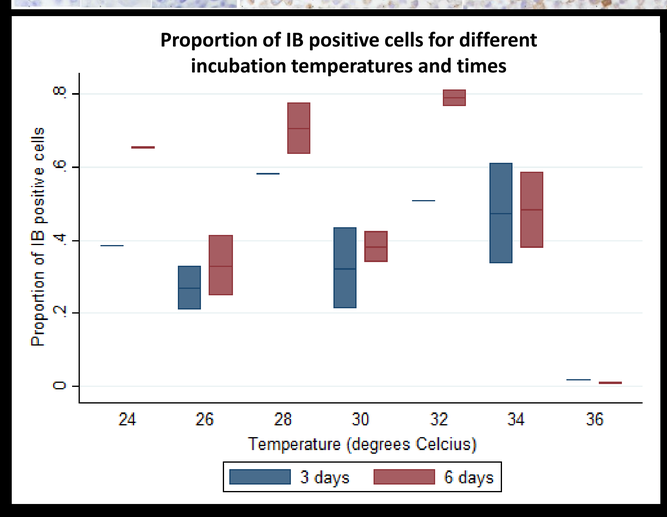




3. IB formation is most intense between 28 and 32°C.



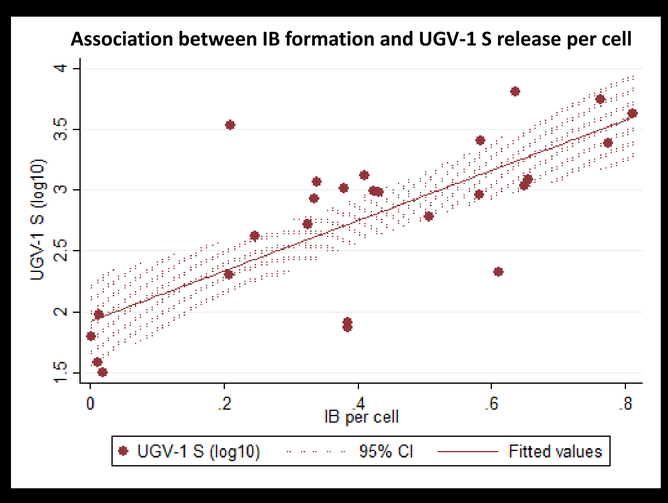
UGV-1 infected *vs* mock-infected I/1Ki cells grown in flasks at different temperatures were pelleted at 3 and 6 dpi and PFA-fixed.
Immunocytochemistry using an antibody against viral NP (main IB component[3]) was performed on paraffin sections of the cell pellets.
Top left: Immunocytochemistry results of viral NP (IB) occurrence at 26, 30 and 34°C at 3 and 6 dpi. IB formation intensifies from 3 to 6 dpi at 26 and 30°C, whereas morphologically appears widely identical at 34°C. Magnification 400x.



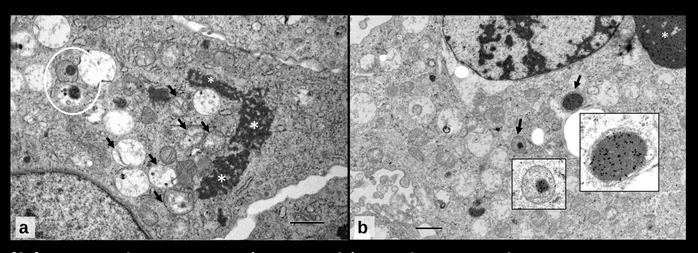
Subsequent morphometric examinations were performed on 10 paraffin sections of each UGV-1 infected cell pellet. For each section, the percentage of cells containing IB with respect to the total number of cells was assessed. For the statistical analyses pairwise mean comparisons were performed using the Bonferroni correction.

4. IB formation positively correlates with virus release.

Linear regression analyses were employed to examine the association at different temperatures between the amount of UGV-1 S segment RNA released per cell and the proportion of IB positive cells in the corresponding sections of cell pellets.
A linear regression model was fitted ($F=29.39$, $R2=0.550$, $p<0.0001$, UGV-1 S = 2.06*IB per cell + 1.92)
CI: confidence interval.



5. The ultrastructural findings correspond to the immunocytochemistry data on IB number and size and reveal limited damage to host mitochondria.



TEM **(a)** and immuno-EM **(b)** of I/1Ki cells at 30°C infected with UGV-1 at 3 dpi. **(a)** Cytoplasmic IBs (asterisks), vacuolated mitochondria (arrows) and partly ruptured mitochondrion with IBs in the matrix (circle).
(b) Cytoplasmic IB (asterisk) and several positive IBs within the mitochondria matrix (arrows). Inserts: higher magnification of the areas indicated by the arrows[5].

Conclusions

The results confirm previous evidence[4] that reptilian cells grow optimally at lower temperatures than mammalian cells (<37°C). They indicate that reptarenavirus infection does not affect cell growth and that virus replication and IB formation is temperature-dependent. Therefore, environmental temperature control could be a tool to limit BIBD development as well as virus replication and, potentially, the spread of reptarenaviruses in captive snake colonies.

References

[1] Stenglein MD *et al.* Identification, characterization, and in vitro culture of highly divergent arenaviruses from boa constrictors and annulated tree boas: candidate etiological agents for snake inclusion body disease. *MBio* **3,** e00180-12 (2012).
[2] Bodewes R *et al*. Detection of novel divergent arenaviruses in boid snakes with inclusion body disease in The Netherlands. *J. Gen. Virol.* **94,** 1206-10 (2013).
[3] Hetzel U *et al*. Isolation, identification, and characterization of novel arenaviruses, the etiological agents of boid inclusion body disease. *J Virol*. **87,** 10918-35 (2013).
[4] Hepojoki J *et al.* Replication of boid inclusion body disease-associated arenaviruses is temperature sensitive in both boid and mammalian cells. *J Virol*. **87,** 1119-28 (2015).
[5] Baggio F *et al*. A subpopulation of arenavirus nucleoprotein localizes to mitochondria. *Sci Rep.* **11,** 21048 (2021).