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Re: Revision WJV Manuscript NO: 36851

Dear Doctor Fang-Fang Ji

Thank you for your letter from November, 30th, that gave us helpful suggestions for the modification of the above designated manuscript. The comments of the reviewers encouraged us to perform a very detailed revision. We carefully followed all comments of the referees, addressed all points in question, and revised the manuscript accordingly. Please find below our detailed point by point reply to the reviewer.

All changes are marked in red and underlined. We hope that with these alterations our manuscript now meets your approval and so is suitable for publication in World Journal of Virology.

Thank you for your time and consideration.

Yours sincerely,

Eberhard Hildt

Point by point reply

Reviewer 1

Main points:

1. **Why the virus was not produced in each specific cell lines and used directly in the experiments? Can the author add some comments?**

To ensure a better comparability of the results we decided to use a single virus stock produced by infected vero cells to perform a single infection experiment. This means in each experiment, all cells were infected with the same virus stock to exclude any inoculum specific effects. The use of an inoculum common tp all infections in one experiments helps to exclude onspecific effects that are triggered by cell line specific factors released in the supernatant that serves as inoculum. Moreover as CHO cells are not susceptible we had to use virus produced in an alternative cell line.

Since in first experiments we did not know how susceptible the cells were and to which extent they were able to produce infectious virus an aliquotated stock of virus was used for all infection experiments.

Moreover, the time of virus inoculum cell-incubation in the cell lines was 16 h. Why so long time ? For example in the titration assay it was used a 2 h time of incubation. The author should explain or comment this differences.

At the present stage of knowledge we have no detailed information about the velocity of the infection process in the different cell culture systems. To avoid effects that reflect potential differences in the velocity of the infection process cells were infected for 16h (overnight) to ensure a high infection level. Compared to the 2h incubation time for plaque identification no acquisition of single plaques was intended in these experiments. This point was clarified on page 5 (lines 125-127) of the revised manuscript.

2. In the paragraph "Analysis of the amount and subcellular distribution of ZIKV envelope protein by confocal immunofluorescence microscopy it is not

clear as the author obtained the percent of susceptibility. How has it been calculated?

In the revised version of the manuscript we included a diagram and a table that summarizes the total number of cells, the number of infected cells and the resulting percentage. This is shown as figure 1e and described in the corresponding figure legend in the revised manuscript (page 25, lines 575/ page 10, lines 251-254).

In two visual fields the total number of cells were determined by counting the number of DAPI-labelled cells. For quantification of ZIKV-positive cells immunofluorescence microscopy was performed using the envelope protein specific antibody 4G2. The amount of ZIKV-positive cells was determined based on the env-staining. The percentage of ZIKV- positive cells was calculated and depicted in a diagram. (page 25, lines 575)

3. Few cell could have a deficient interferon response. How this the authors can comment in their investigation?

The experiments were performed with well characterized cells with low passage number. In light of this it is not very likely that these cells acquired a defect in the interferon response during passaging. It cannot be excluded that few cells acquired a mutation but this would be overcome by the much higher number of non-mutated cells.

Minor points:

1. Introduction, line 4: the authors should explain the name of vector specificity (mosquito ?)

We changed this in the revised version from arbo born virus to mosquito born virus (page 3, line 77 of the revised manuscript)

2. Introduction, line 18: Add the word “virus” after Spondweni.

We performed this correction and added the word “virus” after Spondweni (page 3, line 77).

3. Introduction, lines 20-25: Add references

As requested by the reviwer we included an additional reference:

Kuno G, Chang G-JJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. *Arch Virol* 2007; **152**: 687–696 [PMID: 17195954 DOI: 10.1007/s00705-006-0903-z]

New reference 10 in the revised version.

4. Materials and methods. In RNA isolation and cDNA synthesis it should be explained the amount of RNA used for retrotranscription.

4µg total RNA were used for this. This information was included in the revised RNA isolation and cDNA synthesis part (page 5, line 143).

5. Materials and methods. It should be explained if the qPCR used for the supernatant is an One-step qPCR or not.

Indeed, this is a One-step PCR. The detailed information about the PCR program was included:

…with the following programm. 1. RT-Step: 55°C/ 5min; 2. Denaturation: 95°C/ 5min; 3. Cycling (45 times): 95°/ 5sec, 60°C/ 15sec, 72°C/ 15sec; Cooling: 40°C/ 30sec. This information is provided on page 6, line 160-161 of the revised manuscript.

6**. Materials and methods. The ZIKV strain paragraph should be move after the cell culture paragraph.**

The ZIKV strain paragraph was moved after the cell culture paragraph (page 5, lines 112-115)

7. Figure 1: the difference showed in figure 1a are not to be consider in the reason of that the log difference obtained can be a mere fluctuation of the

quantification. We agree with the reviewer that there are only small differences between the amount of viral genomes found in the supernatant of the various cell lines. This is stated in the manuscript. The small p-values however argue against that this reflects a mere fluctuation of the quantification.

8. Results, line 16: “about 10^5-fold” should be changed in "up to 10^5-fold".
This point was changed asas suggested by the reviewer (page 9, line 222)

Reviewer 2

Main points:

1. Clarification on why ZIKV strain French Polynesia was chosen for the study should be provided.

ZIKV strain French Polynesia was chosen as this strain was found to be associated with increased incidence of microcephaly during the ZIKV pandemic in French Polynesia 2013. Moreover the French Polynesia isolate is closely related to the ZIKV strain isolated during the ZIKV pandemic in South Americas. This is clarified in the revised version of the manuscript on page 12, line 314-315.

Is the most relevant with respect to disease incidence?

As stated above the association between ZIKV infection and microcephaly and GBS was already observed for the ZIKV outbreak in French Polynesia (Baud et al., 2017; Lancet). At present it is still unclear whether virus-specifc or host factors are relevant for ZIKV-associated microcephaly or GBS.

Is this a lab adapted strain or a true low passage clinical strain?

This isolate is a true low passage clinical strain. This is more clearly described on page 5 lines 113-115 of the revised manuscript.

How divergent are the strains of ZIKV? If divergent it would be worth testing multiple strains.

There were three different lines of ZIKV isolates described. The eastern- and the westwrn African isolate and the south east Asian isolate that is represented in our study by the French Polynesia isolate. We agree with the reviewer that a comparative analysis of the different lineages would be interesting but this will be a new project that cannot be performed in the limited time provided for revision of the manuscript.

2. No NS1 is apparent in figure 1c for ZIKV-infected SHY5Y cells in contrast to the statement in the results that lower amounts of NS1 were detected for SH-SY5Y? This needs to be clarified.

We clarified this erroneous statement in the revised version of the manuscript (page 10, line 243).

It would be helpful if the band intensities of NS1 were quantified and normalised to the loading control actin to provide a quantitative figure for comparison with figure 1b.

As requested by the reviewer, we quantified two western blots from two independent experiments. These data were shown as Figure in 1c in and described as follows in the revised version of the manuscript:

(“Two western blots from two independent experiments were quantified using Image J software. The relative NS1 amount represents the ratio between NS1 and actin.”) (Page21, lines 551-553).

3. Given CHO cells do not support ZIKV infection apparently at the stage of entry and the CHO cells were viable it would be extremely interesting to know if they lack the receptor for ZIKV entry. If the receptor is known then an experiment showing lack of surface expression in CHO should be included.

We fully agree with the reviewer that this is a relevant and interesting question. At present there are a couple of receptor (candidates) described and the reports

about this are in some aspects conflicting(Wang et al., J. of Gen. Virol 2017, 98, 2061; Hastings et al., Cell Rep, 2017; 19, 558). However, a detailed analysis of this very interesting question cannot be performed within the limited time given for the revision. Moreover it is not clear at the present stage whether the lack of susceptibility if CHO cells to ZIKV infection is due to an impaired entry or post-entry step.

4. For figure 1d quantification of infected vs non-infected cells should be performed and both raw cell counts and ratios included in a table.

As requested by the reviewer this information is provided as figure 1e in the revised version of the manuscript:

Two visual fields were counted for the total amount of cells according to the DAPI staining and the amount of ZIKV-poitive cells was determined due to the Env-staining. The percentage of positive cells was calculated and depicted in a diagram.

(pages 24/25, lines 576-581)

5. For figure 4a the differences in transfection efficiency between cell lines needs to be accounted for by normalising to a transfected plasmid expressing GFP (can be quantified by flow cytometry for example).

This is an important point. To avoid an impact of differences of the transfection efficiency between the different cell lines we compared for each cell line the uninfected cell line that was arbitrarily set as 1 to the infected cell line. We clarified this point in the revised version of the manuscript (page 28 lines 612-613).

6. Figure 4b requires an uninfected control for comparison (results text actually refers to the uninfected control) and inclusion of the N29.1 cell line which based on figure 4a has an opposite interferon response compared to A549.

An uninfected control for figure 4b was included as requested by the reviewer (page 28, line 618).

7. In the discussion the statement that the findings with 293T cells is in contrast to a published report needs further elucidation? Was the infection levels the same. Can the authors be sure of the integrity of their 293T cell line. STR profiling, where possible, of their cell lines would be highly beneficial.

The 293T cells used in this study were obtained from the DSMZ (German Collection of Microorgnisms and Cell Cultures) ensuring their integrity. However, there are significant differences in the infection protocol. In the study published by Hamel et al., 2015 the cells were infected for 2 h in contrast to the infection time of 16 h in our study.

8. The last two sentences in the 4th paragraph of the discussion make no sense?

We clarified this in the revised version (page 13, lines 323-324).

9. Further explanation on why CHO cells would have reduced interferon response if not infected need to be provided. This could be due to differences in transfection efficiency rather than cell line differences. See point 5 above.

To avoid that differences in the transfection efficiency of infected and uninfected cells affect the assay the cells were transfected prior infection. As we don`t know whether the lack of susceptibility of CHO cells for ZIKV infection is due to impaired attachment, entry or post entry steps there exist a variety of possibilities that could lead to an interference with the interferon signaling. We discussed this in the revised version of the manuscript on page 14, line 380-383:

Minor points:

1. Introduction 2nd paragraph: a reference(s) is required for Zika virus classification and properties.

The additional reference was provided:

Kuno G, Chang G-JJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. *Arch Virol* 2007; **152**: 687–696 [PMID: 17195954 DOI: 10.1007/s00705-006-0903-z]

(Page 3, line 82

2. Materials and methods section on "ZIKV strain" should be immediately after "Cell culture" and "Virus titration assay" should come immediately after "Infection procedure".

The order was changed as suggested by the reviewer (page 5)

3. Information on which region of the Zika virus genome the primers for qPCR actually map to should be provided.

The exact binding sites were included in the description of the PCR procedure:

Zika fwd (5`agatcccggctgaaacactg3`-bp 1924-1943), Zika rev (5`ttgcaaggtccatctgtccc3`-bp 1996-1977). This is described on page 6, line 160-161.

4. RPL27 is not defined?

RPL27 was defined as:ribosomal protein L27 - RPL27 fwd (5` aaagctgtcatcgtgaagaac3`) and RPL27 rev (5` gctgctactttgcgggggtag3`)

This is described on page 6, lines 151-153,

5. In materials and methods under virus titration assay should state "serial dilution of either cell culture supernatant or cell lysates….."

This was changed as requested by the reviewer (page 5, line 131-132)

6. Need to clarify that the Flavivirus group antigen antibody is the same as in used in figure 1d and 4b which is described as virus envelope specific?

This was clarified in the MM-section

(Zika virus envelope protein was stained using anti Flavivirus Group antigen Antibody (clone D1-4G2-4-15 from Merck-Millipore, Darmstadt Germany)). This is found on page 7, lines 175.

7. In materials and methods under "transfection and … assay" need to define components of the "passive lysis buffer"?

The components of the buffer were listed as follows:

Here for cells were lysed in a passive lysis buffer (25mM Tris, 2mM DTT, 2mM EGTA, 10% glycerol (v/v), 1% TX-100 (v/v), pH7.5) for 10 min on ice. This information is included in the revised manuscript on page 7, line 196-197.

8. For figure 1c no mention of westerns or the antibody against NS1 is made in the materials and methods?

As requested by the reviewer we included the detailed information in the the MM-section:

(Western Blot Analysis

The samples were resolved by sodium dodecyl sulfate-polyacrylamid electrophoresis (SDS-PAGE) at 10% and transferred by semi-dry blotting onto a polyvinylidine difluoride membrane (PVDF) (0,45µm; Carl Roth, Germany). The membrane was blocked with 5% skim milk solution and then incubated with anti NS1 specific antibody at a 1:1000 dilution (Biofront, USA) overnight. Then the membrane was incubated with a mouse specific secondary antibody coupled with horseradish peroxidase at a 1:2000 dilution (HRP) (GE Healthcare, UK) and signals were detected with x-ray films (GE Healthcare, UK). Signals were quantified using ImageJ software.). This information is found on page 6/7 lines 162-171 of the revised manuscript.

9. Scale bars should be shown in figure 1d and 4b.

Scale bars were included in the revised figure 4b

10. Figure 4a legend presumably the authors mean uninfected Vero cells as reference?

Here is a misunderstanding in so far, as that each cell line was referred to its uninfected control that was set as one. We added the following description in 4a legend:

Uninfected means that each infected cell line was referred to its uninfected control that was set as one. We clarified this point in the revised version of the manuscript (page 28, lines 612-613).

11. For Table 1 a more appropriate heading than "inventor" would be "origin" or "source"

We agree and accordingly changed the heading to origin. (page 30).

Reviewer 3

No changes were suggested.

Reviewer 4

Major points:

1. The origin of the cells used should be described more clearly. Another important point is to present the passage used in the experiments since this fact can greatly influence the results of the experiments.

The origin of the cell lines is described in table 1. The cells were obtained from the DSMZ (German Collection of Microorgnisms and Cell Cultures) or from the Dept. Virology cell bank. For all cell lines low passage numbers were used.

2. Why were two different methodologies used to evaluate intracellular and extracellular viral RNA?

The viral RNA isolated from cell culture supernatant was transcribed directly in the qPCR as a one-step RT-PCR. Whereas the qPCR from total RNA was performed with reverse transcribed cDNA in a different reaction and the detection was performed using sybr green. In our hands the one-steptRT-PCR is more suitable for the analysis of supernatants , while the “classic” two step procedure gives the more reliable results for the intracellular RNA.

3. The experiment to analyze changes in the Interferon pathway should be reviewed. It is known that DNA transfection into cells can alter various intracellular conditions, and infection immediately after transfection may have been compromised. Luciferase values should be normalized with an uninfected (mock-infected) control.

We clarified this point in the revised version of the manuscript. All luciferase values are normalized to the uninfected control of the respective cell line. We described this in the revised legend to fig. 4a (page 28, line 612-613).

4. In the results it was described that the western blot experiment of NS1 expression was higher or lower in the cell lines tested, however, it has not been described in the methods what methodology was used to quantify the expression of said protein.

As requested by the reviewer a detailed description of the western blot procedure, of the used antibodies and of the quantification is included in the MM-section.:

(Western Blot Analysis

The samples were resolved by sodium dodecyl sulfate-polyacrylamid electrophoresis (SDS-PAGE) at 10% and transferred by semi-dry blotting onto a polyvinylidine difluoride membrane (PVDF) (0,45µm; Carl Roth, Germany). The membrane was blocked with 5% skim milk solution and then incubated with anti NS1 specific antibody at a 1:1000 dilution (Biofront, USA) overnight. Then the membrane was incubated with a mouse specific secondary antibody coupled with horseradish peroxidase at a 1:2000 dilution (HRP) (GE Healthcare, UK) and

signals were detected with x-ray films (GE Healthcare, UK). Signals were quantified using ImageJ software.)
This information is provided on page 6/7 of the revised manuscript.

5. I believe that the LDH methodology should be better described, since it is not such a commonly used method.

We followed the suggestion of the reviewer and included a more detailed description of the LDH methodology. (Upon cellular damage lactate dehydrogenase (LDH) is released into the cell culture supernatant. This release is indirectly measured based on a calorimetric assay detecting an enzymatically formed formazan product. Presto Blue is a red compound that is taken up by the cells and due to the reducing interior environment turns into a red color that is detectable at 570nm.)
This information is provided on page 7, line 183-188 of the revised manuscript.

6. In the fifth line of the discussion it was described that the Polynesian isolate is genetically close to that of Brazil. In fact he is the closest, compared to the African, but it is not so close.

We agree with the reviewer and changed the statement accordingly. ZIKV strain French Polynesia was chosen as this strain was found to be associated with increased incidence of microcephaly during the ZIKV pandemic in French Polynesia 2013. Moreover the French Polynesia isolate is closely related to the ZIKV strain isolated during the ZIKV pandemic in South Americas

(“Since the Brazil epidemic in 2015/2016 [19] ZIKV research has increased dramatically. In this study a Zika virus isolate from French Polynesia that also belongs to the Asian lineage like the ZIKV strain causative for the epidemic in Brazil, was used to characterize its potential to infect various human cell lines with

the aim to provide cell culture models for investigating the Zika virus life cycle in more detail and to test the suitability of various cell culture systems to produce high amounts of this virus [21]. **For the ZIKV outbreak in French Polynesia an association with microcephaly was observed (Baud et al., 2017, Lancet)**
This can be found on page 12, line 314 of the revised manuscript.

Minor points:

1. In the seventh line of the last paragraph of the introduction there is a punctuation error in the sentence.
This mistake was corrected:
Moreover neuronal cells (N29.1 and SH-SY5Y) were of special interest due to the neurological disorders Zika virus infections may cause. Furthermore the infectivity of the well-established standard cell lines… (page 4, line 101)

2. In the third line of the topic "Cell culture", the correct spelling is L-Glutamine
I-Glutamin was changed to L-Glutamine (page 4, line 108).
3. The section describing the viral isolate used in the study should be placed in a more initial part of the material and methods. Also, add in which viral passage the isolate was used - this characteristic may influence the results.
The chapter describing the Zika-strain was placed directly after the cell culture chapter. This clinical low passage strain was kindly provided by Professor Musso, Institute Louis Marlade in Papeete, Tahiti. (Page 5, lines 112-115)
4. Last line of results separate the “293T” from “cells”.
293T was separated from cells. (page 11, line 304).