Response to Reviewer 3 Comments

Thank you kindly for the reviews of our manuscript. Below is a point-by-point response to your comments including line references in the revised version.

Main Comments:

Point 1: Assumptions regarding prior DENV exposure. The authors assertion that any positive PRNT assay against DENV-2 New Guinea C reflects prior DENV exposure is not adequately justified. The authors should cite references when asserting this parameter in their study, i.e. after statements in lines 106 and 176-177. As admitted in lines 189-191 in the discussion, many different flaviviruses can generate cross-neutralizing antibodies, and indeed it may be that prior exposure was not DENV but rather another flavivirus. Though the authors state that patients had recently returned from Caribbean travel (lines 123-124), that can only account for ZIKV infection, and by no means controls for the geographical site of prior flavivirus exposure. Therefore viruses that might confound PRNT results such as Kedougou virus or Spondweni virus (genetically similar to DENV/ZIKV), or indeed prior African- or Asian-acquired ZIKV exposure may be relevant. Indeed, for at least patients 2 and 8, PRNT titres for ZIKV are equal to or greater than DENV (Table 2), arguing that DENV was not the virus these individuals were previously exposed to. As prior DENV exposure was the central defining point segregating patients for comparison, evidence of this needs to be much stronger for this paper to be published. As it stands, the reader cannot be sure that patients should be regarded as having prior exposure to DENV, and so any and all further analyses cannot be seen as trustworthy. Do the authors have access to patent histories that relate confirmed diagnoses of DENV infection (i.e. by qPCR) in the past?

Response 1: Retrospective analysis of testing records indicated that none of these patients were tested previously for dengue virus by Public Health Ontario, the sole testing provider for dengue serology in the province of Ontario in Canada. However, this does not preclude the fact that testing may have occurred in another jurisdiction, or that the patients did not seek medical care. It should be noted that this is often a limitation of clinical studies, as it is not always possible to get health records from other regions. We used the established CDC diagnostic criteria outlined by Rabe and colleagues (Interim Guidance for Interpretation of Zika Virus Antibody Test Results MMWR 65 (21), 543-6 2016) to determine whether patients had previous dengue virus exposure, or a previous exposure with a flavivirus that could be dengue virus, but could not be identified. Therefore, we have made significant changes to the manuscript.

Point 2: Determination of viremia – timepoint and methodology. 5 days post-symptom onset is not the same as 5 days post-ZIKV infection (Table 1) and is likely to be at a point where ZIKV is cleared from human serum (Lessler et al, (2016) Science). Therefore, measurement of ZIKV abundance in patient serum at this timepoint is very likely to be insufficient at relating differences in peak viremia load between groups. Also, could the authors provide a range of days for each cohort that represent time from symptom onset to sample collection, rather than just presenting the mean of 5 days? If there is a lot of variation within groups, presented data becomes even harder to accurately interpret.

We have included the range of days as part of the table. Neither in the results not in figure 1 is viremia described as peak viremia, but is in fact described as the relative magnitude of viremia. We agree that it would be ideal to sample patients at the time of peak viremia, but in the clinical setting they often do not present at this time in the course of their illness. An ideal study would include a much larger number of patients and control for the day after symptom onset that the patient is tested.

Point 3: Regarding the use of the Altona RealStar qRT-PCR kit for viremia interpretation, the manufacturers of this kit state that it is designed for qualitative rather than quantitative determination of ZIKV infection. For citation 15 referred to in this manuscript, the authors of that article used recombinant ZIKV sequences to generate a standard curve, allowing them to report quantitative genome-equivalents in serum. Why did the authors of this current manuscript elect not to use this quantification method and instead report Ct value? Does the Ct value reflect a standard amount of serum, or a standard amount of input cDNA? The assay presented in Figure 1A could be modified as above to produce more robust, reliable results (however, as this is likely so late post-peak viremia, even reporting of genome equivalents by copy-number qRT-PCR will not provide a clear picture of differences between groups).

Response 3: The qRT-PCR was run using a standard amount of serum. The use of Ct value is because these were patient samples run in a diagnostic assay, which was verified as a qualitative assay, and does not report quantitative genome
equivalents, these Ct values are meant to serve as a relative rather than absolute quantity. We acknowledge that ideally the Ct values would be translated into genome equivalents, and is a limitation of this study. However, comparing Ct does allow relative comparison of viral quantities between patient samples. Additionally we have included the PFU equivalents for the Ct values as determined by interpolation to a standard curve as part of figure 1.

Point 4: Symptoms comparison. The illness scores as described does not seem adequate to reflect differences in disease outcomes between patient groups. Rather than each symptom being given a value of 1 for the purposes of generating Figure 1B, each type of symptom should be weighted to reflect severity and dissemination of the infection to the CNS/reproductive tract/non-typical tissues. “Neurological” and “respiratory” symptoms (Table 3) represent outcomes that differ from the typical course of ZIKV infection, and so should be weighted higher than the more typical and less severe “rash” or “aches.” As it stands, Figure 1B does not reflect meaningful differences between groups. Additionally, it seems that the proportion of infected individuals that present any symptoms at all may be a relevant parameter regarding the contribution of prior DENV exposure (i.e. most people infected with ZIKV develop no symptoms at all – does prior DENV exposure lead to less asymptomatic infection?). Do the authors have access to serum samples from family members and/or colleagues of these patients, or other travelers who display no symptoms to assess cryptic ZIKV infection in the context of naïve or prior DENV infection?

Response 4: We acknowledge that an established and validated scoring system for severity would be ideal, however at present no such score exists. Additionally there were limitations to the interpretability of the symptom data available to our study team. Symptom information is limited to that provided on the laboratory test requisition received at our reference laboratory, which is remote from the healthcare institution where the patient is clinically assessed. Each symptom was assigned a score of 1 as we cannot interpret the severity of each symptom individually due to these limitations. For example, respiratory symptoms could be mild (eg. cough) or more serious (eg. shortness or breath or acute respiratory distress). Therefore adding more weight to particular symptoms could lead to erroneous conclusions. Additionally we felt that a greater number of symptoms is a better measure of severity as it would likely reflect involvement of more organ systems. Regrettably, we do not have access to family members or colleagues or of other travelers who display no symptoms. Logistically this would be challenging to do and not possible to do, as it would too burdensome on the healthcare system to request these individuals to present themselves to healthcare providers in order to give a sample. However, we agree, such a research study would be worth doing, but would require dedicated funding and resources that are not available to our team at this time. Additionally we should clarify that the purpose of our study was to assess severity in symptomatic patients, and we have modified the methods to include this (line 78-79).

Minor Comments:

Point 5: The title is far too strong a statement considering the evidence presented in the paper. It should be modified to something along the lines of “Presence of pre-existing dengue virus cross-neutralizing antibodies does not lead to a greater number of symptoms in Canadian travelers infected with Zika virus.”

Response 5: Thank you for this suggestion. This change has been made, and the title modified to:

“Presence of flavivirus antibodies does not lead to a greater number of symptoms in a small cohort of Canadian travelers infected with Zika virus.”

Point 6: The reference list does not reflect all in-text references. For example, in line 83, reference 14 does not seem to match. In line 97, there is no Pongsiri and colleagues paper listed at all in the reference list.

Response 6: Thank you for pointing this out. The reference list has been corrected.

Point 7: The authors continually mention ZIKV endemic areas when relating where patients travelled to. As samples were collected in 2016, it is fair to say that the Caribbean was an epidemic rather than endemic area. As a virus infection cycle becomes stable during establishment of endemcity, once would expect selective pressures to change, possibly affecting antigenic properties, and thus ADE. It is important to make such a distinction.

Response 7: We have modified the methods to state that travellers were from regions where the virus in circulation, and have made similar changes where appropriate throughout the manuscript.