nature portfolio

Peer Review File

Investigating antibody cross-reactivity and transmission dynamics of alphaviruses and flaviviruses using a multiplex serological assay

Corresponding Author: Dr Michael White

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a generally nice analysis of an interesting data set. The inclusion of a range of different antigens within the assay for the different viruses is nice.

The analysis for the flaviviruses is interesting and is a fine approach with the application of mixture models to model cut offs, but it does not really take into account the cross reactivity across viruses which is the most important thing that has to be considered when looking at flavivirus responses. The section looking at correlations of titres is relevant, but is not connected to the mixture model so does not link into the method of estimating seronegativity and seropositivity and which is due to cross-reactivity and which due to infection. This does not mean that the mixture model is wrong or not relevant or useful, it is just not addressing a large amount of the issue in using these types of measurements for dengue and other flaviviruses. It seems more could be made of this with the range of antigens that are included on the assay and their combinations explored, but it is up to the authors whether this is feasible, and the editors whether this is necessary for publication in this journal.

For the alphaviruses, there is more considered, and the inclusion of the competition assay is a nice extension and the analysis including this shows it is important for discriminating estimated past exposure vs cross-reactivity. This part of the analysis alone is very nice.

The paper is nicely written and clear.

(Remarks on code availability)
I did not run the code, but have looked over and as far as I can tell is complete for the analysis.

Reviewer #2

(Remarks to the Author)
This study by Yman et al. measured antibody responses to 9 mosquito-borne flaviviruses and alphaviruses among hundreds of participants in endemic areas, using a novel multiplex assay. They used competitive immunoassays to disentangle true responses from cross-reactive responses, a critical problem that arises when multiple closely-related viruses co-circulate. Strengths of the study are that it addresses an important question using novel methods and a large number of well-characterized clinical samples. The results from three-component mixture models were particularly interesting and raise important questions about the heterogeneity of responses in real-world settings, as discussed well (lines 321-332). Description of asymmetric cross-reactivity among alphaviruses is also a valuable finding. The main challenge I have with the manuscript in its current form is that it seems partly written as a methods paper (which seems well supported) and partly written as an epidemiology study (which seems less well supported given what I presume is secondary use of samples collected from different areas for different purposes). It would be helpful for the authors to reframe the manuscript a bit to address this, e.g. present mostly a methods paper but with some “case study” examples from analysis of specific sample sets.

Major comments:
-How were the 28 antigenic markers selected, especially considering that some have previously been shown to have problems with cross-reactivity?
-I think it is very difficult to conclude that “antibody responses... increases with age” (line 174), since the study included samples from diverse geographic sites at just 1 or 2 cross-sectional points in time; antibody responses would have been affected by many factors including the specific history of virus circulation at each site. This is especially challenging because as the authors say, the results “do not account for cross-reactivity” (line 188). I think this section could be reframed as being descriptive of one or two “case studies” of specific viruses in specific settings at specific points in time, rather than presenting general conclusions.
-Lines 287-301: Please provide more background and justification for the alphavirus transmission models, e.g. why was it assumed that “MAYV transmission in French Guiana has been stable over time” (line 290)? Why were stable-transmission and single-outbreak models compared for Peru but not other sites?

Minor comments:
-line 70, would rephrase “weak health systems”
-line 411, the lack of data on YFV vaccination is an important limitation that would be helpful to discuss earlier when presenting the results about flavivirus cross-reactivity
-line 739, please define how specifically the data will be made available (list the repository, ideally provide a link or accession number here even if it will not be activated until publication)
-Figure 1 – what to make of the fact that the blue and purple curves overlap in the top left panel? Does this suggest that a 2 component model would be as good as 3 component?

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
thanks for the attention to the comments. I commend the extra analysis and the rearrangement of the paper and think it is now a very nice paper.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have addressed my prior comments and questions.

(Remarks on code availability)

Point-by-point response to reviewer comments

Manuscript #: NCOMMS-25-67000

Title: Investigating antibody cross-reactivity and transmission dynamics of alphaviruses and flaviviruses using a multiplex serological assay

Authors: Victor Yman[1,2,3†*], Jason Rosado[1,4†], Noé Ochida[5], Laura Garcia[1], Marie-Fabrice Gasasira[1], Gaëlle Baudemont[1], Estee Cramer[1], Myrielle Dupont-Rouzeyrol[6], Karl Huet[6], Maylis Douine[7], Alice Sanna[7], Yann Lambert[7], Gabriel Carrasco-Escobar[8], Oscar Nolasco[8,9], Dionicia Gamboa[8,9], Gamou Fall[10], Oumar Ndiaye[10], Oumar Faye[10], Cheikh Loucoubar[11], Aissatou Toure-Balde[12],Makhtar Niang[12], Ines Vigan-Womas[12,13], Simon Cauchemez[5], Michael T White[1*]

Below is a point-by-point response to the reviewer comments. References to line numbers in the responses refer to line numbers in the revised manuscript word-file with all tracked changes visible ("all mark-up"). Responses to reviewer comments are highlighted using a blue font colour**.**

Editorial comments

We thank the editors for the invitation to submit a revised version of the manuscript. In particular, the editors requested additional analyses to address concerns related to flavivirus cross-reactivity and to remove broad conclusions on virus epidemiology and antibody responses. In response, we have (i) added new multivariate analyses of the multiplex serological data (described in our response to Reviewer #1) and (ii) reframed the manuscript and removed or tempered sections that could be interpreted as containing broad conclusions about the epidemiology of individual viruses (described in our response to Reviewer #2). All changes are shown with tracked changes in the revised manuscript file.

Reviewer #1 (Remarks to the Author):

This is a generally nice analysis of an interesting data set. The inclusion of a range of different antigens within the assay for the different viruses is nice. The analysis for the flaviviruses is interesting and is a fine approach with the application of mixture models to model cut offs, but it does not really take into account the cross reactivity across viruses which is the most important thing that has to be considered when looking at flavivirus responses.

The section looking at correlations of titres is relevant, but is not connected to the mixture model so does not link into the method of estimating seronegativity and seropositivity and which is due to cross-reactivity and which due to infection. This does not mean that the mixture model is wrong or not relevant or useful, it is just not

addressing a large amount of the issue in using these types of measurements for dengue and other flaviviruses.

It seems more could be made of this with the range of antigens that are included on the assay and their combinations explored, but it is up to the authors whether this is feasible, and the editors whether this is necessary for publication in this journal.

This is a valid and important comment, and we agree that understanding serological cross-reactivity is essential for interpreting flavivirus antibody data. While a fully integrated multivariate extension of the cross-reactivity model would represent an ideal analytical framework, such an approach would constitute a substantial methodological undertaking and lies beyond the scope of the present study (with the methods development alone likely warranting a separate investigation).

To address the reviewer's concerns, we have added an extensive multivariate analysis of the multiplex serological data and a new results figure in the revised manuscript, aimed at further exploring patterns of cross-reactivity and virus-specific structure within the data:

1. **Regularised correlation network analysis.**
   Using EBIC graphical LASSO–regularised correlation networks, we explore conditional relationships between all antigens, providing an explicit multivariate view of antigen–antigen associations and cross-reactivity patterns across the full panel (see results section lines 235–258, Figure 5A, and methods section lines 681-692).
2. **Principal component analysis of NS1 responses.**
   Using PCA applied to flavivirus NS1 antibody responses, we show that the dominant axes of variation correspond to a shared pan-flavivirus dimension, with additional orthogonal dimensions separating DENV- and ZIKV-associated response patterns (see results section lines 260-276, Figure 5BC, and methods section lines 694-710).
3. **Integration with GMM-derived serological phenotypes.**
   We demonstrate how these multivariate dimensions give rise to interpretable serological structure by defining serological response phenotypes based on the univariate GMM results. We show that these phenotypes cluster in multivariate NS1 response space and provide epidemiological context by presenting their age- and site-specific distributions, which are consistent with biologically plausible exposure patterns (see results section lines 260-276, Figure 5BCD and methods section lines 694-710).
4. **Interactive exploratory tool.**
   As a supplementary resource, we have created an online interactive HTML tool that allows readers to explore pairwise relationships across all serological variables, including stratification by site and sex (https://ymanvictor.github.io/arbo_antibody_browser).

Additionally, in response to comments from Reviewer #2 (see below), we have slightly reframed the manuscript as a methodological study, with a focused case study on alphaviruses demonstrating how competitive immunoassays and modelling can be used to disentangle cross-reactive from virus-specific antibody responses. We outline that an analogous framework for flaviviruses represents a logical next step but would require extensive additional analytical and experimental validation. Given the scale of this effort, we consider it beyond the scope of the current manuscript.

For the alphaviruses, there is more considered, and the inclusion of the competition assay is a nice extension and the analysis including this shows it is important for discriminating estimated past exposure vs cross-reactivity. This part of the analysis alone is very nice.

The paper is nicely written and clear.

Reviewer #1 (Remarks on code availability):
I did not run the code, but have looked over and as far as I can tell is complete for the analysis.

The code is available from https://github.com/ymanvictor/arbo_multiplex
All display items can be reproduced from the code and associated data accessible via this link. Additional details are provided within the "Code and Software submission checklist" submitted together with the revised version of the manuscript.

Reviewer #2 (Remarks to the Author):

This study by Yman et al. measured antibody responses to 9 mosquito-borne flaviviruses and alphaviruses among hundreds of participants in endemic areas, using a novel multiplex assay. They used competitive immunoassays to disentangle true responses from cross-reactive responses, a critical problem that arises when multiple closely-related viruses co-circulate. Strengths of the study are that it addresses an important question using novel methods and a large number of well-characterized clinical samples. The results from three-component mixture models were particularly interesting and raise important questions about the heterogeneity of responses in real-world settings, as discussed well (lines 321-332). Description of asymmetric cross-reactivity among alphaviruses is also a valuable finding.

The main challenge I have with the manuscript in its current form is that it seems partly written as a methods paper (which seems well supported) and partly written as an epidemiology study (which seems less well supported given what I presume is secondary use of samples collected from different areas for different purposes). It would be helpful for the authors to reframe the manuscript a bit to address this, e.g. present mostly a methods paper but with some "case study" examples from analysis of specific sample sets.

We agree that this is partly a methodological study. As per the reviewer's suggestion we have revised the manuscript to reframe it as more of a methods paper with an in-depth

epidemiological evaluation using a case study on alphavirus transmission. We have updated the abstract (line 42), the introduction (line 109), the results (lines 277-283), and the discussion (line 443) sections to better reflect this.

The methods section has also been revised to clarify that the study is based on secondary analysis of samples collected for different purposes (lines 539-541, ethics declaration).

Major comments:
-How were the 28 antigenic markers selected, especially considering that some have previously been shown to have problems with cross-reactivity?

The 28 antigenic markers included in the assay were selected from commercially available sources and based on prior evidence supporting their use for sensitive detection of arboviral exposure across multiple virus families. The panel was intentionally designed to include both structural and non-structural proteins, allowing us to capture a broad range of antibody responses while explicitly assessing patterns of cross-reactivity.

For flaviviruses, we included antigens representing the viral envelope (E and VLP antigens), which are known to be highly immunogenic but subject to cross-reactive antibody responses, as well as NS1 and envelope domain III (EDIII) antigens. EDIII antigens were included because they have previously been shown to be less targeted by cross-reactive antibodies and may therefore provide increased specificity for distinguishing between flavivirus exposures.

This has been clarified in the Methods section of the revised manuscript (lines 564-565).

-I think it is very difficult to conclude that “antibody responses… increases with age” (line 174), since the study included samples from diverse geographic sites at just 1 or 2 cross-sectional points in time; antibody responses would have been affected by many factors including the specific history of virus circulation at each site.

This is especially challenging because as the authors say, the results “do not account for cross-reactivity” (line 188). I think this section could be reframed as being descriptive of one or two “case studies” of specific viruses in specific settings at specific points in time, rather than presenting general conclusions.

We appreciate the reviewer pointing this out. This was not intended as a general conclusion about the epidemiology of any given virus at any given site. It was merely intended as an observation of the overall patterns in the data. To avoid confusion between this observation and any conclusions about the site-specific epidemiology of individual viruses the section *“The magnitude of antibody responses to alpha- and flaviviruses increases with age”* has been removed as a separate section and has following substantial revision been incorporated in the subsequent section (lines 175-233). Furthermore, the heading of this section has been revised to *“Magnitude and*

pairwise correlation of antibody responses to alphaviruses and flaviviruses". The discussion has also been revised on lines 417-421.

-Lines 287-301: Please provide more background and justification for the alphavirus transmission models, e.g. why was it assumed that "MAYV transmission in French Guiana has been stable over time" (line 290)? Why were stable-transmission and single-outbreak models compared for Peru but not other sites?

This is an important observation.

At each site there are four theoretical joint transmission patterns of MAYV and CHIKV, stable for both (SS), stable for MAYV but epidemic for CHIKV (SE), epidemic for MAYV but stable for CHIKV (ES) or epidemic for both (EE). With 5 sites this gives 4^5 = 1024 possible transmission patterns. To avoid fitting and comparing 1024 computationally intensive and complex models we made assumptions of site-specific transmission patterns based on external epidemiological evidence.

For MAYV transmission has not been demonstrated outside of Latin America. Therefore, MAYV was assumed to be absent in Senegal and New Caledonia. In French Guiana a previous study by Hoze et al. (reference no. 12) exploring the transmission of both CHIKV and MAYV demonstrated that stable transmission was substantially more likely than epidemic transmission for MAYV and vice versa for CHIKV.

Furthermore, there is substantial evidence to support repeated epidemics of CHIKV in Senegal (see references 37 and 38).

For Peru there is less available published epidemiological data on the transmission of MAYV and CHIKV and therefore we compared different models to identify the most likely historical transmission dynamics at our study site.

Details regarding the transmission assumptions and their epidemiological justification are provided within the methods section on lines (782-790) and discussed on lines 460-472. In the revised manuscript additional detail has also been included in the results section on lines 347-352.

Minor comments:
-line 70, would rephrase "weak health systems"

This has been rephrased to "health systems with limited capacity" (line 70-71)

-line 411, the lack of data on YFV vaccination is an important limitation that would be helpful to discuss earlier when presenting the results about flavivirus cross-reactivity

As per the reviewer's suggestion the paragraph discussing YFV vaccination has been moved to the section discussing flavivirus responses and their cross-reactivity (lines 430-437)

-line 739, please define how specifically the data will be made available (list the repository, ideally provide a link or accession number here even if it will not be activated until publication).

As described within the data availability statement individual-level data are subject to participant confidentiality requirements under GDPR and national regulations in France, Peru, and Senegal, as well as the terms of local ethics approvals. As a result, raw individual-level datasets cannot be made publicly available.
De-identified source data and summary data sufficient to reproduce the analyses and figures is available in a public repository: https://github.com/ymanvictor/arbo_multiplex). Requests for access to individual-level data can be directed to the corresponding authors and will be considered on a case-by-case basis, subject to approval by the relevant ethics committees and the signing of a data use agreement in compliance with applicable regulations.

-Figure 1 – what to make of the fact that the blue and purple curves overlap in the top left panel? Does this suggest that a 2 component model would be as good as 3 component?

In our modeling framework, the Gaussian mixture model is fitted jointly to the data from cross-sectional survey samples and the known unexposed controls. The parameters of the lowest component (mean and standard deviation) are constrained to be shared between the unexposed controls and a subset of the cross-sectional samples, thereby effectively anchoring the seronegative distribution using the control data.

Under this joint modeling strategy, the three-component model provided a superior fit to all antigens (including CHIKV VLP), except WNV EDIII, compared with the two-component model, as indicated by a smaller LOOIC (lines 129–132).

Although there is visible overlap between the lowest and middle components for CHIKV VLP, this does not imply that a two-component model would perform equally well. Rather, the three-component model offers the flexibility needed to capture heterogeneity in background reactivity among seronegative individuals, which is known to vary across populations (as described previously for example with regards to SARS CoV 2, where African donors demonstrate higher background reactivity, please see reference 29: Nkuba Ndaye *et al.*, *Lancet Glob Health* 2021).

In summary, the added flexibility of a three-component model improves both model fit and interpretability across antigens, while remaining conceptually straightforward and robust across datasets.

Point-by-point response to reviewer comments

Manuscript #: NCOMMS-25-67000A

Title: Investigating antibody cross-reactivity and transmission dynamics of alphaviruses and flaviviruses using a multiplex serological assay

Authors: Victor Yman[1,2,3†*], Jason Rosado[1,4†], Noé Ochida[5], Laura Garcia[1], Marie-Fabrice Gasasira[1], Gaëlle Baudemont[1], Estee Cramer[1], Myrielle Dupont-Rouzeyrol[6], Karl Huet[6], Maylis Douine[7], Alice Sanna[7], Yann Lambert[7], Gabriel Carrasco-Escobar[8], Oscar Nolasco[8,9], Dionicia Gamboa[8,9], Gamou Fall[10], Oumar Ndiaye[10], Oumar Faye[10], Cheikh Loucoubar[11], Aissatou Toure-Balde[12],Makhtar Niang[12], Ines Vigan-Womas[12,13], Simon Cauchemez[5], Michael T White[1*]

Below is a point-by-point response to last the comment by Reviewer #2 which was partially omitted from the previous response letter. References to line numbers in the responses refer to line numbers in the revised manuscript word-file with all tracked changes visible ("all mark-up"). Responses to reviewer comments are highlighted using a blue font colour**.**

-Figure 1 – what to make of the fact that the blue and purple curves overlap in the top left panel? Does this suggest that a 2 component model would be as good as 3 component?

In our modeling framework, the Gaussian mixture model is fitted jointly to the data from cross-sectional survey samples and the known unexposed controls. The parameters of the lowest component (mean and standard deviation) are constrained to be shared between the unexposed controls and a subset of the cross-sectional samples, thereby effectively anchoring the seronegative distribution using the control data.

Under this joint modeling strategy, the three-component model provided a superior fit to all antigens (including CHIKV VLP), except WNV EDIII, compared with the two-component model, as indicated by a smaller LOOIC (lines 129–132).

Although there is visible overlap between the lowest and middle components for CHIKV VLP, this does not imply that a two-component model would perform equally well. Rather, the three-component model offers the flexibility needed to capture heterogeneity in background reactivity among seronegative individuals, which is known to vary across populations (as described previously for example with regards to SARS CoV 2, where African donors demonstrate higher background reactivity, please see reference 29: Nkuba Ndaye *et al.*, *Lancet Glob Health* 2021).

In summary, the added flexibility of a three-component model improves both model fit and interpretability across antigens, while remaining conceptually straightforward and robust across datasets.