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# Host range and structural analysis of bat-origin RshSTT182/200 coronavirus binding to human ACE2 and its animal orthologs

Yu Hu, Kefang Liu, Pu Han, Zepeng Xu, Anqi Zheng, Xiaoqian Pan, Yunfei Jia, Chao Su, Lingfeng Tang, Lili Wu, Bin Bai, Xin Zhao, Tian Di, zhihai chen, Jianxun Qi, Qihui Wang, and George Gao
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## Review Timeline:


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| Submission Date: | 24th May 22 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear George,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees appreciate the study but also find that some further analysis is needed for consideration here. The referees find that further functional data using different assays like cell-cell fusion or pseudoviruses is needed to support the key findings of the work. Should you be able to add such data and address the other raised concerns then I would like to invite you to submit a revised version.

I think it would be helpful to discuss the revisions further. I am available to do so via zoom or email if that is helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussion your revisions further with you.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to submit the revised version.

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (3rd Oct 2022).

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

If you require more time to complete the revisions let me know as as I can grant an extension.

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

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Referee #1:

The article by Hu et al. deciphered the structural and functional characterization of the RBD from a bat-CoV - RshSTT182/200 and compared it to the one of SARS-CoV-2 in order to evaluate the potential of emergence. The binding of the RBD on hACE2 is evaluated by FACS and SPR, and the structure of the RBD-hACE2 is obtained by crystallography. The data are validated by a rational point mutagenesis. The results demonstrate the ability of the bat-CoV RBD to bind hACE2 with low affinity whereas the bat-CoV RBD binds to other orthologous ACE2 with higher affinity upon species. Finally, the antigenic properties of the RBD from the Bat-CoV and SARS-CoV-2 are compared.
Overall, the experimental approach and the results are sound and are not over-interpreted, although several weaknesses can be highlighted.

Major comments :

L262 : The study of the affinity with orthologous ACE2 is valuable but a "reference" is missing. Indeed, the bat-CoV were isolated in Rhinolophus shameli and it is useful to have the KD value between RsACE2 and RBD for comparison with the other values, unless one of the ACE2 presented in the study show sequence identity in the binding residues with that of R. shameli.
L290 : This part of the work is interesting but remains artificially linked to the rest of the study. Given the sequence identity between SARS-CoV-2 and the bat CoV RBD, it was expected that sera or Mab would cross-react. An added value would be the demonstration that there is cross-seroneutralization, which would link the structure/function study to the immune response. This could be tackled by pseudotype studies or at least by competition assays between hACE2/RBD and MAbs and sera by biophysical or immunoassay.

Minor Comments :
L22 : Does 92.6% id. with SARS-COv-2 refer to bat CoV 182 or 200 ? It should be clarified
Summary : the binding of RBD on hACE2 should be explicitly presented as one of the features involved in the spillover.
L45 : disasters should replaced (emergence ?)
L65-67 : the introduction of the FCS (furin cleavage site) should be better introduced in order to clarify that 2 features are important for spillover (RBD binding to hACE2 AND presence of a FCS) in the context of SARS-CoV2, then notice that for the mentioned bat-CoV, sequence variations are observed but none of them let to a FCS.
L76 : replace fully conserved by identical amino acid sequences.
L87 : add the relevant reference.
L89-91 : the sentence is not not clear and should rephrased.
L93 : ACE2 is the main receptor
L102 : as compared to what ?
L125 : Compared to SARS-CoV2, four amino acids.
L125 : the authors mention B4 and B5 and B4-B5 loop, but the structure of the RBD has not been introduced. It could be helpful in the introduction to have a short description of SARS-CoV-2 RBD, main structural features and key residues for the binding on hACE2.
L135 : the 3log magnitude could be mentioned to highlight how important is the difference in binding.
L152 : form
L177 : P486 formally did not "lose" interaction, I would write "does not contact with hACE2 in the structure".
L195-L199 : This part is unclear and should be deeply modified so the contribution of the glycosylation and/or the amino acid composition is properly addressed.
L201-203 : I don't understand the notion of recovering (regaining?) the interaction but with increase of the affinity. Maybe all the paragraph from L 193 to L204 has to be modified.
L209 : Further analysis : not correct from a grammatical point of view, modify along the text.
L209 : larger is not appropriate. The sentence should be rephrased for comprehension.
L222 : affinity (...) higher than and not stronger.
L281 : the affinity with the fox ACE2 is interesting and could be compared and discussed (here or in the discussion) with other values between CoV and ACE2, and at least the 18 nM between SARS-CoV-2 and hACE2.

Referee #2:

This manuscript has reported extensive biochemical and structural characterizations of the interactions between the RBD from the bat coronavirus RshSTT182/200 and various ACE2 proteins, including that of humans. The crystal structures of the human ACE2 in complex with the RshSTT182/200 RBD and two mutated RBDs have been determined, and the key contacting residues identified. Mutagenesis studies were also performed to explore potential paths to gain improved binding to human ACE2 using the SACR-CoV-2 as a reference. They have further showed that RshSTT182/200 RBD can recognized many other ACE2 orthologs, but exhibiting lower binding affinities than those by the SARS-CoV-2 RBD. Finally, the authors have demonstrated that antibodies elicited by SARS-CoV-2 can cross-react with the RshSTT182/200 RBD. The data present here indicate the need to monitor the potential spillover of RshSTT182/200, and the closely related coronaviruses for future pandemics.

Overall, the biochemical and structural data are technically sound and solid, and the manuscript has some interesting results that would be significant to the coronavirus field. The main concern is that the entire paper has focused on binding with the purified RBD, and there aren't any functional data from assays, such cell-cell fusion assay or pseudovirus assay, to corelate the RBD binding with receptor usage. Without such data, it would be difficult to be convinced that RshSTT182/200 could infect human cells or cells from any other species. The main figures are overcrowded and it might be better to keep a few representative sets to drive the key point home and move the rest panels to a supplemental figure. There are also grammatical issues in the text, which would need editorial editing.

Referee #3:

In this paper, Hu et al. examine how a bat coronavirus RshSTT182/200 spike receptor-binding domain (RBD) interacts with various animal ACE2 orthologs and identify key residues that account for its weak (by 3 logs) hACE2 binding. The structural and biochemical data presented provide useful insights into the functional difference between RshSTT182/200 and SARS-CoV-2 RBD. Overall, the studies are descriptive but useful in showing possible mechanisms by which RshSTT182/200 RBD could gain higher affinity toward hACE2 and how it can be neutralization by cross-reactive antibodies.

Major points:
While the binding affinity measurements by flow cytometry and SPR for the wt and engineered RshSTT182/200 RBD to hACE2 are informative, it is unknown if the improved affinities by amino acid substitutions, etc. actually enhance the potential to infect human cells expressing ACE2. Would it be possible to perform pseudovirus entry assay to show functionality of the binding?

hACE2 α2-α4 angle is very different (open vs. closed) depending on whether it's bound to SARS-CoV-2 or RshSTT182/200 RBD, which is intriguing. How do these conformations compare to that of free ACE2?
Looking at Fig. EV1-supp panels G-K, ACE2 bound to RshSTT182/200 RBD has the most closed conformation of the substrate-binding cleft, and this might relate to the much lower binding affinity of RshSTT182/200 RBD to hACE2.

Line 160~: How is the number of "contacts" defined? Why are the numbers so large (e.g., 288 contacts for SARS-CoV-2 RBD)? Are the authors counting the pairs of atoms within a distance cutoff?
Table 2 is also confusing. Is this a list of polar contacts with wan der Waals contacts in the parentheses? It's not clear what the "number of wan der Waals contacts" means. The legend should more clearly define what this table shows.

Line 188~: Structural analysis showed that the polarity of D487 was stronger than that of N487, which may pull out F486 from the hydrophobic patch.
This logic is unclear. How does the negative charge of Asp affect the conformation of Phe side chain?
How was the side chain conformation of P486F determined in the hypothetical model shown in Fig. 2D? D487 appears to be modeled as Asp in this figure, which adds to the confusion.

The last sentence from both Abstract and Introduction emphasizes the importance of enhanced surveillance of RshSTT182/200 to prevent potential pandemic. While that's not a bad idea, the following sentence from Discussion appears to better describe its threat to the human health.
"Taken together, RshSTT182/200 is a threat to potentially susceptible animals, but it may need further evolution to obtain strong interspecies transmission abilities like SARS-CoV-2."

Minor points:

In Abstract
The bat-origin CoV RaTG13 shares 96.2% identity with the overall genome (Zhou et al., 2020b).
Is this a comparison to SARS-CoV-2?

Line 159: binding area => buried surface area?

Line 209-211, 228: It is unnecessary to spell out the chemical structures of amino acids arginine, threonine, and lysine.

Awkward or ambiguous sentences:

Emerging and re-emerging viruses are greatly threaten global public health...

These instances remind us that the prevention, detection and control of infectious diseases need to be paid attention at a more rapid pace.

The S protein receptor-binding domain (RBD) of these viruses show highly conservation with SARS-CoV-2...

...the receptor-binding spectra of RshSTT182/200 need to be evaluated to prevent them spillover.

Then, the binding affinity of RshSTT182/200 RBD to hACE2 was detected by surface plasmon resonance (SPR) assay.
detected => determined

α2 and α4 in ACE2 were open which make a distinction conformation in RshSTT182/200 RBD/hACE2 complex

N-glycosylation at the N448 residue may blocked the interaction of RshSTT182/200 RBD-insert1 with hACE2.

Line 213: clash Y449 => clash with Y449

Line 267: were chosen to evaluated
Line 292: to sera from recovered patients or vaccinees
Line 318: but palm-civet ACE2 is hard to detect the binding to SARS-CoV-2 RBD by SPR

**Point-by-point response:**

**Editor's comments:**

**As you can see from the comments, the referees appreciate the study but also find that some further analysis is needed for consideration here. The referees find that further functional data using different assays like cell-cell fusion or pseudoviruses is needed to support the key findings of the work. Should you be able to add such data and address the other raised concerns then I would like to invite you to submit a revised version.**

*Response: Thank you so much for all of your efforts regarding our manuscript. We are so appreciative of the positive comments by all the three referees on our work. The pseudovirus assay data have been added to the revised manuscript. Furthermore, additional functional experiments that the referees asked for have also been performed. All of the typographical errors and the awkward or ambiguous sentences pointed out by referees have been modified.*

**Referee # 1:**

**...Overall, the experimental approach and the results are sound and are not over-interpreted, although several weaknesses can be highlighted.**

*Response: We appreciate the referee's insightful suggestions.*

**Major comments:**

**L262 : The study of the affinity with orthologous ACE2 is valuable but a**

**"reference" is missing. Indeed, the bat-CoV were isolated in Rhinolophus shameli and it is useful to have the KD value between RsACE2 and RBD for comparison with the other values, unless one of the ACE2 presented in the study show sequence identity in the binding residues with that of R. shameli.**

*Response: We measured the binding affinities of R. shameli ACE2 for the RshSTT182/200 and SARS-CoV-2 RBDs. The binding affinity of the RshSTT182/200 RBD for R. shameli ACE2 was 0.93 μM, which was ~7-fold higher than that of the SARS-CoV-2 RBD to R. shameli ACE2 ($K_D$ = 6.67 μM) (**Fig 1B**).*

**L290 : This part of the work is interesting but remains artificially linked to the rest of the study. Given the sequence identity between SARS-CoV-2 and the bat CoV RBD, it was expected that sera or Mab would cross-react. An added value would be the demonstration that there is cross-seroneutralization, which would link the structure/function study to the immune response. This could be tackled by pseudotype studies or at least by competition assays between hACE2/RBD and MAbs and sera by biophysical or immunoassay.**

*Response: We evaluated the neutralization activity of three serum samples from COVID-19 convalescents and three serum samples from donors who were immunized against the SARS-CoV-2 vaccine, ZF2001®. The sera from both COVID-19 convalescents and SARS-CoV-2 vaccinees cross-neutralized RshSTT182 pseudovirus entry into HeLa cells expressing R. affinis ACE2 **(Fig 5B)**. The titer of neutralizing antibodies against RshSTT182 was lower than that of SARS-CoV-2 **(Fig 5B)**.*

**Minor Comments :**

**Referee # 1:**

1. **L22 : Does 92.6% id. with SARS-COv-2 refer to bat CoV 182 or 200 ? It should be clarified**

*Response: Both RshSTT182 and RshSTT200 share 92.6% nucleotide identity to SARS-CoV-2 at the whole-genome level (PMID:34753934). We modified the sentence to "Bat-origin RshSTT182 and RshSTT200 viruses were isolated from Rhinolophus shameli in Southeast Asia (Cambodia), and both of them share 92.6% whole-genome identity with SARS-CoV-2".*

2. **Summary : the binding of RBD on hACE2 should be explicitly presented as one of the features involved in the spillover.**

*Response: We added "Characterizing the interaction between the RBD of coronavirus (CoV) and the ACE2 orthologs is an efficient method to evaluate interspecies transmission" to emphasize that the binding of the RBD to hACE2 is one of the features involved in the spillover.*

3. **L45 : disasters should replaced (emergence ?)**

*Response: Modified.*

**4. L65-67 : the introduction of the FCS (furin cleavage site) should be better introduced in order to clarify that 2 features are important for spillover (RBD**

**binding to hACE2 AND presence of a FCS) in the context of SARS-CoV2, then notice that for the mentioned bat-CoV, sequence variations are observed but none of them let to a FCS.**

*Response: This observation is great. We agree and have updated the text to "The furin cleavage site (FCS) plays a critical role in SARS-CoV-2 infection, and the loss of this site attenuates entry efficiency" (in lines 70-71).*

**5. L76 : replace fully conserved by identical amino acid sequences.**

*Response: Replaced.*

**6. L87 : add the relevant reference.**

*Response: Added.*

**7. L89-91 : the sentence is not not clear and should rephrased.**

*Response: We rephrased this sentence to "Host cell receptor binding is a prerequisite for virus infection. Therefore, characterizing the interaction between the RBD of a CoV and the ACE2 orthologs from a broad range of species is an efficient method to screen for potential hosts" (in lines 94-97).*

**8. L93 : ACE2 is the main receptor**

*Response: Modified.*

**9. L102 : as compared to what ?**

*Response: We modified this sentence to "The GX/P2V/2017 RBD and GD/1/2019 RBD utilize a similar binding mechanism as SARS-CoV-2 and display similar binding affinities as the SARS-CoV-2 RBD for hACE2".*

**10. L125 : Compared to SARS-CoV2, four amino acids.**

*Response: Modified.*

**11. L125 : the authors mention B4 and B5 and B4-B5 loop, but the structure of the RBD has not been introduced. It could be helpful in the introduction to have a short description of SARS-CoV-2 RBD, main structural features and key residues for the binding on hACE2.**

*Response: Thank you for this suggestion. We added the description as "The G446 and Y449 residues on the identical loop in the SARS-CoV-2 RBD form hydrogen bonds with D38 and Q42 of hACE2" (in lines 136-137).*

**12. L135 : the 3log magnitude could be mentioned to highlight how important is the difference in binding.**

*Response: Modified.*

**13. L152 : form**

*Response: Modified.*

**14. L177 : P486 formally did not "lose" interaction, I would write "does not contact with hACE2 in the structure".**

*Response: We modified this sentence to "In the RshSTT182/200 RBD/hACE2 complex, F486 is substituted by P486, which does not form a hydrophobic patch with hACE2 in the structure".*

**15. L195-L199 : This part is unclear and should be deeply modified so the contribution of the glycosylation and/or the amino acid composition is properly addressed.**

*Response: We have rephrased this paragraph and desicribed as the next comment response.*

**16. L201-203 : I don't understand the notion of recovering (regaining?) the interaction but with increase of the affinity. Maybe all the paragraph from L 193 to L204 has to be modified.**

*Response: We rephrased this part as "To evaluate the influence of the deleted amino acids (AAs) in the β4β5 loop, we inserted four AAs (K444, V445, N448 and Y449) into the RshSTT182/200 RBD (RshSTT182/200 RBD-insert1 mutant construction)* ***(Fig 2A)****. Unexpectedly, the RshSTT182/200 RBD-insert1 protein failed to interact with hACE2* ***(Fig EV3B)****. Because an N-linked glycosylation motif was introduced (N448-Y449-S450), SDS-PAGE indicated that the molecular weight of*

*RshSTT182/200 RBD-insert1 was greater than the wild-type RshSTT182/200 RBD* ***(Fig EV3C)****. We hypothesized that N448-glycosylation in the RshSTT182/200 RBD-insert1 may inhibit the interaction of the RBD with ACE2. Therefore, we mutated S450 to N450 (RshSTT182/200 RBD-insert2 mutant construction) to eliminate the effect of this N-linked glycosylation and found that the interaction between the RshSTT182/200 RBD-insert2 protein and hACE2 was re-established, with a similar binding affinity to that of the wild-type RshSTT182/200 RBD* ***(Fig 2B and Fig EV3A)****" (in lines 207-219).*

**17. L209 : Further analysis : not correct from a grammatical point of view, modify along the text.**

*Response: Modified.*

**18. L209 : larger is not appropriate. The sentence should be rephrased for comprehension.**

*Response: Modified.*

**19. L222 : affinity (...) higher than and not stronger.**

*Response: Modified.*

**20. L281 : the affinity with the fox ACE2 is interesting and could be compared and discussed (here or in the discussion) with other values between CoV and**

**ACE2, and at least the 18 nM between SARS-CoV-2 and hACE2.**

*Response: We include the following text in lines 359-364: "The binding affinity of the RshSTT182/200 RBD for fox ACE2 was the highest among the tested ACE2 orthologs. Furthermore, fox ACE2 is broadly bound by the RBDs of many sarbecovirus, such as the SARS-CoV-2 prototype strain and omicron variant, SARS-CoV, RaTG13, GX/P2V/2017 and GD/1/2019. Therefore, surveillance for sarbecovirus in foxes should be stepped up to prevent their spillover".*

**Referee # 2:**

**The main concern is that the entire paper has focused on binding with the purified RBD, and there aren't any functional data from assays, such cell-cell fusion assay or pseudovirus assay, to corelate the RBD binding with receptor usage. Without such data, it would be difficult to be convinced that RshSTT182/200 could infect human cells or cells from any other species. The main figures are overcrowded and it might be better to keep a few representative sets to drive the key point home and move the rest panels to a supplemental figure. There are also grammatical issues in the text, which would need editorial editing.**

*Response: We performed pseudovirus entry assays in the revised manuscript. Moreover, we altered* ***Fig 3 and 4****, and moved the sub-curves to supplemental information. We also addressed the grammatical issues.*

**Referee # 3:**

**... Overall, the studies are descriptive but useful in showing possible mechanisms by which RshSTT182/200 RBD could gain higher affinity toward hACE2 and how it can be neutralization by cross-reactive antibodies.**

*Response: Thank you for your positive comments.*

**Major points:**

**1. While the binding affinity measurements by flow cytometry and SPR for the wt and engineered RshSTT182/200 RBD to hACE2 are informative, it is unknown if the improved affinities by amino acid substitutions, etc. actually enhance the potential to infect human cells expressing ACE2. Would it be possible to perform pseudovirus entry assay to show functionality of the binding?**

*Response: Thank you for your suggestions. We performed pseudovirus entry assays in the revised manuscript, and our data indicate that the RshSTT182 pseudoviruse could successfully enter to HeLa-hACE2 cells (**Fig 2H**). Consistent with their binding features, RshSTT182 insert2-T346R-P486F-D487N-Y496G pseudovirus showed relatively higher entry ability than wild-type RshSTT182 pseudovirus.*

**2. hACE2 α2-α4 angle is very different (open vs. closed) depending on whether it's bound to SARS-CoV-2 or RshSTT182/200 RBD, which is intriguing. How do**

**these conformations compare to that of free ACE2?**

**Looking at Fig. EV1-supp panels G-K, ACE2 bound to RshSTT182/200 RBD has the most closed conformation of the substrate-binding cleft, and this might relate to the much lower binding affinity of RshSTT182/200 RBD to hACE2.**

*Response: We compared the hACE2 in the RshSTT182/200 RBD/hACE2 complex with free hACE2 (**Fig EV2H**) and found the α2-α4 angle of free ACE2 presents as an open state, which is similar to the hACE2 in the SARS-CoV-2 RBD/hACE2 complex. This region is not located in the binding motif of hACE2, and thus, we speculated that it does not influence RshSTT182/200 RBD binding.*

**3. Line 160~: How is the number of "contacts" defined? Why are the numbers so large (e.g., 288 contacts for SARS-CoV-2 RBD)? Are the authors counting the pairs of atoms within a distance cutoff?**

**Table 2 is also confusing. Is this a list of polar contacts with wan der Waals contacts in the parentheses? It's not clear what the "number of wan der Waals contacts" means. The legend should more clearly define what this table shows.**

*Response: The contacts were measured by CCP4. The Van der Waals contacts were analyzed at a cutoff of 4.5 Å and polar interactions at a cutoff of 3.5 Å. We re-edited Table 2 for clarification. The numbers in parentheses of RBD residues represent the numbers of vdw contacts that the indicated residues conferred. The number underlined in bold indicates the number of potential polar interactions between pairs of residues.*

**4. Line 188~: Structural analysis showed that the polarity of D487 was stronger than that of N487, which may pull out F486 from the hydrophobic patch.**

**This logic is unclear. How does the negative charge of Asp affect the conformation of Phe side chain?**

**How was the side chain conformation of P486F determined in the hypothetical model shown in Fig. 2D? D487 appears to be modeled as Asp in this figure, which adds to the confusion.**

*Response: Thank you for your suggestions. The structures in the original* ***Fig 2D*** *are modeled. In the revised manuscript, we deleted this part and added to the discussion as "However, the molecular mechanism by which the RshSTT182/200 RBD D487N substitution enhances hACE2 receptor binding is still not fully elucidated. The structure of RshSTT182/200 RBD D487N in complex with hACE2 needs to be determined in the future".*

**5. The last sentence from both Abstract and Introduction emphasizes the importance of enhanced surveillance of RshSTT182/200 to prevent potential pandemic. While that's not a bad idea, the following sentence from Discussion appears to better describe its threat to the human health.**

**"Taken together, RshSTT182/200 is a threat to potentially susceptible animals, but it may need further evolution to obtain strong interspecies transmission abilities like SARS-CoV-2."**

*Response: We modified the last sentence of the abstract as "Taken together, RshSTT182/200 is a threat to potentially susceptible animals, but it requires further evolution to obtain strong interspecies transmission abilities like SARS-CoV-2" (in lines 34-36).*

**Minor points:**

**1. In Abstract,The bat-origin CoV RaTG13 shares 96.2% identity with the overall genome (Zhou et al., 2020b). Is this a comparison to SARS-CoV-2?**

*Response: Yes, we modified this sentence to "The overall genome of the bat-origin CoV RaTG13 shares 96.2% nucleic acid sequence identity with SARS-CoV-2".*

**2. Line 159: binding area => buried surface area?**

*Response: We modified to "buried surface area".*

**3. Line 209-211, 228: It is unnecessary to spell out the chemical structures of amino acids arginine, threonine, and lysine.**

*Response: Deleted.*

**4. Awkward or ambiguous sentences:**

**Emerging and re-emerging viruses are greatly threaten global public health...**

*Response: Deleted.*

**These instances remind us that the prevention, detection and control of infectious diseases need to be paid attention at a more rapid pace.**

*Response: Deleted.*

**The S protein receptor-binding domain (RBD) of these viruses show highly conservation with SARS-CoV-2...**

*Response: We modified this sentence to "The receptor binding domains (RBDs) of the spike protein of BANAL-52, BANAL-103 and BANAL-236 are >95% identical to SARS-CoV-2 and show similar binding affinity as the SARS-CoV-2 RBD to hACE2".*

**...the receptor-binding spectra of RshSTT182/200 need to be evaluated to prevent them spillover.**

*Response: Deleted.*

**Then, the binding affinity of RshSTT182/200 RBD to hACE2 was detected by surface plasmon resonance (SPR) assay.**

**detected => determined**

*Response: Modified.*

**α2 and α4 in ACE2 were open which make a distinction conformation in RshSTT182/200 RBD/hACE2 complex**

*Response: We modified this sentence to "In detail, we found that this difference was*

*due to hACE2 adopting a different conformation. The α2 and α4 helices of hACE2 in the RshSTT182/200 RBD/hACE2 complex are in the closed state, but in other RBD/hACE2 complex structures and free hACE2, α2 and α4 of hACE2 are in the open state" (lines 164-168).*

**N-glycosylation at the N448 residue may blocked the interaction of RshSTT182/200 RBD-insert1 with hACE2.**

*Response: We modified this sentence to "We hypothesized that N448-glycosylation in the RshSTT182/200 RBD-insert1 may inhibit the interaction of the RBD with ACE2".*

**Line 213: clash Y449 => clash with Y449**

*Response: Modified.*

**Line 267: were chosen to evaluated**

*Response: We modified to "were evaluated".*

**Line 292: to sera from recovered patients or vaccinees**

*Response: We modified this sentence to "we tested the cross-recognition of antibodies in COVID-19 convalescent and vaccinee sera to the RshSTT182/200 RBD with enzyme-linked immunosorbent assays (ELISAs)".*

**Line 318: but palm-civet ACE2 is hard to detect the binding to SARS-CoV-2**

**RBD by SPR**

*Response: We modified this sentence to "but it is difficult to detect the binding of palm-civet ACE2 to the prototype SARS-CoV-2 RBD".*

Dear George,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees and their comments are provided below. As you can see the referees appreciate that introduced revisions. I am therefore very pleased to say that we will accept the manuscript for publication here.

When you submit the revised version will you please take care of the following issues:

- you can only have 5 keywords

-COI needs to be renamed as "DISCLOSURE AND COMPETING INTERESTS STATEMENT". Please also add the following sentence to the statement: "George Gao is an editorial advisory board member." See also https://www.embopress.org/competing-interests

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

- The reference list: The list of authors in the reference list should be cut after 10 et al.

- The synopsis image should be 550 wide by [200-400] high (pixels).

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues.

Let me know if you have any further questions.

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #1:

The authors adequately addressed the reviewer's comments. The current version of the article provides significant insights into the binding mode of environmental CoVs on ACE2 from a wide set of species. These results might contribute to the better understanding of viral spillover and serve as a basis for rational selection of animal species for the surveillance of emergence of bat CoVs.

Referee #2:

The authors have addressed my previous concerns and I do not have additional comments.

Referee #3:

The authors have adequately addressed all comments from this reviewer.

**Point-by-point letter:**

**-you can only have 5 keywords**

*Response: Modified.*

**-COI needs to be renamed as "DISCLOSURE AND COMPETING INTERESTS STATEMENT". Please also add the following sentence to the statement: "George Gao is an editorial advisory board member." See also https://www.embopress.org/competing-interests**

*Response: We have renamed COI as "DISCLOSURE AND COMPETING INTERESTS STATEMENT" and added the statement "George F. Gao is an editorial advisory board member."*

**- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')**

*Response: The Authors Contributions in the manuscript have been removed, and the contributions of each author were provided through the 'author information' section of the manuscript submisssion system.*

**- The reference list: The list of authors in the reference list should be cut after 10 et al.**

*Response: Modified.*

**- The synopsis image should be 550 wide by [200-400] high (pixels).**

*Response: Modified.*

**- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues.**

*Response: We have addressed all the editorial issues raised and responded to the comments in the word file.*

**- Source Data files should be reorganized - one file/folder per figure and ZIPing for each main figure, for EV figures zipping together all source data. The files are not in the correct format, and we cannot open them. Please provide them in PDF format, and zip them as described above.**

*Response: We have re-submitted the source data file.*

Dear George,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at everything and all looks good.

I am therefore very pleased to accept your manuscript for publication here.

Congratulations on a nice study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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## EMBO Press Author Checklist

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|---|
| Corresponding Author Name: George Fu Gao |
| Journal Submitted to: The EMBO Journal |
| Manuscript Number: EMBOJ-2022-111737R |

**USEFUL LINKS FOR COMPLETING THIS FORM**
The EMBO Journal - Author Guidelines
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**Reporting Checklist for Life Science Articles (updated January**
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
**Please note that a copy of this checklist will be published alongside your article.**

**Abridged guidelines for figures**
**1. Data**
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

**2. Captions**
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

**Please complete ALL of the questions below.**
**Select "Not Applicable" only when the requested information is not relevant for your study.**

**Materials**

| Newly Created Materials | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| New materials and reagents need to be available; do any restrictions apply? | Not Applicable | |

| Antibodies | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| For **antibodies** provide the following information:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Yes | In Materials and Methods |

| DNA and RNA sequences | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| **Short novel DNA or RNA including primers, probes**: provide the sequences. | Not Applicable | |

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|---|---|---|
| **Cell lines:** Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID. | Yes | In Materials and Methods |
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| If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants. | Not Applicable | |

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|---|---|---|
| If your work benefited from core facilities, was their service mentioned in the acknowledgments section? | Yes | In Acknowledgements |

**Design**

| Study protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI. | Not Applicable | |
| Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable. | Not Applicable | |

| Laboratory protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Provide DOI OR other citation details if **external detailed step-by-step protocols** are available. | Not Applicable | |

| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Include a statement about **sample size** estimate even if no statistical methods were used. | Yes | In Materials and Methods |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Not Applicable | |
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| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | |
| For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | In Materials and Methods, Figure Legends |

| Sample definition and in-laboratory replication | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
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| In the figure legends: state number of times the experiment was **replicated** in laboratory. | Yes | |
| In the figure legends: define whether data describe **technical or biological replicates**. | Not Applicable | |

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|---|---|---|
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|---|---|---|
| Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section? | Yes | The atomic coordinates have been deposited in the Protein Data Bank (PDB) with accession codes 7XBH, 7XBF and 7XBG |
| Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Not Applicable | |
| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Not Applicable | |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Yes | In Materials and Methods |