Peer Community In Infections

High diversity and evidence for inter-species transmission in astroviruses surveyed from bats in Zibabwae

Tim James *based on peer reviews by 2 anonymous reviewers*

Vimbiso Chidoti, Helene De Nys, Malika Abdi, Getrudre Mashura, Valerie Pinarello, Ngoni Chiweshe, Gift Matope, Laure Guerrini, Davies Pfulenyi, Julien Cappelle, Ellen Mwandiringana, Dorothee Misse, Gori Elizabeth, Mathieu Bourgarel, Florian Liegeois (2023) Longitudinal Survey of Astrovirus infection in different bat species in Zimbabwe: Evidence of high genetic Astrovirus diversity. BioRxiv, ver. 6, peer-reviewed and recommended by Peer Community in Infections. https://doi.org/10.1101/2023.04.14.536987

Submitted: 09 May 2023, Recommended: 03 November 2023

Cite this recommendation as:
James, T. (2023) High diversity and evidence for inter-species transmission in astroviruses surveyed from bats in Zibabwae. *Peer Community in Infections*, 100085. 10.24072/pci.infections.100085

Published: 03 November 2023

Most infectious diseases of humans are zoonoses, and many of these come from particularly species diverse reservoir taxa, such as bats, birds, and rodents (1). Because of our changing landscape, there is increased exposure of humans to wildlife diseases reservoirs, yet we have little basic information about prevalence, hotspots, and transmission factors of most zoonotic pathogens. Viruses are particularly worrisome as a public health risk due to their fast mutation rates and well-known cross-species transmission abilities. There is a global push to better survey wildlife for viruses (2), but these studies are difficult, and the problem is vast. Astroviruses (AstVs) comprise a diverse family of ssRNA viruses known from mammals and birds. Astroviruses can cause gastroenteritis in humans and are more common in elderly and young children, but the relationship of human to non-human Astroviridae as well as transmission routes are unclear. AstVs have been detected at high prevalence in bats in multiple studies (3,4), but it is unclear what factors, such as co-infecting viruses and bat reproductive phenology, influence viral shedding and prevalence.

In this recommended study, Vimbiso et al. (5) study the prevalence and diversity of astroviruses in different insectivorous and frugivorous chiropteran species roosting in trees, caves and building basements across Zimbabwe, a region never investigated for astroviruses. Using both pooled population samples and individual samples from 11 different sites, the authors screened for astrovirus prevalence via RT-PCR and identified bat taxa using mitochondrial gene sequencing. An overall prevalence of 10-14% infection was recorded. No clear association of increased astrovirus and coronavirus coinfection was detected, and although astrovirus infection varied over the season, it did not do so in consistent ways across the two primary sampling sites, Magweto and Chirundu. A phylogeny generated by sequencing all of the astrovirus positive samples showed evidence that most of the viral lineages are transmitting within species but across Zibabwae such that most phylogenetic lineages grouped viruses from the same host species together. However, there was ample evidence for interspecies transmission between bats. Finally, a small percentage of the total astrovirus diversity from Zibabwae clustered with sequences from humans. The timing and direction of the transmission between humans and bats need further investigation. This study provides important baseline data about viral diversity and does an excellent job of capturing the spatial, temporal, host species, and sequence level dynamics of the astroviruses. There are clear limitations on how this study can be interpreted due to different sampling regimes and, in particular, the fact that each of the two primary sites was only explored for temporal variation over a single calendar year. That said, the grand diversity of astroviruses demonstrated in insectivorous bats in Zibabwae shows that we are only seeing the very tip of the iceberg with respect to viral diversity with zoonotic potential. As suggested by the reviewers, more studies like this are needed to understand the basic ecology of viruses and to aid in predicting epidemics.

References:

1. Mollentze N, Streicker DG. Viral zoonotic risk is homogenous among taxonomic orders of mammalian and avian reservoir hosts. Proceedings of the National Academy of Sciences. 2020 Apr 28;117(17):9423-30. https://doi.org/10.1073/pnas.1919176117
2. Carroll D, Daszak P, Wolfe ND, Gao GF, Morel CM, Morzaria S, et al. The Global Virome Project. Science. 2018 Feb 23;359(6378):872-4. https://doi.org/10.1126/science.aap7463
3. Lee SY, Son KD, Yong-Sik K, Wang SJ, Kim YK, Jheong WH, et al. Genetic diversity and phylogenetic analysis of newly discovered bat astroviruses in Korea. Arch Virol. 2018;163(11):3065-72. https://doi.org/10.1007/s00705-018-3992-6
4. Seltmann A, Corman VM, Rasche A, Drosten C, Czirják GÁ, Bernard H, et al. Seasonal Fluctuations of Astrovirus, But Not Coronavirus Shedding in Bats Inhabiting Human-Modified Tropical Forests. EcoHealth. 2017 Jun 1;14(2):272-84. https://doi.org/10.1007/s10393-017-1245-x
5. Vimbiso C, Hélène DN, Malika A, Getrude M, Valérie P, Ngoni C, et al. Longitudinal Survey of Astrovirus infection in different bat species in Zimbabwe: Evidence of high genetic Astrovirus diversity. bioRxiv, 2023.04.14.536987, ver. 6 peer-reviewed and recommended by Peer Community In Infections. https://doi.org/10.1101/2023.04.14.536987

Reviews

Evaluation round #2

DOI or URL of the preprint: https://doi.org/10.1101/2023.04.14.536987
Version of the preprint: 5

Authors' reply, 31 October 2023

By Tim James, 26 Oct 2023 15:03
Manuscript: https://doi.org/10.1101/2023.04.14.536987 version 5
Evaluation of your preprint

I want to thank the authors for carefully revising their paper and fully addressing most of the comments of the reviewers.

Before the final recommendation of the article I would like the authors to make the following changes.

1. Figure 4 is nicely improved. However, Reviewer 2 suggested that you convert this figure to a phylogram with branch lengths. The new figure legend says ”the scale bar(e) represented the number of substitutions per site.” However, this is clearly not the case as there are no meaningful branch lengths in the figure. This needs to either be corrected by using a phylogeny with branch lengths (highly recommended as this shows exactly how close the actual sequences are to each other in terms of nucleotide substitutions), or to delete that part of the legend and say that this is a cladogram and no branch lengths are shown. If you run PhyML on the data you should be able to get this phylogram with branch lengths, and then the bootstrap values are just mapped on top of that phylogram.

Response: Done

2. Since you will revise, please consider changing line 318 to ”nucleotide” instead of ”nucleotidic acid”.

Response: Done

3. Also, lines 305-308 are basically two identical statements, so delete one.

Response: Done

Download author’s reply

Decision by **Tim James**, posted 26 October 2023, validated 27 October 2023

Evaluation of your preprint

I want to thank the authors for carefully revising their paper and fully addressing most of the comments of the reviewers.

Before the final recommendation of the article I would like the authors to make the following changes.

1. Figure 4 is nicely improved. However, Reviewer 2 suggested that you convert this figure to a phylogram with branch lengths. The new figure legend says ”the scale bar(e) represented the number of substitutions per site.” However, this is clearly not the case as there are no meaningful branch lengths in the figure. This needs to either be corrected by using a phylogeny with branch lengths (highly recommended as this shows exactly how close the actual sequences are to each other in terms of nucleotide substitutions), or to delete that part of the legend and say that this is a cladogram and no branch lengths are shown. If you run PhyML on the data you should be able to get this phylogram with branch lengths, and then the bootstrap values are just mapped on top of that phylogram.
2. Since you will revise, please consider changing line 318 to ”nucleotide” instead of ”nucleotidic acid”.
3. Also, lines 305-308 are basically two identical statements, so delete one.

I should be able to finalize the recommendation quickly if you can take care of these issues.

Evaluation round #1

DOI or URL of the preprint: `https://doi.org/10.1101/2023.04.14.536987`
Version of the preprint: 4

Authors’ reply, 09 October 2023

Download author’s reply
Download tracked changes file

Decision by **Tim James**, posted 25 August 2023, validated 25 August 2023

Suggested revision for your article ”Longitudinal Survey of Astrovirus…”

Dear Authors,

Thank you for submitting your manuscript for Peer Review via PCI Infections. Your manuscript has been carefully commented on by two reviewers. You will see they have a number of suggestions which, if taken into consideration, should greatly improve your manuscript. I believe if you are able to address these concerns, I will be able to recommend it via PCI infections. If you choose to revise, please send a coverletter describing your responses to each of the criticisms. Below are the reviews and one reviewer also included a marked up manuscript.

Download recommender's annotations

Reviewed by anonymous reviewer 1, 15 August 2023

In this manuscript, Chidoti et al. performed PCR assays to investigate Astrovirus (AstV) circulation and diversity in insectivorous and frugivorous bats in Zimbabwe through a longitudinal study conducted between 2016 and 2021. They highlighted variations in AstV infection prevalence and reported excretion peaks coinciding with the presence of juvenile bats in colonies. They also took advantage of a previous study investigating the presence of Coronavirus (CoV) in the same samples to report the detection of coinfections with AstV and CoV. Finally, they described a high genetic diversity and identified some viruses clustering with human AstV.

The work and results presented in this manuscript are very relevant. However, I highly recommend further editing and English editing prior any publication to promote the work carried out by the authors. Although all important components are present, the ideas are not well structured. The writing is sometimes clumsy and repetitive. Therefore, the reading is not fluent. I tried to give some advice hereafter, but I couldn't provide modifications for each line. The discussion was way better. We can almost feel that the discussion was not written by the same person. However, I noticed that sometimes the references do not match was it written in the text. I highly recommend the authors to go through the text and check the references. I also suggested to add more references to improve the manuscript.

Also, a general comment, "Astrovirus" is a common name and should not appear in italic. However, "Astroviridae" should always be written in italic, the same for "Mamastrovirus" and "Avastrovirus".

Please, find below some specific points to address:

Abstract:

L39. Replace E. helvum by Eidolon helvum.

L44-45. It is unclear here why you introduced results about coinfection. The manuscript title and the abstract do not mention CoVs before. Maybe you could reformulate and say that you take advantage of a previous study examining the presence of CoV in the same sample to investigate coinfections.

Introduction:

You have introduced AstV= Astrovirus in the abstract but not in the introduction. You should introduce it. Also, you should use the abbreviation more often in the text.

L52. You can remove "(+ssRNA)" which is not useful here. You are not using this abbreviation elsewhere.

L52 to 80. You must simplify the introduction. The information is dispersed. You must structure the ideas and find a balance for the details you give. The writing does not follow a logical progression and some parts are very repetitive. For example, you mentioned AstVs in bats in Europe, Africa, America, and Asia L58, and you have a whole dedicated paragraph on bats L70 to 80. You may maybe give general statements in the first paragraph and keep all information about bats for your second paragraph.

For example:

“Astroviruses (AstVs) are non-envelopped single-stranded positive sense RNA viruses. They have been described in more that 80 vertebrate species and are classified in two genera: Masmastrovirus and Avastrovirus respectively infecting mammals and avian species. Recently, AstVs has also been discovered in fish and insects. Generally, AstVs are responsible for gastrointestinal illness. In humans, they particularly affect children, immunocompromised people, and the elderly. But beyond this well-known clinical manifestation of Astrovirus infections, neurovirulent Astrovirus infections have also been reported in both humans and domestic animals. These viruses seem to be particularly prone to cross species transmission, and some viruses detected in humans closely clustered with strains identified in rodents, minks, feline species and ovine.”

Then you can start your paragraph with bats. “Bats are considered as natural reservoirs for these viruses…”

I suggest adding the following reference:
Fischer, K.; Pinho dos Reis, V.; Balkema-Buschmann, A. Bat Astroviruses: Towards Understanding the Transmission Dynamics of a Neglected Virus Family. Viruses 2017, 9, 34. https://doi.org/10.3390/v9020034

L63-66. I think this level of details can be kept for discussion.

L67. “Astroviruses do not seem to have a common reservoir” versus L56 “Bats and wild birds are considered natural reservoirs of astroviruses”. Maybe you can combine these ideas.

L79. I would replace “minimally symptomatic” with “usually asymptomatic.”

L79-80. What do you mean by “the extent of existence”? Do you mean epidemiology and infection dynamics? I think you can reformulate this sentence to be more impactful.

L81-83. I disagree with this statement. It is too narrow. AstV infection dynamics and more globally infections dynamics are driven by a combination of both biotic and non-biotic factors. The seasonality will for example drive modifications in host fitness and subsequently impact the susceptibility.

Also, I suggest adding the following references:
Altizer S, Dobson A, Hosseini P, Hudson P, Pascual M, Rohani P. Seasonality and the dynamics of infectious diseases. Ecol Lett. 2006 Apr;9(4):467-84. doi: 10.1111/j.1461-0248.2005.00879.x.

Drexler JF, Corman VM, Wegner T, Tateno AF, Zerbinati RM, Gloza-Rausch F, Seebens A, Müller MA, Drosten C. Amplification of emerging viruses in a bat colony. Emerg Infect Dis. 2011 Mar;17(3):449-56. doi: 10.3201/eid1703.100526

L83-84. For shedding pulses favoring spillover you can cite:

Amman BR, Nyakarahuka L, McElroy AK, Dodd KA, Sealy TK, Schuh AJ, Shoemaker TR, Balinandi S, Atimnedi P, Kaboyo W, Nichol ST, Towner JS. Marburgvirus resurgence in Kitaka Mine bat population after extermination attempts, Uganda. Emerg Infect Dis. 2014 Oct;20(10):1761-4. doi: 10.3201/eid2010.140696

Adjemian J, Farnon EC, Tschioko F, Wamala JF, Byaruhanga E, Bwire GS, Kansiime E, Kagirita A, Ahimbisibwe S, Katunguka F, Jeffs B, Lutwama JJ, Downing R, Tappero JW, Formenty P, Amman B, Manning C, Towner J, Nichol ST, Rollin PE. Outbreak of Marburg hemorrhagic fever among miners in Kamwenge and Ibanda Districts, Uganda, 2007. J Infect Dis. 2011 Nov;204 Suppl 3(Suppl 3):S796-9. doi: 10.1093/infdis/jir312

L84-85. “Shedding pulses … higher prevalence is detected”. The sentence is unclear. Can you reformulate?

Material and method:

Legend Table 1. I suggest removing ‘each’ and replacing “at 14 different sites” by “for the 14 selected sites”.

Table 1. Try to harmonize the details you give and keep the same denomination. For the top of the table you miss a capital letter at the beginning of “Type of roost”. Replace “Baobab” by “tree”. I suggest replacing “Date of collect” by “Collection date”. For Chirundu you wrote “December 202” you miss the end of the sampling year.

L108. Remove the second “selected”. Maybe you can reformulate: “visited to collect guano, which is used as a fertilizer, or to hunt bat for consumption purpose”.

L113. As a matter of curiosity, do you have any data regarding the populations size? Also, why only 6g of pooled droppings was collected? This sounds small. Did you subsample the available droppings and pooled them to have a total of 6g? Also, I am interrogative with the fact to keep the plastic sheets overnight. Are you sure to collect only bat droppings? For example, are there rodents living in caves that could have “contaminate” your plastic sheets?

L124. For the individual collection did you also keep the plastic sheets underneath the colony all night long?

Legend of Table 2. Replace “Coinfection” by “coinfection”. Your table is very complete. You should mention in the legend the fact that you provide data regarding reproduction cycle.

Table 2. I am unsure regarding the terms “Reproduction cycle”. Maybe something like “Reproduction cycle stages”.

L162. I suggest “All bats that tested positive for astrovirus were …”.

L167. Be careful “was done … were done”. Correct the sentence.

L176-177. Maybe you can add a sentence to indicate that you migrated your PCR products on agarose gels to visualize your results.

L216-218. Cite your Table 2.

Results:

Globally, the text can be better edited to improve the comprehension and valorize the results.

How do you calculate your confidence intervals in this section?

L222. I suggest replacing the sentence with: "The sampling was carried out from June 2016 to December 2020."

L223. Replace "sampled" by "samples".

L226. When a number is less than 10 usually you will write it in all letters. I suggest replacing "9" by "nine".

L227-228. I suggest reformulation "In Magweto, five of the eight pooled samples (62.5 ± confidence interval) tested positive for AstV. In Bashongwe, all the 72 collected samples tested negative".

L229. Reformulate as for Magweto and Bashongwe.

L227, 229 and 231. Don't forget the confidence interval.

L222-243. With more editing the reading can be improved and the results better valorized.

L235. I suggest "In total, we analyzed 1159 samples coming from from the Magweto cave site, and 1728 samples coming from the Chirundu farm".

L237. I suggest "at Chirundu".

L238. Remove "of".

L239. Do you have more information about the age structure in the colonies. Are there just juveniles? Are they the majority?

L239-240. I suggest "Increased from December 2020 to March 2021, which corresponded to the lactation period, followed by the weaning of four to six months old juveniles."

L241 replace ";" by "," after "(0%)".

Legend of Figure 2. The y-axis represents the percentage of AstV positive samples, not the number. I suggest "x-axis as the sampling collection date". Also, "reproductive season" might not be the most appropriate denomination. Maybe you can change for something like "the corresponding stage withing the reproductive season".

Figure 2. There is a lot of information on your figure. Maybe you can move the table as supplementary material. The graphs are enough clear. How did you calculate the IC on the graph? They seem big and not accurate. Also, I suggest modifying the y-axis label for "Astrovirus prevalence (%)"

Also, if you want ideas to improve the graph representation you can refer to the references below:

Dietrich M, Wilkinson DA, Benlali A, Lagadec E, Ramasindrazana B, Dellagi K, Tortosa P. Leptospira and paramyxovirus infection dynamics in a bat maternity enlightens pathogen maintenance in wildlife. Environ Microbiol. 2015 Nov;17(11):4280-9. doi: 10.1111/1462-2920.12766.

Joffrin L, Hoarau AOG, Lagadec E, Torrontegi O, Köster M, Le Minter G, Dietrich M, Mavingui P, Lebarbenchon C. Seasonality of coronavirus shedding in tropical bats. R Soc Open Sci. 2022 Feb 9;9(2):211600. doi: 10.1098/rsos.211600.

Mortlock, M., Geldenhuys, M., Dietrich, M. et al. Seasonal shedding patterns of diverse henipavirus-related paramyxoviruses in Egyptian rousette bats. Sci Rep 11, 24262 (2021). `https://doi.org/10.1038/s41598-021-03641-w`

L260. Change "5,2%" with "5.2%"

L261. I suggest modifying the sentence. For example: "Results from the GLMM didn't show any effect of bats seasonal reproduction periods on the detection of AstV RNA in samples collected at Magweto".

L280-282. Remove this sentence. See my comment below.

L280-293. The text deserves more editing and English editing. Start with general statement first and then give details on the dynamics. For example:

"The co-infection prevalence was relatively low. In Chirundu, only XX samples out of XX tested positive for the two viruses (Prevalence +/- CI 95%). The highest prevalence was observed"

"In Magweto, a total of XX overt XX tested positive for the two viruses..."

Title of Figure 3. I suggest modifying the title: "Prevalence of astrovirus, coronavirus, and co-infection involving both viruses per month ..."

Figure 3. I suggest modifying the y-axis legend for "Prevalence (%)". Take into consideration my previous comments provided for the figure 2. Also, try to homogenize the figures. Your figure 2 and 3 should apply the same "visual code". Maybe you can find inspiration from the references I provided you.

L300. I would change the title for this section with "Astrovirus genetic diversity".

L301. I suggest modifying the sentence: "All AstV positive samples (N=223) were sequenced, representing 179 samples for Chirundu, 229 for Magweto ..."

L303. I suggest modifications: "Of the 423 samples, 214 were from Hipposideros spp. ... and 76 samples originate from bats for which we couldn't determine the genus/species."

L307. I suggest modifications: "A phylogenetic analysis was conducted with the 155 partial sequences generated during this study. All clustered within the Mamastrovirus genus"

L308. It is not useful to precise of the Astroviridae family. Obviously, we already know this detail.

L309. Replace "1" by "one".

L310. Be careful with "(HuAstVs)". You use this abbreviation earlier in the text. Make sure to make it appear at first use, not here.

L318. I suggest modifications "closely related to astrovirus identified in insectivorous bats."

L319-320. You miss "spp." After "Hipposideros" and "Rhinolophus". Also the sentence needs to be reformulated.

L320-321. “One cluster … Rhinolophus spp.” Unclear what you meant.

L322. “spp.’ Needs to be added after “Nyctalus” and “Verpitillio”.

L323. Did you mean “Another cluster comprised the majority of sequences”? Your sentence is not clear.

L324. You miss “spp.” After Miniopterus.

L327. You mis “spp.” Twice.

Legend Figure 4. Remove “in” after “Honde Valley” L331. L337. Stay consistant, replace “species” by “spp.” I also suggest to move the details fron l341 to L365 in supplementary data to simplify the legend. See my comments of Fig4 below for further details.

Figure 4. To help readers with the tree maybe you can add animal silhouettes for each relevant cluster.

L368. “spp.” should not be in italic.

L369. “spp.” should not be in italic.

L377. “LAOS” should not be in capital. You must replace by “Laos”.

L382. “spp” should not be in italic.

Discussion:

L388. I suggest modifications: “The overall AstVs prevalence was respectively 10.0% and 13.7% in Chirundu and Magweto sites, confirming their circulation in bat populations from Zimbabwe.”

L392. I suggest modifications. “These discrepancies could result from different study designs, longitudinal sampling in our case versus transversal studies in Mozambique and Madagascar.” I also suggest adding a sentence stating that these differences could also results from the studied species and the different localities resulting in subtle changes that may impact the infection dynamics.

L395-401. You could also contrast you results with other longitudinal studies investigating AstV in bat population. I suggest to cite the following work conducted on Reunion Island during four years in which they detect a very low overall prevalence and no variation over time:

Hoarau, A., Köster, M., Dietrich, M., Le Minter, G., Joffrin, L., Ramanantsalama, R., . . . Lebarbenchon, C. (2023). Synchronicity of viral shedding in molossid bat maternity colonies. Epidemiology & Infection, 151, E47. doi:10.1017/S0950268823000171

L405. I suggest to add these other studies for CoV:

Hoarau, A., Köster, M., Dietrich, M., Le Minter, G., Joffrin, L., Ramanantsalama, R., . . . Lebarbenchon, C. (2023). Synchronicity of viral shedding in molossid bat maternity colonies. Epidemiology & Infection, 151, E47. doi:10.1017/S0950268823000171

L426. The reference you cite is not correct. Be careful. Co-infection on Madagascar and Mozambique has been treated in the following paper:

Hoarau, A.O.G., Goodman, S.M., Al Halabi, D. et al. Investigation of astrovirus, coronavirus and paramyxovirus co-infections in bats in the western Indian Ocean. Virol J 18, 205 (2021). `https://doi.org/10.1186/s12985-021-01673-2`

Not in reference [17] and [19] that you are citing.

L428. Again, the references you are cited are not correct.

L429-430. You can implement with Hoarau et al. results from 2021 and 2023.

L433. Better to cite Fischer et al. 2017.

Reviewed by anonymous reviewer 2, 25 August 2023

Dear Editor,

This paper surveys astroviruses from bats in Zimbabwe using PCR of fecal samples. The authors have multiple sampling sites that were visited for multiple years and multiple species were sampled. The rates of prevalence were low to moderate and varied across the seasons, in some ways associated with reproductive cycles. The data were compared to coronavirus prevalence and shown to be non-significant, though the two viruses tend to peak at the same time. All the positive samples were sequenced, and a phylogeny showed a large diversity of sequence types, some of which were related to human astroviruses. The data show that there is low host specificity, with little association with frugivores or insectivores. The data are geographically patchy and viruses from different populations were closely related.

These data are an important contribution to understanding virus diversity in an undersampled region of the world, and the sequences contribute to a better picture of coevolution and transmission among bat species and other mammals. Multiple sampling points give a more comprehensive picture of how viruses vary over space and time and host species.

There are a few major issues that the authors should consider revising. First, the questions the authors are hoping to answer are not made clear, especially in the introduction. Rewriting the introduction to provide the background and to set up the study better with hypotheses, etc., is critical. Specifically, background or hypotheses about reproductive cycles and the emergence of novel species could be added to support the authors' efforts in the Results section. Second the conclusions about reproductive cycles are not supported because there is only one year of sampling, and the two main individual populations are not showing the same result. Lastly, the tree is very useful and informative but could be better presented. For example, a non-circular tree with better labeling of both host and geography would be useful. In addition, branch lengths will be critical for gauging distance from reference sequences to the Zimbabwe sequences. Also, how were the references chosen, i.e., how was taxon sampling from GenBank done?

In addition, the following issues should be addressed:

Figures 2 & 3 are not easy to interpret and redundant with the tables and should be improved.

A conclusion section or paragraph at the end of the Discussion section would be useful.

What is the species-level infection prevalence? This data is not presented clearly but is a valuable output of this study.

Figure 1 also needs work. What is the zoom in rectangle showing?

No mention of biosafety or protocols to reduce cross-contamination are discussed.

In addition, the minor issues highlighted in the manuscript (attached) should be considered.