

Reviews

Reviewed by anonymous reviewer, 15 Aug 2023 20:02

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.

We would like to thank the reviewer for his judicious and constructive comments, which have helped us to improve the quality of this article. We have answered to all comments.

1. 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".

Response: Done

Please, find below some specific points to address:

Abstract:

2. L39. Replace E. helvum by Eidolon helvum.

Response: Done

3. 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.

Response: We added a sentence according to your comments: "By combining data from our previous work on Coronaviruses, we also analyzed co-infection of bats with Coronaviruses and Astroviruses at Magweto and Chirundu sites for which the prevalence of co-infection was 2.6% and 3.5% respectively."

Introduction:

4. 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.

Response: Done

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

Response: Done

6. 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-enveloped single-stranded positive sense RNA viruses. They have been described in more than 80 vertebrate species and are classified in two genera: Mastrovirus 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>

Response: We've reorganized the introduction to take your comments into account

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

Response: We do not agree and we believe that astrovirus taxonomy should be detailed in the introduction.

8. 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.

Response: Done

9. L79. I would replace "minimally symptomatic" with "usually asymptomatic."

Response: Done

10. 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.

Response: Done

11. 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.

Response: We modified this section accordingly

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

12. 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, Tshioko 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

13. L84-85. "Shedding pulses ... higher prevalence is detected". The sentence is unclear. Can you reformulate?

Response: Done, we reformulated: “The varying prevalence of viruses in bats is determined by shedding pulses, where rare or very low prevalence are detected in the absence of shedding pulses and higher prevalence is detected when shedding pulses occur”. We added Amman *et al* and Drexler *et al* reference

Material and methods

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

Response: Done

15. 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.

Response: The table has been modified accordingly

16. 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”.

Response: Noted. Modified.

17. 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?

Response: We have no data on population size. For these first sampling sessions, we used a community-based approach. However, we have obtained new funding for new projects on small mammals in Zimbabwe, and we have integrated the study of bat and rodent communities at the Magweto and Honde valley sites with capture-mark-recapture protocols that will enable us to answer this question among others. We collected 6g of faeces (several drops corresponding to several individuals) per 15 ml tube, mixed with 6 ml of RNAIater and we collected all the faeces present on the plastic sheets. At the laboratory we pooled 3 or 4 tubes.

As for rodents residing in the caves or anywhere near all the sites studied, it is possible. However bat and rodent faeces are distinctly different. For the insectivorous bats, their dropping are much smaller in size compared to rodents. For the frugivorous bats they are all completely different as for fruit bats the faeces are often semi-liquid and very colourful. We also amplified Cytocrome b

gene on all samples that tested positive for the screening of AstVs and CoVs as a way to validate and confirm if indeed the droppings we collected were of bats and not rodents or other animal species. Of all the samples we screened for the mitochondrial gene, we did not have any that came back as rodent species. All were confirmed to be bat faeces.

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

Response: Yes, however please note we only collected faeces that looked very fresh, new and wet, and avoided not too dry ones as it would mean they would have been there for too long.

19. 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.

Response: Done

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

Response: You are correct. We however did explain in detail that these are stages of the reproduction cycle and we added reproduction cycle stage in the legend.

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

Response: Noted. Modified

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

Response: Noted. Rectified

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

Response: Noted. Done

24. L216-218. Cite your Table 2.

Response: Done

Results:

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

Response: We slightly modified the text

26. How do you calculate your confidence intervals in this section?

Response:

The confidence intervals were calculated using Wilson score test. The total number of samples analysed divided by total positives recorded. We did a wilson proportion test as described (<https://epitools.ausvet.com.au/ciproportion>).

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

Response: Done

28. L223. Replace "sampled" by "samples".

Response: Rectified

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

Response: Noted. Done

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

Response: Done

31. L229. Reformulate as for Magweto and Bashongwe.

Response: Done

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

Response: Due to the methodology used, we do not think that Confidence interval is relevant for the the detection of AstV in pooled samples. As we explained above (and in material and methods), on the field we pooled several faeces from different bat individuals into same tube. Then at the laboratory we further pooled four tubes together and we ultra-centrifuged the supernatant. PCR products were cloned (10 clones per PCR product).

In our point of view confidence interval doesn't make sense with this approach as we do not have the actual individual proportions.

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

Response: Noted

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

Response: Done

35. L237. I suggest "at Chirundu".

L238. Remove "of".

Response: Done

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

Response:

1/ Are there just juveniles?

No, there is all ages

/Are they the majority?

Not necessarily the majority as number of adult sexually mature females = number of juveniles as each individual births one pup. However during this time, the sites had a high influx of juveniles following parturition, which according to literature, juveniles have less immunity compared to adults hence the high shedding peaks of viruses, as described in the discussion.

37. 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."

Response: Noted. Modified

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

Response: Noted. Modified

39. 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".

Response: We modified the legend according to your comments

40. 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.

Response: We modified the legend according to your comments

41. 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 (%)"

Response: Please note the IC values are available in the table 1. And with our graphs it is the Prevalence % \pm the IC values. If you for example 10.1% \pm the upper value of 15.7 and lower value of 6.3 then you have the error bars in the graphs.

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>

42. L260. Change “5,2%” with “5.2%”

Response: Done

43. 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”.

Response: Done

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

Response: We modified this section.

45. 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...”

Response: Noted. Modified

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

Response: Noted. Modified

46. 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.

Response: We modified figures 2 & 3

47. L300. I would change the title for this section with “Astrovirus genetic diversity”.

Response: Noted. Modified

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

Response: Thank you for your suggestion. We modified the sentence.

49. 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."

Response: Done

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

Response: Noted. Modified

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

Response: Noted

52. L309. Replace "1" by "one".

Response: Done

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

Response: Done

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

Response: Done

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

Response: Noted. Done

56. L320-321. "One cluster ... Rhinolophus spp." Unclear what you meant.

Response: Rectified

57. L322. "spp.' Needs to be added after "Nyctalus" and "Verpitillio".

Response: Done

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

Response: we reformulated the sentence "Another cluster comprising of the majority of sequences from Chirundu and some from Magweto site, were characterized from Miniopterus spp and Rhinolopus spp. All together, they formed a phylogenetic cluster with strains of bat astroviruses isolated from Miniopterus spp from Madagascar and China (references N, O, R, U,

T), *Rhinolophus* spp from Korea (references P, Q) as well as *Rousettus* spp and *Paratrianops* spp BAsTV from Madagascar (references V, W) (Figure 4)”

59. L324. You miss “spp.” After *Miniopterus*.

Response: Rectified

60. L327. You mis “spp.” Twice.

Response: Rectified

61. Legend Figure 4. Remove “in” after “Honde Valley” L331. L337. Stay consistent, replace “species” by “spp.” I also suggest to move the details from L341 to L365 in supplementary data to simplify the legend. See my comments of Fig4 below for further details.

Response: Noted. Rectified

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

Response: Done. We also modified the tree topology according to the other reviewer’s comments.

63. L368. “spp.” should not be in italic.

Response: Noted. Rectified

64. L369. “spp.” should not be in italic.

Response: Noted. Rectified

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

Response: Noted. Rectified

66. L382. “spp” should not be in italic.

Response: Noted. Rectified

Discussion:

67. 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.”

Response: Noted. Modified

68. 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 result from the studied species and the different localities resulting in subtle changes that may impact the infection dynamics.

Response: Done, modified

69. L395-401. You could also contrast your 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

Response: Done.

70. 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

Response: Done

71. 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. *Virology* 18, 205 (2021). <https://doi.org/10.1186/s12985-021-01673-2>

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

K, Zeus V, Kwasnitschka L, Kerth G, Haase M, Groschup MH, Balkema-Buschmann A. Insectivorous bats carry host specific astroviruses and coronaviruses across different regions in Germany. *Infect Genet Evol.* 2016 Jan;37:108-16. doi: 10.1016/j.meegid.2015.11.010. Epub 2015 Nov 14. PMID: 26584511; PMCID: PMC7106178

Response: Corrected

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

Response: Corrected

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

Response: Done.

74. L433. Better to cite Fischer et al. 2017.

Response: Noted with thanks. Rectified.

Reviewed by anonymous reviewer, 25 Aug 2023 13:29

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.

We would like to thank the reviewer for his judicious and constructive comments, which have helped us to improve the quality of this article. We have answered to all comments.

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.

1. 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.

Response: We rewrote the introduction accordingly

2. 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.

Response: We highlighted this point in the conclusion section

3. 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.

Response: We change the representation of the phylogenetic tree and add the signification of the asterisks and the scale bare in the legend.

4. Also, how were the references chosen, i.e., how was taxon sampling from GenBank done?

Response: References were chosen in order to represent the bat AstV genetic diversity and based on AstV studies previously published.

In addition, the following issues should be addressed:

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

Response: we modified Figures 2 & 3.

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

Response: Done

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

Response: This information is provided in the Results section, paragraph Astrovirus genetic diversity.

“Of the 423 samples, 214 (50.6%) were amplified from *Hipposideros* spp., 63 (14.9%) from *Rhinolophus* spp., 53 (12.5%) from *Miniopterus* ., 10 (2.4%) from *Nycteris* spp., seven (1.7%) from *Eidolon* spp. and for the remaining 76 samples known to originate from bats for which we couldn't determine the genus/species”.

We also discussed these results in the discussion section: “The majority of the astroviruses amplified were from *Hipposideros* and *Rhinolophus* species and these clustered with AstV sequences derived from Mozambique, Madagascar and China of the same families [20,22,23]. The observed trend is due to the two genera being the most dominant species of bats at both sites where sampling was done. Therefore, the probability of higher detection of AstV in these species is expected and evidently observed to be higher than other rare or less dominant species.”

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

Response: We added information in the legend. “In Honde valley, the *Eidolon helvum* colony was established in at least four different sites represented by the rectangle at the right of the figure.”

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

Response: We added a sentence concerning this aspect. “Of note, for all sampling sessions, personnel protection equipment (gloves, FFP3 mask, Tivek coverall, etc) was used in order to respect the biosafety procedures and to protect the sampling teams. Good biosafety practice trainings are regularly dispensed for our sampling teams.

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

Response: All the minor issues highlighted in the manuscript have been considered.