Manuscript Title:

"Bovine ultra-long CDR H3 specific for bovine rotavirus displays potent virus neutralization and therapeutic effect in infected calves"

Reviewer Summary:

The manuscript describes a comprehensive investigation into bovine-derived ultra-long CDR H3 (ULCDRH3) antibody fragments as potential intervention measures against bovine rotavirus (BRV), a leading cause of neonatal calf diarrhea. A single calf was immunized with four doses of BRV (group-A BRV NMG strain), and total RNA was extracted from PBMCs to construct a phage display antibody library, which was subjected to three rounds of biopanning using immobilized BRV. Following round 3 enrichment, 92 randomly selected clones were screened for BRV-binding via ELISA, of which 79 clones showed signs of BRV-specific binding and the 5 clones with the highest signals were selected for further analysis. Additional ELISA confirmed all 5 clones demonstrated binding to BRV, with detection sensitivities in the ng/mL range. Further evaluation of 3 clones via a neutralization assay in MA-104 cells showed ≥50% neutralization of BRV at concentrations ≥20 μg/mL. A BRV challenge model in suckling rats was attempted but was not conclusive due to limited pathological evidence in the positive infection control rats. A more robust bovine in vivo model, using 5-day-old calves intravenously treated with "Clone 84" 1 day after infection, demonstrated therapeutic benefit based on notably increased survival, reduced clinical scores, and significant differences in fecal viral loads. The authors conclude the feasibility of obtaining BRV-specific ultra-long CDR H3 antibodies of bovine-origin via phage display, and propose these as a promising method to prevent or treat BRV calf diarrhea.

Reviewer Assessment:

This manuscript presents a detailed and coherent study on the development and testing of ULCDRH3 for potential use against BRV in neonatal calves. One of the key strengths of this work is the in vivo challenge model in calves, which adds real-world value and relevance and showed encouraging therapeutic potential. The use of a species-matched antibody format derived directly from bovine immune cells and the use of phage display technology to identify new ULCDRH3 brings novelty to the field, especially in the veterinary context. At the same time, there are several structural, content, and presentation issues that currently limit the readability, scientific clarity, and potential impact of the manuscript. The results section is overly segmented and includes a great deal of technical detail that should belong in the methods section, which overall makes it unnecessarily hard to follow the bigger-picture narrative and to interpret the significance of the findings. Similarly, the Discussion contains

substantial redundant background information that was already partly covered in the Introduction, rather than focusing on interpretation of the results and their implications. Obviating this redundant content, the remaining discussion is notably lean and does not sufficiently engage with the study findings or the literature. Combined, these issues make the manuscript harder to follow and weaken the overall narrative.

Given the structural nature of the key issues, I have chosen to structure my review to follow the layout of the manuscript (rather than dividing my comments into "major" and "minor" points as I normally would). However, I have clearly marked the most important comments both explicitly and by color to help the authors identify them more easily.

Overall, this study likely represents a useful contribution to the field, particularly in the area of veterinary antibody therapies. However, the manuscript would greatly benefit from a major revision. I hope my feedback will be helpful to the authors in this process, and I assure them that, despite the extensive nature of my comments, my intention was to be as constructive as possible.

REVIEWER COMMENTS

ABSTRACT:

1. The methods, results and conclusions are mostly well represented, however the aim of the study (or research question) should be more clearly stated
2. Between the sentences in line 19: It would help to give some sense to how many clones were screened/obtained/tested.
3. one of the key findings of this study (increased survival of challenged calves) is not mentioned in the abstract. This should be included.
4. Line 25: “and proposes a new idea” – currently the grammatical subject of this sentence is the CDR H3 fragments. Therefore, this conclusion statement should be clarified and refined. Who proposes what and why?
5. It may be acceptable to summarize/remove non-critical information to compensate for slightly increased abstract length as a result of the above suggestions.

INTRODUCTION:

6. The aim of the study and/or research question is mostly implied rather than explicitly stated. A clearer articulation of the study objective (e.g. rephrasing lines 132-137 or adding a final paragraph or sentence along the lines of: “This study aimed to generate… and evaluate…”) would help to improve the focus and emphasize the relevance/impact of the findings.

7. While the introduction is generally very detailed and complete, it could help to briefly note the relative novelty of applying ULCDR-H3 to treat bovine pathogens rather than more commonly explored uses like cancer/HIV/etc.

8. Lines 86-109: this section provides a well-crafted and detailed overview of ULCDRH3 structural features and implications. However, given this study did not address any structural or mechanistical features, this long technical section seems unnecessary. I encourage the authors to greatly summarize it, and/or refer to a review article, and/or include a schematic as visual that replaces part of the text. Note that reducing this section would be important, given my recommendations about moving large part of the discussion to the introduction (see further below).

RESULTS:

9. **Major Comment:** A major concern with the Results section is its structural organization and narrative style. There is an excessive number of subsections, which also contain extended descriptions of technical procedures, such as primer names, gel electrophoresis conditions, vector construction, etc, which should belong in the methods section. This not only deviates from convention but also greatly dilutes the readability and the impact of the results section, making it read more like a methods section. I strongly recommend a major revision of the entire Results section to focus on reporting and interpreting the conclusion-relevant findings, while moving most unnecessary procedural details to the methods section (if already covered there, then no need to repeat it). Coalescing unnecessary sub-sections, improving data presentation, and summarizing key findings after objective narration of the results, would significantly improve the quality of the manuscript. Just to give one example: all subsections between lines 242-280 could be consolidated into a single section (suckling rat challenge model). Unnecessary detail should be moved to the methods section and leave only a concise but sufficient description of the investigational approach and the experimental findings. This section in particular should be brief, given the findings are not conclusive since the infection model was not validated.

    a. In many results paragraphs, the first sentence(s) introduces a figure and provides excessive detail that should belong in the figure legend. Conversely, a helpful "(Fig.X)" reference is missing at the end of many key results-description statements. As they restructure the results section, I encourage the authors to improve this aspect as well, and focus on a coherent results-driven narrative rather than sub-sectioning based on methods or figures. Introducing figures in the main text is not necessary, since this is what the figure legends are for. However, it would help to add (Fig.X) at the end of relevant statements.

    b. Line 182-192: Ultra-long CDR H3 antibody fragments are described in extensive detail in the introduction and the discussion. However, when the authors describe their experimental approach (in the results and methods sections), it is not immediately clear to the reader what type of protein is expressed as a consequence of ligating the ULCDR-H3 fragment into the pET-22b vector (e.g. whole antibody vs. single-domain structures, or something else). This can be

somewhat inferred from the 13-kDa bands mentioned in lines 191-192) but clarity to the reader is still lacking, especially given reference to "consistent with the expected result as shown in Figure 2B" (it is not clear what the expected results are, nor what they are based on, nor how this is related to the picture of agar plates in figure 2B). Also, regarding "subsequent antibody engineering" (line 19). If the expressed protein products includes any additional scaffold, tags, fusion-proteins, or other supporting domains this should also be specified somewhere in the results narrative section.

c. Line 201-211 and 409-410: while it is a logical interpretation of the results, a definitive conclusion that the tested ULCDRH3 specifically binds to BRV is not entirely supported by the data. Given the use of a secondary antibody staining method, it is unproven whether the IF signal is coming from binding of the primary antibody to a viral protein. Therefore, these conclusive statements should be softened as "indicates that ULCDRH3 likely binds to…" or similar

d. Figure 2: please double-check this figure, since letters in the panel (e.g. B, C etc) do not match what is shown in the figure. Also, it should be clarified whether all clones were tested for immunofluorescence and which clone do the shown images correspond to?

10. Figure 4 (lines 275-279): This could be consolidated as: The pathological changes in the BRV group and control group of the rats were observed by using H&E-stained paraffin sections of the duodenum (C), jejunum (D) and ileum (E).

11. Neutralization results (Line 221-241):

    i. this is perhaps the only section that suffers from the inverse problem (not enough methodological detail provided in order to allow the reader to correctly interpret the results). The neutralization assay should first be briefly described to orientate the reader (see comments about this assay in the methods section of this report)

    ii. Line 224: "the cells were continuously observed for 5 days" – but when was the readout/scoring officially performed? This should be specified in the text and/or Figure3 and/or Table 1.

    iii. Line 229: it is not clear why only 3 of the 5 clones were tested in the neutralization assay. Why these ones?

    iv. Just as a comment: It is unfortunate that the authors did not test sufficient dilutions to get a full neutralization curve that would allow estimating the IC50 value. This is normally critical for this type of studies. However, given the convincing in vivo experiments, it is not a critical flaw in this manuscript but it would have been a very valuable add-on.

    v. Table 1: this table is acceptable but not reader-friendly. A graph with "curves" would be much more helpful (despite the few dilutions tested), or alternatively a simple grid-like plate representation that shows the CPE-

positive/negative wells in different colors (this would be more visual and easier to rapidly interpret).

vi. Line 240: what is meant by "could neutralize"? I would suggest phrasing this as: could neutralize ≥50% of wells at concentrations ≥X µg/mL

12. Line 269-270: how do the authors explain/interpre that the uninfected control group shows intestinal damage?

13. Bovine challenge model (Lines 280-361):

    i. This section should specify which ULCDRH3 Clone was used for treatment and why.

    ii. Line 281-282: the three treatment groups should be introduced with clear definitions, and it should be specified how many calves were in each group. Note that currently there is ambiguity in this section regarding the groups. This is because the BRV group is not the only group that was exposed to BRV, and because the PBS group could have received PBS in lieu of the virus or in lieu of the antibody.

    iii. Line 281-298: please add (Fig.X) at the end of each relevant statement.

    iv. Line 286: a reference ideally should be provided for this clinical scoring method. If this is not possible, the authors should first briefly introduce the rationale behind the scoring method before describing the results.

    v. Line 286-2391: why is the clinical score of the PBS group not described? According to Figure 5C, this group had a clinical score of zero throughout the experiment. How do authors explain/interpret this?

    vi. Line 289-291: it would be critical to specify how many calves died in each group and also this very important data is missing in Figure 5. A good way to present this data would be through a Kapplan-Meier plot.

    vii. Line 203: What is meant by "all the antibody groups"?

    viii. Line 306: "As for the PBS group, BRV was not detectable in the PBS group throughout the whole experiment by RT-qPCR detection." – Is this referring to Figure 5D? The PBS group seems to have the highes viral load here.

    ix. Line 324-326: given the rapid destabilization of the antibody in bovine serum, how do the authors explain/interpret continued in vivo protection up to 6 days post-infection? This should be at least mentioned in the discussion.

    x. In Figure 5, either the legend or the graph should show how many calves were in each group.

    xi. In Figure 5, if calves were injected with antibody every day for 6 days (as suggested in line 605 in the methods section) this should be reflected in the schematic and in the figure legend.

14. Line 342-352: please include (Fig.X) at the end of each relevant statement to guide readers to the corresponding panels of Figure 6.
15. Figure 6: the entire figure legend is incorrect. It seems to describe elements of Figure 5.

DISCUSSION:

16. **Major Comment:** While the information provided in Linea 362-402 is well-written and informative, this content largely overlaps with content presented in the Introduction and more importantly it is not directly related to or indirectly relevant to the results generated in this study. Therefore, I strongly encourage the authors to remove most (if not all) of this content from the discussion and consolidate it with the relevant sections in the introduction. With the removal of the redundant background section, the remainder of the Discussion is disproportionately short and does not provide a sufficient depth of analysis. The authors should substantially expand this section considering (but not limited to) the suggestions below:
17. The discussion section should:
    - **a.** briefly recapitulate key findings and prioritize their interpretation in light of the existing relevant literature.
    - **b.** Highlight strengths and limitations of the study
    - **c.** Modestly emphasize the novelty aspect of the methods/findings/analysis that merit publication of this study as a sufficiently original and impactful contribution to its field.
    - **d.** Discuss or explore the implications for future studies and/or the current state of the field
18. **Limitations:** Currently, no limitations of the study/methodology are discussed. Some that should be considered include:
    i. A specific strain of BRV was used for immunization, however it is unclear which viral strain was used for the neutralization assay. To demonstrate true therapeutic potential, the antibody should ideally have been tested against a panel of different strains based on their global prevalence or local distribution.
    ii. No mechanisitic exploration or epitome mapping was attempted. It is currently unclear which viral protein is bound by the antibody. This would be very helpful to know, as it could enable use of the antibody for diagnostic/research purposes, and also because one could bioinformatically assess the level of conservation of this protein across different strains (to predict cross-neutralization of different strains, given the lack of in vitro data).

iii. Limited statistical power for in vivo challenge model: I must first note that it is perfectly understandable why larger calve groups are logistically challenging, and I applaud the authors' efforts and success in obtaining the dataset presented, which is very convincing. However, , a group size of four calves does limit statistical power, and this limitation should be at liest briefly acknowledged.

iv. The neutralization steps performed in this study were insufficient to obtain full neutralization curves that would allow to calculate IC50 values. Since these values are most commonly used for comparing the potency and utility of different neutralizing agents, this is a small but important limitation of this study.

19. Other potentially discussion-worthy topics (especially when relevant literature is available):

a. Reflections on potential causes for the failure of the suckling rat model.

b. Comparison of ULCDRH3 features (e.g. neutralization potency, serum stability, cost of manufacturing) with those of known standards or similar therapeutic candidates (e.g. IgY).

c. Suitability of ULCDRH3 for practical veterinary use, e.g., route of administration, dosing regimen, cost-effectiveness, likelihood of resistance development, veterinary regulatory landscape, etc.

METHODS:

20. "Line 434-440: Was the virus material used for immunization previously inactivated? If so, this should be specified.

21. Line 484 and 496: which strain was used for biopanning and ELISA?

22. Table 2: PCR primers – this would sit better in the supplementary (optional suggestion)

23. Lines 552-560 (Neutralization assay):

24. Which strain was used for neutralization testing and why?

25. Line 556: were this the concentrations of the antibody prior to mixing with the virus or the final antibody concentrations in the neutralization experiment?

26. Which day after infection were the plates scored for CPE?

27. Line 559: suggest rephrasing as: "PBS+BRV was set as the positive infection control and MA-104 cells were set as the untreated, uninfected blank group.

28. Line 609: which different timepoints, and who did the clinical scoring?

29. Is there a reference/precedent for this clinical scoring system? Or was this developed by the authors?

30. Line 657: “each experiment was independently repeated 3 times” – please revisit this statement and clarify which experiments this applies to (or which are the exceptions).

CONCLUSIONs:

31. Line 662-667: after the major revision of the results and discussion sections has been finalized, this conclusions section should be revisited to ensure that the conclusions are:
    a. Directly related to and supported by the findings in this study, and/or
    b. Been sufficiently discussed in the discussion incl. comparison to relevant literature, and/or
    c. Not overstated and clearly distinguishing between implications for current practice vs. for future work

PHRASING ISSUES:

32. Line 58: “Sometimes the quality of bovine colostrum is not as good as expected because of the cow's own poor immune status” – Both the scientific language and clarity of this statement could be improved.
33. Line 125: “the most promising binders” - should be refined to improve scientific clarity.
34. Line 125-127: “also more conductive to” is slightly unclear. Do the authors mean well-suited to? Also, "small-molecule antibody fragments" is confusing, given that the listed examples are not “small molecules” in the pharmacological sense.
35. Line 117-120: the authors mention “previous studies”, however only 1 article is cited. It would be more helpful to make this statement braoder (briefly covering the key main intended applications of ULCDRH3 in other areas like cancer, HIV, etc) and cite a good review.
36. Line 198: “showed strong binding activity” – it is not clear “strong” compared to what, since no control antibodies/reagents were included in this experiment. One way to support this statement would be to comment on whether the presented ng/mL concentrations are in the nanomolar/picomolar range, considering ULCDR3H molecular weight.
37. Line 218: “The results of the binding of the recombinant ultra-long CDR H3 tBRV were detected by cell immunofluorescence”. This is confusing and inaccurate. Perhaps fuck better: “Binding of the recombinant ultra-long CDR H3 to BRV-infected cells was assessed by indirect immunofluorescence”
38. Line 284: suggest considering rephrasing as: “… as shown in Figure 5B, but both groups maintained…”
39. Line 288: “are worse than calves in the Ab group in terms of…” – this could be improved for scientific language and clarity. E.g.: “showed deteriorated mental state, increased body temperatures, diarrhea, and decreased appetite”.