BRIEF REPORT

Recombinant expression of receptor binding domains of all eight subtypes of botulinum neurotoxin type A for generation of antitoxins with broad reactivity

[version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background

Botulinum neurotoxin type A (BoNT/A) represents a major threat to global public health because of its most potent toxicity with the longest persistence. Several camelid single-domain antibodies (or VHHs) have been reported to exhibit high neutralizing activity against the receptor binding domain (HC) of the BoNT/A subtype used to generate them. However, it remains unclear if these VHHs can neutralize effectively HC of other BoNT/A subtypes. This study aimed to generate HC domains of all eight BoNT/A subtypes and to screen for VHHs with broad reactivity against these domains.

Methods

HC domains of BoNT/A1-A8 were recombinantly produced in *Escherichia coli.* The *bont/HCA1* fragment was amplified from sludge sample and cloned into pET45b vector by Gibson assembly. Expression vectors for HC domains of BoNT/A2-A8 were derived from pET45b-HCA1 by site-directed mutagenesis and/or in-house gene synthesis. Similarly, VHHs were synthesized and cloned into pET22b

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vector. Recombinant protein were purified by Ni-NTA spin columns and analyzed by SDS-PAGE. ELISA was used to confirm the antigenicity of HC domains and to evaluate the reactivity of VHHs to these domains.

Results

SDS-PAGE analysis and ELISA results with commercial polyclonal antibody demonstrated the HC domains of all eight BoNT/A subtypes were correctly produced. ELISA results using a VHH panel indicated that, apart from ciA-C2, a well-characterized VHH specific for HC of BoNT/A1, two new VHHs were found to recognize the HC domains of all BoNT/A subtypes, of which VHH-A3 displayed EC50 values for these domains close to those of ciA-C2.

Conclusion

This study provided a resource to comprehensively identify antitoxins conferring broad protection against BoNT/A.

Keywords
botulinum neurotoxins, botulism, receptor-binding domain, HC, recombinant proteins, neutralization, VHH, nanobody, antitoxin

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Introduction

Botulinum neurotoxins (BoNTs) are the most toxic substances known to the humankind with lethal dose values in the range of nanogram per kilogram body weight scale.[1] Most commonly produced by *Clostridium botulinum*, these toxins are proteins composed of a 50-kDa light chain (LC) linked to a 100-kDa heavy chain (HC) via a disulfide bond. The LC fragment contains a zinc-protease specific domain, whereas the HC consists of an N-terminal translocation domain (H_N) and a C-terminal receptor-binding domain (H_C).[2] The mode of action of BoNTs includes three steps. In the first place, the H_C domain of BoNTs bind specifically to peripheral nerve terminals via polysialoganglioside and synaptic vesicle receptors. Subsequently, BoNTs enter into nerve terminals by endocytosis. Under acidic conditions, the H_N domain translocates the LC into the nerve terminal cytosol where the latter cleaves one of three soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) that are involved in neurotransmitter release, thereby causing nerve paralysis.[2]

BoNTs are traditionally classified into seven serotypes (BoNT/A–BoNT/G), of which BoNT/A represents a great threat to humans because of its most potent toxicity with the longest duration of paralysis.[3] In addition, BoNT/A is categorized into eight subtypes (BoNT/A1–A8) with significant levels of protein sequence differences (up to 12.3%),[4] which complicates the development of a broadly protective monoclonal antitoxin.

Botulinum intoxication is fatal in 5–10% of cases and requires early treatment with antitoxin. Currently, the only available antitoxins for botulism are the heptavalent botulinum antitoxin (HBAT), which contains fragments of immunoglobulins from horses vaccinated with all seven traditionnal serotypes of BoNTs, and BabyBIG, which consists of polyclonal antibodies from human immunized with recombinant botulinum vaccine for serotypes A and B. However, these antitoxin types have limitations due to adverse side effects, limited availability and exorbitant cost.[5] To overcome these drawbacks, neutralizing monoclonal antibodies (mAbs) against BoNT/A, B, E, and F, which cause human botulism, have been generated.[6–9] It has been shown that a combination of several mAbs is required to efficiently neutralize subtypes belonging to a BoNT serotype.[8,9] Another strategy to combat botulism is to develop camelid single-domain antibodies (sdAbs), also referred to as VHHs or nanobodies, that can neutralize BoNTs via the interactions with the functional domains of the toxins. Several VHHs with high affinity against H_C domain of a BoNT/A subtype have been shown to display protective activity when challenged with the same toxin in animal models.[10–12] However, it remains unclear if these VHHs can neutralize effectively other subtypes of BoNT/A. This is because of the lack of a comprehensive toxin resource available for all subtypes of BoNT/A. Here, for the first time, the generation of recombinant H_C domains of BoNT/A1-A8 was described. These proteins were then used to characterize a panel of VHHs targeting BoNT/A1 with unknown binding sites in order to identify novel VHHs with broad reactivity against all subtypes of BoNT/A.

Methods

Materials

Clostridium botulinum genomic DNA carrying the *bont/A1* gene was extracted from an enrichment of a sludge sample from Hanoi, Vietnam.

Oligonucleotides were synthesized by Macrogen (Korea). Expression vectors used in this study comprised pET-45b and pET-22b (Novagen, cat. number 71327-3 and 69744-3, respectively). Hosts used for recombinant protein production were *E. coli* BL21(DE3) and *E. coli* Rosetta™ 2(DE3) (Novagen, cat. number 69450 and 71397, respectively).

All other reagents were from Thermo Scientific™, New England Biolabs, Merck, Qiagen, Tetracore, Immunology Consultants Laboratory, Vazyme, and Himedia unless otherwise stated.

Construction of expression vectors for BoNT/A1-A8 production

The *bont/H_CA1* fragment (residues 871-1296 of BoNT/A1) was amplified from extracted *C. botulinum* DNA and cloned into pET45b vector with an N-terminal His-tag by NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, cat. number E2621L).[13] Expression vectors for H_C domains of BoNT/A2-A8 (Table 1) were derived from pET45b-H_CA1 by site-directed mutagenesis and/or in-house gene synthesis.[14–20] All vectors were sent for sequencing to verify the accuracy of the constructs. Sequencing results can be found in the Sequence Read Archive under accession number PRJNA1206782.

Construction of expression vectors for VHH production

Genes encoding VHHs (A1, A3, A16, A17, A18, and ciA-C2) from previously reported studies[10,21] were codon-optimized for expression in *E. coli* and synthesized by Genscript. These genes were inserted into the pET22b expression vector which was modified to carry a FLAG tag (DYKDDDDK) at the C-terminal end for detection.[22] All vectors were

Table 1. Accession numbers of BoNT/A subtypes used in this study.

BoNT/A subtypes	Accession numbers
A2	WP_061323842.1
A3	WP_012301031.1
A4	WP_012720356.1
A5	WP_078992015.1
A6	ACW83608
A7	AFV13854
A8	AJA05787

sent for sequencing to verify the accuracy of the constructs. Sequencing results can be found in the Sequence Read Archive under accession number PRJNA1206786.

Expression and purification of recombinant proteins

H_C domains of BoNT/A1-A8 and VHHs were produced in *E. coli* Rosetta 2(DE3) and *E. coli* BL21(DE3), respectively. Bacteria carrying expression vectors were cultured at 37°C in LB medium (Himedia, cat. number 81254) supplemented with appropriate selecting antibiotics until $OD_{600} \sim 0.6 - 0.8$ and then induced with 0.5 mM IPTG (Thermo Scientific, cat. number R0392) at 20°C for 12 hours. The His tagged recombinant proteins were purified by affinity chromatography using Ni-NTA spin columns (Qiagen, cat. number 31014) under native conditions according to the manufacturer's instructions and then analyzed by SDS-PAGE.

ELISA assays

The reactivity of VHHs and commercial Rabbit Anti-Botulinum Toxin A and B IgG (Tetracore, cat. number TC-7007-001) against purified recombinant H_C domains of BoNT/A1-A8 were tested by ELISA. High Bind Stripwell™ Microplates (Corning, cat. number 07-200-24) were coated with 2 µg/mL recombinant antigens in carbonate buffer (Thermo Scientific, cat. number CB01100) at 4°C overnight. Plates were then blocked by 1% bovine serum albumin (Sigma-Aldrich, cat. number A2058) for 2 h at 37°C. VHHs in serial dilutions and commercial polyclonal antibody were added to the wells and incubated for 2 h at 37°C. After washing, anti-DYKDDDDK (Flag) Antibody Rabbit - HRP Conjugated (for VHHs) and HRP Conjugated Goat anti-Rabbit IgG h+l Antibody (for polyclonal antibody) (Immunology Consultants Laboratory, cat. number RFLG-45P-Z and GGHL-15P, respectively) were added and incubated for 1 h at 37 °C. Plates were then washed six times with PBST (Sigma-Aldrich, cat. number P3563) and reactions were developed with TMB substrate (Abcam, cat. number AB171523) and read at 450 nm. EC_{50} values were calculated via non-linear regression analysis using GraphPad Prism.

Results and discussion

Due to the extremely high toxicity of BoNTs, the development of antitoxins against them is of major interest for therapeutic applications. The target for antitoxin can be each of the three structural domains of BoNTs: (i) H_C responsible for receptor binding; (ii) H_N for toxin translocation; and (iii) LC for cleavage of SNARE proteins. Among these domains, the H_C fragment is the target of choice for the generation of antidote to BoNT intoxication,[23] as well as for the development of vaccines[24] and for intracellular delivery of cargo molecules specifically to neurons.[25] In the present study, we focused on the production of the H_C fragments of all subtypes of BoNT/A because of its extremely high toxicity, long persistence, and high sequence divergence among subtypes. However, only *bont/A1* gene was available in our group. Consequently, we opted for an approach that involved both site-directed mutagenesis from H_CA1 construct and in-house gene synthesis to generate expression vectors for H_C domains of BoNT/A2-A8.[14–20] These recombinant proteins were expressed in the *E. coli* Rosetta 2(DE3) and purified by nickel affinity chromatography. SDS-PAGE analysis showed that only one band was observed for all purified samples at the expected molecular mass of 50 kDa (Figure 1), suggesting that H_C fragments of BoNT/A1-A8 were successfully expressed and prepared. The purified proteins were then evaluated by ELISA for antigenicity using the Rabbit Anti-Botulinum Toxin A and B IgG. All samples were recognized by the commercial polyclonal antibody,[26] which indicated that the receptor binding domains of all BoNT/A subtypes were correctly expressed in *E. coli* Rosetta 2(DE3).

To our knowledge, only three VHHs neutralizing BoNT/A by binding to the H_C domain have been described in the literature,[10–12] of which ciA-C2 have been extensively characterized for the inhibition mechanism on the BoNT/A1.[11] In a separate report, 18 VHHs have been identified for their specific recognition of BoNT/A1[21] but their binding sites

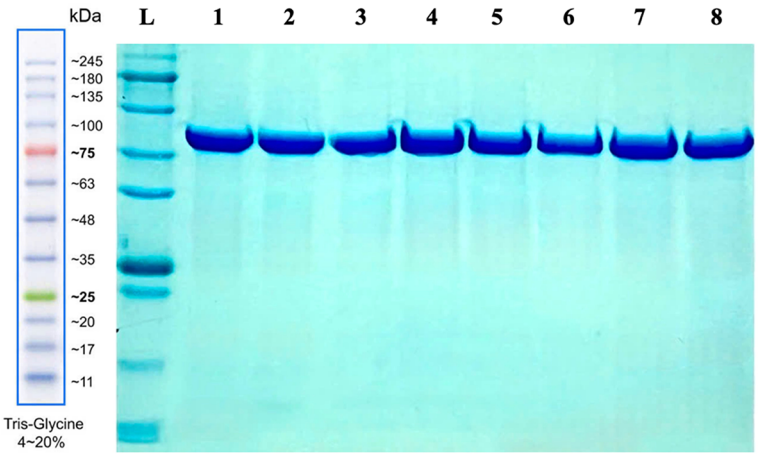

Figure 1. SDS-PAGE analysis of purified recombinant BoNT/A H_C domains.

were unknown. We therefore performed a systematic screening of a panel of five most promising VHHs from this report and ciA-C2, on the H_C fragments of BoNT/A1-A8 by ELISA. All the VHHs were successfully expressed and purified.[22] Screening results clearly indicated that apart from ciA-C2, two nanobodies (VHH-A1 and VHH-A3) could recognize the H_C domains of all BoNT/A subtypes with VHH-A3 exhibiting significantly higher affinity than VHH-A1.[27] Consequently, only ciA-C2 and VHH-A3 were characterized in subsequent experiments. According to the EC_{50} values calculated using ELISA (Table 2),[28] both ciA-C2 and VHH-A3 exhibited the highest affinity for the H_CA1 (EC_{50} = 11.0 and 24.0 nM respectively). This is not surprising, because these two VHHs were generated based on the selection with BoNT/A1.[11,21] VHH-A3 displayed an intermediate affinity for H_CA4 (EC_{50} = 46.0 nM) and comparably low affinity for the remaining subtypes. In comparison to ciA-C2, VHH-A3 displayed similar EC_{50} values for H_C domains of BoNT/A2, A3, A5, A6, A7, and A8. Concerning ciA-C2, the binding mechanism of this VHH to H_CA1 involves a cation-π interaction and multiple hydrogen bonds between CDR1 and residues K289, N318 and D419 of H_CA1. In addition, CDR2, CDR3, FR2, FR3, and FR4 of ciA-C2 also participate in the binding to H_CA1 through hydrogen bonds with residues T193, H194, Y242, T276, E423 and a hydrophobic interaction with P425 of the domain.[11] Consistent with these structural observations, the affinity of ciA-C2 was least affected for H_CA4 (EC_{50} = 26.2 nM) with only a T193 to P193 replacement,[16] whereas it was most affected for H_CA2, H_CA3 and H_CA8 ($EC_{50} \geq 80.4$ nM) containing three major substitutions T193P, H194R, and P425S.[14,15,20] These data underline the importance of the structural studies of VHHs in order to generate antitoxins with a broad protection to BoNTs. Furthermore, considering the sequence divergence among H_C domains of BoNTs[29] and most studies so far use BoNT/A1 as the selection agent to generate VHHs, it would be of interest to include a divergent H_C domain, for instance, H_CA2, H_CA3 or H_CA8, during the selection steps in order to obtain VHHs having high affinity against these domains. Similarly, these recombinant fragments could be combined with H_CA1 for the development of vaccines or polyclonal antitoxins with broad potency compared to conventional approach using only one BoNT/A subtype for immunization.

Table 2. EC_{50} values of ciA-C2 and VHH-A3 against H_C domains of BoNT/A1-A8.

BoNT/A subtypes	ciA-C2	VHH-A3
A1	11.0	24.0
A2	80.4	93.5
A3	83.8	88.9
A4	26.2	46.0
A5	51.4	67.6
A6	77.5	97.1
A7	62.3	73.3
A8	109.3	98.2

In summary, this study provided recombinant H_C domains of all BoNT/A subtypes, which could be used for the development of antitoxins and vaccines against BoNTs. This study also identified two new nanobodies, VHH-A1 and VHH-A3, capable of binding to all BoNT/A H_C domains. However, one question remains unsolved in this study, whether the VHH-A1, VHH-A3 and ciA-C2 would bind to a distinct, non-overlapping epitope. Further research is on-going to resolve this question and to improve neutralizing activity of ciA-C2 through the generation of heterodimers.

Ethics and consent

Ethical approval and consent were not required.

Data availability

Underlying data

Figshare: Raw data of ELISA results for reactivity of H_CA1-H_CA8 to VHHs and Rabbit Anti-Botulinum Toxin A and B IgG; for determination of EC_{50} values of ciA-C2 and VHH-A3 against HCA1-HCA8. Doi: https://doi.org/10.6084/m9.figshare.28171994.[30]

This project contains the following underlying data:

- ELISA raw data.xlsx

Dataset from NCBI Sequence Read Archive: Sequencing results of H_C-BoNT/A subtypes.

Accession number PRJNA1206782;

https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1206782.

Dataset from NCBI Sequence Read Archive: Sequencing results of VHHs against H_C-BoNT/A subtypes. Accession number PRJNA1206786;

https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1206786.

Extended data

Figshare: Supplement I for "Recombinant expression of receptor binding domains of all eight subtypes of botulinum neurotoxin type A for generation of antitoxins with broad reactivity".

Figshare: Construction of pET45b-HCA1 plasmid. Doi: https://doi.org/10.6084/m9.figshare.28159514.v1.[13]

This project contains the following extended data:

- Supplement I.pdf

Figshare: Construction of pET45b-HCA2 plasmid. https://doi.org/10.6084/m9.figshare.28159544.v1.[14]

This project contains the following extended data:

- Supplement II.pdf

Figshare: Construction of pET45b-HCA3 plasmid. https://doi.org/10.6084/m9.figshare.28159553.v1.[15]

This project contains the following extended data:

- Supplement III.pdf

Figshare: Construction of pET45b-HCA4 plasmid. https://doi.org/10.6084/m9.figshare.28159559.v1.[16]

This project contains the following extended data:

- Supplement IV.pdf

Figshare: Construction of pET45b-HCA5 plasmid. https://doi.org/10.6084/m9.figshare.28159574.v1.[17]

This project contains the following extended data:

- Supplement V.pdf

Figshare: Construction of pET45b-HCA6 plasmid. https://doi.org/10.6084/m9.figshare.28159586.v1.[18]

This project contains the following extended data:

- Supplement VI.pdf

Figshare: Construction of pET45b-HCA7 plasmid. https://doi.org/10.6084/m9.figshare.28159592.v1.[19]

This project contains the following extended data:

- Supplement VII.pdf

Figshare: Construction of pET45b-HCA8 plasmid. https://doi.org/10.6084/m9.figshare.28159598.v1.[20]

This project contains the following extended data:

- Supplement VIII.pdf

Figshare: Construction of pET22b-VHH plasmids. https://doi.org/10.6084/m9.figshare.28159622.v1.[22]

This project contains the following extended data:

- Supplement IX.pdf

Figshare: Reactivity of HCA1-HCA8 to the Rabbit Anti-Botulinum Toxin A and B IgG. https://doi.org/10.6084/m9.figshare.28159754.v1.[26]

This project contains the following extended data:

- Supplement X.pdf

Figshare: Reactivity of HCA1-HCA8 to VHHs. https://doi.org/10.6084/m9.figshare.28159982.v1.[27]

This project contains the following extended data:

- Supplement XI.pdf

Figshare: Determination of EC_{50} values of ciA-C2 and VHH-A3 against HCA1-HCA8. https://doi.org/10.6084/m9.figshare.28160042.v1.[28]

This project contains the following extended data:

- Supplement XII.pdf

Figshare: The overall amino acid sequence identity among BoNT/A subtypes using, A1 as the benchmark. https://doi.org/10.6084/m9.figshare.28160051.v1[29]

This project contains the following extended data:

- Supplement XIII.pdf

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Peer Review Status: ? ✓ ?

Version 1

Reviewer Report 04 March 2025

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Charles B Shoemaker
Tufts University, Medford, Massachusetts, USA

In this manuscript, the author describe the creation of a set of Botulinum neurotoxin serotype type A (BoNT/A) heavy chain domain (AHc) representing each of the eight reported BoNT/A subtype natural variants. They use this resource to screen the AHc subtype specificity of several single-domain antibodies (sdAbs) that are reported to bind to AHc. Comprehensive methods and construction details are provided and the work appears well performed.

General comments:

- The authors suggest the primary accomplishment is their creation of "a resource to comprehensively identify antitoxins conferring broad protection against BoNT/A" which seems a substantial overstatement as the resource is simply a panel of expressed receptor-binding domain subunits of known amino acid sequence. Most capable labs could create a similar set by ordering and cloning a panel of synthetic coding DNA into an expression vector. Furthermore, identifying broad subtype antibody binding to AHc does not demonstrate their antitoxin potential as the VHH binding site must also be at a site that interferes with toxin binding which requires neutralization studies.
- Authors claim that the ciA-C2 specificity for AHc is unknown though information on subtype specificity was provided in the manuscript cited as the source of this information.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biotechnology.mmunotherapeutic discovery, engineering and development.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 03 March 2025

https://doi.org/10.5256/f1000research.176528.r366733

Jianlong Lou
University of California San Francisco, San Francisco, California, USA

"Recombinant expression of receptor binding domains of all eight subtypes of botulinum neurotoxin type A for generation of antitoxins with broad reactivity" is a well-written original research report with clear citation of some current literature in the BoNT countermeasure development field.

The work has some academic merit and potential field application, it is accurately presented with sufficient details, so other researchers should be able to replicate the experiments if desired. The relative simple software generated statistical analysis and its interpretation seems to be appropriate. And the conclusions drawn for in vitro activity were adequately supported by the results, but the author should not expand or imply the potential application for in vivo anti-toxin countermeasure efficiency without solid animal test data.

In vitro binding data and in vivo biological function data are somewhat related but of different dimensions. The authors also need to explain the size of HC showing in Figure 1. Why does it look more like 100KD instead of 50KD if the molecular marker on the left side is correct? Although the designed fragment with 426 AA should have the molecular size close to 50KD? Furthermore, Some publications such as the ones added below that discusses VHH specific for BoNT/A-LC or clinical trial using antibody based counter measure for BoNT/A will help reader to grasp the status and current situation of antitoxin development better.

References
1. Dong J, Thompson AA, Fan Y, Lou J, et al.: A single-domain llama antibody potently inhibits the enzymatic activity of botulinum neurotoxin by binding to the non-catalytic alpha-exosite binding region.*J Mol Biol*. 2010; **397** (4): 1106-18 PubMed Abstract | Publisher Full Text
2. Nayak SU, Griffiss JM, McKenzie R, Fuchs EJ, et al.: Safety and pharmacokinetics of XOMA 3AB, a novel mixture of three monoclonal antibodies against botulinum toxin A.*Antimicrob Agents Chemother*. 2014; **58** (9): 5047-53 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Human antibody and animal based antibody engineering for BoNT and other antigens.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 28 February 2025

https://doi.org/10.5256/f1000research.176528.r366732

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In general, the article is interesting for the target audience. However, the article contains very little

data. In my opinion, it is not entirely correct to draw conclusions based only on ELISA data. Characterization of the interaction between the VHH and HC domain of BoNT/A using SPR or BLI methods would be very useful. I would recommend that the authors add at least one alternative method for characterizing the interaction of VHH with HC domain of BoNT/A.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: mAbs, VHH, recombinant proteins, Adenoviral vectors, rAAV, gene therapy.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.