

In Vivo Neutralization of Botulinum Neurotoxins Serotype E with Heavy-chain Camelid Antibodies (VHH)

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Abstract Ingestion of botulinum neurotoxin (BoNT) results in botulism, a severe and frequent fatal disease known in the world. Current treatments rely on antitoxins, such as equine antitoxin and human botulism immunoglobulin. In some cases, side effects have been reported, including early anaphylactic shock and late serum sickness. Thus, diagnosis and treatment measure of BoNT are necessary and crucial. In the present study, a single-domain variable heavy-chain (VHH) antibody fragment was obtained from an immune dromedary phage display library against the putative binding domain of botulinum neurotoxin E (BoNT/E), a non-toxic 50-kDa fragment. The characteristics of nanobody VHH include excellent production, superior heat stability and specific binding capacity to soluble antigen without cross-reaction to other relevant or irrelevant antigens. A total of 150 ng/Kg of nanobody entirely neutralized 3LD₅₀ of the BoNT/E in an in vivo challenge of the mice. This phenomenon indicates BoNT/E toxin neutralizing capacity of the produced

nanobody. These results also suggest possession of unique properties by the nanobody applicable in diagnostics or therapeutic purposes.

Keywords BoNT/E · Binding domain · Phage display · Nanobody

Introduction

Botulinum neurotoxin (BoNT) generated by an anaerobic, spore-forming *Clostridium botulinum* is an etiologic factor responsible for botulism. Botulinum neurotoxin (BoNT) serotypes A, B and E are often associated with human botulism [2, 11]. These toxins may cause life-threatening systemic toxicity by natural factor such as food poisoning, infant botulism and wound [24]. Botulinum neurotoxin is the most toxic substance known to exist [17] and considered among the most severe (CDC Category A) of the existing bioterrorism threats.

The holotoxin is about 150 kDa consisting of three functional domains composed of a light-chain segment and two heavy-chain segments [26, 40] that are individually responsible for neuron receptor binding, translocation and catalysis. The C-terminal region of the heavy chain (Hc) is the cell-binding domain, which binds the toxin to the ganglioside receptors on pre-synaptic neurons, leading to endocytosis of the toxin into the cell [8, 30]. Botulinum neurotoxins (BoNT) operate on the peripheral nervous system to suppress the release of acetylcholine from pre-synaptic nerve ends at the neuromuscular connection, causing acute flaccid paralysis [44]. Thus, diagnosis and treatment measure of botulinum neurotoxins (BoNT) are necessary and crucial. Antibody products, such as equine antitoxin and human immunoglobulin, are used for treatment of adults. These treatment

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measures do have side effects such as early anaphylactic shock and late serum sickness [5, 22]. Several methods such as ELISA, microsphere arrays, evanescent wave fiber optics and electrochemiluminescence have been used to improve assays to detect botulinum toxins [16, 29, 36, 39]. However, measurements using conventional antibodies often lack stability to chemical or heat exposure [45]. The application of various IgG derivatives (e.g., Fab, Fab'2 and scFv) has also been proven to be problematic according to reduced stability compared to the conventional antibodies and restricted solubility [21, 43]. In addition, in vivo application of mouse-derived scFv constructs may induce an immune response in human. These limitations may cause serious obstacle to their further development as therapeutic agents [23]. Therefore, the current limitations with anti-BontE in diagnosis and therapeutic application have forced us to think for a new antibody format with better stability, faster tissue distribution, higher binding affinity and neutralizing capacities of BontE toxin. VHHs are a new class of single-domain antigen-binding fragments derived from heavy-chain antibodies found in sera of the camelidae (i.e., camels, dromedaries and llamas) [18]. Unlike a conventional antibody, the antigen-binding domain (VHH) of these antibodies consists of a single domain, and each heavy chain contains four framework regions (FRs) and three CDRs with high antigen-binding specificity and affinity [33, 34]. VHHs are the smallest known natural single-antigen-binding fragments (~15 kDa) [1]. The VHH domain of heavy-chain antibodies is characterized by several unique features such as ability to identify and bind a wide variety of epitopes [6, 13, 35]; resistance to acid and alkaline pH, temperature and to proteolytic effects [12, 20]; and ease of cloning and expression in *Escherichia coli* and *Saccharomyces cerevisiae* [1, 14]. Replacement of hydrophobic amino acid with hydrophilic ones in VHHs results in more solubility and resistance to denaturation [18, 19, 33]. Camelid-derived VHHs share a high sequence identity with human VH of family III, the most widespread VH family [34, 46], and are also used as immunotherapeutic agent [4, 20]. Vaccination with the non-toxic-binding domain (Hc) of BoNT/E has already proved to protect mice against toxin [15]; it is therefore anticipated that neutralizing antibodies could be produced using this domain as an antigen. In order to achieve this, we made an attempt to produce and identify recombinant VHHs derived from the camel immunized with Hc of BoNT/E.

Materials and Methods

Antigen Purification and Identification

Synthetic gene coding for Hc was cloned to pET-28a vector (Novagen, Madison, WI, USA) as described

previously [15]. The recombinant gene was then expressed in *E. coli* BL21 (DE3) at 28 °C overnight. Hexahistidine-tagged BoNT/E Hc was purified by IMAC using Ni-NTA agarose (Qiagen) under native conditions followed by cation-exchange chromatography. The purification of recombinant BoNT/E Hc protein was analyzed by SDS-PAGE. The binding activity of the purified Hc to BoNT/E was estimated by ELISA with horse anti-BoNT/E antiserum as described previously [15].

Dromedary Immunization

The purified recombinant BoNT/E Hc protein was used to immunize a 1-year-old male dromedary (*Camelus dromedarius*). A dromedary was injected intramuscularly with ascending amounts of recombinant BoNT/E Hc. A total of 1 mg/ml of antigen was mixed with 1 ml of Freund's Complete Adjuvant (Sigma, St. Louis, MO) at the first immunization. Four booster doses of 1.5, 2, 2.5 and 3 mg/ml of antigen with an equal volume of Freund's Incomplete Adjuvant (Sigma) were given on days 15, 30, 45 and 60, respectively. Pre-immune serum collected before the first injection served as the negative control. A total of 15 ml of blood was collected 10 days after each immunization, and the sera were stored in aliquots at -20 °C. Antibody titer was determined by ELISA. 96-well plates were coated with 2 µg of recombinant BoNT/E Hc proteins in 100 µl PBS per well and incubated overnight at 4 °C. Wells were washed with PBS + 0.05 % Tween 20 (PBST) and blocked for 2 h at room temperature with 5 % skimmed milk in PBS. After washing with PBST, immune (from boosters 3 and 4) and non-immune sera were serially diluted from 1:500 to 1:3,000 with PBS, added to the wells in duplicate and incubated at room temperature for an hour. Wells were washed, and rabbit anti-camel IgG (1:16,000 in PBS) was added and incubated for 90 min at room temperature. After washing with PBST, mouse anti-rabbits IgG-HRP conjugate (1:5,000 in PBS) was added for 1 h at room temperature. After a final wash, the wells added with TMB as a substrate and incubated at room temperature for 15 min. The reaction was stopped with 3 M H₂SO₄, and absorbance was read at 450 nm.

Library Construction

Lymphocytes from peripheral blood samples collected 10 days after the fifth immunization were used for library construction. White blood cells were isolated by centrifugation on a Ficoll discontinuous gradient and stored at -80 °C until further use. Total RNA was isolated from 10⁸ leukocytes by Total RNA Purification Kit (Roche). Using oligo dT as a primer, the total RNA was converted to cDNA. The VH and VHH genes were amplified with the

gene-specific primers using cDNA as a template. The forward and reverse pair of primers used in this experiments were 5'-GTCCTGGCTGCTCTTCTACAAGG-3', 5'-GATGTGCAGCTGCAGGCGTCTGGRGGAGG-3' and 5'-GGTACGTGCTGTTGAACTGTTCC-3', 5'-CGCCATCAAGGTACCAGTTGA-3', respectively. The doublet PCR bands between 600 and 700 bp (VHH-CH2 without CH1 exon and corresponding to heavy-chain antibodies) were separated from the 900-bp fragment (VH-CH1-CH2 exons, derived from heavy chain of dromedary classical antibodies) [34, 46] by agarose gel and purified using the Gel Purification Kit (Bioneer). DNA fragments encoding nanobodies were amplified by nested PCR with nested primers annealing at the extremity of the framework-1 (5'-ACTGGCCCAGGCGGCCGAGGTGCAGCTGSWGS AKTCKG-3') and framework-4 (5'-ACTGGCCGGCCTGGCCTGAGGAGACG GTGACCWGGGTC-3') regions with incorporated SfiI restriction sites at the ends of the VHH genes.

The amplified VHH fragments along with pComb3x phagemid DNA were digested with SfiI restriction enzyme (New England Biolabs) overnight at 50 °C. VHH DNA was then ligated into the pComb3x vector at the SfiI restriction site. Ligation product was transformed into electro-competent *E. coli* TG1 cells. Several electroporations were performed, and the transformants were plated on LB-Agar ampicillin plates [37]. The transformed bacteria were scraped from the plates and stored at -80 °C in LB medium supplemented with glycerol until further use. Library size was calculated by plating serial dilution aliquots on LB-Agar ampicillin plates and incubated overnight at 37 °C. At least 30 random clones were picked and characterized by restriction digestion of the vectors.

Panning and Selection of a BoNT/E-Specific Nanobody

The library was expressed on phage by infection with M13K07 helper phage (Amersham Pharmacia Biotech). Phages were prepared as described by [1]. In order to enrich the Nanobodies, the phage library was panned five consecutive rounds of in vitro selection on microtiter plates coated with 10 µg/well of purified recombinant BoNT/E Hc. Descending incubation time and extensive washing with PBS/0.1 % Tween 20, plates were used in subsequent panning cycles for selection of higher affinity phage to the target protein. Bound phage particles were recovered with 200 µL of 200 mM glycine buffer (pH 2.2). The elution solution was immediately neutralized with 1 M Tris-HCl (pH 7.4) and used to infect exponentially growing *E. coli* TG1 cells. Phages were purified according to the method [37]. The purified phage was used as an input for the next round of panning. Phage titers of the input and output in all steps of panning were estimated by infection of TG1 cells

and plating on LB ampicillin plates. After the fifth round of panning, polyclonal phage ELISA was used to determine the enrichment of antigen.

96-well plate (Wallach, Gaithersburg, MD) was coated with 1 µg/well of the purified recombinant BoNT/E Hc in 5 µg/ml in 0.15 M carbonatebicarbonate buffer pH 9.6 and incubated overnight at 4 °C. The same concentration of bovine serum albumin (BSA) was used as a non-specific-binding (NSB) control. The wells were washed 3x with PBST (PBS/0.05 % Tween 20), blocked with 150 µL of 3 % solution of BSA in PBS for 2 h at 37 °C and rinsed 3x with PBST. 10¹² transducing units of phage purified from each round of panning were added to the well and incubated while shaking for 1.5 h at 37 °C. The wells were washed five times with PBST. A total of 100 µl of 1:8,000 mouse anti-M13/HRP monoclonal antibody (Roche) was added to each well and incubated on a shaker for 1 h at 37 °C. Finally, the wells were washed five times with PBST, added with 100 µL of substrate TMB and incubated for 10–15 min at room temperature. The reaction was stopped by the addition of 3 M H₂SO₄, and the absorbance was read at 450 nm. After five rounds of panning, individual phage colonies were randomly picked and antigen-binding specificity was determined by monoclonal phage enzyme-linked immunosorbent assay (ELISA) according to the standard procedures [1]. Unique clones yielding high phage ELISA signals were selected and sent for DNA sequencing.

Expression and Purification of Nanobodies

Following the selection of high-affinity clones against recombinant BoNT/E Hc, plasmids were purified using Plasmid Mini Extraction Kit (Bioneer) and transformed into *E. coli* non-suppressor strain Top10 (Invitrogen) in order to express nanobodies as soluble periplasmic proteins. The clones bound firmly to antigen were screened with ELISA according to procedures described by [25]. Two positive clones (BMR2 and BMR14) with unique sequences were produced in larger scale. Production in shaker flasks was performed by growing the bacteria in SB [37] supplemented with ampicillin until the optical density reached 0.6–0.9. Cultures were induced with 1 mM IPTG for 6 h on a rotary shaker at 28 °C. After pelleting the cells, the periplasmic proteins were extracted by sonication. Hexahistidine-tagged VHH protein was loaded on a Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen). The bound proteins were eluted with 200 mM imidazole. Soluble nanobody was dialyzed against phosphate buffer (10 mM, pH 7.2) after confirmation of expression by SDS-PAGE and Western blotting. The nanobody genes (BMR2 and BMR14) scored positive in ELISA was subcloned into the pET28a expression vector using the restriction enzymes

EcoRI and *BamHI*. Transformed *E. coli* BL21 (DE3) cells expressed VHHs in the periplasm after induction by IPTG 1 mM for 6 h at 25 °C. Purification of nanobody was performed as described above.

Affinity Determination

The affinity of the nanobody selected against recombinant BoNT/E Hc was determined by ELISA. Microtiter plates were coated with 20, 10, 5, 2.5 and 0 µg of recombinant BoNT/E Hc and incubated at 4 °C overnight. The same concentrations of BSA were used as non-specific binding control (NSB). The wells were then washed with PBS + 0.05 % Tween 20 (PBST) and blocked for 2 h at 37 °C with 5 % non-fat powdered milk in PBS followed by a wash with PBST. Each recombinant BoNT/E Hc-coated well at the above concentrations was added with 100 µl of nanobody from each of 20, 10, 5, 2.5 and 1.25 µg/ml concentrations. The plate was incubated for 2 h at 37 °C followed by five washes with PBST. Anti-hemagglutinin tag (HA tag) conjugated to HRP (Roche) was added to each well and incubated for 1 h at 37 °C. Finally, wells were washed three times with PBST and 100 µL of substrate, TMB, was added to the wells and incubated for 10–15 min at 37 °C. The enzyme reaction was stopped using 100 µL of 3 N H₂SO₄, and absorbance was read at 450 nm. Affinity of nanobody was determined by measuring the amount of bound enzyme activity. The association constant (K_a) of nanobody to antigen was calculated using the following mathematical expressions according to procedure of Beatty et al. [3].

$$[Ag]/[Ag'] = n$$

$$K_a = n - 1/2(n[Ag'] - [Ag])$$

Determination of Binding Specificity

Percent cross-reaction of some related proteins, such as BoNT/A and BoNT/B, and non-related proteins, such as BSA, UreC from *Helicobacter pylori*, PSMA, Bap from *Acinetobacter baumannii*, LPS from *Salmonella typhi* and *Vibrio cholera*, and CS3 from *Enterotoxigenic E. coli*, was tested using coated microtiter plates with 10 µg/ml concentrations of each cross-reacting molecule. All assays were carried out in duplicate. A total of 20 µg/ml of purified recombinant nanobody (BMR2) was added to each well. The assay was performed as explained in the previous section.

In Vivo Challenge of The Mice

To evaluate the neutralization capability of the BMR2 nanobody, mice were intraperitoneally injected (i.p.) with

BoNT/E toxin and different concentrations of the nanobody. The LD₅₀ of the BoNT/E toxin was determined as 50 ng/Kg in BALB/c mice. Mice were divided into 10 groups, each consist of 6 mice. Mice were initially injected with BoNT/E toxin in one flank and 1 h later, with BMR2 nanobody. Mice in groups two and three received 100 ng/Kg of BoNT/E (2 LD₅₀) toxin and 200 and 100 ng/Kg of BMR2, respectively. Each mouse in groups four to eight was injected with 150 ng/Kg (3LD₅₀) of BoNT/E and 200, 150, 100, 70 and 50 ng/Kg of BMR2 nanobody, respectively. Groups nine and ten received 200 ng/Kg (4 LD₅₀) of BoNT/E and 400 and 200 ng/Kg of BMR2 nanobody, respectively. The survival of the mice was monitored after 72 h.

Heat Stability

The purified nanobody prepared in PBS (pH 7.0) at 1 mg/ml concentration was exposed for 10 min to a temperature range of 60–90 °C at 10 °C increments. The binding activities were compared with unheated sample (control). Microtiter plates coated with each of 20, 10, 5, 2.5 and 0 µg/ml concentrations of the recombinant BoNT/E Hc were incubated at 4 °C overnight. The wells were washed with PBST and blocked for 2 h at 37 °C with 5 % non-fat powdered milk in PBS. A total of 20 µg/ml of the heated and control nanobody was added to each antigen concentration in duplicate and incubated for 2 h at 37 °C. The assay was performed as explained in the “Affinity Determination” section.

Statistical Analysis

Statistical analysis was carried out by Student's *t* test using GraphPad Prism(Graphpad, San Diego, California) software. Experiments were performed in duplicate or triplicate, and results were statistically significant for *p* ≤ 0.05.

Results

Production of Recombinant BoNT/E Hc

The soluble fraction of recombinant BoNT/E Hc was purified by immobilized affinity chromatography (Ni-NTA resin). Average yield of purified Hc was 2.3 mg per liter of bacterial culture. A band corresponding to 54 kDa was detected in SDS-PAGE (Fig. 1). The antigen was also identified by reaction with the anti-BoNT/E antibody. The recombinant BoNT/E Hc was strongly and specifically recognized by anti- BoNT/E antibody (Fig. 2).

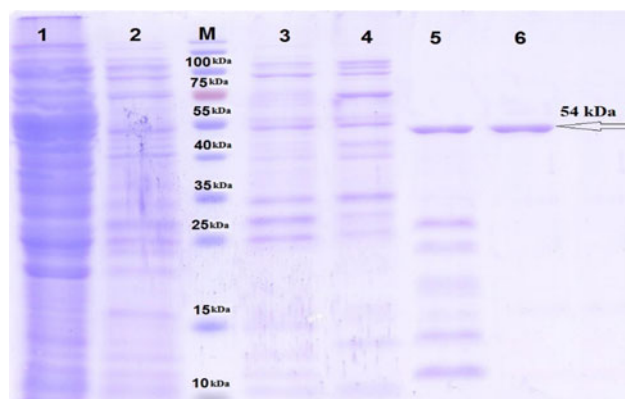


Fig. 1 SDS–PAGE analysis of purified Hc. Lane M is molecular weight marker. Clear extract of induced cells was subjected to Ni–NTA column, followed by washing and several elutions. Proteins were visualized by Coomassie brilliant blue staining. Lane 1 is the follow-through; lane 2 is washing output; lanes 3, 4, 5 and 6 indicate consecutive elution steps with increasing concentration of imidazole. Band pertaining to Hc is pointed by the arrows on the right of panel, respectively. Purified Hc was observed at fractions 6 (Color figure online)

Selection of BoNT/E-specific Nanobody

Polyclonal Response

In order to prove the immunization efficacy, blood sera from dromedary were collected and analyzed by ELISA prior to and after immunization. The results confirmed significant increase in the antibody titer after booster immunization and showed strong antigen-specific response in recognizing BoNT/E Hc (data not shown).

Libraries Construction

VHH phage display library was produced from B cells of an immunized dromedary. A library of 3.2×10^7 independent clones was obtained. More than 98 % of clones contained an insert of the expected 400-bp size for VHH gene. The VHH repertoires of the library were successfully expressed on phages infected with M13K07 helper phage.

Panning

The recombinant phage-displayed VHH library was used through consecutive rounds of panning against BoNT/E Hc and BSA. The strongest enrichment occurred at the fifth round of panning and decreased upon subsequent rounds. Remarkable enrichment during the consecutive round of panning was observed as a result of the presence of high-affinity antibody fragments against antigen (Fig. 3). A total of 20 individual colonies randomly picked from plates after the fifth round of panning were tested by phage ELISA for their binding capacity to BoNT/E Hc. Two colonies

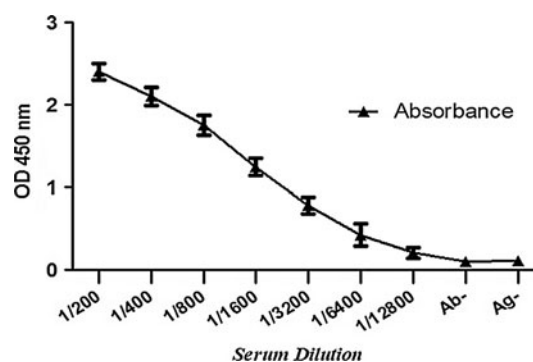


Fig. 2 ELISA analysis of purified Hc with anti-BoNT/E. Wells were coated with Hc; negative controls are designated as Ab⁻ (no antibody) and Ag⁻ (no antigen). The results are expressed as absorbance at 450 nm $\pm$ SD

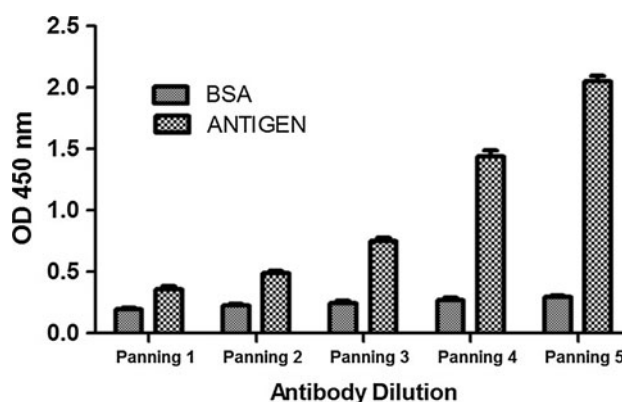


Fig. 3 Polyclonal phage ELISA on antigen using enriched phage after each round of panning. Wells were coated with Hc and BSA (as the negative control). Anti-M13/HRP monoclonal antibody at 1:8,000 was used. The results are expressed as absorbance at 450 nm $\pm$ SD

(BMR2 and BMR14) revealed high specificity and strong binding capacity to antigen. Since the specificity and binding capacity of the BMR2 was higher compared to those of the BMR14, the latter was not studied further.

Expression of Nanobody in a Soluble Form

Prior to expression of soluble nanobody, a single dominant phagemid (BMR2) encoding a VHH was sequenced. The data obtained from sequencing (Fig. 4) were aligned by NCBI BLAST tools for homologous genomic sequences. The (BMR2) VHH sequences were highly homologous with at least ≥ 90 % identity with the Camelus dromedaries anti-VSG (EM_OM:AY263489) sequence and *C. dromedarius* IgVH (EM_OM:AB091890). BMR2 VHH clone was expressed in a non-suppressor *E. coli* Top10 strain. Following cell disruption and precipitation of the cells debris, the periplasmic nanobody was purified using nickel affinity chromatography column. A single band of approximately 17 kDa was detected on SDS polyacrylamide gel (Fig. 5).

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CCC GGG ACC CAT GGC GGG GCC CAC GCT GAT AGC GTG TGC GGT CAT GGA GGG TCT CTG
P G T H G G A H A D S V C G H G G S L
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
AGA CTC GCC TGT GCA GCC TCA GCC TCT GGA TAC ACC GAA AGT GTG AAG TGG ATG GGC
R L A C A A S A S G Y T E S V K W M G
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
TGG TTC CGC CAG GCT CCA GGG CAG GAG CGC GAG GGG GTC GCA GTT ATT TCT ATT CCT
W F R Q A P G Q E R E G V A V I S I P
39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57
GGT GGT AGT ACA TAC TAT GAC GAC GAC GTG AAG GGC CGA TTT ACC ATC TCC CAA GAC
G G S T Y Y D D D V K G R F T I S Q D
58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76
AAC GCC AAG AAC ACG GTG TAT CTG CAA ATG AAC AGT CTG AAA CCT GAG GAC ACT GCC
N A K N T V Y L Q M N S L K P E D T A
77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95
ATG TAC TAT TGC GCA GCG CGC AAT GCG GGG GGG CGT TTT CGG CCT TCG GCC GCT GGT
M Y Y C A A R N A G G R F R P S A A G
96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114
GGG TAT AAT TAT TGG GGC CAG GGG ACC CTG GTC ACT GTC TCC TCA GGC CAG GCC GGA
G Y N Y W G Q G T L V T V S S G Q A G
115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133

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Fig. 4 Nucleotide and deduced amino acid sequences of the BMR2

A yield of 5 to 7 mg of purified nanobody was calculated per liter of *E. coli* culture. In order to produce excess amount of selected nanobody, the 400-bp BMR2 encoding sequence was subcloned into pET28a expression vector. The expressed nanobody in *E. coli* BL21 (DE3) was purified by IMAC column. A single band of the purified protein is shown on SDS–PAGE (data not shown) with the yield ranging from 55 to 57 mg/L bacterial culture.

Specificity and Affinity Characterization

Specific binding and high immunoreactivity of VHH toward antigen with no cross-reaction to BSA was noted (Fig. 6a). The measured affinity constant indicated the K_a of around 5.7×10^{-7} for the selected specific nanobody according to the procedure of Beatty et al. [3]. The nanobody also showed specific binding to BoNTE and no significant cross-reactivity with structurally and functionally related (BoNT/A, BoNT/B) or unrelated immobilized antigens. Interestingly, the results demonstrated highly significant difference in immune reactivity of nanobody toward whole toxin compared to other family of botulinum neurotoxins (Fig. 6b).

In Vivo Challenge of the Mice

In vivo challenge with 150 ng/Kg of BMR2 nanobody per mouse completely neutralized 3LD₅₀ of BoNT/E toxin (150 ng/Kg) in BALB/c mice. The administration of 100 and 70 ng of BMR2 nanobody per Kg of mouse resulted in 50 % survival of the tested mice (Fig. 7).

Thermostability of the Nanobody Molecules

The nanobody endured a temperature of 90 °C while maintaining a higher percentage of its binding capacity with recovery of specific binding activity upon cooling. There was no significant decrease in binding activity upon exposure to 60 or 70 °C. A non-significant decreased

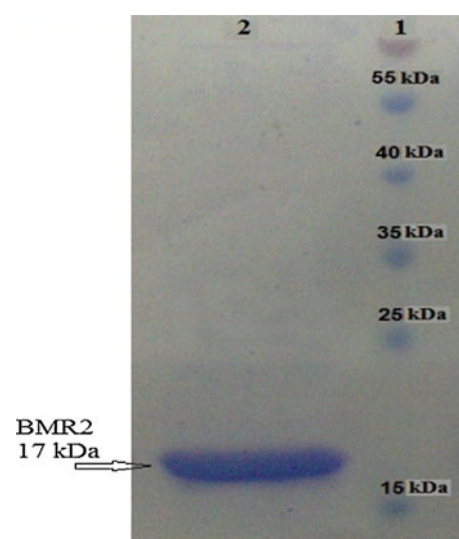


Fig. 5 SDS–PAGE analysis of purified BMR2. Lane 1 is molecular weight marker. Lane 2 is purified nanobody (BMR2). Arrow on the left indicates 17-kDa nanobody

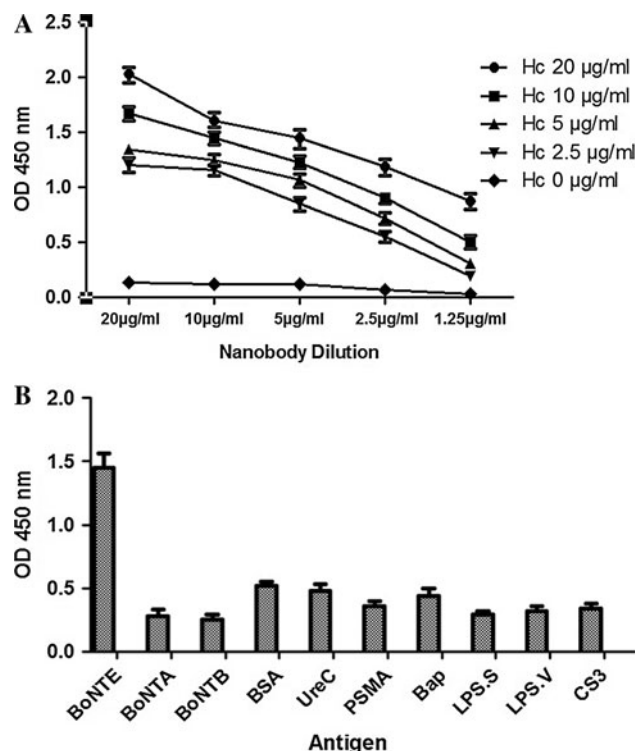


Fig. 6 **a** ELISA binding assay of nanobody to recombinant BoNT/E Hc proteins. Wells were coated by serial twofold dilutions of Hc. **b** ELISA determination of nanobody-binding specificity. Wells were coated with related and non-related proteins. After incubation with nanobody, binding capacity was detected by anti-hemagglutinin tag (HA tag) conjugated to HRP. The results are expressed as absorbance at 450 nm $\pm$ SD

binding activity was noted at 80 °C. Immunoreactivity of the nanobody was not significantly affected by incubation under different heated conditions (Fig. 8).

Fig. 7 In vivo neutralization of BoNT/E with BMR2. LD₅₀ of BoNT/E was determined as 50 ng/Kg in BALB/c mice. BoNT/E toxin was injected through i.p. route in one flank of mice and BMR2 nanobody in the other flank after 1 h. The amount of BMR2 nanobody needed for complete neutralization of the threefold toxin was 150 ng/Kg of each mouse

in vivo neutralization of BoNT/E with BMR2

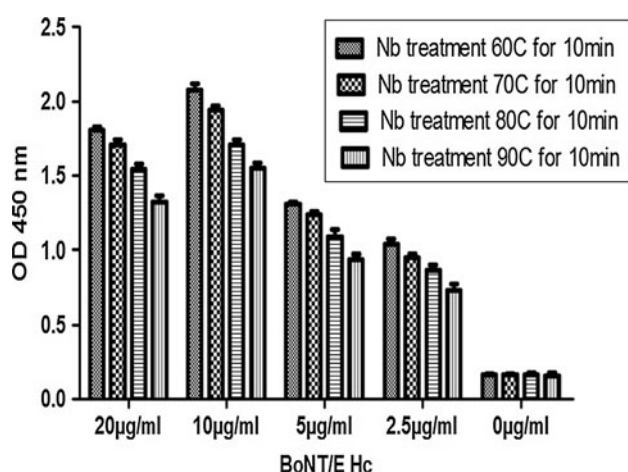
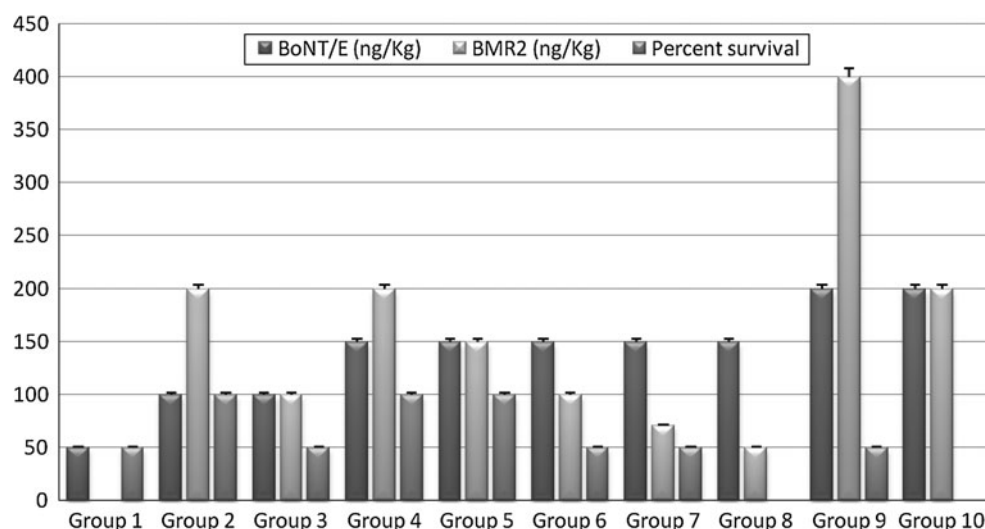


Fig. 8 The effect of heat treatment on binding activities of nanobody by ELISA. Wells were coated by serial twofold dilutions of Hc. After treating at different temperatures, nanobodies were added to the wells, and binding was detected by anti-hemagglutinin tag (HA tag) conjugated to HRP. The results are expressed as absorbance at 450 nm $\pm$ SD

Discussion

A, B and E serotypes of botulinum neurotoxins (BoNT) are associated with food-borne, infant and wound botulisms [41, 42]. The toxins act pre-synaptically at the peripheral nervous system to block release of neurotransmitter, causing flaccid paralysis. Botulinum neurotoxin has severe toxicity and thus is a NIAID Category, a Biodefense Priority Pathogen [44]. Presently, the diagnosis of BoNT in laboratory is carried out using mouse bioassay. This method is problematic and takes several days [38, 42]. This justifies the necessity of developing a rapid and sensitive assay for the detection of BoNT. So far as the treatment is

concerned, the currently available method is based on the production of antibodies in horses immunized with the toxin. These antibodies can cause severe reactions following patient's response to the animal sera and disturbances in the immune system that may lead to death [5]. In the present research, we used recombinant 50-kDa BoNT/E Hc domain instead of whole BoNT/E toxin in order to eliminate toxicity of the whole toxin [15] and ease of working. Moreover, recombinant BoNT/E Hc has powerful antagonists of the corresponding BoNT/E neurotoxins [28]. The present study describes successful isolation and cloning of a single domain (nanobody) VHH gene from hyper-immunized dromedaries' library, followed by soluble expression and characterization of the nanobody. To our knowledge, this is the first report on the development of a single-domain VHH BoNT/E Hc binder.

The size of the library constructed from lymphocytes in the present research was calculated as 3.2×10^7 . This size is comparable to those reported in other researches [9, 44]. For in vitro selection of anti-BoNT/E Hc binders, five rounds of panning were done against BoNT/E Hc with increased stringency of the washing buffer. The best candidate nanobody gene was selected and subcloned (BMR2). BMR2 nanobody gene was well expressed within *E. coli*. High yield of biologically active constructs was achieved at about 57 mg/L of bacterial culture. This is greater than the yields reported for recombinant scFv (0.3 mg/L), Fab and diabody (100 µg/L) against other toxins [7, 32]. The expressed nanobody yield was higher than those reported for scFv and Fab [31]. The affinity of our nanobody was moderately reasonable; however, higher affinities have also been reported for nanobodies directed against related antigen such as BoNT/A [9, 44] and other antigens [10, 23]. Our nanobody specifically recognizes

BoNT/E toxin without cross-reactivity with closely related structures of the members of this family (BoNT/A, BoNT/B) (Fig. 6b). Our data indicate that nanobody at 150 ng/Kg concentration entirely neutralized 3LD50 of the BoNT/E upon i.p. injection in challenged mice. This is fairly adequate concentration to neutralize BoNT/E toxin normally produced at lower concentrations in food stuff. Unlike conventional antibodies, VHHs tend to bind cavities and clefts [6]. Nanobody produced against BoNT/E Hc could penetrate canyon of toxin and prevent BoNT/E from binding to normal target neurons. It is well documented that VHH antibodies could resist high-temperature conditions [13, 27]. Thermal stability and ability to recover secondary structure and function of our nanobody indicated that BMR2 is thermally stable for 10 min at 90 °C. The nanobody still retained 80 % of its initial binding activity (Fig. 7). Immunoreactivity of the nanobody was markedly less affected by incubation at higher temperatures. Our results suggest that BMR2 may provide more rugged and reliable reagents for immunodiagnostic applications as well as pasteurization step in the manufacture of VHH antitoxin.

In conclusion, the recombinant VHH finds their applications in the diagnostic tools and treatment measures for their very stable, highly soluble, specific response and high affinity [19]. They also share extra sequence homology with human VH domains and are so well tolerated in human beings. These facts make our nanobody an appropriate candidate for the development of drugs to treat botulism. Specificity of this nanobody to BONT/E makes it a suitable diagnostic tool for the detection of *C. botulinum* neurotoxin type E.

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