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Staphylococcus aureus β-hemolysin-neutralizing single-domain antibody isolated from phage display library of Indian desert camel

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ABSTRACT

Objective: To isolate and characterize *Staphylococcus aureus (S. aureus)* β-hemolysin-neutralizing dAbs from phage display library of Indian desert camel. **Methods**: Phage display library of 5×10^7 dAb clones of LPS-immunized Indian desert camel constructed in our laboratory was used for selection of *S. aureus* exotoxin-specific clones by panning technique. Enrichment of Ag-specific clones in successive rounds of panning was assessed by phage-ELISA and phage titration. Different dAb clones binding to *S. aureus* exotoxin Ags were expressed with C-terminal 6×His tag in *E. coli* and purified by Ni-chelate chromatography. The expression was verified by SDS-PAGE and western blotting. The purified clones were tested for inhibition of 'hot-cold' hemolytic activity *in vitro*. Resistance to thermal inactivation of the dAb clones was studied by observing the effect of heat treatment from 50 ℃ to 99 ℃ for 30 mins on the 'hot-cold' hemolytic activity *in vitro*. **Results**: Several dAb clones binding to *S. aureus* exotoxins were isolated and enriched by three rounds of panning. The soluble dAb clones were approximately ~16 kDa in size and reacted with 6×His tag specific murine monoclonal antibody in western blot. One of the Ni-chelate affinity purified dAb.6×His clones, inhibited *S. aureus* β-hemolysin activity *in vitro* and resisted thermal inactivation upto 99 ℃. **Conclusions**: An *S. aureus* β-hemolysin-neutralizing dAb clone of possible therapeutic potential has been isolated.

1.Introduction

Antibodies (Abs) are produced by traditional method of animal immunization and modern methods based on hybridoma and recombinant DNA (rDNA) technologies for applications in diagnosis, treatment and bioscience research [1-3]. Several monoclonal antibodies (mAbs) have been developed by hybridoma technique and have been suitably modified for use as therapeutic agents in humans during the past three decades[4]. Although none yet licensed for use, Abs produced by modern rDNA technologies (rAbs) are cost-effective and promising to have wide ranging applications in areas such as therapeutics, diagnostics, micro-arrays and proteomics, biosensors, novel drug delivery systems, *in vivo* tumor targeting, disease-resistant plant varieties, abzymes, enzyme inhibitors, etc. [2,5]. Various formats of rAbs, such as single-chain Fv (scFv), single-domain Ab (dAb), diabody, triabody, minibody, bi-specific Ab, etc. have been produced [6].

Phage display technology employs a set of rDNA techniques to produce Ab libraries representing a huge number of Ag-specific clones displayed on phage particles, subsequent selection of a desired Ag-specific Ab clone and its further genetic modification for achieving intended effector function [7-8]. Numerous libraries of scFv fragments derived from Ig gene segments of humans and animal species have already been constructed [9]. Although other display systems based on yeast, bacteria, viruses and ribosomes have been developed [6], phage display remains most popular.

Unique features of 'heavy chain antibodies' (HCAbs) in camelids and sharks have allowed production of the smallest Ag-binders, composed of only one variable domain of Ab, by phage display technology [10]. HCAbs differ from their full-length counterparts in having two identical shorter heavy chains and lacking light chains in their structure, and occur naturally in members of the family Camelidae[11] and sharks. The so-called single-domain Abs (dAbs) or 'nanobodies' are derived from variable domain of HCAb designated as VHH gene segments and have attracted great attention in recent years for applications in medicine and biology [12-13]. Nanobodies

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are highly expressed in microbial cell culture, possess favorable biophysical properties including solubility and thermal stability, and are amenable to affinity maturation and other genetic modifications[14]. Nanobodies against several clinically and industrially useful Ags have already been produced [12-13]. Necessity of production of dAbs and other suitable Ab formats that neutralize bacterial toxins has also been felt for making available cost-effective and alternative therapeutic agents for toxinosis[6,15].

Staphylococcus aureus is the causative agent of clinically important human and animal diseases, such as, food poisoning, toxic shock syndrome, abscesses, osteomyelitis, endocarditis, mastitis, etc. [16-17]. Pathogenic staphylococci produce several exotoxins such as haemolysins(α , β , γ , δ), enterotoxins (SEA-SEE, SEG-SEI), toxic shock syndrome toxin-1 (TSST-1), leucocidin, etc. as important virulence determinants [18]. Some of the staphylococcal toxins are known to cause disease, and all such toxinoses can possibly be treated with antibodies. Anti-SEB and anti-TSST-1 dAbs have already been produced from phage display library of the Arabian camel [15] and anti- α -hemolysin dAb from shark phage display library [19]. The present study was undertaken with an objective to select and characterize *Staphylococcus aureus* β -hemolysin-neutralizing single-domain antibodies from phage display library of Indian desert camel produced in our laboratory.

2. Materials and methods

2.1. Preparation of S. aureus Hlb-rich exoproteins

Coagulase- and β -hemolysin (Hlb)-positive *Staphylococcus aureus* isolated from a clinical bovine mastitis case was used for production of total exoproteins in the culture supernatant. The culture was grown in one litre of brain-heart infusion broth using standard bacteriological procedures and the supernatant was harvested by centrifugation at 5 000×g for 30 mins at 4 °C. Grams staining of the culture was done to confirm the presence of Gram-positive staphylococci. Hlb titre in the crude supernatant was determined[20].Then, Hlb-rich total exoproteins were prepared from the supernatant by precipitation with 65% saturated ammonium sulfate solution and dialysis against phosphate-buffered saline (PBS), pH 7.0[21]. The resultant partially purified Hlb-rich total exoprotein preparation was thus 100× concentrated from the original culture.

2.2. Electro-elution of S. aureus exoproteins from polyacrylamide gel

S. aureus exoproteins, further concentrated 25-fold by trichloroacetic acid (TCA) precipitation, dissolved in Laemmli´s sample buffer and resolved by sodium dodecyl sulphate-polyacryamide gel electrophoresis (SDS-PAGE) under reducing conditions in 15% resolving gel[22]. The 39 kDa, 27 kDa and 23 kDa size polypeptide bands were electro-eluted from the gel[23]. After dialysis against PBS, pH 7.0, the eluates were stored at -70 °C. The molecular size (Mr) and purity of the eluates were verified by SDS-PAGE in 12.5% resolving gel.

2.3. Determination of protein concentration

Protein concentrations in various preparations were determined against bovine serum albumin standard[24] using a commercial kit (Genei, Bangalore, India) in a microtitre plate version.

2.4. SDS-PAGE

SDS-PAGE of Hlb-rich exoproteins, electro-eluted exoproteins, expressed dAbs in periplasmic contents of *E. coli* host and purified dAbs was done under reducing conditions using Laemmli´s discontinuous buffer system in 12.5% or 15% resolving gel and 4% stacking gel in a vertical gel electrophoresis apparatus[22].

2.5. Western blotting

Western blotting was performed essentially according to the method of Towbin and his colleagues (1979)[25]. Briefly, polypeptides resolved by SDS-PAGE were electro-blotted from the gel to methanol-pretreated polyvinyl difluoride (PVDF) membrane (0.22 μm pore size) in a semi-dry trans-blot apparatus (Atto® Corporation, Japan) at 1.0 mA/cm^2 of the gel for 1 hr at RT. Immunoblots were developed using following steps: i) washing in PBS-0.05% tween 20 (PBST) for 15 min, ii) blocking with the PBST-3%BSA for 1.5 hr, iii) 3×5 min washings, iv) reaction with antibody [mouse anti-HA mAb (Cell Signal Technology® , USA) at 1:500 dilution or with rabbit anti-dAb hyperimmune serum (HIS) at 1:3000 dilution for expressed dAb detection; purified dAb clones for exotoxins detection], 2 hrs at RT, v) 3×5 min washings, vi) reaction with anti-species conjugate [anti-mouse or anti-rabbit HRPO conjugate at 1:3 000 dilution] or rabbit anti-dAb followed by anti-rabbit conjugate for one hr at RT, vii) 4×5 min washings as above and the 5 min last one in citrate-phosphate buffer, pH 5.0 (CPB), viii) blot development with DAB/H_2O_2 substrate solution at RT in the dark until the background started just appearing, ix) stopping colour development with deionized water, x) drying the blots for photography.

2.6. Selection and enrichment of S. aureus exoproteins-specific dAb-displaying phages from the phage display library

S. aureus exoproteins-specific dAb-displaying phages from the display library were selected and enriched by the panning technique[15]. The phage display library of LPS-immunized Indian desert camel constructed in our laboratory was employed in the present study. The phage display library was a collection of >5×10^7 M13/fd filamentous phages, each displaying a dAb on its minor coat protein, pIII, and encapsidating its corresponding VHH-pHEN4 DNA. Three rounds of panning of the phage display library were performed to select and enrich *S. aureus* exoproteins-binding dAb-displaying M13 phages.

S. aureus exoprotein Ags (1.0 mg/ mL) in 50 mM PB, pH 7.0 were coated 100 μL/well at 4 °C O/N. After washing 3 ×3 min in PBS, pH 7.0, the Ag-coated wells were loaded with 100 μL/well of >10^{11} virions/mL of the original phage library. The phages were allowed to bind at RT for 1 hr. and after extensive washing, eluted with triethylamine solution and neutralized with 1M Tris-HCl, pH 7.4. The Ag-specific phage eluates were designated as Pan-I/SAE. Pan-I/SAE phages were titrated by infecting TG1 cells. The Pan-I/SAE phages were rescued with M13K07 helper phage and

amplified in TG1 cells to produce post-Pan-I phages for the second round of panning. In this manner, three rounds were completed.

Enrichment of Ag-specific phages in successive rounds of panning was determined by phage titration by TG1 infection [26] and phage-ELISA of post-Pan-I, -II, -III phage preparations [15]. Exponentially growing TG1 cells were infected with phages eluted at each round and plated onto LB/ampicillin/glucose agar plates for titre determination. The phages from infected TG1 cells in LB/ampicillin/glucose broth were rescued with helper phage superinfection and prepared 10^{11} phages/mL for phage-ELISA, using *S. aureus* total exoproteins as coated Ag (1.0 mg/mL) and rabbit anti-M13K07 hyperimmune serum followed by anti-rabbit IgG-HRPO conjugate for phage detection.

2.7. Expression of selected Ag-specific soluble dAb.HA clones in WK6

Amber non-suppressor WK6 cells were infected with the rescued post-Pan-III/SAE phages and 10 VHH-PCR tested clones of soluble dAb with C-terminal HA tag (dAb. HA) were expressed from WK6 transformants obtained on LB/ampicillin agar plates. The periplasmic contents were released by hypotonic shock treatment[15], collected by high speed centrifugation, mixed with phenyl methyl sulphonyl fluoride (PMSF) to a final conc. 1 mM, and kept at -70 ℃ until further use.

2.8. Subcloning of Ag-specific VHHs in pHEN6c vector, WK6 transformation and expression of dAb.6×His in WK6 transformants

Standard protocols in molecular biology were used [27]. VHH clones, dAb/SAE Cl-3, Cl-6, Cl-7 and Cl-9, from selected Ag-specific VHH-pHEN4 clones, of 370-450 bp size were amplified by PCR, gel extracted and ligated with pHEN6c DNA into *PstI* and *BstEII* (*Eco91I*) sites at 16 ℃ O/N using T4 DNA ligase. The ligated VHH-pHEN6c transformed into chemi-competent WK6. The vector, pHEN6c is a derivative of pHEN4 that allows IPTG-inducible expression of soluble dAb with C-terminal 6× His tag in WK6 periplasm. A total of 14 transformant WK6 colonies were screened by VHH-PCR and the positive transformants used for expression of dAb.6×His clones in periplasm. The periplasmic expression was assessed in 12.5% resolving gels in SDS-PAGE and Ag-specificity of dAb.6×His clones detected by indirect ELISA, using *S. aureus* total exoproteins Ag (10 μg/mL) coated onto 96-wells ELISA plate (Nunc Maxisorb® , Denmark). Soluble dAb.6 ×His clones in WK6 periplasmic contents were detected using rabbit anti-dAb HIS followed by anti-rabbit IgG -HRPO conjugate.

2.9. Purification of soluble dAb.6×His clones

Three Ag-specific dAb.6×His clones showing relatively higher A492 values in indirect ELISA were selected further for expression in WK6 periplasm and subjected to purification by Ni-TED affinity column chromatography according to manufacturer´s protocol (USB®, USA) or Ni-NTA affinity chromatography in batch-mode (Invitrogen, USA) followed by ultra-filtration through 30 kDa cut-off membrane (Amicon® , USA).

2.10. Characterization of the purified dAb.6×His clones

Ag-specific purified dAb.6×His clones were tested for Ag specificity by indirect ELISA and western blotting. For indirect ELISA, *S. aureus* total exoproteins (20 μg/mL) and three gel-extracted *S. aureus* exoproteins, viz., SAE-p39, SAE-p27 and SAE-p23 (5 μg/mL each) were coated onto wells of an ELISA plate (Nunc Maxisorb® , Denmark), blocked with PBST-10% horse serum, reacted with purified dAb.6×His clones, viz., Cl-7-1, Cl-7-5 and Cl-9-2 O/N at 4 ℃ , and detected with rabbit anti-dAb IgG followed by conjugate. Western blotting of the four Ags was done with all the three dAb.6×His clones.

2.11. Neutralization of S. aureus Hlb activity in vitro

Staphylococcus aureus was grown fresh and the supernatant containing exoproteins was harvested from the culture and Hlb activity in the culture supernatant titrated. For demonstration of Hlb neutralization with three dAb.6×His clones (i.e., Cl-7-1, Cl-7-5 and Cl-9-2), serial 2-fold dilution starting from neat to 1∶128 of each purified dAb.6 ×His clone was made using ´toxin diluent buffer´ (PBS, pH 7.4 with 10 mm $MgCl_2$) buffer in a volume of 50 μL/well in a 96-wells U-bottom microtitre plate. Then, Hlb-containing culture supernatant was diluted in the toxin diluent buffer so as to contain five hemolytic units of Hlb and added in a volume of 50 μL/well in all the wells of different dAb.6× His dilutions. BHI broth and toxin diluent were the negative control. The plate was then incubated at 37 ℃ for half an hour to allow Hlb-dAb.6×His interactions.

One percent sheep RBC suspension was added 50 μL/well in all the wells, mixed with Hlb dilutions and incubated for one hr at 37 ℃. Sheep RBCs settled in the wells were resuspended and the plate transferred to refrigerator at 4 ℃ for two hrs. Sheep RBC buttons at the bottom of wells were taken as indicator of Hlb neutralization by dAb6×His clones. Hlb neutralization titre was calculated as reciprocal of the highest dilution of the purified dAb clone(s) that completely inhibited hemolysis by five Hlb units.

2.12. Examination of thermal inactivation

For determination of thermal inactivation of purified dAb.6 ×His clones, each clone was incubated in a temperature gradient thermal cycler at 37 ℃, 50 ℃, 60.5 ℃, 70.9 ℃, 80 ℃, 89.9 ℃ and 99 ℃ for 30 min followed by cooling to 4 ℃ for 5 min. Then, 2-fold serial dilutions of each clone were made in toxin diluent buffer in a U-bottom microtitre plate and neutralization of two hemolytic units of Hlb recorded as indicator of resistance to thermal inactivation.

2.13.DNA sequencing of S. aureus Hlb-neutralizing dAb clone

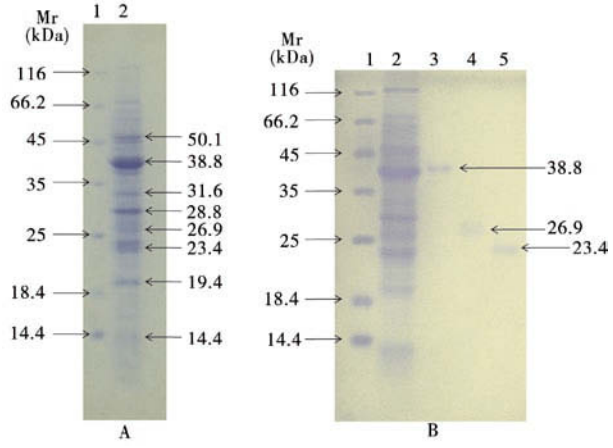
DNA sequence of anti-*S. aureus* Hlb dAb Cl7-5 was determined using automatic sequencing facility at Chromous Biotech Pvt. Ltd., Bangalore, India and the sequence submitted to NCBI GenBank [GenBank via Bankit:http://www.ncbi.nlm.nih.gov/Banklt/]. Immuno- and bioinformatics techniques were used to reveal the molecular biological features of the clone.

3. Results

3.1. S. aureus exoproteins profiling by SDS-PAGE

The polypeptide profile of the Hlb-rich total exoproteins obtained in 15% resolving gel by SDS-PAGE under reducing conditions is shown in Figure 1A. Several major bands were revealed and Mr size ranged between 10 kDa and 150 kDa. The most prominent band was of 38.8 kDa size approximately, called SAE-p39. Other major bands of 50.1 kDa (SAE-p50), 31.6 kDa (SAE-p32), 28.8 kDa (SAE-p29), 26.9 kDa (SAE-p27), 23.4 kDa (SAE-p23), 19.4 kDa (SAE-p19), 14.4 kDa (SAE-p14), etc. were also visible in the CBBR-stained gel as shown in the figure.

SAE-p39, SAE-p27 and SAE-p23 polypeptides, electro-eluted from SDS-polyacrylamide gel, appeared as single bands of ~39 kDa, ~27 kDa, and ~23 kDa size in 15% resolving gel of SDS-PAGE (Figure 1B).



A: Lane 1: Protein Mr markers, 2: *S. aureus* total exoproteins, B : Lane 1: Protein Mr markers, 2: *S. aureus* Hlb-rich total exoproteins, 3: SAE-p39, 4: SAE-p27, 5: SAE-p23

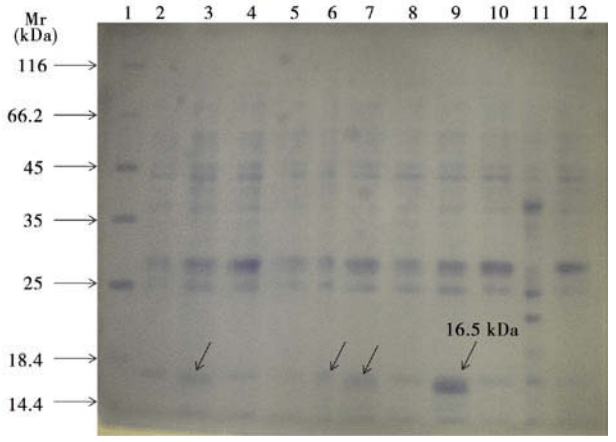
Figure 1 A-B. SDS-PAGE profile of ammonium sulfate precipitated *S. aureus* total exoproteins (A) and gel electro-eluted purified exoproteins (B).

3.2. Selection and enrichment of Ag-specific dAb-displaying phages from the phage library

Pan-I, -II and -III eluted phage titration and phage-ELISA results are shown in Table 1. Difference of CFU counts obtained with phages after the first and the third round of panning showed an enrichment factor of 168. A_{492} values in phage-ELISA using Ag-specific phages increased in successive rounds of panning. Put together, the results indicated successful selection of Ag-specific phages from the original library.

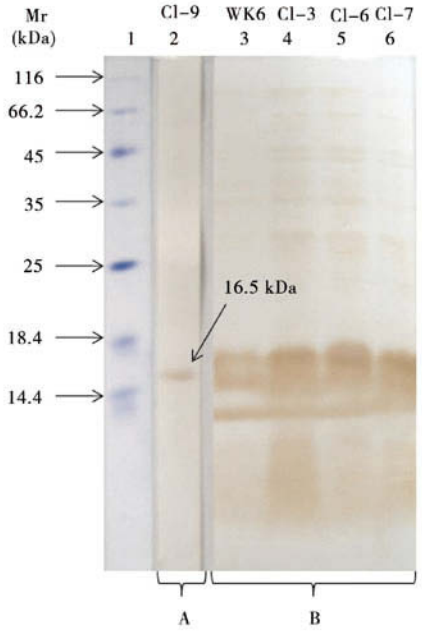
3.3. Expression of soluble dAb.HA in WK6 periplasmic contents

All the nine VHH-positive WK6 transformants were found to express dAb.HA of approximately 16.5 kDa size in their periplasm as analyzed by SDS-PAGE in 15% resolving gel (Figure 2). However, only four clones, designated as dAb.HA/SAE Cl-3, Cl-6, Cl-7, and Cl-9 had relatively higher levels of expression of dAb.HA. Immunoblot of dAb.HA Cl-9 developed with murine anti-HA monoclonal antibody showed only one brown band of ~16.5 kDa size (Figure 3A). Whereas, immunoblots of other three clones i.e., Cl-3, Cl-6, and Cl-7 developed with rabbit anti-dAb HIS showed ~16.5 kDa band, along with some additional bands in each, indicating the presence of anti- *E. coli* proteins exoproteins antibodies in rabbit anti-dAb HIS (Figure 3B). These findings confirmed the expression of expected size of dAbs with HA tag in the clones tested.



Lane 1: Protein Mr markers, 2-10: dAb.HA expressed in periplasmic content of Cl-1 & Cl-3 to Cl-10, 11-12: WK6 periplasmic contents as negative controls.

Figure 2. SDS-PAGE of periplasmic contents of WK6 clones expressing dAb.HA in 15% resolving gel.



Lane 1: Coomassie blue stained PVDF strip of Protein Mr markers, 2: immunoblots of dAb.HA Cl-9 with mouse anti-HA monoclonal Ab, 3-7: immunoblots of dAb.HA with rabbit anti- dAb.HA His, 3:WK6 control, 4: Cl-3, 5: Cl-6, 6: Cl-7.

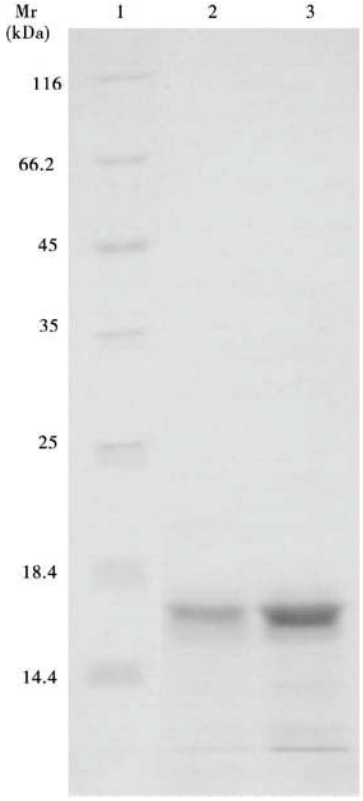
Figure 3 A-B. Immunoblots of periplasmic content of WK6 expressing dAb.HA developed with: (A) mouse anti-HA monoclonal Ab and, (B) rabbit anti- dAb.HA His.

3.4. Expression of Ag-specific dAb.6×His clones

All the 14 VHH-positive WK6 transformants expressed dAb.6×His of 16.5 kDa size in their periplasm as revealed by SDS-PAGE using 12.5% resolving gel, wherein WK6 control did not showed any corresponding band. However, the levels of expression of dAb.6×His clones were lower than those of dAb.HA. Only 4 of the 14 clones tested gave relatively higher A_{492} values as compared to WK6 control in indirect ELISA.

3.5. Purification and characterization of dAb.6×His clones

The dAb.6×His clones purity achieved by Ni-TED affinity chromatography under native conditions was assessed by SDS-PAGE in 12.5% resolving gel. Of the three serial eluates from nickel chelate chromatography column, the first two eluates, i.e., El-I and El-II had a band of 16.5 kDa dAb.6xHis in both the clones, i.e., Cl-7-5 and Cl-9-2 in CBBR-stained gel (Figure 4), indicating satisfactory level of purity achievement. Eluate III of both the clones did not show any band, indicating that all the bound dAb.6×His was completely/largely eluted with first two mL lots of the elution buffer used under native conditions.



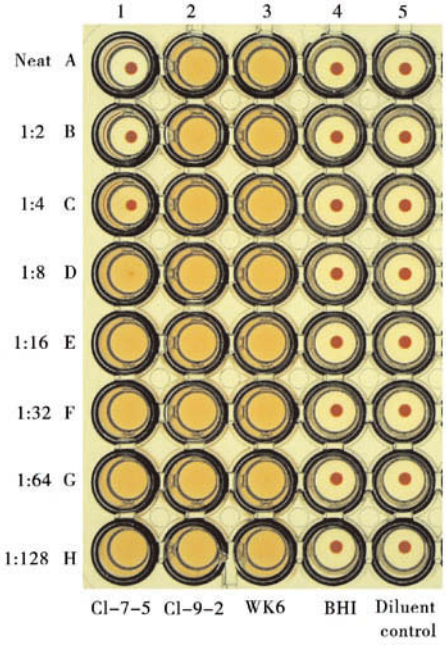
Lane 1: Protein Mr marker, 2-3: dAb.6×His Cl-7-5 eluates El-I and El-II from Ni-TED column.

Figure 4. SDS-PAGE in 12.5% polyacrylamide gel showing dAb.6× His clones purified by Ni-TED chromatography.

Cl-7-1 and Cl-7-5 purified with Ni-NTA batch-mode chromatography under native conditions followed by ultrafilteration through 30 kDa cut-off membrane showed two major bands, one of ~16.5 kDa dAb.6×His and the other of about 29 kDa in CBBR-stained gel. The purity achieved was thus not 100%, but substantial for further work using suitable control for the contaminant band.

The indirect ELISA results confirmed the Ag-specificity of the three clones used. The clones reacted with total exoproteins as well as SAE-p39 preparation, and did not react to SAE-p27 and SAE-p23 Ags. The immunoblots of four different *S. aureus* Ags, viz. Hlb-rich total exoproteins, SAE-p39, SAE-p27, SAE-p23 developed with each of the three purified dAb.6xHis clones showed the specific band of SAE-p39, but not SAE-p27 and SAE-p23.

Of the three clones tested, only one clone, i.e., Cl-7-5 upto its 1:4 dilution completely inhibited five Hlb hemolytic units *in vitro* as indicated by SRBC button formation in the microtitre plate (Figure 5). The neat Cl-7-1, however, showed partial inhibition of two Hlb units only, whereas complete hemolysis occurred in all the wells having five Hlb hemolytic units mixed with serially 2-fold diluted Cl-9-2, WK6, BHI, and the diluent control. The Cl-7-5 clone, heat-treated for 30 mins. at 37℃, 50℃, 60.5 ℃, 70.9 ℃, 80℃, 89.9℃, and 99℃ followed by cooling to 4℃ for 5 mins, inhibited two hemolytic units of β-hemolysin, thereby indicating its resistance to thermal inactivation.



Columns 1-5: serial 2-fold dilutions starting from neat; Col. 1: dAb.6xHis Cl-7-5; Col. 2: dAb.6×His Cl-9-2; Col. 3: WK6 negative control; Col. 4: BHI negative control; Col. 5: toxin diluent as negative control.

Figure 5. Inhibition of β-hemolytic activity by purified dAb.6×His clones in microtitre plate.

3.6. DNA sequence and molecular biological features of S aureus Hlb-neutralizing camel dAb Cl7-5

Comparison of deduced amino acid sequences of three clones from the same phage display library revealed that all had the key features of camelid VHH i.e., S11, F37, E44, R45 and G47 [anti-*S. aureus* dAb Cl7-5 (GenBank Accession no.: GU014816)]/E47 [randomly chosen Cl2 (Genbank Accession no.: EU429319)]/K47 anti-LPS dAb Cl26 (GenBank Accession no.: EU861212)] (Kabat numbering of VHH domain). However, CDR1 and CDR3 were of exceptionally large size, having 14 and 27 amino acids respectively in GU014816. All the three also gave a 3-D structure based on 1jtpA (anti-lysozyme camel dAb) template using Swiss 3D modeling.

Table 1
Successive enrichment of antigen-specific phages in three rounds of panning indicated by eluted phage titres, VHH-PCR and Phage-ELISA.

Panning round	Input dAb-displaying phages	Output Ag-specific phages	VHH-PCR positive TG1 transformants(% positive)*	Phage ELISA (A_{492}values)**
I	4.5×10^{11} (original library)	8.0×10^{6} (Pan-I/SAE)	15/16 (93.7%)	0.661
II	7.0×10^{11} (post- Pan-I phages)	6.0×10^{8} (Pan-II/SAE)	16/17 (94.1%)	0.763
III	8.7×10^{11} (post- Pan-II phages)	2.6×10^{9} (Pan-III/SAE)	16/17 (94.1%)	1.477

* About 80% of TG1 transformants in the original library were VHH-PCR positive; ** A_{492}value of M13KO7 helper phage control & original phage library was 0.197 and 0.345, respectively.

4. Discussion

A local isolate of *S. aureus* from bovine mastitis case was used. The isolate was positive for coagulase, catalase and some exoproteins including β-hemolysin (unpublished observations). Hlb or β-toxin was verified by demonstration of the 'hot-cold' hemolytic activity in the culture supernatant with a titre of 128 and the presence of the most abundant protein of 39 kDa size (SAE-p39) in the 65% ammonium sulphate precipitated preparation resolved by SDS-PAGE. According to Projan and colleagues (1989) [28], the molecular size of the full-length Hlb was 39 kDa. Later on, it was suggested that the mature secreted product had a molecular size of 35 kDa[18]. The findings of the present study, however, agree with that of Projan and his colleagues (1989)[28]. Most of the *S. aureus* isolates from bovine mastitis secrete β-hemolysin along with a wide variety of other exoproteins[17].

Hlb was chosen for several reasons: i) it is secreted by most bovine strains of *S. aureus*, and its role in pathogenesis of udder infections is implicated, though not clearly understood, ii) Hlb-neutralizing dAb or other rAb formats have yet not been reported in the literature, iii) it has sphingomyelinase C activity, having homology with sphingomyelinases from several bacterial species, such as *Bacillus cereus, Listeria ivanovi, Leptospira interrogans*, etc. [29], to cause damage of cells of the hematopoietic origin, brain and other tissues of animal species having high sphingomyelin content. Neutralizing antibodies against this enzyme should therefore be useful in diminishing or preventing the damage caused by Hlb during *S. aureus* infection.

However, the levels of expression were not high, which pointed out the need for increasing the expression levels by trying different conditions of expression in a prokaryotic system[30] or eukaryotic system[31]. Neutralization of the β-hemolytic activity was the most revealing of the bio-activity of the expressed clones. Out of the three clones tested, dAb/SAE Cl-7-5 completely neutralized five haemolytic units of Hlb *in vitro*. Whereas, dAb/SAE Cl-7-1 only partially neutralized only two units of Hlb and dAb/SAE Cl-9-2 did not at all neutralize Hlb. This observation suggested that the three clones were probably detecting three different epitopes on the same antigen i.e. Hlb. However, possibility also existed that dAb/SAE Cl-7-1 and dAb/SAE Cl-7-5 that originated from Cl-7 ancestor were detecting one and the same antigen but with different affinities. Interestingly, a dAb clone was found to resist thermal denaturation even up to 99 ℃. Thermal resistance of dAbs has been explained by some investigators [32-33]. Small size, additional Cys residues in the amino acid sequence and ability to rapidly renature are supposed to be responsible for this feature of dAbs, which the dAbs in the present study also share. In addition, subjecting the dAb clones to a range of elevated temperatures, followed immediately by cooling to 4 ℃ prior to their mixing with Hlb, might have rapidly renatured the clone. Another interesting point that emerges from these findings is that if the dAb is neutralizing Hlb as an enzyme, then it must have some special features in its paratope[34]. The antibody must be able to bind in the active site or nearby to cause stereic hindrance to the substrate binding.

One dAb clone, i.e., anti-SAE-p39 showing neutralization of *S. aureus* β-toxin *in vitro* was the most significant finding in the present study. Another application that the dAb clone could find is its use in developing immunosensor-based diagnostic test for detection of Hlb-secreting *S. aureus* isolates. Only the dAbs have been reported to be suitable for immobilization on the biosensor chip, because of their small size, exceptional stability and good affinity. Immunosensor-based diagnostics are proving better than ELISA technique due to their being highly sensitive (fM/mL), rapid giving result within 10 mins and linear over a wider range of concentrations [35].

In conclusion, camel single-domain antibody that neutralizes *S. aureus* β-hemolysin has been isolated and partially characterized, and suggested to have potential for applications in *S. aureus* disease diagnosis, therapeutics and research.

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References

[1] Winter G, Milstein C. Man-made antibodies. *Nature* 1991; **349**: 293-9.

[2] Little M, Kipriyanov SM, Le Gall F, Moldenhauer G. Of mice and men: hybridoma and recombinant antibodies. *Immunol Today* 2000; **21**: 364-70.

[3] Shibasaki S, Ueda M. Therapeutic antibodies and other proteins obtained by molecular display technologies. *Recent Paten Biotechnol* 2009; **3**(1): 19-27.

[4] Reichert J, Pavlou A. Monoclonal antibodies market. *Nature Drug Discovery* 2004; **3:** 383-4.

[5] Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Adv Drug Deliv Rev* 2006; **58**: 640-56.

[6] Likhacheva A, Ulanova T. Recombinant Antibodies. In: Khudyakov YE, editor. *Medicinal Protein Engineering*. Boca Raton: Taylor & Francis; 2009, p. 353-83.

[7] Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. *Annu Rev Immunol* 1994; **12**: 433-55.

[8] Smith GP, Petrenko VA. Phage display. *Chem Rev* 1997; **97**: 391-410.

[9] O´Brien PM, Aitken R. Broadening the impact of antibody phage display technology: Amplification of immunoglobulin sequences from species other than humans or mice. In: O´Brien PM, Aitken R, editors. *Antibody phage display: methods & protocols*. Totowa, NJ: Humana Press Inc; 2002, p. 73-86.

[10] Ghahroudi MA, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett* 1997; **414**: 521-6.

[11] Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Bajyana SE, et al. Naturally occurring antibodies devoid of light chains. *Nature* 1993; **363**: 446-8.

[12] Gibbs WW. Nanobodies. *Scientific American* 2005; **293**(2): 67-71.

[13] Harmsen MM, De Haard HJ. Properties, production and applications of camelid single-domain antibody fragments. *Appl Microbiol Biotechnol* 2007; **77**(1): 13-22.

[14] Muyldermans S. Single domain antibodies: current status. *Rev Mol Biotechnol* 2001; **74**: 277-302.

[15] Muyldermans S, Wyns L. Antibody molecules which interact specifically with the active site or cleft of a target molecule. European Patent EP0937140 (patent) 2007.

[16] Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998; **339**: 520-32.

[17] Jonsson P, Wadstrom T. *Staphylococcus*. In: Gyles CL, Thoen CO, editors. *Pathogenesis of bacterial infections in animals*. 2nd ed. Ames: Iowa State University Press; 1993,p.21-35.

[18] Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 2000; **13**: 6-34.

[19] Liu JL, Anderson GP, Goldman ER. Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library. *BMC Biotechnol* 2007; **19**: 78.

[20] Bernheimer AW. Assay of hemolytic toxins. *Meth Enzymol* 1988; **165**: 213-20.

[21] Maheswaran SK, Smith KL, Lindorfer RK. Staphylococcal β-hemolysin. I. Purification of β-hemolysin. *J Bacteriol* 1967; **94**(2): 300-5.

[22] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 1970; **227**: 680-5.

[23] Singh A, Singh A, Chaudhri SC. Visceral schistosomiasis of domestic animals in India: humoral immune status of infected cattle, sheep and goats against major polypeptide antigens of *Schistosoma indicum* and *S. spindale*. *Parasite Immunol* 2004; **26:** 167-75.

[24] Lowry OH, Roseberg NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-75.

[25] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylammide gels to nitrocellulose sheets, procedure and some applications. *Proc Nat Acad Sci USA* 1979; **76**: 4350-4.

[26] Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G. *Nucleic Acids Res* 1991; **19**: 4133-37.

[27] Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. 3rd ed. New York: Cold Spring Harbour Laboratory Press; 2001, p.74-8.

[28] Projan SJ, Kornblum J, Kreiswirth B, Moghazeh SL, Eisner W, Novick RP. Nucleotide sequence of the β-hemolysin gene of *Staphylococcus aureus*. *Nucleic Acids Res* 1989; **17**: 3305.

[29] Goni FM, Alonso A. Sphingomyelinases: enzymology and membrane activity. *FEBS Lett* 2002; **531**: 38-46.

[30] Ghahroudi MA, Tanha J, MacKenzie R. Prokaryotic expression of antibodies. *Cancer Metast Rev* 2005; **24**: 501-19.

[31] Li E, Brown SL, Dolman CS, Brown GB, Nemerow GR. Production of functional antibodies generated in a non-lytic insect cell expression system. *Protein Express Purif* 2001; **21**(1): 121-8.

[32] Goldman ER, Anderson GP, Conway J, Laura J, Sherwood MF, et al. Thermostable llama single domain antibodies for detection of botulinum A neurotoxin complex. *Anal Chem* 2008; **80**(22): 8583-91.

[33] Omidfar K, Rasaee MJ, Kashanian S, Paknejad M, Bathaie Z. Studies of thermostability in *Camelus bactrianus* (Bactrian camel) single-domain antibody specific for the mutant epidermal-growth-factor receptor expressed by Pichia. *Biotechnol Appl Biochem* 2007; **46**(1): 41-9.

[34] Lauwereys M, Ghahroudi MA, Desmyter A, Kinne J, Holzer W, De Genst E, et al. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* 1998; **17**: 3512-20.

[35] Saerens D, Huang L, Bonroy K, Muyldermans S. Antibody fragments as probe in biosensor development. *Sensors* 2008; **8**: 4686-99.

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