Preparation and Identification of a single domain antibody specific for the Adenovirus Vectors

Yi Cheng
Inner Mongolia Agricultural University
Yanxia Hao
Inner Mongolia Agricultural University
Huimin Zhang
Inner Mongolia Agricultural University
Yanlong Liu
Inner Mongolia Agricultural University
Kexin Ao
Inner Mongolia Agricultural University
Shan Fu
Inner Mongolia Agricultural University
Qiyao Wu
Inner Mongolia Agricultural University
Zhi Wang
Inner Mongolia Agricultural University
Fuxiang Bao (baofuxiang@imau.edu.cn)
Inner Mongolia Agricultural University https://orcid.org/0000-0003-4807-6277

Original article

Keywords: Adenovirus, Single domain antibody, Phage display library, Immunofluorescence, Immunoaffinity purification

Posted Date: January 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1216048/v1

Abstract

Adenovirus belongs to the family of Adenoviruses, as a vaccine carrier, it not only has high safety, but also can stimulate the body to produce cellular immunity and humoral immunity. In this study, We aimed to prepare the adenoviral vectors specific single domain antibody to use in adenovirus identification and purification. We successfully constructed a single domain antibody phage display library with the capacity of 1.8×10^9 by immunizing and cloning the VHH gene from the Bactrian camel. After second round of biopanning, the clones specific for adenovirus were screened by Phage ELISA. Twenty-two positive clones were obtained, and two clones with the highest binding affinity from ELISA were selected and named as sdAb 5 and sdAb 31 respectively for further application. The recombinant single domain antibody was soluble expressed in *E. coli*, and can specifically bind to adenovirus rAd26, ChAd63 and Had5 in ELISA assay and in live cell immunofluorescence. At last, we established an effective method for immunoaffinity purification of adenovirus by immobilizing the single domain antibody to Sepharose beads, and it can be used to selectively capture Adenoviruses from cell culture medium. The preparation of the adenovirus specific single domain antibody was laying a foundation for the one-step immunoaffinity purification and identification of Adenoviruses.

Introduction

Adenovirus (Ad) is a promising vector platform that can be used for vaccine development due to their high safety and stimulation of robust cellular response and humoral immunity in multiple species (Antrobus et al., 2014; Barnes et al., 2012; Coughlan et al., 2018). Adenovirus belongs to the family of Adenoviruses and is mainly isolated from five major vertebrates including birds, mammals, reptiles, amphibians and fish. Currently, about 200 non-human Ad serotypes have been identified by scientists. The diameter of the adenovirus shell is 70-90 nm, which is composed of three main structural proteins: fiber, the penton base and the hexon. The genome of Adenovirus a linear double-stranded DNA without envelope (Trentin et al., 1962). Adenovirus can well stimulate the body's natural immune response without requiring immune adjuvants (Molinier-Frenkel et al., 2002). It has a broad spectrum of host cell tropism, and it can infect host cells regardless of cell division (Chang, 2021). Adenovirus exhibits some desirable characteristics, for instance, their genomes are stable and easy to manipulate, making them particularly suitable for the development of preventive vaccines. They can be amplified and produced high titers using a variety of complementary cell lines that comply with Good Clinical Practice (GCP) (Kovesdi and Hedley, 2010). They are now used as safe immunogenic vaccine vectors in clinical trials and exhibiting outstanding performances (Gurwith et al., 2013; Liebowitz et al., 2020; Sebastian and Lambe, 2018).

In order for adenovirus vectors to be used safely and effectively in animals and humans, researchers have modified their genomes. By deleting the *E1/E3* region to obtain the first-generation adenovirus vector, it can insert a transgene with replication defective properties and improved immunogenicity characteristics (Bett et al., 1994). However, the first-generation adenovirus vector genome is homologous to the *E1* part inserted into the packaging cell line HEK293, which may result in the occurring of

replicating-competent adenovirus (RCA). Researchers solved this problem by using minimal homology cell lines (Kovesdi and Hedley, 2010). In the process of the second-generation adenoviral vector research, it was found that by additionally deleting the *E2/E4* site can increases transgenic ability and decreases the possibility of RCA formation. On the other hand, the second-generation adenoviral vector has a decreased replication ability in producer cell lines (Wang and Finer, 1996), and the overall production yield of adenovirus vectors is lower than that of the first-generation adenovirus vectors. The third-generation adenoviral vectors, as known as helper-dependent gutless adenoviral vectors, were obtained by deleting almost all genomic sequences except those necessary for packaging (Kochanek et al., 1996; Parks et al., 1996). Therefore, multiple transgene expression cassettes can be inserted, but with increasing difficulty of manufacturing. In addition, the immunogenicity of these vectors is also reduced due to the deletion of the sequence and there is a possibility of helper virus contamination.

When adenovirus is used as a vaccine carrier, whether it is prevalent in human needs to be paid attention during the research process. The pre-existing immunity will reduce the effectiveness of the vaccine. Studied have found that the globally most prevalent adenovirus vectors human adenovirus serotype 2 (HAd2) and HAd5 have demonstrated variable results in clinical studies and about 80% of the population have neutralizing antibodies against HAd5 (Farina et al., 2001). Therefore, scientists have tried to prepare adenovirus vectors by using human adenovirus serotypes with low prevalence in the population and hunting for adenoviruses of other species such as chimpanzees and rhesus monkey.

The prevalence of these alternative Ads is very low compared with Ad2 and Ad5 vectors, for example in group D adenovirus (such as Ad26, Ad48, Ad24, and Ad49). Data showed that neutralizing antibodies against Ad26 only exist in individuals in South Africa and Southeast Asia, while Ad5-specific neutralizing antibodies have detected very high levels in all regions (Shenk, 1996). Similarly, research results showed that compared with rhesus monkeys vaccinated with Ad5, rhesus monkeys vaccinated with Ad26 can induce higher levels of interferon-γ (IFN-γ), interleukin-1 receptor agonist (IL-1RA), IL-6 and inducible protein-10(IP-10) cytokines, so rAd26 can play a better role in the field of vaccine research and development.

In addition to studying the use of human adenovirus, researchers also isolated adenovirus from non-human species, constructing and carrying out clinical applications (Lewis and Rowe, 1970). The main purpose of applying adenoviruses from non-human species is to avoid the existing immunity to human adenovirus serotypes. The diseases caused by non-human adenoviruses are species-specific, so these viruses will not harm humans. Studies have shown that when chimpanzee adenoviruses (ChAd3, ChAd6, ChAd7, ChAd63, and ChAd68) are used in clinical trials in mice, non-human primates and human, these adenovirus vectors can evade the specific immunity of human adenovirus serotypes (Wold and Toth, 2013). These results make ChAd63 becoming a good candidate for gene transfer applications (Ison and Hayden, 2016)

Single domain antibodies have only one heavy chain variable region domain called variable domain of the heavy chain of heavy chain antibody (VHH), and also referred to as nanobody, which is extracted

from the Camelidae serum, and can tightly binding to the specific antigens. On the other hand, the relative molecular weight of nanobody is only about 15 kDa, its small molecular weight allows them to have a strong tissue penetration ability (Hu et al., 2017). It has good stability, can be stored for a long time at -80°C, and can even withstand the high temperature, high pressure and extreme pH environment (Salvador et al., 2019). Thence it could be stockpiled as therapeutic treatment options in case of an epidemic. Not only the simple molecular structure makes it easier to express in large quantities in yeast, *E. coli* and other microbial expression systems, and makes it possible for industrialized large-scale production (Lafaye and Li, 2018), but also compared with traditional antibodies, nanobody does not have an Fc segment, and the sequence information encoding VHH is highly homologous to human VH families 3 (Ahmadvand et al., 2008), with a very low immunogenicity in human (Vincke et al., 2009). The part of the hydrophobic amino acids of nanobody are replaced by hydrophilic amino acids, which improves the solubility of nanobody (Beghein and Gettemans, 2017). In addition, the CDR1 and CDR3 of nanobody are longer than the conventional monoclonal antibody VH, which can penetrate into the interior of the antigen, and can has the better binding activity to the concave structure of antigen (Padlan, 1996). Based on the above-mentioned excellent characteristics, nanobody has been used as a therapeutic and diagnostic agent in multiple research and clinical trials (Beghein and Gettemans, 2017).

Materials And Methods

Bactrian camel immunization

A healthy 3-year-old female Bactrian camel was selected for immunization with adenovirus rAd26 and ChAd63 (gifted by Professor Jinsheng He from Beijing Jiaotong University). The adenoviruses were diluted from the original concentration of 1×10^{13} vp/ml to 2×10^{11} vp/ml to 1×10^{9} vp/ml for the immunization. 5 ml of blood was collected from the jugular vein and left to stand until it coagulated naturally before immunization. The serum was taken and stored at -20°C. The camel was immunized four times, and the interval between immunizations was two weeks. A week after each immunization, blood was collected from the jugular vein, and placed at 4°C to separate the serum used for ELISA assay to detect antibody titer. Two weeks after the fourth immunization, 50 ml of blood was collected from the jugular vein of the immunized camel for the separation of peripheral blood lymphocyte. The camel was farmed in the pasture that located in the suburb of Hohhot city, Inner Mongolia, and was provided free access to water and food. All experimental procedures were performed in accordance with the institutional and national guidelines and regulations and were approved by the Animal Care and Use Committee of Inner Mongolia Agriculture University.

Construction and screening of Bactrian camel phage display antibody library

Construction and screening of single domain phage display library as shown in Fig.1. The peripheral blood monocyte (PBMC) was prepared by the test method of the animal blood lymphocyte separation kit (TBD science, purchased from Tianjin Haoyang Biological Products Technology Co. Ltd.). Using TRIzol reagent (Ambion, USA) to extract total RNA from PBMC, and reverse transcripted to cDNA with ted reverse

transcription kit (Promega, USA) by using random hexamer as primer. The VHH fragment was amplified by the nested PCR method using the primers as listed in Table 1. In the first round of PCR, using P_1 and P_2 as primers, the antibody gene sequence from leader peptide to CH2 region was amplified from the cDNA. The PCR products were run on agarose gel, and the VHH containing band (600 bp) was recovered by cutting and extracting from the gel and used as the template for the second round of PCR. The full-length VHH gene from FR1 to FR4 with a fragment size of about 400 bp was amplified from the second round of PCR using P_3 and P_4 as primers. The PCR amplified product was digested with restriction enzymes *Nco*Ⅰand *Not*Ⅰ (New England Biolabs, UK) and ligated into the pMECS plasmid vector (a gift from Professor Serge Muydermans of the Vrije Universiteit Brussel, Belgium) with T4 ligase (New England Biolabs, UK), and transformed the ligation product into *E. coli* TG1 competent cells (GE Healthcare, USA) to construct the antibody library. Estimate the capacity of the antibody library by calculating the colony forming units (CFU) of 10 series of multiple dilutions on a 2×YT-AG plate. The transformation efficiency and the insertion of VHH was evaluated by PCR method with the sequencing primers of pMECS plasmid vector MP57 and GIII as listed in Table 1.

The antibody library was added to 200 ml of 2×YT medium and the OD600nm value of the bacterial solution does not exceed 0.3, then culture the library in a shaker at 37 °C, 250 r/min for 2 h; Added M13K07 helper phage (GE Healthcare, USA) and incubated at 37 °C for 1 h for infection; The cultured bacteria solution was centrifuged at 2 000×g for 10 min at 4 °C; discard the supernatant, resuspended, and inoculated the solution into 2×YT-AK medium containing ampicillin and kanamycin at 37 °C, 220 r/min shaking overnight; The cultured medium was centrifuged at 4°C, 7197×g for 15 min in the next day, and take the supernatant, then add 20% PEG8000 and centrifuge at 7197×g at 4°C for 25 min to precipitate the phage; Discarded the supernatant, and resuspended the pellet in PBS to obtain the recombinant phage library. Adding the phage library to the 96-well microtiter plate coated with the adenovirus rAd26 at a concentration of 2×10^7 vp/ml, and binding at 37°C for 1h; eluting the bound phage with 100 μl 100 mM triethylamine, sealing, incubating at room temperature for 10 min, and neutralizing with 50 μl 1 M Tris-HCl. The eluted recombinant phage was used to infect *E. coli* TG1 (OD600nm = 0.6), and M13K07 helper phage was added for rescue. The phages were harvested, purified and used for a new round of enrichment. The *E. coli* TG1 infected with the eluted phages from the final enrichment were grown on 2×YT-AG plates. 92 clones were randomly picked from the 2×YT-AG plate and added them to the 2×YT-AG liquid medium, incubated at 37°C, 250 r/min overnight. The next day, the M13K07 helper phage was added and infected for 1 h; after centrifugation at 14000 r/min for 5 min, the bacteria were resuspended in 2×YT-AK medium, and incubated at 37°C, 250 r/min overnight. The medium was centrifuged at 14000 r/min for 5 min in the next day, and the supernatant was collected, 20% PEG8000 was added, and the mixture was centrifuged at 7197 × g for 20 min. The recombinant phage was added to an ELISA plate pre-coated with a concentration of 2×10^7 vp/ml adenovirus rAd26, and the M13K07 helper phage was used as a negative control, PBS buffer was used as a blank control, and binding for 2 h at room temperature; HRP/Anti-M13 Monoclonal Conjugate (GE Healthcare, USA) diluted at 1:5000 and added to each well, binding for 1 h at room temperature; TMB solution was added for color development,

and determine the OD450nm value. The absorbance of the experimental group/negative control≥2.1 was regarded as positive.

Expression and purification of the sdAb

The plasmids of the positive clones were isolated from the phage-ELISA, and double digested with restriction enzymes *Nco*Ⅰand *Not*Ⅰ. The digested VHH fragments were ligated into the pET-22b (+) –SBP (The pET-22b (+) vector carried a streptavidin binding protein that constructed and preserved by the Public Health Department of the School of Veterinary Medicine, Inner Mongolia Agricultural University) plasmid that digested by the same restriction endonuclease with T4 DNA ligase. The products were transformed to *E. coli* BL21 (DE3) competent cells (TransGen Biotech, Beijing, China). The expression of recombinant sdAb should be induced by IPTG (Solarbio Life Sciences, Beijing, China) for 8-12 h. Then the cells were collected and sonicated. Afterwards, the precipitate and supernatant were collected and analyzed by SDS-PAGE. Ni-NTA Sefinose™Resin (Sangon Biotech, ShangHai, China) was used to purify the expressed recombinant single domain antibodies, and the purified sdAbs were analyzed by SDS-PAGE.

Binding activity and specificity of the sdAb

In order to determine the binding activity of the purified recombinant sdAbs, the purified clone 5 and 31 sdAbs were diluted and added as a primary antibody to a 96-well plate coated with adenovirus rAd26, ChAd63 and HAd5 at a concentration of 2×10^7 vp/ml. At the same time, the sonicated *E. coli* BL21 (DE3) that transformed with empty pET-22b (+) vector was used as negative control, and PBS buffer was used as blank control. The HRP-conjugated 6*His-tag Mouse monoclonal antibody (Proteintech Group, WuHan, China) was used as the secondary antibody at a concentration of 1:10000, and after added the TMB solution for color development, and determine the OD450nm value on microplate reader.

Immunofluorescence assay

One microliter per well of polylysine was added to a 24-well cell culture dish, and incubated for 10 min, discarded the liquid, and dry in the super clean bench. HEK293A cells were incubated with a 10^5 cells/well in a 24-well cell culture dish at 37°C and 5% CO_2 for about 24 h; the cell culture medium was discarded, and added 1 ml of DMEM medium to each well; the adenovirus rAd26 was diluted to an concentration of 2×10^7 vp/ml, and added 100 μl of virus diluent to each well, and added DMEM to the control group, and cultured in an incubator for 24 h; the medium was discarded and added 1 ml of paraformaldehyde to each well to fixing for 20 min at room temperature, then aspirating the fixative, and rinsed with PBS 3 times for 5 min each time. Adding 100 μl 0.3% TritonX-100 dropwise to each well, and incubated at room temperature for 20 min, and rinsed with PBS 3 times, each time for 5 min. Adding 100 μl 5% BSA solution to each well, and incubated at room temperature for 30 min, prepared the primary antibody working solution with PBS to the concentration of 200 μg/ml, and added 100 μl to each well, and incubated overnight in a refrigerator. The 24-well cell culture dish was taken out of the refrigerator, placed at room temperature to rewarm for 10 min, and PBST was added to the shaker and rinsed 3 times, each for 5 min.

The CoraLite®488-conjugated 6*His His-Tag Mouse Monoclonal antibody (Proteintech Group, WuHan, China) was used as the secondary antibody at a concentration of 1:150, and added 100 µl of the working solution of the secondary antibody, incubated at room temperature for 1 h, added PBST to the shaker and rinse 3 times, each time for 5 min. 100 µl ready-to-use DAPI working solution was added and incubated at room temperature for 4 min, and rinsed with PBS 3 times, 5 min each time. The fluorescence signal was detected by a confocal microscope (ZEISS, LSM-800).

Immunoaffinity purification of adenovirus from cell culture medium with immobilized sdAb

The conjugation of sdAb to the NHS-Activated Sefinose™ 4 Fast Flow (BBI, Beijing, China) was done according the manufactures instruction. One microgram of purified sdAb was dissolved in 1 ml of coupling buffer ($NaHCO_3$ and NaCl solution). One microliter of NHS-Sepharose FF was added into the gravity column, and take 5 mL of 1 mM HCl to wash the column three times to remove the preservation solution, and added antibody solution immediately after washing, and shake overnight at 4°C for better coupling. The filtrate was collected in the next day, and rinsed the coupled sepharose with 5 ml coupling buffer, and use 1 ml of blocking buffer to shake at room temperature for 4 h to block uncoupled agents. After removing the blocking buffer, the sepharose was washed three times with 2 ml of coupling buffer. The recombinant human adenovirus HAd5 expressing enhanced green florescent protein (EGFP) (prepared in Department of Veterinary Public Health Faculty) was diluted in cell culture medium with the titer of 10^7 vp/ml, and added to the prepared immunoaffinity column, and combined for 30 min at room temperature. The bound virus was eluted with 2 M and 4 M NaCl solution, and the eluent was added to the cultured HEK293A cell for reinfection. The presence of the recombinant virus in the eluent and in the reinfected cell was visualized under the confocal microscope (ZEISS, LSM-800) by the expression of the EGFP protein.

Results

Bactrian camel immunization

The basal serum collected before the immunization was used as negative control, and anti-His-labeled goat anti-alpaca was used as secondary antibody for detection in ELISA assay. The OD450nm value of basal serum was 0.375, and when the dilution of immune serum specific for rAd26 and chAD63 was 1:8000 and 1:10000 respectively, the OD450nm value was still 2.1fold higher than the negative control as shown in Fig.2, so we determined the titer of immune serum was 1:8000 and 1:10000.

Construction and screening of Bactrian camel phage display antibody library

The VHH gene fragment was amplified from the cDNA that obtained from total RNA of camel PBMC by nested PCR method. In the first round of PCR, using P_1 and P_2 as primers, the antibody leader peptide to CH2 gene fragment was amplified from the cDNA, and detected by 1% agarose gel electrophoresis, as shown in Fig.3 (a), and successfully amplified a gene fragment of about 600 bp. The 600 bp product was recovered by cutting the gel and used as the template for the second round of PCR. When performing the

second round of PCR, P_3 and P_4 primers was used to amplify the full-length VHH gene FR1 to FR4, and the fragment size is about 400 bp as shown in Fig.3(b).

The VHH gene fragment was digested and ligated into pMECS vector, then electro-transformed into competent *E. coli* TG1. The bacterial solution was diluted from 10^1 to 10^{10} and coated on 2×YT-AG solid medium, and incubate overnight at 37°C, and the calculated phage repertoire with capacity is 1.8×10^9. Twenty-four clones were randomly selected from the 2×YT-AG solid medium for colony PCR identification. After 1% agarose gel electrophoresis, 22 strains of target bands of about 600 bp were obtained and shown in Fig.4. The correct insertion rate of antibody fragment in the library was 91.6%.

The recombinant phage specific for adenovirus was identified by Phage ELISA. 92 randomly selected recombinant phage clones were added to the microtiter plate coated with adenovirus vector rAd26, and HRP/anti-M13 monoclonal antibody was used to detect the bound phage. M13K07 helper phage was used as negative control, and PBS was used as blank control. The results were shown in Fig.5. The measured OD405nm value of the negative control is 0.07, and the highest values in the selected recombinant phage clone was 1.133, and we identified 38 clones with the OD450nm values were greater than 2.1fold of the negative control. Two clones with the highest absorbance value were selected and named as 5 and 31 for the further identification.

Expression and purification of the sdAb

The plasmids of the clone 5 and 31were extracted, and digested with restriction enzymes *Nco*Ⅰand *Not*Ⅰ, and detected by 1% agarose gel electrophoresis, as shown in Fig.6. A target band of about 400 bp was obtained. The VHH gene fragment was ligated into pET-22b (+) –SBP, and then transformed into *E. coli* BL21 (DE3). After induction by IPTG, the recombinant sdAbs were expressed mainly in the supernatant of bacteria lysate by analyzing with SDS-PAGE (data not shown), and the sdAbs were applied to Ni-NTA Sefinose™ Resin for purification. The result of SDS-PAGE analysis on the purified sdAb showed that there was a protein band about 20 kDa, which was consistent with the expected result as shown in Fig.7.

Binding activity and specificity of the sdAb

The purified recombinant sdAb was used as the detection antibody to verify the binding activity to different adenoviruses in ELISA assay. The recombinant sdAb 5 and 31 showed strong binding activity to human adenovirus Ad5, rAd26, and chAd63 viruses in ELISA assay at the concentration of 5 μg/ml as shown in Fig.8, when PBS is used as a blank control, and *E. coli* BL21(DE3) cell lysate that transformed with pET-22b (+) empty vector was used as a negative control.

Immunofluorescence assay

The results of immunofluorescence assay were shown in Fig.9. The HEK293A cells were infected with rAd26 adenovirus, and recombinant sdAb 5 was used as detection antibody, and then visualized with CoraLite®488-conjugated 6*His His-Tag Mouse Monoclonal antibody under an Inverted fluorescent microscope. When using 488nm as excitation light, we can find a green fluorescence signal from the cells that incubated with sdAb, and mainly stained the cytoplasm of cells when merged with the image of DAPI staining as shown in Fig.9A. But we didn't observe any fluorescence signal from the control group that without incubation with sdAb as shown in Fig.9B. The results showed that sdAb can specifically bind to the adenovirus infected and propagated in HEK293A cells.

Immunoaffinitypurification of adenovirus from cell culture medium with immobilized sdAb

The recombinant human adenovirus Ad5 expressing EGFP was diluted in cell culture medium with the titer of 10^7 vp/ml, and added to the prepared immunoaffinity column that coupled with purified sdAb, and combined for 30 min at room temperature. The bound virus was eluted with 2 M and 4 M NaCl solution and PBS, and the eluent was added to the cultured HEK293A cell for reinfection. The presence of the recombinant virus in the eluent and in the reinfected cell was visualized under the confocal microscope by the expression of the EGFP protein as shown in Fig.10. We observed that the green florescence was detected in both 2 M and 4 M NaCl solution eluent infected cells as shown in Fig.10A and B, but didn't detect in PBS eluent infected cells and non-infected cells as shown in Fig.10C and D. These results indicated that the immobilized sdAb can specifically bind to the adenoviruses and isolate them from culture medium, and both 2 M and 4 M NaCl solution can elute the bound virus from the antibody.

Discussion

Adenovirus is a linear double-stranded DNA, which is enclosed in a capsid without envelop structure. Human adenoviruses are one of the causes of acute respiratory infections in children. So far, no safe and effective prevention method has been developed, and there are more than 100 serotypes of human adenovirus. Caused the diseases by various serotypes are different and diverse disease severity is also observed, so the research of a related antibody is a great significance for clinical diagnosis and therapy. Human adenoviruses (Ads) have long been studied in the basic virology field and are exploited as vectors for gene therapy, vaccination, and oncolytic therapy. Not only because it has three upstream regions that can insert foreign genes without affecting the replication of the virus in the cell, but also the relatively thorough research on its structure and characteristics (Gao et al., 2019).

The outbreak of new coronavirus (2019-nCoV) infection poses a serious threat to the global public health, and vaccination is an effective means to prevent viral infections. With the efforts of scientific researchers from difference countries, adenoviruses vector vaccine, such as Ad5, Ad26 and ChAdOx1 that encoding the SARS-CoV-2 major antigen was developed, and already in clinical trials. The clinical trail showed that the adenovirus vector vaccine were proven to be a safe and effective strategy for prevention and controlling of SARS-CoV-2 infection, and some of them have received conditional marketing authorization for SARS-CoV-2 (Logunov et al., 2021; Madhi et al., 2021; Zhu et al., 2021).

The recombinant dificient adenovirus have been widely used as a delivery system to express functional protein for gene therapy. Two Ad5 based vectors expressing the tumor suppressor P53 were named as Advexin and Gendicine, and have obtained a great deal of clinical data from its application. Advexin has been evaluated in different cancers, such as head and neck squamous cell carcinoma (HNSCC), colorectal cancer, hepatocellular carcinoma (HCC), and Advexin has demonstrated a consistent safety profile and clinical efficacy as a monotherapy, as well as in combined modality regimens with chemotherapy and radiation (Senzer and Nemunaitis, 2009).Gendicine was approved in 2003 by the State Food and Drug Administration (SFDA) in China for intratumoral treatment of HNSCC in combination with chemotherapy (Tian et al., 2009).

Oncolytic adenoviruses have attracted many researchesrs interest by their peculiar tumor selectivity, safety, and transgene-delivery capability. The adenoviruses that armed with different Immunostimulatory Cytokines and Chemokines have been developed. ONCOS-102, a adenovirus that armed with granulocyte macrophage colony stimulating factor (GM-CSF) have showed a synergistic anti-tumor effect in the humanized mice treated with the combination of ONCOS102 and pembrolizumab (Kuryk et al., 2019). In recent studies, various adenoviruses that armed with Immune-Activating Ligands and Bispecific T-Cell Engager (BiTE) Molecules have been developed and tested in clinical studies (Freedman et al., 2017; Malmstrom et al., 2010).

In principle, the recombinant adenovirus can ben produced in cell culture and purified by ultracentrifugation in CsCl density gradients, followed by desalting by size exclusion chromatography (sepharose columns) or dialysis. However, the limitation of using the CsCl density gradients ultracentrifugation are time consuming, and the purification process is complex and the virus activity is inconsistent between batches (Altaras et al., 2005). Immunoaffinity chromatography using single domain antibody as capturing liand is regarded as a powerful tool for single step purification of recombinant virus and proteins from culture medium with high purity and yields. Compare to the convinient antibody, the single domain antibody is lack of Fc domain of antibody, and can avoid the existence of antibody heavy and light contamination when used as immunoaffinity ligand, and has been used widely in single step process (Ren et al., 2020; Verheesen et al., 2003).

In this study, we successfully constructed a single domain antibody phage display library with the capacity of 1.8×10^9 by immunizing the Bactrian camel with adenoviruses. The phage antibody was rescued, and the specific phages were enriched by adenoviruses, finally, 22 positive clones were screened, and two single domain antibodies with highest binding activity and different sequences were selected for further expression and identification. Two recombinant sdAbs were soluble expressed in the bacteria and purified with Ni-NTA agarose. Two recombinant sdAbs could specifically binding to adenoviruses both in ELISA and immunofluorescence assay. Subsequently, we established a method for immunoaffinity purification of adenovirus, which greatly saved purification time and cost. This method can be used to selectively capture Ad from vaccines or other virus culture mixtures, laying a foundation for the one-step immunoaffinity purification and identification of Adenovirus.

Declarations

Author's contributions

BFX and WZ conceived and designed the experiments. CY, HYX, LYL, ZHM, WQY and AKX carried out the experiments. BFX, CY, HYX analyzed the data. BFX, CY, HYX, WQY, AKX, and FS contributed reagents/materials/analysis tools. BFX, WZ , CY and HYX wrote the paper. BFX and WZ contributed to the study, interpretation of the studies, analysis of the data and review of the manuscript. BFX and WZ supervised the project.

Acknowledgements

The authors sincerely thank Professor Serge Muydermans, Vrije Universiteit Brussel, Belgium for kindly providing pMECS plasmid vector and Professor Jinsheng He, Beijing Jiaotong University, China for providing the adenovirus rAd26 and ChAd63.

Competinginterests

The authors declare that they have no conflict of interest.

Availability of data and material

Please turn to authors for all requests.

Consent for publication

Not applicable.

Ethics statement

Blood samples from a three years old female Bactrian camel that farmed in a pasture in the southern suburbs of Hohhot, Inner Mongolia Autonomous Region, were collected according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. The current study was approved by the Animal Care and Use Committee of Inner Mongolia Agriculture University.

Funding

This study was funded by National Natural Science Foundation of China (No. 81660297), The Innovation Fund for Young Talent at College of Veterinary Medicine, Inner Mongolia Agricultural University (No. 2015QNJJ01).

References

1. Ahmadvand, D., Rahbarizadeh, F., Vishteh, V.K., 2008. High-expression of monoclonal nanobodies used in the preparation of HRP-conjugated second antibody. Hybridoma 27, 269-276.

2. Altaras, N.E., Aunins, J.G., Evans, R.K., Kamen, A., Konz, J.O., Wolf, J.J., 2005. Production and formulation of adenovirus vectors. Advances in biochemical engineering/biotechnology 99, 193-260.
3. Antrobus, R.D., Coughlan, L., Berthoud, T.K., Dicks, M.D., Hill, A.V.S., Lambe, T., Gilbert, S.C., 2014. Clinical Assessment of a Novel Recombinant Simian Adenovirus ChAdOx1 as a Vectored Vaccine Expressing Conserved Influenza A Antigens. Mol Ther 22, 668-674.
4. Barnes, E., Folgori, A., Capone, S., Swadling, L., Aston, S., Kurioka, A., Meyer, J., Huddart, R., Smith, K., Townsend, R., Brown, A., Antrobus, R., Ammendola, V., Naddeo, M., O'Hara, G., Willberg, C., Harrison, A., Grazioli, F., Esposito, M.L., Siani, L., Traboni, C., Oo, Y., Adams, D., Hill, A., Colloca, S., Nicosia, A., Cortese, R., Klenerman, P., 2012. Novel Adenovirus-Based Vaccines Induce Broad and Sustained T Cell Responses to HCV in Man. Sci Transl Med 4.
5. Beghein, E., Gettemans, J., 2017. Nanobody Technology: A versatile Toolkit for Microscopic imaging, Protein-Protein interaction Analysis, and Protein Function exploration. Frontiers in Immunology 8.
6. Bett, A.J., Haddara, W., Prevec, L., Graham, F.L., 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci U S A 91, 8802-8806.
7. Chang, J., 2021. Adenovirus Vectors: Excellent Tools for Vaccine Development. Immune Netw 21, e6.
8. Coughlan, L., Sridhar, S., Payne, R., Edmans, M., Milicic, A., Venkatraman, N., Lugonja, B., Clifton, L., Qi, C., Folegatti, P.M., Lawrie, A.M., Roberts, R., de Graaf, H., Sukhtankar, P., Faust, S.N., Lewis, D.J.M., Lambe, T., Hill, A.V.S., Gilbert, S.C., 2018. Heterologous Two-dose Vaccination with Simian Adenovirus and Poxvirus Vectors Elicits Long-lasting Cellular Immunity to Influenza Virus A in Healthy Adults (vol 29, pg 146, 2018). Ebiomedicine 31, 321-321.
9. Farina, S.F., Gao, G.P., Xiang, Z.Q., Rux, J.J., Burnett, R.M., Alvira, M.R., Marsh, J., Ertl, H.C., Wilson, J.M., 2001. Replication-defective vector based on a chimpanzee adenovirus. Journal of virology 75, 11603-11613.
10. Freedman, J.D., Hagel, J., Scott, E.M., Psallidas, I., Gupta, A., Spiers, L., Miller, P., Kanellakis, N., Ashfield, R., Fisher, K.D., Duffy, M.R., Seymour, L.W., 2017. Oncolytic adenovirus expressing bispecific antibody targets T-cell cytotoxicity in cancer biopsies. Embo Mol Med 9, 1067-1087.
11. Gao, J., Mese, K., Bunz, O., Ehrhardt, A., 2019. State-of-the-art human adenovirus vectorology for therapeutic approaches. FEBS Lett 593, 3609-3622.
12. Gurwith, M., Lock, M., Taylor, E.M., Ishioka, G., Alexander, J., Mayall, T., Ervin, J.E., Greenberg, R.N., Strout, C., Treanor, J.J., Webby, R., Wright, P.F., 2013. Safety and immunogenicity of an oral, replicating adenovirus serotype 4 vector vaccine for H5N1 influenza: a randomised, double-blind, placebo-controlled, phase 1 study. Lancet Infect Dis 13, 238-250.
13. Hu, Y., Liu, C., Muyldermans, S., 2017. Nanobody-Based Delivery Systems for Diagnosis and Targeted Tumor Therapy. Front Immunol 8, 1442.
14. Ison, M.G., Hayden, R.T., 2016. Adenovirus.Microbiol Spectr.
15. Kochanek, S., Clemens, P.R., Mitani, K., Chen, H.H., Chan, S., Caskey, C.T., 1996. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both

full-length dystrophin and beta-galactosidase. Proc Natl Acad Sci U S A 93, 5731-5736.

16. Kovesdi, I., Hedley, S.J., 2010. Adenoviral producer cells. Viruses 2, 1681-1703.
17. Kuryk, L., Moller, A.W., Jaderberg, M., 2019. Combination of immunogenic oncolytic adenovirus ONCOS-102 with anti-PD-1 pembrolizumab exhibits synergistic antitumor effect in humanized A2058 melanoma huNOG mouse model. Oncoimmunology 8, e1532763.
18. Lafaye, P., Li, T.F., 2018. Use of camel single-domain antibodies for the diagnosis and treatment of zoonotic diseases. Comp Immunol Microb 60, 17-22.
19. Lewis, A.M., Jr., Rowe, W.P., 1970. Isolation of two plaque variants from the adenovirus type 2-simian virus 40 hybrid population which differ in their efficiency in yielding simian virus 40. Journal of virology 5, 413-420.
20. Liebowitz, D., Gottlieb, K., Kolhatkar, N.S., Garg, S.J., Asher, J.M., Nazareno, J., Kim, K., McIlwain, D.R., Tucker, S.N., 2020. Efficacy, immunogenicity, and safety of an oral influenza vaccine: a placebo-controlled and active-controlled phase 2 human challenge study. Lancet Infect Dis 20, 435-444.
21. Logunov, D.Y., Dolzhikova, I.V., Shcheblyakov, D.V., Tukhvatulin, A.I., Zubkova, O.V., Dzharullaeva, A.S., Kovyrshina, A.V., Lubenets, N.L., Grousova, D.M., Erokhova, A.S., Botikov, A.G., Izhaeva, F.M., Popova, O., Ozharovskaya, T.A., Esmagambetov, I.B., Favorskaya, I.A., Zrelkin, D.I., Voronina, D.V., Shcherbinin, D.N., Semikhin, A.S., Simakova, Y.V., Tokarskaya, E.A., Egorova, D.A., Shmarov, M.M., Nikitenko, N.A., Gushchin, V.A., Smolyarchuk, E.A., Zyryanov, S.K., Borisevich, S.V., Naroditsky, B.S., Gintsburg, A.L., Gam, C.-V.V.T.G., 2021. Safety and efficacy of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine: an interim analysis of a randomised controlled phase 3 trial in Russia. Lancet 397, 671-681.
22. Madhi, S.A., Baillie, V.L., Cutland, C.L., Voysey, M., Izu, A., 2021. Safety and efficacy of the ChAdOx1 nCoV-19 (AZD1222) Covid-19 vaccine against the B.1.351 variant in South Africa.
23. Malmstrom, P.U., Loskog, A.S., Lindqvist, C.A., Mangsbo, S.M., Fransson, M., Wanders, A., Gardmark, T., Totterman, T.H., 2010. AdCD40L immunogene therapy for bladder carcinoma–the first phase I/IIa trial. Clin Cancer Res 16, 3279-3287.
24. Molinier-Frenkel, V., Lengagne, R., Gaden, F., Hong, S.S., Choppin, J., Gahery-Segard, H., Boulanger, P., Guillet, J.G., 2002. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. Journal of virology 76, 127-135.
25. Padlan, E.A., 1996. X-ray crystallography of antibodies. Adv Protein Chem 49, 57-133.
26. Parks, R.J., Chen, L., Anton, M., Sankar, U., Rudnicki, M.A., Graham, F.L., 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 93, 13565-13570.
27. Ren, J., Zhang, C., Ji, F.L., Jia, L.Y., 2020. Characterization and comparison of two peptide-tag specific nanobodies for immunoaffinity chromatography. J Chromatogr A 1624.
28. Salvador, J.P., Vilaplana, L., Marco, M.P., 2019. Nanobody: outstanding features for diagnostic and therapeutic applications. Anal Bioanal Chem 411, 1703-1713.

29. Sebastian, S., Lambe, T., 2018. Clinical Advances in Viral-Vectored Influenza Vaccines. Vaccines-Basel 6.
30. Senzer, N., Nemunaitis, J., 2009. A review of contusugene ladenovec (Advexin) p53 therapy. Curr Opin Mol Ther 11, 54-61.
31. Shenk, T.J.F.V., 1996. Adenoviridae : the viruses and their replication. 2.
32. Tian, G., Liu, J.L., Sui, J., Zhou, R.M., Chen, W.H., 2009. Multiple hepatic arterial injections of recombinant adenovirus p53 and 5-fluorouracil after transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma: a pilot phase II trial. Anti-Cancer Drug 20, 389-395.
33. Trentin, J.J., Yabe, Y., Taylor, G.J.S., 1962. The Quest for Human Cancer Viruses: A new approach to an old problem reveals cancer induction in hamsters by human adenovirus. 137, 835-841.
34. Verheesen, P., ten Haaft, M.R., Lindner, N., Verrips, C.T., de Haard, J.J., 2003. Beneficial properties of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography. Biochim Biophys Acta 1624, 21-28.
35. Vincke, C., Loris, R., Saerens, D., Martinez-Rodriguez, S., Muyldermans, S., Conrath, K., 2009. General Strategy to Humanize a Camelid Single-domain Antibody and Identification of a Universal Humanized Nanobody Scaffold. J Biol Chem 284, 3273-3284.
36. Wang, Q., Finer, M.H., 1996. Second-generation adenovirus vectors. Nat Med 2, 714-716.
37. Wold, W.S., Toth, K., 2013. Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. Curr Gene Ther 13, 421-433.
38. Zhu, F., Jin, P., Zhu, T., Wang, W., Ye, H., Pan, H., Hou, L., Li, J., Wang, X., Wu, S., Wang, Y., Gou, J., Huang, H., Wu, H., Wang, X., Chen, W., 2021. Safety and immunogenicity of a recombinant adenovirus type-5-vectored COVID-19 vaccine with a homologous prime-boost regimen in healthy participants aged 6 years and above: a randomised, double-blind, placebo-controlled, phase 2b trial. Clin Infect Dis.

Tables

Table 1 The Primers

Primers	Sequences	Products
P_1	5'-GGTACGTGCTGTTGAACTGTTCC-3'	600 bp900 bp
P_2	5'-GTCCTGGCTGCTCTTCTACAAGG-3'	
P_3	5'-AGTTGTTCCTTCTATGCGGCCCAGCCGGCCATGGCTGAKGTBCA GCTGGTGGAGTCTGG-3'	
P_4	5'-ATTGCGTCAGCTATTAGTGCGGCCGCTGAGGAGACRG TGACCWGGGTCC-3'	400 bp
MP57	5'-TTATGCTTCCGGCTCGTATG-3'	
GIII	5'-CCACAGACAGCCCTCATAG-3'	600 bp

Figures

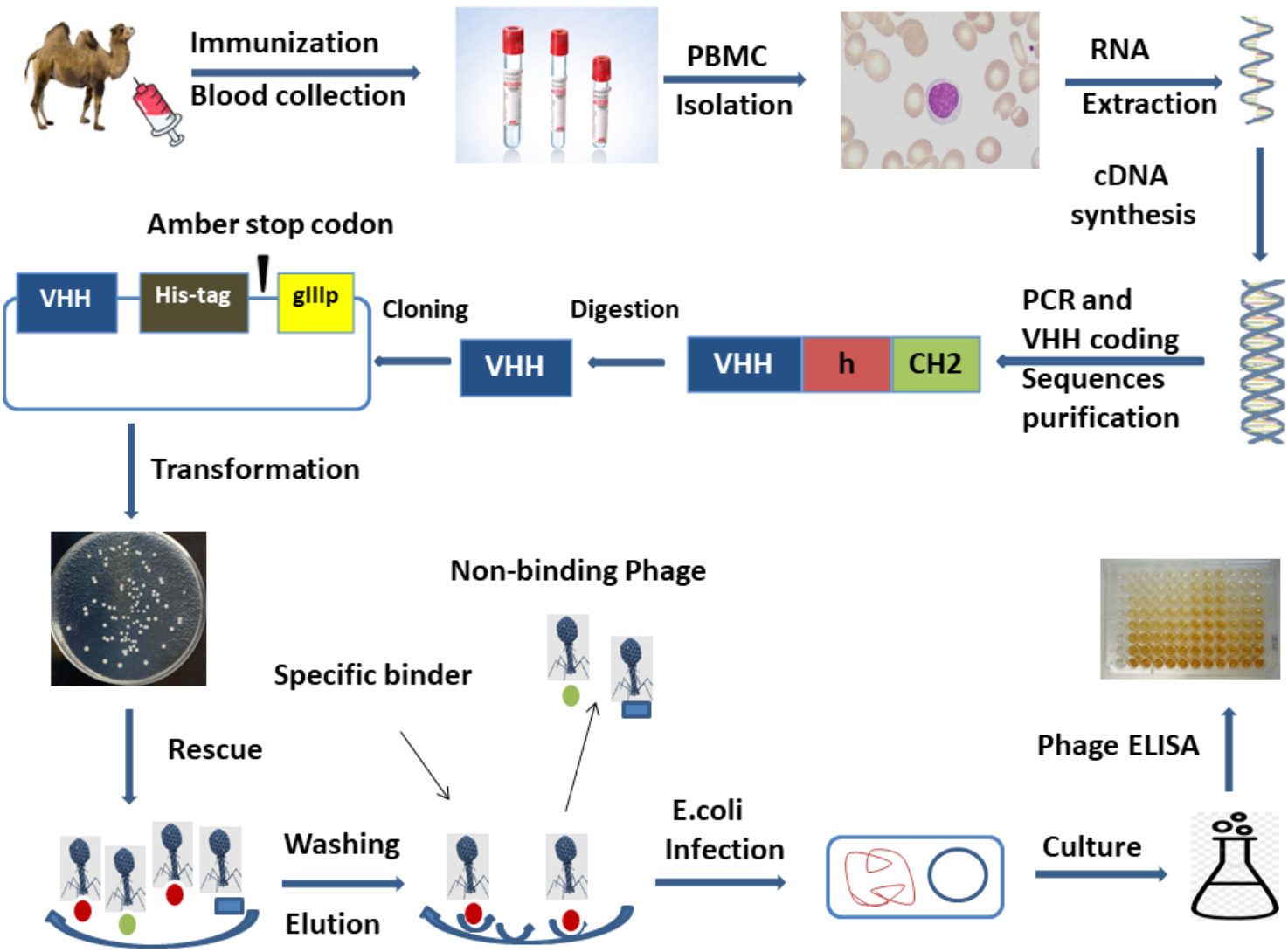

Figure 1

Schematic diagram of construction and screening of single domain phage display library. The Bactrian camel was immunized with adenovirus rAd26 and ChAd63, and the blood was collected to isolated Peripheral blood mononuclear cell (PBMC). The total RNA extracted from the PBMC and reverse transcript to cDNA. The VHH gene fragments were amplified with PCR. In the first round of PCR, the fragment of about 600 bp of leader sequence to CH2 domain was amplifiedfrom the cDNA. The full-length VHH gene from FR1 to FR4 amplified with P5 and P6 primers in the second round of PCR. The PCR amplified product was digested with restriction enzymes *Nco* and *Not*. The VHH gene was ligated into pMECS plasmid and transformed into *E.coli* TG1. After rescued by the M13k07 helper phage, theVHH gene was displayed on the phage surface and the specific phages were enriched and selected phage ELISA.

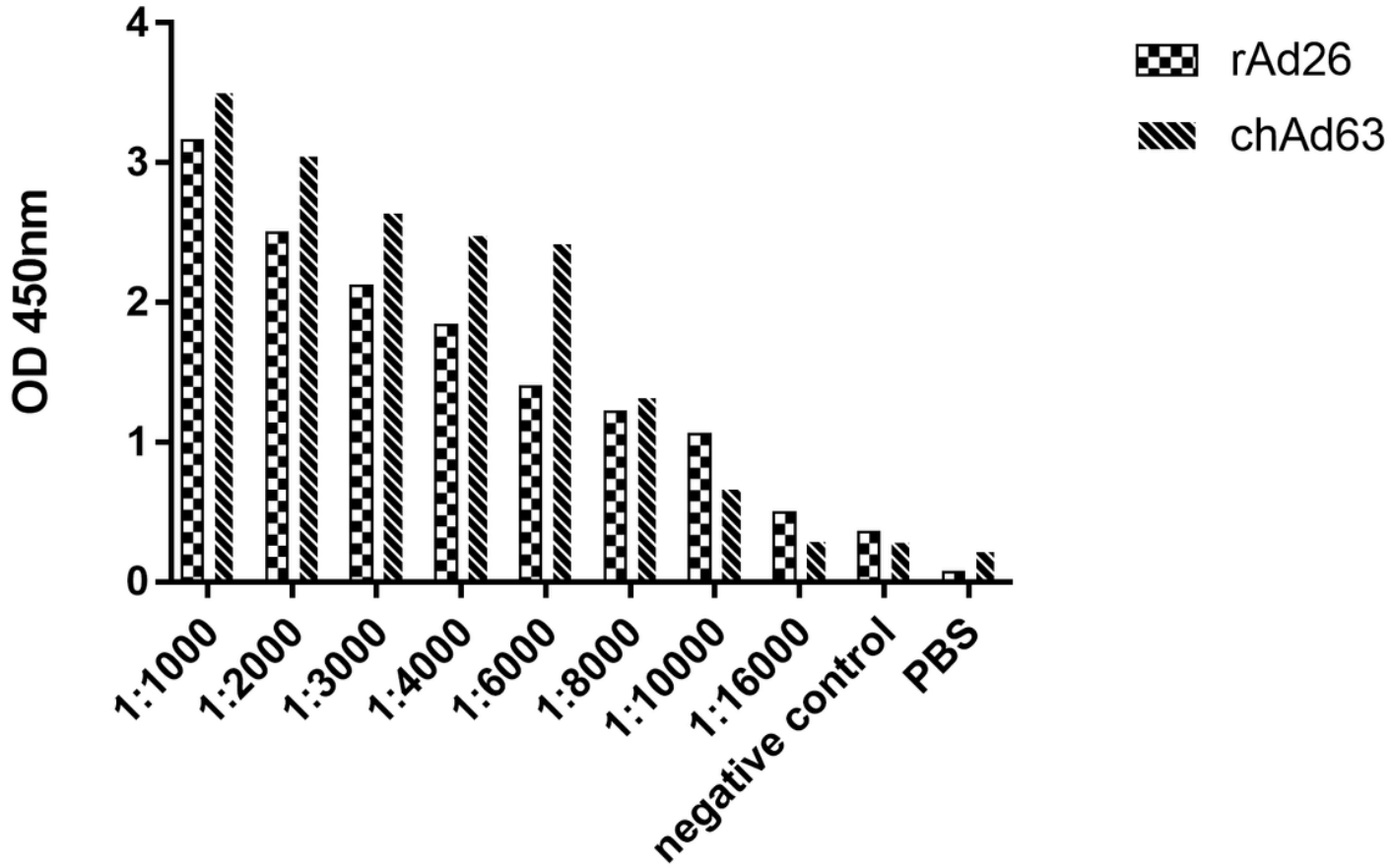

Figure 2

The serum antibody titer of immunized Bactrian camel was measured with ELISA assay. Wells of polystyrene microplate was coated with rAd26 and chAd63 viruses. The final immune serum of camel was diluted with PBS, and basal serum was used as negative control, PBS was used as blank control. The binding activity of each well was detected with HRP-labeled goat anti-alpaca at 1:10000 concentration and visualized with the TMB solution. The plate was read at 450nm in a microplate reader.

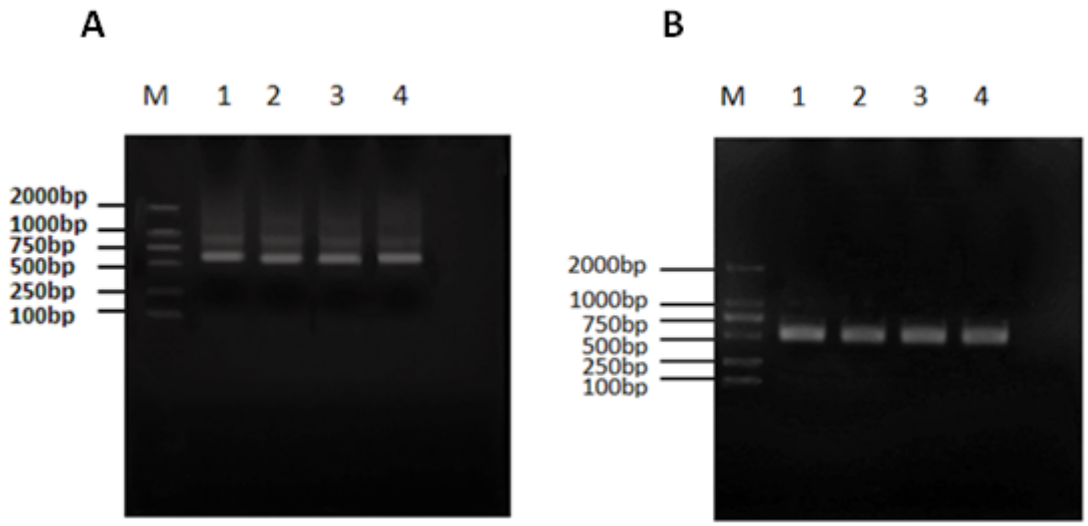

Figure 3

Amplification of VHH gene. cDNA was used as a template, and a fragment containing the leader sequence to the CH2 region of the IgG (900 bp for VH and 600 bp for VHH) was amplified with primers P_1 and P_2. The result was shown in Figure 3(a). The 600 bp fragment was purified by 1% agarose gel electroporation and used as the template for the second round of PCR with primers P_3 and P_4, which anneal to the VHH FR1 and FR4 regions respectively. The target bands of about 400 bp were successfully amplified as shown in Figure 3(b).

Figure 4

Identification of phage display antibody library. The electro-transformed TG1 bacterial solution from 10^1 to 10^{10} with fresh 2×YT/AG media and grown in 37°C.The next day, calculated volume of the phage antibody library is $1.8×10^9$. 24 single clones were randomly selected from 2×YT/AG plate for colony PCR identification. After 1% agarose gel electrophoresis, 22 clones with the amplicons of 600 bp fragment were obtained, and we calculated the recombination rate of antibody library was 91.6%.

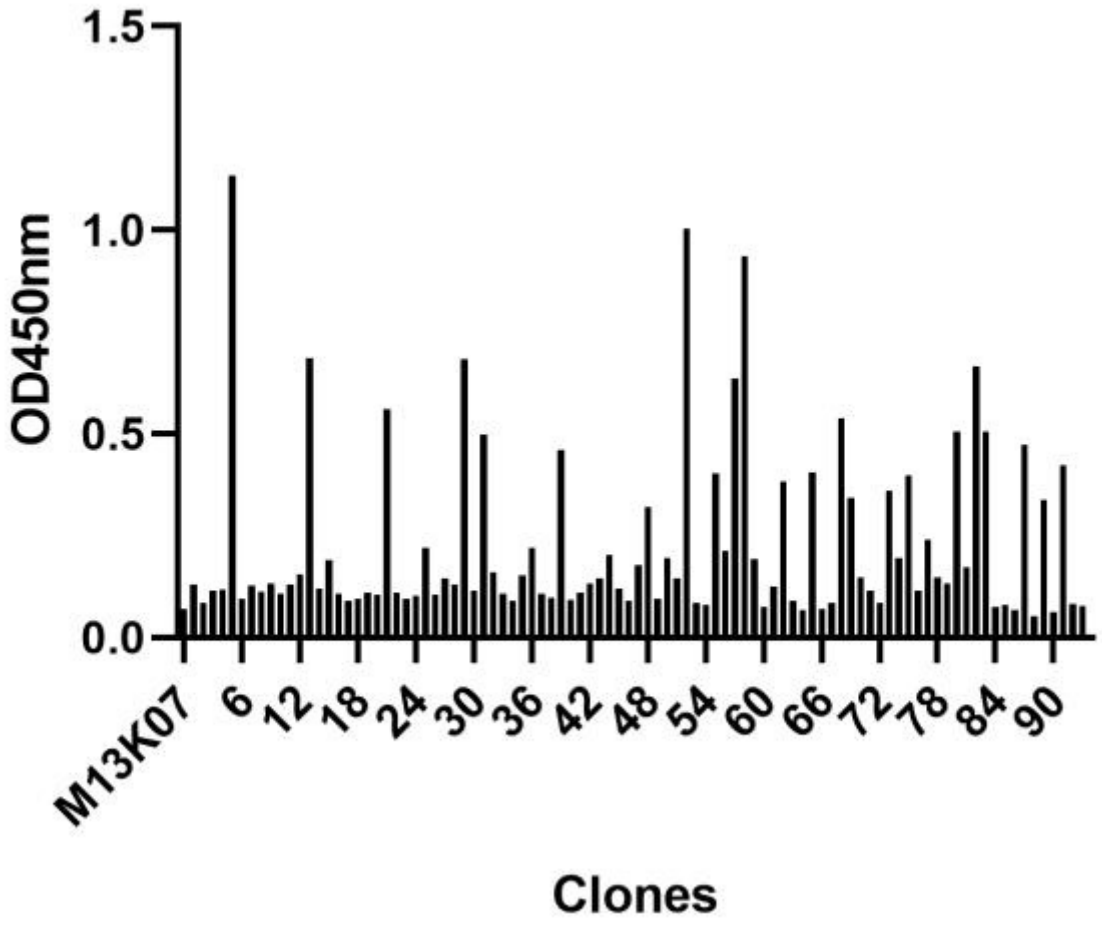

Figure 5

Detection of immune activity of Recombinant Phage against adenovirus by Phage ELISA. Recombinant phages prepared from 48 randomly picked clones were added to the Microplates coated with adenovirus vector rAd26. In parallel, the M13K07 helper phage as a negative control, and PBS as a blank control. The HRP/anti-M13 monoclonal antibody was used to detect the bound phage. The highest OD450nmvalue of recombinant phage is 1.133, and the lowest values is 0.052, while the negative control is 0.07. Two positive clones with highest OD450nm value were selected and named sdAb5 and sdAb31 respectively. The two clones were expressed, purified and identified in the next step.

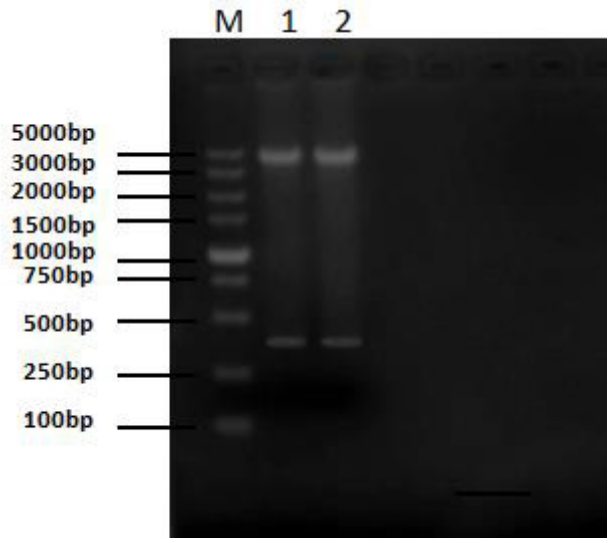

Figure 6

Recombinant plasmid digestion identification. The plasmids of the positive clones from phage ELISA were isolated, and digested with *Nco*Ⅰand *Not*Ⅰ, and detected by 1% agarose gel electrophoresis. A target band of about 400 bp was obtained.

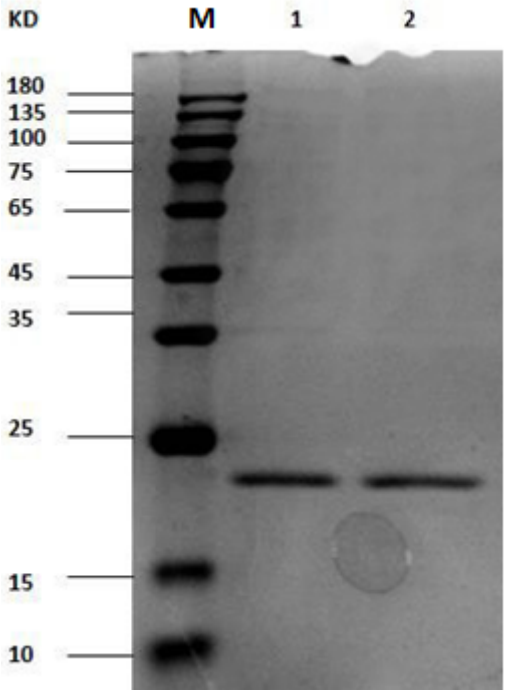

Figure 7

Expression and purification of the recombinant sdAbs. The plasmids of the sdAb 5 and sdAb 31 from phage ELISA were isolated, and the VHH gene was digested with *Nco*Ⅰand *Not*Ⅰ and ligated to a pET-22b (+) −SBP. SDS-PAGE results showed that the recombinant sdAb 5 and sdAb 31 antibody were expressed in a soluble form with an expected molecular weight of 20 kDacan successfully purified from the Ni−NTA Agarose.

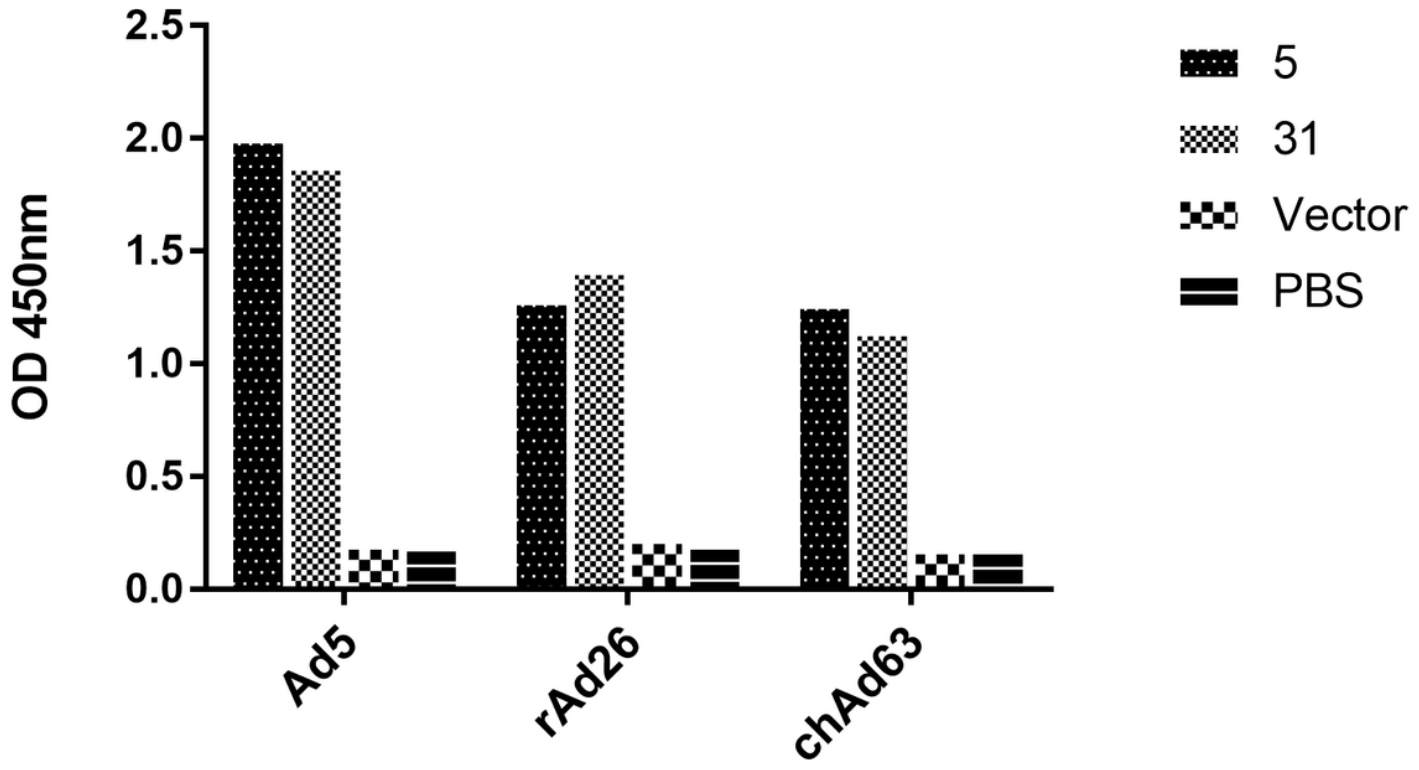

Figure 8

Binding activity and specificity was analyzed by indirect ELISA. The recombinant sdAb 5 and 31 showed strong binding activity to human adenovirus Ad5, rAd26, and chAd63 viruses in ELISA assay at the concentration of 5 μg/ml. The protein extracts from *E. coli* that transformed with empty pET-22b(+) vector was used as negative control and PBS solution was used as blank control.

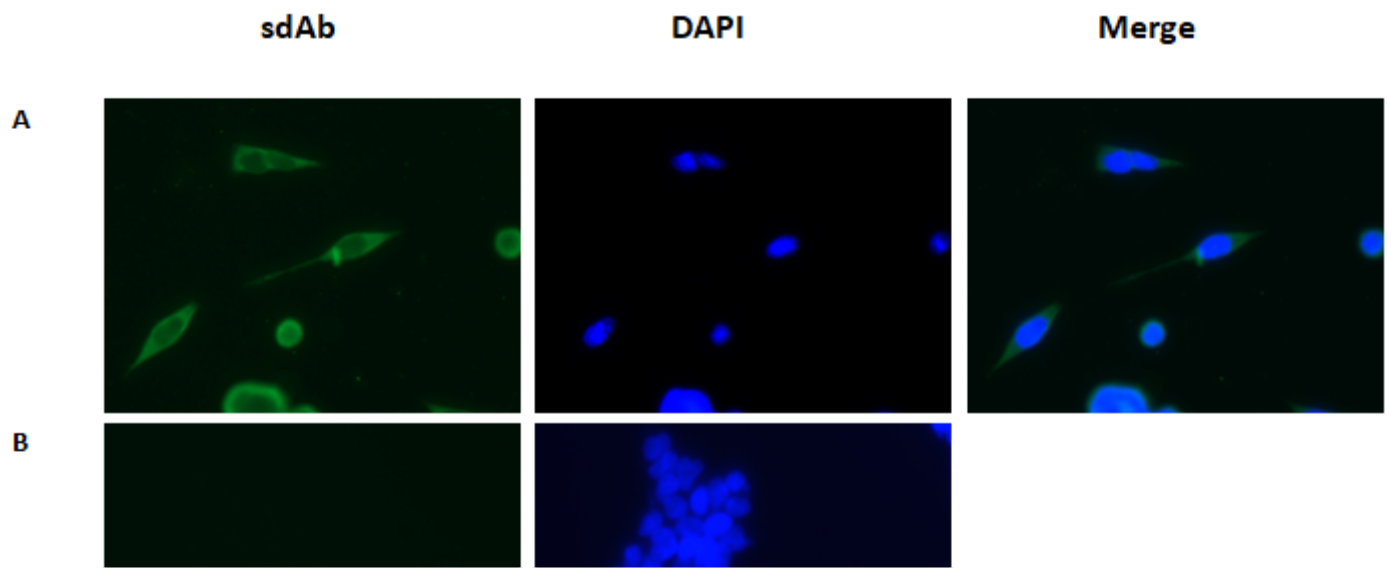

Figure 9

Immunofluoresence assay. Adenovirus-infected HEK293A cells were incubated with sdAb 5 antibody, and visualized with CoraLite®488-conjugated 6*His His-Tag Mouse Monoclonal antibody. In parallel, the HEK293A cell uninfected with adenovirus rAd26 was used as negative control. The cell nucleus were stained with DAPI. The fuorescent signal and images were obtained through confocal microscopy. The merged images showed that the localization of adenovirus rAd26 in HEK293A cells (Fig 9A), but there is no fluorescent signal in the uninfected HEK293A cells (Fig 9B).

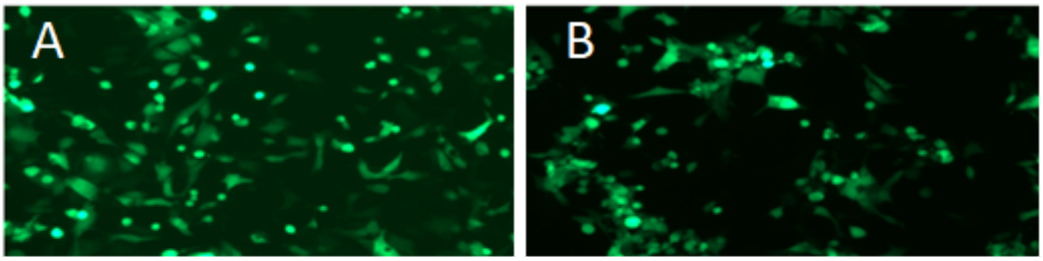

Figure 10

Immunoaffinity purification of adenovirus. The bound viruses were eluted with 2 M, 4 M NaCl solution and PBS and the eluent was added to the cultured HEK293A cell for reinfection. Meanwhile, set up a blank and negative control group. After inoculation, they were placed at 37°C, 5% CO_2 incubator for 24 h to 48 h, then observed and counted. A: 2 M eluent test group; B: 4M eluent test group; C: blank control group; D: PBS buffer test group. The green florescence was detected in both 2 M and 4 M NaCl solution eluent infected cells in Fig.10A and B but didn't detect in PBS eluent infected cells and non-infected cells as shown in Fig.10C and D. These results indicated that the immobilized sdAb can specifically bind to the adenoviruses and isolate them from culture medium,