NANOBODY AGAINST N PROTEIN OF CANINE DISTEMPER VIRUS AND ANTIVIRAL APPLICATION THEREOF

TECHNICAL FIELD

The invention relates to the fields of biotechnology and veterinary biomedicine, in particular to a nanobody against N protein of canine distemper virus and its antiviral application.

BACKGROUND

Canine distemper is a highly contagious and multisystem-involved viral disease caused by canine distemper virus. Clinically, canine distemper can be manifested as fever, respiratory symptoms, digestive tract disorder and hyperkeratosis of the skin, and in severe cases, nervous system disease or even death occurs, with a mortality rate as high as 50%-80%, which poses a serious threat to the health of companion animals, animal husbandry safety and biodiversity protection.

Antibody biological agents show broad prospects in the field of antiviral therapy. Traditional IgG antibody has high molecular weight, poor tissue penetration and high production cost, and it is difficult to enter the key link of targeted virus replication in cells. In addition, the process of canine-derived or humanized transformation is complex, which is easy to cause immunogenicity problems.

Nanobody, that is, a single-domain antibody derived from the variable domain of heavy chain antibody (VHH) in camel family, has become an ideal candidate for a new generation of diagnostic molecules because of its unique advantages such as small molecular weight, simple structure, high affinity, strong stability, easy engineering and cell membrane penetration.

However, up to now, nano-antibodies with high affinity, broad-spectrum recognition and both therapeutic and diagnostic functions against CDV N protein have not been reported publicly. Most of the existing research focuses on the development of neutralizing antibodies against F protein or H protein, while ignoring the key value of N protein as an intracellular target in inhibiting virus replication.

To sum up, the existing technology has the following main shortcomings: the lack of effective treatment means: there is no approved specific anti-CDV drugs, especially the lack of biological agents that can enter cells to interfere with virus replication;

The performance of diagnostic tools is limited: the traditional antibody has poor stability and incomplete coverage of mutant strains, so it is difficult to popularize molecular detection methods at the grassroots level;

The existing antibody platform is not suitable: conventional IgG is difficult to target intracellular virus components, and the production and transformation costs are high;

Application blank of nanobody: the functional nanobody against CDV N protein has not been systematically developed and verified.

Therefore, it is urgent to develop a nanobody with high affinity, high stability and broad spectrum to recognize CDV N protein, so as to meet the dual needs of accurate diagnosis and effective treatment at the same time and fill the key technical gap in the current canine distemper prevention and control system. Therefore, a nanobody against N protein of canine distemper virus and its antiviral application were proposed.

SUMMARY

The invention provides the following technical scheme: a nanobody against N protein of canine distemper virus, wherein the nanobody is a single-domain antibody derived from the variable domain of heavy chain antibody (VHH) of Camelidae, can specifically bind the nucleocapsid protein (N protein) of canine distemper virus (CDV), and has biological activity of inhibiting the replication of CDV in a host cell.

As the preferred technical scheme of the invention, the binding affinity KD value for N protein of canine distemper virus is not higher than 10 nM.

As a preferred technical scheme of the invention, the amino acid sequence comprises three complementary determining regions (CDR1, CDR2 and CDR3), wherein the CDR3 region is 12-18 amino acids in length and contains at least one aromatic amino acid or amino acid residue with positive charge, so as to mediate high affinity binding with N protein conserved epitopes.

As the preferred technical scheme of the invention, the N protein binding activity is still not less than 80% after being treated for 1 hour in the temperature range of 4°C to 60°C.

A pharmaceutical composition for resisting canine distemper virus comprises a therapeutically effective amount of the nanobody according to claim 1, and a pharmaceutically acceptable carrier, excipient or stabilizer.

As the preferred technical scheme of the invention, the administration form is selected from injection, nasal spray, inhalation or modified oral preparation for preventing or treating canine distemper virus infection.

A canine distemper virus detection kit comprises the nanobody of claim 1, and is used for capturing or detecting N protein of canine distemper virus in a biological sample.

As the preferred technical scheme of the invention, the detection method is one of enzyme-linked immunosorbent assay (ELISA), colloidal gold immunochromatography, immunofluorescence staining or Western blot.

A fusion protein, the fusion structure of the nanobody and effector molecules, wherein the effector molecules are selected from human or canine IgG Fc fragments, alkaline phosphatase, horseradish peroxidase, green fluorescent protein (GFP) or membrane-penetrating peptide sequences.

A nucleic acid molecule encoding the nanobody, an expression vector containing the nucleic acid molecule and a host cell transformed with the expression vector, wherein the host cell is an Escherichia coli, a yeast or a mammalian cell.

Compared with the prior art, the invention has the beneficial effects that:

The nanobody of the invention has nano-molecular binding affinity for canine distemper virus (CDV) N protein (KD≤2 nM), and does not cross-react with common canine pathogens such as canine parvovirus, canine adenovirus and canine coronavirus, thus ensuring the accuracy and reliability of detection or intervention in complex biological samples.

The nanobody of the invention can effectively identify N proteins of various CDV epidemic strains, including Asia, 1, America, 1, Europe, 2 and other genotypes, indicating that its targeting epitope is highly conservative, which is suitable for the prevention and control of canine distemper in different regions of the world and overcomes the problem of diagnosis or treatment failure caused by virus variation.

The nanobody of the invention can effectively inhibit the replication of CDV in host cells, reduce the viral load by more than 98%, significantly relieve clinical symptoms in infected animal models, improve the survival rate, show good therapeutic potential, and is especially suitable for early disease intervention.

The nanobody of the present invention maintains structural integrity in the range of 4°C to 60°C, and still maintains high binding activity under the condition of pH 4.0-9.0, which is convenient for normal temperature transportation and long-term storage, and is especially suitable for application in resource-limited environments such as grass-roots veterinary stations, remote areas or wildlife protection sites.

No obvious toxicity, allergic reaction or adverse effects on hematology and biochemical indexes were observed in single and repeated drug administration tests in dogs, and the immune response of the existing CDV live vaccine was not affected, which had a safe basis for clinical transformation.

In the diagnosis aspect, the invention is suitable for various platforms such as ELISA, colloidal gold test strips, chemiluminescence and the like, has high sensitivity (LOD is less than or equal LOD≤0.2 ng/mL), is simple and convenient to operate, and meets the dual requirements of on-site rapid screening and laboratory accurate detection; by fusion with Fc fragment, fluorescent label or cell-penetrating peptide, it can be expanded into a long-acting therapeutic agent, visual probe or intracellular delivery carrier, and realize a closed loop of diagnosis, treatment and monitoring.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a block diagram of the core characteristic data of a N protein of canine distemper virus nanobody provided by the present invention;

Fig. 2 is a block diagram of derivative application data based on nanobody provided by the present invention;

Fig. 3 is a data block diagram of subdivision parameters of nanobody derivative products provided by the present invention.

DESCRIPTION OF THE INVENTION

In order to make the purpose, technical scheme and advantages of the embodiment of the invention more clear, the technical scheme in the embodiment of the invention will be described clearly and completely with the attached drawings. Obviously, the described embodiment is a part of the embodiment of the present invention, not the whole embodiment.

Example 1: The full-length open reading frame of N protein coding gene (GenBank accession number: AF378805) of canine distemper virus (CDV, Onderstepoort strain) was cloned into prokaryotic expression vector pET-28a(+), and the recombinant plasmid pET28a-CDV-N was constructed. The plasmid was transformed into competent cells of E. coli BL21(DE3), cultured at 37°C until OD_{600}≈0.6, and IPTG with a final concentration of 0.5 mM was added, and induced to express at 16°C for 16 hours. Bacteria were collected, ultrasonically crushed and purified by Ni-NTA affinity chromatography to obtain high-purity soluble His-tagged N protein. SDS-PAGE and Western blot showed that its molecular weight was about 55 kDa, and it could be specifically recognized by anti -His antibody and CDV positive dog serum for subsequent immunization and screening.

Example 2: Construction and screening of nanobody immune library: Healthy adult alpacas (Vicugna pacos) were selected, and the purified CDV N protein (50μg/time, emulsified with Freund's adjuvant) was immunized subcutaneously for 4 times, with an interval of 14 days. Peripheral blood was collected on the 7th day after the last immunization, lymphocytes were isolated, total RNA was extracted, and cDNA was synthesized by reverse transcription. The VHH gene fragment (about 400 bp) was amplified by specific primers, then ligated into the phage display vector pComb3XSS, and transformed into E. coli TG1, and an immune phage display library with a capacity of about 1×10^{9}CFU was constructed.

The immune tube was coated with purified CDV N protein, and four rounds of biopanning were carried out: the first round used 10μg/mL N protein, and the subsequent rounds were gradually reduced to 100 ng/mL to improve the screening rigor. After the fourth round, 96 monoclonal antibodies were randomly selected for ELISA screening. The results showed that 23 clones were strongly positive for CDV N protein, and did not bind to BSA or unrelated virus proteins (such as canine parvovirus VP2). Five clones with the strongest signal were selected for sequencing, and candidate nanoantibody sequences were obtained, among which clone Nb-CDV-N1 was selected as the representative molecule for subsequent research.

Example 3: Expression and Purification of Nanoantibody Nb-CDV-N1

The VHH coding sequence of Nb-CDV-N1 was subcloned into the expression vector pET-22b(+), and the C-terminal was fused with 6×His tag. Transformed into Escherichia coli BL21(DE3), cultured at 37°C to logarithmic growth stage, and induced to express at 25°C with 0.2 mM IPTG for 6 hours. Collecting thalli, ultrasonically cracking, taking supernatant, purifying by Ni-NTA affinity chromatography, and finely purifying by Superdex 75 gel filtration chromatography to obtain high-purity monomer nanobody. SDS-PAGE showed a single protein band with a molecular weight of about 15 kDa. Dynamic light scattering (DLS) analysis shows that it has good monodispersity and no obvious aggregation.

Example 4: Affinity and Specificity Identification of NB-CDV-N1

The binding kinetics of Nb-CDV-N1 and CDV N protein was determined by biological layer interference technique (BLI, Octet RED96 system). His-tagged N protein was immobilized on a Ni-NTA sensor probe, and NB-CDV-N1 was diluted gradient (0.1–100 nM) for binding and dissociation analysis. The results show that its equilibrium dissociation constant KD=1.8 nM, and it has high affinity of nanomolar order.

Cross-reaction experiments showed that Nb-CDV-N1 did not bind to the corresponding structural proteins of canine parvovirus (CPV), canine adenovirus type 2 (CAV-2), canine coronavirus (CCoV) or rabies virus (RABV) under the same conditions, which proved its high specificity.

Example 5: Thermal Stability and pH Tolerance Test of NB-CDV-N1

The purified Nb-CDV-N1 was incubated at 4°C, 25°C, 37°C, 50°C and 60°C for 1 hour respectively, and its binding activity to CDV N protein was detected by ELISA after cooling to room temperature. The results showed that 87% of the binding capacity remained after treatment at 60°C, indicating its excellent thermal stability.

In addition, Nb-CDV-N1 was incubated in buffer with pH of 3.0–10.0 for 2 hours at room temperature, and the activity was detected after being adjusted back to neutral pH..

In the range of pH 4.0-9.0, the retention rate of binding activity is higher than 82%, which proves that it has good pH tolerance and is beneficial to the development and storage of preparations.

Test example 1: Cross-binding ability test of nanobody with N protein of different CDV strains

Objective: To evaluate the broad-spectrum recognition ability of nanobody Nb-CDV-N1 to N protein of canine distemper virus from different geographical sources or genotypes.

Methods: N proteins of five representative CDV strains were expressed and purified, including Onderstepoort (vaccine strain), America-1 (classic wild strain), Asia-1 (Asian epidemic strain), Europe-2 (European wild strain) and Siberia (Siberian strain). By indirect ELISA, the enzyme-labeled plate was coated with each N protein (1μg/mL), and Nb-CDV-N1 with the same concentration (1μg/mL) was added, and HRP was used to label the anti-His secondary antibody, and the OD_{450} value was determined.

Results: Nb-CDV-N1 showed strong positive signals for all five CDV N proteins (OD_{450}>1.8), and there was no significant difference (P>0.05), indicating that its recognition epitope was highly conserved, and it was suitable for the detection and intervention of CDV infection worldwide.

Test example 2: Evaluation of diagnostic efficacy of nanobody in clinical samples of canine distemper

Objective: To verify the accuracy of detection reagent based on Nb-CDV-N1 in real clinical environment.

Methods: 60 dog samples suspected of canine distemper (including 30 serum samples, 20 nasopharyngeal swabs and 10 cerebrospinal fluid samples) were collected simultaneously:

(i) the double nanobody sandwich ELISA of the present invention;

(ii) commercial RT-QCPR kit (targeting CDV N gene);

(iii) virus isolation and culture (gold standard).

Based on the positive virus isolation, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ELISA were calculated.

Results:

Positive samples of virus isolation: 42;

ELISA was positive: 40 samples, 2 false negative samples (all early cerebrospinal fluid samples);

False positive: 0 copies.

Sensitivity =95.2%(40/42), specificity =100%(18/18), PPV=100%, NPV=90.0%.

The results show that the nanobody diagnosis system has high reliability, especially suitable for the scene where there is no molecular detection condition at the grass-roots level.

The above embodiments are only used to illustrate the invention, but not to limit the technical solutions described in the invention. All technical solutions and improvements that do not depart from the spirit and scope of the invention are included in the scope of the claims of the invention.