Current Science
CS 5 (3), 2310-2316 (2025)
Received 16 Feb 2025 | **Revised 12 March** 2025 | **Accepted 15 April**. 2025 | **Online Available 13 May** 2025
https://doi.org/ 10.52845/CS/2025-5-3-21

Current Science

Current Science

OPEN ACCESS

ISSN(O)2795-8639

Original Article

Isolation of Nanobodies Targeting the Secreted DPS Protein of *Brucella*

Lina He[1], Heiya Na[1], Yaoxin Zhang[2], Tianyu Guo[3], Huanmin Zhou[4]*, Fang Wan[5]*

[1]**College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, China**

[2]**College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, China**

[3]**College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, China**

[4]**College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, China**

[5]**College of Life Sciences, Inner Mongolia Agricultural University, Hohhot, China**

[*]**Corresponding Author: Fang Wan, Huanmin Zhou**

Abstract:
In this study, we identified and characterized two nanobodies, Nb29 and Nb45, that specifically target the secreted DNA-binding protein Dps of *Brucella* using a yeast surface display library. Following four rounds of screening with magnetic bead-based selection, Nb29 and Nb45 were isolated and validated by flow cytometry and ELISA. Nb29 exhibited a binding affinity of 0.468 μM, while Nb45 showed moderate affinity with 1.384 μM. Upon fusion via a glycine-serine linker, the resulting nanobody dimer demonstrated enhanced avidity (0.1912 μM). These nanobodies, displaying specificity for Dps, owing to their small molecular size and improved avidity in dimeric form, hold promise as molecular tools for diagnostic development and potential therapeutic strategies against Brucellosis. Our findings provide a foundation for further engineering and application of nanobody-based technologies in targeting *Brucella* virulence factors.
Keywords : Brucellosis ; Brucella secretory protein Dps ; Nanobody

Introduction

Brucellosis, caused by bacteria of the genus *Brucella*, is a major zoonotic disease with significant public health and economic burdens worldwide(P. Sun et al., 2024). Transmission to humans and animals typically occurs through direct contact with infected animals or contaminated animal products, such as unpasteurized dairy, raw meat, wool, or animal care materials (Mukhametova et al., 2024). The pathogen's ability to survive and replicate within host macrophages is central to its virulence. *Brucella* utilizes a type IV secretion system (T4SS) to deliver a range of effector proteins into host cells, facilitating intracellular survival, immune evasion, and chronic infection. To date, at least 15 T4SS-dependent effector proteins have been identified(Celli, 2019), many of which disrupt host cellular pathways, including protein secretion, iron metabolism, and autophagy(Myeni et al., 2013).

Among these effectors, the DNA-binding protein from starved cells (Dps) plays a dual role in *Brucella* pathogenesis. Internally, Dps is a nucleoid-associated protein critical for genome organization and resistance to acid and oxidative stress(S. Anbazhagan et al., 2023). Externally, secreted Dps has been detected in the supernatant of cultured *B. melitensis* and shown to interfere with host iron metabolism by binding cytosolic iron, reducing reactive oxygen species (ROS) production, and promoting bacterial survival and host cell necrosis(Hop et al., 2023; Lee et al., 2014). Importantly, Dps elicits strong immune responses in infected animals but not in those vaccinated with *B. abortus* S19, making it a promising candidate for differentiating infected from vaccinated animals (DIVA) (Prachita Nandini et al., 2023).

Currently, nanobody screening libraries include phage display, yeast surface display (YSD), and

bacterial surface display libraries. YSD technology allows for high-throughput screening by displaying proteins on yeast cell surfaces, using *Saccharomyces cerevisiae*. YSD is a powerful platform for nanobody selection, offering advantages such as proper eukaryotic folding, large library sizes (up to 10^9 variants), and efficient screening via fluorescence- or magnetic-activated cell sorting (FACS/MACS).

This method involves fusing a protein-encoding gene with a yeast cell wall gene, followed by transformation and growth under selective conditions, Additionally, YSD simplifies the process by placing proteins directly on the cell surface, eliminating purification steps and reducing costs, it has emerged as a valuable tool for the selection of nanobodies, facilitating the generation of nanobodies with affinities ranging from micromolar to nanomolar levels(Chen et al., 2024; Y. Sun et al., 2024).This technology is also suitable for constructing antibody libraries, and developing whole-cell biocatalysts, biosensors, and oral vaccines, demonstrating significant potential in biotechnology and biomedicine.

2. Materials and Methods

2.1 *B. melitensis* Dps Sequence Analysis

The *Dps* sequence was obtained from the National Center for Biotechnology Information（NCBI）. Signal sequence predictions were made using the SignalP 4.1 server. Homologous sequence analysis was conducted using BLAST and Clustal Omega. Phylogenetic analysis was performed with MEGA 6 software.

2.2 Expression and Purification of pET28a-*Dps*

The *Dps* gene from *B. melitensis* was synthesized by GenScript and inserted into the pET28a(+) expression vector in-frame. Protein expression was induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 6 hours. The recombinant protein was purified using affinity chromatography. Protein purity was assessed by 15% SDS-PAGE, and concentration was determined using the BCA assay.

2.3 Expression Test of Yeast-Surface Display Library

The Yeast-surface Display Nanobody Library (NbLib) was purchased from Kerafast, Inc. One milliliter of NbLib was diluted into 500 mL of Ygal4.5-Trp medium supplemented with 1% Pen-Strep and incubated at 28°C with shaking (230 rpm) for approximately 60 hours. A total of 1.5×10^7 yeast cells were harvested. In the experimental group, 0.5 μg anti-HA antibody and 1 μM labeled protein were added and incubated at 4°C for 60 minutes. The cells were then centrifuged, washed 2–3 times, and resuspended in 500 μL PBS for flow cytometry analysis.

2.4 Screening of Dps-Specific Yeast Clones

Cultured yeast cells were counted and transferred to a 10 mL tube, centrifuged, resuspended, and incubated with FITC/PE cocktail and rapid spheres. The cells were captured using a magnet, centrifuged again, and resuspended. Labeled protein was added, and the mixture was incubated. After incubation, the protein-antibody complexes were sorted, and the selected cells were transferred into fresh medium for further culture. The cultures were expanded in Yglc4.5-Trp medium.

2.5 Amplification of Positive Nanobody clones

Following multiple rounds of screening, single clones were transferred into 15 mL tubes containing 3 mL Ygal4.5-Trp medium and cultured at 28°C with shaking at 230 rpm for 24 hours. A 100 μL aliquot of the yeast suspension was centrifuged, and 5 μL of lysis buffer was added. The mixture was incubated at 98°C for 15 minutes to lyse the cells. After centrifugation, 2 μL of the supernatant was used as the template for PCR amplification. The resulting PCR products were purified and sequenced to obtain the nanobody sequences.

Table.1 Primer sequences

Primer name	Primer sequence
F	CGCTGCTAAGGAAGAAGGTGTT
R	GGAAGACGATGAACTGGACGAA

2.6 Detection of Nanobodies-Dps Affinity by Flow Cytometry

After sorting, positive cells were transferred into 5 mL of -Trp + Galactose medium and cultured at

30°C with shaking at 230 rpm for 48–72 hours. The culture was diluted 100-fold with PBS, and cell density was determined using a hemocytometer. A total of 10^8 cells were transferred into two 1.5 mL tubes, centrifuged, and resuspended in 100 µL selection buffer. To one tube, 1 µM PE-labeled target protein was added, while the second tube served as a negative control. The cells were incubated at 4°C for 40 minutes, followed by flow cytometry analysis using CytExpert 2.0 software.

2.7 Interaction of Dps with Nanobodies

Dps-nanobodies was expressed by adding 0.1 mM IPTG and incubated for 16 hours at 16°C. The protein was purified using Ni resin. A 96-well plate was coated overnight at 4°C with 5 µg Dps protein. Following blocking with 1% BSA, nanobodies (diluted 5-fold from 2.6 mg/mL) were added and incubated for 1 hour at 37°C. After washing, a diluted enzyme-conjugated secondary antibody was added and incubated for 1 hour. The reaction was developed using TMB substrate, and absorbance at 450 nm was measured to determine titer.

3. Results

3.1 Identification of *B. melitensis* Dps Homologue

The nucleotide and deduced amino acid sequences of the full-length Dps protein consist of 165 amino acids (Fig. 1A). Multiple sequence alignments of *B. melitensis* Dps with homologous Dps proteins from various *Brucella* species were performed using Clustal Omega (Fig. 1B). The amino acid sequence identity between *B. melitensis* Dps and its orthologs in other *Brucella* species was highly conserved. Phylogenetic analysis revealed that *B. melitensis* Dps clustered with Dps proteins from other *Brucella* species, forming a distinct clade separate from other bacterial species (Fig. 1C). These findings suggest that Dps is highly conserved across *Brucella* species and may play a similar role in their pathogenicity.

3.2 Recombinant Protein Expression, Purification, and Characterization

The *B. melitensis* Dps gene was subcloned into the pET28a(+) vector and expressed in *Escherichia coli* BL21(DE3). The soluble recombinant pET28a-*Dps* protein was purified using Ni-affinity chromatography. SDS-PAGE analysis revealed a specific band at approximately 20 kDa, corresponding to the expected size of the recombinant protein (Fig. 1D and Fig. 1E).

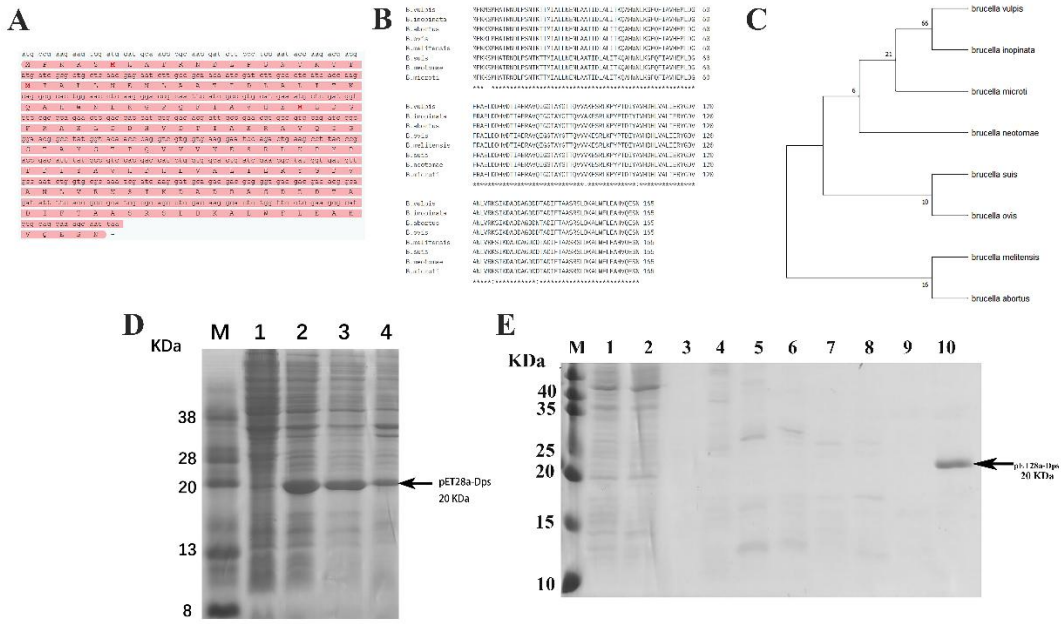

Figure 1 Conservative analysis, expression and purification of Dps

A: Nucleotide and deduced amino acid sequences of Dps from *B.melitensis*. The putative signal sequences were shaded. B: Multiple amino acid sequence alignments between Dps of *B. melitensis*

and other Dps of *Brucella* species (*B. neotomae, B. vulpis, B. suis, B. ovis, B. microti, B. inopinata and B.abortus*) was performed by Clustal Omega program. Asterisks denote identical amino acids. C: Phylogenetic tree of representative Dps members from *Brucella* species as described above. The neighbor-joining distance tree was constructed based on the entire amino acid sequence alignments of Dps members. Branch confidence levels were based on 1000 bootstrap reveal an evolutionarily ancient split into the Brucella Dps branch. D-E: SDS-PAGE analysis of the recombinant pET28a-*Dps* protein. The protein separation was visualized with Coomassie brilliant blue R-250. M: stained protein standards; 1: Whole cells of pET28a-*Dps* without IPTG induction for the expression of pET28a-*Dps* protein; 2: Whole cells of pET28a-*Dps* with 1mM IPTG induction for the expression of pET28a-*Dps* protein; 3: Supernatant from lysed *E.coli* cells induced with 1mM IPTG for the expression of Dps protein; 4: Pellet from lysed *E.coli* cells induced with 1mM IPTG for the expression of pET28a-Dps protein. E1: Flow-though; E3- E10: Imidazole eluents with different concentration gradients.

3.3 Nanobody Expression Test in NbLib

To assess the binding capacity of the nanobodies, flow cytometry analysis was performed after each round of sorting. As the concentration of Dps decreased, the nanobody positivity rate progressively increased. By the third round of sorting, resulting in a final nanobody positivity rate of 3.81% of the library (Fig. 2).

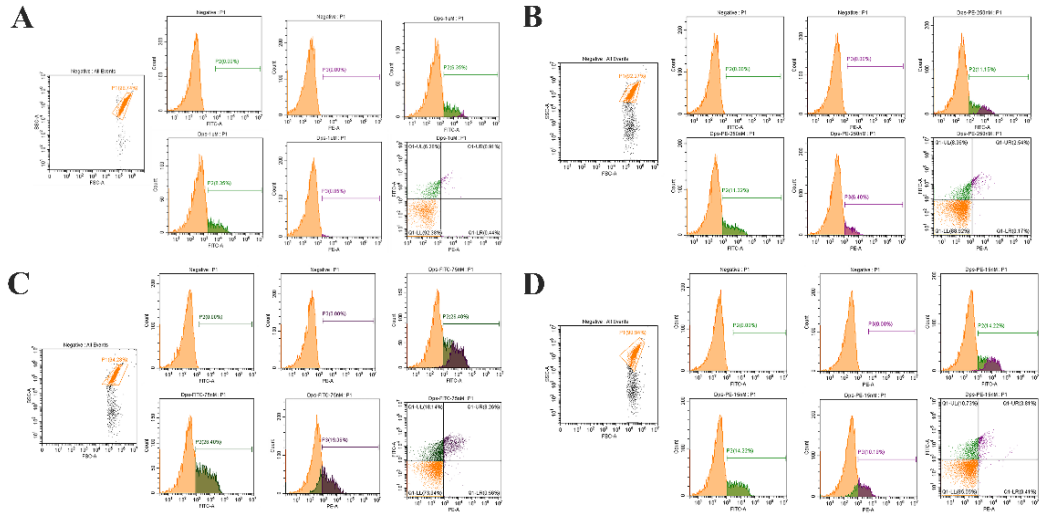

Figure 2 Analysis of the positive rate of nanobody sorting

A: Analysis of the positive rate of the first round of nanobody sorting ; B: Analysis of the positive rate of the second round of nanobody sorting ; C: Analysis of the positive rate of the third round of nanobody sorting ; D: Analysis of the positive rate of the fourth round of nanobody sorting ;

3.4 Identification of Dps-Nanobodies

Nanobodies were identified using colony PCR. Target bands were detected by agarose gel electrophoresis, and the samples matching the expected fragment size were selected for DNA recovery. These samples were subsequently sequenced to confirm the identity of the nanobody. The affinity of Dps-Nanobodies was further characterized by flow cytometry (Fig. 3).

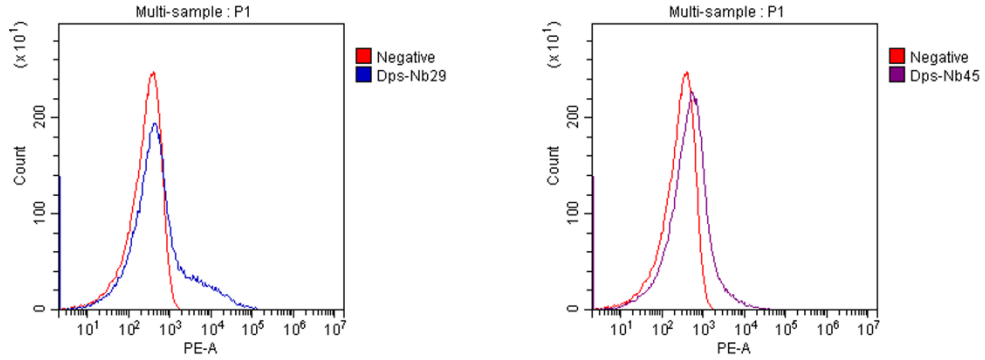

Figure 3 Flow cytometry was used to analyze the affinity between nanobodies and antigen

3.5 Determination of the Affinity Between Dps and Dps-nanobodies

The binding affinity of Dps-Nb29、Dps-Nb45 and dimer for Dps was assessed using ELISA . ELISA results revealed a dissociation constant of 0.468 μM for Dps-Nb29,1.384 μM for Dps-Nb45,0.1912 μM for dimer (Fig. 4).

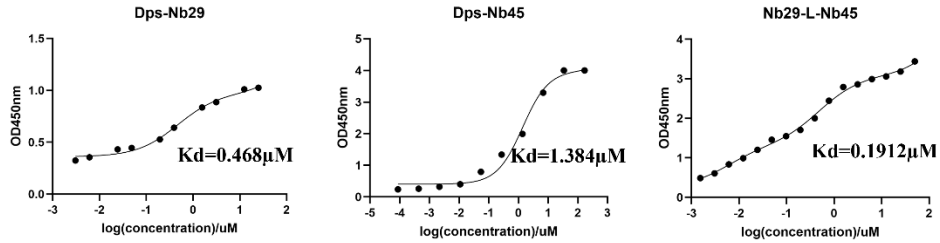

Figure 4 ELISA was used to detect the affinity between nanobodies and Dps

4. Discussion and Conclusion

Brucellosis is a global zoonotic disease with significant economic and public health implications, affecting both animals and humans(Qureshi et al., 2023). It can lead to acute and chronic health complications, underscoring the need for a better understanding of its molecular mechanisms to develop effective control strategies(Huang et al., 2024). Despite ongoing research, the molecular targets for therapeutic interventions remain limited, highlighting the importance of identifying new targets such as *Brucella* Dps.

One of the most compelling findings in our study was the significant enhancement in binding affinity of Dps nanobodies upon dimerization. While the monomeric Nb45 displayed a binding affinity of 1.384 μM, its dimerized form exhibited a marked improvement, with an affinity of 0.1912 μM. This increase highlights the potential of nanobody dimerization as a strategy for enhancing binding affinity, which could substantially improve the effectiveness in both diagnostic and therapeutic applications. The observed increase in affinity following dimerization is likely attributed to the multivalent nature of the interaction, which enables stronger and more stable binding to the target antigen. These results are in line with previous studies demonstrating that the dimerization or multimerization of small binding domains, such as nanobodies, can significantly enhance binding strength and specificity, making them more suitable for applications that require high-affinity interactions(Liu et al., 2023; Weiss & Verrips, 2019).

In conclusion, we have identified and characterized two nanobodies, Nb45 and Nb29, that specifically bind to Dps. Notably, despite the moderate affinity of individual nanobodies like Nb45, their dimerized forms hold considerable promise for therapeutic applications. The ability to modulate the affinity of nanobodies by engineering them into multimeric forms may provide a key strategy for the development of highly specific and potent therapeutics. This finding opens avenues for further optimization of nanobody-based therapies, as modifications aimed at increasing binding affinity could enhance their therapeutic efficacy in treating Brucellosis or

other *Brucella*-related diseases.

Given that Dps typically exists as a dodecamer(Orban & Finkel, 2022), the capacity of nanobodies to form dimers greatly boosts binding affinity. This sets the stage for further development of nanobody polymers, especially those built on dimers, which can be tailored to interact with different polymer forms of Dps. Such advancements not only highlight the broad potential of nanobody engineering in elevating diagnostic and therapeutic applications but also mark a novel direction in this field, as there have been no prior reports of nanobodies targeting Dps. These explorations could be instrumental in creating more effective diagnostic tools and targeted therapies for Brucellosis.

Acknowledgments

We would like to express our gratitude to Dr. Andrew C. Kruse and Edward Paul, Harvey, PhD at Harvard University for providing the synthetic yeast surface display nanobody library via Kerafast, Inc., and for his valuable technical support. This work was supported by the Prairie Talent Leadership Program(DC2000000754), Inner Mongolia Natural Science Foundation(2024LHMS08055), Research funds of universities directly under the Inner Mongolia Autonomous Region(BR22-14-02).

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

References

1. Celli, J. (2019). The intracellular lifecycle of Brucella spp. *Microbiology Spectrum*, *7*(2), . https://doi.org/10.1128/microbiolspec.BAI-0006-2019
2. Chen, F., Liu, Z., Kang, W., Jiang, F., Yang, X., Yin, F., Zhou, Z., & Li, Z. (2024). Single-domain antibodies against SARS CoV-2 RBD from a two-stage phage screening of universal and focused synthetic libraries. *BMC Infectious Diseases*, *24*(1), 199. https://doi.org/https://doi.org/10.1186/s12879-024-09022-8
3. Hop, H. T., Huy, T. X. N., Lee, H. J., & Kim, S. (2023). Intracellular growth of Brucella is mediated by Dps-dependent activation of ferritinophagy. *EMBO reports*, *24*(9), e55376. https://doi.org/10.15252/embr.202255376 |
4. Huang, S., Xu, J., MenghanLu, QingZhen, Han, X., HaoWang, Ma, T., ZhuoLi, Wang, Y., Song, R., Zhang, Y., Hao, J., & Shui, T. (2024). Updatedtherapeutic options for human brucellosis: A systematic review and network meta-analysis of randomized controlled trials. *PLoS neglected tropical diseases*, *18*(8), e0012405. https://doi.org/https://doi.org/10.1371/journal.pntd.0012405
5. Lee, J. J., Lim, J. J., Kim, D. G., Simborio, H. L., Kim, D. H., Reyes, A. W. B., Min, W., Lee, H. J., Kim, D. H., Chang, H. H., & Kim, S. (2014). Characterization of culture supernatant proteins from Brucella abortus and its protection effects against murine brucellosis. *Comparative Immunology Microbiology and Infectious Diseases*, *37*(4), 221-228.
6. Liu, H., Wu, L., Liu, B., Xu, K., Lei, W., Deng, J., Rong, X., Du, P., Wang, L., Wang, D., Zhang, X., Su, C., Bi, Y., Chen, H., Liu, W. J., Qi, J., Cui, Q., Shuhui Qi 10, Fan, R., . . . Wang, Q. (2023). Two pan-SARS-CoV-2 nanobodies and their multivalent derivatives effectively prevent Omicron infections in mice. *Cell Reports Medicine*, *4*(2), 100918. https://doi.org/10.1016/j.xcrm.2023.100918.
7. Mukhametova, L. I., Zherdev, D. O., Eremin, S. A., Sclyarov, O. D., Babicheva, O. V., Motorygin, A.
8. V., Kuznetsov, A. N., Yudin, V. I., Tsvetkov, Y. E., Krylov, V. B., & Nifantiev, N. E. (2024). Applying a Fluorescence Polarization Assay for Detection of Brucellosis in Animals Using the Fluorescently Labeled Synthetic Oligosaccharides as Biosensing Tracer. *Biosensors (Basel)*, *14*(8),404. https://doi.org/10.3390/bios14080404
9. Myeni, S., Child, R., Ng, T. W., III, J. J. K., Wehrly, T. D., Porcella, S. F., Knodler, L. A., & Celli, J. (2013). Brucella Modulates Secretory Trafficking via Multiple Type IV Secretion Effector Proteins. *PLOS Pathogens*, *9*(8). https://doi.org/10.1371/journal.ppat.1003556
10. Orban, K., & Finkel, S. E. (2022). Dps Is a Universally Conserved Dual-Action DNA-Binding and Ferritin Protein. *JOURNAL OF BACTERIOLOGY*, *204*(5), e0003622. https://doi.org/ 10.1128/jb.00036-22.
11. Prachita Nandini, Padmaja Jakka, Subathra Murugan, Varadendra Mazumdar, Deepak Kumar, Richa Prakash, Sukhadeo B. Barbuddhe, &

Girish Radhakrishnan. (2023). Immuno-profiling of Brucella proteins for developing improved vaccines and DIVA capable serodiagnostic assays for brucellosis. *Frontiers in Microbiology*, *14*, 1253349. https: //doi.org/10.3389/fmicb.2023.1253349

12. Qureshi, K. A., Parvez, A., Fahmy, N. A., Hady, B. H. A., Kumar, S., Ganguly, A., Atiya, A., elhassan, G. O., Alfadly, S. O., Parkkila, S., & Aspatwar, A. (2023). Brucellosis: epidemiology, pathogenesis, diagnosis and treatment–a comprehensive review. *Annals of Medicine*, *55*(2), 2295398. https://doi.org/ https://doi.org/10.1080/07853890.2023.22953 98
13. S.Anbazhagan, K.M. Himani, R. Karthikeyan, Lakshmi Prakasan, M. Dinesh, Sonu S. Nair, Jonathan Lalsiamthara, Abhishek, S.G. Ramachandra, V.K. Chaturvedi, Pallab Chaudhuri, & Prasad Thomas. (2023). Comparative genomics of Brucella abortus and Brucella melitensis unravels the gene sharing, virulence factors and SNP diversity among the standard, vaccine and field strains. *International Microbiology*, *27*(1), 101-111. https://doi.org/10.1007/s10123-023-00374-w
14. Sun, P., Yang, X., Liu, J., Bao, Y., Qi, J., Han, X., Liu, G., Wang, S., & Tian, M. (2024). Development and Application of Colloidal Gold Test Strips for the Rapid Detection of Canine Brucellosis. *Biosensors (Basel)*, *14* (8), 388. https:// doi.org/https://doi. org/10.3 390/bios14080388
15. Sun, Y., Martinez-Ramos, C., Chen, E., Osawa, Y., & Zhang, H. (2024). A General Method to Screen Nanobodies for Cytochrome P450 Enzymes from a Yeast Surface Display Library. *Biomedicines*, *12*(8), 1863. https://doi.org/https://doi.org/10.3390/biomedi cines12081863
16. Weiss, R. A., & Verrips, C. T. (2019). Nanobodies that Neutralize HIV. *Vaccines*, *7*(3), 77. https://doi.org/10.3390/vaccines 703 0077.