Central Asian Journal of Medical Sciences
CAJMS

Original Article

Cent Asian J Med Sci. 2020 September 6(3):159-166. https://doi.org/10.24079/CAJMS.2020.09.007

Isolation of Heavy Chain Antibodies from *Camelus Bactrianus* Serum

Enkh-Ariun Altantulga[1], Lkhagvamaa Erdene[1], Ariun Narmandakh[1,2], Janbolat Ashim[1], Bayarjargal Munkhuu[1], Zolzaya Majigsuren[3], Batjargal Batdorj[4], Gan-Erdene Tudev[1]

[1]Laboratory of Biochemistry, Institute of Chemistry and Chemical Technology, Ulaanbaatar, Mongolia; [2]Biocomputation Group, School of Computer Science, University of Hertfordshire, United Kingdom; [3]Laboratory of Physiology and Pathology of Young Animals, Institute of Veterinary Medicine, Ulaanbaatar, Mongolia; [4]Department of Biology, National University of Mongolia, Ulaanbaatar, Mongolia

Submitted: August 25, 2020
Revised: September 1, 2020
Accepted: September 28, 2020

Corresponding Author
Gan-Erdene Tudev, Sc.D
Laboratory of Biochemistry,
Institute of Chemistry and,
Chemical Technology,
Mongolian Academy of Sciences,
Ulaanbaatar 13330, Mongolia

Tel: +976-9911-1209
E-mail: tudeviinganerdene@gmail.com

Objectives: The IgG antibodies from species of Camelidae are named heavy-chain antibodies that lack L-chains of typical antibodies. The antigen-binding site of the dromedary HCAb is located in the single-chain domain referred to as the nanobody. Nanobodies are distinguished from other conventional antibodies by their unique properties, which lead to numerous biopharmaceutical and medicinal applications. Therefore, this study aimed to isolate IgG protein by isoforms from the blood serum of Mongolia's two-humped camels and determine the contents of isomers of the antibody to enable further *C. bactrianus* nanobody research. **Methods:** IgG subclasses were separated by two different affinity chromatography steps using the ÄKTA Prime fast protein liquid chromatography method. The immunoglobulin fractions were determined using assays by the Bradford method and absorbance at 280 nm. The purity of IgG fractions was verified by the SDS-PAGE electrophoresis method. **Results:** Camel blood serum IgG subclasses were isolated by Protein A and G affinity chromatography columns, and the ratios of IgG1, IgG2, and IgG3 isotypes were 40.5 ± 4.5 kDa, 24.0 ± 8.0 kDa, and 35.5 ± 5.1 kDa, respectively. IgG2 and IgG3 subclass heavy chain bands on SDS-PAGE showed different molecular weights: 45 kDa and 43 kDa. **Conclusions:** Our findings suggest that IgG protein isotypes in the domestic *Camelus Bactrianus* serum that we identified were statistically different from *C.Dromedarius* serum.

Keywords: Serum, Affinity Chromatography, *Camelus Bactrianus*, Immunoglobulin G, Antibodies.

Introduction

The Camelidae family of species have acquired various unique attributes, such as surviving long periods without any food and water, tolerating a high dietary salt intake without developing hypertension, and tolerating high blood glucose levels without developing diabetes to adapt to the harsh climate conditions of deserts or semi deserts [1-3]. Bactrian camels are distributed mainly in Central Asian countries, including Mongolia, China, Kazakhstan, northeastern Afghanistan, Russia, Crimea and Uzbekistan [4]. The IgG antibodies from species of Camelidae (i.e. *Camelus dromedarius, Camelus bactrianus, Lama glama, L. guanicoe, L. alpaca* and *L. vicugna*) have what are called heavy-chain antibodies (HCAbs), that lack the light L-chain [5]. The absence of CH1 in the H-chain and lack of L-chains in IgG are the main characteristics of camelid HCAbs. Therefore, their architecture is compact and smaller, with a molecular weight of 90 kDa rather than 150 kDa for conventional antibodies [6].

The antigen-binding site of the Arabian camel's heavy-chain antibodies consists of one single domain. It is referred to as the variable domain of HCAb (VHH) or nanobody (Nb) [5]. The nanobody's hallmarks include small size (~15 kDa, 4 nm in length and 2.5 nm in width), high solubility, stability, specificity and affinity, ease of cloning and thermal and chemical resistance. Moreover, recombinant production in microorganisms is cost-efficient, and nanobodies can become the building blocks for multi-domain constructs [7]. Nanobodies are distinguished from other conventional antibodies by their unique properties of size, solubility, intrinsic stability, easy tailoring into pluripotent constructs, recognition of unique or hidden epitopes, binding into cavities or active sites of enzyme and inhibitor targets [8], allowing binding to a wide range of antigens [9] ease and speed of drug discovery, and ease of manufacture. These features are useful in several biopharmaceuticals and medicinal applications in which nanobodies excel over other antibody formats [8]. For example, Wrapp et al. [10] found that camelid specific immunoglobulins neutralized beta-coronary viruses. And an increasing number of studies show efficient epitope isomers of nanobodies that exhibit Covid-19 spike protein blocking [11].

Moreover, variously engineered nanobodies have been identified that play crucial roles in designing diagnostic tools in addition to being medicinal drugs, including cancer therapies [12]. Engineered nanobodies are used in immunohistochemical (IHC) staining, a powerful method that can reveal the neoplasms' biologic behavior and thus provide useful prognostic information [13]. Production of specific and sensitive immunoreagents requires developing methods for selection and generation of recombinant antigen-binding antibody fragments. The first of these was variable regions from conventional antibodies, cloned by PCR, then evolved, and selected by phage display methods. More recently, single-chain antibodies from camelids or selachians have been used for research, diagnostic, and therapeutic purposes [14]. Llama serum HCAbs contain as much as is 50% total IgG and camel serum as much as 75% [15], indicating that the prevalence of HCAbs varies among the species. Recent studies have revealed the genetic diversity, population structure, genome sequence of heavy chain antibodies from the milk of Mongolia's domesticated *Camelus bactrianus* [16-17], even though there is a lack of information on the ratio of IgG isoforms in *Camelus bactrianus* serum.

Therefore, this study aimed to isolate IgG protein by isoforms from the blood serum of Mongolia's two-humped camels and determine the contents of isomers of the antibody to enable further *Camelus bactrianus* nanobody research.

Materials and Methods

Blood sampling

Serum was collected from four castrated healthy male camels *Camelus bactrianus* camels housed at Sukhbaatar Soum, Sukhbaatar province, Mongolia. About 5 ml of camel blood was drawn from the jugular vein using a sterile needle into a vacuum blood collection tube. The serum was prepared by centrifuging for 10 min at 3000 rpm, transferred to a thermally-insulated icebox for transport, and stored at -20°C until the purification steps by affinity chromatography.

Protein determination using bradford assay

The Coomassie dye-binding assay, as described by Bradford, was used to measure low protein concentrations with high sensitivity in purified fractions. The protein content of the blood serum and immunoglobulin fractions were estimated using the method of Bradford. Each 5 µl samples and 200 µl Bradford reagent were vortexed gently to mix thoroughly, and the samples were incubated at room temperature for 5 min followed by the absorbance measure at 600 nm in a microplate reader. Bovine

serum albumin (Sigma Aldrich) was used as standard. The sample's protein concentration was determined by comparing the net absorbance value obtained at 600 nm against the standard curve [18].

Protein determination using absorbance at 280 nm

The determination of protein concentration by ultraviolet absorption depends on aromatic amino acids in proteins. The equation to estimate protein concentration is:

$$Protein\ concentration\ (mg/ml) = 1.55 \times D_{280} - 0.76 \times D_{260}$$

where D_{280} and D_{260} are the ultraviolet absorption at 260 nm and 280 nm, respectively [19].

Fractionation of IgG subclasses

Separation of different IgG subclasses from camel serum was conducted using ÄKTA prime fast protein liquid chromatography (ÄKTA FPLC™, GE Healthcare). This procedure was performed by differential adsorption of proteins on Hitrap Protein-A and Hitrap Protein-G affinity columns (GE Healthcare). Briefly, 1 ml camel serum was diluted with an equal volume of phosphate buffer (20 mM phosphate, pH 7.0) and loaded at a flow rate of 1 ml/min on the Protein-G column (1 ml) equilibrated with phosphate buffer previously. After one column-volume washing with phosphate buffer, eluting buffer A (100 mM acetate, pH 3.6) and eluting buffer B (100 mM glycine–HCl, pH 2.7) were used for unbound proteins and other serum ingredients. The column was washed intensively with phosphate buffer and was reloaded onto the column to remove the residual IgG subclasses in the flow-through fraction (the initial eluent or first fraction of a chromatography). The flow-through was captured and loaded onto the phosphate buffer-equilibrated Protein A column, then eluted by using buffer C (100 mM acetate, pH 3.0). Monitoring the UV absorption at 270 nm was facilitated for fractionation and sample collection. Once eluted, all IgG fractions were neutralized with 1.5 M Tris-HCl pH 8.8 by dialyzing in a phosphate buffer followed by quantifying and were stored at −20°C [20].

SDS-Polyacrylamide gel electrophoresis

The integrity and the purity of IgG fractions were verified by loading 2 µg protein (denatured and reduced) onto a 12.5% polyacrylamide SDS gel, followed by Coomassie blue staining [21]. All protein fractions were determined by absorbance at 280 nm and Bradford assay. We used two different protein markers, M1 (Lambda biotech – AccuRuller Plus G02102), M2 – protein marker mixed with BSA (67 kDa), Ovalbumin (45 kDa), Trypsin inhibitor (21 kDa), and Lysozyme (14 kDa) (all from Sigma Aldrich).

Statistical analysis

The mean and standard deviations (M ± SD) for all measured variables were calculated for the statistical analysis. The independent sample t-test was used to compare the means of our two IgG subclass different protein determination methods in this study. The one-sample t-test was used to compare the mean of our measurements to the peer-reviewed published values. Statistical significance was considered as $p<0.05$ and was adjusted using the Bonferroni correction to compensate for the increased risk of type 1 statistical error associated with multiple comparisons. All analyses were performed using SPSS (Version 25).

Ethics statement

Samples were collected following the "Guidelines for Ethical Conduct for Use and Care of Animals in Research" (American Psychological Association, 2012) and approved by the Ethics Care Committee, Mongolian University of Life and Sciences, Mongolia (NoMEBUS-19/02/04).

Results

Fractionation of IgG subclasses from *Camelus bactrianus*

All serum samples were filtered through a 0.22 µm membrane filter. The serum protein content was 60.6 ± 8.1 mg/ml, determined by Bradford assay. The IgG subclasses (IgG-1, 2 and 3) were fractionated from the camel serum by differential adsorption on the protein-G and protein-A column elution fractions performed with 6 repetitions (Figure 1, 2).

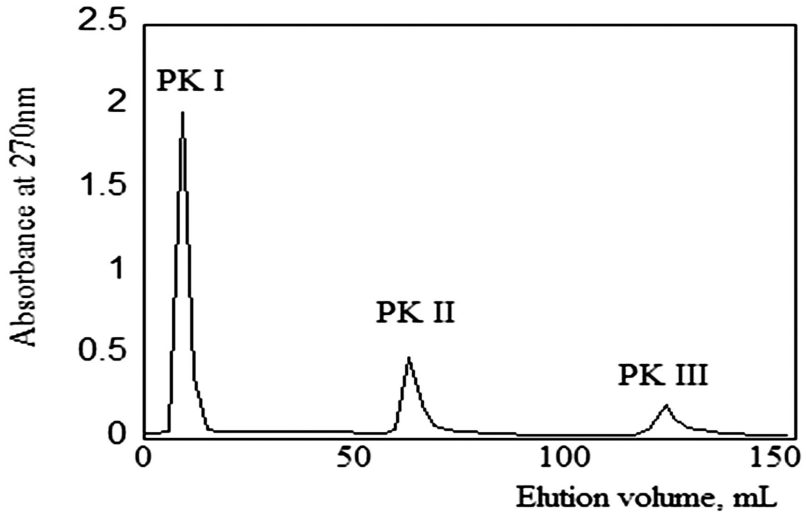

Figure 1. Purification profile of IgG1 and IgG3 from *Camelus bactrianus* serum by HiTrap Protein G column chromatography. (From 2 mL serum sample containing 22.6 mg/mL protein; Column: HiTrap Protein G HP, 1 mL; Loading buffer: 20 mM phosphate, pH 7.0; A buffer: 100 mM citrate, pH 3.6; B buffer: 100 mM glycine, pH2.7; Flow rate: 1 mL/min; Reader speed: 2 mm/min)

The PK I peak corresponding to the camel serum unbound protein fraction to Protein G column, which contains the IgG-2 subclass of antibodies, was eluted with 20mM phosphate buffer pH 7.0. Then IgG-3 was eluted with buffer A (100 mM acetate buffer, pH 3.6), which showed PK II. IgG-1 was then eluted with buffer B (100 mM glycine–HCl, pH 2.7), as illustrated in PK III (Figure 1). All peak fractions were collected, and their protein contents and molecular weights were determined.

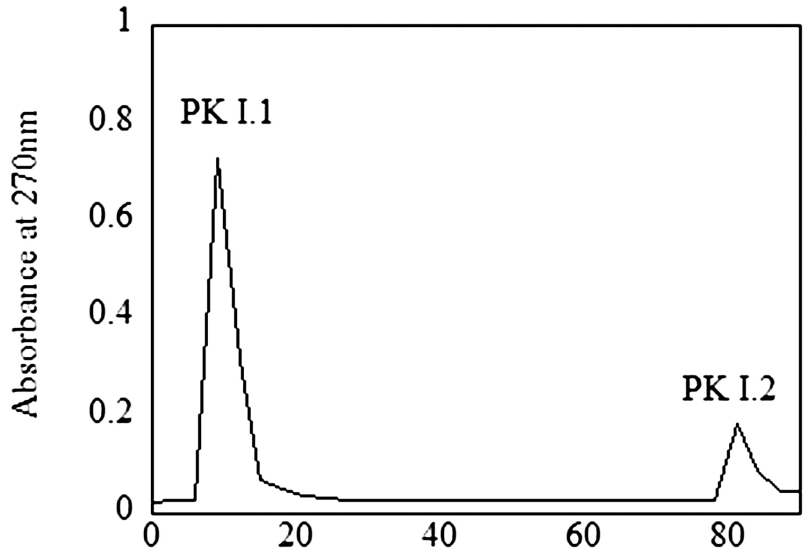

Figure 2. Purification profile of IgG2 from *Camelus bactrianus* serum by HiTrap Protein A column chromatography. (From camel serum unbounded protein fraction to Protein G with 7.5 mg/mL protein concentration; Column: HiTrap Protein A HP, 1 mL; Loading buffer: 20mM phosphate, pH 7.0; C buffer: 0.1 M citrate, pH 3.0; Flow rate: 1 mL/min; Reader speed: 2mm/min)

IgG2 fractions were purified from PK I by affinity chromatography on a 1 ml HiTrap Protein A column. Binding was then performed in 20 mM sodium phosphate (pH 7), and the IgG-2 subclass of antibody was eluted with 0.1 M citric acid (pH 3) presented as PK I.2 (Figure 2). The eluted IgG was collected and immediately neutralized to pH 7.0.

Molecular weight determination of IgG subclasses from *Camelus bactrianus*

The results of further purification detected by SDS PAGE gel electrophoresis (Figure 3) showed that each of the two protein bands appeared near the position of 43 kDa in the second and fourth lanes, which were consistent with the molecular weights of the Bactrian camel heavy chain's two IgGs subclasses.

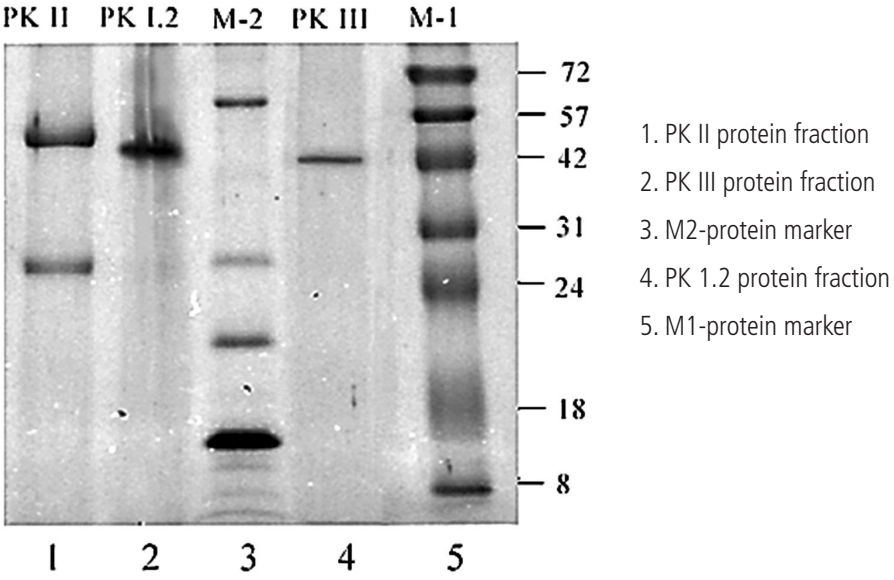

Figure 3. Characterization and purification of *Camelus bactrianus* IgG subclasses on Protein A and Protein G by the SDS-PAGE electrophoresis.

The molecular weight of HiTrap Protein G fractions, PK II and PK III (first and fourth lane respectively) and HiTrap Protein A fraction PK I.2 (second lane) were compared with the marker proteins. The PKII fraction contained 55 kDa and 25 kDa chains, consistent with the molecular weights of the Bactrian camel light chain's IgG1 subclass.

All fractions from HiTrap Protein G and HiTrap Protein A columns were determined in *C. bactrianus*, two-humped camel serum IgG subclasses ratio by their volumes and protein concentration. To evaluate the concentrations of the different IgG isotypes in serum from the Bactrian camel, we performed Bradford protein determination and absorbance at 280 nm serum collected from castrated healthy male camels. The results are summarized in Figure 4. As expected, HCAbs represented ~65% of the total serum IgG. According to our Bradford and A280 assays, the IgG3:IgG1:IgG2 ratios were 35:40:24 and 30:43:26, respectively. Independent sample t-tests compared our two IgG subclass different protein determination methods, and none were statistically significant ($p > 0.050$).

The content of our serum IgG subclasses samples were compared with *Camelus dromedarius* [24] and *Lama glama* [25] from recent studies using the one sample t-test as shown in Table 1. The IgG3 subclass concentration from *Camelus bactrianus* (Bradford method) was significantly different from *Lama glama* ($p = 0.001$). Likewise, the IgG3 subclass concentration from *Camelus dromedarius* was significantly

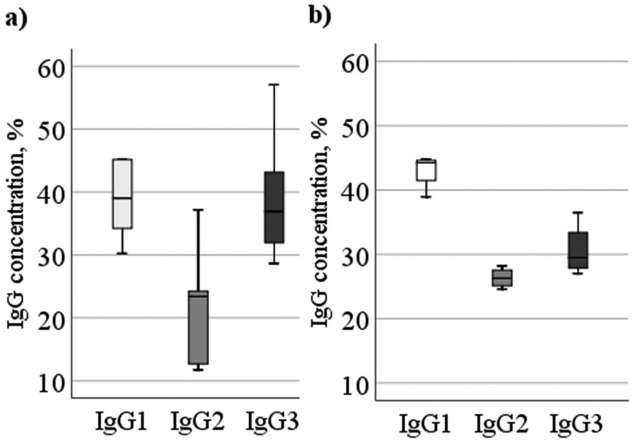

Figure 4. The percentage of concentrations of IgG isotypes in serum from *Camelus bactrianus* by different methods, a) Bradford method, b) spectrophotometric method (Absorbance at 280nm)

different from *Lama glama* (p = 0.010). The *Camelus bactrianus* IgG1 subclass (by both Bradford and A280) differed from *Lama glama* (p = 0.000 and p = 0.001, respectively) and *Camelus bactrianus* (by A280) differed from *C. dromedarius* (p = 0.000).

Our IgG3 and IgG1 subclasses concentrations (by A280 method) were not significant different from *Camelus bactrianus* (p = 0.045), and C. dromedarius (p = 0.01), because our Bonferroni-adjusted critical p-value was p ≤ 0.016. The differences in the concentration of IgG1 and IgG3 subclasses between *Camelus bactrianus*, and *Lama glama* were statistically significantly different.

Table 1. The content of serum IgG subclasses of *Camelus bactrianus* compared with *Camelus dromedarius* and Lama glama by different methods by a) Bradford method, b) spectrophotometric method (Absorbance at 280 nm)

	IgG concentration, %			
	Camelus bactrianus			
	Bradford (Mean ± SD)	**A280** (Mean ± SD)	***Camelus dromedariusa***	***Lama glamab***
IgG3	35.5 ± 5.1a	30.6 ± 3.4	30 - 45	5 – 15[a]
IgG1	40.5 ± 4.5b	43.0 ± 2.5c,d	~25d	55 – 75[b,c]
IgG2	24.0 ± 8.0	26.3 ± 1.4	25 - 30	20 – 30

The values are reported as mean ± SD (n = 6). Values with superscript letters a - d indicate significant differences (p < 0.050/3 or 0.016) within rows: [a]p = 0.001, [b]p = 0.0001, cp = 0.001 and dp = 0.01; [a]Content of serum IgG subclasses of *Camelus dromedarius* from Hamers-Casterman et al. [24]; [b]Content of serum IgG subclasses of *Lama glama* from Linden et al. [25]

Discussion

Camelus bactrianus serum IgG subclass antibodies, which originate against *Staphylococcus aureus* and Streptococcal bacteria with varying affinities, bind to Protein A and G affinity columns [22]. The IgG protein subclasses IgG1 and IgG3 in the serum of two-humped camels bind to Protein A and Protein G affinity columns, while the IgG2 isotype binds only to the Protein A affinity.

The molecular weight of *C. bactrianus* IgG1 is approximately 150 kDa, and its heavy and light chain molecular weights are 52 - 55 kDa, 20-29 kDa, respectively, according to Tillib [9] and Zhang [23]. Our study identified that *C. bactrianus* IgG1 subclass consists of heavy and light chains with the molecular weight 55 kDa and 30 kDa, respectively, which is PK II from the Protein G fraction (seen in Figure 3, lane 1).

The molecular weights of *C. bactrianus* IgG2 and IgG3 are approximately 80 ~ 90 kDa, and their heavy chain molecular weights are about 40 ~ 47 kDa, according to Zhang [23]. Our PK I.2 and PK III analysis found slightly different molecular weights, 45 kDa and 43 kDa (Figure 3, lane 4, 2), in SDS-PAGE. This protein band was detected as lightly stained because in the Bactrian camel, IgG subsets have only IgG1 with light chains and IgG2 and IgG3 naturally lack the light chains[23].

Due to the lack of previous studies on serum IgG biochemical characteristics from two-humped camels, we compared our results for two-humped camels with those of one-humped camels and llama deer. However, the differences in the ratio of IgG1 and IgG3 subclasses between *Camelus bactrianus*, and *Lama glama* were statistically different, as shown in Table 1 (for IgG3 p = 0.001 (Bradford method), for IgG1 p = 0.000 (Bradford method) and p = 0.001 (A280 method).

In 2015, Zhang reported that HCAbs accounted for 60% ~ 80 % of the Bactrian camel total IgG, [23] compared to ~ 65 % in our study. This difference was not statistically significant (p = 0.05).

The proportions of IgG1, IgG2, and IgG3 isotypes in the blood serum of the three *Camelidae* are significantly different. This is likely because of yet undetermined individual physiological characteristics of each species. Further studies will address *C. bactrianus* IgG3 and IgG2 specific antibodies that differ from other species.

Further research will increase the sample size to address our study's statistical limitations and to verify the IgG subclass proteins' contents and ratios. Also, the molecular weight determinations using size-exclusion chromatography demonstrated the need for additional molecular mass precision specific to Mongolian *Camelus bactrianus* IgG subclass proteins.

Conclusions

Three isotypes of immunoglobulin G were isolated from Mongolian domestic *Camelus bactrianus* serum using Protein A and G affinity columns, and the ratios of IgG1, IgG2, and IgG3 isotype molecular weights were identified as 40.5 ± 4.5 kDa, 24.0 ± 8.0 kDa, and 35.5 ± 5.1 kDa, respectively. The proportion of HCAbs was found to be approximately 60% in the serum.

The ratio of IgG protein isotypes in the two-humped camel serum we identified was statistically different from the published values for *Lama glama* serum. The isolated subclasses' purity and molecular weight were determined to be 55 kDa and 25 kDa single spots for IgG1 and 43 kDa for IgG2 and IgG3, respectively.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

This research was supported by the Mongolian Foundation of Science and Technology, funded by the Ministry of Education, Culture, Science and Sports of Mongolia (Project No 2018/13). The submitted manuscript will be Enkh-Ariun Altantulga's thesis for a Master's degree in Biology at the National University of Mongolia.

References

1. Emmanuel B, Nahapetian A. Fatty acid composition of depot fats, and rumen wall of the camel (Camelus dromedarius). Biochem Mol Biol 1980; 67(4): 701-4.
2. Al-Ali A, Husayni H, Power D. A comprehensive biochemical analysis of the blood of the camel (Camelus dromedarius). Biochem Mol Biol 1988; 89(1): 35–7.
3. Yao H, Zhang M, Li Y, Yao J, Meng H, Yu S. Purification and quantification of heavy-chain antibodies from the milk of bactrian camels. Anim Sci J 2017; 88 (9): 1446-50.
4. Burger PA, Ciani E, Faye B. Old world camels in a modern world – balancing act between conservation and genetic improvement. Anim Genet 2019; 50(6): 598-612.
5. Muyldermans S, Baral TN, Cortez Retamozzo V, Baetselier PD, Genst ED, Kinne J, et al. Camelid immunoglobulins and nanobody technology. Vet Immunol Immunopathol 2009; 128(1-3): 178-83.
6. Muyldermans S. Nanobodies: Natural single-domain antibodies. Annu Rev Biochem 2013; 82: 775-97.
7. Van Audenhove I, Gettemans J. Nanobodies as Versatile Tools to Understand, Diagnose, Visualize and Treat Cancer. EBioMedicine 2016; 8: 40-8.
8. Deffar K, Shi H, Li L, Wang X, Zhu X. Nanobodies-the new concept in antibody engineering. African J Biotechnol 2009; 8(12): 2645-52.
9. Tillib SV. "Camel nanoantibody" is an efficient tool for research, diagnostics and therapy. Mol Biol 2011; 45: 66-73.
10. Wrapp D, Vlieger DD, Corbett KS, Torres GM, Wang N, Breedam WV, et al. Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. Cell 2020; 181(5): 1004-15.
11. Schoof M, Faust B, Saunders RA, Sangwan S, Rezelj, V, Hoppe N, et al. An ultra-high affinity synthetic nanobody blocks SARS-CoV-2 infection by locking Spike into an inactive conformation. BioRxiv 2020. doi:10.1101/2020.08.08.238469.
12. Chanier T, Chames P. Nanobody engineering: toward next generation immunotherapies and immunoimaging of cancer. Antibodies 2019; 8(1): 13.
13. Enkhee O, Bolortuya D, Tuul B, Bayarmaa E. Prognostic value of immunohistochemical biomarkers in patients with astrocytic brain tumor. Cent Asian J Med Sci 2018; 4(3): 211-9
14. Leandro LM, Damila CT, Maria GLM, Maria ASP. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomarker Insights 2010; 5: 9-20.
15. Daley LP, Gagliardo LF, Duffy MS, Smith MC, Appleton JA. Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. Clin Diagn Lab Immunol 2005; 12(3): 380-6.
16. Chuluunbat P, Charruau P, Silbermayr K, Khorloojav T, Burger PA. Genetic diversity and population structure of Mongolian domestic bactrian camels (camelus bactrianus). Anim Genet 2014; 45(4): 550-8.
17. Jirimutu Y, Wang Z, Ding G, Chen G, Sun Y, Sun Z, et al. Genome sequences of wild and domestic bactrian camels.

Nat Commun 2012; 3(1): 1202-9.
18. Bio-Rad Laboratories. Bio-Rad protein assay (bradford). Bio-rad 2010; 1-24.
19. John MW, The protein protocols handbook. Totowa, NJ, USA: Humana press inc; 2009. p 3-6.
20. Haddad M, Soukkarieh C, Khalaf HE, Abbady AQ. Purification of polyclonal IgG specific for camelid's antibodies and their recombinant nanobodies. Open Life Sci 2016; 11(1): 1-9.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227 (5259): 680-5.
22. Conrath KE, Wernery U, Muyldermans S, Nguyen VK. Emergence and evolution of functional heavy-chain antibodies in Camelidae. Dev Comp Immunol 2003; 27(2): 87-103.
23. Zhang WD, Wang WH, Jia S. Distribution of immunoglobulin G antibody secretory cells in small intestine of Bactrian camels (Camelus bactrianus). BMC Vet Res 2015; 11(222): 4-11.
24. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. Nature 1993; 363(6428): 446-8.
25. Linden R, Geus BD, Stok W, Bos Wassenaar DV, Verrips T, Frenken L. Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of llama glama. J Immunol Methods 2000; 240(1-2): 185-95.