REVIEW / ARTÍCULO DE REVISIÓN

Production of nanobodies in Andean camelids and their most common applications: A general review in the medical field

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Abstract: The heavy chain fraction present in Camelidae antibodies is so-called nanobodies. They have different characteristics when compared to immunoglobulin G, like more diminutive size, higher affinity, shorter half-life in serum, etc. These proteins are codified by B lymphocytes cDNAs and can be produced in different hosts like *Escherichia Coli, Pichia Pastoris*, plant cells and even insect cells. Andean camelids have been mainly used in the Andean region of South America as transport means and source of raw materials like fibers and meat, then being of great economic importance. However, in Ecuador, the potential of these animals as a source of biomedical products has not been investigated or exploited yet. Due to the scarce information related to these molecules and their industrial production in the country, this review aims to remark on the most common medical application of nanobodies produced from Andean camelids; also, industrial applications are described.

Key words: Cancer, Coronavirus, VHH, production, treatment, diagnosis.

Introduction

Camelid produces antibodies composed of heavy chain constant domains and a single variable domain; this variable domain is known as nanobody (Nb). It possesses a single antigen-binding domain called VHH[1,2]. Antibodies with a single variable chain, known as vNARs, have also been reported in the fish subclass Elasmobranchii to which sharks and rays belong[3-5]. Phylogenetic studies have shown that antibodies from Camelidae and Elasmobranchii evolved independently[6,7]. In recent years, Nb has been used as therapeutic agents, in diagnostic tests and as research tools[8-10]. Camelidae and Elasmobranchii possess different kinds of antibodies[6], but only the antibodies that lack the light chains are of interest in this review.

Camelidae is a family that includes the old-world camelids: -*Camelus dromedarius* (dromedaries), and *C. bactrianus* (camels)- and the new-world *camelids*[11-13]. There are four species of new-world *camelids* in South America[14,15]: *Lama glama* (llamas), *L. guanicoe* (guanacos), *Vicugna vicugna* (vicuñas) and *V. pacos* (alpacas). All of them represent a genetic resource of great importance, not only from a scientific point of view[16-18], since they could represent an opportunity for economic, social and technological development in Ecuador.

Nbs were first discovered and described by the Hamers-Casterman team[19] at the Free University of Brussels while analyzing *C. dromedarius* serum. The potential use of Nbs in the medical field is focused on the prevention, diagnosis and disease treatments[20,21]. Additionally, several preclinical and clinical studies regarding their use in phases 1 and 2[22,23]. The development of a drug at the laboratory scale until its sanitary registration takes about 10 years[24-26]. In fact, in 2019, the Food and Drug Administration (FDA) approved for the first time the therapeutic use of a humanized Nb[27]. Due to the great potential of Nbs, Steeland *et al.*[28] concluded that these molecules could be positioned as pharmaceuticals in a short period for daily use.

The main objective of this review is to describe the most common medical application of nanobodies produced from *Andean camelids* to demonstrate the potential of these molecules as a medical product. In addition, a little overview of its recombinant production in different biotechnological hosts and other industrial fields is described.

Immunoglobulin G VS Nanobodies

Structurally, immunoglobulins G possess 2 light and 4 heavy chains. Heavy chains contain 3 constant domains and 1 variable domain (VH), while the light chains contain 1 constant and 1 variable domain (VL)[29,30]. However, Camelidae antibodies lack the light chain[31], and their 3 chains are composed of 2 constant domains and 1 variable domain (Nb)[32,33]. In addition, *Camelidae*-like shark antibodies (which lack a light chain and possess 7 chains) are called New Antigen Receptors (NAR) antibodies[34]. They comprise 5 constant domains and 1 variable domain (vNAR)[6,35]. Figure 1 shows the structural comparison between mammalian IgG and the IgG-like antibodies from Elasmobranchii and Camelidae. Figure 2 shows the alignment of Elasmobranchii vNAR, Camelidae Nb and a *Mus musculus* heavy chain antibody sequences.

The figure shows the amino acid variation between vNAR, VHH and mAb heavy chains. In parenthesis protein name according to DATA bank information.

Despite the biotechnological importance of immunoglo-

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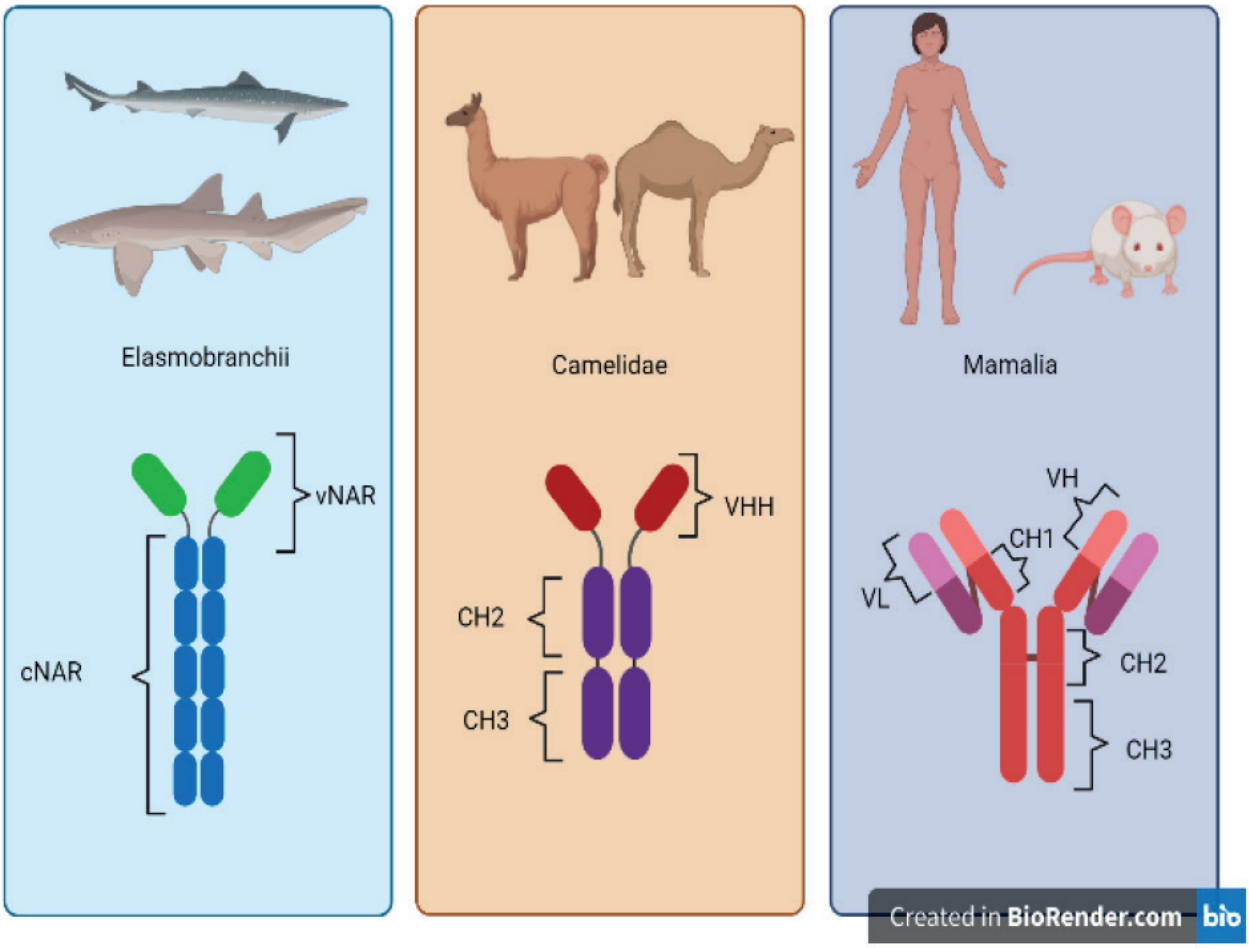

Figure 1. Structural comparison between IgG-like Elasmobranchii, Camelidae and Mammalian antibodies. *Elasmobranchii* antibodies possess a variable region called vNAR and a constant region composed of five constant domains (cNAR). *Camelidae antibodies* are conformed by one variable heavy chain domain (VHH) and 2 constant heavy chain domains (CH2 and CH3). Mammalian antibodies are composed of one variable heavy chain domain (VH), three constant heavy chain domains (CH1, CH2, CH3) and a constant light chain domain (VL).

bulin G, Nbs have certain differences over IgG, which are summarized in Table 1.

The main Nbs disadvantage is their low half-life in human blood serum because they are filtered by the kidney in 26 to 60 minutes[46-48]. However, they have low toxicity and can be eliminated from the human body by filtration by the kidneys[49]. Nbs have a low immunogenicity risk profile, which drives their use to develop potential clinical applications[50]. Using Nbs conjugated with small molecules like drugs, toxins, enzymes and imaging agents allows an approach to target sites with low systemic toxicity[51]. In addition, Nbs do not present the solubility and aggregation problems typical of conventional antibodies due to their hydrophilicity[36,52].

The size of a camelid antibody is around 95 kDA[53-55] due to the absence of the light chain; Elasmobranchii antibodies size is about 175 kDA[56] while the variable antigen-binding domains (Nbs and vNAR) measure between 12 and 15 kDA[46,56,57]. There is a vast difference between the IgG medical development and the research performed on Nbs and vNAR molecules[58]. Nevertheless, Nb and vNAR have similar advantages to the IgG (more specificity, smaller capacity to bind crypto antigens, more stability)[59,60]. Figure 3 represents the 3D structure of a Nb, a vNAR and a Mus musculus monoclonal antibody (mAb). This figure shows that the Nb and vNAR structures are smaller and more straightforward than the *M. musculus* mAb, facilitating their production[58].

How are Nanobodies produced?

The development of Nb libraries can be achieved from a naive or synthetic source or by immunization. All of them are great ways to get the appropriate Nb that best meets the final research goal. For this reason, Nbs are expressed and produced at a large scale in the chosen host[64]. Libraries obtained by immunization are the most widely used and consist in injecting the animal with the correct antigen[65]. Native libraries are developed from non-immunized camelids blood[66], and synthetic libraries are developed from a determined sequence framework[67,68] or the target antigen[69], from which randomization of oligonucleotides in the hypervariable regions is performed[70].nThe yields in the production of Nbs from immune libraries and synthetic libraries can vary significantly[71].

Figure 1 shows a general diagram for Nb production by immunization. This process starts with the inoculation of a young-adult camelid with 50 to 200 µg of the chosen antigen[72]. After the first immunization, the animal must be injected from 4 to 8 times during 2 months[64]. Once the immunization process is completed, the camelid's blood must be extracted and B lymphocytes purified. Further, B lymphocyte mRNA is isolated, purified and recovered to finally get the total complementary cDNA using polymerase chain reaction (PCR) techniques[73,74].

Then, a Nb library is generated from the above-obtai-

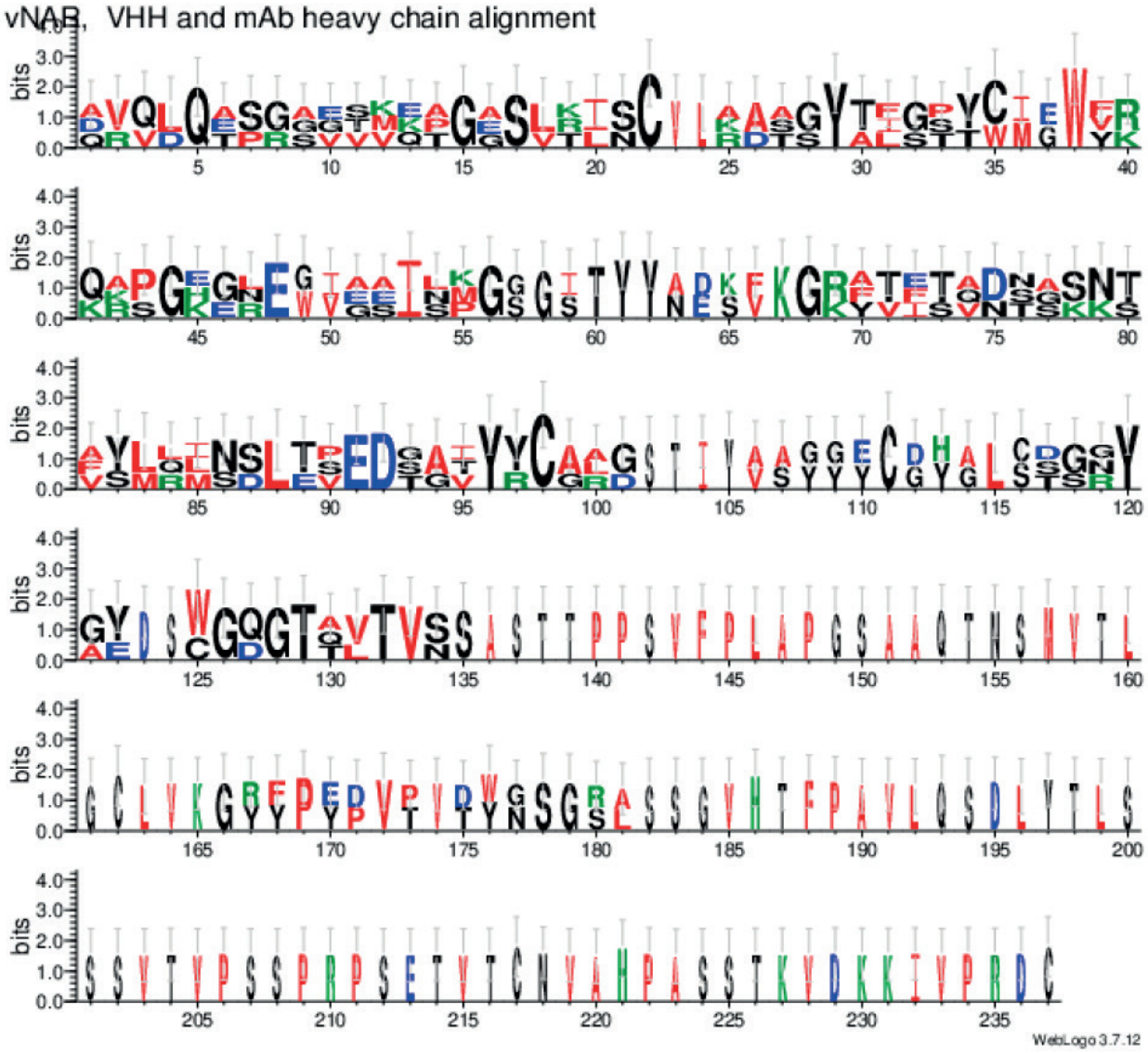

Figure 2. Sequence logo plot of the alignment of vNAR (1T6V), VHH (1MEL) and monoclonal *Mus musculus* heavy chain (1MLC).

ned cDNA, for which the VHH regions are amplified using specific primers[73,75]. Muyldermans (2021) research suggests that the amplification of the VHH regions should be performed using mainly the nested PCR technique[64].

From the PCR products, a gene library is generated[67]; then the fragments must be chosen by a display technique; the most common technique is the phage display[64]. The phage display library consists of bacteriophages with the antigenic molecule (Nb) in their coat; then, all the library is exposed to the specific antigen[76]. Finally, the selected fragments are screened and then chosen those that produce specific Nbs for the particular antigen[64,73,75,77].

The selected VHH sequences are cloned at the industrial level in appropriate hosts like bacteria such as *Escherichia coli*[64,78,79] or yeasts such as *Pichia pastoris*[75]. Mammalian cells[73], insects or plants[71] are also excellent options as industrial production hosts[80]. Table 2 exposes the kind of expression and yields of the different host cells. Finally, the proteins are extracted and purified, generally using the ammonium sulfate precipitation method in conjunction with other types of chromatography[73] as shown in Figure 1.

Applications of camelid nanobodies in Human Health

The small Nb size, joined with their high stability, solubility and a great capacity to recognize crypto-antigens place them as excellent alternatives for the prevention[86], diagnosis[87,88] and human diseases treatment[46,89,90].

Disease diagnosis using Nbs points to:1- the generation of images from tissues and organs[57,91]; and 2- the performance of immunoassays to detect pathogen antigens[89,92,93]. Meanwhile, nanobodies as a therapeutic agents seek to attack an antigen, mainly to block virus replication[94,95] or bacteria growth[96].

Another novel application is their use in antiphonic serums as neutralizers and blockers of toxins present in different types of poisons[97-99].

Finally, a search was made for medical applications of nanobodies developed in Andean camelids. About 518 articles were found using the "Publish or perish" program where Andean camelids were used. The reported applications belonged mainly to the health branch and articles published in the last 5 years including 50 scientific publications that were not review articles.

Using descriptive statistics and frequency histograms, it

Immunoglobulin G	Nanobodies
It has two variable domains: VH and VL. The latter provides stability and antigen-binding specificity.	The only variable domain it possesses is the VHHs; they have high stability despite lacking VLs.
They have a large size (150 kDa).	They are one-tenth the size of traditional antibodies (15 KDa), increasing the permeability of the molecule.
Low tissue permeability.	It can be entered into different types of tissues.
They may have flat surfaces, making them difficult to attach to specific antigen sites.	Its configuration allows it to bind to crypto antigens like protein cleavages and enzyme active sites.
It has low resistance to proteolysis and thermal denaturation.	They have a high resistance to proteolysis and thermal denaturation.

Information is taken from [36–45]. VH=Variable Heavy domine, VL= Variable Light domine.

Table 1. Comparison between conventional antibodies and nanobodies.

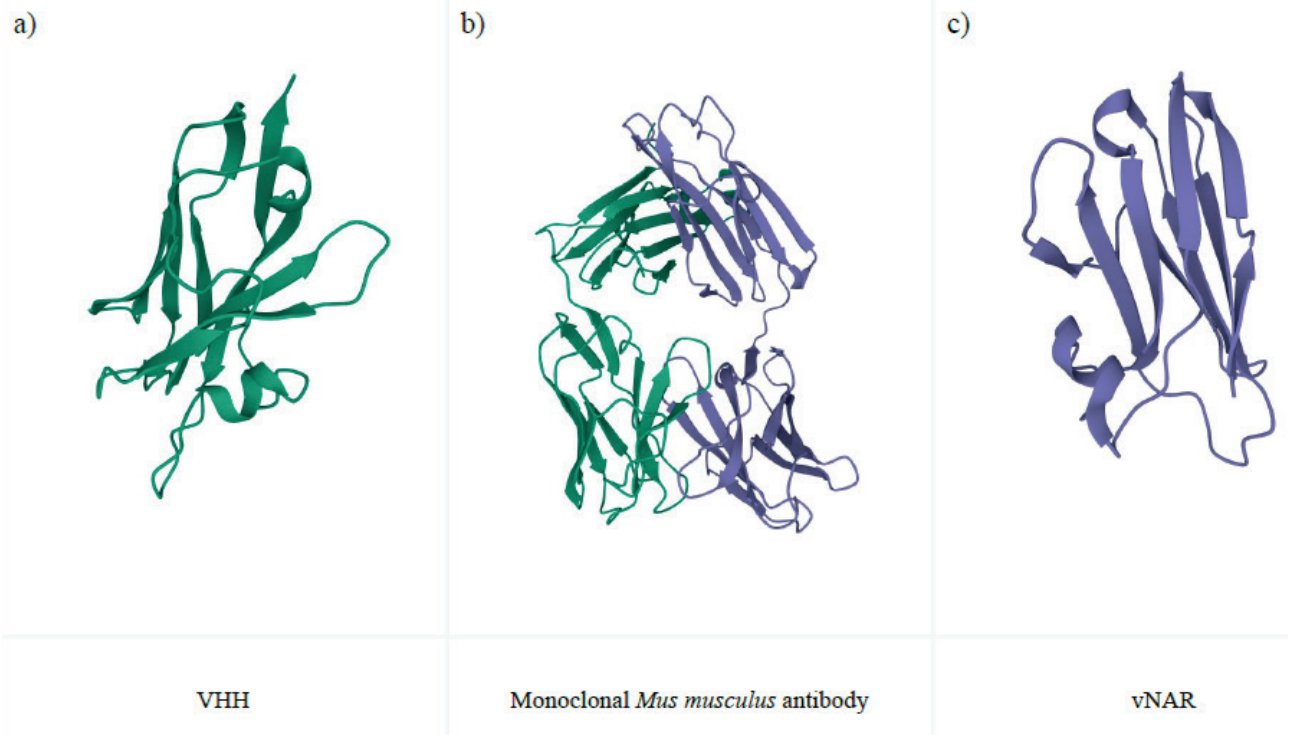

Figure 3. 3D structure of antibody domains. a) VHH (1MEL). b) mAb of *Mus musculus* (1MLC). c) cNAR (1T6V). Information and images are taken from[61-63].

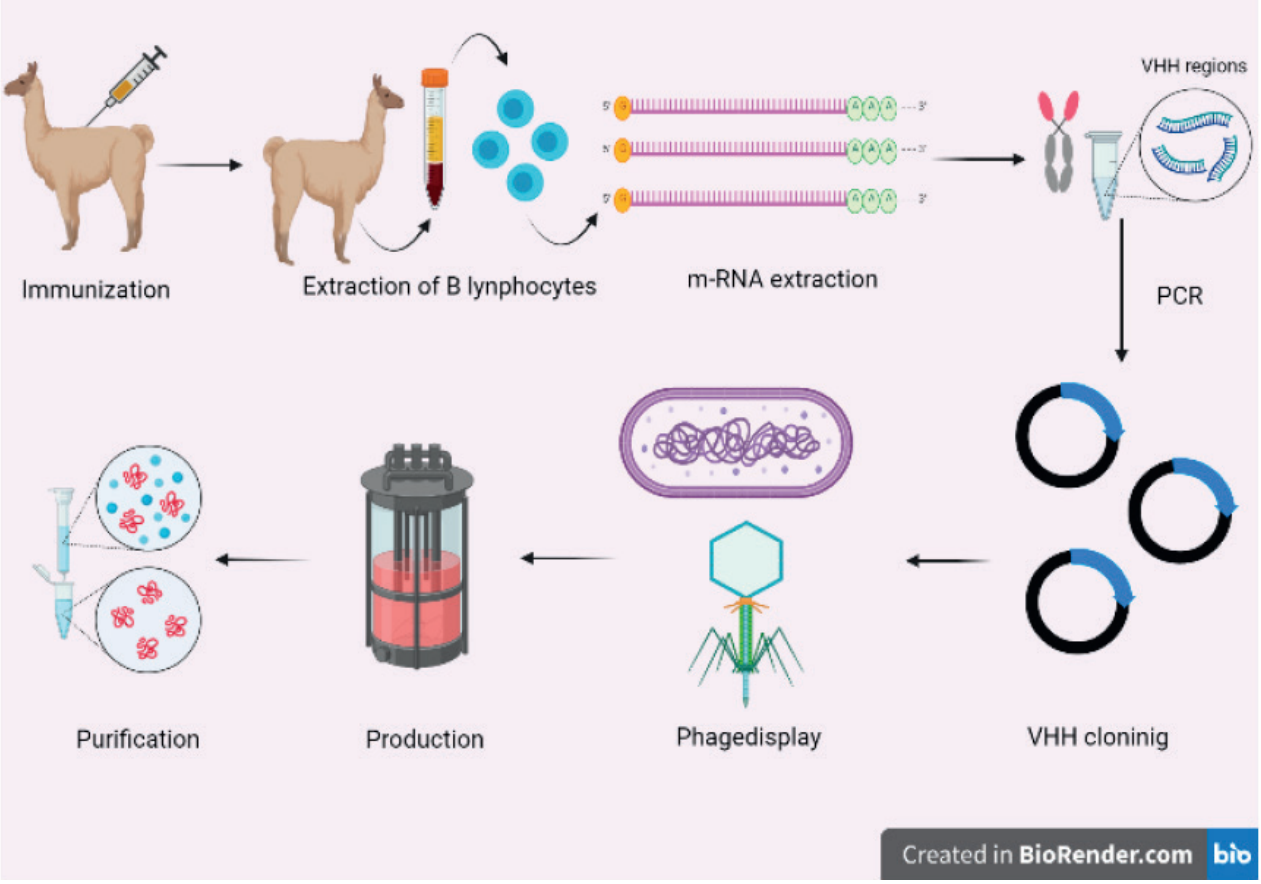

Figure 4. Production process of recombinant nanobodies. The first step is to immunize the camelide. Then, the B lymphocytes must be extracted and purified to extract m-RNA. The VHH sequences are obtained by PCR. Thereafter, the nanobody sequences are selected by phage display (or any display method). Finally, the chosen nanobodies are produced recombinantly and purified.

Author	Host	Kind of Expression	Yield
T. Iwaki, *et al* (2020)	*E. coli*	Extracellular	1.67-13.3 mg/L
Ruano-Gallegos, *et al* (2019)	*E. coli*	Extracellular	0.3–1.3 mg/L
Chen, *et al* (2018)	*P. pastoris*	Extracellular	46.68-51.71 mg/L
Sheikholeslami, *et al* (2010)	*P. pastoris*	Extracellular	0.3–0.5 mg/L
Shokrollahi, et al (2021)	insect cells	Intracellular	equal as bacterial systems
Wang *et al,* (2021)	Plant cells	Intracellular	1.7%of total soluble proteins 30% f total leaf protein

Information is taken from [37,81–85]

Table 2. Comparison between conventional antibodies and nanobodies.

was determined that the most common application of Nbs is the treatment and diagnosis of infectious diseases (Figure 3). However, the condition for which the largest number of Nbs have been developed was cancer (Figure 4).

The 2020 pandemic greatly spurred the development of Nbs and other pharmaceuticals for the treatment and/or diagnosis of COVID-19[100,101]. However, Nbs development focused on diagnosis, as shown in table 3; cancer treatment has been developed since before 2020.

Cancer is a disease in which uncontrolled cell multiplication can spread to other parts of the body[102]. Alternatives for cancer prevention have existed since the 1700s[103], and their treatments include surgeries, chemotherapies, radiotherapies, immunotherapies and hormone therapies[104]. Nbs have a good synergy with these treatments[105], so they could be used together, although the full range of Nbs applications in cancer has not yet been explored thoroughly[106,107].

On the other hand, COVID-19 caused by SARS-CoV-2 generates affections on the respiratory system[108]. On March 11 of 2020, the World Health Organization (WHO) declared a world pandemic for this infection[109]. There is still no defined treatment for this disease, although monoclonal antibodies[100,110] and nanobodies seem to be the best option to fight the virus[111-113]. The future evolution of the virus must be awaited as it could generate less infectious and virulent variants or more aggressive strains resistant to the current preventive treatments[114].

Applications of camelid nanobodies in different branches of industry

The main focuses of Nbs in plants are to protect crops by providing immunity against pathogens[72,85] and rapid

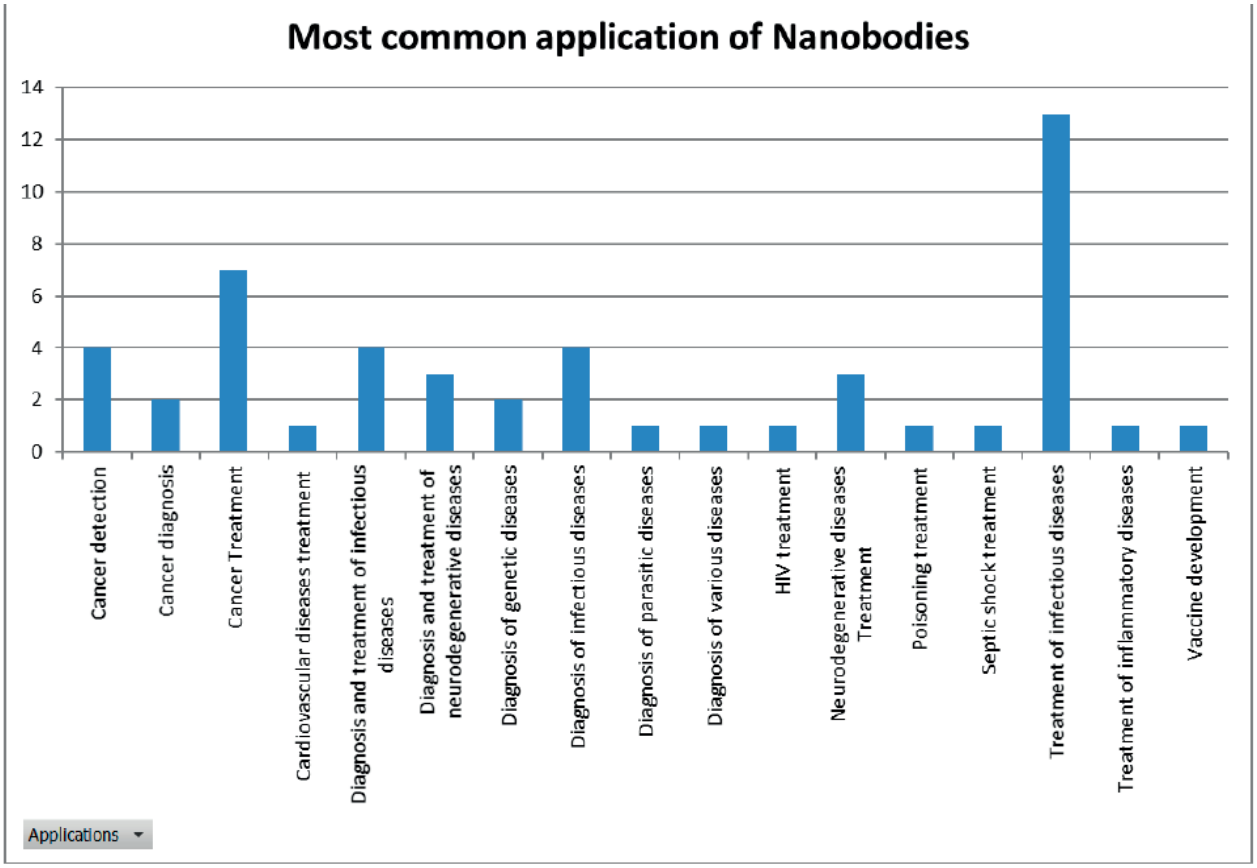

Figure 5. Most common applications of nanobodies.

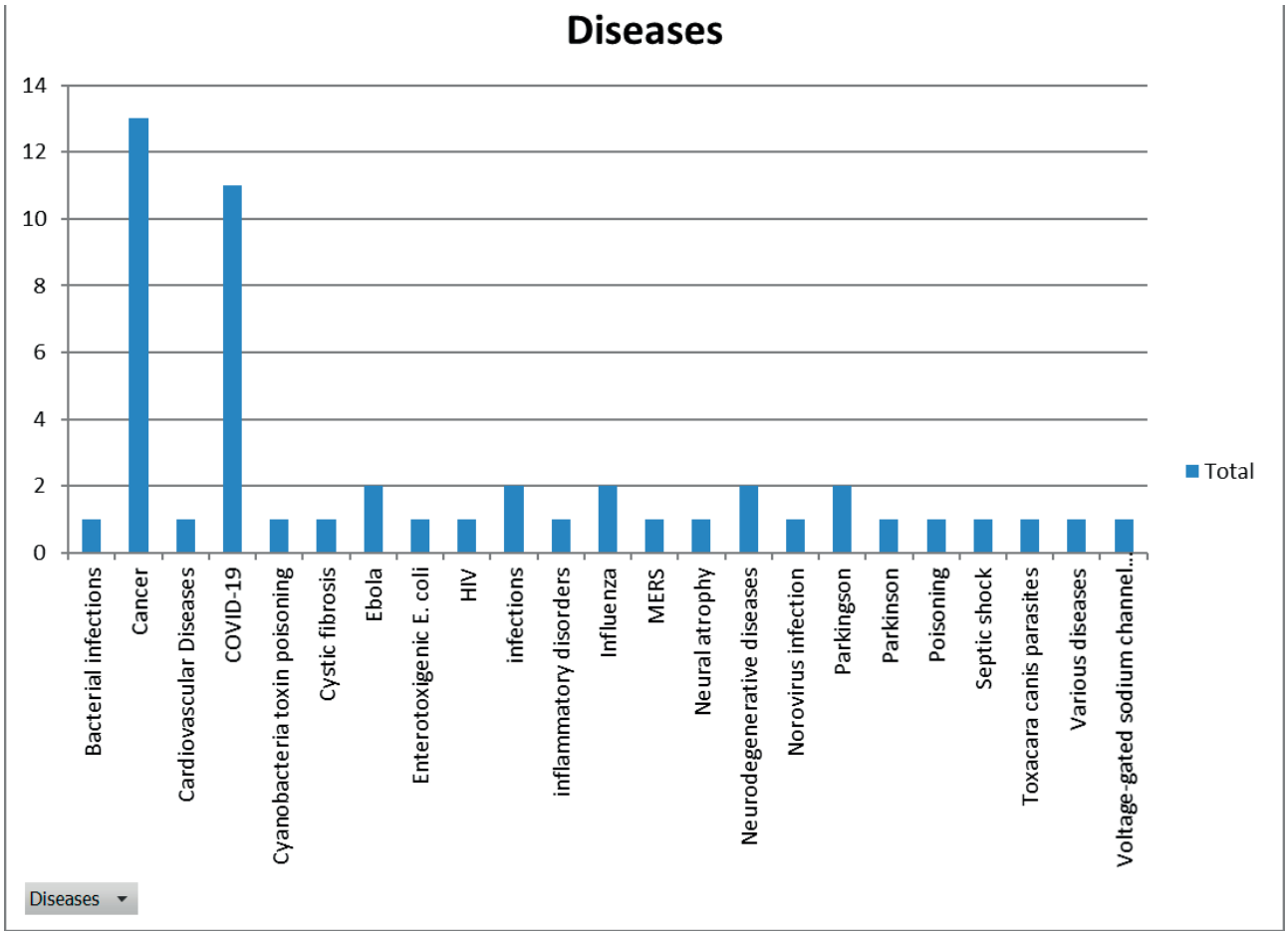

Figure 6. Diseases for which nanobodies were developed.

		Year of nanobodies development.					
Published papers on the development of nanobodies in Andean camelids	**Disease**	**2018**	**2019**	**2020**	**2021**	**2022**	**Total**
	Cancer	5	2	4	1	1	13
	COVID-19	0	0	2	8	1	11

Table 3. Comparison between published papers reporting the development of Nanobodies in Andean camelids for cancer and COVID-19.

detection of toxins that may affect food safety[115]. Passive immunity is the use of external antibodies to protect a patient[116-118]; this concept can be extrapolated to plants, where antibodies would protect them against pathogens[85,119,120]. On the other hand, Nbs can identify toxins in absorbance, fluorescence or Enzyme-Linked ImmunoSorbent Assay (ELISA) tests, depending on the toxin to be tested[121-123].

In the livestock sector, the main Nbs application is on early disease detection[124,125] to avoid zoonoses[3,126,127]. Nbs can also provide passive immunity in animals, especially those sensitive to diseases after weaning, such as piglets[128]. This is very important to avoid using antibiotics that could generate resistant bacterial strains[89,129].

Nanobodies used in water and soil allow the quick and accurate identification and detection of toxins and contaminants to the nanogram levels so, becoming a handy tool for these purposes[130-132].

The ability of nanobodies to bind to specific compounds makes them good alternatives in high-affinity chromatography[133,134]. They can also help to determine the protein structure[135] with dynamic properties in case of proteins with various conformations and shapes which are difficult to solubilize[64,136]. Another application is identifying protein functions and protein-protein interactions by mass spectrometry that could replace conventional antibodies by Nbs[137].

The use of intrabodies that consist of nucleic acid sequences with the genetic coding information of an antibody or Nb[138,139] that can be expressed inside a cell to modify cellular activity[140,141]. This biological application could be used to alter cellular functions[142], blocking or reducing the activity of endogenous proteins[143].

Future Perspectives and Conclusions

This mini-review presented applications of nanobodies that could promote the use of Andean camelids in Ecuador as new sources of products of high social, economic and public health interest.

The SARS-CoV-2 virus generated a global health crisis. Therefore, several methods for the diagnosis of COVID-19 were rapidly developed[144]. Nonetheless, there is still no defined treatment[100,110]. Becker's study in 2020[145] e mentions that a great deal of research is being carried out, mainly focused on blocking the replication and translation of the virus RNA; moreover, some treatments seek to block the binding of the virus and its ACE2 receptors[146-148] so, Nbs could be used as therapeutic molecules against COVID-19.

Ecuador has the greatest number of reptile species per unit area; one of the groups of greatest interest are snakes. Among them, about 35 species are reported to be venomous and are distributed along the Ecuadorian coast and Amazon[149]. On the other hand, scorpions, of which 40 species are known to be venomous, have been little studied[150]. In the past, serums to treat poisonings were produced by the "Instituto Nacional de Investigación en Salud Pública" INSPI, but production was suspended in 2014 so only antivenoms were imported[151]. In 2022, INSPI produced a batch of 300 experimental antivenom sera[152]. The Nbs could be used to act as high-affinity antivenom.

The incidence of cancer in Ecuador is 61% in women while 41% in men[153]; the types of cancer most reported in male patients are prostate, lymphoma and stomach[154], while for female patients, they are breast cancer, cervix and lymphoid leukemia[153]. Nanobodies could be an interesting alternative for treating these types of cancer in Ecuador. However, the fact that they are rapidly filtered by the kidney makes them less attractive as a therapeutic alternative[58].

In conclusion, the most common application of Nbs after analyzing more than 50 articles was the treatment and diagnosis of infectious diseases in cancer, so the future development of nanobodies should be linked to the treatment and diagnosis of these diseases. It is suggested that Nbs that achieve a balance between tissue permeability and a longer half-life in serum be developed for treating diseases such as cancer. However, other exciting applications in other fields are also not ruled out for the use of nanobodies.

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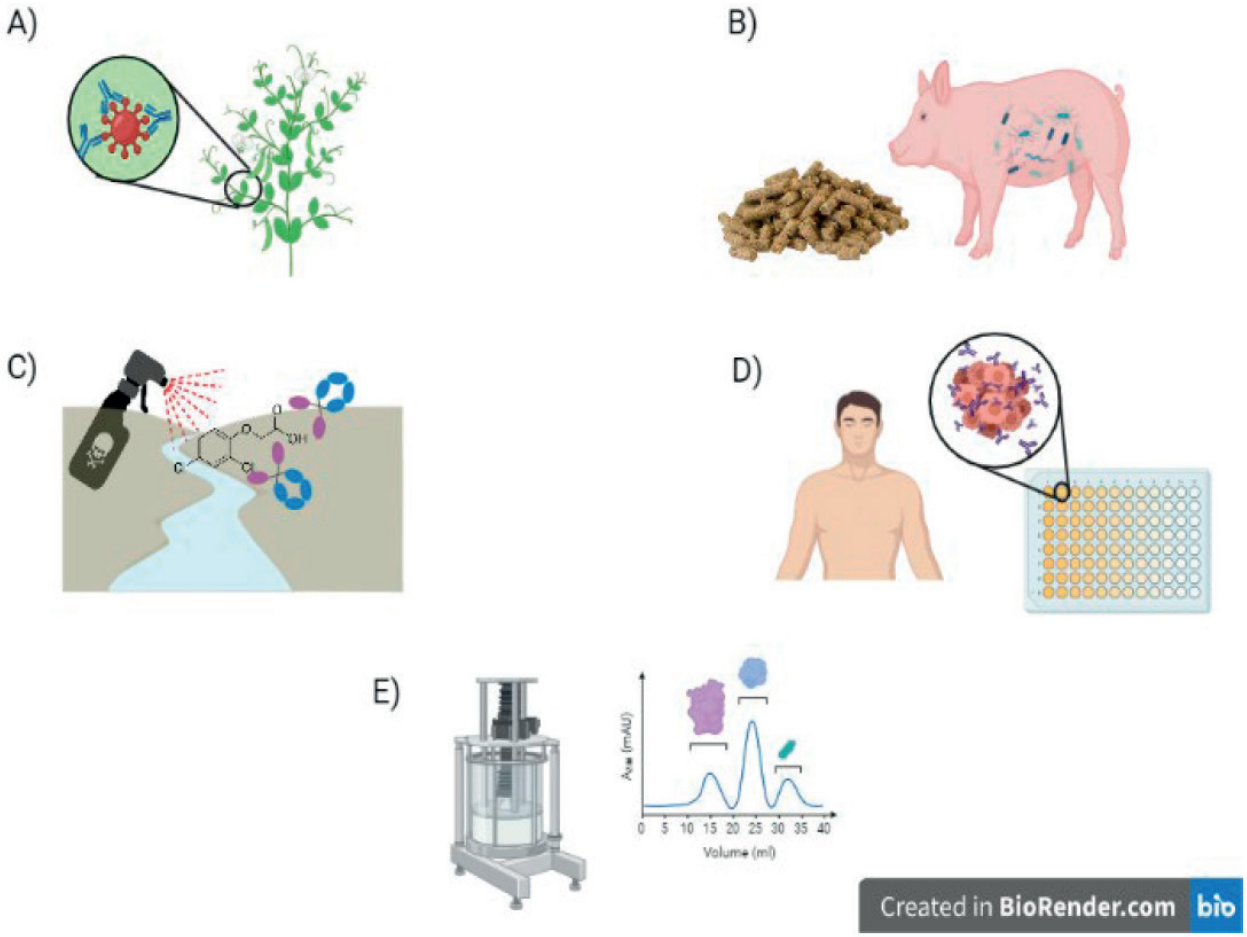

Figure 7. Applications of Nanobodies (Nb). A) Passive immunity in plants. B) Passive immunity in animals. C) Identification of contaminants in soil and water, D) Identification of human diseases. E) Nbs as a solid phase in chromatography.

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