Use of camel single-domain antibodies for the diagnosis and treatment of zoonotic diseases

Pierre Lafaye[a,*], Tengfei Li[b]

[a] Institut Pasteur, Plate forme d'Ingénierie des Anticorps, C2RT, Paris, France
[b] Université Paris Diderot, Paris 7, France

ABSTRACT

Camelids produce both conventional heterotetrameric antibodies and homodimeric heavy-chain only antibodies. The antigen-binding region of such homodimeric heavy-chain only antibodies consists of one single domain, called VHH. VHHs provide many advantages over conventional full-sized antibodies and currently used antibody-based fragments (Fab, scFv), including high specificity, stability and solubility, and small size, allowing them to recognize unusual antigenic sites and deeply penetrate tissues. Since their discovery, VHHs have been used extensively in diagnostics and therapy. In recent decades, the number of outbreaks of diseases transmissible from animals to humans has been on the rise. In this review, we evaluate the status of VHHs as diagnostic and therapeutic biomolecular agents for the detection and treatment of zoonotic diseases, such as bacterial, parasitic, and viral zoonosis. VHHs show great adaptability to inhibit or neutralize pathogenic agents for the creation of multifunctional VHH-based diagnostic and therapeutic molecules against zoonotic diseases.

1. Introduction

The generation of monoclonal antibody (mAb) triggered a revolution in biotechnology. Therapeutic mAbs belong to the fastest-growing branch of biotechnology. However, the large size of the molecules may hinder efficient tissue penetration. This obstacle can be overcome using Fab or single-chain Fv (scFv) fragments, as they are three to six times smaller than full-sized antibodies. However, cloning these fragments, consisting of heavy and light chain variable regions linked by disulfide bridges or a linker, is challenging and they are not always efficiently expressed [1].

An alternative approach to avoid these pitfalls is the use of functional fragments of heavy chain antibodies, which are present in the serum of animals belonging to the Camelidae family. They interact with the antigen by virtue of one single variable domain referred to as VHHs, single-domain antibodies (sdAbs), or nanobodies™ (trademark of Ablynx). They combine the advantages of both immunoglobulins and small molecules and provide an alternative to conventional antibodies and binders derived from alternative scaffolds. Sharks also produce a unique heavy-chain only immunoglobulin that does not associate with light chains, referred to as IgNAR. V domains of IgNAR are often applied in biotechnological and biomedical fields (for a review [2]).

The Camelidae family (order: Artiodactyla) consists of six species: dromedary (Camelus dromedarius), Bactrian camel (Camelus bactrianus),

llama (Lama glama), guanaco (Lama guanicoe), alpaca (Vicugna pacos), and vicuna (Vicugna vicugna). Animals belonging to this family are well adapted to live in harsh environments, such as deserts or high altitudes [3].

2. Structure and sequence of camel heavy chain antibodies

Ungar-Warom et al. [4] and Azwai et al [5] have isolated low molecular weight Ig-like proteins from dromedary serum. However, the detailed characterization and demonstration of the potential usefulness of these proteins stemmed from the work of the Hamers laboratory in the Free University of Brussels [6]. The authors demonstrated that these unusual proteins are "heavy-chain only antibodies" (HCAbs), devoid of light chain. These antibodies form homodimers and interact with antigens by virtue of only one single variable domain, referred to as VHH (VH domain of heavy-chain antibodies) to distinguish it from conventional VH [7]. In contrast to conventional antibodies, HCAbs do not possess the CH1 domain [6].

The active antigen-binding fragment of heavy chain antibodies can be cloned and expressed in the form of VHH, which consists of only one domain (Fig. 1).

The percentage of HCAbs in the bloodstream of camelids varies greatly among species. It can reach a relatively high level in camels, ranging from 50% to 80%, whereas the maximum level is 45% in South

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* Corresponding author.
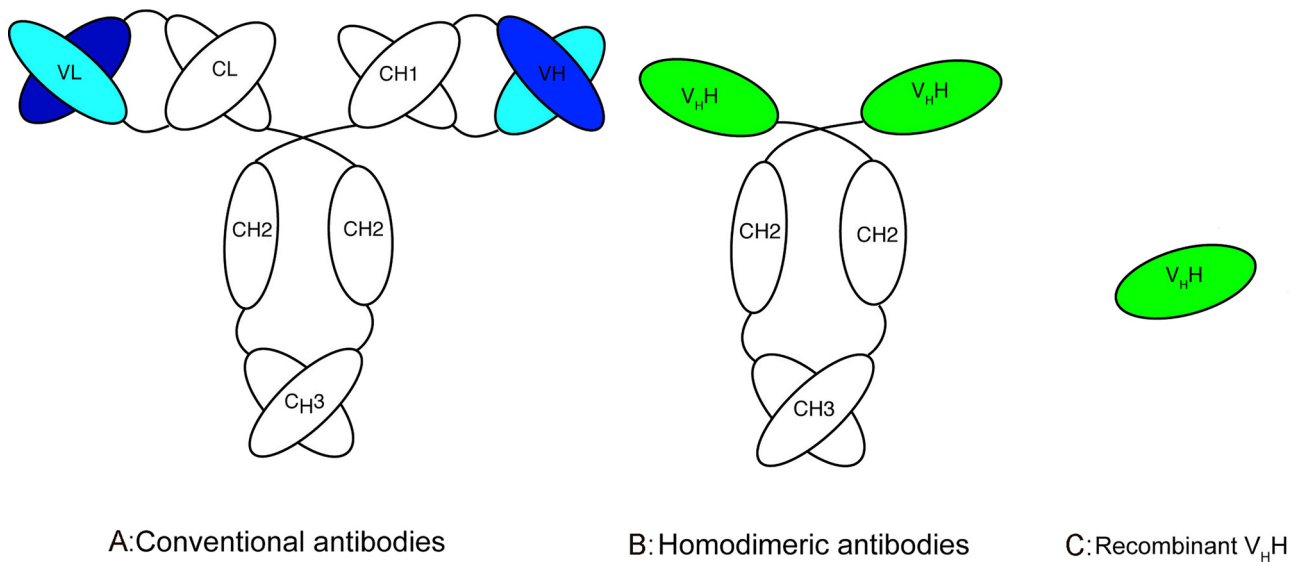E-mail address: plafaye@pasteur.fr (P. Lafaye).

Fig. 1. Schematic diagram of different types of antibodies (adapted from [23]). A The common structure of an IgG antibody, which composed of two heavy and two light chains, B the structure of homodimeric camelid antibody only composed of heavy chains, C recombinant antibody-binding domain (VHH).

American camelid species [8].

Despite the absence of light chains, the heavy-chain antibodies exhibit a broad antigen-binding repertoire. They exhibit specific characteristics, such as the substitution of three to four hydrophobic residues (which interact with the VL in conventional antibodies) by more hydrophilic amino acids. VH possess the conserved Val37, Gly44, Leu45 and Trp47 while in VHH, these amino acids are often substituted (Val37Phe or Val37Tyr, Gly44Glu, Leu45Arg and Trp47Gly) [9–11]. Furthermore, the complementarity determining regions (CDRs) of VHHs, and especially CDR3, are statistically longer than those of conventional VH-VL antibodies [12].

3. Unique properties of VHH fragments and their use in biotechnology

Over the last decades, VHHs have received progressively greater interest from the pharmaceutical and biotechnology industries, due to their specific properties. Indeed, VHHs provide the following advantages over conventional antibodies and their recombinant fragments:

- VHHs are weakly immunogenic in humans, because the genes encoding them share high sequence homology with genes belonging to human VH families 3 and 4 [13,14].
- VHHs consist of only one domain. They can thus be easily engineered, cloned, and expressed with high yields using various expression systems [15].
- VHHs are highly soluble and stable, even under denaturing conditions or high temperatures [16].
- The high variability of the length and sequence of VHHs allows them to recognize a variety of protein epitopes, located not only on the surface of a protein [17], but also buried deep in the clefts [18]. VHHs have been shown to recognize a wide range of epitope types, from small haptens [16,19] to the binding sites of enzymes [17,20], and can bind epitopes that cannot be recognized by conventional antibodies [21].
- The small size and basic isoelectric point of VHHs allows them to penetrate tissues, pass through barriers, such as the blood-brain barrier [22–26], and bind intracellular antigens.
- VHHs can be efficiently functionalized [27–29] and are widely used for imaging [24,26,30].

There has been an explosion in the number of publications concerning applications of VHHs that cover their use in pharmaceutical development or as biotechnological tools [26,31–35]. Here, we mainly focus on the potential use of VHHs for the diagnosis and the treatment of zoonotic diseases.

4. Zoonotic diseases

Zoonotic diseases (ZDs) are infections that can be naturally spread between vertebrate animals and humans. Several recent outbreaks, such as Ebola and Zika have emphasized the serious impact of these diseases on human health [36]. Their expansion is related to global trade, human migration, and climate change. Up to now, the outbreaks have been contained by the control of human populations in the affected areas, the use of antibiotics, and the development of rapid diagnostic tests. However, the emergence of ZDs is still a major challenge and there is a crucial need for new tools for diagnosis and therapy. Camelid VHHs could offer an attractive possibility for the development of such tools in the future.

4.1. Bacterial zoonosis

4.1.1. Campylobacteriosis

Campylobacteriosis is caused mostly by *Campylobacter jejuni* or *Campylobacter coli*. Poultry are naturally infected, without clinical signs, and it is the leading cause of foodborne gastroenteritis in humans worldwide [37].

A VHH that binds *C. jejuni* flagella was isolated by Riazi et al. [38] and engineered for greater thermal and proteolytic stability. Hussack et al. [39] obtained a highly stable VHH through the use of error-prone polymerase chain reaction and disulfide-bond engineering. This VHH, directed against the flagella, can potently inhibit *C. jejuni* motility and is being studied for the prevention or significant reduction of *C. jejuni* colonization in the gastrointestinal tract of chickens. Recently Vanmarsenille et al [40] described the isolation and characterization of 6 VHHs against multiple *Campylobacter* strains. These VHHs which bind with the major outer membrane protein (MOMP) interacted with 23 *C. jejuni* isolates and 5 *C. coli* isolates. They could potentially be used in therapy and as a diagnostic tool.

4.1.2. Escherichia coli

Escherichia coli is a facultative gram-negative bacterium that belongs

to the family *Enterobacteriaceae*. Although it is normally commensal in nature and animals, many strains are food and waterborne zoonotic pathogens. Shiga toxin (Stx)-producing *E. coli* (STEC) bacteria (which include enterohemorrhagic *E. coli* [EHEC]) cause no discernible disease in their animal reservoirs; however, diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) are common in humans [41]. The major virulence determinants of STEC are mainly caused by the Shiga toxins Stx1 and Stx2 [42].

VHHs that neutralize Stx1 and/or Stx2 have recently been obtained [43–45]. The VHH 2vb27 specific of Stx2 was unable to give protection against a lethal dose of toxin in mice while a trivalent molecule (two copies of VHH 2vb27 and one copy of anti-albumin VHH) presented an extended half-life and was able to neutralize the *in vivo* effect of toxins in mouse models [43]. Tremblay et al [44] showed that VHH heterodimers, containing two linked neutralizing VHHs, generally neutralized Stx much more efficiently than a pool of individual monomers. Moreover co-administration of an effector Ab substantially improved the ability of Stx toxin-neutralizing VHHs to prevent death or kidney damage in mice following challenge with Stx1 or Stx2.

4.1.3. Listeriosis

Listeriosis is a bacterial infection caused by the gram-positive bacterium *Listeria monocytogenes*. The major source of infection is contaminated food. The disease primarily affects elderly people, immunocompromised patients, pregnant women, causing abortion, and newborns [46].

VHHs specific for the invasin, internalin B, of L. *monocytogenes* have been isolated [47,48] and can inhibit Listeria invasion *in vitro*. A crystal structure between one VHH and internalin B shows that the VHH competes with c-met, the target cell receptor of this invasin, explaining the protective effect [48]. Two VHHs that specifically bind to three L. *monocytogenes* serotypes (1/2a, 1/2b, and 4b) have been isolated from a non-immune library [49]. A sandwich ELISA was developed using a mAb for antibody capture and one VHH, L5-79 for detection, with a detection limit of 1×10^4 colony forming units (CFU)/ml of bacteria in milk.

4.1.4. Anthrax

Bacillus anthracis, the causative organism of anthrax, is a spore-forming Gram-positive bacillus commonly found in the soil of endemic areas. Herbivores can be infected while grazing. Recently, anthrax was detected in Siberia after degradation of the permafrost due to global warming [50]. B. anthracis is also one of the most important biological warfare agents, because of its pathogenic nature and spore-forming capacity [51]. The bioterrorist events in the United States in 2001 revealed that treatment with antibiotics is not always sufficient to prevent patient deaths, due to the effects of toxins produced by the bacteria. Neutralizing mAbs may therefore be of great therapeutic value, complementing antibiotic treatment to prevent the toxin-dependent symptoms of anthrax [52].

Anthrax is caused by a toxin consisting of protective antigen (PA), lethal factor (LF), and edema factor (EF). Several VHHs directed against the three components of the toxin have been shown to be efficient against Anthrax disease [53,54]. Gene therapy with an adenoviral vector expressing a bispecific VHH, consisting of two linked VHHs targeting different PA-neutralizing epitopes, was tested in mice, and found to protect them from anthrax toxin challenge and anthrax spore infection [55].

4.2. Parasitic zoonosis

4.2.1. Taeniasis

Taeniasis occurs in the human host, after ingestion of undercooked pork infected with cysticerci. Cysticercosis is caused by the larval stage of the pork tapeworm *Taenia solium*. Humans are the definitive host, harboring the adult tapeworm in the intestine, but both pigs and humans can be infected with the cysticerci [56].

The mAbs obtained thus far are not genus-specific, preventing definitive diagnosis of infection by *T. solium* [57]. Indeed, specific binders are needed. Deckers et al. [58] have isolated VHHs specific for a glycoprotein of *T. solium* that do not cross react with other Taenia species and a sandwich ELISA has been developed. This serodiagnostic test could be helpful in pigs for epidemiological studies and monitoring the efficacy of control programs.

4.2.2. Trypanosomiasis

African trypanosomiasis (AT), or sleeping sickness, is mainly caused by a unicellular flagellated protozoan parasite, *Trypanosoma brucei gambiense*, belonging to the genus Trypanosoma. AT affects mainly remote rural areas and its distribution coincides mostly with the habitat of the hematophagous insect vector, *i.e.*, the tsetse fly (*Glossina sp*) [59].

Multiple VHHs have already been generated against the parasite (for a review, [60]). Trypanolytic VHH An46 disturbs the endocytic machinery of the parasite in the flagellar pocket of the parasite. Non-trypanolytic VHH An33 was made more potent by linking the nontoxic prodrug cephalosporin mustard (CCM) onto the highly toxic PDM at the surface of the parasite [61]. Linking this VHH to apolipoprotein L-I resulted in an immunotoxin that lyses almost all trypanosomes [18]. Another approach is to couple pentamidine, a first-line anti-trypanosomiasis, to VHH An33 to effectively target the drug to the parasite. *In vivo*, a ten-fold lower dose than the minimal full curative dose of free pentamidin incorporated into this conjugate cured all infected mice, wheras a 100-fold lower dose cured 60% of them [62]. Parasite development in the tsetse fly and subsequent spread of the parasite can be controlled through the expression of trypanolytic VHHs in genetically modified tsetse fly symbionts [63]. In addition, VHHs that target the paraflagellar rod protein of varioius trypanosomes have been described, but are mainly useful as diagnostic markers of trypanosiomasis [64]. A VHH (Nb474) directed against *T. congolese* aldolase (*Tco*ALD) has been developped for a sandwich immunoassay. This VHH is highly specific and did not recognize other trypanosomes such as *T. brucei brucei*, *T. vivax* and *T. evansi* [65,66].

4.3. Viral zoonosis

4.3.1. Influenza

Influenza A viruses (IAV), members of the RNA family *Orthomyxoviridae*, consist of up to 144 subtypes, depending on the variation/combination of the surface glycoproteins, hemagglutinin and neuraminidase. IAV are further classified as human, swine (SIV), bat, equine, or avian influenza viruses (AIV). SIV and AIV are transmitted from pigs or birds to humans, respectively, mostly *via* direct contact with infected animals. The infection in humans ranges from mild self-limiting respiratory-like illness to death. However, pandemic outbreaks remain unpredictable, as illustrated by the 2009 H1N1 virus (also named Mexican flu) and H5N1 virus. Occasional zoonotic infections with these viruses and their high propensity to reassort with SIV have earmarked them as a major pandemic threat [67].

Several VHHs specific for influenza viruses have been raised against the nucleoprotein [68] and M2 ion channel protein [69] of Influenza A, and the neuraminidase [70] and hemaglutinin [71] of H5N1. Most of these VHHs can neutralize influenza viruses. Ploegh et al. [57] exploited the ability of VHHs to bind the intracellular nucleoprotein protein to block viral replication, leading to the possibility of creating new therapeutic molecules to prevent viral escape due to antigenic variation. An alternative approach has been to create multivalent VHHs to increase their antiviral potential: dimers made by the fusion of two neutralizing VHHs [68,72] or a VHH–fused to an immunoglobulin Fc region [68]. *In vitro* antiviral potency was increased from 1 to 2 logs relative to the monomeric VHH.

4.3.2. Rabies

The rabies virus (RABV) belongs to the genus Lyssavirus of the RNA family *Rhabdoviridae*, within the order *Mononegavirales*. Despite the availability of a vaccine against rabies virus (RABV), rabies continues to claim 55,000 human lives per year, mostly in developing countries in Asia and Africa [73,74]. The vaccine is mostly used therapeutically as a post-exposure treatment. For infection combined with a seriously bleeding injury, the WHO recommends complementing vaccination with local instillation of human or equine rabies immunoglobulins (RIG) to neutralize the RABV load *in situ*. There is currently a critical shortage worldwide and the WHO is exploring alternative approaches, such as cocktails of human or humanized neutralizing mAbs [75].

Neutralizing anti-RABV VHHs directed against glycoproteins have been raised from a VHH phage library generated from the immunization of a llama with inactivated rabies vaccine (genotype 1, Sanofi Pasteur MSD) [71]. The IC$_{50}$s of the CVS-11 (genotype 1) strain ranged from 7 to 325 nM. These VHHs were fused to an anti-albumin VHH to extend its serum half-life and were able to neutralize the virus at picomolar doses [76]. A combined treatment based on VHH and vaccine (Rabipur, Novartis) acted synergistically to protect mice in an intranasal rabies infection model [77]. However, the principal difficulty of an antiviral approach against rabies resides in the specific neurotropism of RABV, which makes it not readily accessible once it has accessed the CNS. At this stage, only molecules capable of crossing the blood brain barrier and penetrating into neurons would be able to inhibit the infection.

4.3.3. Foot-and-mouth disease

Foot-and-mouth disease (FMD) is a contagious viral disease that affects cattle, swine, sheep, and approximately 70 wildlife species (including llama and camel), with a potential for rapid spread between susceptible animals. The disease has been identified worldwide whereever livestock are raised. In the last 20 years, there have been massive outbreaks of FMD in countries formerly free of the disease, such as the United Kingdom in 2001 [78] and Taiwan in 1997 [79]. Seven antigenically distinct serotypes of FMD viruses have been identified: O, A, C, Asia 1, SAT1, SAT2, and SAT3 [80]. Emergency vaccination can be used as an effective control measure for FMD outbreaks in FMD-free regions, such as the European Union, but the development of novel antiviral therapies that confer rapid protection against FMD is still needed. Moreover, it is important to develop a rapid diagnostic test for identification of the various serotypes of the viruses involved in FMD outbreaks.

VHHs directed against serotype O have been raised. They have provided only limited protection to pigs. Trimers consisting of two VHHs specific for FMDV and one VHH specific for porcine Ig have been constructed to increase their potency and half-life. These trimers provided better protection to pigs and delayed FMD transmission [81]. Specific VHHs raised against FMD Asia 1 virus have also been obtained. They have been used to develop diagnostic assays by conjugating them to either quantum dots [82] or carboxyl-magnetic beads [83].

5. Conclusion

In this review, we provide evidence for the possible use of VHHs as valuable biomolecules for the diagnosis and treatment of ZDs, including bacterial, parasitic, and viral zoonosis. VHHs provide many advantages over conventional antibodies and currently used antibody based fragments. The ease of high-level production, small size, and high stability make VHHs extremely reliable for genetic and chemical modification, such as the production of VHH-based fusion proteins to increase the persistence of VHHs in serum or confer additional functions. Construction of multivalent VHHs consisting of two or more linked VHHs targeting various epitopes leads to an increase in their ability to neutralize toxins [43,44] and viruses [68,72,76,81], suggesting the development of these approaches for VHHs targeting other viruses not

yet tested.

Presently available anti-viral therapeutic mAbs, at various stages of pre-clinical evaluation, mostly target surface antigens that are often diverse or variable in sequence (*e.g.* HIV gp160, influenza HA): their efficiency thus requires challenging protein engineering efforts to obtain antibodies that are either broadly neutralizing or robust against viral escape through antigenic variation (drift). An alternative is the use of "broadly neutralizing antibodies (bNAb)", which are antibodies found in infected mammals able to neutralize most strains of a given highly antigenically variable pathogen. bNAbs have been isolated from infected humans against HIV [84], influenza [85], and dengue viruses [86]. Camelids could also be a source of bNAbs. Indeed antibodies against MERS-Coronaviruses (MERS-CoV) [87–89], Crimean Congo hemorrhagic fever virus (CCHFV) [90,91], Rift Valley fever (RFV) [90], *Toxoplasma gondii*, and Rickettsia sp. [92] have been found in the sera of infected camels, whereas antibodies against rabies virus, vesicular stomatitis virus, and FMD virus have been detected in llamas [93] and could lead to the possible isolation of specific broadly neutralizing VHHs.

Many neutralizing VHHs that bind to different sites on the same target, including hidden antigenic sites, can be isolated from immunized or infected camelids. These VHHs can be engineered in various ways to improve their diagnostic and/or therapeutic properties and efficacy. In addition, VHHs readily and rapidly penetrate tissues, even the brain, and it is likely that specific VHH-based constructs will be developed that can neutralize agents involved in brain infections, such as influenza [68], Zika, or rabies virus. Overall, VHHs or VHH-based molecules are potentially valuable diagnostic and therapeutic reagents to treat ZDs.

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