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Expression and Purification of Brucella-Specific Nanobodies

Abdul Qader Abbady [1,*], Ayman Al-Mariri [1], Moutaz Zarkawi [2]

[1] Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria
[2] Department of Agriculture, AECS, P. O. Box 6091, Damascus, Syria

*Corresponding author: Abdul Qader Abbady, Department of Molecular Biology and Biotechnology, AECS, P. O. Box 6091, Damascus, Syria. Tel.: +96-311213580, Fax: +96-3116112289, E-mail: ascientific@aec.org.sy.

ABSTRACT

Background: Brucellosis is still considered as one of the major zoonosis afflicting Syrian health and economy. This disease is caused by members of the genus Brucella which are gram-negative bacteria living facultatively within mammalian cells during infection.
Objectives: In this paper, a strategy was developed to introduce a new generation of binders called Nanobodies (Nbs) in our combat against Brucella. Nbs are genetically engineered camelid-derived single-domain antibody fragments that are very stable and highly soluble, making them promising tools in numerous biotechnological and medical applications.
Materials and Methods: In our previous work, three Nb-displaying phages (Nb-phage), referred to as NbBruc01, 02 and 03, were retrieved by a phage display panning of a Nb library constructed from Brucella- immunized camel. In this work, soluble Nbs were produced after recloning their genes in protein expression plasmid followed by purification with affinity chromatography.
Results: Interestingly, two of these soluble Nbs (NbBruc02 and 03) were able to detect Brucella antigens from two main Brucella species (*B. abortus* and *B. melitensis*) and distinguish them from those of Yersinia. This is remarkable as the camel IgG failed in such antigen discriminations. High similarity, mainly in the structure of lipopolysaccharides (LPS) of these different types of bacteria, causes regular serum cross reactivity and thus lack of specificity urging the need for more accurate diagnostic techniques, e.g. a multiplex PCR. Furthermore, NbBruc02 and 03 targets may represent Brucella immunodominant proteins as shown by immunoblotting.
Conclusions: In addition to their own importance, identifying these antigenic targets will open new perspectives for diagnosis, vaccines and treatment of Brucellosis.

Keywords: Brucella; Camel; Nanobody; Yersinia

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▸Implication for health policy/practice/research/medical education:
This article is recommended for general practitioners and those who are involved in medical research and drug development.

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1. Background

Camelids produce antibodies that are naturally devoid of light chains (1). The variable domain of such heavy chain antibody (HCAb) is fully capable of antigen binding. The recombinant variable domain of the HCAb referred to as Nanobody (Nb) is a monomeric structure that possesses a high physicochemical stability and solubility; with a high production level in *E. coli* or yeast (2). Thus, Nbs offer several advantages over intact antibodies for biotechnological or research purposes and medical applications (3-6). For example, they might be an efficient alternative to the engineered antigen-binding domains of conventional antibodies which are referred to as a single chain antibody variable fragment (scFv) (7, 8). Indeed, similar to classical antibodies and their engineered scFvs, which have a longstanding history as diagnostic and therapeutic agents against toxins and pathogens, the impact of Nbs is gradually rising (7-9). Nbs were successfully generated against numerous pathogens including intact bacterial cells, viruses and toxins (5, 10-13). Brucella is a small invasive, non-motile, gram-negative coccobacillus that multiplies and survives within a broad range of eukaryotic cells (14). Brucellosis, also known as undulant fever or Mediterranean fever, is caused by this genus of bacteria and is still one of the major, highly transmissible, zoonotic diseases afflicting people and animals (15). While it has been successfully eradicated from most developed countries, the situation of this disease in certain countries of the Near East and Middle East (e.g. Syria) is becoming even worse. It still has a major impact on the health of both human beings and domestic animals, particularly cattle, sheep, goats and camels with concomitant economic consequences (16, 17). While classical serological techniques are widely used for diagnosing human and animal brucellosis, they depend mainly on the detection of circulating host serum antibodies to lipopolysaccharide (LPS), the immunodominant surface antigen (18). However, the immunologically recognized feature of Brucella LPS, the O:9 epitope, is also shared by some Yersinia. As a result, many existing anti-Brucella sera exhibit cross-reactivity with Yersinia (15). This shortcoming of anti-LPS antibodies has fueled an increasing interest in the detection of antibodies alternative to Brucella antigens, since it is important to distinguish these two organisms as double infections are common (15). Phage display is considered as one of the most successful applications for the identification of new diagnostics and therapeutics (Nbs or ScFvs) for a wide range of disease causing agents including bacteria (19). This approach was used recently to isolate Brucella specific scFvs, despite the solubility and stability problems these antibody fragments might show (20). Considering all the advantages of Nbs, we have recently established a new Nb-phage display library from an Arabian camel immunized with Brucella (21). This immune library was sufficiently large and competent to retrieve new Brucella specific Nbs. After three rounds of phage display panning on total bacterial lysate, three different Nbs were isolated. In this study, we report on the purification and characterization of soluble Nbs against Brucella. Unlike purified IgG from camel serum, Nbs were able to discriminate Brucella from Yersinia antigens, providing an efficient tool to surmount the problem of cross-reactivity between these two types of bacteria.

2. Objectives

Brucella specific Nbs are of particular importance since there is a definite need for better diagnostic and therapeutic tools to combat this infectious agent in the Near and Middle East.

3. Materials and Methods

3.1. Antigens and Antibodies

Brucella and *Yersinia* strains were provided by Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium. Prior to inactivation, *B. melitensis* strain Rev. 1 and *B. abortus* strain 2308 NalR were grown in Brucella broth medium (HIMEDIA Laboratories) supplemented with 5% heat inactivated horse serum (PAN Biotech GmbH) for at least 48 h in a 37°C incubator. The inactivation step was performed by washing the bacteria twice with cold phosphate buffered saline (PBS) followed by heat inactivation by incubation at 60°C for 30 min. Inactivation was confirmed by inoculating 1 mL of bacterial suspension on Brucella agar solid media. *Y. enterocolitica* strain O9 was used mainly to test the antibody cross-reactivity. In order to prepare lysate from different types of bacteria, 1 gram of wet weight bacteria was re-suspended in 5 mL of ice cold PBS and protease inhibitor solution (1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 × complete-protease inhibitor from Roche supplemented with 1 $mg.mL^{-1}$ of lysozyme), and was incubated for 30 min on ice followed by a 5 times sonication (15 sec pulses) on ice. Bacterial debris was eliminated by centrifugation (15000 ×g, 15 min, 4°C) and the supernatant was saved. The protein concentration in the supernatant was estimated by the Bradford colorimetric assay. For ELISA tests, rabbit anti-camel (Bethyl Laboratories Inc.), Horseradish peroxidase (HRP) conjugated goat anti-rabbit (BioRad) and mouse anti-his-HRP (Roche) antibodies were all used according to manufacturer's instructions.

3.2. Recloning of Nb genes in an Expression Vector

Nb genes from three previously isolated clones (21) were PCR-amplified using forward FR4For38 (3'-GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT) and reverse A6E (3'-GAT GTG CAG CTG CAG GAG TCT GGA GGA GG) primers. Amplified Nb genes were recloned into the protein

expression vector pHEN6 (22), using the restriction enzymes PstI and BstEII and the plasmid constructs were transformed into *E. coli* WK6 cells. Nbs expressed from this vector were supplemented with 6× his-tag necessary for further purification steps using nickel charged columns.

3.3. Purification of Soluble Nbs

A large-scale production of recombinant Nbs was performed in 250 ml shake flasks by growing the bacteria in Terrific broth medium (1.2 % tryptone, 2.4 % yeast extracts, 0.8 % glycerol, 17 mM KH2PO4, 72 mM K2HPO4) supplemented with 0.1% glucose and 100 μg.mL^{-1} ampicillin till an optical density (at 600 nm) of 0.6 to 0.9 was reached and then expression was induced with 1 mM IPTG for 16 h at 28°C according to previous reports (23). After pelleting the cells, the periplasmic proteins were extracted by osmotic shock (24). Using fast protein liquid chromatography (FPLC) AKTAprime plus system (GE lifescience), this periplasmic extract was loaded on a 1 mL column of nickel charged nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were eluted with a 250 mM imidazole buffer. The eluted fraction was concentrated on Vivaspin concentrators with a molecular mass cutoff of 5 kDa (Vivascience) and loaded on a Superdex-75 10/30 gel filtration column (Pharmacia). The purity of the fragments was evaluated in a Coomassie-stained SDS-PAGE. The absorption at 280 nm and the extinction coefficient as calculated from the amino acid sequence of each Nb (Figure 1) were used to determine protein concentrations.

3.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Maxisorb 96-well plates (Nunc) were coated with antigens by overnight incubation (at 4°C) with 100 μL/well of bacterial lysates, or pure LPS, prepared at 0.01 - 1 mg.mL^{-1} in PBS. Residual protein binding sites in the wells were blocked for 2 h at 37°C with 5% skimmed milk powder in PBS. Dilutions of soluble Nbs and IgGs, previously purified from the serum of Brucella immunized and controled camels (21), were added to the wells according to what was indicated in each experiment. Detection of antigen-bound Nbs or IgG was performed with mouse anti-his peroxidase conjugated antibody, and rabbit anti-camel antibodies, respectively. Subsequent detection of the rabbit antiserum was done with an anti-rabbit-HRP conjugate. The absorption at 450 nm was measured after adding peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB ready-to-use from Sigma) followed by stopping buffer (1 M of H2SO4) in order to terminate the enzymatic reaction of peroxidase.

3.5. Immunoblotting

SDS-PAGE (10 %) separation was performed for 10 mg of bacterial extract and separated proteins were blotted onto two 0.45 μm nitro-cellulose membranes (BioRad) using 1 × blotting buffer (25 mM Tris-base, 200 mM Glycine, 0.1 % SDS and 20 % Methanol). After incubation in the blocking buffer (3 % skimmed milk, 1 % BSA in 1 × PBS), one of the membranes was incubated with 1:500 dilution of NbBruc02 and the other with 1:50 dilution of NbBruc03 for an hour at room temperature. After several washes with TBS-tween-20 (20 mM Tris-base, 150 mM NaCl, 0.05 % Tween-20, pH 7.5), they were incubated with 1:1000 dilution of mouse anti-6 × His antibody HRP for 1 h at room temperature. Bands revelation was achieved by adding AEC (3-amino-9-ethylcarbazole) chromogen substrate in acetate buffer in the presence of hydrogen peroxide.

3.6. Multiplex PCR

Multiplex PCR reactions were achieved directly on aliquots of heat-killed bacteria. Before use, the killed organisms (see above) were rinsed once with distilled water and were then resuspended in distilled water at an optical density at 600 nm of 0.15 to 0.20 (approximately 10^9 cells per mL). Two μL of bacteria suspension were used in each PCR reaction. Brucella and Yersinia Multiplex PCRs were performed using multiple pairs of primers able to amplify a specific genome locus, based on the work of Imaoka et al. (25) with minor modifications.

4. Results

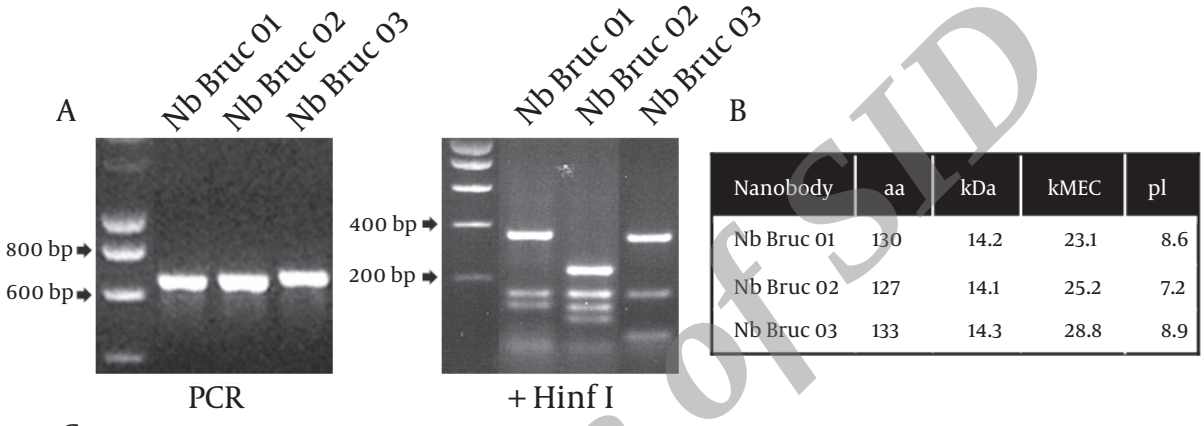
4.1. Characteristics and Sequences of the New Nbs

After immunizing a dromedary with heat-killed *B. melitensis* bacteria, we cloned the Nb genes from its blood lymphocytes and constructed an 'immune' library. This Nb library was displayed on phage and panned on *B. melitensis* bacterial lysate. After three rounds of panning, three isolated clones were able to produce virions with a Nb at their tip that recognized the crude bacterial lysate of Brucella (21). The genes of these Nbs were amplified with PCR from three independent clones in TG1 *E. coli* cells. The amplicons of these three clones have a similar size; however, differences in their nucleotide sequences could be easily discerned by restriction digestion with HinfI followed by gel-electrophoresis. This restriction fragment length polymorphism (RFLP) analysis revealed three distinctive cutting profiles (*Figure 1A*). These (intact) amplicons were sequenced and the deduced encoded soluble proteins were analyzed (*Figure 1B*). Their amino acid sequences were aligned (*Figure 1C*) and compared with a consensus Nb sequence. Minor changes were noticed in the protein size and molecular weight between the three Nbs and these differences were mainly related to the amino acid composition and the length of CDR3 loop. Obviously, among the three clones, the other CDRs (1 and 2) have the same length but have differences in their amino acid

sequence. Therefore, these clones reflect independent B-cell lineages that were at the origin of their expressed Nbs (6). Nevertheless, it is clear that all three Nbs were derived from the VHH germline genes, as they contained the hallmark amino acid substitutions in frameworks 1 and 2. As such, they possess arginine instead of leucine residues at position 50 (4, 9, 26). In addition to the conserved bridge between Cys23 and Cys104, that is present in all the clones, an extra interloop disulfide bridge, frequently occurring between CDR1 and CDR3 in camel Nbs, was observed in NbBruc02 (*Figure 1C*).

ported into the periplasmic space of *E. coli*. Purification of Nbs using periplasmic extract was done by immobilized-metal affinity chromatography, using a nickel-charged NTA column installed on AKTAprime system. The UV-detector, supplemented with this system, enabled the real-time monitoring of the different steps of Nb purification (*Figure 2A*). Column purification yielded 95% pure Nbs (14.5 kDa) as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with coomassie blue dye (*Figure 2B*, lane 3), which totally disappeared in the purification flow-through of each sample (*Figure 2B*, lane 2).

Figure 1. Molecular Characterization of the New Nbs



B

Nanobody	aa	kDa	kMEC	pI
Nb Bruc 01	130	14.2	23.1	8.6
Nb Bruc 02	127	14.1	25.2	7.2
Nb Bruc 03	133	14.3	28.8	8.9

C

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                1........10........20........30........40........50........60........7
IMGT            ^*********^*********^*********^*********^*********^*********^*********^
Regions         <-----------FR1----------><---CDR1---><------FR2------><--CDR2--><----
Nb Bruc 01      QVQLQESGG~GSVQAGGSLRLSCTASGSTY~~~~SRKRVGWFRQAPGKEREGVAIIYTAD~~GSAHYADS
Nb Bruc 02      QVQLQESGG~GSVQAGGSLRLSCAASGLTD~~~~STCGMAWYRQAPGKERELVSTIIT~D~~GNTYYADX
Nb Bruc 03      QVQLQESGG~GSVQSGGSLRLTCLASGHIR~~~~SGNVMGWFRQAPGKEREGIAAIYVS~~GGSTYYADS
Consensus       QVQLQESGG~GLVQAGGSLRLSCAASGGTF~~~~SIYTMGWFRQAPGKEREFVAAITRS~~~GASDYADS

                0........80........90........100.......110.....................120......
IMGT            *********^*********^*********^*********^*123456*123456*******^********
Regions         ----------FR3-----------------><---------CDR3----------><---FR4--->
Nb Bruc 01      VK~GRFSISQDNAKNTIYLQMNSLKPEDTAMYYCAASPHDY~~~YSLSG~~~~~SGFMYWGQGTQVTVSS
Nb Bruc 02      VK~GRFTISQDNAKNRVFLQMNSLKPEDTAMYYCNSWSESK~~~~RLC~~~~~~LPDNYWGQGTQVTVSS
Nb Bruc 03      VK~GRFTISLDNAKNTVYLQMNSLKPEDTAIYYCAAKGSAY~~AGSSWYRA~~~ADFGYWGQGTQVTVSS
Consensus       VK~GRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAADKTTGWNI~~~~~~~~~~~~YDYWGQGTQVTVSS
```

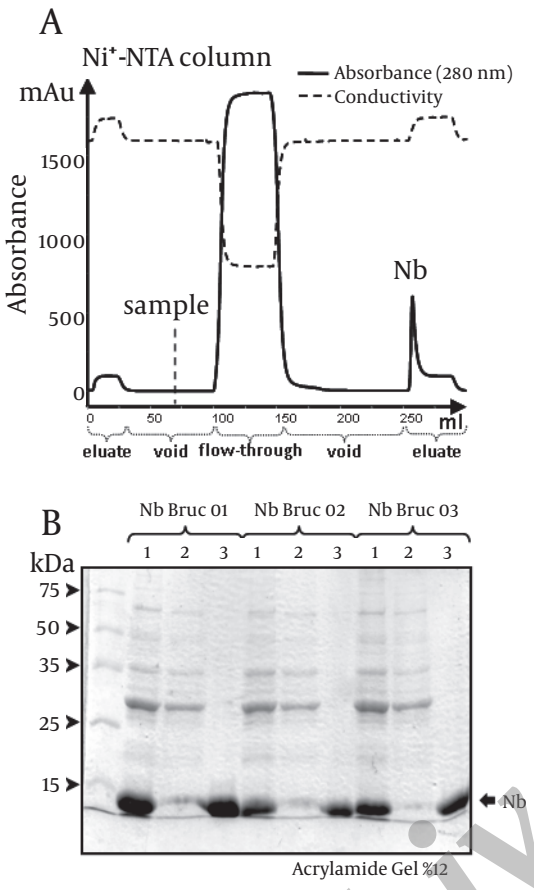

(A) Agarose gel migration of DNA fragments, containing Nb genes, after being amplified by PCR from their respective colonies. Internal sequence polymorphisms were revealed by digestion of the DNA fragments with HinfI restriction enzyme resulting in three distinct profiles on agarose gel (right). (B) Major protein characteristics of the three predicted Nbs including, fragment size (aa), theoretical molecular weight (kDa), isoelectric point (pI) and extinction coefficient (kMEC). This last property was used to calculate the concentrations of Nbs by spectrophotometer. (C) Alignment of amino acid sequence of the three predicted Nbs. Framework and hypervariable regions (CDR) are indicated. Numbers are according to IMGT numbering (27).

4.2. Expression and Purification of Soluble Nbs

Soluble Nbs were obtained after ligation of their amplicons into the expression vector pHEN6 and transformation into *E. coli* (22). In this system the expressed Nbs, carrying a 6× his tag to facilitate purification, were trans-

The yield of the purified product varied from 100, 80 and 40 mg.L^{-1} of bacteria culture for NbBruc01, 03 and 02, respectively. The purified proteins were present as monomers, since only a single band co-migrating with proteins of molecular mass of 14,000 Da could be seen after SDS-PAGE (*Figure 2B*).

Figure 2. Expression and Purification of Soluble Nbs



(A) Diagram of purification procedure using Ni-NTA column installed on FPLC AKTAprime system. Continuous line represents the absorbance of the eluate; different purification steps are shown below and peaks of the flow-through sample and of the purified Nb are indicated. The dashed line represents conductivity of the eluate. (B) Protein migration in SDS-PAGE (12%) of Nb fractions obtained after different steps of purification. Total periplasmic extract (1); flow-through sample from Ni-NTA column (2); and purified Nb protein (3) for each of the three Nbs. The location of Nbs in the gel is indicated and defined as 14.5 kDa by comparison to the protein molecular weight ladder in the first lane (M).

4.3. Brucella Recognition by Purified Nbs

Nbs were tested for binding to Brucella lysate in a solid phase indirect ELISA using serial dilutions of each of the three Nbs (*Figure 3*). NbBruc02 and 03 were both able to recognize Brucella antigens at high and moderate dilutions, respectively. At dilutions from 1×10^{-3} onwards, the NbBruc03 gradually lost its binding capacity toward Brucella. Interestingly, NbBruc02 showed a higher reactivity since it still had its full binding capacity at high dilutions of up to 2×10^{-4}. Unexpectedly, soluble NbBruc01 failed to give a positive signal in ELISA even when used at a high concentration. To optimize the quantity of Brucella lysate necessary for recognition by the different Nbs at their optimal dilutions, an ELISA test was performed using serially diluted concentrations of Brucella lysate ranging from 0 to 100 μg per well (*Figure 4*). NbBruc02 and 03 were able to recognize Brucella antigens even at 1 μg/well and this reactivity reached a steady state level starting from 5 μg/well, onwards. NbBruc01 failed to detect Brucella lysate even at high antigen quantities of 100 μg/well (*Figure 4*).

4.4. Identification of Nbs Purification and Antigen Detection Problems

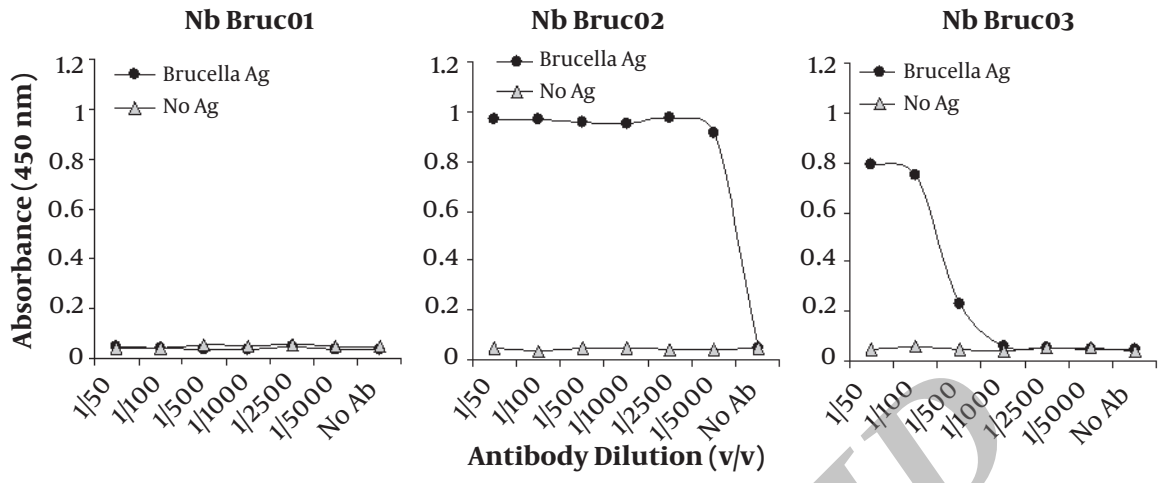
We anticipated that the conformational structure of the Nb might hinder the accessibility of its 6× his-tag resulting in low ELISA signal when using an anti-his antibody for detection. To examine if this could be a plausible explanation for the low NbBruc03 binding capacity relative to that of NbBruc02, we used different Nb detection strategies in our ELISA (*Figure 5A*). In addition to anti-his-HRP conjugated antibody, a rabbit anti-camel antibody followed with goat anti-rabbit-HRP conjugated antibody was employed to detect the Nbs attached to the immobilized (coated) Brucella antigens (*Figure 5B*). Rabbit-anti-camel antibody was able to recognize NbBruc02 and NbBruc03 which are derived from camel heavy chain antibodies (*Figure 5B*). In addition, the difference in reactivity between NbBruc02 and NbBruc03 observed with anti-his system disappeared when this anti-camel antibody was used. The NbBruc01 was probably not binding to Brucella antigens, since it was undetectable with either of these approaches (*Figure 5B*).

NbBruc01 was isolated from Brucella Nb library by the phage display method, where it was expressed as fusion with the phage coat protein (GIII). Apparently, the production of a single form of NbBruc01 by cloning its coding sequence into the expression vector pHEN6 resulted in an unstable protein that may have lost antigen recognition. Therefore, we surmise that the malfunction of NbBruc01 could be related to a misfolding problem, as the crude NbBruc01 periplasmic extract has low binding capacity to the immobilized Brucella antigens and this capacity is totally lost after purification (*Figure 5B* inset). In agreement with the last finding and unlike NbBruc02 and 03, the NbBruc01 was found to form a precipitate in the elution buffer after the chromatographic purification from periplasmic extract.

4.5. Specificity of the New Nbs Toward Brucella Species

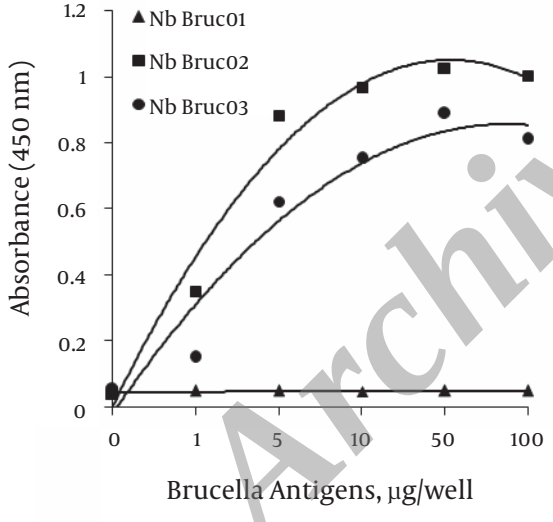
Purified Nbs were tested in an ELISA for their specificity to antigens from different species of Brucella, and their cross-reactivity toward Yersinia antigens (*Figure 6*). In order to ensure the identity of the used bacterial strains, Brucella and Yersinia multiplex PCRs were performed directly on bacterial suspension and the resulting PCR products were separated through agarose gel (*Figure 6A-*

Figure 3. Titration of the Nbs



Indirect ELISA test of serial dilutions (v:v of PBS) of each Nb (at 1 mg.mL^{-1}), in the absence or presence of Brucella lysate coated at 10 μg/well

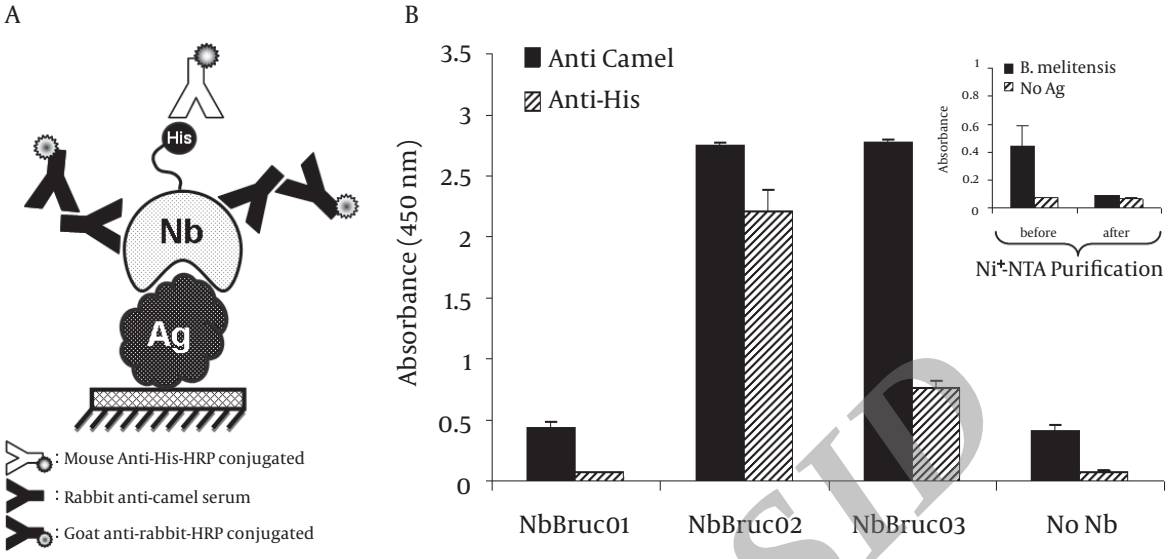
Figure 4. Optimization of the Quantity of Brucella Lysate for ELISA Test



One single dilution (1/100) of each Nb was tested in ELISA against increasing concentrations of Brucella lysate, ranging from 0 to 100 μg/well.

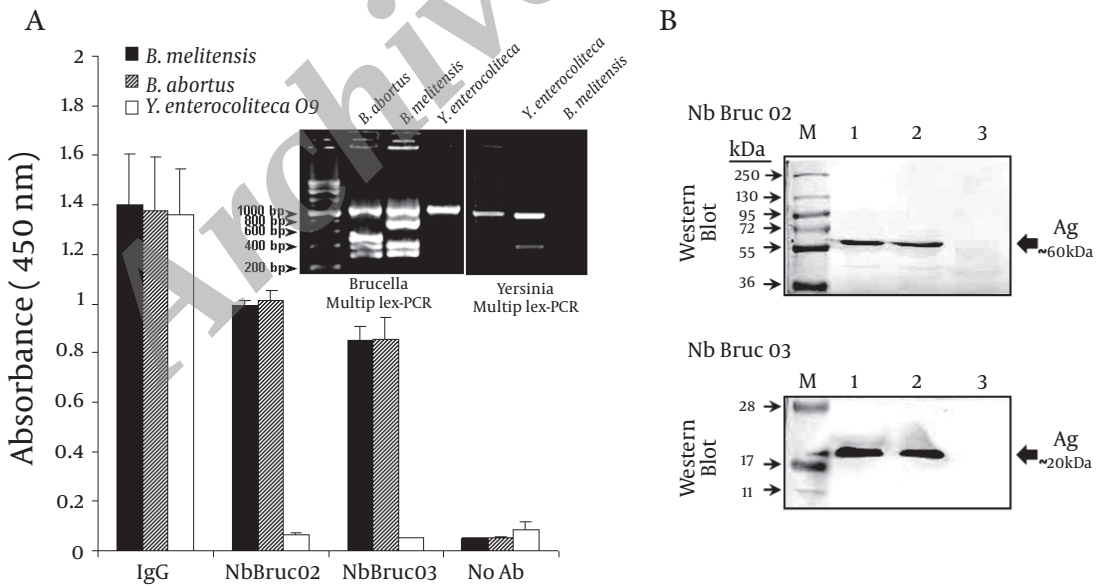
inset). With the amplified DNA bands from Brucella, we could define the Brucella genus by the presence of a pair of bands at approximately 300 and 400 bp. Brucella species of each sample were identified by the presence of a single band above the duplicate, 500 bp for *B. abortus* and 700 bp for *B. melitensis*. One universal band amplified from the highly conserved 16s rRNA locus could be observed in all tested DNAs at 1000 bp and was considered as our control for the presence of genomic bacterial DNA in the PCR reaction. Another multiplex PCR was performed to identify Yersinia. In this reaction, the characteristic bands for Yersinia, absent in Brucella sample, were clearly visualized at 950 bp (for the genus Yersinia) and at 300 bp (for the species *Y. enterocolitica*). Total bacteria lysates were prepared from these PCR-checked strains, immobilized on ELISA plates and tested for recognition by soluble Nbs, NbBruc02 and NbBruc03, that had reacted positively in previous tests (*Figure 6A*). We found that both Nbs were able to detect antigens from *B. melitensis* and *B. abortus* lysates giving absolutely no cross-reactivity to those from *Y. enterocolitica*. This clear antigen discrimination was completely abolished when the polyclonal camel IgG was used instead of the monoclonal Nbs, since this 'immune' polyclonal IgG sample reacted to the same level with the antigens present in the different types of bacteria (*Figure 6A*). To identify the specific antigen of NbBruc02 and NbBruc03 within Brucella lysate, we performed an immunoblotting assay. To this end, proteins from cell lysates of *B. melitensis* and *B. abortus* were separated onto 10% SDS-PAGE and then transferred to nitro-cellulose membranes in order to be revealed either by NbBruc02 (*Figure 6B*, top) or NbBruc03 (*Figure 6B*, bottom). Attached Nbs on the membranes were detected by incubation with mouse anti-6× His-HRP conjugated antibody. This experiment demonstrated that, NbBruc02 antigen was a protein of about ~ 60 kDa and NbBruc03 antigen was a smaller protein of about ~ 20 kDa and both proteins were detected in the lysates of the two strains of Brucella.

Figure 5. Inspection ofNb Detection Problems



(A) Schematic representation of two models for the detection of the interaction between Nbs and Brucella antigens (Ag). Either by anti-his antibody conjugated to HRP or by rabbit anti-camel serum antibody followed by goat anti-rabbit antibody conjugated to HRP. (B) ELISA test of the interaction between Nbs (1/100) and Brucella total lysate (10 μg/well) using the two indicated approaches of detection. Signal backgrounds of both systems are shown in the absence of Nbs (No Nb). Reactivity of NbBruc01, before and after chromatography purification, in the absence (No Ag) or the presence of Brucella lysate (10 μg/well) was ELISA tested (inset).

Figure 6. Specificity of Nbs Toward Antigens From Different Brucella Species



Diluted Nbs (1/500 for NbBruc02 and 1/50 for NbBruc03) or Brucella-immunized (+) or control (-) camel-purified IgGs (1/1000) were tested by ELISA and backgrounds of each antigen condition in absence of Nbs or IgG are shown (No Ab). (A) ELISA was performed in the presence of immobilized total extract (10 μg/well) from different sources (B. melitensis, B. abortus and Y. enterocolitica as negative control). Identity of used bacteria strains were confirmed by Brucella and Yersinia multiplex PCRs which were performed directly on bacterial suspension. PCR products, containing different DNA fragments according to the genus and the species of each type of bacteria, were separated through 1.5% agarose gel (A-inset). (B) Immunoblotting of 10μg of protein extracts from B. melitensis (lane 1), B. abortus (lane 2) or no antigens (lane 3), using NbBruc02 (Top; 1:500 v:v) or NbBruc03 (Bottom; 1:50 v:v).

5. Discussion

The data presented here report the purification and the characterization of three Nbs which were previously isolated from a large *Brucella* 'immune' Nb library. In fact, phages carrying these Nbs were highly enriched after panning with *Brucella* total antigens (21). This study confirms the findings from a previous report describing the feasibility to isolate a panel of infectome-specific binders from an immunized camel without having prior knowledge of the antigens involved (4). In contrast to their apparent equivalent size, the internal amino acids sequences of the Nbs are quite different, especially in their CDRs, indicating the use of different V-germline within their respective B-cells. Furthermore, the extra disulfide bond in NbBruc02, observed between the first and the third antigen-binding loop, is expected to confer an increased stability to the protein structure and thus can assist in its high reactivity toward *Brucella* antigens even at a low concentration. All three Nbs were successfully recloned, expressed and purified in large yields from *E. coli* culture suggesting being an ideal system for continuous mass production as required for medical or commercial purposes. Dissimilar to monomeric behaving Nbs, which exist as a single polypeptide chain, scFvs were produced by the random joining of the two variable domains (V) of conventional antibody; those of the heavy (VH) and light (VL) chains. This splitting and then artificial rejoining of the natural antigen-binding partners usually result in binders with suboptimal affinity and stability (1). In fact, previous research that worked to produce soluble *Brucella* specific scFv have encountered multiple problems of solubility and degradation (20). ELISA tests have shown that both NbBruc02 and 03 could recognize two of the main *Brucella* species, *B. melitensis* and *B. abortus* belonging to a monospecific genus with interspecies homology above 87 percent (15). Despite the shared antigens of this genus with types of Yersinia, mainly the LPS O-ring epitope, both Nbs were able to distinguish between these two genera. As expected, purified polyclonal IgG from camel, immunized with *Brucella*, was unable to make such distinction. This cross-reactivity is seen regularly in sera collected from immunized or naturally infected animals. The protein nature of the antigens targeted by NbBruc02 and 03 was presumed since these Nbs showed no reactivity to *Brucella* LPS despite them being the main immunogenic part of bacteria (28). This strategy of avoiding LPS-specific antibodies while using Nbs to detect relatively rare but unique and specific epitopes should allow rapid definition of bacteria within mixed populations and be of application to environmental sensing. Our results demonstrated that beside multiplex PCR, which is considered as the most accurate molecular technique (25), *Brucella* could also be successfully identified by Nbs. In this context, it would be interesting to continue with this current *Brucella* 'immune' Nb library and explore more advanced panning strategies to retrieve a wider range of species-specific Nbs. For example, a subtractive panning could be introduced here as it was already successfully employed to identify Taenia species-specific Nbs (5). Such an approach could be adapted in order to obtain Nbs able to distinguish the different species of *Brucella* and thus be used for different diagnostic purposes. Apart from the employment of Nbs in diagnostic tests, a therapeutic utilization might be envisaged as well. In fact, using proteomic techniques such as immunoprecipitation and MALDI-TOF-MS (29), the 60 kDa protein antigen of NbBruc02 (GenBank accession no. JF313902), was isolated and identified as the chaperonin GroEL (30). A similar approach should be done in order to identify the 20 kDa protein antigen of NbBruc03. Studying the importance of these antigens in *Brucella* physiology and life cycle, including infection, will be of great importance. These antigens are presumed to be immunodominant, not only because the phages displaying these Nbs were rapidly enriched during panning but also since a small quantity of *Brucella* total lysate was sufficient to be recognized by their purified proteins (i.e. high sensitivity of detection). In case these antigens turn out to be novel markers, we could invest in their utility to be developed for a sensitive and specific serological diagnostic tool of *Brucella* infection and even to develop a new vaccine against Brucellosis.

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Authors' Contribution

The work presented here was carried out in collaboration between all authors which have contributed to, seen and approved the manuscript.

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References

1. 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
2. Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, Frenken LG, et al. Single-domain antibody fragments with high conformational stability. *Protein Sci*. 2002;**11**(3):500-15
3. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, Kinne J, et al. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. *Proc Natl Acad Sci U S A*. 2006;**103**(12):4586-91
4. Saerens D, Conrath K, Govaert J, Muyldermans S. Disulfide bond introduction for general stabilization of immunoglobulin heavy-chain variable domains. *J Mol Biol*. 2008;**377**(2):478-88
5. Deckers N, Saerens D, Kanobana K, Conrath K, Victor B, Wernery U, et al. Nanobodies, a promising tool for species-specific diagnosis of Taenia solium cysticercosis. *Int J Parasitol*. 2009;**39**(5):625-33
6. Muyldermans S, Baral TN, Retamozzo VC, De Baetselier P, De Genst E, Kinne J, et al. Camelid immunoglobulins and nanobody technology. *Vet Immunol Immunopathol*. 2009;**128**(1-3):178-83
7. Van Bockstaele F, Holz JB, Revets H. The development of nanobodies for therapeutic applications. *Curr Opin Investig Drugs*. 2009;**10**(11):1212-24
8. Wesolowski J, Alzogaray V, Reyelt J, Unger M, Juarez K, Urrutia M, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Med Microbiol Immunol*. 2009;**198**(3):157-74
9. Saerens D, Ghassabeh GH, Muyldermans S. Single-domain antibodies as building blocks for novel therapeutics. *Curr Opin Pharmacol*. 2008;**8**(5):600-8
10. El Khattabi M, Adams H, Heezius E, Hermans P, Detmers F, Maassen B, et al. Llama single-chain antibody that blocks lipopolysaccharide binding and signaling: prospects for therapeutic applications. *Clin Vaccine Immunol*. 2006;**13**(10):1079-86
11. Abderrazek RB, Hmila I, Vincke C, Benlasfar Z, Pellis M, Dabbek H, et al. Identification of potent nanobodies to neutralize the most poisonous polypeptide from scorpion venom. *Biochem J*. 2009;**424**(2):263-72
12. Harmsen MM, van Solt CB, Fijten HP. Enhancement of toxin- and virus-neutralizing capacity of single-domain antibody fragments by N-glycosylation. *Appl Microbiol Biotechnol*. 2009;**84**(6):1087-94
13. Lam AY, Pardon E, Korotkov KV, Hol WG, Steyaert J. Nanobody-aided structure determination of the EpsI:EpsJ pseudopilin heterodimer from Vibrio vulnificus. *J Struct Biol*. 2009;**166**(1):8-15
14. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis*. 1997;**3**(2):213-21
15. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. *N Engl J Med*. 2005;**352**(22):2325-36
16. Darwish M, Benkirane A. Field investigations of brucellosis in cattle and small ruminants in Syria, 1990-1996. *Rev Sci Tech*. 2001;**20**(3):769-75
17. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis*. 2006;**6**(2):91-9
18. Forestier C, Moreno E, Meresse S, Phalipon A, Olive D, Sansonetti P, et al. Interaction of Brucella abortus lipopolysaccharide with major histocompatibility complex class II molecules in B lymphocytes. *Infect Immun*. 1999;**67**(8):4048-54
19. Hoogenboom HR. Selecting and screening recombinant antibody libraries. *Nat Biotechnol*. 2005;**23**(9):1105-16
20. Hayhurst A, Happe S, Mabry R, Koch Z, Iverson BL, Georgiou G. Isolation and expression of recombinant antibody fragments to the biological warfare pathogen Brucella melitensis. *J Immunol Methods*. 2003;**276**(1-2):185-96
21. Abbady AQ, Al-Mariri A, Zarkawi M, Al-Assad A, Muyldermans S. Evaluation of a nanobody phage display library constructed from a Brucella-immunised camel. *Vet Immunol Immunopathol*. 2011;**142**(1-2):49-56
22. Conrath KE, Lauwereys M, Galleni M, Matagne A, Frere JM, Kinne J, et al. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. *Antimicrob Agents Chemother*. 2001;**45**(10):2807-12
23. Arbabi Ghahroudi M, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett*. 1997;**414**(3):521-6
24. Skerra A, Pluckthun A. Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science*. 1988;**240**(4855):1038-41
25. Imaoka K, Kimura M, Suzuki M, Kamiyama T, Yamada A. Simultaneous detection of the genus Brucella by combinatorial PCR. *Jpn J Infect Dis*. 2007;**60**(2-3):137-9
26. Nguyen VK, Hamers R, Wyns L, Muyldermans S. Camel heavy-chain antibodies: diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. *EMBO J*. 2000;**19**(5):921-30
27. Lefranc MP, Pommie C, Ruiz M, Giudicelli V, Foulquier E, Truong L, et al. IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev Comp Immunol*. 2003;**27**(1):55-77
28. Tumurkhuu G, Koide N, Takahashi K, Hassan F, Islam S, Ito H, et al. Characterization of biological activities of Brucella melitensis lipopolysaccharide. *Microbiol Immunol*. 2006;**50**(6):421-7
29. Borch J, Roepstorff P. SPR/MS: recovery from sensorchips for protein identification by MALDI-TOF mass spectrometry. *Methods Mol Biol*. 2010;**627**:269-81
30. Abbady AQ, Al-Daoude A, Al-Mariri A, Zarkawi M, Muyldermans S. Chaperonin GroEL a Brucella immunodominant antigen identified using Nanobody and MALDI-TOF-MS technologies. *Vet Immunol Immunopathol*. 2012;**146**(3-4):254-63