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Research Article

Construction of a Nanobodies Phage Display Library From an *Escherichia coli* Immunized Dromedary

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Background: Diarrhea caused by *Escherichia coli* is a major cause of morbidity and mortality in young animals. Few treatment options are available, mainly antibiotic therapy increasingly limited by resistance to commonly used drugs.
Objectives: The aim of this work was to develop immunotherapy based on the use of camel VHH antibody fragments, or nanobodies, to target pathogenic *E. coli* surface antigens.
Material and methods: We immunized a camel with a killed strain we had previously isolated from a diarrheic camel calf and identified as expressing the F17 fimbriae antigen.
Results: The immunized animal developed an anti-*E.coli* immune response including heavy-chain antibodies. Lymphocytes from this animal were purified and RNA isolated to create a VHH library by phage display with a size of about 10^9 individual transformants. Panning on live *E. coli* cells resulted in the isolation of VHH fragments specific to the cell surface antigens.
Conclusion: The identification of these antigens can lead to the development of new diagnostic and therapeutic tools against diarrhea.
Key words: *Escherichia coli*; diarrhea; nanobodies; phage display

1. Background

Neonatal diarrhea is a major cause of death in herds and is responsible for considerable economic losses. *Escherichia coli* (*E. coli*) is a major etiological agent of diarrhea in young animals. It is part of the intestinal commensal microflora of most warm-blooded animals (1). However, *E. coli* can also be an opportunistic or obligate pathogen, able to multiply and persist in the host's digestive tract by bypassing immune defenses and inducing cell damage (2).

The study of the different modes of interactions between the host and the bacterium during infection makes it possible to classify *E. coli* strains into several pathogenic variants called pathovars or pathotypes. This classification is based on the combination of particular properties associated with virulence factors expressed by a strain, the infection route and the associated clinical signs (3).

There are currently nine pathovars of pathogenic *E. coli* that can be classified into two groups: Intestinal *E.coli* comprising seven pathovars responsible for intestinal disorders: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic (EHEC) (or Shiga toxin-producing *E. coli*, STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC or EAggEC) and diffuse adhesion *E. coli* (DAEC). Extra-intestinal *E. coli* including two pathovars, *E. coli* associated with neonatal meningitis (NMEC) and uropathogenic *E. coli* (UPEC) (4).

Recently, we demonstrated that out of 120 fecal samples from camel calves aged 1 day to 3 months (62 healthy and 58 diarrheic), 70 strains of *E. coli* were isolated with a prevalence of 67% and 50% in diarrheic and healthy calves, respectively. Molecular identification of *E. coli*-associated virulence genes, using the PCR technique, showed that the prevalence of the gene encoding F17 fimbriae was significantly higher in diarrheic than in clinically healthy calves (46.5% vs. 14.5%)(5).

In addition to conventional antibodies, camelids (camels and llama) produce antibodies devoid of the light chain and the CH1 domain called heavy chain antibodies (HCAbs) (6). VHH fragments or nanobodies are derived from these HCAbs. They are highly soluble, physicochemically stable and can be produced with high yields in bacteria, fungi or plants (7, 8). Thanks to their monomeric structure, VHH are easily engineered to create multimers or to be more stable, especially to proteases(9) These favorable properties have led to the development of several nanobodies for use in a wide variety of research, diagnosis or therapeutic applications (10).

In diarrhea, VHH fragments have been used to target rotavirus antigens like E2 protein (11) or the F4 and F18fimbriae antigens(12). Tremblay et al. (13) described the development of VHH fragments against Shiga toxins produced by STEC.

2. Objective

The goal of this work was to create an immune VHH library from a dromedary immunized with killed *E. coli* isolated from diarrheic camel calves in order to select nanobodies for original antigens.

3. Materials and Methods

3.1 Production of Polyclonal Antibodies in Rabbits

Camel IgG subclasses (IgG1, IgG2 and IgG3) purified by protein A affinity chromatography and purified M13KO7 helper phage were used for the production of antibodies in two rabbits. Each rabbit received, subcutaneously, the first injection of a mixture of 1mL antigen (0.3 mg of IgG or 10^9 phage) and 1mL of complete Freund adjuvant followed by four boosts with incomplete Freund adjuvant. At the end of the immunization program, animals were bled, the blood was left to coagulate and then centrifuged to recover the serum. The antibodies were purified from rabbit serum by the ÄKTApurifier 10 (General Electric Healthcare, Sweden) Fast Protein Liquid Chromatography (FPLC) system on a HiTrap protein A FF 5ml column (GE Healthcare). After equilibration of the column with 15 ml of phosphate buffer, the rabbit IgG was eluted with 0.1M citrate buffer pH3, quantified at 280 nm and stored at -30°C.

3.2 Immunization of the Camel With Killed E. coli

A strain isolated from diarrheic camel calf with the virotype: *f17/afa/EastI/papC/iroN/iss/iucD* and serotype O64 and belonging to the phylogenetic group B1 was used for the immunization. A colony of the *E. coli* strain was cultured overnight with stirring of 200 rpm in 5 mL of Luria broth (LB) at 37°C. From this preculture, a 1/100 dilution was made in 200 mL of LB medium in 1L Erlenmeyer flask and incubated at 37°C for 48 hours without shaking. This culture was centrifuged and the pellet was washed twice in 20 mL PBS. Formaldehyde was added to resuspended bacteria in PBS to a final concentration of 0.4% and incubated overnight at 4°C. The cell pellet was recovered in 5mL PBS after 30 min of centrifugation at 6000g. Five boosts at 10 days intervals were performed subcutaneously by a mixture of 1mL of this preparation and 1mL of incomplete Freund adjuvant. A new culture was used for every boost.

3.3 Purification of Serum Immunoglobulin G

Camel antibodies were purified by FPLC on Protein A and G affinity columns with the ÄKTApurifier 10. One milliliter of serum was loaded on a 5mL protein G column equilibrated with phosphate buffer (pH 7) at a flow rate of 3 mL/min. The bound IgG3were eluted with 0.15M NaCl/0.58% acetic acid (pH 3.5). Then, after washing, IgG1 was eluted with 0.1M glycine buffer(pH 2.7). IgG2 not bound by the G protein column are purified on a protein A column and eluted with 0.15M NaCl 0.58% acetic acid (pH 3.5). The eluate of each IgG fraction was neutralized with 1M Tris-HCl pH 8.8. The purity of the eluted fractions was evaluated by SDS-PAGE (Supplementary figure 2).

3.4 Library Construction

Total RNA was extracted from the peripheral blood lymphocytes with RNAqueous midi kit (Ambion) and the cDNA was prepared using MMLV reverse transcriptase (Invitrogen) with the CALL002 primer (5'-GGT ACG TGC TGT TGA ACT GTT CC-3'). VHH coding sequences were amplified by PCR with primers FR1For (5'-GAT GTG CAG CTG CAG GAG TCT GGG GGA GG-3') and FR4Rev (5'-GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT-3') annealing at the framework-1 and framework-4 regions flanking the CDRs of heavy chain antibodies, respectively. The PCR fragments were ligated into the phagemid vector pHEN4 (14) after cutting with the restriction enzymes *Pst*I and *Not*I. Ligated materials were transformed by electroporation in freshly prepared *E. coli* TG1 cells, plated on 24×24 cm plates containing 2YT medium (16g tryptone, 10g yeast extract and 5g NaCl) supplemented with ampicillin and glucose(2YT–AMP–GLU) and incubated overnight at 37°C. The electroporation was performed with the Eporator (Eppendorf, Germany) in 1mm gap cuvettes (Sigma) at 1700Kv. Colonies were scraped from the plates and stored at −80°C in 2YTmedium supplemented with 50% glycerol.

To establish the library size, dilution series (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) were prepared from electroporated cells in a liquid medium and 100 µL were plated onto 9 cm diameter 2YT–AMP–GLU plates and incubate overnight at 37°C.

3.5 VHH Phage Library Panning

Two milliliters of TG1 bacteria from the library were cultured in one liter of 2YT medium containing ampicillin (100 µg/mL) with shaking at 37°C. Once the optical density (OD) at 600nm reached 0.5, the bacteria were infected with M13KO7 helper phage and incubated with shaking at a low speed at 37°C for one hour. Then,

kanamycin was added to a final concentration of 50 µg/mL and the culture incubated overnight at 37°C with shaking. The next day, phage precipitation was performed by polyethylene glycol 6000/NaCl (20%/2.5M) and resuspended in a total volume of 3 mL PBS.

For the first round of panning, 1mL of the produced phages (4×10^{11} particles) were incubated for 2 hours at room temperature under rotation with live *E. coli* F17+ bacteria (approximately 10^{10} CFU / mL). After 10 washes with PBS, the phages bound on the bacteria are eluted for 10 minutes in 1 mL of 100 mM triethylamine (pH 11) then neutralized by the addition of 1 mL of 1M Tris-HCl (pH 7.4).

Output phages were used to infect TG1 bacteria in the exponential phase and incubated at 37°C for one hour with shaking and plated on 2YT ampicillin/glucose plates. Enrichment of bacteria-specific phages was assessed by the ratio of the number of eluted phages to incubated phage for panning at every round. Individual colonies were picked at the end of the third round to be assayed by ELISA.

3.6 Enzyme-linked immunosorbent assay

The *E. coli* F17+ strain was cultured in LB medium overnight at 37°C. The bacteria were centrifuged at 4000×g for 15 min and the pellet was resuspended in PBS. A 96-well microplate (Greiner Bio-One) was coated overnight at 4°C with 100 µL per well of the bacterial suspension (about 10^6 cells/well). The microplate was emptied and dried with an air dryer and the residual binding sites were blocked for two hours with 200 µL of 3% BSA in PBS. A plate was assayed either with serum and purified IgG with the anti-camel antibody or with monoclonal phages with the anti-phage antibody. An anti-rabbit HRP conjugate (Invitrogen) was used (100µl from 1/5000 dilution) as a secondary antibody and 100 µl of TMB (Invitrogen) as a substrate.

4. Results

4.1 Production and Purification of Anti-Camel IgG and Anti-Helper Phage Antibodies in Rabbits

Two rabbits were immunized with antibodies purified by FPLC from a non-immunized dromedary or purified M13KO7 helper phage. We performed 5 injections of purified camel IgG or 10^9 purified phage with Freund's complete adjuvant for the first immunization and incomplete for the 4 boosts. Four days after the last boost, the sera obtained after centrifugation of blood from the two rabbits were purified by affinity chromatography and assayed by ELISA on purified camel IgG and M13KO7 helper phage (**Supplementary Figure 1**).

4.2 Kinetics of the Immune Response of Different IgG Subclasses

A 2-year-old female camel was immunized 5 times with killed bacteria at 10-day intervals. Blood samples were collected at each immunization in order to evaluate the *E. coli* specific immune response. At the end of the immunization and after purification by FPLC (**Figure 1**), the kinetics of the production of the different IgG subclasses were assayed individually. The final dilution of the samples was 1/5000 for the different subclasses taking into account the dilution factor during purification by FPLC following elution. For example, a fraction eluted in 5 mL of buffer from 1 mL of injected serum was diluted1/1000 to obtain a final dilution of 1/5000. For immune sera, the dilutions were 1/10000. The ELISA results (**Figure 2**) showed that the camel developed a specific immune response against *E. coli*. For the serum we can notice a fast increase for the immune response between the first and the second immunization, suggesting that the animal has been already exposed to an *E. coli* infection. The reactivity toward *E. coli* was high for IgG1 and IgG3 and weak for IgG2.

4.3 VHH Library Construction

Total RNA, purified from 3×10^7 lymphocytes, isolated from 100 mL of blood collected from the immunized camel four days after the last boost, was reverse transcribed with CALL002 primer into cDNA. The cDNA was used as a template to amplify VHH gene fragments by PCR. The PCR fragments were ligated to pHEN4 after digestion with *Pst*I and *Not*I (**Figure 3**). Ligation products were electroporated to freshly prepare competent *E. coli* TG1 cells. The resulting library had a size of about 10^9 individual clones. By a PCR screening on isolated clones, 85% harbor VHH genes of the proper size.

4.4 Panning of VHH Fragments anti-E. coli F17+

The isolation of *E. coli*-specific VHH was performed by three consecutive rounds on live cells suspended in PBS. To produce phages carrying on their surface the VHH, the bacteria from the library were infected with the M13KO7 helper phage, containing a non-replicative phagemid but coding for all the proteins necessary for the generation of new phages. Approximately 4×10^{11} phage particles were incubated with about 10^8 live *E. coli* cells. Washing was performed by 10 centrifugations with 10mL PBS each time. The eluted phage was used to infect exponentially growing TG1 cells, spread on 2YT plates containing glucose and ampicillin and scraped for the next round of panning. For each round of panning, an aliquot of phage-infected cells is taken, serially diluted

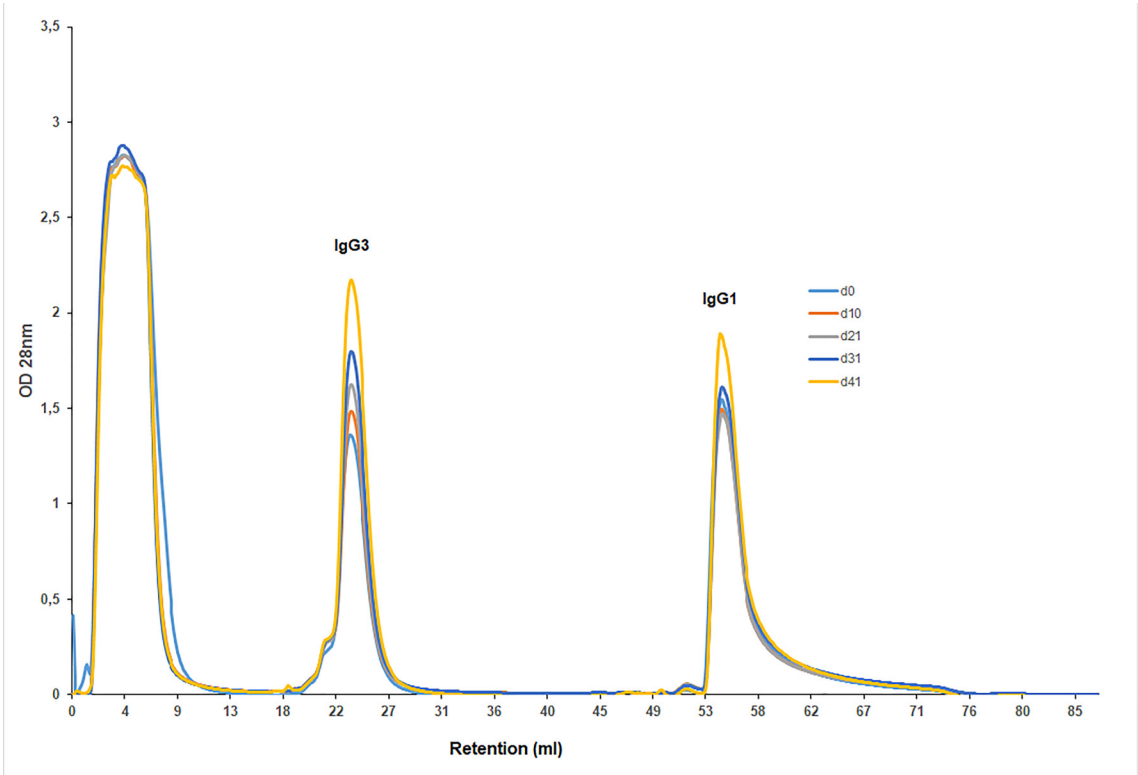

Figure 1. Purification of different IgG subclasses of immunized camel serum by Fast protein liquid chromatography using ÄKTA purifier 10 system. For each boost, one mL of camel serum was adsorbed onto HiTrap Protein G column and washed with phosphate buffer to remove the unbound proteins (first peak), before eluting the IgG3 (second peak) and then the IgG1 (third peak) fractions.

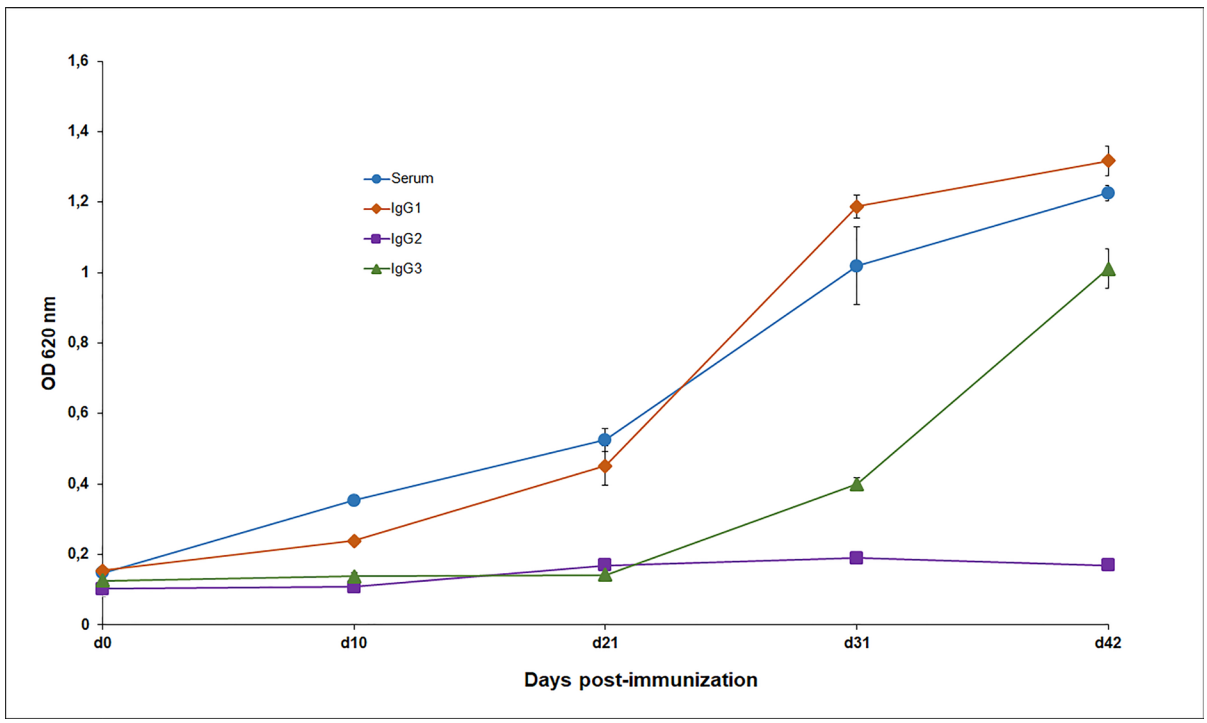

Figure 2. Evaluation of the camel immune response raised against *E. coli* by ELIS Aassay. Reactivity against *E. coli* cells of serum samples and purified IgG taken from the immunized camel at several time points from the first injection onwards. Serums were diluted to 1/10000 and purified IgG were diluted to 1/5000. Results represent the mean of three duplicates.

and spread on 2YT Petri dishes to estimate the number of output phage. The ratio of the number of output to input phage (**Table 1**) indicated a clear enrichment of the library with *E. coli* specific phage.

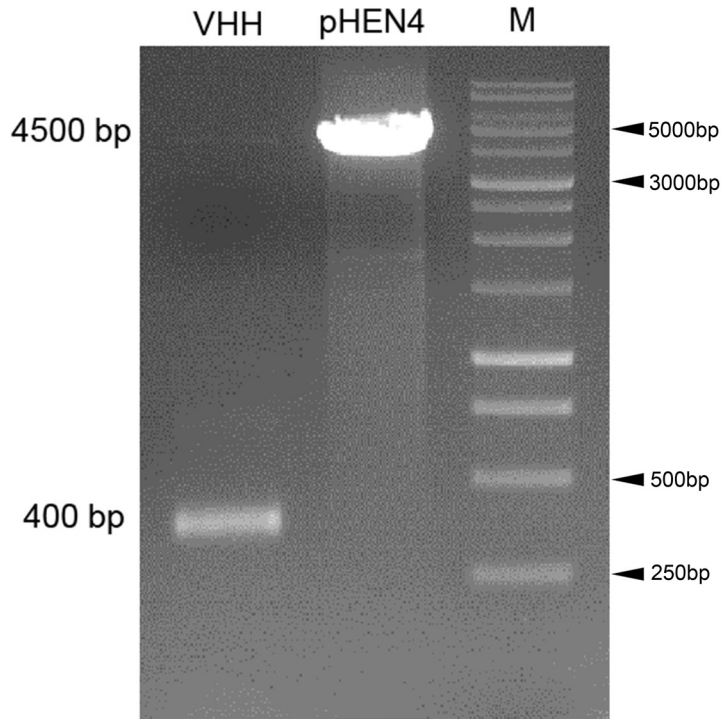

Figure 3. Agarose gel electrophoresis of the VHH PCR fragments and the pHEN4 phagemid digested with *Not*I and *Pst*I restriction enzymes before ligation and transformation of TG1 bacteria.

Table 1. Library enrichment during panning cycles. Input corresponds to phage incubated with cells and output number of phage eluted from each panning cycle.

Panning Cycle	Input (CFU*)	Output (CFU*)	Enrichment (Output/ Input)
1	4.6×10^{11}	1.4×10^{6}	0.3×10^{-5}
2	4.6×10^{11}	5×10^{9}	1.1×10^{-2}
3	4.6×10^{11}	8.6×10^{10}	1.86×10^{-1}

*CFU (colony forming units)

4.5 Monoclonal Phage Analysis by ELISA

After the third round of panning, 95 TG1 bacterial colonies from the Petri dishes serving for output count, were randomly picked to 3 numbered Petri dishes. Every colony was cultured in 100µL 2YT medium with ampicillin in a 96 well culture plate for 2 hours at 37°C with shaking. Bacteria were infected by helper phage and incubated at 37°C without shaking then kanamycin was added and the plate incubated overnight with shaking. The culture supernatant was used for ELISA on plates coated with *E. coli* cells as previously described. All the tested clones were able to bind the *E. coli* cells (**Figure 4**). Since working with coated bacteria is difficult because during washing steps they tend to detach from the wells, the ELISA was repeated two times and gave comparable results (data not shown).

5. Discussion

Diarrhea is a major health problem in camel rearing, as for most other animals. Morbidity can range from 10% to 30% depending on the farming system and can even reach 80% in some farms. In camels, the only treatment for this disease is antibiotic therapy, which is not always effective because many strains develop resistance to commonly used antibiotics (5). In this work, we have considered developing nanobody-based immunotherapy against *E. coli* infections in newborn camel calves. The aim was to develop an anti-*E. coli* VHH library from a camel immunized with killed bacteria. Recently, we showed that the F17 fimbriae are detected with a predominant prevalence in the analyzed fecal samples derived from diarrheic camel calves (5). In the literature, several

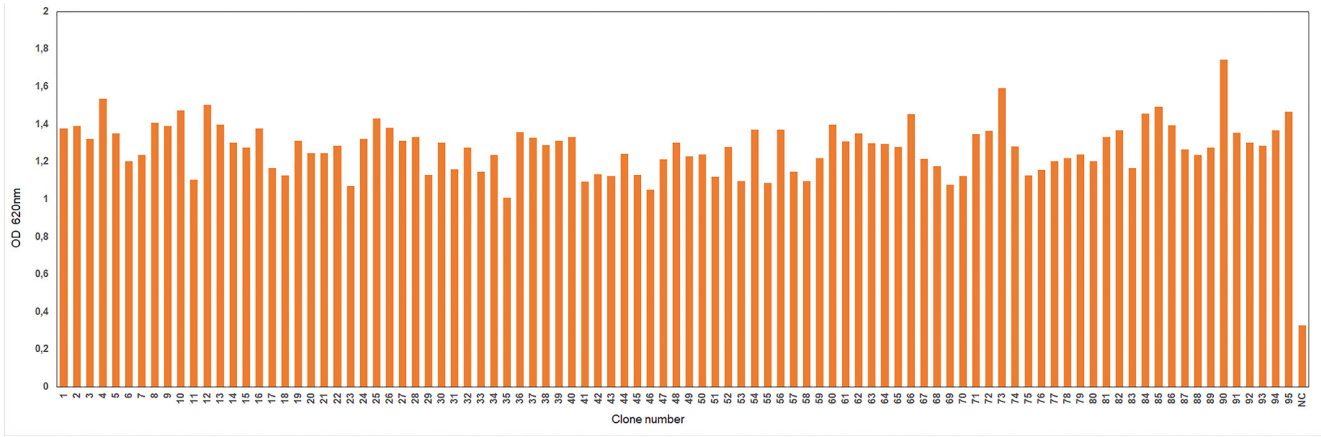

Figure 4. ELISA assay of the randomly picked clones expressing VHH-phage on E. coli live cells. NC; negative control was incubated only with primary and secondary antibodies.

studies have proved the interest of antibody fragments (nanobodies) in the treatment of pathologies including diarrhea. Indeed, nanobodies have been selected against different antigens of *E. coli* responsible for diarrhea (LT toxin, F4 and F18 fimbriae ...) (12, 15, 16). The selection of VHH against certain *E. coli* antigens and other causes of diarrhea has shown promising results for the treatment of this disease (13, 17).

Immunization with purified antigens is not always a prerequisite for successful panning. It is possible, and sometimes necessary, to immunize with complex antigens to identify original targets. Odongo et al. (18) selected VHH specific to *Trypanosoma congolense* from llama immunized with the parasite's whole proteome. Abbady et al. (19) selected VHH from a dromedary immunized with heat killed *Brucella melitensis* and *Brucella abortus*. Saerens et al. (20) selected 16 VHH fragments from a dromedary infected with *Trypanosoma evansi*.

Here we describe the immunization of a dromedary with formalin killed *E. coli* strain causing diarrhea in camel calves and the selection of VHH fragments by panning on live bacteria. Our ELISA results on intact cells showed that vaccination elicits a strong immune response against cell surface antigens involving conventional and heavy chain antibodies.

From the 3×10^7 isolated lymhocytes we isolated RNA, transformed it into cDNA and amplified VHH DNA sequences then we constructed an immune library in the pHEN4 phagemid(14). The transformation of TG1 bacteria by the cloning product allowed us to obtain a library of about 10^9 clones. The size of libraries derived from immunized animals can vary from 10^7(21), 5.10^7(22) to 10^8(19).

We performed Panning on whole bacteria and not on a lysate in order to select anti-surface antigens VHH able to inhibit the adhesion of bacteria to epithelial cells, a key step in the infection process. Attempts have been made to produce diagnostic and therapeutic antibodies against a wide range of pathogens, including bacteria (23). One of the most successful applications has been the isolation of a single-chain variable region fragment (scFv) against *Brucella melitensis* (24). Very similar work to our study was carried-out with *Brucella melitensis* and *Brucella abortus* (19). Immunization was performed with heat-killed bacteria. The study showed that the immunized camels developed a specific immune response against the two strains. The panning was performed on the lysate of both strains and allowed to isolate three different VHH clones. In further work, the authors identified, by the MALDI-TOF-MS technique, the target antigen to be the chaperone protein GroEL (25).

We carried-out three panning rounds on live *E. coli* F17+ in suspension yielding a high enrichment rate from round to round. Some clones are probably directed against original or major antigens of the cell surface. The positive clones should be sequenced and produced as soluble VHH in order to identify their antigens and determine their affinity and develop the most interesting ones as diagnosis or therapeutic tools. For diagnostic purposes, VHH-based biosensors can be developed (26). For therapy, VHH can be administered directly to animals (27) or expressed by a Lactobacillus strain. This approach was used to express VHH directed against the TcdB toxin from *Clostridium difficile* (28).

Since F17 is the major fimbriae antigen found in *E. coli* strains isolated from diarrheic camel calves in Tunisia (5), dairy calves in Uruguay (29) and Argentina (30), it will be interesting to develop VHH fragments against its major A subunit.

6. Conclusion

Diarrhea affects young animals whose immune systems are not yet developed. Therefore, vaccination will have no effect. Our study demonstrated that immunizing an

adult camel with killed bacteria could lead to a very strong immune response. Using phage display technology, we were able to select VHH antibodies fragments that recognize cell surface targets. The identification of these *E. coli* surface antigens could lead to innovative immunotherapies for diarrhea caused by these bacteria.

Acknowledgment

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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