

Cairo University  
Faculty of Veterinary Medicine  
Department of Microbiology

# **Some studies on Camel Nanobodies**

**A Thesis Presented By**

**Wafy Eid Hamed Farhan**

**(B.V.Sc. - Cairo University, 2007)**

**For**

**M.V.Sc in Microbiology**

**(Bacteriology- Mycology - Immunology)**

**Under Supervision of**

**Prof. Dr. Rafik T. M. Soliman**

**Prof. of Microbiology  
Faculty of Veterinary Medicine,  
Cairo University.**

**Prof. Dr. Khaled Farouk M. El-Amry**

**Prof. of Microbiology  
Faculty of Veterinary Medicine,  
Cairo University.**

**Dr. Nasser Abass Sherif**

**Chief Researcher – Head of viral poultry vaccine department  
Control laboratory for evaluation of veterinary biologics  
Abassia, Cairo.**

**2017**

**Cairo University**  
**Faculty of Veterinary Medicine**  
**Department of Microbiology**

### **Approval Sheet**

This is to certify that the dissertation presented by **Wafy Eid Hamed Farhan** to Cairo University for the **Master** degree in Veterinary Science (Bacteriology, Immunology and Mycology) has been approved by the examining committee:

**Prof. Dr. Fawzy Reyad El-seidy**  
Professor of Microbiology  
Faculty of Veterinary Medicine  
Beni-souif University

**Prof. Dr. Ahmed Samir Mohamed**  
Assistant Professor of Microbiology  
Faculty of Veterinary Medicine  
Cairo University

**Prof. Dr. Rafik Tawfik M. Soliman (Supervisor)**  
Professor of Microbiology  
Faculty of Veterinary Medicine  
Cairo University

**Prof .Dr. Khaled Farouk M. El-amry (Supervisor)**  
Professor of Microbiology  
Faculty of Veterinary Medicine  
Cairo University

**Dr. Naser Abass Sherif (Supervisor)**  
Chief Researcher – Head of viral poultry vaccine  
department Control laboratory for evaluation of  
veterinary biologics Abassia, Cairo.

**2017**

## **Supervisors**

### **Prof.Dr.RafikTawfik Mohamed Soliman**

Professor of Microbiology,  
Faculty of Veterinary Medicine, Cairo University

### **Prof. Dr. Khaled Farouk Mohamed El-amry**

Professor of Microbiology,  
Faculty of Veterinary Medicine, Cairo University

### **Dr. Nasser Abass Sherif**

Chief Researcher – Head of viral poultry vaccine department  
Control laboratory for evaluation of veterinary biologics  
Abassia, Cairo.

Cairo University  
Faculty of Veterinary Medicine  
Department of Microbiology

**Name: Wafy Eid Hamed Farhan**

**Nationality: Egyptian**

**Date of birth: 27/12/1985**

**Scientific degree: Master degree (Bacteriology- Mycology- Immunology)**

**Title of thesis: Some studies on Camel Nanobodies.**

**Supervision: Prof. Dr. Rafik Tawfik Soliman**

**Professor (Emeritus) of Microbiology, Faculty of Veterinary Medicine, Cairo University.**

**Prof. Dr. Khaled Farouk Mohamed El-amry**

**Professor of Microbiology, Faculty of Veterinary Medicine, Cairo University.**

**Dr. Nasser Abass Sherif**

**Chief Researcher – Head of viral poultry vaccine department  
Control laboratory for evaluation of veterinary biologics  
Abassia, Cairo.**

*Key words: Camel antibodies, Nanobodies, SDS-PAGE, prophylactic and therapeutic effect of Nanobodies*

### **Abstract**

Camel antibodies is unusual simplified non canonical antibodies (HCAb) which devoid of CH1 domain and light chain which gave it great chance for different application in diagnosis and treatment. In this study, immunization of Egyptian camel with tetanus toxoid for studying the structure and features of camel nanobodies, the serum were collected and purified and found that the main antibody in serum of camel is HCAb (75-80kDa) which appeared by SDS-PAGE. The prophylactic and therapeutic effects of nanobodies were tested in groups of Mice injected with tetanus toxin. The camel antibodies can enter the nerve cell and neutralize the toxin and relieve the signs of toxicity.

## *Dedication*

*To my lovely Wife*

*To my little son, Mohamed*

*To my spirit of my father*

*To all my family*

## *Acknowledgement*

*First of all, I would like to express my all- embracing gratitude and praise to **ALLAH**, glorified is he, for his for his unmitigated support and graceful benevolence in carrying out this humble thesis.*

*I would like to seize the opportunity to show off my greatest indebtedness and sincere loyalty to my scientific father **Prof. Dr. Rafik Tawfik Soliman** professor of Microbiology, Faculty of Veterinary Medicine, Cairo University for his initiating power, effective scientific supervision, continuous encouragement and his generous help.*

*I shall not forget the highly appreciated advices and most esteemed constructive criticism of **Prof. Dr. Khaled Farouk Mohamed El-amry** professor of Microbiology, Faculty of Veterinary Medicine, Cairo University.*

*I would like to express else my gratitude to **Dr. Nasser Abass Sherif** chief researcher head of viral poultry vaccine department, Control laboratory for evaluation of veterinary biologics, Abassia, Cairo.*

*I would like to express else my gratitude to **Dr shaymaa Abdelmalek Mohamed** Lecturer of Microbiology, Faculty of Veterinary Medicine, Cairo University for her support .*

*Yours,*

*Wafy Hamed*

## List of Contents

|                 | <b>Item</b>                                                                                           | <b>Page</b> |
|-----------------|-------------------------------------------------------------------------------------------------------|-------------|
| <b>1.</b>       | <b>Introduction</b>                                                                                   | <b>1</b>    |
| <b>2.</b>       | <b>Review of Literature:</b>                                                                          | <b>4</b>    |
| <b>2.1.</b>     | <b>Camel Immunoglobulins.</b>                                                                         | <b>4</b>    |
| <b>2.2.</b>     | <b>Why Camel immunoglobulins were considered non canonical immunoglobulins?</b>                       | <b>9</b>    |
| <b>2.3.</b>     | <b>Heavy chain antibody</b>                                                                           | <b>10</b>   |
| <b>2.4.</b>     | <b>Nanobodies.</b>                                                                                    | <b>14</b>   |
| <b>2.4.1.</b>   | <b>Separation and purification of nanobodies.</b>                                                     | <b>28</b>   |
| <b>2.4.2.</b>   | <b>Interesting features of nanobodies.</b>                                                            | <b>29</b>   |
| <b>2.4.2.1.</b> | <b>High expression yield and ease of purification.</b>                                                | <b>29</b>   |
| <b>2.4.2.2.</b> | <b>High stability, solubility and affinity.</b>                                                       | <b>30</b>   |
| <b>2.4.2.3.</b> | <b>Alternative epitope recognition.</b>                                                               | <b>31</b>   |
| <b>2.5.</b>     | <b>Application of nanobodies.</b>                                                                     | <b>32</b>   |
| <b>2.5.1.</b>   | <b>Nanobodies in cancer diagnosis and therapy.</b>                                                    | <b>32</b>   |
| <b>2.5.2.</b>   | <b>Nanobodies application in other diseases.</b>                                                      | <b>35</b>   |
| <b>3.</b>       | <b>Material and Methods.</b>                                                                          | <b>38</b>   |
| <b>3.1.</b>     | <b>Materials:</b>                                                                                     | <b>38</b>   |
| <b>3.1.1.</b>   | <b>Animals:</b>                                                                                       | <b>38</b>   |
| <b>3.1.2.</b>   | <b>Tetanus toxoid vaccine.</b>                                                                        | <b>38</b>   |
| <b>3.1.3.</b>   | <b>Blood samples from immunized camel.</b>                                                            | <b>38</b>   |
| <b>3.1.4.</b>   | <b>Materials used for separation and purification of camel Igs</b>                                    | <b>38</b>   |
| <b>3.1.5.</b>   | <b>Materials used for concentration and measurement.camel Igs</b>                                     | <b>39</b>   |
| <b>3.1.6.</b>   | <b>Materials used for Agar Gel Double immune diffusion assay.</b>                                     | <b>39</b>   |
| <b>3.1.7.</b>   | <b>Materials used for separation and characterization of camel immunoglobulins using SDS-PAGE of.</b> | <b>40</b>   |
| <b>3.1.8.</b>   | <b>Tetanus toxin (VACSERA).</b>                                                                       | <b>44</b>   |

|                 |                                                                                                                                                                                                |           |
|-----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>3.1.9.</b>   | <b>Tetanus toxin specific camel antibodies.</b>                                                                                                                                                | <b>44</b> |
| <b>3.1.10.</b>  | <b>Commercial antitetanic sera (VACSERA).</b>                                                                                                                                                  | <b>44</b> |
| <b>3.1.11.</b>  | <b>Instruments</b>                                                                                                                                                                             | <b>45</b> |
| <b>3.2.</b>     | <b>Methods:</b>                                                                                                                                                                                | <b>46</b> |
| <b>3.2.1.</b>   | <b>Immunization of Camel with tetanus toxoid vaccine.</b>                                                                                                                                      | <b>46</b> |
| <b>3.2.2.</b>   | <b>Collection of camel blood for serum preparation.</b>                                                                                                                                        | <b>46</b> |
| <b>3.2.3.</b>   | <b>Determination of specificity of the prepared camel antibodies using agar gel Double immune diffusion assay.</b>                                                                             | <b>46</b> |
| <b>3.2.4.</b>   | <b>Separation and purification of Immunoglobulin.</b>                                                                                                                                          | <b>47</b> |
| <b>3.2.5.</b>   | <b>Immunoglobulin concentration and measurement.</b>                                                                                                                                           | <b>48</b> |
| <b>3.2.6.</b>   | <b>SDS-PAGE.</b>                                                                                                                                                                               | <b>48</b> |
| <b>3.3.</b>     | <b>Evaluation of the prophylactic and therapeutic potentiality of the prepared tetanus-specific camel antibodies and evaluation its resistance to heat, acid and alkaline.</b>                 | <b>50</b> |
| <b>3.3.1.</b>   | <b>Experimental design</b>                                                                                                                                                                     | <b>50</b> |
| <b>3.3.1.1.</b> | <b>Experiment No. 1:<br/>Evaluation of the prophylactic potential of the prepared tetanus toxoid-specific camel nanobodies:</b>                                                                | <b>50</b> |
| <b>3.3.1.2.</b> | <b>Experiment No 2:<br/>Evaluation of the therapeutic effect of the prepared tetanus-specific camel nanobodies in intoxicated mice after appearance of the clinical signs of intoxication:</b> | <b>51</b> |
| <b>3.3.1.3.</b> | <b>Experiment No 3:<br/>Evaluation of the effect of heat, acids, alkaline treatment of the prepared tetanus-specific camel nanobodies on its neutralizing efficacy of in mice</b>              | <b>52</b> |
| <b>4.</b>       | <b>Results:</b>                                                                                                                                                                                | <b>53</b> |
| <b>4.1.</b>     | <b>Immunization of camel with tetanus toxoid vaccine.</b>                                                                                                                                      | <b>53</b> |
| <b>4.2.</b>     | <b>Purification of camel serum to obtain camel Ig by ammonium Sulphate purification method.</b>                                                                                                | <b>53</b> |
| <b>4.3.</b>     | <b>Concentration of purified camel Ig.</b>                                                                                                                                                     | <b>54</b> |
| <b>4.4.</b>     | <b>Results of determination of the molecular weight of</b>                                                                                                                                     | <b>54</b> |

|             |                                                                                                                                                                  |           |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
|             | <b>separated Ig using SDS-PAGE.</b>                                                                                                                              |           |
| <b>4.5.</b> | <b>Specificity of the prepared camel Ig using Double immune diffusion assay.</b>                                                                                 | <b>57</b> |
| <b>4.6.</b> | <b>In vivo testing of the specificity and neutralizing potential of the prepared camel immunoglobulins to tetanus toxin:</b>                                     | <b>58</b> |
| <b>4.7.</b> | <b>Result of evaluation of the prophylactic effect of the prepared tetanus specific camel Igs (VHH Ig) in mice:</b>                                              | <b>59</b> |
| <b>4.8.</b> | <b>Results of the evaluation of the therapeutic effect of produced and purified camel Ig (VHH) in mice after manifesting clinical signs of tetanus toxicity.</b> | <b>60</b> |
| <b>4.9.</b> | <b>Effect of heat, acid and alkaline treatment of camel antibodies on its neutralizing efficacy in tetanus intoxicated mice.</b>                                 | <b>62</b> |
| <b>5.</b>   | <b>Discussion:</b>                                                                                                                                               | <b>65</b> |
| <b>6.</b>   | <b>Summary</b>                                                                                                                                                   | <b>70</b> |
| <b>7.</b>   | <b>References</b>                                                                                                                                                | <b>72</b> |
| <b>8.</b>   | <b>Arabic summary الملخص العربي</b>                                                                                                                              | <b>88</b> |

## List of tables

|                | <b>Item</b>                                                                                                                                                                            | <b>page</b> |
|----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| <b>Table 1</b> | <b>Nanobodies types in field according to FDA</b>                                                                                                                                      | <b>36</b>   |
| <b>Table 2</b> | <b>Experiment 1: Evaluation of the prophylactic potential of the prepared tetanus toxoid-specific camel nanobodies.</b>                                                                | <b>59</b>   |
| <b>Table 3</b> | <b>Experiment 2: Evaluation of the therapeutic effect of the prepared tetanus-specific camel nanobodies in intoxicated mice after appearance of the clinical signs of intoxication</b> | <b>61</b>   |
| <b>Table 4</b> | <b>Experiment No 3: Evaluation of the effect of heat, acids, alkaline treatment of the prepared tetanus-specific camel nanobodies on its neutralizing efficacy of in mice.</b>         | <b>68</b>   |

## List of figures

|        | Item                                                                                                                                 | Page |
|--------|--------------------------------------------------------------------------------------------------------------------------------------|------|
| Fig.1  | Structure of classical antibodies                                                                                                    | 5    |
| Fig.2  | The camel nanobodies                                                                                                                 | 6    |
| Fig.3  | Standard protein ladder (BIORAD)                                                                                                     | 43   |
| Fig.4  | The one year old camel used for preparation of tetanus toxin-specific antibodies                                                     | 53   |
| Fig.5  | The molecular weight of the separated camel Ig using SDS-PAGE (non reducing treatment)                                               | 55   |
| Fig.6  | Reducing treatment with DTT for fractionation of camel Ig showing fraction of Ig including small fraction about 19-20kD (nanobodies) | 56   |
| Fig.7  | Double immune diffusion assay of camel Ig towards tetanus toxin showing white line of precipitation                                  | 57   |
| Fig.8  | In vivo testing of the specificity and neutralizing efficacy of tetanus toxin specific camel Ig                                      | 58   |
| Fig. 9 | Tetanus toxicity signs, stiffness of ear, tail and limbs then ends with death                                                        | 60   |
| Fig.10 | After 1 hr complete relieve of signs and return to eat                                                                               | 62   |

## 1. Introduction

Antibodies or immunoglobulins (Igs) are soluble glycoproteins in blood and tissue fluids that play a central role in the humoral immune response of vertebrates. Antibodies are synthesized by B-lymphocytes in response to foreign biological and chemical substances (antigens) of great structural verity with a purpose to neutralize them. Owing to their high specificity and high affinity of antigen binding and to the possibility of antibody generation against practically limitless repertoire of antigens, antibodies and their derivatives are among the most important reagents for the basic, applied and medical research (**Honegger A, Plückthun A 2001**).

The conventional antibody molecule consists of 4 polypeptide chains; two are called heavy chains and the other two having low molecular weight are called light chains (**Utsumi and Karush 1964 & Burton, 1985**). Although the antibodies have proven their worth protection against infections and as therapeutic drugs, they do suffer from a number of technical problems, including their slow clearance rate after injection into the body. Also the antibody molecular size (e.g., IgG) is over five times larger than the average therapeutic protein and if used against solid tumors, will not fully penetrate the cancerous tissue to destroy it. This has prompted the search for much smaller proteins that retain both the antigen-binding capability of the conventional antibodies and their ability to remove invading organisms.

In 1993, a group of Belgium scientists has published an important discovery (**Hamers et al., 1993**) indicating that in addition to classical

antibodies, there is a significant amount of unusual non-canonical antibodies with simplified structure in blood of camelids (camel, lama, vicugna). These nanobodies consist of a dimer of only one shortened heavy chain, and the light chains are absent (heavy chain antibodies HCAb).

These nanobodies represent a new generation of antibody proteins that retains the variable (V) region sequences within a much smaller protein framework. As an example is the single domain nanobodies developed by **Ablynx** Company, which has antigen-binding capability in a molecule of around 12,000 Da.

Nanobodies, derived from naturally occurring single-chain antibodies, are the smallest fragments of naturally occurring heavy-chain antibodies that have evolved to be fully functional in the absence of a light chain. It is the smallest known protein with the ability to specifically recognize an antigen (**Fridy et al., 2014**).

The nanobody technology was originally developed following the discovery that camelidae (camels and llamas) possess a unique repertoire of fully functional antibodies that lack light chains. Like conventional antibodies, nanobodies show high target specificity and low inherent toxicity; however, like small-molecule drugs, they can inhibit enzymes and can access receptor clefts. Their unique structure consists of a single variable domain (VHH), a hinge region, and two constant domains; CH2 and CH3 (**Maggi and Scotti, 2017**).

The discovery of the single-domain antibody's potentials has stimulated their use in an increasing variety of fields. The rapid accumulation of articles describing new applications and further

developments of established approaches has made it. At present, the possibility to use nanobodies as enzyme inhibitors (**Martin *et al.*, 1997; Lauwe reys *et al.*,1998; Conrath *et al.*,2001; De Genst *et al.*, 2006; Koch-Nolte *et al.*, 2007**), affinity ligands (**Kloosteret *et al.*, 2007**) ; intrabodies (**Gueorguieva *et al.*, 2006; Rothbauer *et al.*, 2006; Verheesenet *et al.*, 2006**), as probes in biosensors (**Pleschberger *et al.*, 2004; Huang *et al.*, 2005a; Saerens *et al.*, 2005**), as tools to trace antigens in live cells (**Rothbauer *et al.*, 2006**) and to study protein–protein interactions (**Huang *et al.*, 2005b**) has been demonstrated. Owing to their small size and reduced complexity of paratopes, Nanobodies are ideal candidates to design small peptide mimetic (**Marquardt *et al.*, 2006**). Nanobodies can also be used in consumer products. For example, Nanobodies prevent infection of lactic acid bacteria by phage, thereby accelerating fermentation in cheese production (**Ledeboer *et al.*, 2002; de Haard *et al.*, 2005**) or they can prevent dandruff when supplied in shampoo (**Dolk *et al.*, 2005**).

**The aim of the present work was to elucidate the therapeutic potential of tetanus toxin-specific nanobodies in clinically tetanus toxin intoxicated mice. It is planned to achieve this aim by:**

- 1. Immunization of Egyptian camel with tetanus toxoid vaccine.**
- 2. Separation of immunoglobulins from camel sera.**
- 3. Study the nature of camel immunoglobulins by SDS-PAGE and colorimetric identification by Commassie blue.**
- 4. Determination of the heat stability and pH stability of the prepared tetanus toxin- specific camel nanobodies.**
- 5. Evaluation of the immune-prophylactic and immune-therapeutic potential of the prepared tetanus toxin-specific camel nanobodies.**

## 2. Review of Literature

### 2.1. Camel immunoglobulins:

**Utsumi and Karush (1964)** found in the conventional antibody, the variable region of the light chain (VL) and the variable region of the heavy chain (VH) are combined to make the antigen binding site, although, in some cases, the heavy chain alone can also bind antigen (Fig.1)

**Burton (1985)** recorded that papain protease can split antibody into two fragments: Fab (Fragment antigen binding) and Fc (Fragment crystallizable). Correspondingly, one part of the antibody molecule (Fab) defines its antigen specificity, and the other part (Fc) is responsible for effector functions, which lead to the antigen elimination. They recorded also that the hinge region located between CH1 and CH2 domains of H-chain is responsible for mobility of Fab-fragment. Papain cut immunoglobulin right in the hinge region above inter-chain disulfide bonds. CH2-domain is the place of carbohydrates attachment and of complement binding. CH3-domain interacts with the Fc-receptor at the surface of cells involved in immunological reactions. Right combination of heavy-chain variable region (VH) and light-chain variable region (VL) make the antigen binding site, and constant antibody domains do not participate directly in the antigen recognition. Single-chain protein construct (scFv) composed of both heavy chain and light chain variable domains that are connected by a linker sequence is the minimized derivative of the antigen-binding domain of conventional antibodies. The constant domains of antibodies however, are not involved in antigen recognition; the heavy chain constant regions CH2 and CH3 (Fc) are