MOLECULAR IDENTIFICATION OF 20 *Escherichia coli* STRAINS ISOLATED FROM DEAD NEONATAL CAMELS (*Camelus dromedarius*) IN UAE AND GENERATION OF A SINGLE-DOMAIN ANTIBODY (sdAb) AGAINST *E. coli*

Faysal Abdishakur H Hassan

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Biotechnology

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เอกลักษณ์ระดับโมเลกุลของเชื้อ *Escherichia coli* 20 ชนิด จากซากอูฐแรกเกิดในสหรัฐอาหรับเอมิเรตส์ และการผลิตแอนติบอดีสายเดี่ยวต่อเชื้อ *E. coli*

นายไฟย์ซาล อับดีชาคูร์ เอช ฮาซซาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาเทคโนโลยีสุรนารี
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Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ไฟย์ซาล อับดีชาคูร์ เอช ฮาซซาน : เอกลักษณ์ระดับโมเลกุลของเชื้อ *Escherichia coli* 20 ชนิด จากซากอูฐแรกเกิดในสหรัฐอาหรับเอมิเรตส์ และการผลิตแอนติบอดีสายเดี่ยวต่อเชื้อ *E. coli* (MOLECULAR IDENTIFICATION OF 20 *Escherichia coli* STRAINS ISOLATED FROM DEAD NEONATAL CAMELS (*Camelus dromedarius*) IN UAE AND GENERATION OF A SINGLE-DOMAIN ANTIBODY (sdAb) AGAINST *E. coli* อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 118 หน้า.

เชื้อ *Escherichia coli* เป็นแบคทีเรียในวงศ์ Enterobacteriaceae หลายชนิดพบเป็นจุลชีพในลำไส้และระบบทางเดินอาหารของลูกสัตว์ โดยส่วนใหญ่จะไม่ก่อโรคยกเว้นในกรณีที่สัตว์มีภาวะภูมิคุ้มกันบกพร่อง ในการศึกษานี้เราได้ทำการเก็บตัวอย่างเชื้อจากลูกอูฐ (*Camelus dromedarius*) ช่วงอายุ 1 ถึง 2 สัปดาห์ ที่ตายด้วยภาวะ colisepticemic และ colibacilosis ในการทดลองแรก เพื่อจำแนกเชื้อ *E. coli* ก่อโรคที่เป็นสาเหตุการตายของลูกอูฐแรกเกิด โดยนำเชื้อ *E. coli* จำนวน 20 ตัวอย่าง ที่เพาะแยกได้จากตัวอย่างซากลูกอูฐ ทำการเพิ่มจำนวนของยีน 16SrRNA *E. coli* ด้วยเทคนิค conventional PCR และใช้เทคนิค real-time PCR ร่วมกับชุดน้ำยา Power Chek Diarrheal *E. coli* 4-plex Real-time PCR Kit I and II (Kogenebiotech, Seoul, Korea) เพื่อตรวจยีน 8 ชนิดที่จำเพาะต่อเชื้อ *E. coli* ก่อโรค พบว่าทุกตัวอย่างแสดงผลเป็นลบ จากนั้นทำการวิเคราะห์ลำดับเบสเพื่อเทียบกับฐานข้อมูล GenBank ซึ่งแยกเชื้อ *E. coli* ออกเป็น 6 กลุ่ม และพบว่าเชื้อตัวอย่างมีลำดับเบสตรงกับเชื้อ *E. coli* ที่เป็นจุลชีพประจำถิ่นที่ไม่ก่อโรค แต่เนื่องด้วยระบบภูมิคุ้มของลูกอูฐแรกเกิดที่ยังไม่สมบูรณ์ และการได้รับเชื้อเพิ่ม ทั้งทางน้ำนมแม่ที่ปนเปื้อนหรืออาหารที่ไม่ถูกสุขลักษณะทำให้เชื้อ *E. coli* ส่งผลต่อร่างการสัตว์ให้ได้รับความเสียหายและตายในที่สุด

การทดลองที่สอง เป็นการผลิตนาโนบอดียับยั้งลิโปโพลีแซคคาไรด์ของเชื้อ *E. coli* (anti *E. coli* lipopolysaccharide (LPS) nanobody) โดยการเพิ่มจำนวนยีนควบคุมการแสดงออกของลิโปโพลีแซคคาไรด์ของเชื้อ *E. coli* จากอูฐที่ถูกกระตุ้นภูมิคุ้มกัน โดยอาศัย Flexi® vector (Promega. USA) เพื่อทำการสร้าง VHH library และตัวอย่าง RNA ทั้งหมดที่แยกได้จาก preverbal blood lymphocytes (PBLs) ของอูฐที่ถูกกระตุ้นภูมิคุ้มกันนาน 70 วัน สามารถผลิต DNA ขนาด 400bp จาก VHH primer จากนั้นทำการเชื่อมชิ้นส่วน DNA กับ pF1AT7 Flexi® Vector (Promega USA) ตัดจำเพาะด้วยเอนไซม์ SgfI และ PmeI เพื่อให้ได้ JM109 *E. coli* competent cells ขนาด 6.9 x 10^4 cfu/µg ตาม VHH library จากนั้น สุ่มเลือกเชื้อจำนวน 48 โคโลนี เพื่อเพาะเลี้ยงและทำการวิเคราะห์ด้วยเทคนิค ELISA โดยใช้ inclusion bodies และ periplasmic protein ต่อลิโปโพลีแซคคาไรด์ของเชื้อ *E. coli*

ในขณะเดียวกัน plasmid DNA ถูกสกัดจาก 48 โคโลนีตัวอย่างและย่อยด้วยเอนไซม์ SgfI และ PmeI หลังจากทำ PCR พบว่า มี 11 โคโลนีให้ผลบวกต่อ VHH gene และสามารถวิเคราะห์

ลำดับเบสด้วย Big Dye terminator kit (Applied Bio systems. USA) ได้จาก 6 โคโลนีตัวอย่าง โดยใช้ฐานข้อมูล GenBank และ IMGT และจากนั้นทำการเปรียบเทียบลำดับเบสของ DNA และโปรตีน โดยใช้ Clustal Omega software ซึ่งพบว่าตรงกับลำดับเบสของ VHH gene

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

FAYSAL ABDISHAKUR H HASSAN : MOLECULAR IDENTIFICATION OF 20 *Escherichia coli* STRAINS ISOLATED FROM DEAD NEONATAL CAMELS (*Camelus dromedarius*) IN UAE AND GENERATION OF A SINGLE-DOMAIN ANTIBODY (sdAb) AGAINST *E. coli*. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 118 PP.

ESCHERICHIA COLI/16SrRNA *E.coli*/LIPOPOLYSACHARIDE LPS/VHH LIBRARY/NANOBODY

Escherichia coli is a member of the *Enterobacteriaceae* family. Many *E. coli* strains are predominantly found as commensal flora in the intestine that colonise the gastrointestinal tract of new-born animals. The majority do not cause any disease unless the host is immuno-suppressed. In this study, we fucued microbial sample from dead camel calves (*Camelus dromedarius*) (aged one or two weeks) from postmortem samples identified as colisepticemic or colibacillosis. The first phase of this study was to identify whether any pathogenic *E. coli* is the causative agent of neonatal camel death. Twenty *E. coli* isolates were collected and successfully amplified with conventional PCR of the 16SrRNA *E. coli* gene. Subsequently, we ran the commercial real-time kits Power Chek Diarrheal *E. coli* 4-plex Real-time PCR Kit I and II, which target eight pathogenic *E. coli* genes known to produce diseases, which give negative results for all samples. This occurred even after sequencing and blasting against the GenBank database with the six known pathogenic *E. coli* strains excluded and GenBank data matching only commensal *E. coli* flora. Subsequently, the immune system of new born camels is weak and immature. Therefore, infections of the normal flora may be caused by ingestion of

contaminated mother's milk or unhygienic food where the commensal *E. coli* easily evade host defences leading to host damage or even death.

The second phase of this study involved production of an anti *E. coli* lipopolysaccharide (LPS) nanobody from the pF1AT7 Flexi® expression vector (Promega. USA) and construction of a VHH library obtained from immunised camels (*Camelus dromedaries*). Total RNA was isolated from peripheral blood lymphocytes (PBLs) collected 70 days after immunization with the experimental camel, 400bp DNA fragment from the VHH primer pair was amplified and ligated into the pF1AT7 Flexi® vector (Promega USA) after digestion with *SgfI* and *PmeI*, two rarely-cutting restriction endonucleases, followed by ligation and transformed by heat shock into JM109 *E. coli* competent cells (Promega USA.). The ligated vector was successfully transformed, resulting in VHH library at the size of 6.9 x 10^4 cfu/µg. Forty-eight randomly picked colonies were cultivated and productive expression was induced then tested by ELISA using inclusion bodies and periplasmic protein on *E. coli* lipopolysaccharide coated plates. Simultaneously, plasmid DNA was isolated from a duplicated set of 48 picked colonies and digested with *SgfI* and *PmeI* enzymes. Then, PCR of the VHH gene was performed and with 11 colonies and six of them were positive, followed by successful sequences with the BigDye terminator kit (Applied Bio systems. USA). Further, samples were analysed with the GenBank database, IMGT database, and multiple sequencing alignment of both DNA and protein using Clustal Omega software on the EBI web site. All analyses were similar to those of VHH in data sequenced before.

School of Biotechnology Student's Signature _____

Academic Year 2017 Advisor's Signature _____

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Faysal Abdishakur H Hassan

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LIST OF ABBREVIATIONS

Ab	=	Antibody
Amp	=	Ampicillin
BSA	=	Bovine serum albumin
C. bactrianus	=	*Camelus bactrianus*
C. dromedarius	=	*Camelus dromedarius*
DAEC	=	Diffusely adherent *E. coli*
DNA	=	Deoxyribonucleic acid
E. coli	=	*Escherichia coli*
EAEC	=	*E*ntroaggregative *E. coli*
EHEC	=	*E*ntrohemorrhagic *E. coli*
EIEC	=	*E*ntroinvansive *E. coli*
ELISA	=	Enzyme-linked immunosorbent assay
EPEC	=	Entropathogenic *E. coli*
ETEC	=	Entrotoxigenic *E.coli*
FAO	=	Food and Agriculture Organisation of the United Nations
GIT	=	Gastrointestinal tract
IPTG	=	Isopropyl β-D-thiopgalactopyranoside
KN	=	Kanamycin
L. glama	=	*Lama glama*
L. guanicoe	=	*Lama guanicoe*
L. pacos	=	*Lama pacos*

LIST OF ABBREVIATIONS (Continued)

LB	=	Luria Bertani broth
LPS	=	Lipopolysaccharide
LT	=	Heat-labile enterotoxin
Nb	=	Nanobody
PBLs	=	Preverbal blood lymphocytes
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PMN	=	Polymorphonuclear leucocytes
PP	=	Post-partum
PID	=	Primary immunodeficiencies
RBC	=	Red blood cells count
RNA	=	Ribonucleic acid
SOC	=	Super optimal broth with catabolite repression
ST	=	Heat-stable enterotoxin
UAE	=	United Arab Emirates
UV	=	Ultraviolet
VH	=	Variable domain of the heavy chain
VHH	=	Variable domain of the heavy chain only antibody
VL	=	Variable domain of the light chain
VTEC	=	Verotoxinogenic *Escherichia coli*
WBC	=	White blood cell
WHO	=	World Health Organisation

CHAPTER I

INTRODUCTION

1.1 Background

Theodor Escherichia discovered *Escherichia coli* in 1885. It is a member of the genus *Escherichia,* within the family *Enterobacteriaceae,* gram negative, motile, and a facultative anaerobe among the commensal flora present in the gut of warm-blooded animals (Kaper et al., 2004b). *Escherichia coli* also ferment sugars. These organisms are often motile because of the presence of peritrichous flagella (flagella that are distributed around the entire cell). Many strains are wide spread intestinal pathogens in mammals and birds. It is also the most commonly encountered pathogen in the *Enterobacteriaceae* (Deborah Chen and Frankel, 2005). They are oxidase negative, catalase positive, and are capable of reducing nitrate to nitrite. They cause a variety of diseases including diarrhoea, urinary tract infections (UTIs), and nosocomial infections. Normal *E. coli* flora colonise the intestine during the first hour of life (Nataro and Kaper, 1998). Normally pathogenic strains attached to the mucosal site of intestine via pili, fimbriae, or fibrils and colonise the ileal mucosa followed by multiplication and expression of pathogenic factors to evade the host defences leading to host damage or even death (Kaper et al., 2004a).

Virulence factors like adhesins, toxins, plasmids, and phages play roles in pathogenesis and a combination of these factors determine the degree of pathogenicity and severity of the disease (Ho et al., 2013).

In camels, *E. coli* causes major neonatal diarrhoea resulting in up to a 40% loss of camel calves, especially when they are not treated well during the first few days of their life (Wernery and Kaaden, 2002; Farah et al., 2007). Insufficient colostrum intake and poor management increases calf disease mortality (Abbas and Omer, 2005). Immature immunity, insufficient colostrum, and contaminated soil contribute to increased camel calf mortality and have economic impact on all camel breeders (Mohammed, 2003).

E. coli causes diarrhoea by six different mechanisms, each one of which has different virulence and pathotype results in terms of septic infection, acute enteritis, haemorrhagic colitis, and diarrhoea. These pathotypes include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Kaper, 2004; Donnenberg and Kaper, 1992a). Colibacillosis and colisepticemia occur mostly in one- to four-week-old camel calves causing high fever, yellowish watery diarrhoea, dehydration, and mostly the death during their first week of life. The objective of this study is to identify a pathogenic *E. coli* strain that kills camel calves, focusing on postmortem confirmed colibacillosis or colisepticemia cases using molecular diagnostic methods.

The word antibody was first mentioned by Emil von Behring and Shibasaburo Kitasato in late 1890 in their classic paper “On the mechanism of immunity to diphtheria and tetanus in animals”. This paper was the birth of immunology. In their results they indicate that antibodies are able to eliminate tetanus and diphtheria toxins in addition to preventing the symptoms of the diseases (Kantha,1991). They furthermore confirmed antibody specificity, as the diphtheria antitoxin will not react

with tetanus toxin and vice versa. In 1901 Von Behring was awarded The first Nobel Prize in Medicine for his diphtherial serum therapy findings, which started a new way of treatment against tetanus and diphtheria (Kantha, 1991). However, the first immunoglobulin Isotype G (IgG) was discovered in 1939 by Arne Tiselius and Elvin Kabat (Tiselius and Kabat, 1939). Additionally, in 1948 Astrid Fagraeus defined antibody production in plasma B cells (Fagraeus, 1948). The structure of antibodies was independently described in 1959 by Gerald Edelman and Rodney Porter, for which they shared the Nobel Prize in 1972 (Kantha, 1991;Philip D Hodgkin, 2007). However, the clonal selection theory was established in 1957 by Frank MacFarlane Burnet and David W. Talmage extended this theory in 1976 with Susumu Tonegawa's description of the recombinant Ig genes. Further, 1975 was the first generation of hybridomas as well as the development of monoclonal antibodies carried by Georges Köhler and César Milstein. The phage display technique was developed 15 years later by John McCafferty and co-workers (Hodgkin, 2007; Cohn et al., 2007).

In mammals there are two major systems that try to respond to pathogen invasion in the body. First, the innate immune system delivers an immediate and non-specific reaction after the invasion of microbes (Medzhitov, 2007; Beutler, 2004). The second phase of the immune system is antigen-dependent and specific, developed over the lifetime of an individual (Delves, 2000). In these various cells the molecules play different roles, among which lymphocytes and antibodies are the significant fundamentals, specially T and B lymphocyte cells. T cells are accountable for cellular immunity, while B cells produce the humoral immune response. In both together, the immune response is inspired by specific antigens that could be as small as lipids, sugars, and peptides in highly complex molecules, such as proteins. (Delves, 2000)

(Collis, 2003) In humoral immunity, B cells play both the roles of permanent protection and specificity of microbial pathogens (Harwood and Batista, 2010). Normally antigens bind to the surface of a B cell through the B cell receptors (BCRs), and are internalised and processed into peptides (Parker, 1993). Through the Major Histocompatibility Complex (MHC) II the treated antigen is returned to the surface of B cells and presented to antigen specific helper T cell (TH cell) recognition (Noelle, 1992). The production of specific interleukins include IL-4, IL-5, and IL-6 to facilitate contact between B and TH cell activation, which in turn activates B cells (Parker, 1993; Noelle, 1992). IL-4 stimulates clonal proliferation, whereas IL-5 and IL-6 induce the differentiation of B cells. The activated B cells either differentiate into memory B cells providing permanent protection against secondary infection or insertion into antibody secreting plasma cells (Reth, 1992).

Antibodies act as the first line of defence in the body, as they are responsible for recognition and binding of antigens. They protect the host in many ways. Toxin neutralization, facilitating phagocytosis, and helping natural killer (NK) cells eliminate antibody-coated cells. The antibody molecules were referred to as immunoglobulins (Ig), contain of two heavy (H) chains and two light (L) chains. Although two types of light chains (κ and λ) and five types of heavy chains (μ, δ, γ, ε and α) can be distinguished. Heavy and light chains are covalently linked by disulfide bonds (Porter, 1963). Together, the heavy and light chains are composed of two regions with a distinct amino acid sequence. The variable (V) region at the N-terminus and the constant (C) region at the C-terminus. Both light chains consist of one variable domain (VL) and one constant domain (CL). Each heavy chain, however, is comprised of one variable domain (VH) and up to four constant domains (CH). The hinge region is

situated between variable and constant regions of the heavy chain forming the typical Y-shaped antibody structure (Cohen, 1967; Hilschmann, 1965; Johnson, 2000).

Immunoglobulins can be differentiated into five classes IgM, IgD, IgG, IgE, and IgA, which are distinguished by their heavy chain (CH) classes (isotypes) named as μ, δ, γ, ε, and α. After B cell activation, IgM is the first type of antibody to be secreted (Harriman and Volk, 1993). IgM forms a soluble pentamer for the antigen binding process, called class switch recombination (CSR) (Li et al., 2004), which contains the heavy chain μ. Additionally, its gene coding for CH was changed from Cμ to Cδ, Cγ, Cε, or Cα, switching from IgM to either IgD, IgG, IgE, or IgA. However, using papain protease, the immunoglobulin molecules can be cut into separate fragments that cleave the molecule into two regions, Fab (fragment antigen binding) and Fc (fragment crystallisable). The Fab region retains antigen binding activity while the Fc region contains most of the constant regions of the heavy chain (Delves, 2000; Padlan, 1994).

Normally IgGs recognise and bind to antigens via variable regions located at the N-terminus of the antibody and varies between immunoglobulins, but the sequence variability is not distributed equally in variable regions but focused in three regions called hypervariable regions (Johnson, 2000). When VH and VL domains are paired in the antibody, three hyper variable loops from each domain generate a single hyper variable site known as the antigen-binding site. The hyper variable loops form the complementarity determining regions (CDRs) CDR1, CDR2, and CDR3, which are flanked and called framework regions (FRs) (Wu et al., 1993). However, mutations in FRs have an impact on binding, as they play an important role in structure defence, folding yield, and stability (Cohn et al., 2007; Padlan, 1994). The CDRs of the light chain are about six to ten amino acids long, whereas the heavy chains are between five

and fifteen amino acid residues in length (Wu et al., 1993; Johnson, 2000) and they vary in length between species (Collis, 2003; Padlan, 1995). This is particularly true in the CDR3 of heavy chains, which often elicit a response in antigen binding (Padlan, 1994).

Genetically modified antibody formats include immunoglobulin are produced by genetic engineering in place of naturally occurring antibodies. This technology is used to create antibody libraries based on their diversified gene segments. As recombinant antibodies are generated outside an organism, it is possible to find antibodies not being provoked by the immune system (Nizak, 2003). There are different recombinant antibody fragments but the most prominent are the single-chain variable fragments (scFvs) and antigen binding fragments (Fabs) (Vincke et al., 2009).

The VH or VL domains are smallest antigen-binding fragments in the Fv regions. In contrast, VHs have a hydrophobic interface that stabilises the dimer with the VL (Chothia et al., 1985). The other smaller and more stable binding unit is the scFv that represents the minimal recombinant antigen-binding fragment of antibodies, which consists of two naturally variable domains, VH and VL, which have hydrophobic interactions (Hudson, 1998; Winter, 1991). However, with the help of a hydrophilic and flexible linkers, the Fv fragments can be engineered by linking the two domains to create a single-chain Fv fragment. scFvs are comparatively small and unglycosylated proteins can be synthesised in heterologous expression systems like bacteria or lower eukaryotes (Huston et al., 1988; Skerra et al., 1988).

Compared to complete antibodies the scFvs deliver fast and effective tissue penetration for *in vivo* imaging and demonstrate a low non-specific background. Furthermore, scFvs demonstrate short circulating half-lives in the organism because of

quick absorbance and excretion by the kidney. Additionally, therapeutic and diagnostic applications are still troubled by stability problems. Subsequently, scFvs still have a strong tendency towards hydrophobic bonds in a hydrophilic atmosphere (Cattaneo et al., 1999; Sanz et al., 2005). Similar to scFvs, Fab and F(ab)2 fragments also use recombinant technology of antibody fragments. Fabs are synthesized by proteolytic enzyme digestion of full length antibodies that are commonly used for diagnostic imaging, specifically *in vivo* imaging (Behr et al., 1995), and recently were successfully tested for *in vivo* imaging (Els et al., 2009). As described above, recombinant antibodies are from immunoglobulin libraries, which are a synthetically generated libraries like the Fab library (Knappik et al., 2000). These libraries provide powerful tools for obtaining antibodies. Recombinant antibodies can be selected *in vitro* from completed libraries derived synthetically or from immunised animals (Haard et al., 1999; He et al., 1999).

The specific binding of antibodies makes them one of the most valuable tools in diagnostics, therapy, and biomedical investigation. A major advance occurred in 1975 when Georges Köhler and César Milstein developed the hybridoma technology. Earlier, polyclonal antibodies were used, which recognise many epitopes of the identical antigen and are susceptible to batch-to-batch variability. Through the combination of antibody-producing B cells from an immunised mouse with a myeloma cell line, Köhler and Milstein were able to produce a memorable hybridoma cell capable of generating unlimited amounts of antibodies specific to a single epitope, called monoclonal antibodies (Kohler, 1975). In scientific research, immunoglobulins play a major role in many technologies from proteomics to cell analyses. The most prominent examples include Western blotting for identification of specific proteins,

immunofluorescence for viral diagnostics, and immunohistochemistry, which is the "gold standard" for tumour diagnosis. Owing to their high specificities it was also used with antibody-based immunoassays. Other diagnostic tests to detect even very small amounts of drugs or hormones, the exclusive features of antibodies permit the development of accurate qualitative and quantitate assays as well as detect markers associated with specific diseases. In addition, antibodies are an important tool used in diagnostic imaging, particularly, radio labelled antibodies employed to locate antigens on the surface of positive tumour cells (Borrebaeck, 2000; Holliger and Hudson, 2005; Porter, 1988).

1.2 Research objectives

1.2.1 To isolate the *E. coli* strains that cause neonatal camel diarrhoea from postmortem samples of dead camel calves.

1.2.2 To identify *E. coli* strains using a molecular identification system (PCR, real-time PCR, and sequencing).

1.2.3 To construct a VHH library of anti-*E. coli* lipopolysaccharide from an immunised camel and to generate the specific nanobodies to the isolated *E. coli* strain.

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CHAPTER II

LITERATURE REVIEWS

2.1 The camelids

2.1.1 Taxonomic and classification

Camels belong to the taxonomic order *Artiodactyla* (even-toed ungulates), sub-order *Tylopoda* (pad-footed). They are ruminants like giraffes, deer, cattle, sheep, goats, and antelopes. They belong to the family of Camelidae with two different genera, namely old-world and new-world camelids. The old-world represented by dromedary camels and Bactrian camels, while the dromedary has one hump the Bactrian has two, which is the primary distinguishing factor. Whereas the new-world camelids include llama, alpaca, guanaco, and vicuna, (Wernery and Kaaden, 2002) 95% of the old world camelids are dromedaries and the remaining 5% are domestic Bactrian camels (there are less wild Bactrian camels). In the new world, around 47% of camelids are llamas, 41% alpacas, 8% guanacos, and 4% viçunas (Don Wilson et al., 2011).

2.1.2 Origin and distribution

Camels originally came from North America during the Eocene period, 40-45 million years ago, and later become extinct later from their original locations (Cui et al., 2007). The camels that would give rise to the dromedary and Bactrian camel are spread across Asia, Europe, and as far west as Spain (Cui et al., 2007; Kohler Rollefson, 1991).

Taxonomy of camels

Classification	Name of Classification
Kingdom	Animalia- Animal
Phylum	Chordata- Internal Skeleton
Class	Mammalia- Mammal
Order	Artiodactyla- Even Toed Ungulate
Family	Camelidae- Camels, Llamas, Vicunas
Genus	Camelus- Camels
Species	Camelus dromedarius- Dromedary camel Camelus bactrianus- Bactrian Camel

Figure 2.1 Camel Taxonomic and classification.

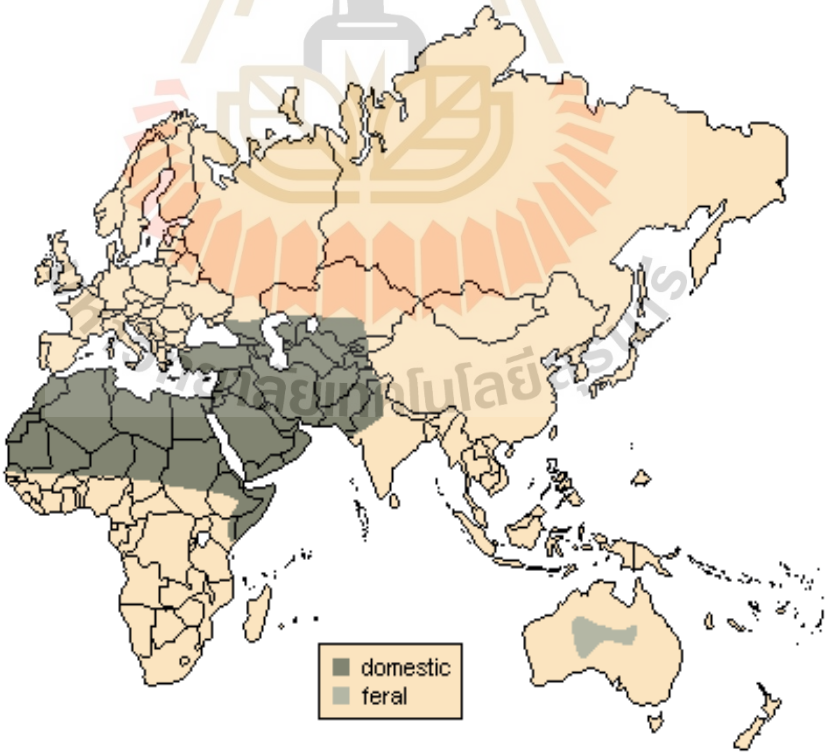

Figure 2.2 Worldwide distribution of domestic and wild *C. dromedaries* (Kohler, 1991).

The dromedary or Arabian camel (*Camelus dromedaries*) tend to be covered in soft, short woollen hair that is lighter in colour. Additionally, they have teeth on both the upper and lower jaws. The hump of these animals plays a significant role and makes them adaptable to harsh desert conditions. They also have very high-water consumption and they can drink up to 100 L of water within 15 min. They are mainly found in northern Africa, the middle east, Somalia, Sudan, and northern Kenya. Whereas the Bactrian camels (*Camelus bactrianus)* are typically found in cold regions of Eurasia such as China, Eastern Mongolia, and Central Asia overlapping areas of Southwest Asia like Afghanistan and Pakistan (Don Wilson et al, 2011).

The llama (*Lama glama*), alpaca (*Lama paco*), guanaco (*Lama guanicoe*), and vicuña (*Lama vicuna*) species live mainly in Southern America. However, their use as pets has become more prominent so that they can be found in North America and Europe (Fowler, 1998).

2.1.3 Old world versus new world camelids

Old world camels have characteristic dorsal humps and represent the largest species of camelids, weighing up to 1500 kg. One-humped dromedary may grow up to 2.5 meters and have padded feet and hairy coats. In contrast, the new world camels include the guanaco, llama, vicuna, and alpaca, which are much smaller. The wild species of the new world camels are guanaco and vicuna. Additionally, the guanaco has longer ears and tail then the vicuna. Their domesticated species are llamas, which are larger than alpaca and have double-coats (Fowler, 1998). Camelids are phylogenetically related to each other and have 74 chromosomes (2 x 36 = 72 autosomes and one pair of sex chromosomes) (Potts, 2004).

Figure 2.3 New world and Old world Camels. Characteristic features of New (1-4) and the Old (5 and 6) World camelids. (1) llama; (2) guanaco; (3) vicuña; (4) alpaca; (5) Bactrian and (6) dromedary.

The Food and Agriculture Organization (FAO) statistical year book for 2013 estimates the total population of camels in the world as approximately 25.89 million, of which 89% are dromedary camels and 11% are Bactrian camels generally found in the cold deserts of Asia. Over 80% of the world's camel population is found in Africa, with the highest concentration in East Africa with 63% of the world camel population. Somalia is the largest camel population in the world (FAO, 2013).

2.1.4 Ecology and important of camelids

Camelids dromedaries are drought tolerant, as they played an important roles in a variety of human societies They have been use in multi-purpose unlike any other domesticated animal thrive in dry zones of many countries in the world and provide food, transport, meat, milk, fat, and fuel, as well as soft wool for clothing and tents. Therefore, there has even been an increasing interest in the dromedary in desert countries, where other domesticated animals have difficulties to survive.

The main economic value of camels is derived from their milk, meat, use in transportation, and sports (racing) (Farah et al., 2007; Faye et al., 2008). Nevertheless, camels are essential source of animal protein to human in rough areas, they mostly contribute to the rural activities of geo-economic development (Faye et al., 2008). Their contribution to the developing countries are very less because of underestimate their true value (Ahmad et al., 2010).

2.1.5 Camel milk

Camel milk is one of the most valuable food resources it has very high nutrition comparing cow milk it contains niacin, high Vitamin C, more iron and very high calcium, It also contains only 2% fat molecules joined to protein which contains higher concentrations of long-chained fatty acids and is therefore healthier. Even though the lactose in camel milk is high like in cow milk, for unknown reason camel milk l intolerance of lactose does not occur the reason is not yet known (Wernery, 2006).

Camel milk is one of the best diet alternative for individuals that show allergic reactions to the protein fraction of cow, ewe or goat milk, as it does not contain ß-lactoglobulin and the content of alpha-casein is much lower than the other

milks (Restani et al., 1999). Camel milk reduce blood sugar level and manage quality of life of the people having type I diabetes mellitus some research has shown reduction of the insulin dose when camel milk is consumed daily (Agrawal et al., 2005).

Camel milk is considered a comprehensive diet and can be used as daily food having all nutritional requirements especially in arid regions and can contribute to a better income for the pastoralists (Agrawal et al., 2005) Their role as source of milk is a common place phenomenon which drives most of all other species of animals out of milk specially at times of drought Camel milk does not coagulate easily. It passes the acid stomach unchanged and reaches the intestines for absorption (Farah et al., 2004).

The significant features of dehydrated camels is their capability of secreting high diluted milk which contains about 90% of water (Yagil, 2000). There are few countries who produce pasteurized camel milk like Saudi Arabia, UAE, Kazakhstan and Mauritania in national market (Wernery et al., 2002).

2.1.6 Camel meat

In addition to milk, meat is one of the most important camel products. Camel meat production is estimated to be much lower than other farm animals. However, camels are still not systemically farmed for meat production in many regions, as camels are valuable. Therefore, males and infertile females are used as slaughter animals. Yet the sale of these camels for meat production is a main source of income (Morton, 1984; Farah et al., 2004). There has been a growing demand for camel meat in urban areas, especially arid societies, which mainly slaughter young animals.

2.2 *Escherichia coli*

2.2.1 Bacterial characteristics

E. coli was named after a German scientist, Theodor Escherichia, who first identified it in 1885 from human faeces. *E. coli* is a member of the family *Enterobacteriaceae* and is a gram negative, motile, non-spore-forming, and facultative anaerobe. Some strains have flagella, which allow bacteria to move among the commensal flora present in the gut of animals. The outer cell membrane contains phospholipids, membrane proteins, and lipopolysaccharide (LPS). The lipophilic inner layer of LPS is Lipid-A, which is a toxic effect of LPS also known as endotoxin (Hogan and Smith, 2003; James, 1996). On the outer layer, they may have fimbrias that protrude from the cell wall. They may have a capsule composed of a thick polysaccharide layer on their surface, which represents the K-antigens that play a role in differentiating antigenic structures. *E. coli* strains can be divided into O:H:K serotypes (James, 1996). However, some specific *E. coli* are wide spread intestinal pathogens in mammals and birds. Normally these strains represent primary pathogens with a higher potential to cause disease with specific virulence attributes. These virulence attributes are normally encoded in the genetic material that define these pathotypes, which are genetically-encoded in plasmids, chromosomal DNA, or phages. Pathogenic *E. coli*, like any other mucosal pathogen, first colonise the mucosal surface, overcome the host immune responses, and secrete virulence factors that help with the colonization and persistence that can be exchanged between different strains and cause a related disease using the same set of virulence factors (Rodriguez-Siek et al., 2005).

2.2.2 Pathogenic classification

Pathogenic *E. coli* have been classified into two main categories: extraintestinal pathogenic *E. coli* and diarrhoeagenic *E. coli*. Within the diarrhoeagenic *E. coli* classification, there are six categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusively adherent *E. coli* (DAEC) (Russo and Johnson, 2000; Kaper et al., 2004b; Donnenberg and Kaper, 1992b). Extraintestinal pathogenic *E. coli* are epidemiologically and phylogenetically distinct from diarrhoeagenic *E. coli*. They have the capability to colonise many organs causing various infections outside the gastrointestinal tract, of which urinary tract infections are the most common (Johnson et al., 2003).

2.2.3 Virulence factors

Pathogenic *E. coli* are typically specific to the nature of the disease they cause and to the animals they infect. They produce factors involved in the pathogenesis of specific diseases (China and Goffaux, 1999). Bacterial virulence factors are required to colonise and infect the host to overcome host defence systems. The main *E. coli* virulence factors include cell adherence (adhesins), toxins, secreted proteins from bacteria, polysaccharide capsules, and other mechanisms to resist killing by the complement or to scavenge iron. Bacteria do not produce virulence factors continuously but only upon obtaining the bacterial genome that may exist in plasmids, even though the virulence factor is not produced (Harel and Martin, 1999).

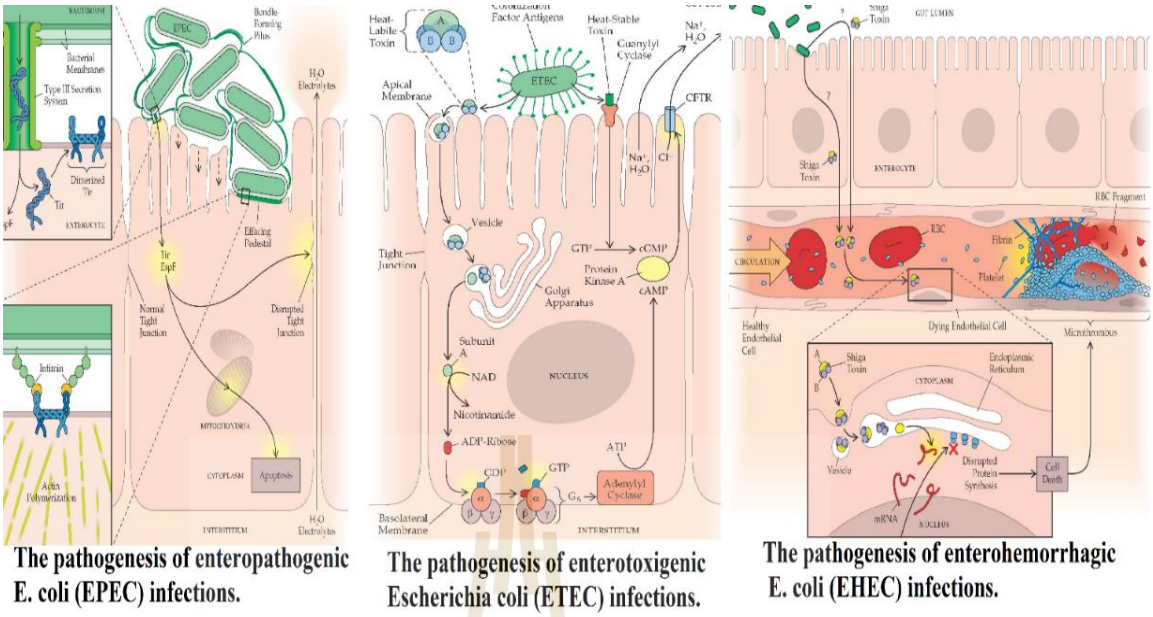

Figure 2.4 Mechanisms of *E. coli* pathogenesis.

2.2.4 Adhesins

Adhesins are either pili, fimbriae, or afimbrial adhesins associated with the cell surface because of colonization of the host cell surface (Soto and Hultgren, 1999). Several adhesins have been discovered in *E. coli* isolates from bovines. For example, F17 fimbriae, which are located on the chromosome expressed by bovine enterotoxigenic *E. coli*, while F17b is from *E. coli* isolated from septicaemic, diarrhoeic calves, and lambs. F17c expressed septicaemic *E. coli* strains and F17d was discovered in bovine enterotoxigenic *E. coli* and *E. coli* isolated from diarrhoeic calves (Le Bouguenec and Bertin, 1999; Bertin et al, 1996). Due to aerobactin production, about half of the F17-positive *E. coli* strains were resistant to complement (Pohl and Mainil, 1995).

Mostly S and P fimbriae are found in strains of *E. coli* causing urinary tract infection. Adherence of bacteria to urinary tract epithelia are suggested to be involved in urinary tract infections (Schilling et al., 2001).

2.2.5 Toxins

Pathogenic *E. coli* strains produce a variety of toxins with different activities including heat-labile (LT) and heat-stable (ST) enterotoxins produced by Enterotoxigenic strains. These toxins act upon separate intracellular targets, adenylate cyclase in the case of LT and guanylate cyclase in the case of ST, both leading to host cell secretions resulting in diarrhoea (Sears, 1996). Shiga toxins are produced by enterohaemorrhagic *E. coli* strains (Kaper et al., 2004b) resulting in non-bloody, watery diarrhoea similar to infections caused by *Shigella* spp. (Nataro and Kaper, 1998). These organisms are related to *Shigella* spp. pathogenicity, (Pupo et al., 2000; Slanec et al, 2009). Enteroinvasive *E. coli* invade and multiply in colonic epithelial cells with encoded genes on the plasmid pInv (Small and Falkow, 1988) and spread by either releasing bacterial apoptotic factors or migrating between epithelial cells through actin that are synthesised at one pole of a bacteria, which is mostly similar to *Shigella* and *Listeria* (Nataro and Kaper, 1998).

2.3 Conventional antibodies

A conventional antibody, immunoglobulin (Ig), is a large Y-shaped glycoprotein (Maverakis et al., 2015) with a molecular weight of 150 kDa mainly produced in plasma cells used in research experiments and medical applications. Normally they are classified according to their heavy chain isotypes, IgG, IgM, IgE, IgA, and IgD, which vary in both structure and function. Usually antibodies are tetramers containing two identical heavy chains and two identical light chains, which play roles in the antigen binding site based on their flat and concave shape. Each heavy chain comprises the N-terminal variable VH domain tailed by two or more constant (CH) domains, while the

light chain comprises only one variable domain. The basic functional unit of each antibody is an immunoglobulin monomer. It can form a dimer with two Ig units in a Y shape that consists of four polypeptide chains with two H chains and two L chains connected by disulfide bonds (Woof and Burton, 2004). They all occur in two physical forms; a free soluble form secreted from the cells into the blood stream and a membrane-bound form on the surface of a B cell, referred to as the B-cell receptor (Borghesi and Milcarek, 2006).

Each chain is composed of a structural domains called immunoglobulin domains that contains about 70 to 110 amino acids that can be classified according to their variable and constant regions as well as their sizes and functions (Barclay, 2003). The heavy chain has two regions: the constant region, which is constant with all immunoglobulins, and the variable region, which differs based on B cells and each heavy chain has about 110 amino acids composed of a single immunoglobulin domain (Woof and Burton, 2004).

In animals, there are two types of immunoglobulin light chains, lambda and kappa. They have about 211 to 217 amino acids with two domains, constant and variable. Each light chain possesses one constant domain (CL) whereas 3 or 4 constant domains (CH1-CH3/CH4) are found in antibody heavy chains. Each CH1 is linked to CH2 by a flexible proline-rich stretch of amino acids referred to as the hinge region. The CH2-CH3 (or CH2-CH4) tail domains of the antibody comprise the Fc domain. Induction of effector responses upon antigen binding is mediated by the Fc domain. The disulfide linked VL-CL and VH-CH1 arms were generated *in vitro* by proteolytic cleavage of the Y-shaped antibody referred to as the Fab fragments.

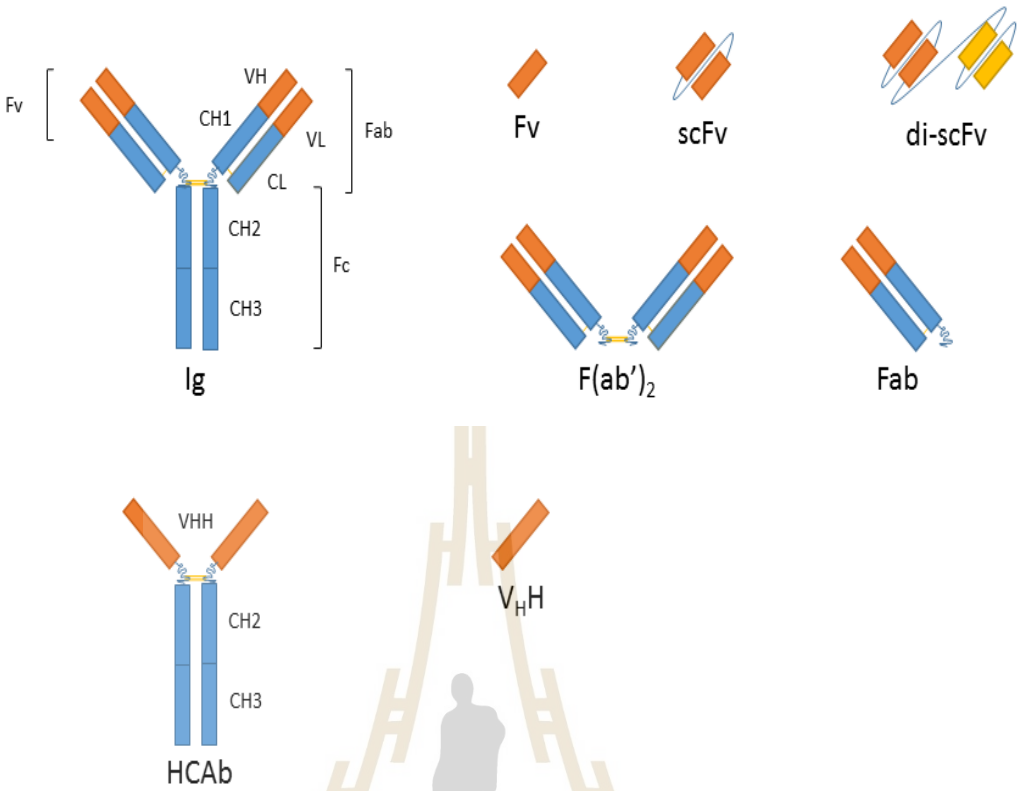

Figure 2.5 Structure of camel immunoglobulins and antibody fragments.

2.4 Camel antibodies

In camelids there are three IgG isotypes. IgG1 shows the typical conventional antibody structure while IgG2 and IgG3 are referred as Heavy chain antibody (HCAbs) they lacks L chains as well as CH1 and their nomenclature based on biophysical properties of IgG isotypes i.e separation of chromatography into three isotypes (Hamers-Casterman et al., 1993).

2.4.1 Characteristics

More than two decades have passed when Hamers- Casterman and coworkers in University of Brussels was first published in nature in 1993 while they were investigating the immune system of camel dromedaries they discover an unusual single domain heavy chain antibody (nanobody) in dromedary camels and other

camelid species (Hamers-Casterman et al., 1993) which have a refolding capacity, small in size up to (15 kD), have high solubility, thermal stable as well as good tissue penetrations (Hamers-Casterman et al., 1993). They also lack CH1 domain which the heavy chain antibody (V-domains) are directly connected to the hing region. This single N-terminal domain can bind antigen without other domain (Riechmann and Muyldermans, 1999). Unlike conventional antibody the heavy chain antibody (camelid antibodies) can bind antigen with a single heavy chain. Researches showed a lot of interest on camlid antibody in the recent past as it demonstrate a powerful immuno therapeutic and diagnostic use as well as biosensor tools, particular in targeting cancer cells, inflammatory, autoimmune and in viral diseases (Wesolowski et al., 2009).

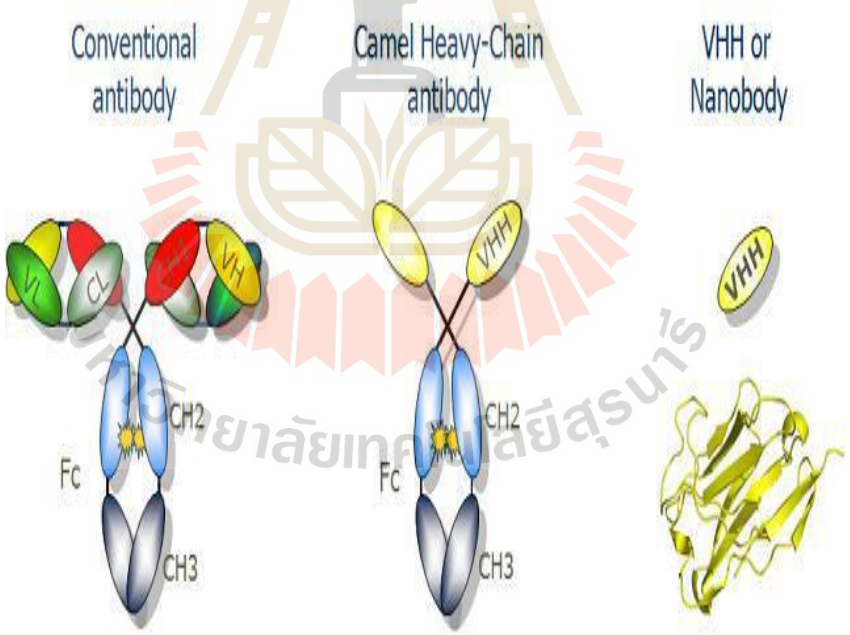

Figure 2.6 Structures of antibody, camel heavy-chain and nanobody (Muyldermans et al., 2009).

Antibodies with specificities and strong affinities for cognate antigens are widely used for the treatment of diseases including bacterial infections, autoimmune disorders and cancer (You et al., 2013). Camel nanobody has a high specific antigen binding capacity which provides key defenses against pathogenic organisms and toxins. It also has penetration capacity into the cells targeting internal antigens like enzyme activity site (lysozyme) and viral particles with rapid elimination of the kidney, neutralizing of toxins, cytokines and blood clotting components.

These nanobodies are single domain soluble and are very good for sensitive immunological tests as well as a neurosensors (Holliger and Hudson, 2005). The heavy chain antibody consists of only two heavy chains and lacks the two light chains. This antigen can only bind with VH domain camelids and are the only mammals who poses heavy chain antibodies (Conrath et al., 2003) that lacs the light chain and one of their constant domains (CH1). In affinity chromatography, Protein A and G separate into three antibody fractions, one with two light and two heavy chains and other two lacking light chains (Nguyen et al., 2001). These are named IgG1, IgG2, and IgG3 according their molecular weights (Nguyen et al., 2001). The conventional camel IgG has a molecular wt of 90,000 Da rather than the 150,000 Da of other antibodies. HCAbs have a lower molecular weight (90 kDa) than conventional antibodies (150 kDa), which should facilitate tissue distribution. VHHs can be cloned and recombinantly expressed as the smallest antibody molecule with antigen binding properties (Muyldermans, 2001). Owing to their small size, these single domain antibodies are also known as Nanobodies® (Nbs). The recombinant expression of Nbs as water soluble protein molecules results in the replacement of 4 hydrophobic amino acids at the former interface with the VL domain to hydrophilic residues accounts for

the high solubility and stability of VHH domains at what would be the light/heavy chain interface (Nguyen et al., 1998; Vu et al., 1997). They are thermally stable and capable of binding to antigens at a very high rate at about 90ºC. Where conventional antibodies lose affinity for their antigens (van der Linden et al., 1999). This ability to withstand high temperatures has been employed to purify VHH from VH domains and to detect some drugs in hot drinks such as caffeine (Graef et al., 2011). The purification process is simple and expression yields are high. Nbs are effortlessly expressed in *E. coli* as periplasmic proteins or in eukaryotic cells as secretory proteins with high yields and low costs (Holliger and Hudson, 2005; Wesolowski et al., 2009). VHH domains usually have many useful features. Primarily, the centre of the antigen binding paratope, such as the complementarity determining region 3 (CDR3), is much longer and diverse in VHH domains than in the domains of conventional antibodies (Harmsen et al., 2000; Wu et al., 1993). Secondly, the conventional antibodies generally form planar interaction surfaces with their antigen but the CDR3s of heavy chain antibodies could form finger-like supplements that could form convex paratopes. Thus, the CDR3 regions of VHH are longer than conventional antibodies reaching up to 26 amino acids (Decanniere et al., 1999). These long regions can form finger-like structures that reach the protein surface to block enzymatic activity (De Genst et al., 2006). The CDR3 of heavy-chain antibodies is able to reach and fill out on proteins that are often unreachable by conventional antibodies (Koch-Nolte et al., 2007). Third, the VHH surface is comprised of hydrophilic amino acids that guarantee VHH domain solubility. Fourth, owing to their small size VHH domains, CDR3 show good tissue penetration *in vivo* (Cortez-Retamozo et al., 2004; Muruganandamn et al., 2002).

2.4.2 Structure of HcAbs

HcAbs of camelids originate from IgG antibodies and are about 50% of the circulating immunoglobulins in dromedary camels (Muyldermans, 2001). In general the structure of the hcAbs resembles that of conventional immunoglobulins with a number of significant deviations. The heavy chain of hcAbs is composed of three rather than four domains and the V domain is linked directly to the hinge region and the Fc (Hamers-Casterman et al., 1993). Whereas the two constant domains at the C terminus (CH2 and CH3) are extremely homologous to the Fc domains of conventional antibodies, the CH1 domain is missing in hcAbs. Secondly, the light chains are completely absent in hcAbs. The Fab is compacted to a single variable domain known as the VHH (variable domain of heavy chain antibodies) domain or Nanobody. Antigen binding happens within this single domain only, demonstrating the smallest intact antigen binding fragment (~15 kDa) derived from a functional immunoglobulin. Camelid antibodies comprise only two heavy chains (VHH, CH2, and CH3). They lack the light chains and the CH1 domain. Antigen binding occurs through one domain only, the VHH domain. Compared to the human VH domain sequences the VHH FRs show 80 % homology (Muyldermans, 2001). However, in the FR2 region the VHHs have four amino acid substitutions: Val37Phe or Val37Tyr, Gly44Glu, Leu45Arg or Leu45Cys, and Trp47Gly (Muyldermans et al., 1994)

2.5 Genetic of nanobody

The presence of functional hcAbs required some alterations in the configuration of an antibody molecule in order to modify the antibody deletion of the CH1 domain, which was first quite obviously adjusted and involved in the gathering process of

heavy and light chain antibodies. Normally the absence of the CH1 domain in hcAbs is caused by a mutation in the CH1 exon splice site(Nguyen., 1999; Nguyen et al., 2002; Woolven, et al., 1999). The VHH FRs shows 80 % sequence homology when compared with the human VH domains (Muyldermans, 2001). However, VHHs show four typical amino acid substitutions in the FR2 region: Gly44Glu, Leu45Arg or Leu45Cys, Val37Phe or Val37Tyr, and Trp47Gly. In conventional antibodies the VH residues interact with the VL domain through hydrophobic connections. In the absence of a VL domain, these hydrophobic residues would likely cause an hcAbs accumulation but the VH residues are more hydrophilic in VHHs (Muyldermans, et al., 1994).

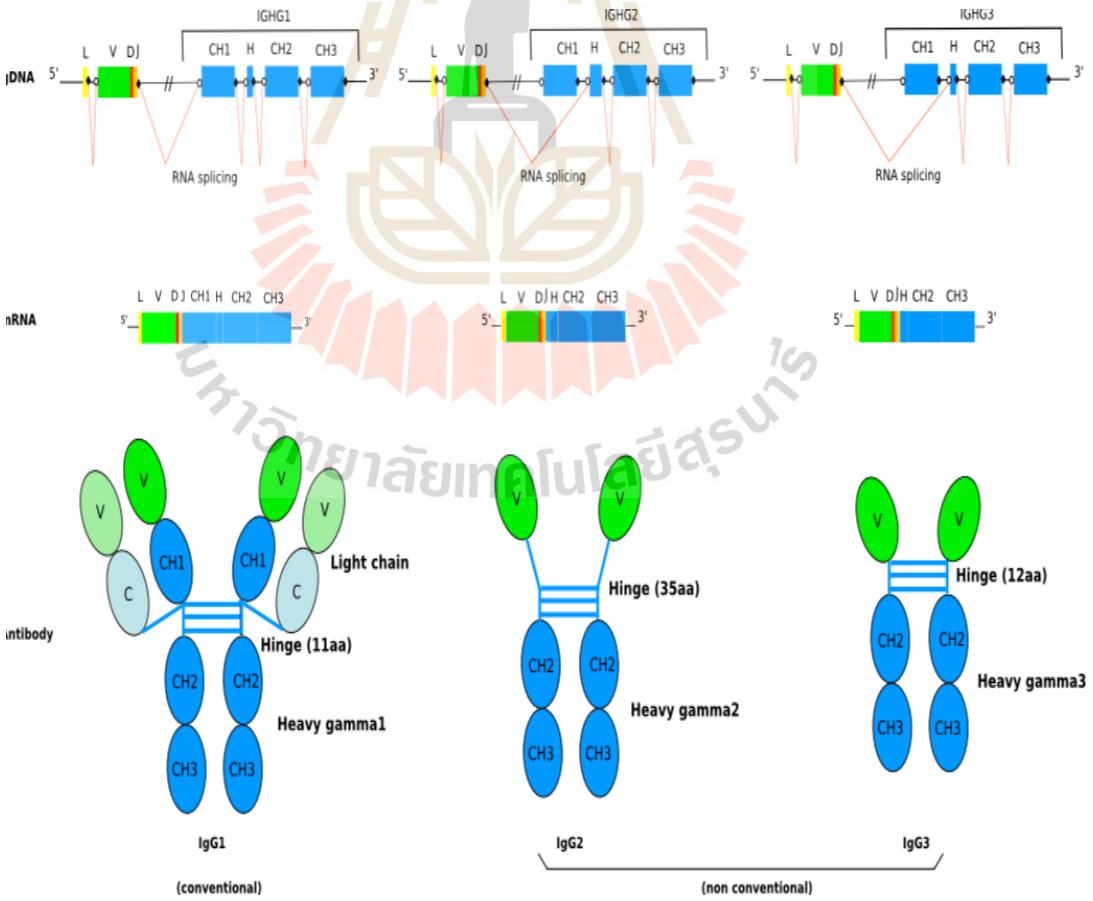

Figure 2.7 Structure of IgG1, IgG2 and IgG3 of camel immunoglobulin.

Nanobody specifically recognizing lysozyme targeted tumours transgenic for lysozymes (Revets et al., 2005). Additionally, VHHs can differentiate isoforms of the prostate-specific antigen (PSA) and, therefore, distinguish different steps of prostate cancer (Saerens et al., 2004). Furthermore, the VHHs show potential in therapeutic areas including neurology, inflammation, and pulmonary diseases (Cortez-Retamozo et al., 2004; Roovers et al., 2007). VHHs have features comparable to those of conventional antibodies. Numerous advantages, starting with their natural single-domain, their small size (15 kDa), quick blood clearance, and tissue penetration. Meanwhile antigen binding arises only through one domain that is set by a single exon, so that there is no requirement for recombination of the VH and VL domains. VHHs are remarkably stable, especially at very high temperatures and long incubation times at 37°C while maintaining their functionality at a very high pH (van der Linden, et al., 1999; Arbabi, et al., 1997; Verheesen, et al., 2006).

2.6 Cloning

Cloning of amplified DNA is difficult to perform but several techniques have been advanced. Selecting an appropriate method depends on the aim of the cloning experiment, the length of the PCR product, and the DNA polymerase used. If, for example, a DNA polymerase with proofreading activity is used for a site-directed mutagenesis study, blunt-end cloning would be appropriate. For protein expression, a method that permits directional cloning is suitable. This short review gives a summary of some of the cloning approaches developed for cloning PCR products.

Terminal transferase activity is a property of Taq DNA polymerase that preferentially adds adenine to the 3' ends of PCR products (Hu, 1993; Clark, 1988).

PCR products with a single 3' adenylate extension were cloned into a vector containing complementary 3' thymidine overhangs (TA cloning) (Kwak, 1995). TA cloning may also be used for the products of Tth DNA Polymerase and the Expanded PCR Systems. For example, T-vectors can be easily cloned using a rare recognition sequence restriction enzyme (Xcm I) that creates a 3' end with a single base protruding (Borovkov and Rivkin, 1997; Mead et al, 1991; Harrison, 1994). However, T-vectors can be prepared by an enzymatic addition of a single thymidylate with terminal transferase and ddTTP (Holton and Graham, 1991) or Taq DNA Polymerase and dTTP (Marchuk et al., 1991). Polymerases used for the TA cloning method should have terminal transferase activity and lack 3'-5' exonuclease activity. In addition to Taq DNA Polymerase, several DNA polymerases have these characteristics and can be used for TA cloning (Tth DNA Polymerase).

Moreover, DNA polymerases with 3'-5' exonuclease activity produce blunt-ended PCR products and remove mispaired nucleotides from the 3' ends of double-stranded DNA (Hu, 1993). The PCR products generated by these proofreading polymerases can be cloned into vectors by blunt-end ligation (Lohff and Cease, 1992). Polishing methods permit the removal of a single nucleotide extension generated with Taq DNA Polymerase. These polished PCR products can be cloned by blunt-end ligation into an appropriate vector (Costa and Weiner, 1994; Lohff and Cease, 1992). Blunt-end cloning of PCR products into plasmid vectors is less efficient than sticky-end cloning. Therefore, a more efficient variation of the blunt-end cloning method has been developed. In this method, a rare cutter restriction enzyme is added to the ligation mixture to linearise any self-ligated vector formed during the ligation reaction (Costa et al., 1994).

Additionally, directional cloning of the PCR products is one of the most common methods for efficient cloning, which introduces additional restriction sites at the 5' end of each primer. As amplification proceeds, these primers were incorporated into the PCR product. After PCR, the amplified DNA fragment was digested with the suitable restriction enzymes and ligated into multiple cloning sites of a linearised vector (Scharf et al., 1986). However, there are two problems with this method. Some restriction enzymes may fail to cleave near the ends of linear double-stranded DNA (Jung et al., 1990; Kaufman, 1990). However, the sequence the PCR product is often unknown. The chosen restriction enzyme could potentially recognise and cleave sites within the product itself (Aslanidis and de Jong, 1990; Shuldiner et al., 1991). Exonuclease-III-mediated directional cloning is an example of directional cloning that does not require special primers. The 3'-5' exonuclease activity of Exonuclease III can degrade the PCR product from the 3' end of both strands. The resulting modified PCR product can easily be cloned into a linearised vector with complementary bases (Walls et al., 1993; Kaluz et al., 1992).

Generally blue/white screening is used to detect transformed bacteria with recombinant plasmids. This method allows non-recombinants and recombinants to be differentiated by colony colour. However, in-frame cloning of the PCR fragment into the X-gal gene occasionally allows the cloned gene to be expressed, leading to faulty phenotypes and differences in the colour intensity of blue colonies. This aspect leads to the generation of false negative colonies. To obtain maximal transformation efficiency, numerous positive selection vectors were developed. Most of the positive selection vectors are based on lethal genes (Schlieper et al., 1998; Yazynin et al., 1996; Bernard et al., 1994; Henrich, 1995). The insertion of foreign DNA alters

expression of the lethal gene, so only those clones that contain an insert survive. The use of such positive selection vectors is limited by their restricted host range and by the need for mutagenic agents or complex media (Kast, 1994). However, Schlieper and co-workers developed a positive selection vector that can be employed with almost all commonly used strains of E. coli without requiring complex media or induction conditions. By this method, blunt-end DNA fragments are ligated into the Mlu NI restriction site within the multiple cloning sites of the vector, disrupting expression of the *Crps* gene. Only positive recombinants grow after transformation, since cells that harbour the non-recombinant lethal gene are killed during transformation.

2.7 Flexi® Vector systems

The Flexi® Vector Systems (Promega Madison, WI USA) were used this study, which simplify the study of multiple aspects of protein function by decreasing the cloning burden of shuttling protein-coding regions between two or more vectors with different functional abilities (Blommel et al., 2009). All Flexi® Vectors can act as an acceptor of a protein-coding sequence flanked by SgfI and PmeI sites. However, the two rare-cutting restriction endonucleases, SgfI and PmeI, have the fewest restriction sites in the protein-coding regions of human cDNA sequences. This enzyme pair also cuts infrequently into the open reading frames of many organisms. Most (>98%) known human open reading frames are not affected by the use of these restriction enzymes for directional cloning. The presence of SgfI or PmeI sites within the protein-coding region will interfere with the cloning of the full-length protein-coding region. If the protein-coding region contains these sites, RecA protein can be used to protect

the SgfI or PmeI sites within the protein-coding region from digestion (Schoenfeld et al., 1995). Otherwise, PCR-based site-directed mutagenesis methods are good to mutate restriction enzyme sites without changing the amino acid sequence of the protein-coding region (Higuchi et al., 1988; Ho et al., 1989). However, the Flexi® Vectors have a lethal gene, barnase, that should be replaced with an insert into the clone. This insert allows high-efficiency transfer of a protein-coding region between vector backbones (Paul et al., 2006). Antibiotic resistance genes carried on the Flexi® Vectors facilitate the transfer of protein-coding regions between vectors. In Flexi® Vector systems either ampicillin or kanamycin-resistant plasmids are employed. Any vector of this system can act as an acceptor of a protein-coding region flanked by *SgfI* and *Pme*I sites. The *SgfI* site is upstream of the start codon of the protein coding region and depends upon the Flexi® Vector used for cloning. This allows the expression of a native (untagged) protein or an amino-(N-) terminal-tagged protein by read through of the *SgfI* site. However, the PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the carboxy (C)-terminus of the protein (Hartley, 2006; Blommel et al., 2009). Protein-coding on other Flexi® Vectors, following digestion with *SgfI* and *PmeI*, allows easy adaptation to high-throughput formats. C-terminal Flexi® Vectors allow expression of C-terminal-tagged proteins. These vectors act as acceptors of a protein-coding region flanked by *SgfI* and *PmeI* sites, they lack a PmeI site and contain a different blunt-end site, *Eco*ICRI. When the blunt PmeI and *Eco*ICRI ends are joined, the stop codon is not recreated, allowing read-through into the C-terminal peptide sequence. However, this sequence cannot be cut by either PmeI or EcoICRI, so the protein-coding region cannot be removed from the C-terminal Flexi® Vectors and transferred to other Flexi® Vectors.

This indicates non-reversible transfer into C-terminal Flexi® Vectors (a one-way exchange) (Blommel et al., 2009; Festa et al., 2013).

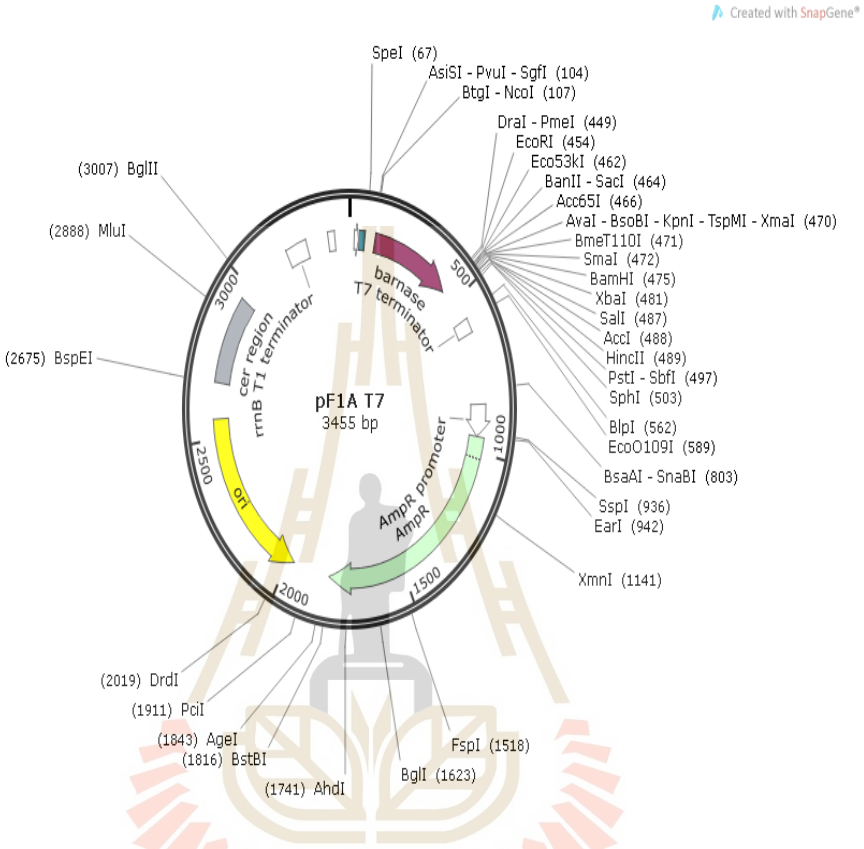

Figure 2.8 pF1A T7 Flexi Vector with having SgfI and Pmel restriction sites (promega USA).

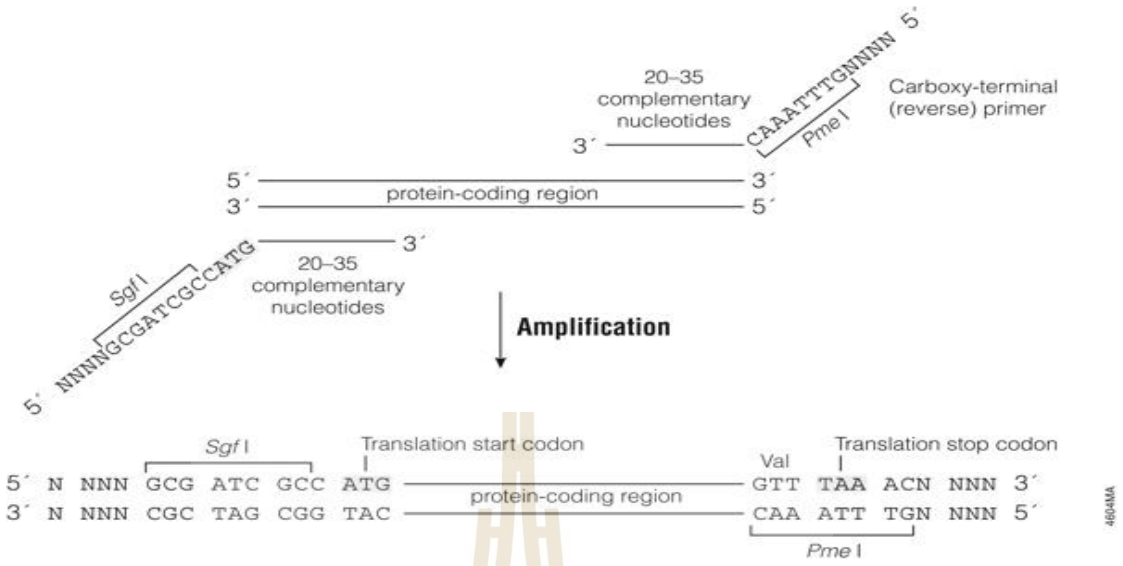

Figure 2.9 SgfI and Pmel restriction sites (promega USA).

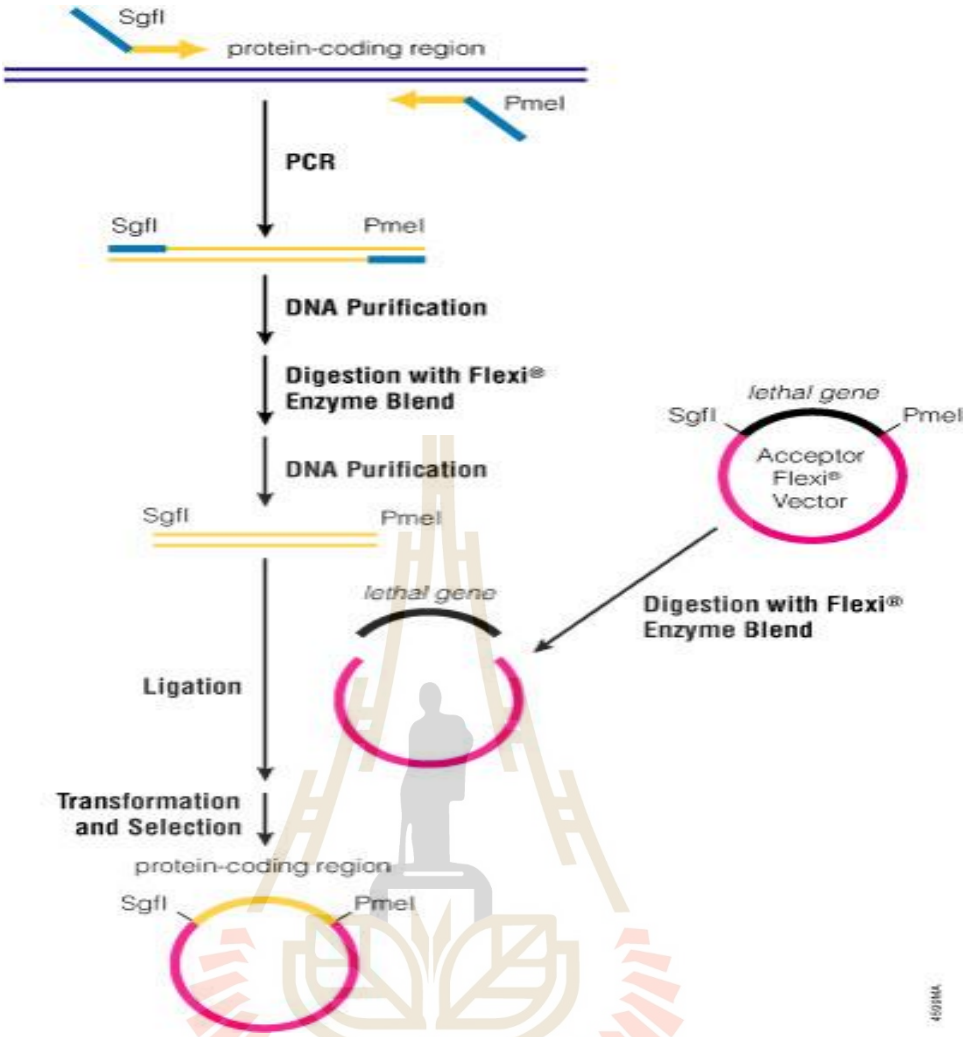

Figure 2.10 Flexi vector ligation and transformation procedure (promega USA).

2.8 ELISA (Enzyme Linked Immunosorbent Assay)

ELISA (Enzyme Linked Immunosorbent Assay) is one of the most generally used methods in laboratory analyses of hormones, peptides, proteins, antibodies, and lipopolysaccharides. It can be used for qualitative and quantitative assessments of low concentrations among a multitude of other substances. Moreover, ELISAs are rapid, sensitive, and cost-effective. An ELISA is used in a broad variety of assays (direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA). Nevertheless, all

ELISA assays are based on the same principle, the binding of either an antigen or antibody to a solid surface and the selective interaction between both assay components. After washing, the assay can be detected by labelling or linked to an enzyme (direct ELISA). Otherwise, a secondary antibody conjugate can be used (indirect ELISA). The colour is developed by adding a substrate to get a measurable signal. The strength of the colour is directly proportional to the quantity of analytes in the sample (Huberlab, 2008; Vos et al., 1982). PBS or Tris buffer are buffers primarily used during ELISA. During assessment of ionic strength and pH of the buffer play a significant role. Carbonate buffer (0.05 M) at pH 9.6 is the most common buffer for coating plastic surfaces. However, the alternative coating buffers are 10 mM PBS at pH 7.4 or an acetate buffer at pH 5.5. It is essential to block unoccupied binding sites on the solid surface in a blocking step after coating the plate to reduce non-specific binding and to avoid background problems during assay steps. BSA, casein, or non-fat dried milk powder in Tris or PBS buffer are the blocking agents normally used for ELISAs. Washing steps are important to remove unbound reagents after incubation steps. Insufficient washing may result in a high background, whereas excessive washing may lead to debonding and decrease the sensitivity of the assay (Vos et al., 1982; Yolken, 1980).

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CHAPTER III

MOLECULAR IDENTIFICATION OF 20 *ESCHERICHIA COLI* ISOLATES FROM DEAD NEONATAL CAMEL CALVES (*CAMELUS DROMEDARIUS*) IN THE UNITED ARAB EMIRATES

3.1 Abstract

Escherichia coli is a member of the *Enterobacteriaceae* family. Their cells are typically rod shaped, about 2 µm long, and 0.5 µm in diameter. Optimal growth occurs at 37°C and growth can be supported by aerobic and anaerobic respiration. Many *E. coli* strains are predominantly found as commensal flora in the intestine that colonise the gastrointestinal tract of new-born animals within the first hour of their life. The majority do not cause any disease unless the host is immuno-suppressed or the gastrointestinal barriers are breached. In contrast, even commensal *E. coli* can cause disease. In this study, we concentrate on the gut microbiota from dead neonatal camel calves (one or two weeks old) collected from the postmortem of individuals identified as colisepticemic or colibacillosis. First, we collected 20 *E. coli* isolates. All of them were successfully amplified by conventional PCR of the 16SrRNA *E. coli* gene, and then we ran the commercially made real-time kits PowerChek Diarrheal *E. coli* 4-plex Real-time PCR Kit I and II (Kogenebiotech, Seoul, Korea) that target eight pathogenic *E. coli* genes. Results of the 20 samples analysed were negative for all pathogenic *E.*

coli strains. Even after sequencing and blasting against the GenBank database, all six pathotype strains were excluded and all GenBank data were matched with commensal flora of *E. coli.* Since the immune system of newly born camels is weak and their immune response is immature, infection might result from ingestion of contaminated mother's milk or unhygienic food where the commensal *E. coli* easily evades the host defences leading to host damage or even death.

3.2 Introduction

Theodore *Escherichia* discovered *Escherichia coli* in 1885. It is a member of the genus *Escherichia* within the family *Enterobacteriaceae*, gram negative, motile, and a facultative anaerobe among the commensal flora present in the gut of warm-blood animals (Kaper et al., 2004b). Many strains are wide spread intestinal pathogens of mammals and birds and it is the most commonly encountered pathogen in the *Enterobacteriaceae* (Chen and Frankel, 2005). Normal *E. coli* flora colonise the intestine during first hour of life (Nataro and Kaper, 1998). With the help of bacterial antigens, the pathogenic strains are attached to the mucosal portion of the intestine via pili, fimbriae, or fibrils. Then, they colonise the ileal mucosa, multiply, and express pathogenic factors to evade the host defences leading to host damage and death (Kaper et al., 2004a). Virulent factors like adhesins, toxins, plasmids, and phages are involved in pathogenesis and a combination of these factors determine the degree of pathogenicity and severity of the disease (Ho et al., 2013). In camels, *E. coli* causes major neonatal diarrhoea resulting in camel calf losses of up to 40%, especially during the first few days of their life, if it is not treated well (Wernery and Kaaden, 2002; Farah et al., 2007). Insufficient colostrum intake and poor management increases the

disease based mortality (Abbas and Omer, 2005). Immature immunity, insufficient colostrum, and contaminated soil also contribute to increased camel calf mortality and economic impacts on all camel breeders (Mohammed et al., 2003).

E. coli causes diarrhoea via six different mechanisms, each with different virulence and pathotype results including septic infection, acute enteritis, haemorrhagic colitis, and diarrhoea. These pathotypes include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli (*EAEC), enteroinvasive *E. coli* (EIEC*),* and diffusely adherent *E. coli (*DAEC*)* (Kaper, 2004; Donnenberg and Kaper, 1992a). Colibacillosis and colisepticemia primarily occur in one to four-week-old camel calves and cause high fever, yellowish watery diarrhoea, and dehydration. Death primarily occurs in the first week of their life. The objective of this study is to identify a pathogenic *E. coli* strain that causes death in camel calves, focusing on postmortem confirmed colibacillosis or colisepticemia cases using molecular diagnostic methods

3.3 Material and Methods

3.3.1 Sample collection

Twenty *E. coli* isolates were used in this study. All the samples were dead camel calves (one or two weeks old) isolated from postmortem samples that showed septicaemic and systemic *E coli*. Bacterial cultural and biochemical analysis were performed in the following centres, seven of which were isolated from the Advanced Scientific Group breeding center, Abu Dhabi; three from Al Wathba veterinary clinic, Abu Dhabi; and ten from the Central Veterinary Research Laboratory, Dubai. All twenty samples were taken from these centres contained pure

E. coli culture. PCR were performed with primers for the 16s rRNA gene of *E. coli* genera.

Table 3.1 List of *E. coli* isolates and their diagnostics.

S No	Lab No	Sample	Diagnostic	Bacteria	Center
1	MB4068	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
2	MB3881	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
3	MB3882	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
4	MB3668	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
5	MB3839	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
6	MB3860	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
7	MB3871	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
8	MB3768	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
9	MB3859	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
10	MB3864	Postmortem tesue	Colisepticemia	*E. Coli*	CVRL
11	VRC0765	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
12	VRC0588	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
13	VRC0110	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
14	VRC0234	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
15	VRC0973	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
16	VRC0298	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
17	VRC0654	Postmortem tesue	Colisepticemia	*E. Coli*	VRC
18	WA2880/13	Postmortem tesue	Colisepticemia	*E. Coli*	WATHBA
19	WA3081/13	Postmortem tesue	Colisepticemia	*E. Coli*	WATHBA
20	WA2634/13	Postmortem tesue	Colisepticemia	*E. Coli*	WATHBA

3.3.2 Molecular Identification of *E. coli*

3.3.2.1 DNA Extraction

DNA were extracted using two different methods: boiling method and G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology Korea). DNA was extracted according to the iNtRON G-spin™ Extraction Kit protocol. Whereas the boiling method was carried out by taking a few colonies from a MacConkey Agar plate and diluting them in 500 µL of distilled water. Samples were they centrifuged for 10 min at 16.000 xg and room temperature. The supernatants were discarded. The remaining pellets were re-suspended in 100 µL of distilled water and boiled for 15 min at 100°C in a water bath. The samples were centrifuged again for 10 min at 16.000 xg and room temperature. Finally, the supernatant was transferred into a sterile tube.

3.3.2.2 PCR Primer

E. coli samples (20) were identified using the 16SrRNA gene with the *E.coli* primer pair where the forward primer is 5'CCC CCT GGA CGA AGA CTG A3' and the reverse is 5'ACC GCT GGC AAC AAA GGA3' (Wang et al., 2002; Brandal et al., 2007).

3.3.2.3 Conventional PCR

The 16SrRNA gene of the *E. coli* primer pair were used in this study. The forward primer is 5'CCC CCT GGA CGA AGA CTG A3' and the reverse is 5'ACC GCT GGC AAC AAA GGA3' (Wang et al., 2002; Brandal et al., 2007). The PCR assay was carried out in 25 µL of reaction mixture consisting of PCR buffer (20 mM Trisp-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$), 0.4 mM of dNTPs (Promega, Madison, USA), 0.2 µM of each primer (Alpha DNA Canada), 1 unit of hot start *Taq*

polymerase (Promega USA), and 2 µL of the DNA template. The reaction mixture was run on a thermal cycler (Veriti Thermal Cycle Applied Biosystems, CA USA) with an initial denaturation of 95ºC for 5 minutes, then 30 cycles of 95ºC for 1 minute, 55ºC for 30 seconds, and 72ºC for 40 seconds. The final extension was performed at 72ºC for 5 minutes. The PCR products were analysed by gel electrophoresis with 10 µl of sample added to the 2% agarose gel, which was prepared with 5 µL of 20.000X RedSafe nucleic acid staining solution (iNtRON Biotechnolgy, Korea) and electrophoresed at 90 V for 40 minutes in 1X TAE buffer (0.04 M Tris/acetate, EDTA 1 mM). A 100 bp ladder (Promega, Madison, USA) was used as a standard marker, then the gel was visualised on a UV transilluminator and photographed.

Table 3.2 PCR mixture of16SrRNA gene of *E.coli* primer pair.

Content	**Volume (µl)**
5x Buffer	2.4
MgCl2 (25mM)	0.9
dNTP (10mM)	0.4
For Primer (10pmol)	2
Rev Primer(10pmol)	2
Go Taq	0.1
DNA	2
Water	2.2

3.3.2.4 Gel Electrophoresis

The PCR products were analysed by gel electrophoresis with 10 µL of sample applied to a 2% agarose gel prepared with 5 µL of 20.000X RedSafe nucleic acid staining solution (iNtRON Biotechnolgy, Korea) and electrophoresed at

90 V for 40 minutes in 1X TAE buffer (0.04 M Tris/acetate, EDTA 1 mM). A 100 bp ladder (Promega, Madison, USA) was used as a standard marker. Then, the gel was visualised with a UV transilluminator and photographed.

Table 3.3 PCR condition of16SrRNA gene of *E.coli* primer pair.

Steps	Tempreture	Time	No. Cycles
Int Den	95°C	5 min	1
Den	95°C	15 sec	
Ann	55°C	30 sec	30
Ext	72°C	30 sec	
Final Ext	72°C	10 min	1

3.3.2.5 Realtime PCR

Multiplex real-time PCR were carried out using PowerChek Diarrheal *E. coli* 4-plex Real-time PCR Kits I (VT1, VT2, LT, ST) and II (eaeA, aggR, bfpA, and ipaH) (Kogenebiotech, Seoul, Korea) according to the manufactures protocol. Assay was performed on a Rotor-Gene Q Real-time PCR (QIAGEN, Hilden Germany) with initial denaturation of 95ºC for 10 minutes, then 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute where fluorescent detection was carried out at the end of the second step of each cycle with appropriate probe dyes according to the kit protocol.

3.3.2.6 Sequencing 16SrRNA gene

The PCR product for the 16SrRNA gene primers were purified with a QIAquick PCR purification kit (QIAGEN, CA, USA) and directly sequenced with PCR primers, forward and reverse, separately. Sequencing was carried out with Bigdye Terminators on an ABI 3730 genetic analyser (Applied Biosystems, CA, USA) according to the manufacturer's protocol.

3.4 Results and Discussion

DNA were extracted from subcultures of 20 *E. coli* samples, isolated from dead camel calves and amplified successfully with 16SrRNA gene of *E. coli*. All samples were shown PCR positive giving a single band of 400bp on 2% agarose gel electrophoresis. *E. coli* strain JM109 competent cells (Promega, Madison, USA) were used as a PCR positive control, which also gave a clear band of 400bp the extraction and negative controls did not have any band. 100bp ladder were used as a molecular weight marker (Fig. 3.1).

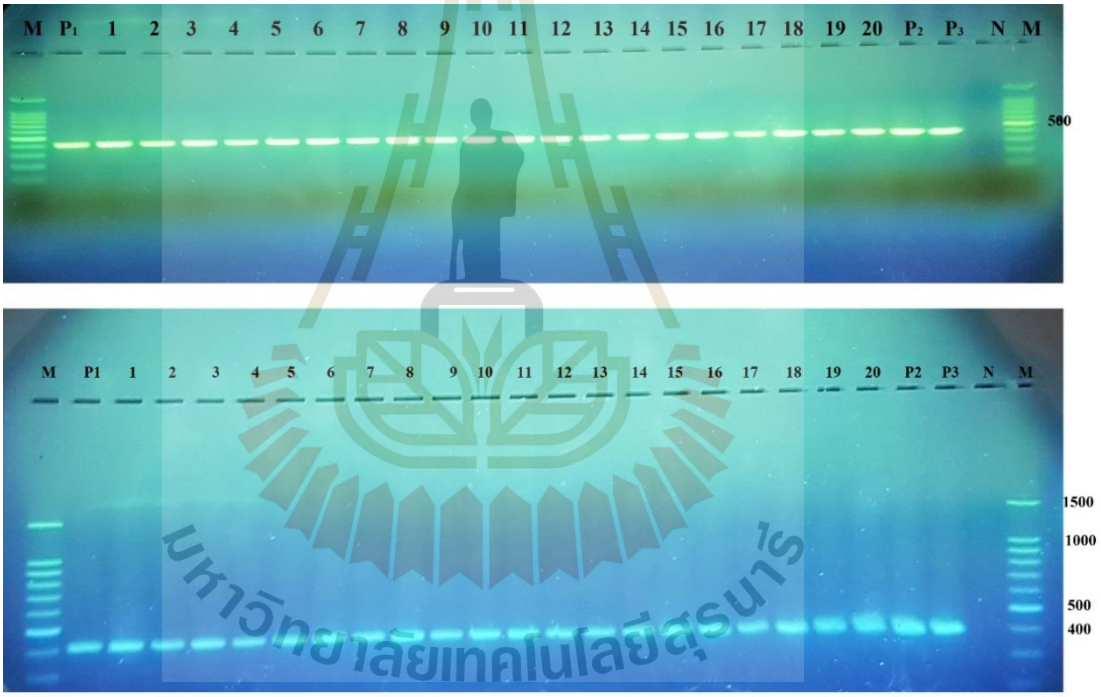


Figure 3.1 Agarose Gel electrophoresis 1- 20 E. coli isolates. P is Positive control. N is Negative control. M is 100bp leader.

In this study, we ran commercial made real-time kits for *E. coli* pathogenic strains. Two kits were run simultaneously that target eight genes, namely VT1 (stx1), VT2(stx2), LT, ST, eaeA, aggR, bfpA, and ipaH. PowerChek Diarrheal *E. coli* 4-plex Real-time PCR Kits I and II (Kogenebiotech, Seoul, Korea) were performed with only

two multiplex real-time PCRs. All 20 *E. coli* DNA samples were run on a Rotor-Gene Q Real-time PCR (QIAGEN, Hilden, Germany.) according to the manufactures protocol. The fluorescent curves of the first kit were analysed relative to their corresponding genes. The VT1 and VT2 genes were read under the FAM and ROX channels, respectively, which are specific to detect the presence or absence of enterohaemorrhagic *E. coli (EHEC).* The same reaction detected the heat stable (ST) and heat labile (LT) toxic producing genes of enterotoxigenic *E. coli (ETEC)* and were read with the HEX and Cy5 channels. All 20 samples showed no amplification whereas the fluorescent curve of the positive control threshold after 17 cycles and the negative control showed no amplification. As shown in (Fig. 3.2)

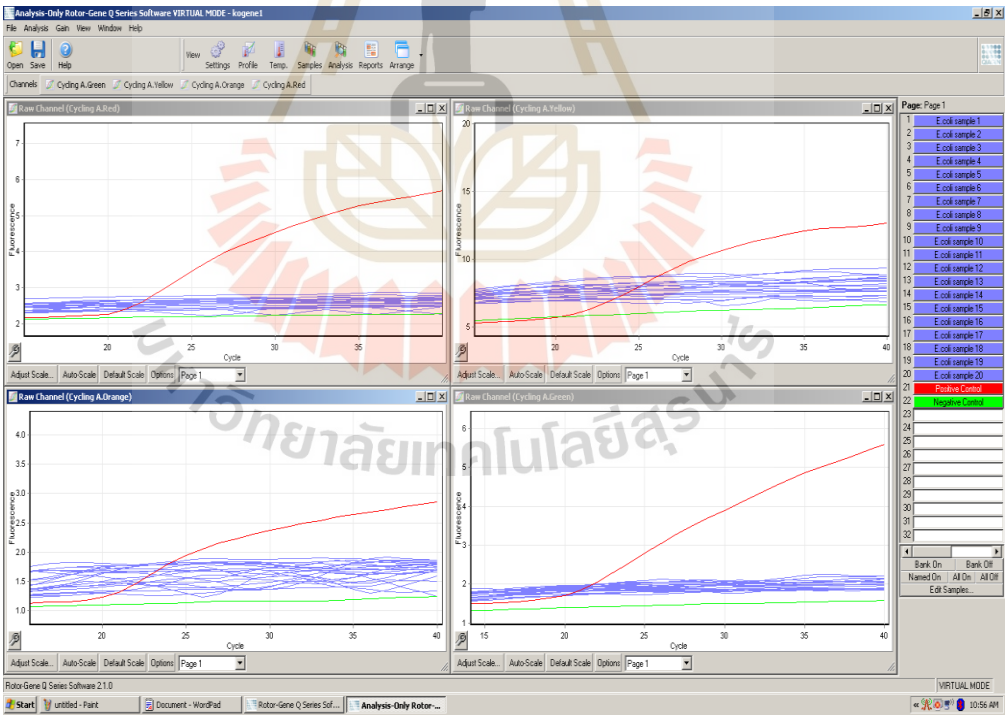


Figure 3.2 Result of E coli isolates 4-plex Real-time PCR Kit I amplifying VT1, VT2, LT and ST genes (Red=positive, green= negative, blue= samples).

Furthermore, the second kit (PowerChek Diarrheal *E coli* 4-plex Real-time PCR Kit II) was carried out to detect the bfpA and eaeA genes for enteropathogenic *E. coli* (EPEC), ipaH gene for enteroinvasive *E. coli (*EIEC*)*, and aggR for enteroaggregative *E. coli (*EAEC). The fluorescent curves were read on VIC, ROX, Cy5, and FAM channels, respectively. All 20 *E. coli* samples were negative for bfpA, eaeA, ipaH, and aggR genes. The positive control amplified well and crossed the thresholder line at 17 cycles. The negative control showed no amplification. As shown in (Fig. 3.3)

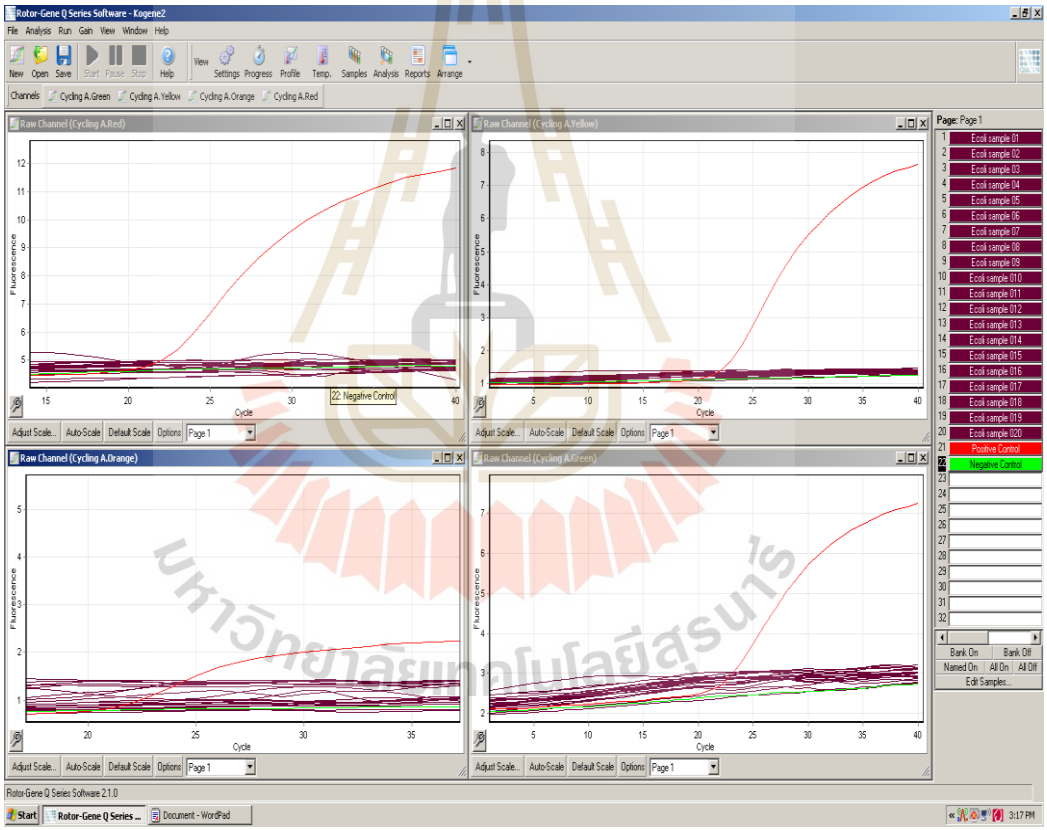


Figure 3.3 Result of *E coli* isolates 4-plex Real-time PCR Kit II amplifying eaeA, aggR, bfpA, and ipaH genes (Red=positive, green= negative, purple= samples).

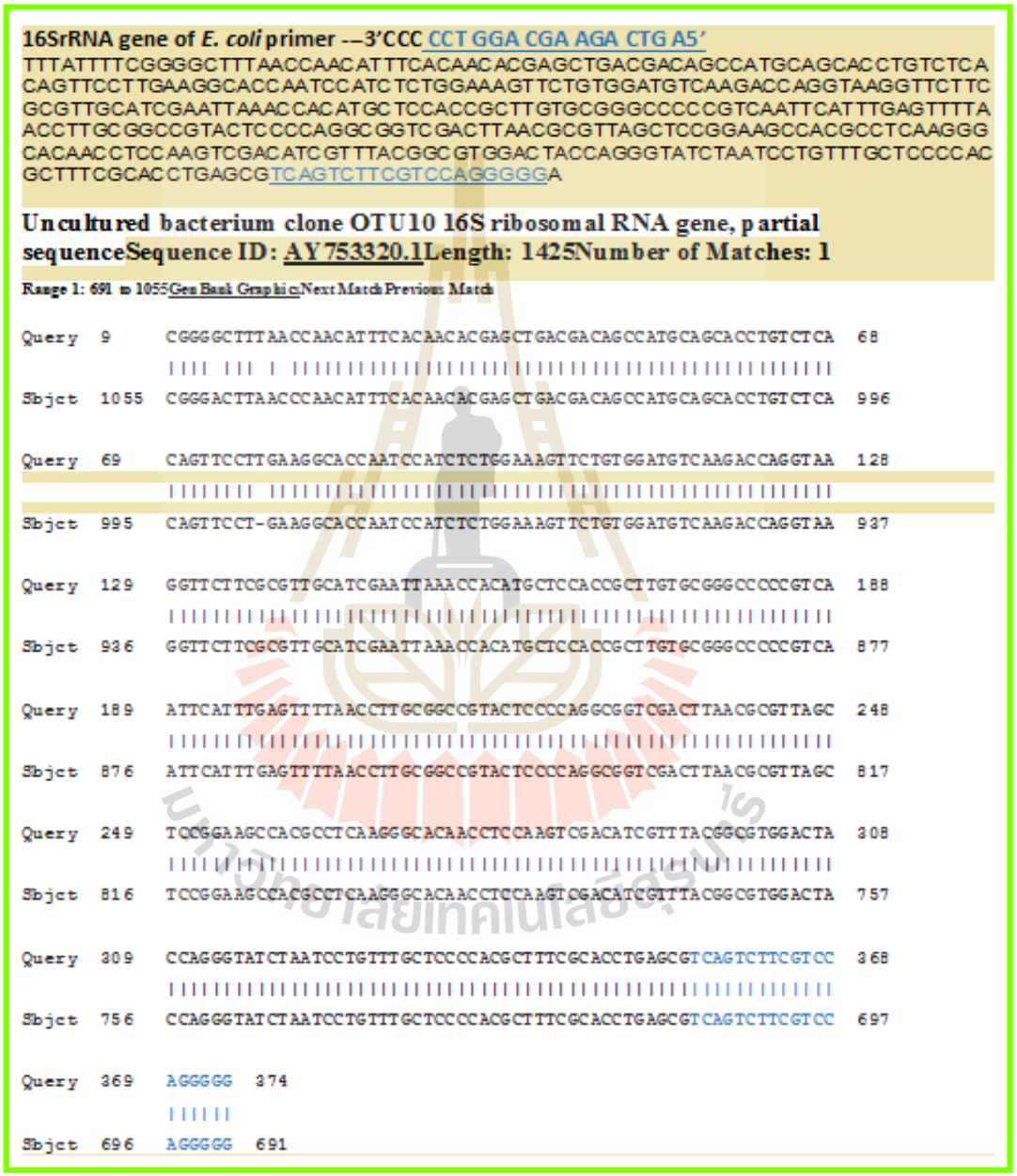
E. coli is a major causative agent for neonatal camel death, which mostly occur in the first week of their lives. Some studies showed that the loss might reach 40% (Wernery and Kaaden, 2002), which result in heavy losses in the camel breading industry. *E. coli* eruption in our breeding centre resulted in high mortality in neonatal camels in 2013. In 2013, we (Advanced Scientific Group ASG) lost about 30% of our newly born racing camels within their first 10 days of life (Anouassi at el unpublished data) diarrhoeal samples mostly show *E. coli* culture growth. Later the postmortem report indicated that colisepticemic or colibacillosis was the cause of death. In this study, we focused on verifying the *E. coli* strains isolated from dead calves. Pure *E. coli* cultures from septicaemic cases were collected postmortem and sub-cultured into MacConkey agar plates. Samples were grown overnight and the DNA was extracted for PCR amplification with 16SrRNA gene primers (Wang et al., 2002; Brandal et al., 2007) to confirm all twenty *E. coli* isolates.

First, the 16SrRNA gene was amplified from the cultures. We then focused on six known pathotype *E. coli* strains, namely enteropathogenic *E. coli*, enterohaemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and diffusely adherent *E. coli* (Kaper, 2004). To outline the causative strain, we used in this study, two commercial multiplex real-time PCR kits for diarrhoeagenic *E. coli* PowerChek Diarrheal *E. coli* 4-plex Real-time PCR Kits I and II (Kogenebiotech, Seoul, Korea). The first kit is 4-plex real-time PCR specific to identify the VT1 (stx1) and VT2 (stx2) genes of enterohaemorrhagic *E. coli*. A phage encoded Shiga toxins (Stx1 and Stx2), the virulence factor that plays a role in the pathogenicity of this strain. They are closely related Shiga toxins but are genetically and immunologically different. Stx2 has higher toxicity than Stx1 (Ho et al., 2013). The kit could detect the

heat labile (LT) and heat stable (ST) toxin genes of enterotoxigenic *E. coli*, which are encoded by the same or different plasmids (Begum et al., 2016). Four genes (Stx1, Stx2, LT and ST) were run with real-time PCR and analysed but none of them were amplified in the 20 samples. Simultaneously, the second 4-plex was run to detection the bfpA and eaeA plasmid encoded genes of enteropathogenic *E. coli* (Hu and Torres, 2015). The ipaH chromosomal or plasmid encoded enteroinvasive *E. coli* (EIEC) genes (Venkatesan et al., 1989) at the last aggR gene of enteroaggregative *E. coli*. None of the 20 analysed samples produced an amplification curve, which excluded all pathogenic strains of *E. coli* except the diffusely adherent *E. coli* strains not included in this kit. However, to rule out this strain and to discover whether any other possible strains might cause death. Sequencing of the 16S rRNA gene of 20 *E. coli* isolates was performed then compared and aligned with the GenBank sequencing data. Amplicons (400 bp) were used as a query to search for the homologous sequence in GenBank using Nucleotide BLAST (Basic Local Alignment Search Tool) in the GenBank database (Zhang et al., 2000).

The blast results showed no homogony with any of the six *E. coli* pathogenic strains in the GenBank database. Further searches were performed with Blast for three of the isolates. Isolates 5, 7, and 13 were 99% matched with the coliform uncultured bacterium clone OTU10 16S ribosomal RNA gene and a partial sequence was isolated from giant panda faeces (*Ailuropoda melanoleuca*) GenBank: AY753320.1.(Wei et al., 2007) in Shanghai China (Fig. 3.4). Another case was a 99% BLAST match with *E. coli* isolate 2 and uncultured bacterium clone 16S ribosomal RNA gene, which was partially sequenced and submitted by Oh (2013) GenBank: KF109400 from the skin of patients with primary immunodeficiencies (PID) as shown in (Fig. 3.5) (Oh et al.,

2013). These results were similar to our isolates. However, *E. coli* might result in the death of neonatal camels with immature immunity if they do not get good colostrum and a hygienic environment.



16SrRNA gene of *E. coli* primer ---3'CCC CCT GGA CGA AGA CTG A5'
TTTATTTTCGGGGCTTTAACCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCA
CAGTTCCTTGAAGGCACCAATCCATCTCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTC
GCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTA
ACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGG
CACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC
GCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGA

Uncultured bacterium clone OTU10 16S ribosomal RNA gene, partial sequence Sequence ID: AY753320.1 Length: 1425 Number of Matches: 1

Range 1: 691 to 1055 GenBank Graphics Next Match Previous Match

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Query  9     CGGGGCTTTAACCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCA  68
             |||| ||| | ||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  69    CAGTTCCTTGAAGGCACCAATCCATCTCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAA  128
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  995   CAGTTCCT-GAAGGCACCAATCCATCTCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAA  937

Query  129   GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA  188
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Sbjct  936   GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA  877

Query  189   ATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGC  248
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Sbjct  876   ATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGC  817

Query  249   TCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTA  308
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  816   TCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTA  757

Query  309   CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCC  368
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Sbjct  756   CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCC  697

Query  369   AGGGGG  374
             ||||||
Sbjct  696   AGGGGG  691
```

Figure 3.4 GenBank blast results for the fifth *E. coli* isolate similar to *E. coli* isolated from giant panda faeces (Ailuropoda melanoleuca) GenBank: AY753320.1.

CGGGAGGGGGGGGGGAAAATTGGGGGGGGGGGTGTGGGGGGTTTGGGATTCCACATTTAAACCGAG
CTGACGACGCCATGCGCACCTGTCTCACCGTTTCCGAAGGTACATTCTCATCTCTGAAAACTTCCGTG
GATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCG
GGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGT
TATCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGAAGT

Uncultured bacterium clone nck330c03c1 16S ribosomal RNA gene, partial sequence
Sequence ID: KF109400.1 Length: 1363 Number of Matches: 1
Range 1: 708 to 1058 GenBank Graphics Next Match Previous Match

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Query  52    ACATTT-A-AAC-CGAGCTGACGAC-GCCATGC-GCACCTGTCTCACCGTTTCCGAAGGT  106
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Sbjct  1058  ACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACGGTTTCCGAAGGC  999

Query  107   ACATTCTCATCTCTGAAAACTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGC  166
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  998   ACATTCTCATCTCTGAAAACTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGC  939

Query  167   ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTT  226
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  938   ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTT  879

Query  227   AACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTATCTCCGGAAGCCACGCC  286
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Sbjct  878   AACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCC  819

Query  287   TCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC  346
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Sbjct  818   TCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATC  759

Query  347   CTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGG  397
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758   CTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGG  708
```

Figure 3.5 GenBank blast results for the fifth *E. coli* isolates showing similarity with *E. coli* isolated from the skin of patients with primary immunodeficiencies.

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CHAPTER IV

GENERATION OF A SINGLE-DOMAIN ANTIBODY (sdAb) AGAINST ISOLATED *E. coli*

4.1 Abstract

Antibodies and antibody fragments, especially nanobodies, are important tools in diagnostics, research, and therapeutics involving conventional antibodies where the light and heavy chains contribute to the antigen binding site. Furthermore, the old and new world camels have conventional and heavy chain antibodies (hcAbs) and lack the light chain antibodies that unusually bind to the antigen with a single domain, the VHH domain, which characterises the smallest antigen binding fragments that also have high solubility, stability, and specificity.

In this thesis we aimed to produce a VHHs library against *E.coli* Lipopolysaccharide using the camel immune system. Principally, we isolated *E. coli* strains from dead camel calves to extract the lipopolysaccharide and immunise 5-year-old female camels after we isolated lymphocytes for RNA extraction and amplification of the VHH gene, we cloned the PCR product into the pF1AT7 Flexi vector (Promega Cat No C8441) and transformed by heat shock into JM109 *E. coli* competent cells (Promega, USA), resulting in a comprehensive VHH library with 6.9 x 10^4 cfu/µg. Furthermore, we expressed the VHH and screened with ELISA and PCR. Eleven colonies were positive by PCR, six of which were sequenced and compared with

GenBank data to confirm the production of nanobodies with similarity greater than 90%. Finally, SDS-PAGE was performed as an additional confirmation.

4.2 Introduction

Antibodies are essential tools in diagnostics, basic research, and therapy. The conventional antibody (immunoglobulin Ig) is a large Y-shaped glycoproteins (Maverakis et al., 2015) with a molecular weight of 150 kDa mainly produced in plasma cells they are used in research experiments and medical applications. Normally they are classified according to their heavy chain isotypes into IgG, IgM, IgE, IgA, and IgD, which vary in both structural and function. These antibodies contain two heavy and two light chains with both chains contributing to the antigen-binding site. Along with these conventional antibodies, camelid species possess a heavy chain antibody (hcAbs) that lacks light chains. This single N-terminal domain can bind antigens without other domain (Riechmann and Muyldermans, 1999). Unlike conventional antibodies the antigen binding site of the heavy chain antibody (camelid antibodies) is as it is formed by a single domain that can bind antigens with a single heavy chain, the so called VHH domain. Research has focused on the camelid antibody in the recent past, as it demonstrate a powerful potential immuno-therapeutic, diagnostic, and biosensor. This biosensor function is particularly useful for targeting cancer cells, inflammatory response, autoimmune diseases, and viruses. Overall, the camel nanobody has a high specific antigen binding capacity that provides key defences against pathogenic organisms and toxins (Nguyen et al., 2002). The VHH domain characterises the smallest antigen binding fragment (~15 kDa) with high stability, solubility, and specificity. In this chapter, we aimed to generate and characterise VHHs

suitable for *E. coli* lipopolysaccharide. We used the immune system of *dromedary camel* to generate VHH libraries against *E. coli* lipopolysaccharide. The libraries were screened with specific VHH primers and sequenced. as Additionally, ELISAs were performed for antigen specific VHHs.

4.3 Methods

4.3.1 Molecular identification of *E. coli*

4.3.1.1 Preparation of *E. coli* lipopolysaccharide

LPS extraction kit was used to get pure lipopolysaccharide (Intron biotechnology Korea).

1) *E. coli* samples were cultured on LB broth and incubated overnight at 37ºC.

2) Culture suspension (1 mL) was placed in a 1.5 mL tube then centrifuged for approximately 30 sec at 10,000 G. and the supernatant was then removed.

3) Lysis buffer (1 ml) was added to the samples and they were vortexed vigorously until the cell clump dissolved.

4) Chloroform (200 µL) was added and the samples were vortexed vigorously for 10-20 sec. Then, the samples were incubated at RT for 5 min.

5) The mixture was then centrifuged at 13,000 rpm for 10 min at 4ºC and 400 µL of the supernatant was transferred to a new 1.5 mL tube.

6) Purification buffer (800 µL) was added and the samples were mixed well. Then, they were incubated for 10 min at -20ºC .

7) The samples were then centrifuged at 13,000 rpm for 15 min at 4ºC.

8) The resulting LPS pellets were washed with 1 mL of 70% Ethanol and dried completely at room temperature.

9) Tris-HCl buffer (70 µL at 10 mM and pH 8.0) was added to the LPS pellet and the pellet was dissolved by boiling for 1 min.

10) The extracted LPS yields of approximately 30 µg were obtained from the pellet of 2 mL of culture according to the kit.

4.3.1.2 Camel immunization

Two-year-old female camels (*Camelus dromedaries*) were used in this experiment. To start the experiment, we separated the experimental camel with other 5 camels to overcome the stress that may cause immunosuppression in the camel (Pardon et al., 2014). This was performed with the help of experienced veterinary technicians. Then, 50 mL of blood sample was collected before giving animal the *E. coli* LPS (immunogen) and the blood was allowed to clot for two hours at room temperature. Clotted blood was centrifuged for 5 min at 3,000 x*g*. Then, the supernatant (serum) was stored at -20°C and used as the ELISA negative control to compare the titre of the pre-immune serum and post-immune plasma when measuring the serum alteration induced by each antigen. Serum was made by four-fold serial dilutions.

The camel was immunised six times with the freshly prepared LPS (from Step 4) at two-week intervals. Each immunization included six 0.2 mL intradermal injections of *E. coli* LPS (1 mg/mL) (Li et al., 2012). The LPS was gently mixed with an equal volume of complete Freund's adjuvant (Santa Cruz Biotechnology

USA) to make an emulsion, then injected intradermally. The first, second, third, and fourth immunizations were performed in the camel neck base, near the bow lymph node. The booster doses of fifth and sixth LPS immunizations were emulsified with incomplete Freund's adjuvant in the pre-scapular region (Koch-Nolte et al., 2007).

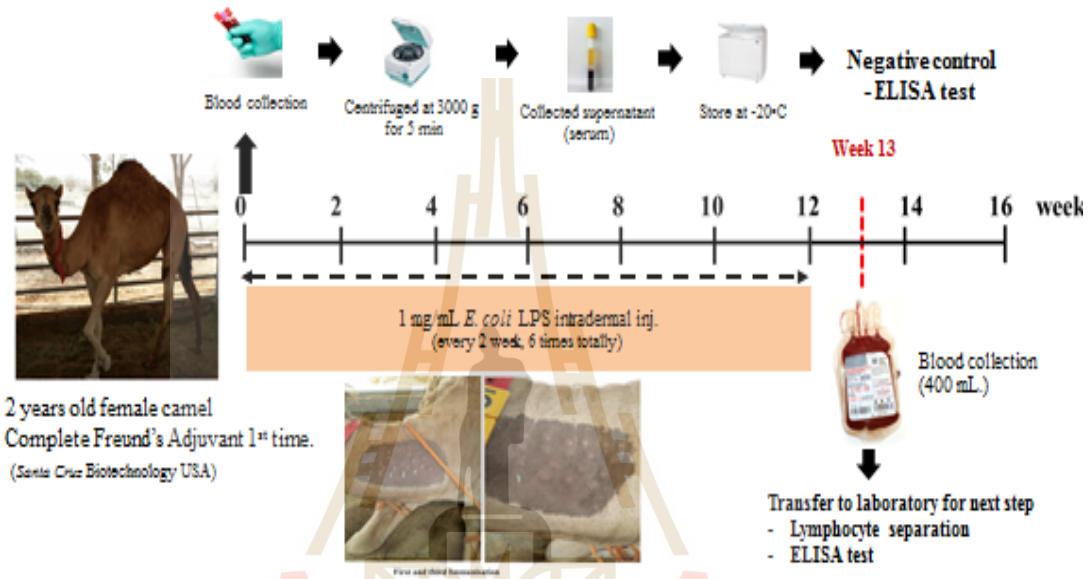

Figure 4.1 immunization schedule.

One week after completion of a 70 day immunization regimen, 400 mL of peripheral blood was collected, with sodium citrate as an anticoagulant, then immediately transferred to the lab for assessment.

4.3.1.3 ELISA Procedure

1) Microtiter plate wells were coated with 100 µL of the *E. coli* lipopolysaccharide antigen at a concentration of 10 µg/mL in a coating buffer.

2) The plate wells were covered and incubated four hours at room temperature and then overnight at 4°C.

3) The plate wells were washed with 200 µL of washing buffer three times. As a final step, the plates were taped on paper towels to remove excess washing buffer.

4) All wells were blocked with 200 µL of blocking solution. Then, the plates were sealed and incubated for 2 hours at room temperature on a rocking platform and washed three times with 200 µL of washing buffer.

5) Three different dilutions of serum samples (1:100, 1:500, and 1:1000) were prepared and 200 µL of each were added and incubate for 2 hours at room temperature.

6) The plates were washed three times with 200 µL of washing buffer.

7) Then 100 µL of HRD Rabbit Anti-Camel IgG was added to the plates, at a concentration of 300 ng/mL, and incubated for one hour.

8) The plates were washed three times with 200 µL of washing buffer.

9) The plates were filled with 100 µL/well of substrate solution and incubated at room temperature for 20 min.

10) After colour production the reaction 100 µL of 0.5 M sulphuric acid was added to each well and the microplate was read with a spectrophotometer at 450 nm.

4.3.1.4 Lymphocyte separation (Lymphoprep™ solution)

1) Collected blood (20 mL) was diluted with an equal volume of 0.9% NaCl in a 50 mL tube.

2) Diluted blood (6 mL) was carefully layer over 3 mL of Lymphoprep™ solution in a 15 mL centrifuge tube. Then, the tubes were caped to prevent the formation of aerosols.

Figure 4.2 Lymphocyte separation (up slide) before and (down slide) after Lymphoprep™ solution.

3) Tubes were centrifuge at 800 xg for 20 minutes at room temperature. The mononuclear cells were removed from the distinct band at the sample/medium interface using a Pasteur pipette, without removing the upper layer. Lymphocyte cells were then diluted with 0.9% NaCl to reduce the density of the solution in the 2 mL tube and centrifuged for 10 minutes at 250 xg. Then, the pellet was stored at -20ºC.

4.3.1.5 RNA extraction

RNA were extracted using TRIZOL® Reagent

4.3.1.5.1 Phase separation

1) The frozen pellets were incubated for 5 minutes at room temperature.

2) Chloroform (0.2 mL) and TRIZOL Reagent (1 mL) were added and tubes were caped securely.

3) The mixture was homogenised by shaking the tubes vigorously for 15 seconds. Then, homogenised samples were incubated for 5 minutes at room temperature.

4) The samples were centrifuged at 12,000 ×g for 15 minutes at 4°C until the mixture separated into the lower red, phenolchloroform phase, interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.

5) The upper aqueous phase of the sample was carefully removed by angling the tube at 45°C and pipetting the solution out without disturbing the interphase. The aqueous phase was moved into a fresh 1.5 mL tube.

4.3.1.5.2 RNA preparation and wash

1) The aqueous phase of RNA was precipitated by mixing with 0.5 mL of 100% isopropyl alcohol and incubation at room temperature for 10 minutes. Then, centrifugation at 12,000 xg for 10 minutes at 4°C.

2) The supernatants were completely removed and 1 mL of 75% ethanol was added into the samples, which were mixed by vortexing and centrifuged at 7,500 g for 5 minutes at 4°C.

3) The above washing procedure was repeated once.

4) The samples were air-dried for 10 minutes at room temperature and dissolved in DEPC-treated water by passing the solution through a pipette tip a few times.

5) The samples were then quantified by measuring OD at 260 nm. Approximately 20 µg of RNA were obtained.

4.3.1.6 cDNA synthesis

cDNA were synthesized using GoScript™ Reverse Transcription System (promega USA)

1) The GoScript™ Reverse Transcriptase, GoScript™ 5X Reaction Buffer, and PCR Nucleotide Mix were thawed on ice before use for 10 min.

2) Each component was mixed and briefly centrifuged before use and the following components were employed:

Experimental RNA (up to 5 µg/reaction)	
Primer [Oligo(dT)] (0.5 µg/reaction)	2 µL
Random Primer (0.5 µg/reaction)	2 µL
Nuclease-Free Water	1 µL

Final volume	5 µL

3) Each tube of RNA was closed tightly and placed into a preheated 70°C heat block for 5 minutes. Samples were immediately chilled in ice-water for 5 minutes. Then, samples were centrifuged for 10 seconds and kept on ice until the reverse transcription reaction mix was added.

4) The reverse transcription reaction mix was prepared by combining the following:

Nuclease-Free Water	5.0 µL
GoScript™ 5X Reaction Buffer	4.0 µL
$MgCl_2$ (final concentration 2.5 mM)	4.0 µL
PCR Nucleotide Mix	1.0 µL
GoScript™ Reverse Transcriptase	1.0 µL
Final volume	15.0 µL

5) Aliquots (15 µL) of the reverse transcription reaction mix were added carefully to the reaction tube on ice.

6) RNA and primer mix (5 µL each) were added to the reaction for a final reaction volume of 20 µL in tube.

7) Annealing: Tubes were placed in a temperature-controlled heat block equilibrated at 25°C and incubated for 5 minutes.

8) The tubes were incubated on a temperature-controlled heat block at 42°C for one hour.

9) The extension temperature may be optimised between 37°C and 55°C.

10) To inactivate reverse transcriptase, the reaction tubes were incubated in a temperature-controlled heat block at 70°C for 15 minutes.

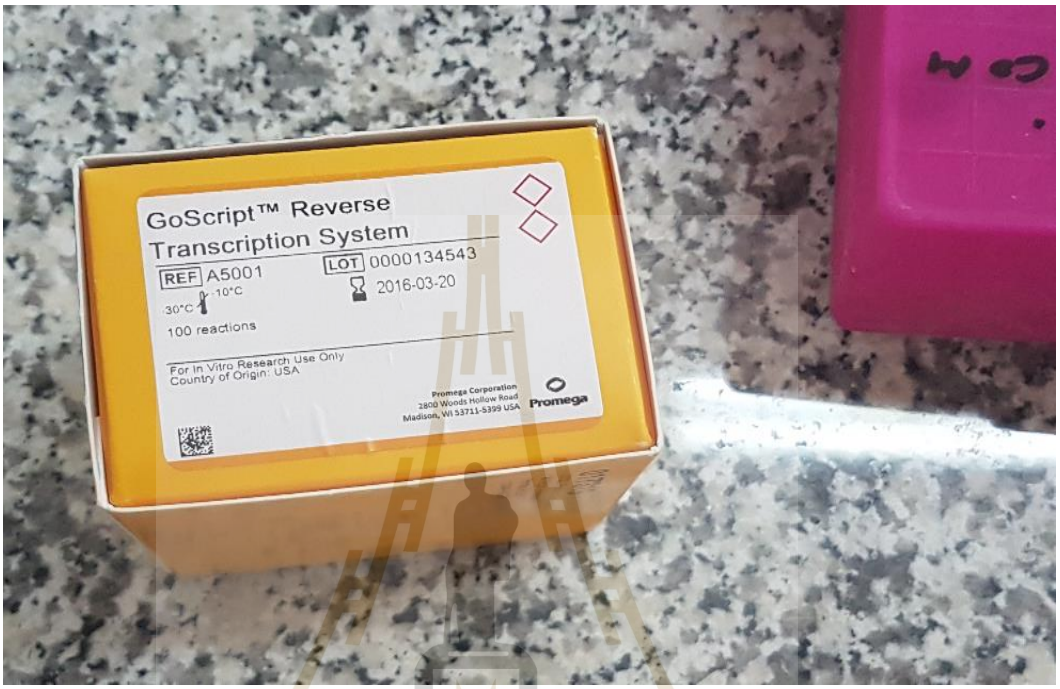

Figure 4.3 GoScript RT system kit used in this study.

4.3.2 Construction of library

The variable domains of heavy chains (VHs and VHHs) were amplified from the cDNA with two modified gene-specific primers, CALL001 and CALL002 (Vincke et al., 2012). CALL001: 5′-GTCCTGGCTGCTCTTCTACAAGG-3′; CALL002: 5′-GGTACGTG CTGTTGAACTGTTCC-3′; VHH-Rev: 5′- CAA ATT TG--GATGTGCAGCTGCAGGAGTCT GGRGGAGG-3′ (PmeI); VHH-For: 5′-CTAGTGCGGCCGCTGGAGACGGTGACCTGGGT--GCG ATC GCC ATG-3′ (SgfI) (Vincke et al., 2012; Pardon et al., 2014). The nested primers were added to

these customised primers that were made by Alpha DNA (Canada) and constituted in 10 mM Tris-HCl buffer (pH 8.5) to make 100 µM stock solutions.

4.3.2.1 First PCR

1. PCR reactions (8) were performed, each reaction in a total volume of 25 µL of reaction mixture containing dNTPs mix (0.4 µM), CALL001 and CALL002 primers (0.4 mM each), GoTaq DNA polymerase (1 U) (Promega Madison, WI USA), PCR buffer supplied by manufacturer, and 2 µL of first-strand cDNA reaction.

2. The reaction was incubated at 95°C for 5 min in a thermal cycler to activate the enzyme and denature the DNA.

3. Thirty-two PCR cycles were performed, each cycle consisting of 45 s of denaturation at 94°C, 45 s of annealing at 55°C, and 45 s of extension at 72°C. After 32 cycles, the final extension step was performed for 10 min at 72°C. Then, all PCR reactions were pooled.

4. The pooled reactions (8 µL) were used for agarose gel analytics. Then, PCR products were analysed by gel electrophoresis with 10 µL of sample in a 2% agarose gel prepared with 5 µL of 20.000X RedSafe nucleic acid staining solution (iNtRON Biotechnolgy, Korea) and electrophoresed at 90 V for 40 minutes in 1X TAE buffer (0.04 M Tris/acetate, EDTA 1 mM). A100 bp ladder (Promega, Madison, USA) was used as the standard marker. Then, the gel was visualised on a UV transilluminator and photographed.

5. PCR reactions (200-300 µL) were loaded onto a 1.5% agarose gel (RedSafe nucleic acid staining solution) and the gel was run until the PCR products were well separated. The gel was put on a UV trans-illuminator and PCR

bands matching the VHHs bands, those lower than 600 bp were cut out of the gel with a sterile scalpel.

6. The DNA fragments from agarose were purified using the Agarose Gel DNA Extraction Kit (Roche Cat No 11696505001). The DNA was eluted in elution buffer and measured by Nanodrop spectrophotometry.

Table 4.1 PCR mixture of CALL primers of Camel immunoglobulin.

Component	Volume (ul)
cDNA	2
MgCl2 (25mM)	1.8
5x buffer	5
CALL for (10pmol)	2
CALL rev (10pmol)	2
dNTPs (10 mM each)	0.8
GoTaq DNA polymerase	0.2

4.3.2.2 DNA Extraction from agarose gel

1) Electrophoresis was ran with the PCR product on an agarose gel in TAE buffer for 45 minutes.

2) With a sharp scalpel, the DNA band was removed from the gel, taking only the band and agarose around the band.

3) The excised gel slice was weighed in a pre-weighed 1.5 mL microfuge tube and 300 µL of Agarose Solubilization Buffer was added for each 100 mg of agarose gel in the slice in the tube.

4) Homogenised silica suspension (10 µL) was added to the tube containing the slice, and then the mixture was incubated for 10 min at 56-60°C and vortexed every 2-3 min.

5) The tubes were centrifuged in a tabletop centrifuge at maximum speed for 30 seconds and the supernatant were discarded

6) Nucleic acid binding buffer (500 µL) was added to the silica suspension, the tubes were centrifuged at the maximum speed for 30 seconds, and the supernatant was discarded.

7) The pellet was washed with 500 µL washing buffer solution and centrifuged at the maximum speed for 30 seconds. The tube and the suspension were discarded as in the previous step.

8) All the liquid was removed with a pipette and these tubes were inverted on an adsorbent tissue to allow the pellet to dry at room temperature for 15 minutes.

9) The dried pellets were eluted with 40 µL of TE buffer (pH 8.0), resuspend by vortexing, and incubated for 10 minutes at 15 to 25°C. The extracted DNA was transferred to a new tube.

4.3.2.3 Second PCR

The second PCR were amplified with modification of the original sequencing and endonuclease restriction sites introduced using flowing protocols.

1) PCR reactions (24) were performed, each in a total volume of 25 µL. Each 25 µL reaction contains dNTPs mix (0.2 mM each), VHH-Rev, and VHH-For primers (0.4 mM each), GoTaq DNA polymerase (1 U) (Promega Madison,

WI USA), PCR buffer supplied by manufacturer, and 10-50 ng of purified first PCR products obtained previously.

2) The reaction was incubated at 95°C for 5 min in a thermal cycler in order to activate the enzyme and denature the DNA.

3) PCR cycles (20) were performed; each cycle consisting of 45 s of denaturation at 94°C 45 s of annealing at 55°C and 45 s of extension at 72°C followed by final extension step for 10 min at 72°C.

4) All PCR reactions were polled and 8 µL of the pooled sample were analysed for gel electrophoresis on a 2% agarose gel in 1x TBE buffer with DNA 100 bp ladder (Promega) as DNA molecular weight marker.

Table 4.2 Second PCR reaction set of VHH gene for construction of the nanobody library.

Component	**Volume (ul)**
cDNA	2
MgCl2 (25mM)	1.8
5x buffer	5
VHH for (10pmol)	2
VHH rev (10pmol)	2
dNTPs (10 mM each)	0.8
GoTaq DNA polymerase	0.2
water	11.2
Total	**25**

5) After getting clear band of about 400 bp, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and DNA was eluted in elution buffer. The concentration was measured by NanoDrop spectrophotometry.

4.3.2.4 MEGAquick–spin Total Fragment DNA Purification Kit Protocol

1) Buffer PB (500 µL) was added into 100 µL PCR sample and mixed well till the colour of mixture turned yellow.

2) Maxture were transferred into the QIAquick column in a 2 mL collection tube and centrifuged for 60 s at the maximum speed.

3) The flow-through was discarded and placed the QIAquick column back into the same tube.

4) Buffer PE (750 µL) was added to the QIAquick column and centrifuged for 60 s at maximum speed. Then, the flow-through was discarded and the QIAquick column was placed back in the same tube. The column was centrifuged for an additional 1 min.

5) A QIAquick column was placed in a clean 1.5 mL microcentrifuge tube and DNA was eluted with 50 µL of elution Buffer.

4.3.2.5 Restriction digest

Restriction digest of second PCR product and acceptor Flexi® Vector

Digestion reactions for the PCR product and the acceptor Flexi® vector were performed simultaneously. The 5X Flexi® digest buffer, the acceptor Flexi® vector and nuclease free water were thawed vortexed and then stored on ice

before use. The following reaction mix was used to cut the PCR product with SgfI and PmeI.

Table 4.3 SgfI & PmeI Enzyme Digestion with PCR product.

Components	**Volume**
5X Flexi® Digest Buffer	4µl
Purified PCR product	8µl
Flexi® Enzyme Blend (SgfI & PmeI)	4µl
Nuclease-Free Water	4µl
Total	20µl

Table 4.4 SgfI & PmeI enzyme digestion with acceptor Flexi® vector.

Components	**Volume**
5X Flexi® Digest Buffer	4µl
acceptor Flexi® Vector (200ng)	2µl
Flexi® Enzyme Blend (SgfI & PmeI)	2µl
Nuclease-Free Water	12µl
Total	20µl

Both the reactions were incubated at 37°C for 30 minutes and then heated at 65°C for 20 minutes to inactivate the restriction enzymes. Then, digested products were purified using the QIAquick PCR purification kit (Qiagen) and stored on ice.

4.3.2.6 DNA Ligation

Ligation of PCR product and acceptor Flexi® vector. The following reaction components were assembled for ligation of the digested PCR product and digested acceptor Flexi® vector.

Table 4.5 DNA ligation between acceptor Flexi® Vector and PCR product.

Components	**Volume**
2X Flexi® Ligase Buffer	10µl
Digested acceptor Flexi® Vector (50ng)	5µl
Digested PCR product (100ng)	3µl
T4 DNA Ligase (HC) (20u/µl)	1µl
Nuclease-Free Water	1µl
Total	**20µl**

The reaction mixture was incubated at room temperature for 1 hour then processed for transformation.

4.3.2.7 Transformation

The ligation products were transformed into high-efficiency *E. coli* competent cells, specifically JM109 competent cells (Promega), and selection for transformants was carried out on LB plates supplemented with 100 µg/mL ampicillin suitable for the acceptor pF1AT7 Flexi® vector with the letter A in the name, indicating that it contains the ampicillin resistance gene, were used for this transformation protocol.

1) 10 plates were equilibrated for transformation at room temperature.

2) The tubes containing the ligation reactions were quick-centrifuged to collect the pellet and 2 µL of each ligation reaction was added to a sterile 1.5 mL microcentrifuge tube in an ice bath.

3) One tube of frozen high-efficiency JM109 competent cells was removed from -80°C storage and placed in an ice bath for 5 minutes until just thawed and the cells were mixed gently flicking the tube.

4) Carefully 50 µL of cells was transferred into 1.5 mL tube and gently flicked the tubes to mix and placed them on ice for 20 minutes.

5) The cells were heat shocked for 50 seconds in a water bath at 42°C without shaking, and then immediately transferred back to ice bath for 2 minutes.

6) Room-temperature LB broth (950 µL) was added to the tube and incubated for 1.5 hours at 37°C on a shaking rack at 150 rpm.

7) Transformants (50 µL) were plated onto duplicate plates of LB agar containing 100 µg/mL ampicillin to screen for the acceptor Flexi® vector.

8) The plates were incubated overnight (16-24 hours) at 37°C.

4.3.2.8 Screening of the culture by PCR

1) The cultured plates were acquired after 24 h and 48 colonies were randomly selected.

2) The colonies were divided into two groups, 48 each, and subcultured in LB broth in a 96 well plate overnight.

3) Plasmid DNA extraction was carried out using the plasmid DNA extraction kit (Intron, Korea).

4) DNA was digested with SgfI and PmeI enzymes and PCR was performed using VHH primers.

4.3.3 Protein Expression

1) Each colony were transferred to 10 ml of media and incubate at 37°C (shaking) for 3 to 5 hours.

2) Once the culture has reached 0.6 OD one ml of culture were taken and centrifuge down at max speed for 5 min. in a 1.5ml tube.

3) The supernatant were discarded and expanded the culture by adding the 10 ml of LB with Ampicillin antibiotic (room temp) and incubate for 3 hours at 37°C (shaking)

4) The culture were induced by adding IPTG to a final concentration of 0.5 mM

5) The induced culture were kept for 4 hours at 37°C with shaking.

6) The cells were transferred 15 ml tube and centrifuged at 3,500 x g for 20 min.

7) The supernatant were discarded and resuspend cells in ICE COLD PBS and re-centrifuged at 3,500 x g for 20 min

8) The supernatant were removed and the protein were extracted using Smart Bacterial Protein extraction kit (Intron Korea) as well as Periplasmic protein extraction method according manufacturer's protocol.as well as manual periplasmic extraction using Cold Osmotic Shock method as following.

4.3.4 Periplasmic extraction

The other 48 subcultures were re-subcultured in 10 mL LB broth, after an overnight incubation, the culture was centrifuged and the plates were used for periplasmic protein extraction.

4.3.4.1 Cold Osmotic Shock

1) Release of periplasmic protein fraction from *E. coli* by cold osmotic shock was done using the modified protocol of Neu & Heppel, 1965.

2) *E. coli* cell suspension was centrifuged for 5 min at 14,000 xg and 4°C to collect the cells

3) The entire supernatant was discarded.

4) The cells were resuspended in ice-cold Cell Fractioning Buffer 1.

5) The cells were incubated for 20 min on ice. The suspension was inverted at regular intervals to counteract sedimentation.

6) The cell suspension was centrifuged for 15 min at 14,000 xg and 4 °C.

7) The entire supernatant was discarded.

8) The cells were resuspended in ice-cold Cell Fractioning Buffer 2.

9) Then, the cells were incubated for 20 min on ice with regular inversion.

10) The cell suspension was centrifuged for 15 min at 14,000 xg and 4°C.

11) The supernatant was retained and contained the periplasmic proteins and membrane.

4.3.5 SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophorese, is the most commonly used technique to separate proteins in a complex mixture. In the presence of electricity, proteins travel towards the negative anode inside the poly-acrylamide gel under denaturing conditions. In SDS-PAGE, the detergent SDS and heating regulate the electrophoretic mobility of a protein, affected by its molecular weight, in the porous acrylamide gel. Preparation of the SDS PAGE electrophoresis system are consisted of:

1. Tank
2. Lid with power cables
3. Electrode assembly
4. Cell buffer dam
5. Casting stands
6. Casting frames
7. Combs (usually 10-well or 15-well
8. Glass plates (thickness 0.75mm or 1.0mm or 1.5mm)

The SDS PAGE gel in a single electrophoresis run can be divided into the stacking gel and resolving gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted into the stacking gel. The acrylamide percentage in an SDS PAGE gel depends on the size of the target protein in the sample, as shown below, and Ammonium Persulfate and TEMED were added right before each use. Separating gel was prepared as follows:

1. Casting frames was placed (clamp two glass plates in the casting frames) on the casting stands.

2. The gel solution was prepared (as described above) in a small beaker.

3. The appropriate amount of separating gel solution was pipetted (listed above) into the gap between the glass plates.

4. The top of the separating gel was filled in water into until it overflowed.

5. The gel was left for 20-30 min until it solidified.

Stacking gel follow as

1. Water was discarded from the top of the form.

2. The stacking gel was pipetted until it overflowed out of the form.

3. The well-forming comb was inserted, without trapping air under the teeth.

4. The gel was left for 40 min until it solidified.

5. The set of glass plates was taken out of the casting frame and placed in the cell buffer dam.

6. The running buffer was poured (electrophoresis buffer) into the inner chamber until the buffer surface reached the required level for the outer chamber.

4.3.5.1 SDS-PAGE to analyze the expression:

A 15 % polyacrylamide SDS gel was prepared using Intron polyacrylamide. Then, 18 µL of prepared periplasmic fractions or Bacterial protein extract was added to 6 µL of 4x SDS-PAGE loading buffer. The samples were boiled with a low molecular weight marker for 10 min at 95°C and spun down at 14000 rpm for 5 min. They 15 µL of each sample was loaded into the SDS-PAGE and run at 90 V for 15 min and 200 V for 40 min. The gel was stained with SenSafe™ Fast Protein Staining Solution (31194, Intron, Korea)

1. Samples (100 µL) were mixed with 20 µL of loading buffer (Intron Biotechnology, Korea).
2. The mixture was heated at 100ºC for 10 min.
3. The samples were centrifuged at 14000 rpm for 10 min.
4. The supernatant was loaded into the wells.
5. Protein Marker was loaded into the last well.
6. The tank was covered and the anodes connected.
7. The voltage was set for 90 V for 15 min then 200 V for 40 min.

4.4 Results and Discussion

These results identify antigen specific VHHs. The first step was to generate antigen specific binding molecules, so a female camel was immunised with an *E. coli* lipopolysaccharide antigen isolated from a dead neonatal camel. Subsequently, the VHH repertoire was amplified with nested PCR and then cloned into the pF1AT7 Flexi vector (Promega Cat No C8441), resulting in a comprehensive VHH library. To screen for antigen specific VHHs, ELISA were performed, resulting in the selection of antigen specific VHHs. Additionally, PCR was screened using VHH specific primers and then sequenced.

4.4.1 Selection of antigen

The most significant steps in generating antigen specific VHHs is the immune response after immunization. Therefore, we used *E. coli* lipopolysaccharide. Throughout the immunization period the camel received weekly intradermal injections. On the bleeding day, 300-400 mL blood was collected from the immunised camel. To screen the immune response of the camel, an ELISA assay was performed on the

immobilised antigen using serum samples from day 0 as the control. The outcomes of these experiments show strong reactivity of the serum post-immunization but not pre-immunization, as the camel serum was tested before and after the immunization. (Figure 4.6)

4.4.2 Amplification of the VHH

Following the ELISA test, that shows that the camel generated antibodies against the *E. coli* LPS antigen, the VHH gene of the immunised animal was amplified to produce a comprehensive VHH library.

4.4.3 RNA extraction and cDNA synthesis

To select antigen specific VHHs, B cells from immunised camels were used as a foundation to generate a comprehensive VHH library, since they should specifically contain mRNA encoding for LPS antigen specific VHHs. One important step to ensure a large and diverse library is to maximise the mRNA yield.

Mainly, a large number of B cells from peripheral blood is required. In brief, 300-400 mL of heparinised blood from the immunised camel was used. Blood samples (15 mL) were split between 6 50 mL falcon tubes and diluted with an equal volume of 0.9% NaCl. Then, 30 mL of the diluted blood was carefully layered over 15 mL of Lymphoprep™ solution. The process to separate B cells from other blood components was achieved by centrifugation, as mentioned in the materials and methods section. Subsequently, total RNA was extracted from the isolated B cells using the triazole method. Finally, RNA samples were tested with NanoDrop Spectrophotometry, indicating 2499 μg/mL RNA. Then, the extracted RNA was used as a template for cDNA synthesis using oligo dT primers.

4.4.4 PCR amplification

cDNA was used as a template to amplify genes encoding the variable domains of the heavy chain antibodies. First, the CALL1 and CALL2 PCR primer pair was used to obtain the specific product of VHH-CH2 700 bp exons. Then, VHH encoding gene fragments were amplified in the second PCR, which was purified from an agarose gel after cutting the 700 bp band obtained from first PCR using VHH primer pairs. A 400 bp DNA fragment was generated.

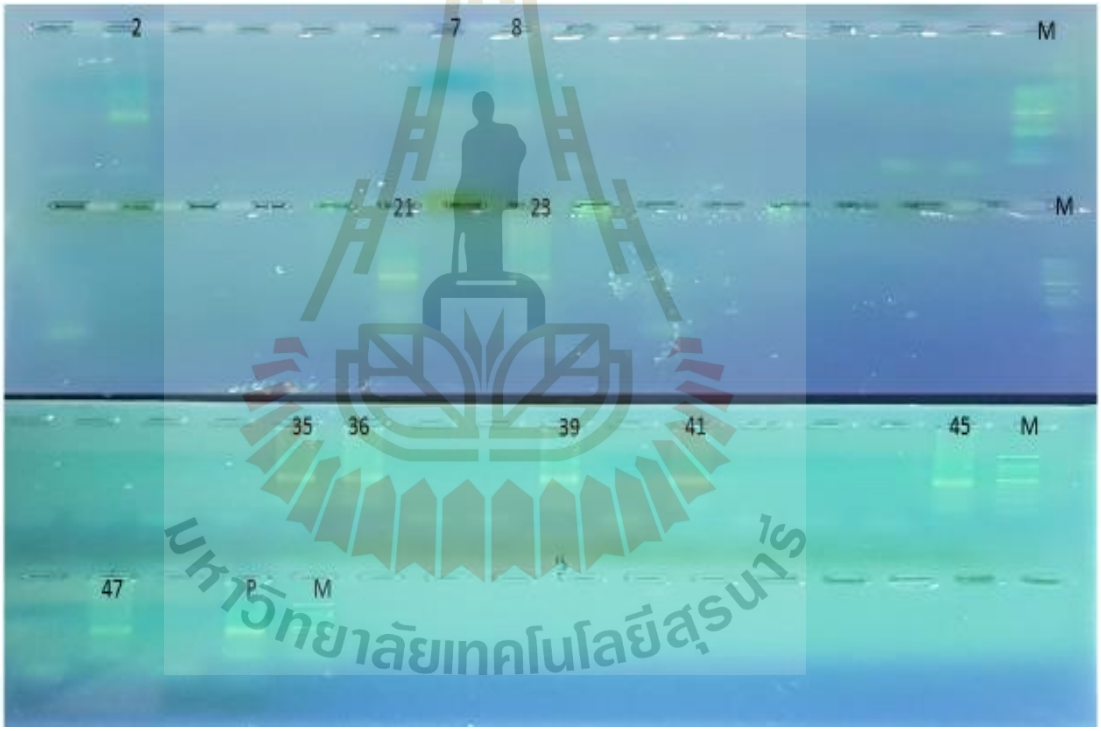

Figure 4.4 PCR of VHH gene from plasmid DNA of transformation 11 of them shows positive.

4.4.5 Cloning of VHH library in pF1A T7

To obtain a large VHH library, the ligation steps of the VHH amplicon and the corresponding pF1AT7 flexi vectorare, as well as next transformation steps, are very important. First, the VHH encoding gene fragment and the vector were digested by SgfI and PmeI, two rare-cutting restriction endonucleases, followed by ligation and transformed by heat shock into JM109 *E. coli* competent cells (Promega USA.). To increase the VHH library size, the absolute amount of DNA ligated, and the number of successful transformations are very important factors that must be considered. The cloning of the PCR amplicon, with the corresponding restriction enzymes, results in an open reading frame of the pF1AT7 flexi vector to the putative VHH domain that facilitates transportation of the expressed protein to the bacterial periplasm to ensure the correct formation of essential disulfide bonds. To determine the size of the library when *E. coli* cells were transformed with the pF1AT7 flexi vector, transformation efficiency was calculated using the TE equation where TE = colonies/µg/dilution. The library was 6.9×10^4 cfu/µg.

Forty-eight randomly picked single clones from the library were subcultured into 96 x 2 mL well plates for further analyses by PCR and protein extraction. Plasmid DNA extraction was carried out with the PCR group whereas periplasmic extraction was performed with the other group as mentioned in the materials and methods section. Plasmid DNA was digested with SgfI and PmeI enzymes and PCR was performed with 11 amplified VHH primers, of which 6 were selected for protein experetion.

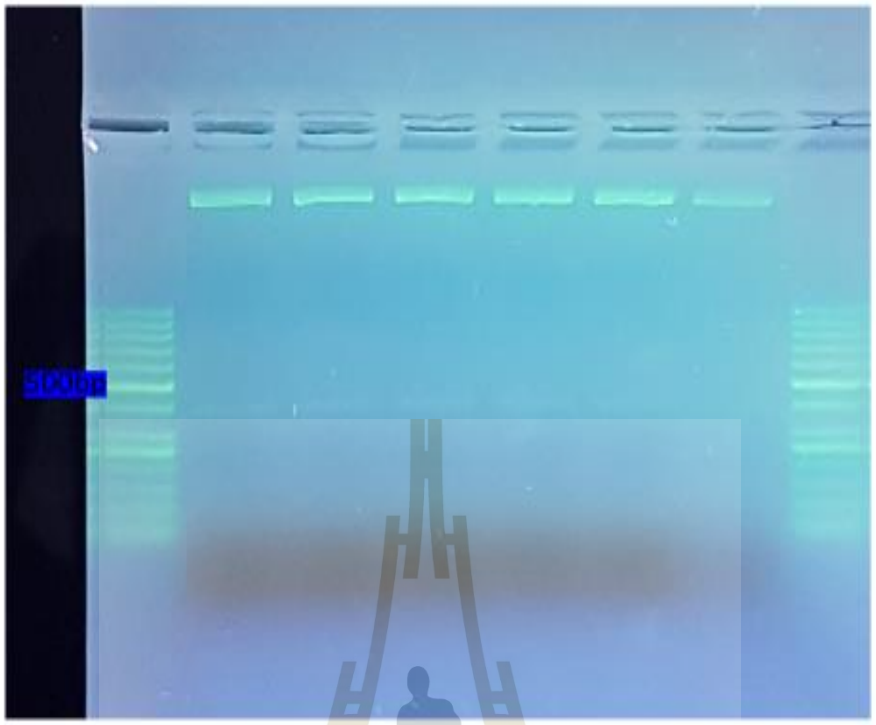

Figure 4.5 Plasmid extraction and vector insert digestion.

4.4.6 Sequencing of VHH

PCR amplified VHH products (6) were purified with QIAquick (QIAGEN, CA, USA) and directly sequenced with only a forward primer pair. Sequencing was carried out with Bigdye Terminators on an ABI 3730 genetic analyser (Applied Biosystems, CA, USA) according to the manufacturer's protocol. However, all sequences displayed more than 95% similarity between colonies 1 and 3, showing unique sequencing. VHH PCR amplicon isolates (6) were tested and then compared and aligned with GenBank sequencing data. Amplicons (400 bp) were used as a query for searching for a homologous sequence in GenBank using Nucleotide BLAST (Basic Local Alignment Search Tool) in the GenBank database. (Zhang et al., 2000) The blast results showed a similarity of about 90% and no sequence matched more than 91% in

the GenBank database. Further searches were done in Blast comparing many immunoglobin heavy chain sequences from dromedary camels, Lamas, and vocuna.

The VHH sequence alignments of single clones are illustrated in Figure 4.7. In order to select a specific VHHs *E. coli* lipopolysaccharide antigen to evaluate the success of process we used an ELISA (enzyme-linked immunosorbent assay) measurement as a semi-quantitation assessment to verify this result and exclude unspecific VHHs. The pre-immunised serum were tested as a negative control in parallel.

Figure 4.6 Sequencing of six transformed colonies showed positive of VHH gene PCR.

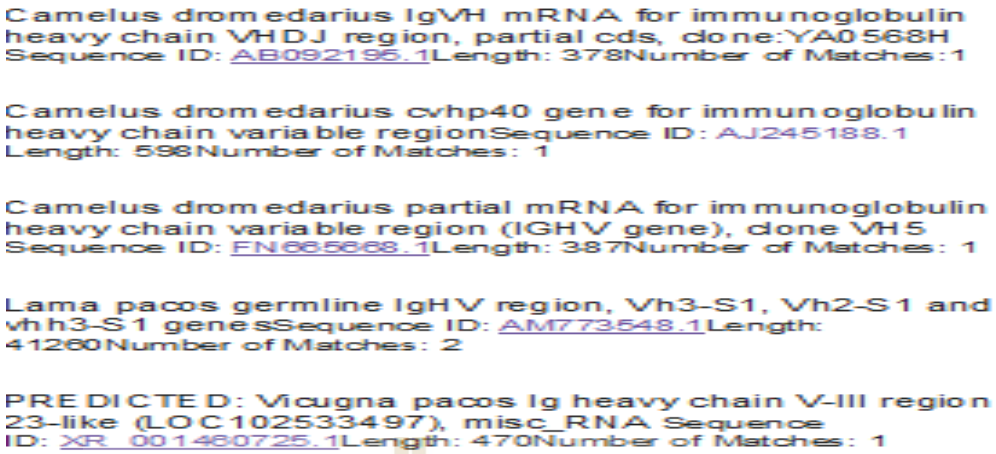

Figure 4.7 GenBank data showing similarity about 90% with first clone.

E. coli libraries were constructed with VHHs obtained from immunised camels (*Camelus dromedaries*) with *E. coli* lipopolysaccharide. Total RNA was isolated from peripheral blood lymphocytes (PBLs) collected after 70 days of immunization and ligated into the pF1AT7 Flexi vector (Promega Cat No C8441). Using High-efficiency competent *E. coli* cells (JM109 Promega), the ligated vector with an Ampicillin resistance gene was transformed and incubated in LB plates supplemented with 100 µg/mL ampicillin at 37°C for 24 hours. Independent clones (48), that overexpressed the resistance gene on the LB agar plate were randomly picked, sub-cultured and duplicated to 48x2 into LB broth medium in a 96 well plate with 2 mL wells. Cells were incubated overnight in a shaking incubator and the culture was transferred into a 15 mL tube with 10 mL of LB broth medium. After 24 hours the tubes were centrifuged at 10000 rpm for 5 minutes then 10 mL of fresh LB broth medium was added and induced with IPTG to a final concentration of 0.5 mM. The culture was shaken in a 37° incubator four hours after the log phase. The culture was then centrifuged at 3,500 xg for 20 min.

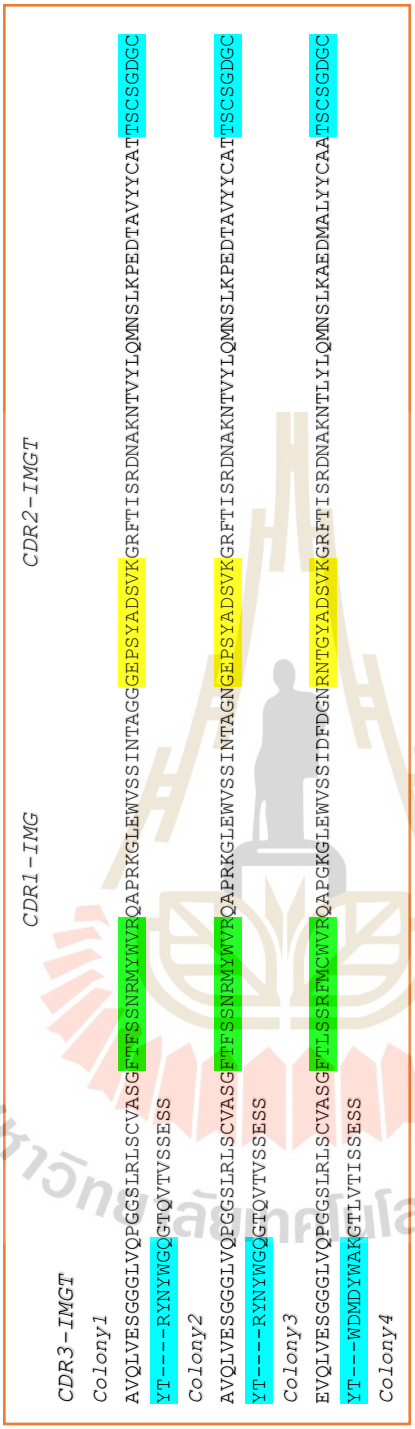

Figure 4.8 Multiple Protein sequencing alignments results of six colonies ClustalW software.

The supernatant was discarded and the total protein was extracted from the resulting cell pellet using the Smart bacterial protein extraction kit (Entron Korea) as well as periplasmic protein extraction using the cold osmotic shock technique as mentioned in the material and methods section then 100 µL of supernatant were used for ELISA screening. This was subsequently used to test the antigen-binding activity of the produced VHHs as a general method for screening obtained VHHs. Thus, reaction of the antigen-specific VHH from an immune library (Yan et al., 2015). However, nine sub-cultured colonies produced colour on the ELISA plate, generating Nbs and demonstrating that these Nbs recognised their specific antigens screened for their ability to react with fixed antigens on the ELISA plate. Lipopolysaccharide coated the plate. Thus, these data demonstrated nanobody production. To determine the size of the VHH libraries, *E. coli* cells were transformed with the flexi vector library and plated in serial dilutions where colony forming units (cfu) were counted and calculated using the Transformation efficiency equation TE=Colonies/µg/dilution and the library size was 6.9×10^4 cfu/µg.

To produce nanobodies specific to lipopolysaccharides of the *E. coli* cell wall antigens, we chose to use the expression cloning vector Flexi® Vector (Promega, USA.) with a protein-coding sequence flanked by SgfI and PmeI sites. These two rare-cutting restriction endonucleases help to clone the VHHs gene acquired from immunised camels after amplifying with a previously tailed VHH primer pair with a recognition site for SgfI and PmeI endonucleases. The SgfI site is upstream of the start codon of the protein coding region, which induces the expression of a protein by reading the SgfI site. While the PmeI site comprises the stop codon for the protein-coding region and attaches a single valine residue to the protein.

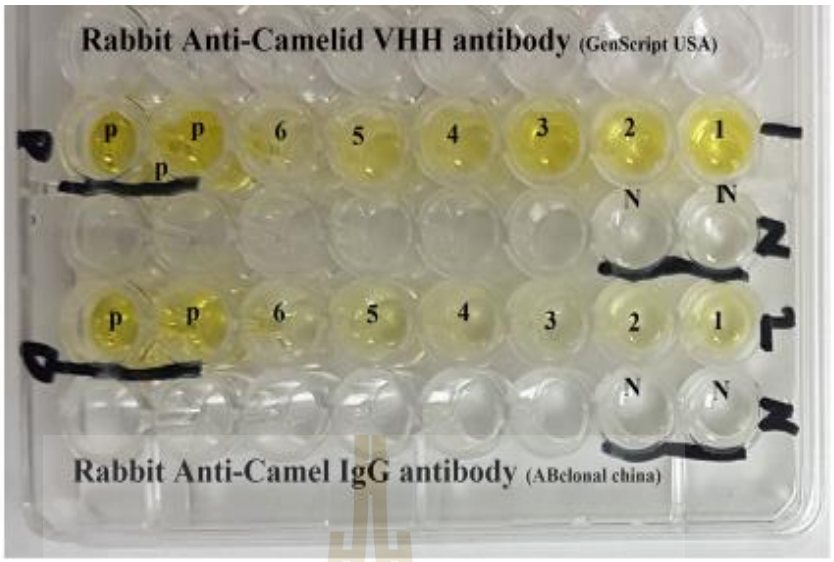

Figure 4.9 Result of ELISA test for VHH production of six colonies using two diffirent antibodies.

In this study, we demonstrate the production of anti *E. coli* LPS nanobodies from the expression cloning vector based on ELISA positive results that led to recognition of nine colonies of expression nanobodies and bound the LPS antigen previously coated plate.

However, eleven colonies were shown positive for VHH primers via PCR on extracted plasmid DNA, including six which approximately matched with the positive ELISA. From all the PCR products, these six were sequenced using only the forward primer with a ABI 3730 genetic analyser (Applied Biosystems, CA, USA) and Bigdye Terminators kit according to the manufacturer's protocol. Furthermore, we demonstrated positive PCR results via SDS_PAGE to confirm the production of nanobodies. These nanobodies were simply manufactured in milligram quantities in *E. coli* and can stored for prolonged periods (Muyldermans, 2013; De Meyer *at el*, 2014).

These types of production normally help to create a bank of nanobodies with numerous applications for cell biology research (Keiji and Sergei, 2014).

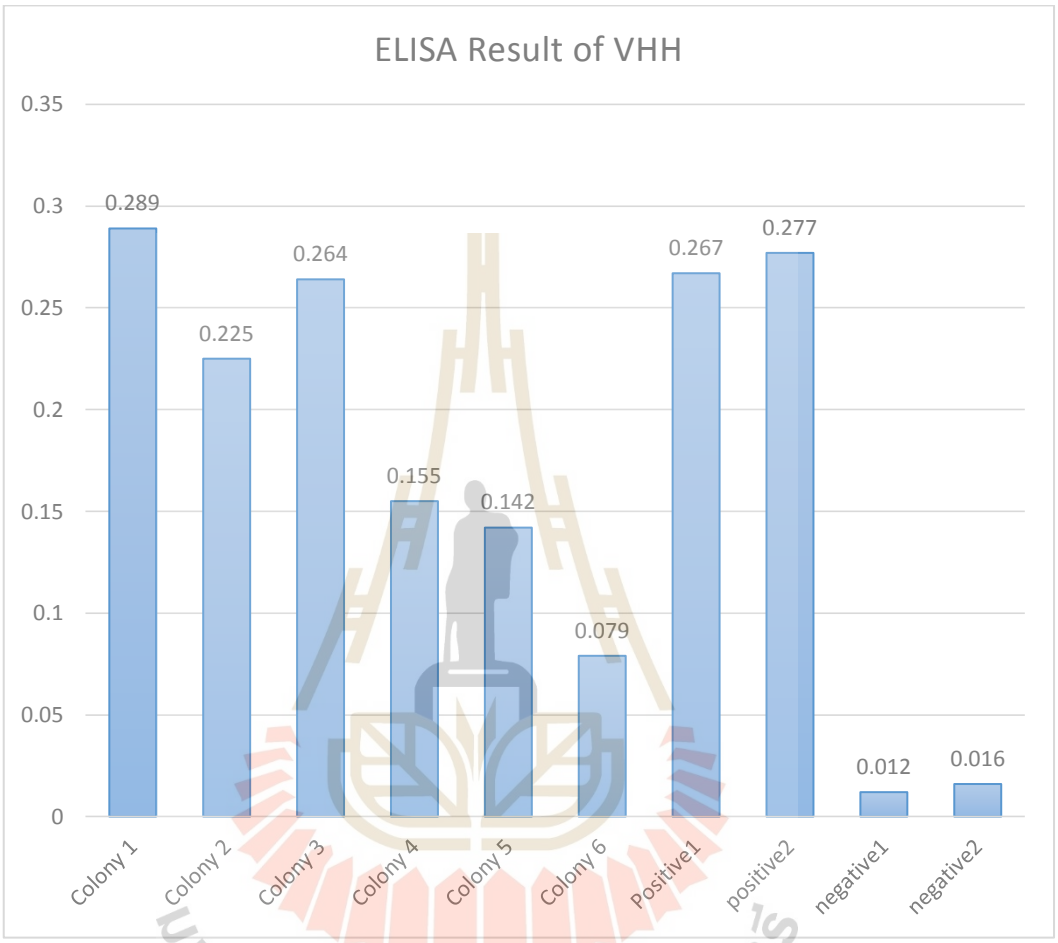

Figure 4.10 Result of ELISA graph shows antibody OD against six expressed colonies.

More than two decades have passed since the discovery of naturally occurring single chain antibodies and have been used as diagnostic tools in a number of studies (De Meyer et al, 2014; Muyldermans, 2013). A number of different nanobodies have been generated against many diverse individual antigens, like tumour receptors (Cortez-Retamozo et al, 2004; Roovers et al, 2007), HIV (Vercruysse et al., 2010), Clostridium botulinum neurotoxin, (Conway et al., 2010) and many other microbial

antigens. The production of nanobodies against *E. coli* to use as passive immunization for camel calves are very important, especially during their first week of their life since they have no antibodies to fight disease without an adequate supply of mother's colostrum as a source of nutrients they are very susceptible to infection from its surrounding environment. Pathogenic *E. coli* strain K99 (Snodgrass et al, 1982) is largely used as a passive immunization in cattle, horse, and camel farms, specifically in their first week of life. This type of production could prevent a lot of camel calf deaths and act as passive immunization. Good hygiene is essential. It is extremely important to make sure the environment is kept as clean as possible.

4.5 References

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CHAPTER V

OVERALL CONCLUSION

Although pathogenic *E. coli* strains cause infection in some animals, they can be commensal flora for others. Since immune system activation in new born camels is weak, specifically in the first days of their life, infections with opportunistic microbial flora can result from ingestion of contaminated mother's milk or unhygienic food where the host defence mechanisms, particularly antimicrobial immune response, are immature. This study demonstrated that all 20 of the *E. coli* isolates that killed neonatal camels were non-pathogenic.

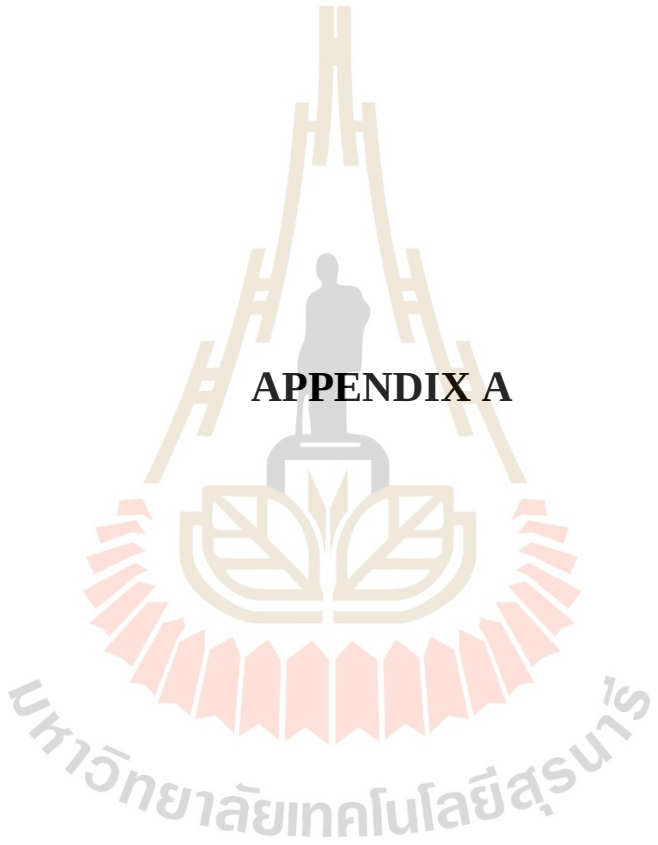
My second experiment generated a VHH library using lipopolysaccharide from *E.coli* to immunise camels. To get specific camel IgG the camel received intradermal injections weekly after bleeding and RNA was extracted from animal I. cDNA was amplified and VHH was amplified with an encoding gene fragment using the pF1AT7 flexi vector. I successfully cloned and transformed nanobody families against *E. coli* lipopolysaccharide.

I performed three different methods to randomly screen nanobody production. I subcultured 48 colonies that doubled when I performed periplasmic protein extraction for one set and plasmic DNA extraction for another. I carried out an ELISA for the first set and 6 of them were positive compared with pre-immunization samples. Whereas 11 samples produced a clear positive for VHH primers assessed with a gel. I have also done SDS-PAGE to demonstrate the VHH band.

In conclusion, I constructed the VHH library against the LPS of *E.coli*. This can be used for passive vaccination. Therefore, it is essential that camel calves consume an adequate amount of high quality colostrum and orally administrated nanobodies to ensure that newborn animals achieve maximum immune response and minimum mortality.

APPENDICES

APPENDIX A



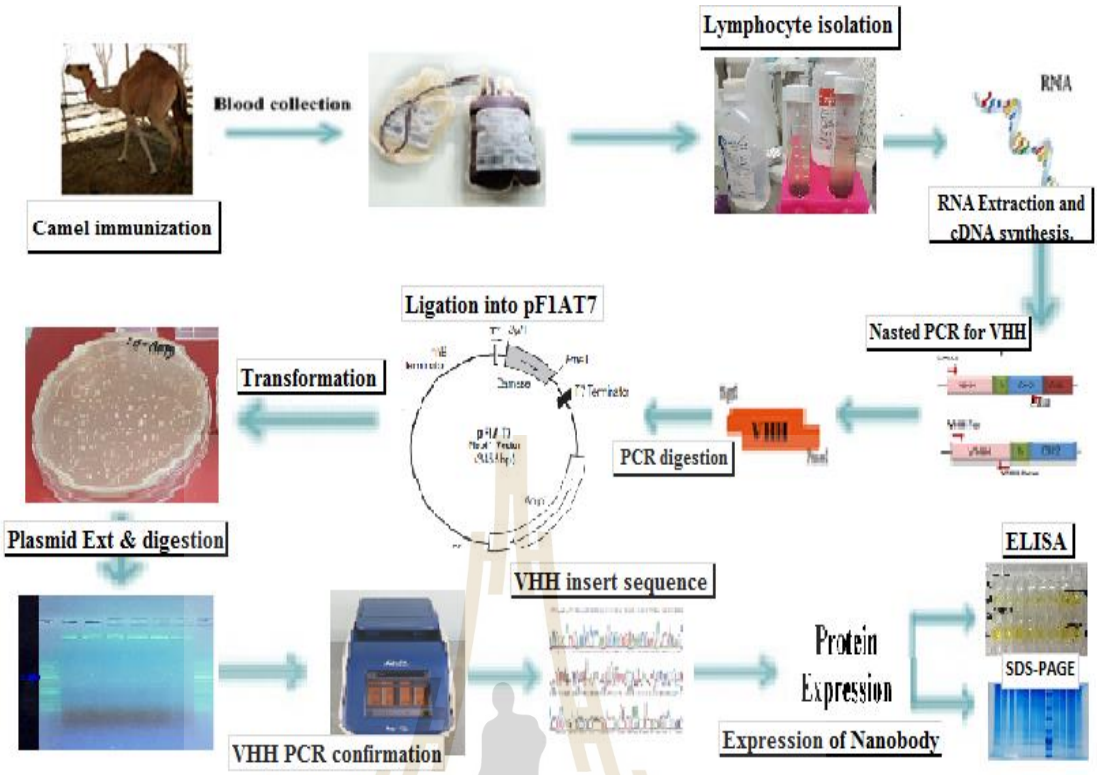


Figure 1 Workflow for the generation of of a single-domain antibody (sdAb).

Figure 2 First and third immunization injections with *E. coli* LPS.

Figure 3 Bleeding of Camel after Immunization.

Figure 4 Female camel used in this research.

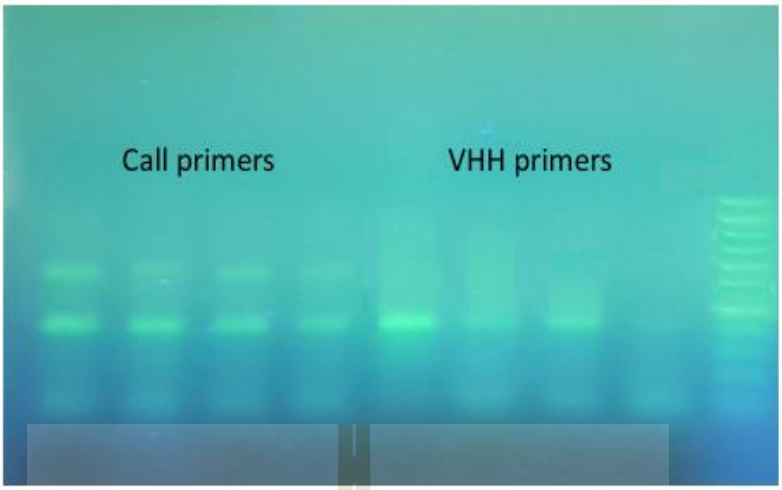


Figure 5 Call primers400 & 700bp band and VHH primers only 400bp agarose gel electrophorases.

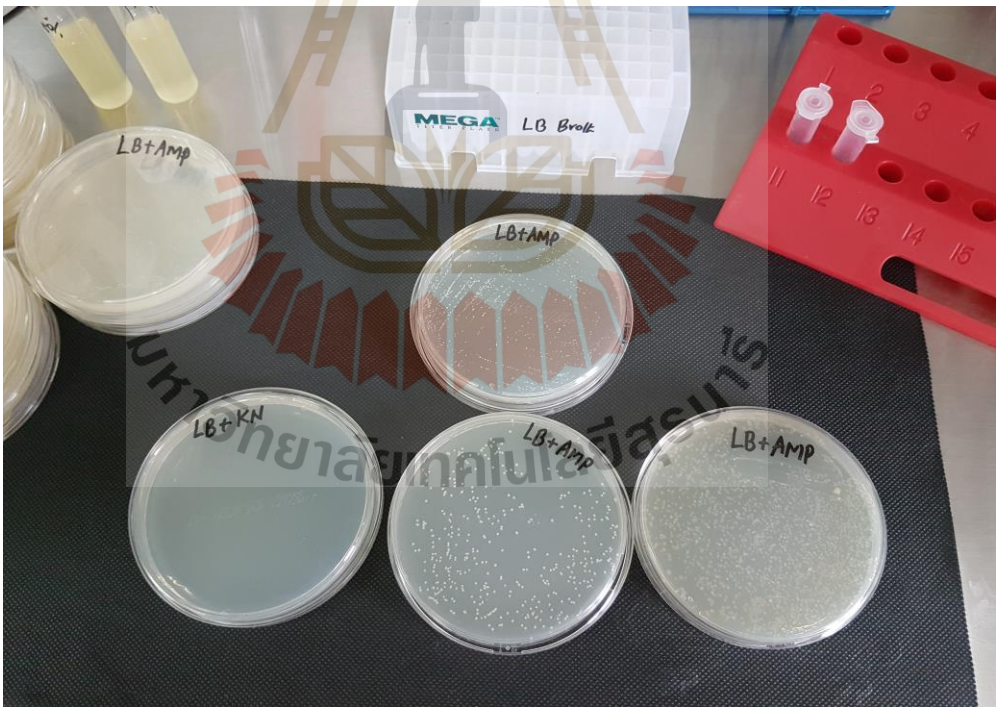


Figure 6 Three LB agar with Ampicillin *E. coli* Transformation left No Growth LB agar with Kanamycin.

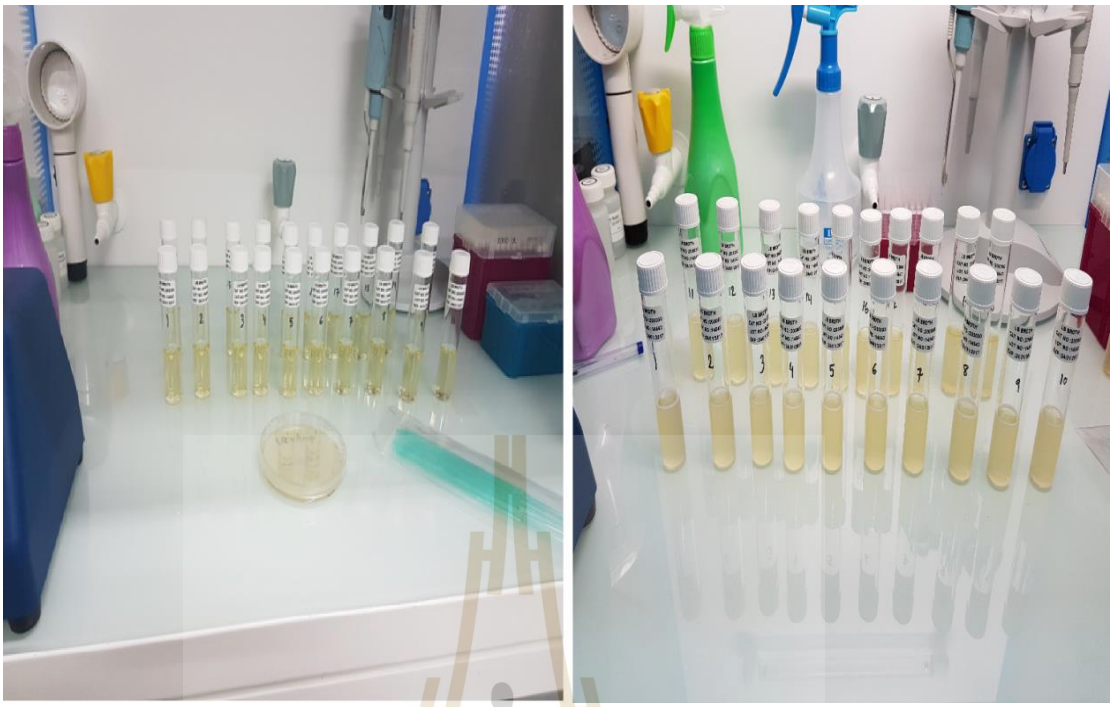
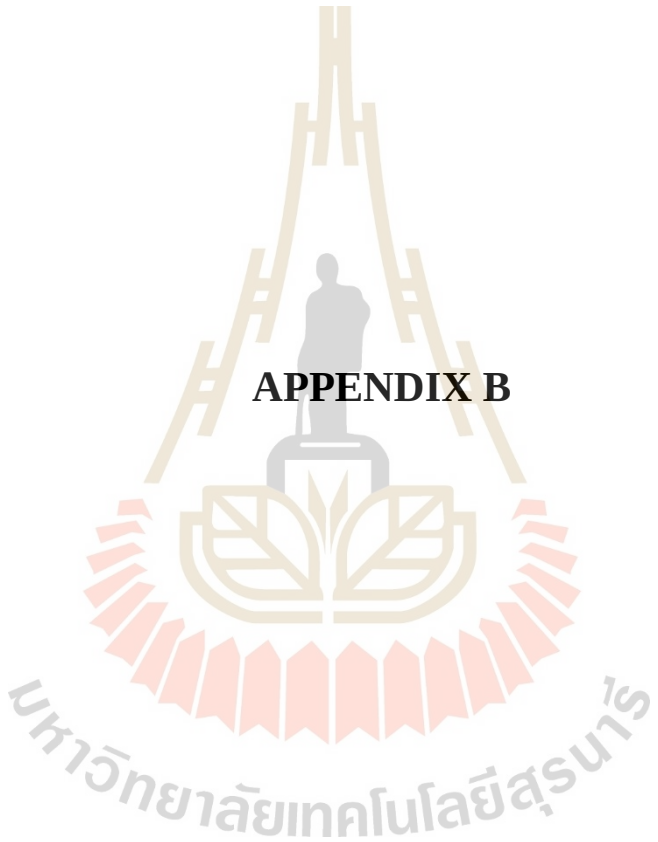


Figure 7 LB broth subculture from VHH library for screening of Nanobody production. Left Before inoculation Right After production.

APPENDIX B



CHEMICALS AND REAGENTS

1. Chemicals

1.1 Acetic acid, glacial (A9967, Sigma, Germany)

1.2 10% ammonium persulfate (ba013, Intron Biothecnology, Korea)

1.3 4-nitrophenyl phosphate disodium salt hexahydrate (DNPP) substrate (34064, Thermo, USA)

1.4 5x Go-Taq Polymerase buffer (M3008, Promega, USA)

1.5 Acrylamide/Bis-acrylamide (ba004, Intron Biothnology, Korea)

1.6 Agarose (V2831, Promega, USA)

1.7 $CaCl_2 \cdot 2H_2O$ (449709, Sigma, Germany)

1.8 Chloroform (288306, Sigma, Germany)

1.9 dNTP mix (U1515, Promega, USA)

1.10 *E. coli* competent cells (JM109, Promega, USA)

1.11 EDTA (4884,Sigma, Germany)

1.12 ELISA-blocking reagent

1.13 Ethanol (24102, Sigma, Germany)

1.14 Flexi Vector System (C8640, Promega, USA)

1.15 Glycine (G8898, Sigma, Germany)

1.16 GoScrpt Reverse Transcription System (A5001, Promega USA)

1.17 Go-Taq DNA polymerase. (M3008, Promega, USA)

1.18 Growth media and agar plates (U1090, Pan Gulf, UAE)

1.19 HRP-conjugate substrate (1706431, Biorad, USA)

1.20 Isoamyl alcohol (W205702, Sigma, Germany)

1.21 isopropyl β-D-thiopgalactopyranoside IPTG (367-93-1, Sigma, Germany)

1.22 K_2HPO_4 (P3786, Sigma, Germany)

1.23 KCl (7447-40-7, Merck, Germany)

1.24 KH_2PO_4 (1551139, Sigma, Germany)

1.25 LB +Amp (U1270, Pan Gulf, UAE)

1.26 LB +Kn (U1280, Pan Gulf, UAE)

1.27 LB broth (U1230, Pan Gulf, UAE)

1.28 $MgCl_2$ (M8266, Sigma, Germany)

1.29 $MgSO_4$ (746452, Sigma, Germany)

1.30 $Na_2HPO_4 \cdot H_2O$ (RES20908-A7, Sigma, Germany)

1.31 NaCl (7647-14-5, Merck, Germany)

1.32 $NaH_2PO_4 \cdot H_2O$ (S9638, Sigma, Germany)

1.33 $NaHCO_3$ (S5761, Sigma, Germany)

1.34 RedSafe Nucleic Acid Staining Solution (21141, Intron Biotechnology, Korea)

1.35 SMART Bacterial Protein Extraction Solution (17511, Intron Biotechnology, Korea)

1.36 Sodium acetate$\cdot 3H_2O$ (S2889, Sigma, Germany)

1.37 Sodium dodecyl sulfate SDS (151-21-3, Sigma, Germany)

1.38 SenSafe™ Fast Protein Staining Solution (31194, Intron, Korea)

1.39 T4 DNA ligase, 1 U/µL (M1801, Promega, USA)

1.40 TEMED (TEM001, Intron Biotechnology, Korea)

1.41 Tris-HCl (1185-53-1, Sigma, Germany)

2. Restriction Enzymes

2.1 Flexi® Enzyme Blend (SgfI & PmeI) (Promega, USA)

2.2 GoTaq® DNA polymerase (5 U/uL) (Promega, USA)

2.3 T4 DNA Ligase (HC) (20 U/µL) (Promega, USA)

3. DNA Purification kits

3.1 QIAquick PCR purification kit (Qiagen, Germany)

3.2 Agarose Gel DNA Extraction Kit (Roche, Germany).

4. Antibodies

4.1 HRP-Rabbit Anti Camel IgG (ABclonal China)

4.2 HRP-Anti-Camelid VHH antidody (GeneScript USA)

Both Rabbit Anti-Camel antibodies were HRP-conjugated. The stock contained 1000 mg/mL at a 1:5000 dilutions before the working solution (300 ng/mL). Both the stock and working solution were aliquoted and stored at -20°C.

5. Antibiotics

5.1 Ampicillin

Ampicillin stock solution of 100 mg/ml contained 1 g of ampicillin dissolved in 10 mL of ddH_2O, sterilised through a 0.22-µm filter, and then stored at -20°C.

5.2 Kanamycin

Kanamycin stock solution of 25 mg/mL contained 0.25 g of kanamycin dissolved in 10 L of ddH_2O and sterilised through a 0.22-µm filter stored at -20°C

6. Primers

6.1 CALL001 and CALL002

CALL001: 5′-GTCCTGGCTGCTCTTCTACAAGG-3′; CALL002: 5′-GGTACGTGCTGTTGAACTGTTCC-3′;

6.2 VHH-For and VHH-Rev

VHH-rev5′-CAA ATT TG--GATGTGCAGCTGCAGGAGTCT GRGGAGG-3′ (PmeI); VHH-For: 5′-CTAGTGCGGC CGCTG GA GA CGGTGACCTGGGT--GCG ATC GCC ATG-3′ (SgfI) (Vincke et al., 2012; Pardon et al., 2014).

6.3 16SrRNA

The 16SrRNA gene of the E.coli primer pair with the forward primer 3'CCC CCT GGA CGA AGA CTG A5' and the reverse primer 3'ACC GCT GGC AAC AAA GGA. (Wang et al., 2002; Brandal et al., 2007).

7. Buffers and Reagents

7.1 LB medium

LB medium contained 25 g of LB medium mixed in 1 litre of ddH_2O. The medium was autoclaved and stored at 4°C.

7.2 20% glucose

20% glucose, containing 20 g of glucose, were dissolved in 100 mL of ddH_2O by heating and then autoclaving the solution, which was then stored at room temperature.

7.3 LB-ampicillin glucose plates

LB-ampicillin glucose plates with 25 g of high salt LB medium and 15 g of agar were dissolved in 900 mL of ddH_2O. Then, the solution was autoclaved and cooled to 50°C. Then, 100 mL of 20% glucose and 1 mL of 100 mg/mL of ampicillin (sterilised 0.20-µm-filter) were added and poured into plates.

7.4 Tris-borate for electrophoresis (TBE)

Tris-borate for electrophoresis (TBE) contained 54 g of Tris base and 27.5 g of boric acid dissolved in 800 mL of ddH_2O. Then, 20 mL of 0.5 M EDTA (pH 8.0) and ddH_2O were added to adjust the volume to1L. TBE was stored at room temperature in a glass bottle. Buffer was diluted 5 times with ddH_2O for agarose gel electrophoresis.

7.5 10×PBS Buffer

10×PBS Buffer contained 2.4 g of KH_2PO_4, 14.1 g of $Na_2HPO_4 \cdot H_2O$, 2 g of KCl, and 80 g of NaCl dissolved in 1 L of ddH_2O. Then, the buffer was diluted to 1× and pH 7. The buffer was autoclaved and stored at room temperature.

7.6 Washing buffer PBST

Washing buffer PBST, 100 mL of 10× PBS, and 500 µL of Tween 20 were dissolved in 900 mL of ddH_2O. PBST can be stored for months at room temperature.

7.7 Coating buffer

Coating buffer contained 1.5 g of Na_2CO_3 and 2.93 g NaH CO_3 dissolved in 1 L of ddH_2O, adjusted to pH 9.6 with 1 M HCl, and stored at 4°C.

7.8 Blocking solution

Blocking solution contained 1 g of Bovine serum albumin (BSA) (dissolved in 100 mL of PBS buffer) with gentle stirring and stored at 4 C.

7.9 TBS Buffer

TBS Buffer contained 8.0 g of NaCl, 0.2 g of KCl, and 3.0 g of Tris base were dissolved in 800 mL of distilled water. The pH was adjusted to 8.0 with 1 M HCl and then water was added to a final volume of 1 L. The solution was autoclaved and stored at room temperature.

7.10 Chloroform/isoamyl alcohol solution

Chloroform/isoamyl alcohol solution contained 24 volumes of chloroform and 1 volume of isoamyl alcohol that were mixed and stored at 4°C in a tightly closed bottle.

7.11 2M of sodium acetate

Sodium acetate (2 M) and 27.2 g of sodium acetate trihydrate were dissolved in 100 mL of ddH_2O, adjusted to a pH of 4.0, and then stored at room temperature.

7.12 Lipopolysaccharide Antigen

Lipopolysaccharide Antigen_Stock of 100 µg/mL was prepared using a LPS extraction kit (Intron biotechnology Korea) with a working solution of 10 µg/mL made with a 1:10 dilution of stock to get 10 µg/mL with Carbonate-Bicarbonate buffer.

7.13 HRD Substrate

Stock solution: 3,3',5,5'-Tetramethylbenzidine (TMB) 10 mg/mL in DMSO (Josephy et al., 1982). Working solution were prepared with a 1: 100 dilution of the stock solution in Citrate/Acetate Buffer (Marquez and Dunford, 1997).

7.14 Citrate/Acetate Buffer

Citrate/Acetate Buffer with 0.1 M Sodium Acetate was titrated with 0.1 M Citric Acid to a final pH of 6.0 and stored frozen at -20°C.

7.15 Stopping solution

Stopping solution was prepared with 0.5 M sulphuric acid.

BIOGRAPHY

Faysal Abdishakur H Hassan was born in Mogadishu, Somalia on May 5th, 1972. He finished his secondary school at Jamal Abdelnaser Secondary school in Mogadishu. In 1998 he received his bachelor's degree of Chemistry, Microbiology, and Zoology from Osmania University, Hyderabad, India. In 2002 he finished his MSc degree of Medical Laboratory Technology at the Birla Institute of Technology and Science (BITS) Pilani, India. Following his postgraduate degree, he began his career as a Joiner Scientist in the Molecular Biology and Genetics unit of Central Veterinary Research Laboratory, Dubai, UAE, where he was responsible for Molecular pathogen identification of animals and DNA typing of horses and camels. During his seven years at CVRL he was involved in many research projects and obtained excellent experience in molecular biology techniques. Late 2009 he joined the Advenced Genetics Center in the Advanced Scientific Group, Abudhabi, UAE as a Senior laboratories until 2015 when he became Head of the Advanced Genetic Center. In 2012 he applied for a PhD. degree program at the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology with Assoc. Prof. Dr. Rangsun Parnpai. During the five years of his education, he performed his experiments in the laboratory of Assoc. Prof. Dr. Rangsun Parnpai and the Advanced Scientific group laboratories.