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Use of the RS-ATL8 NFAT Reporter System for Diagnosis of Hydatid Disease

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Abstract

Despite major advances in the diagnosis of infections for many pathogenic organisms, there is still a problem obtaining an accurate immunodiagnosis of *Echinococcus* infection due to its serological cross-reactivity with other species of taeniid cestodes or at higher taxonomic levels. For this reason, there are ongoing efforts to develop a better method for diagnosis of echinococcosis, especially when the parasite has a crucial role in hypersensitivity reactions. Like other helminthic infections, one of the immunological hallmarks is an elevated serum concentration of parasite-specific IgE.

Our aim was to assess the use of IgE reporter system as a possible new method for diagnosis *Echinococcus spp* infection, using RS-ATL8 NFAT Reporter System, which is a humanised rat basophilic leukaemia (RBL) cell line that can be used to detect the presence of specific human IgE directed against *Echinococcus* allergens and cross-link their receptors, depending on luciferase generation as an indication of presence of parasite-specific IgE, pointing to infection.

Towards this goal, we first optimised the use of the humanised RS-ATL8 Reporter System. This was achieved by optimisation of experimental conditions, such as cell density, stimulation time, optimum conditions for sensitising factors and stimulant optimum concentration. Once a robust standard operating procedure had been elaborated, the second goal was to choose a few *Echinococcus* antigens for investigation into their immunogenic properties and potential diagnostic value and to express them recombinantly for testing through the RS-ATL8 NFAT Reporter System. The chosen antigens were EF1-alpha, EgAg5, AgB2, Cyclophilin, Eg19, EgTeg, and EgTPx.

The recombinant *Echinococcus* genes were successfully engineered into four different vectors (as verified by sequencing) for wheat germ lysate, bacterial and mammalian expression systems. Expression using wheat germ lysate (WGL) indicated that is some

cases expression had been successful, but yields were very low. Therefore, all genes were sub-cloned into the pF1A-T7 Flexi Vector for expression into BL-21(DE3) *E. coli*, without success potentially due to rare codons usage. Our attempts to express these proteins in the Rosetta strain did not overcome this issue for unknown reasons. Thus our next step was to purchase synthetic cDNA for three of these genes (EF1-a, Ag5 and Cyclophilin), to optimise the codons for bacterial expression. These genes were then successfully cloned into pET-3a vector and expressed in BL-21(DE3); however, it was difficult to obtain soluble proteins. For this reason, our next step was to produce the *Echinococcus* antigens in HEK293-6E cell line, after cloning into pTT5 vector. The expressed Cyclophilin and Eg19 were identified by SDS-PAGE and Western Blotting in the cell supernatant and purified using IMAC. The purified recombinant antigens were finally assessed using the optimised RS-ATL8 NFAT Reporter System with the available sera of echinococcosis patients. The preliminary data of RS-ATL8 assay determined that 16 μg/ml and 3.2 μg/ml of Cyclophilin significantly elevate the cell activation response comparing to other concentrations.

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List of Abbreviations

% (w/v)	Percentage weight per volume
bp	Base pair
cDNA	Complementary DNA
CMV	Cytomegalovirus
CO2	Carbon dioxide
Con A	Concanavalin A
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides 5' Triphosphate
dNTP	Deoxynucleoside triphosphate
DPBS	Dulbecco's Phosphate Buffered
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
EXILE	IgE cross-linking inducing luciferase expression
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
HCl	Hydrochloric acid
HEK293-6E	Human Embryonic Kidney Cells 293-6E
HRP	Horseradish Peroxidase
HsFcεRI	Human Fc epsilon IgE receptor
IgE	Immunoglobulin E
IL	Interleukin
kb	Kilo base
kDa	Kilo Dalton
kV	Kilovolt
L	Litre

M	Molar concentration
mA	Milliampere
MEM	Minimum Essential Medium
mRNA	messenger Ribonucleic acid
MTA	Material transfer agreement
NFAT	Nuclear Factor of Activated T- Cells
NRSB	Non-reducing sample buffer
PAGE	Polyacrylamide gel electrophoresis
Parj2	*Parietaria judaica* (pollen)
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PHA-E	*Phaseolus vulgaris* erythroagglutinin
RBL	Rat Basophils Leukaemia cells line
RBL	Rat basophilic leukaemia
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSB	Reducing sample buffer
SNA	*Sambucus nigra* agglutinin
SNP	Single nucleotide polymorphism
TBE	Tris-borate-EDTA buffer
TBE	Tris Borate EDTA
v/v	Volume per volume

Chapter 1: General Introduction

1.1 Echinococcosis Background

Echinococcosis refers to hydatid disease or echinococcal disease and it is one of the most serious zoonotic diseases which affects both humans and other animals; common animal hosts are sheep, cows, goats, dogs and foxes [1]. There are three types of echinococcosis; hydatid disease or cystic echinococcosis caused by the species *E. granulosus*, alveolar echinococcosis caused by the species *E. multilocularis*, and polycystic echinococcosis caused by both *Echinococcus vogeli* and *Echinococcus oligarthrus*. *Echinococcus granulosus* is more common and can be found in The Middle East, Argentina, South Africa, Australia, China and many parts of Europe (such as Italy, Spain, Russia, Greece), and the West of United States especially in California, Mexico and Arizona. Hydatid disease or cystic echinococcosis is caused by the definitive host, which is dog, and intermediate hosts such as sheep, cattle and pigs. The intermediate hosts are infected by ingesting eggs of the adult worm [2, 3]. *Echinococcus multilocularis* is less common and the parasite circulating between the definitive hosts carnivores (such as wolves and foxes), which belong to the taxonomic Family *Canidae*, and rodents as intermediate hosts. In terms of alveolar hydatid disease, humans are considered as an accidental host [4].

Nutrients are absorbed through the tegument, the outer membrane of the adult worm, and diffuse very easily and quickly to all the cells due to the small volume of the flatworm compared to the surface [2, 3]. With regard to symptoms, the most common symptoms that accompany *Echinococcus* infection are abdominal pain and vomiting when the cysts are formed in liver, while cysts in the lungs cause chronic cough, chest pain, and shortness of breath. The other main symptom of echinococcosis are the allergic phenomena, such as anaphylactic reactions [5, 6].

The serious symptoms of echinococcosis can start when the cyst ruptures spontaneously or due to surgery leading to secondary infection and anaphylactic shock which is induced by spreading of cystic fluid and the protoscoleces, followed by elevating the IgE level in the infected patients [7, 8].

There are tight-links, at the same time, an ambiguous relationship between the parasitic disease and allergy. Studying and comparing these two diseases give a better understanding of how the immune system works and the pathological effects which may cause by these diseases. In addition, collecting more information about the immunological response, characteristics of allergy and echinococcosis, and immunomodulation lead to find modern techniques to design new medicines, vaccines and preventative factors [8].

1.2 Morphology and Pathogenicity

Adult stage of *Echinococcus spp.* measure 2-11 mm in length. *Echinococcus* can be divided into scolex, neck and 3-4 proglottids. A scolex has four suckers and a double circlet of hooks. The parasite can insert into the crypts of Lieberkühn in the host intestine using these claw-shaped spines, so they enable the worm to fix itself tightly [9]. The second part is a neck, which is short, non-segmented and contains strong muscles, this part is followed by the last section called proglottids. The first proglottid immature and contains a germinal mass. Whereas, the second proglottid is mature and has hermaphrodite sexual organs (ovary and testes). The last proglottid is the gravid proglottid which has a uterus full of fertilised eggs (Figure 1.1) [5, 10].

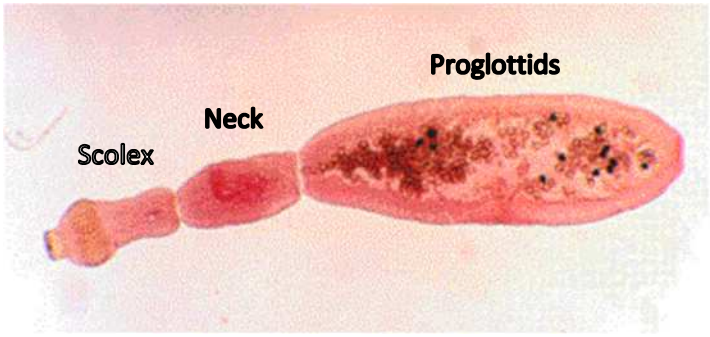


Figure 1.1: Adult of *Echinococcus*, which consists of three segments scolex, neck and proglottids. Source DPD CDC.

The egg is spherical in shape and ranges from 22 µm to 50 µm in size. An egg consists of two layers: the outer shell is a thin membrane, and inner shell which is thicker and has a radially striated embryophore. The latter is the more physical protection to the embryo which is called oncosphere. Eggs are released with the faeces of an infected definitive host and are characterised by being extremely resistant to the environmental temperatures and conditions (Figure 1.2) [5].



Figure 1.2: Egg of *Echinococcus* containing outer shell, inner shell and oncosphere. Source: DPD CDC

When the egg of *Echinococcus* is digested by the intermediate host, it hatches in the stomach or small intestine of the host, leading to oncosphere release. After that, oncosphere penetrates the wall of the host intestine using its hooks, body movements and the secretions of penetration glands. Once the oncosphere passes into the mucosa, it directly enters the bloodstream or lymphatic vessels and migrates to the anatomical organs of the host [11]. The oncosphere settles there and then develops during the first 10-14 days, leading to the formation of the metacestode. The modifications involve a series of reorganizational processes; such as cellular multiplication, oncospheral hooks disintegration, circulatory system reticulation, and formation of central cavity and growth of both germinal and laminated layers. The metacestode is subspherical in shape and filled with fluid, it grows up to 10 cm, and it is full of fluid. After that, it develops and forms as a structure known as hydatid cyst (Figure 1.3) [12].

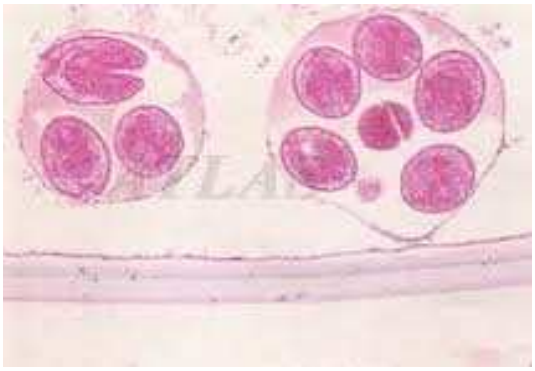


Figure 1.3: Hydatid Cyst of *Echinococcus*. A section from muscle of experimentally infected mice shows two hydatid cysts containing protoscoleces. Source: DPD CDC

Hydatid cysts contains two laminated membranes, an outer layer which is a thick, tough, elastic, acellular membrane and an inner membrane which is called a germinal or nucleated layer that is the parasite itself. The outer layer of the hydatid cyst is an absorptive layer, so it can readily absorb the nutrients by diffusion. The host's fibrous

layer is the result of the cellular inflammatory reaction of the host during the initiation stage of metacestode development [13]. The germinal layer (inner layer) consists of thin epithelial membranes, the epithelial cells start to grow and form brood capsules. The brood capsule later produces protoscoleces, which are the larva stage of the parasite [14].

1.3 Life Cycle

As most flatworms, *Echinococcus* needs at least two hosts to complete its life cycle, which are definitive (final) hosts, such as dogs, and intermediate hosts, such as sheep, cattle, camel, swine, goat and horses. The strobilar stage of the parasite which resides in the small intestines of the definitive host releases eggs from its gravid proglottid, each containing a single embryo or oncosphere. These eggs are passed with the faeces of a definitive host into the environment. They are able to survive in the environment for several months. The intermediate hosts are infected with the parasite by ingesting the embryonated eggs. Eggs hatch in the small bowel of the intermediate host, leading to a release of oncospheres, which attach to the intestinal crypts. Consequently, oncospheres penetrate the epithelium, and enter arterioles and venules migrating through the circulatory system and are able to settle in various organs, especially in the liver and lung. Each oncosphere develops into a metacestode (hydatid cyst) in these organs, then the later produce protoscoleces by asexual multiplication. The hydatid cyst enlarges rapidly and makes numerous daughter cysts to fill the lumen of the hydatid cyst. Definitive hosts are infected with the parasite by ingesting the organs of the infected intermediate hosts, which contain the cyst stage of the parasite. In the small intestine of the final host, the cyst ruptures and protoscoleces release. During 32-80 days, protoscoleces grow and develop into a mature adult, see figure 1.4 [5, 15].

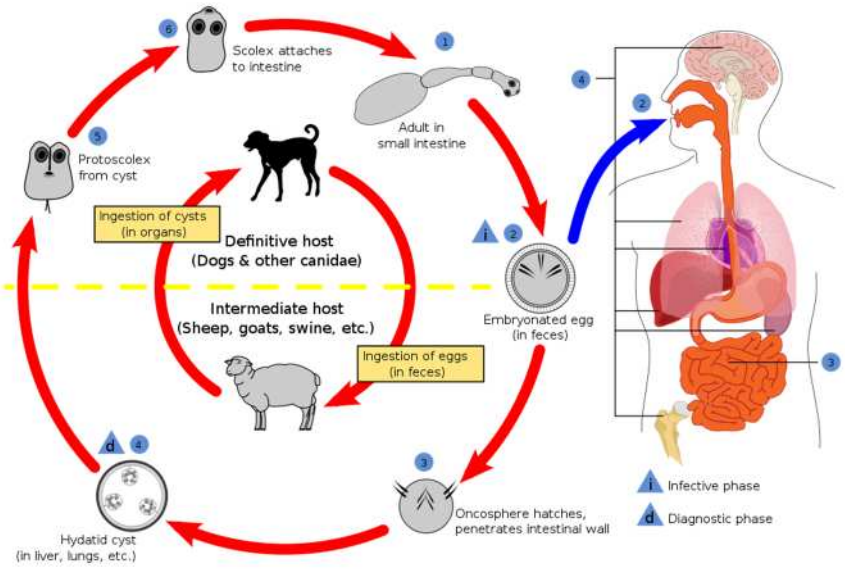


Figure 1.4: Life Cycle of *Echinococcus*. Source: DPD CDC

1.4 Immunological Characterisation of *Echinococcus* Infection

Vuitton (2003) [16] determined that the immunity status of the host has a crucial role in the occurrence and severity of the infection and it is related with the range of IL-10 secretion which is the main signs of chronic echinococcosis. He also pointed out that the Th-1 related immune response was associated with protection against the parasite infection, whereas, Th-2 related immune response was associated with the growth of the parasite. The experiments which have been done have shown that the cellular immunity which is induced by Th1 in the host has the ability to kill the metacestode stage of the parasite at the initial stages of development. In addition, antigenic proteins of the metacestode and oncosphere have the ability to connect with the antigen presentation

and cell activation leading to produce the cytokines, such as IL-10, by the immune cells and these mediators could inhibit the innate immune response. Moreover, the effect of the parasite can extent on the host and associate deeply with the immunogenic characteristics of the host. It has been noticed that the *Echinococcus spp.* have related with the Th2-linked IgE production and the activation of mast cells and basophils which are responsible for the allergic reactions in the host [8].

1.5 *Echinococcus* Proteins

Proteins represent the highest percentage of the cell components, and perform different cellular functions, including the cell cytoskeleton, the formation of contractile systems and controlling on the transportation system of the molecular particles. In addition, Protein acts as hormones, different enzymes and toxins. Moreover, it acts as antigens, which some of them are allergens. *Echinococcus* proteins have been divided into soluble and insoluble proteins depend on their properties. The soluble protein composed of glycoprotein, whereas, the insoluble protein composed of lipoprotein [5].

1.6 *Echinococcus* Antigens

An antigen is a specific protein that binds to the respective antibody. Actin, is an example of *Echinococcus spp.* protein which is forms the skeletal structure of the parasite. It has been noticed that there are two genes, in the genome of the parasite, responsible to the transcription of actin [17]. Immunoglobulin against actin and other cytoskeletal proteins have been detected in humans infected with both species of *Echinococcus, granulosus* and *multilocularis* [18].

Another *Echinococcus granulosus* protein is keratin, which can be found in protoscolex hooks and Keratin is the major component of the oncosphere [19].

Antigen B (AgB), antigen A (AgA) and antigen 5 (Ag5) have been found in the hydatid cyst fluid of *Echinococcus granulosus*, and they have been considered as candidates for specific immunodiagnostic techniques of the cystic echinococcosis in humans and animals. Antigen B is thermos-stable antigen and resistant to the boiling degree for 15 minutes without losing its antigenicity. While, antigen A and antigen 5 are heat labile [5].

March *et al.* (1991) [20] noticed that the antigen Ag5 binds to concanavalin A, and has a large amount of carbohydrates and polypeptide. The terminal N of Ag5 links with oligosaccharides (PNGase F-sensitive), so the terminal N does not link with acetyl-D-glucosamine (N-acetyl-D-glucosaminidase H-non-sensitive). On the other hand, he pointed out that AgB does not bind to concanavalin A, and it is not sensitive to digestion with PNGase F and N-acetyl-D-glucosaminidase. Whereas, Shepherd *et al.* (1991) [21] determined that there is no carbohydrate in AgB components. The antigen AgB is very resistant to the high temperature and can be boiled for 15 minutes without losing its antigenicity [5].

Makni *et al.* (1992) [22] noticed that *Echinococcus granulosus* has another antigen, which is created in protoscoles tegument and stored in the fluid of hydatid cyst, it is called P1 antigen. This antigen has ability to diffuse through the laminated layer of the hydatid cyst to the surrounding tissue to be phagocytosed by the host monocytes.

With regards to *Echinococcus multilocularis,* Leducq *et al.* (1988) [23] discovered that the cyst of the species *multilocularis* has the complex glycoconjugates (glycoprotein is one of the carbohydrate components) which can be found in the tegumentary structure of the cyst. The function of these glycoconjugates is unknown, but it may be released to inhibit the immune response of the host against *Echinococcus multilocularis* [24].

1.7 *Echinococcus* Allergens

An allergen is a specific antigen that produces an abnormally strong immune response, and has ability to induce immunoglobulin E (IgE) production. The term allergy was introduced by Clemens von Pirquet in 1906 to describe symptoms in patients who have hypersensitivity to specific substances such as some pathogens, pollen, dust and some food [25-27]. Allergy occurs by producing IgE in response to the allergens. Allergen is received by antigen presenting cells which are mast cells, basophils, and dendritic cells. In 1963, hypersensitivity reactions describe as excessive immune response to antigens which may cause deterioration to the host [28].

It has been stated that the both species of *Echinococcus granulosus* and *multilocularis* have a tight link, at the same time, an ambiguous relationship with allergy and anaphylactic reactions in human [7, 8]. Several *Echinococcus* allergens have been described: AgB, Ag5, cyclophilin, and EgEF1-alpha/ beta/ delta [8]. Antigen B (AgB), Cyclophilin and antigen 5 (Ag5) have been detected in the hydatid cyst fluid of *Echinococcus granulosus,* and they have been considered as candidates for specific immunodiagnostic detection of cystic echinococcosis in humans and animals [5].

1.6 Immunoglobulin E (IgE)

In 1966, Ishizaka [29] was the first scientist who identified the immunoglobulin E (IgE), the latest subtype of immunoglobulin that has been found. IgE represents a very small proportion of the total of antibodies in human serum, amounting to less than 1% of total immunoglobulin. IgE presents approximately at 150 ng/ml in the human serum. The very small amount of IgE is not affecting its function due to the effectiveness of this antibody [30]. There are several factors affect the total level of IgE which are environmental factors,

immune system status, genetic status and human race [31]. In addition, IgE has the shortest half-life comparing with the other immunoglobulins, which is very rarely more than two days. IgE has a crucial role in mediated allergic reactions. Also, a high level of IgE has been tested with some of parasitic infection [32].

It has been reported that the all patients with echinococcosis have specific IgE for *Echinococcus* allergens and this related with severity of the disease. Stimulating basophils with *Echinococcus spp.* allergens lead to release histamine in patients with echinococcosis [8].

1.6.1 The Structure of IgE

IgE comprises of two main regions, Fab region and Fc region (Figure 1.5). The Fab region of the IgE composes of two equal portions; each portion has a heavy (H) chain and a light (L) chain. Both the heavy and the light chains in Fab region have two molecules which are variable domains (V) and the first pair of constant domains (C). Both variable and constant domains of the light chains, (C_L) and (V_L), consist of approximately 110 amino acids each, whereas each heavy chain has around 110 amino acids in its variable domains (V_H) and between 330 to 440 amino acids in its constant domains (Cε1). The amino acids bind with each other by disulphide covalent bonds.

The Fc region of IgE comprises of the remaining constant domains of the heavy chains (Cε2, Cε3 and Cε4). The Fc region has the cell receptor binding sites which are FcεRI and FcεRII (CD23) (Figure 1.5) [33, 34].

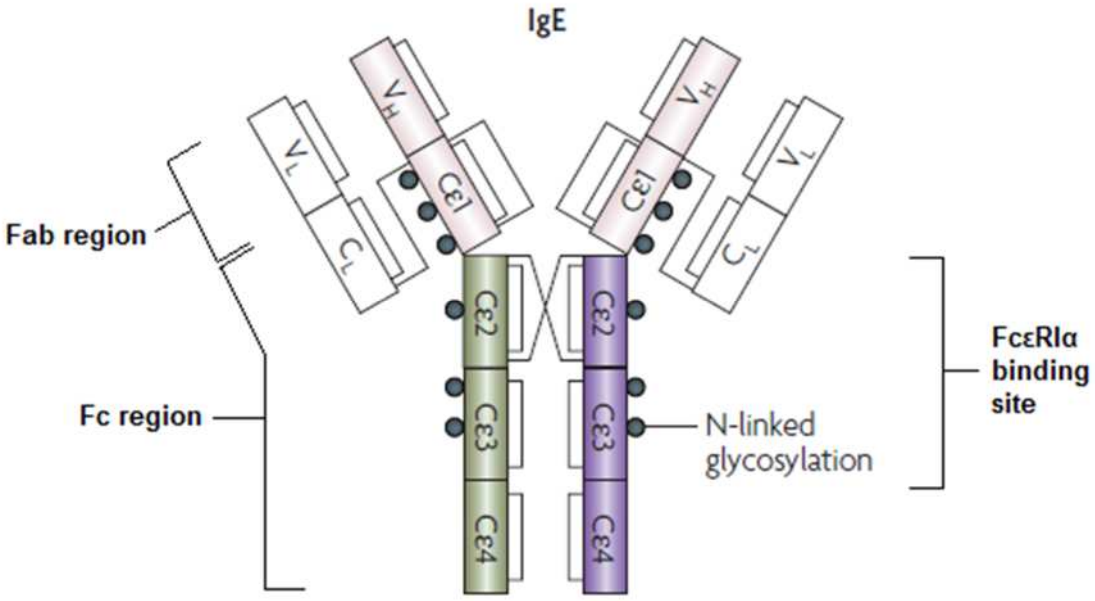


Figure 1.5: The figure shows IgE molecule which composes of Fab and Fc regions. Source: DPD CDC.

1.6.2 IgE Receptor

There are two types of IgE receptors can bind to the Fc region of IgE: the high–affinity receptor FcεRI and the low-affinity receptor FcεRII (CD23) (Figure 1.6) [31]. There are approximately 2 million high–affinity receptor FcεRI on the membrane of the cell, they cover the surface of the mast cells, basophils and dendritic cells [35]. After activation, these cells start release mediators such as histamine (Figure 1.7) [30]. It has been reported that the high–affinity receptor of IgE can been found on the surface on some antigen presenting cells such as monocytes in atopic individuals but in much less abundant level comparing with mast cells and basophils; and it is not accompanied by releasing of any mediators [35, 36].

1.6.3 IgE high-affinity receptor FcεRI

The high-affinity receptor (FcεRI) plays an important role in IgE mediated allergic reactions and it consists of four chains, an alpha (α) chain, a beta (β) chain and two gamma (y) chains (Figure 1.6). FcεRI can be found as a two different forms depend on the type of the holder cells; Tetrameric (αβy2) on mast cells and basophils, and Trimeric (αy2) on antigen-presenting cells such as dendritic cells, monocytes which have no β chain in their FcεRI receptor [37, 38].

1.6.3.1 The α Chain of FcεRI

The α chain of the receptor FcεRI has the IgE binding site, so it is binding IgE (Figure 1.6). There are two extracellular domains of α chain; α(1) and α(2) which are homologous to each other [39, 40]. An acute angle has been formed by the special position of these two domains, α(1) and α(2), together [41, 42]. The domain α(2) holds the IgE's Fc binding site [43, 44].

1.6.3.2 The β Chain of FcεRI

The β chain of the receptor FcεRI has two extracellular loops and one cytoplasmic loop (Figure 1.6) [39]. β chain plays a crucial role in amplification of the signal transduction though the cross-linking of the receptors [45]. As well as, the FcεRI receptors increase in the surface on the mast cells and basophils and amplify signalling due to the expression of β chain [31].

1.6.3.3 The y Chain of FcεRI

The two y chains comprise of a trans membrane domain with a little extracellular domain and they attached with each other by disulphide bond. The both subunits of y chains have a role in signal transduction cascade and promote cell activation (Figure 1.6) [39].

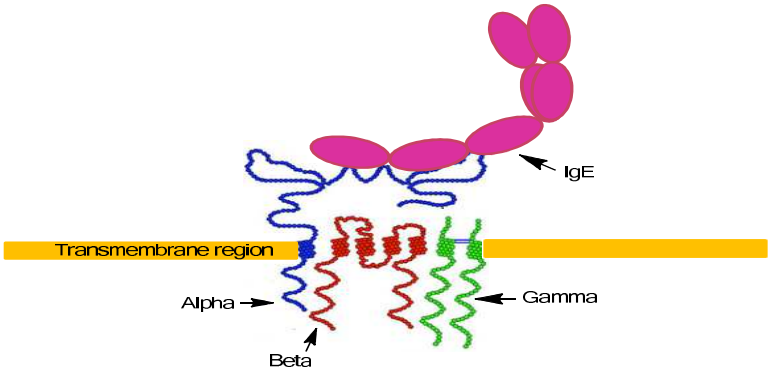


Figure 1.6: Schematic diagram illustrating the α, β and γ chains of IgE FcεRI receptor (Image source: Franco H. Falcone).

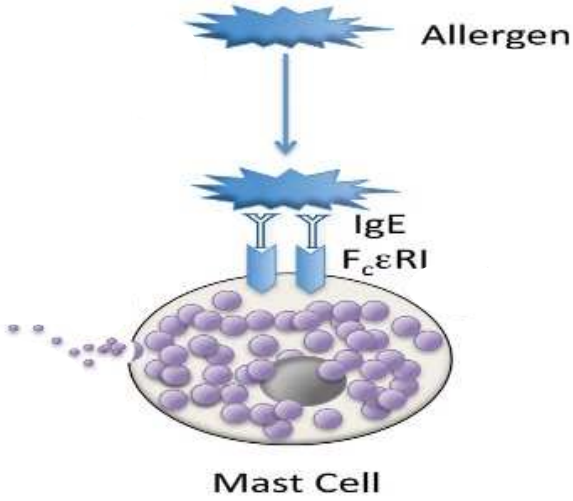


Figure 1.7: The role of IgEs to cross-link the IgEs bound receptors by binding an allergen and release of mediators, such as histamine. Source: DPD CDC.

1.8 Immunological and Molecular Diagnosis of Echinococcosis

Diagnosis of infection with *Echinococcus spp.* is an interest of many scientists because the disease is prevalent and widely observed. The infection combines humans and animal.

There are different methods used to diagnose echinococcosis which can be summarised as the following:

1.8.1 Physical Method

Physical method such as palpation can readily detect the cysts of *Echinococcus spp*. in very late stage by the clinician, and it could be an active method for fully or nearly-fully developed cysts, but it cannot detect the initial stages of the disease [46, 47].

1.8.2 Medical Imaging

The imaging techniques use in this diagnosis method, such as a magnetic resonance imaging (MRI) scan can visualise the hydatid cysts among tissues, radioisotopic, computerized axial tomography (CT) Scanning, and ultrasonic scanning. The medical imaging method can identify the location and size of the cyst. In addition, it can determine the physical characterization of the disease, such as calcification, fibration, lesion and necrosis. Still this method is not suitable for the early stage of the disease [47, 48].

1.8.3 Antibody Detection (Immunodiagnostic Techniques)

It is known that any damage to the cyst can lead to leaking of its fluid and spreading of the protoscoleces. The protoscoleces have the ability to form the secondary cysts and triggering anaphylaxis in infected patients. So, it is difficult to take a biopsy or sample from the cyst to do more investigations. Consequently, immunological diagnosis has played a crucial role to reach a definitive diagnosis for the disease [47, 48].

Researchers have paid more attention to develop the immunodiagnostic techniques for *Echinococcus spp.* infection. In spite of this, using serodiagnostic methods to distinguish between *Echinococcus granulosus* and *Echinococcus multilocularis* is difficult, although it has been detected a specific antibody against *E. multilocularis,* Em2 antigen and recombinant antigen 11/3-10. Unfortunately, there is no corresponding antigen in *Echinococcus granulosus* that has been discovered to distinguish between these two species. Although, there are several immunodiagnostic methods available to diagnose echinococcosis, still numerous complications can be faced due to the serological cross-reactivity with other species of taeniid cestodes. In addition, interference can be happened between the four different species of *Echinococcus,* because of the difficulty to recognise the specific immunoglobulin for each species of *Echinococcus*; *granulosus, multilocularis, vogeli* and *oligarthrus*, especially when more than one of these species can exist in the same area. Moreover, there is further problematic regarding the immunodiagnostic methods of *Echinococcus* infection in other intermediate hosts, which is the weakness in the immune response and the formation of specific antibodies in animals. This is in complete contrast to humans, which can be found with a strong response of the immune system. A high level of specific antibodies can be determined in humans who are infected with *Echinococcus spp.* The development of immunodiagnostic studies of echinococcosis has a great and positive impact to discover more information

about the characterization of the parasite antigens. Identification of the specific antigens of *Echinococcus spp.* still remains a crucial idea for the scientists to take full advantage of immunodiagnostic assays in humans and animals [5, 49, 50].

The most widely used serological techniques for diagnosis of echinococcosis; the indirect haemagglutination assay (IHA), the complement fixation test (CFT), the latex agglutination (LA) tests, but they are considered as insensitive and nonspecific assays. Therefore, they have been replaced by the enzyme linked immunosorbent assay (ELISA), the enzyme linked immunofluorescence assay (ELIFA), and immunoprecipitation tests for instant immunoelectrosyneresis (IEP) and immunoblotting (IB) [47, 51].

A great difference can be observed between these various immunological techniques in terms of their sensitivity (the capacity to detect specific antibody, or the ability of the test to give a positive result if patient has the disease), and specificity (the ability to distinguish between the patients with echinococcosis and patients with different parasites or who are infected with other pathogens). Although some of these immunodiagnostic methods are sensitive, they frequently give false positives or false negative. Finding out more about the *Echinococcus* antigens is necessary to get a sufficient specificity and taking more advantage of the greater sensitivity of the assay. The diagnostic sensitivity and specificity of immunological tests depend on two aspects which are (i) the quality and purity of the antigen, (ii) the nature of immunoglobulin, (iii) the principle of the chosen test [5, 50].

The development of immunodiagnostic studies of echinococcosis has a great and positive impact to treat the disease in the early stage of infection and to discover more information about the characterisation of the parasite antigens. Identification of the specific antigens of *Echinococcus spp.* still remains a crucial necessity for the researchers to take full advantage and get a sufficient specificity and sensitivity of immunodiagnostic assays in humans and animals. Most serological techniques for immunodiagnostic

echinococcosis use parasitic antigens to detect the antibodies in sera. Protoscoleces, fluid and membranes of the cysts have been used as an easy source of *Echinococcus* antigens for diagnostic purposes. IHA assay and ELISA have been used to detect the antibodies against the parasitic antigens. Although some of these immunodiagnostic methods are sensitive, they frequently give false positives or false negative. Recently, *Echinococcus* antigens have been used to detect the antibody of infected humans using some techniques such as Immunoelectrophoresis (IEP), monoclonal antibodies, Western Blotting and immunoprecipitation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) [5, 52].

1.8.4 Polymerase Chain Reaction (PCR)

Molecular diagnostic tools are another technique to diagnose *Echinococcus spp.* and characterise *Echinococcus* antigens to use recombinant DNA procedures. The modern diagnostic methods constructed on DNA hybridization and amplification of the specific genes of various species of *Echinococcus* using polymerase chain reaction (PCR) is starting to be practiced for the investigation and detection of *Echinococcus spp*. infection [5, 49].

To take advantages and disadvantages of serological diagnostic assays, it needs to make comparison between different diagnostic techniques to know to what extent these techniques are sensitive and specific. The table below shows the previous studies to diagnose echinococcosis based on different antigens.

Table 1: Summary of the antigens, antibodies, tests which have been used to diagnose *Echinococcus spp.*

Ref.	Antibody	Antigen	Accession Nu. (NCBI)	Test	Principle	Result
[53]	N/A	Hydatid cyst antigen (P1)	DNA AJ508037	IEP	Isolated fraction from cyst fluid as antigens and reacted with the sera of patients infected with echinococcosis. The immunoreactive component purified using Diethylaminoethyl (DEAE) cellulose to analyse the antigens	N/A
[54]	N/A	Hydatid cyst antigen (P1)	DNA AJ508037	IEP	Analyse the antibodies response in infected human	analysis the antibodies against *Echinococcus* antigen
[55]	N/A	Hydatid cyst Antigen A & Antigen B	Antigen B mRNA HM357145	IEP	Purified the antigens from cyst fluid	They could purify the two antigens Antigen A & Antigen B
[56]	N/A	Hydatid cyst Antigen A & Antigen B	Antigen B mRNA HM357145	IEP		Detecting antibodies in the sera of patients with hydatid cysts antigens A & B
[57]	N/A	Hydatid cyst Antigen 4 & Antigen 5	Ag5 mRNA HM055370	IEP & SDS PAGE	Evaluated the immunoreactivity of the antigen 4 & 5 with the sera of patient with hydatid infection. Purification of these two antigens.	Detecting antibodies in the sera of patients with hydatid cysts antigens 4 & 5. Negative reaction to the antigen 4 with the sera of some patients.
[58]	N/A	Hydatid cyst Antigen Band 7	N/A	Salt precipitation & Density sedimentation	Measure the sensitivity and specificity of Band 7 antigen.	Band 7 Fraction is very useful for serological diagnosis and gives a high sensitivity and specificity in diagnosis.
[59]	IgG	Hydatid cyst Antigen CAg	N/A	ELISA & IEP	Immunized rabbits with the CAg antigen and measure the level of specific IgG. To measure the specificity of the IgG reaction.	IgG titter was high
[60]	IgG, IgE & IgA	Alveolar cyst Em2 antigen	DNA AY324068	ELISA	Measuring the antibodies titter before and after treatment with mebendazole.	Diagnostic sensitivity for the detection of Specific IgE was low 69 %. IgG decreased after two years of treatment. While, IgE & IgA disappeared.
[61]	N/A	Hydatid cyst antigens	N/A	Western blotting	Practical the sensitivity of immunochemical technique of Western blotting with antigen and immunoassay with antibodies in the sera of infected human.	N/A
[62]	N/A	Hydatid cyst Antigen 5	mRNA HM055370	Monoclonal Antibodies & IEP & Western blotting	Generate a series of monoclonal antibodies against Antigen 5	Generate a series of monoclonal antibodies against Antigen 5

[63] [64]	Antibodies	Alveolar cyst Em2 Antigen	DNA AY324068	ELISA	To detection the disease in early stage	Antibodies level decreased after removing the cyst by surgery. The test shows high specificity and sensitivity
[65]	IgE	*Echinococcus* granulosus antigens & *Echinococcus* multilocularis antigens		Radio-allergo-sorbent test	Demonstrate parasite- specific IgE. Circulating and basophil-bound specific IgE against *Echinococcus* antigens in patients with alveolar echinococcosis. Measuring specific degranulation and histamine release The abilities of E. granulosus and E. multilocularis antigens to reveal bound and circulating IgE antibodies were also assayed	Diagnostic sensitivity for the detection of Specific IgE was very low 61 %. Determined the clinical symptoms of immediate-type hypersensitivity (ITH) in patients with E. granulosus but NOT in patients with E. maltilocularis. Basophil-bound sIgE were revealed in every patient by histamine release and degranulation tests. E. multilocularis antigenic preparation induced a histamine release considerably higher than E. granulosus. The sensitivity of the tests used to detect basophil-bound sIgE was higher than that of the usual serological tests, and the basophil degranulation test could be used to confirm diagnosis of alveolar echinococcosis.
[66]	IgG & IgM	Hydatid cyst antigens	N/A	Western blotting & SDS PAGE	Investigate if there is any antibodies can pass through the membranes of hydatid cyst radiolabelled antigen and analysis of the immunoprecipitates	Found that the IgG in sera (NOT IgM) penetrate in the hydatid cyst fluid.
[67]	N/A	Hydatid cyst fluid antigens	N/A	ELISA & IHA	Evaluation the validation of ELISA and IHA in term of sensitivity and specificity	The sensitivity and specificity of ELISA were high 96.7% & 97.5% respectively. The sensitivity and specificity of IHA were less than ELISA 86.7%, &95% respectively.
[68]	N/A	*Echinococcus* multilocularis protein & *Echinococcus* multilocularis antigen B8/1 & E. granulosus protien	mRNA AY191243	ELISA	Measure the sensitivity and specificity of ELISA to diagnose and differentiate between E. granulosus and E. multilocularis	Peptides identified unsuccessful to differentiate between *E. multilocularis* and *E. granulosus* infection. The best peptide performing was 57% sensitivity and 94% specificity. E. multilocularis antigen B8/1 presented strong reactivity to sera from patients infected with E. multilocularis and E. granulosus. Single peptide antigen cannot offer appropriate diagnostic sensitivity while several peptides antigens improv e sensitivity.
[69]	IgG & IgG4	Hydatid cyst antigens AgB antigen	DNA AY871019	ELISA	Evaluation the sensitivity and specificity of ELISA	Anti-hydatid cyst fluid IgG ELISA gave 92.4% sensitivity and 92.6% specificity. Low sensitivity of anti AgB IgG ELISA (84%), and the best specificity 94.4% with less cross-reaction with sera

						of infected patients with other parasites.
[70]	N/A	AgB2 & Recombinant AgB2	mRNA HM357146	Tajima's D test & ELISA	Evaluation the sensitivity and specificity of ELISA	Tajima's D test proved negative selection pressure. The sensitivity and specificity of the Recombinant AgB2 ELISA was 96.7 and 94.7 respectively. No cross reactivity was found with other parasites because cross reactive antigen was expressed intracellularly. AgB2 is the appropriate immunological indicator for detection, diagnosis and development of the disease.
[71]	N/A	Hydatid cyst fluid antigens Native antigen B & Recombinant antigen B8/1 (RAgB)	Antigen B DNA AF362442 B8/1 DNA AF362443	ELISA	Comparing the sensitivity and specificity of immunoserological assay using two *Echinococcus* granulosus antigens; native antigen B & recombinant antigen B8/1 (RAgB)	Both native antigen B and RAgB had high sensitivity, but RAgB showed a brilliant specificity in comparison with antigen B. Recombinant antigen showed a high diagnostic preference ratio in comparison with other evaluated antigen for diagnosis of hydatid disease.
[72]	N/A	Subunit of antigen B (AgB8/1)	DNA AF362443	using of synthetic peptidesp176 as antigens & ELISA (p176 ELISA)	Examined the performance of the synthetic peptide p176 for the diagnosis of lung hydatid disease	AgB8/1 has shown hopeful for diagnosis of hydatid disease. Positive serum antibody reactions were related with the existence of synthetic peptidesp176and with the number of cysts. P176 ELISA achieves well for the diagnosis of lung hydatid disease and adds a readily reproducible diagnostic assay to the existing diagnostic tests. ELISA test is very sensitive and specific.
[73]	N/A	*Echinococcus* multilocularis metacestode Antigens Em	DNA AY265908	ELISA	Analyse the cellular production and plasma levels of pro-inflammatory cytokines lL-17A, lL-17B, lL-17F and their receptors lL-17RA (slL-17RA) and IL-17RB (sIL-17RB)	Increasing the levels of IL-17B and slL-17RB were pointedly observed. Whereas, lL-17F and slL-17RA were decreased in patients with AE. The production of lL-17F and slL-L7RA in response to E. multilocularis antigens was low in patient's sera. Levels of slL-17RB were high in patient's sera.
[74]	N/A	*Echinococcus* granulosus EgEno gene	N/A	ELISA & Western blotting & SDS PAGE	To clone and express EgEno gene of *Echinococcus* granulosus, and to examine the immunogenicity and diagnostic importance of recombinant EgEno.	EgEno of E. granulosus was cloned and expressed in E. coli BL21 (DE3) successfully, and could be used as candidate antigen of immunodiagnosis for hydatid disease. Western blotting indicated that the antigenicity of the protein was specific and the sensitivity of diagnosis by ELISA for hydatid cyst was very high

The table above shows that the ELISA technique, nowadays, is the best method for diagnosing and measuring the antibodies level against *Echinococcus*. The technique shows a satisfying sensitivity and specificity when using some of *Echinococcus* antigens. However, it still does not give a high specificity due to the cross-reactivity with the other parasites. In addition, it is not possible to diagnose the disease in the early stage of infecti on using the previous methods.

1.9 Current Treatment of Echinococcosis

In the past, surgery was the only treatment for cystic echinococcosis. Chemotherapy and PAIR (percutaneous aspiration, injection of chemicals and re-aspiration) have been used to replace surgery as effective treatments for *Echinococcus* infection. However, chemotherapy and PAIR may not be effective for very late infection. Therefore, surgery remains the most active treatment to remove the cyst in this case. Two benzimidazolic are the only antihelmintics drugs can destroy the parasite, mebendazole and albendazole. Albendazole and mebendazole are both effective but show different efficiency. Albendazole is considerably more active than mebendazole in the treatment of echinococcosis. Treating *Echinococcus* infection continuously is more preferable and has been administered for periods of up to two years without substantial side effects [46, 75-80]. *Echinococcus* cysts that are less than 5 cm can be treated with albendazole alone. However, cysts that are more than 5 cm cannot be treated with albendazole alone and required further treatments which are PAIR or surgery [46]. Overall, albendazole has been verified to be a valuable improvement in the treatment of echinococcosis. Praziquantel has recently been suggested to be used additionally with albendazole [81].

Hence, a definitive diagnosis of echinococcosis in the early stage of infection is significant important to rule out PAIR and surgery treatment.

1.10 RS-ATL8 Reporter Assay

Nakamura *et al.* (2010) [82] and (2012) [83] developed a new technique for measuring the activation of basophils and mast cells through the cross-linking of specific IgE (sIgE) bound to the high affinity receptor FcεRI on mast cells and basophils in the presence of allergen (example egg white and environmental allergens). In this technique, rat basophil leukaemia cells (RBL-SX38), created by Wiegand *et al.* 1996 [84], were used for indicating the presence of allergen-specific IgE (sIgE) in the tested blood samples. Because RBL cells do not bind human IgE, Wiegand group started transfecting the cells with three human IgE FcεRI receptor cDNA encodings; α, β and γ chains. Moreover, Nakamura and co-authors transfected the RBL-SX38 cells with nuclear factor of activated T-cells (NFAT) regulated luciferase reporter gene, which was taken from firefly to express luciferase as an indicator of activation the cells (Figure 1.8). After transgenic the RBL-SX38 cells with these different cDNA encodings, the cells were named RS-ATL8 and the technique was called EXILE (IgE cross-linking (X) Inducing Luciferase Expression) assay which is used as a new method for measuring the sensitisation of basophils and mast cells using a patient's IgE and an allergen (the principle of the assay will be illustrated in details in chapter 6).

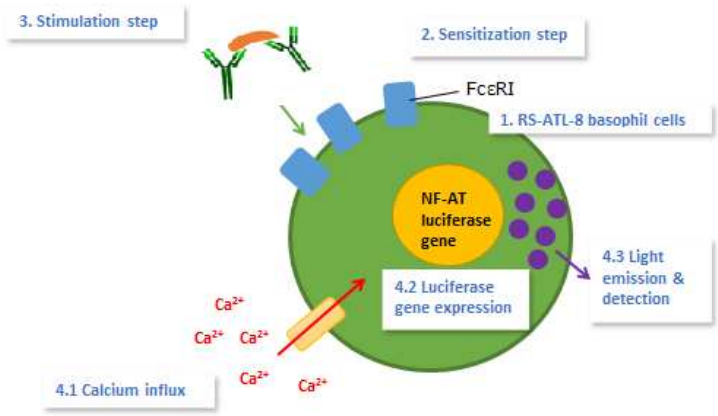


Figure 1.8: Schematic diagram illustrating the principle underlying the use of humanized RBL (RS-ATL8) reporter system for detection of sIgE in patients'sera. Reporter cell line are incubated overnight with diluted sera to be tested and stimulated the next day with the suspected allergen. In the presence of sIgE, FcεRI crosslinking by the allergens will result in activating a signal transduction cascade ultimately resulting in the nuclear translocation of NFAT, which activates the luciferase reporter gene. This can be measured after 3 h of stimulation with the suspected allergen. (Source: Franco H. Falcone).

1.11 Project Aim and Objectives

The motivation behind this study was to establish a workflow which is suitable for screening of *Echinococcus* allergens and use this method as a novel diagnostic tool for echinococcosis.

It is well known that *Echinococcus* has a great role for inducing IgE and triggering allergy in the infected patients. Also, it has been reported that *Echinococcus*-specific IgE is present in all the patients and related with the severity, and the histamine release by circulating basophils stimulated with *Echinococcus* allergens is present in all patients with both species of *Echinococcus*; *granulosus* and *multilocularis*. Therefore, the RS-ATL8 reporter assay could also be used for *Echinococcus* infection diagnosis.

Hence, the main aim of this study was to assess the use of RS-ATL8 Reporter System for detection of the specific human IgE against *Echinococcus spp*. that binds to the parasite's allergens and is ability to activate the reporter cell line, and this technique may overcome the limitations of current echinococcosis diagnosis methods and give a better specificity and higher sensitivity, and may also diagnose the disease in the early stage of infection.
This aim will be achieved through the following specific objectives:

1) Optimising conditions of the RS-ATL8 Reporter System.
2) Surveying the literature to identify which antigens are used for immunodiagnosis purposes and which of these are potential allergens suitable for use in the EXILE System. Hence, a list of *Echinococcus* candidate proteins was chosen for the investigation into their diagnostic value. These proteins are EF1-α, Ag5, AgB2, Cyclophilin, Eg19, EgTeg and EgTPx. EF1-α, Ag5, AgB2 and Cyclophilin were chosen because they have been reported as allergens and have potential relevance to

producing IgE antibody in the host. While, Eg19, EgTeg and EgTPx were chosen to be used as a control.

3) Incorporation of two tags (C-Myc and His) at the C-terminal of *Echinococcus* antigens using PCR. This step allows for the detection and purification of the antigens.
4) Expressing the *Echinococcus* antigens and choosing the suitable expression system.
5) Purification of *Echinococcus* antigens using immobilized metal ion affinity chromatography (IMAC) method.
6) Testing these antigens with available sera of *Echinococcus* infected patients using RS-ATL8 Reporter System.

Chapter 2: General Materials and Methods

This chapter reports general procedures used throughout this project. The specific details to each chapter can be found within their own Materials and Methods section.

During this study, a wide range of techniques was used to achieve every single objective. The first aim of this study was to express and purify seven *Echinococcus* recombinant proteins which are EgEF1-a, EgAg5, Cyclophilin, EgAgB2, Eg19, EgTeg and EgTPx in different expression hosts. To reach this aim, mammalian and bacterial cell culture, standard molecular biology and chromatographic techniques were used. Maps of the specific expression vectors used are illustrated in Section 2.5.

The second aim of this study was to assess the RS-ATL8 reporter cell line for diagnosis of *Echinococcus* infection, using the recombinant expression antigens and sera from *Echinococcus* infected individuals.

2.1 Preparation of Chemically Competent Cells

Escherichia coli DH5-α, XL-10, BL-21(DE3) and Rosetta 2(DE3) strains (Invitrogen, UK) were grown overnight at 37˚C with a shaking speed of 250 rpm, after inoculation in 10 ml of SOB medium (Tryptone 20 g, yeast extract 5 g, NaCl 500 mg, KCl 10 ml, adjust to 1 L with dH_2O). The next day, 5 ml of overnight culture was inoculated in 125ml of fresh SOB medium in 1 litre flask and incubated at 37˚C until OD_{600} reached 0.5. The culture was then placed on ice and split into 4 centrifuge tubes, each contains 30ml. The cells in the 4 tubes were then centrifuged at 2500 xg for 10 minutes at 4˚C. The bacterial pellet from one centrifuge tubes was re-suspend in 20 ml of Inoue Buffer ($MnCl_2$ $4H_2O$ 10.88 g, $CaCl_2$ $2H_2O$ 2.2 g, KCl 18.65 g, 0.5M HEPES 20ml, adjust to 1 L with dH_2O), followed by transferring the re-suspended bacteria into the second centrifuge tube and re-suspended

again. This step was repeated for the other two centrifuge tubes to obtain 40ml in total of bacterial cells in Inoue Buffer, in two centrifuge tubes. The two tubes were then centrifuged again at 2500 xg for 10 minutes at 4˚C, and re-suspended in 5ml of Inoue Buffer each and then pooled together to obtain 10ml of cell-containing buffer in total. 750µL of DMSO was added to the cells and mixed gently, and the bacterial cells were incubated for 10 minutes at 4˚C. Finally, aliquots were prepared by applying 100µl of cell-containing buffer into pre-chilled Eppendorf tubes and they were frozen in liquid nitrogen and then kept at -80˚C freezer until use.

2.2 Bacterial Culture and Growth Conditions

E. coli was grown in Luria-Bertani agar or broth media (Sigma-Aldrich) with appropriate antibiotics at 37°C (unless mentioned) in a shaking incubator (Barnstead Lab-Line MaxQ 4000) at 250 revolutions per minute (rpm). Antibiotics used were ampicillin (100 µg/ml) and/or chloramphenicol (100 µg/ml) (Sigma-Aldrich). *E. coli* was used for the reproduction of plasmids and for expression of 6X histidine-tagged recombinant proteins. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega) 0.25 mM, 0.5 mM or 1 mM was used to induce the bacteria for protein expression.

2.3 Molecular biology

Molecular biology experiments were performed using nuclease-free molecular biology water (HyClone, UK), pyrogen-free filter tips, PCR tubes (Starlab, UK), DNA polymerase (New England Biolabs, UK).

2.3.1 Data Collection

A number of *Echinococcus* proteins were chosen taking into account two criteria:

1- Their known allergenicity or capability to bind IgE, according to the published literature, such as EF1-α, Ag5, AgB2 and Cyclophilin.

2- Putative exposure to the host immune system, such as secretory or tegumental proteins.

3- Three other *Echinococcus* antigens, which have not been reported as allergens, were chosen to be used as a control. These antigens were Eg19, EgTeg and EgTPx.

The sequences of genes and proteins were obtained from http://www.ncbi.nlm.nih.gov/. The genes were selected depending on information as mentioned previously. Around thirty genes were chosen, but after collecting enough relevant information it was confirmed that some of these genes have no relationship with allergenicity. Thus, only seven genes were taken into account. For more information about the selected proteins see table 2.1.

Table 2.1: Summary of the name, Accession Number, size, location and function (where known) of *Echinococcus* selected proteins (putative allergens).

Name of Protein	Other Name	DNA / Protein Accession Number	Predicted size amino acid	Expression stage	Function	Ref.
Antigen 5	Ag5	JF970202 mRNA AFI71096 Protein	484 aa	Hydatid fluid	Serine protease and relevant in the development of the metacestode	[8] [85]
Elongation Factor-1 alpha	EF1-a	AB306934 DNA BAF63677 Protein	448 aa	Protoscoleces	Responsible for the GTP- dependent binding of minoacyl-tRNAs to ribosomes	[8]

Cyclophilin	EA21	AF431734 DNA AAN62875 Protein	162 aa	Protoscoleces and Hydatid fluid	Cyclophilin exhibits peptidyl-prolyl cis-trans isomerase (PPiase) activity, and it plays a role in the folding of many essential proteins.	[8, 86, 87]
Antigen B2	AgB2	HM357146 DNA ACO55060 protein	90 aa	Hydatid fluid	A protease inhibitor and a strong Th-2 inducer	[8]
Eg19	Eg19	DQ840455 mRNA ABI24154 Protein	146 aa	protoscoleces	Unknown	[85]
Tegumental protein	EgTeg	AAX20156 Protein	105 aa	protoscoleces	Unknown	[85]
Thioredoxin Peroxidase protein	EgTPx	EU696759 DNA ACD70342 Protein	193 aa	Protoscoleces and brood capsules	Antioxidant role. It has a role in protection against oxidative damage by metal-catalyzed oxidation	[85, 88]

2.3.2 Designing Primers

After collecting the genomic and proteins information about the genes of interest using http://www.ncbi.nlm.nih.gov/, signal peptide sequences at 5' end were scanned using SignalP 4.1 Server and then removed from the protein sequences of the parasite, before designing primers.

For each gene, both forward and reverse primers were designed using either http://www.promega.com/techserv/tools/FlexiVectorTool/ or Snap Gene software. Also, the software has been used to determine the presence of unique binding sites within the gene sequence. Moreover, NEB Tm Calculator was used to investigate primer sequences for characteristics such as melting temperature. Before the primers were ordered, a His-Taq (6X Histidine), and C-Myc Tag were added at 3' end. Finally, stop codon was added to the reverse primers in order to stop the translation processes.

2.3.3 Polymerase Chain Reaction (PCR)

After ordering the primers which had been designed previously, the *Echinococcus* genes EgAg5, EgEF1-alpha, Cyclophilin, EgAgB2, Eg19, EgTeg and EgTPx were amplified from larva stage of *Echinococcus* cDNA library which was obtained as a kind donation from Estela Castillo, University of the Republic, Uruguay.

The enzymes used for PCR reaction were Taq Polymerase Hot Start Master Mix Ready Mix™ Red Taq (Sigma-Aldrich), Phusion High-Fidelity DNA Polymerase and Proofreading Q5 Hot Star Master Mix (New England Biolabs, UK). 7.5 µl of enzyme was used for each PCR reaction, and was added to 0.75 µl of relevant Forward or Reverse oligonucleotide primers (10 µM), followed by adding 1 µl of cDNA library (template) and 5 µl of nuclease free molecular biology water to complete the volume of PCR reaction to 15 µl. The cycling reaction for PCR using Taq Master Mix, Proofreading Hot Star Q5 and Phusion High-Fidelity polymerases was as illustrated in Tables 2.2 and 2.3.

Table 2.2: Thermocycling conditions for a routine PCR using Taq Polymerase Hot Start Master Mix Red Taq.

Step	Temperature	Time
Initial Denaturation	94°C	30 seconds
35 Cycles	94°C	10 seconds
	40-64°C	30 seconds
	72°C	30 seconds/kb
Final Extension	72°C	1:30 minute
Hold	4°C	

Table 2.3: Thermocycling conditions for a routine PCR using Proofreading Q5 Hot Star Master Mix and Phusion High-Fidelity DNA Polymerase.

Step	Temperature	Time
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C	10 seconds
	40-64°C	10 seconds
	72°C	30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4°C	

The annealing temperature gradient was used to identify experimentally the optimal annealing temperatures for all primers used in this study, and have been illustrated in tables in the relevant chapters. PCR tubes (Starlab, UK) and a PTC-200 Thermal Cycler (MJ Research, Canada) were used to carry out the reactions. The PCR was repeated again using the best conditions after optimisation for amplification of the target genes.

2.3.4 Agarose Gel Electrophoresis

The PCR products were separated using agarose Gel Electrophoresis. Tris Borate EDTA (TBE) 10x buffer (Fisher BioReagent, UK) was diluted to 0.5x in deionised water, and used for preparing the gel and as a running buffer for gel electrophoresis. Agarose gel was prepared an hour before running the gel, consisting of 1% w/v agarose (Sigma-Aldrich) in 0.5x Tris EDTA Borate buffer and stained with 10 µg/ml Ethidium Bromide (Thermo Fisher Scientific, USA) or Cybr Gold nucleic acid gel stain (Thermo Fisher Scientific, USA). The gel was added in a gel tray and suitable comb were set in the gel to create wells for sample loading. Then, the gel tray was left at room temperature for 30 minutes. 10 µl of the PCR

products were mixed with 2µl of 6× loading dye (New England Biolabs) before loading in the gel. Afterwards, the DNA fragments were run in the gel at 110 mA for approximately 60 minutes. The gel was visualised using a Syngene® Gene Genius Imaging System (Syngene, UK). Finally, the size of DNA molecules was defined by comparing with the 100bp or 1Kb DNA molecular weight markers (New England Biolabs).

2.3.5 PCR Products Extraction and Cleaning Up

After running the gel, the amplicons bands of the genes of interest Ag5, EF1-alpha, Cyclophilin, EgAgB2, Eg19, EgTeg and EgTPx were excised from the agarose gel using a sterile scalpel blade under UV radiation, followed by extraction using Wizard SV Gel Extraction Kit (Promega, UK), according to the manufacturer's protocol.

The concentrations and purity of the target DNAs were determined using Nanodrop™ ND-1000 spectrophotometer (NanoDrop Technologies-Thermo Fisher Scientific) by measuring the absorbance ratio at 260nm and the purity of the DNA was measured at 280nm.

2.3.6 Restriction Endonuclease Digestion and Ligation of DNA

PCR products and plasmids have been cleaved at specific nucleotide sequences using appropriate restriction endonuclease enzymes (See Table 2.4) from New England Biolabs or Promega according to the manufacturer's protocols (the details of the appropriate utilised enzymes are mentioned in their own chapters). Afterwards, the digested DNAs were incubated with T4 DNA ligase (Promega, UK) for ligation the genes of interest into appropriate plasmids.

Table 2.4: Summary of the names and the sequences of the restrictions enzymes which used in this study.

Restriction Enzymes	Sequences
SgfI	GCGATCGC
PmeI	GTTTAAAC
NedI	CATATG
BamHI	GGATCC
EcoRI	GAATTC
XbaI	TCTAGA

2.3.7 Bacterial Transformation and Colonies Screening

The ligated plasmid DNA with the gene of interest was transformed into previously prepared chemically competent *E. coli* DH5-alpha or XL-10 cells (Invitrogen, UK) using a heat shock technique as follows: 100 µl of *E. coli* bacteria was thawed in ice for few minutes, then ~5µl (500 ng) of plasmid DNA was added to bacterial cells and mixed gently by flicking the tube. The mixture was incubated on ice for 30 minutes and then the cells were heat shocked in a water bath at 42°C for 90 seconds. After that, the cells were placed on ice for 2 more minutes, directly after heat shocking. Afterwards, 1ml of SOC medium (Sigma) (10 mM sodium chloride, 10 mM magnesium chloride, 20 mM glucose, 2% tryptone and 0.5% yeast extract) was added to the transformed bacteria and then incubated in a shaking incubator (Barnstead Lab-Line MaxQ 4000) at 37°C and 250 rpm for 1 hour, for expression of antibiotic resistance genes. Finally, 100 µl of the bacterial culture was spread on a petri dish containing LB agar and appropriate antibiotic

(100µg/ml ampicillin or/and 100µg/ml chloramphenicol), for selection of transformed bacteria. The petri dishes were then incubated at 37°C upside down, overnight.

The next day, PCR colony screening was performed by picking single colonies from the petri dishes using sterile loops and inoculated into 20µl of LB medium containing appropriate antibiotic, in PCR tubes. 1µl of each colony was used as template for PCRs colony screening.

2.3.8 Plasmid Purification and Sequencing

After confirming the sizes of the ligated plasmids using agarose gel electrophoresis, the colonies which gave positive results were inoculated in 20ml of LB medium containing appropriate antibiotic and grown in a shaking incubator at 37°C and 250 rpm overnight, for glycerol stocks and plasmid purification using QIAprep Spin Miniprep Kit, QIAgen (Promega, UK) according to the manufacturer's instructions. The concentration and purification of the plasmid were determined using NanoDrop 3000 spectrophotometer (Thermo Scientific, USA).

The plasmids were sequenced and analysed using Sanger Sequencing Service (Source Bioscience, Nottingham) to confirm that the target recombinant genes were inserted into the plasmids successfully and the sequences are lacking for any point mutations which could have been introduced during the PCR reactions and cloning steps. The National Centre for Biotechnology Information (NCBI) database was used to compare the deduced amino acid sequences with the reference amino acids sequences.

2.4 Recombinant Protein Expression

In this study, three different expression systems with varying expression hosts and plasmids were used to produce the *Echinococcus* allergens and antigens, all this has been illustrated in the relevant chapters (See sections 3.2.2, 4.2.1.3, 4.2.2.3, 4.3.2, and 5.2.3).

2.5 Expression Vectors Used in This Study

A brief summary of the functional features of the plasmids utilised for expression of recombinant *Echinococcus* proteins is mentioned below.

2.5.1 pF3A WG (BYDV) Flexi Vector

pF3A WG (BYDV) Flexi Vector (Promega, UK) is designed to be used with the Flexi System, which allow easy entering and transferring of a gene from one Flexi Vector to another to express in different expression system (gene shuttling). pF3A WG (BYDV) Flexi Vector contains translation enhancer (TE) sequences from RNA plant virus which is barley yellow dwarf virus (BYDV). This translation enhancer sequence enhances the production of protein in wheat germ extract. The vector also contains SP6 promoter which assists transcription of the downstream gene of interest. In addition, the vector has several different restriction sites including SgfI and PmeI for insertion and transferring of the target gene from pF3A WG (BYDV) Flexi Vector to other Flexi Vectors for different expression system. Moreover, the vector holds a lethal barnase gene for positive selection of the insert. Also, the vector carries ampicillin-resistance gene for selection of the plasmid in *E. coli.*

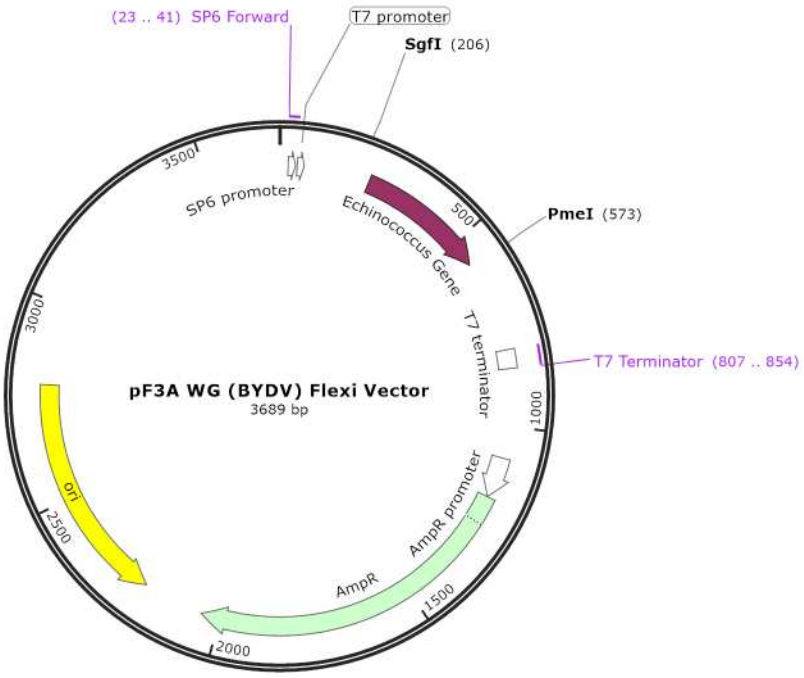


Figure 2.1: pF3A WG (BYDV) Flexi Vector Map. The lethal barnase gene was removed and replaced with the gene of interest (*Echinococcus* gene).

2.5.2 pF1A T7 Flexi Vector

pF1A T7 Flexi Vector (Promega, UK) contains T7 RNA polymerase binding site promoter for bacterial expression. In addition, the vector carries a lethal barnase gene for positive selection of the insert and ampicillin resistance gene for selection of the plasmid. Moreover, the vector contains several restriction sites including SgfI and PmeI. Inserts can effortlessly be transferred from the pF1A T7 Flexi Vector to other Flexi Vectors for expression in different hosts. T7 promoter and T7 Terminator were used for sequencing the inserted genes and colonies screening.

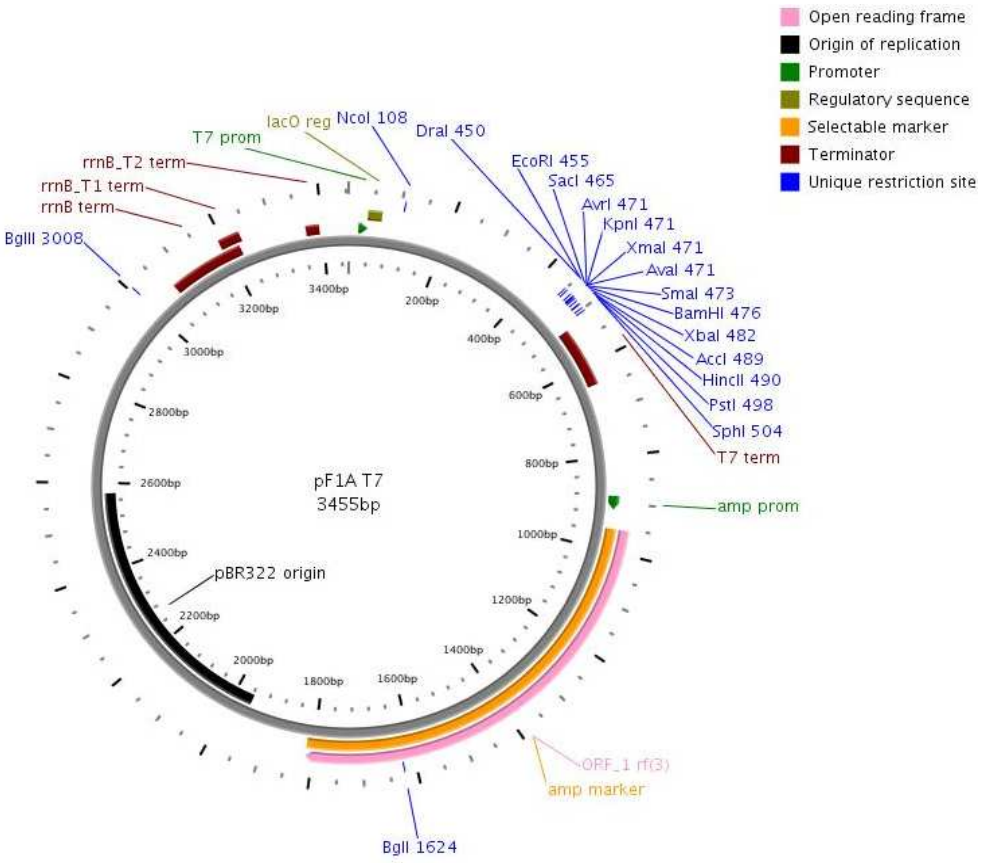


Figure 2.2: pF1A T7 Flexi Vector Map.

2.5.3 pET-3a Bacterial Expression Vector

Expression of the genes of interest cloned into pET-3a Vector (Novagen) occurs under the driving of T7 promoter, the binding site of T7 RNA Polymerase. Thus, the production of the proteins can be very efficient in hosts that have a source of T7 RNA polymerase, such as *E. coli* BL21 (DE3). In addition to T7 promoter (T7P), the vector carries T7 Terminator (T7T) as well. T7P forward and T7T reverse primers were used for sequencing the inserted genes and for PCR colony screening. pET-3a also carries ampicillin resistance gene for plasmid selection (See figure 2.3).

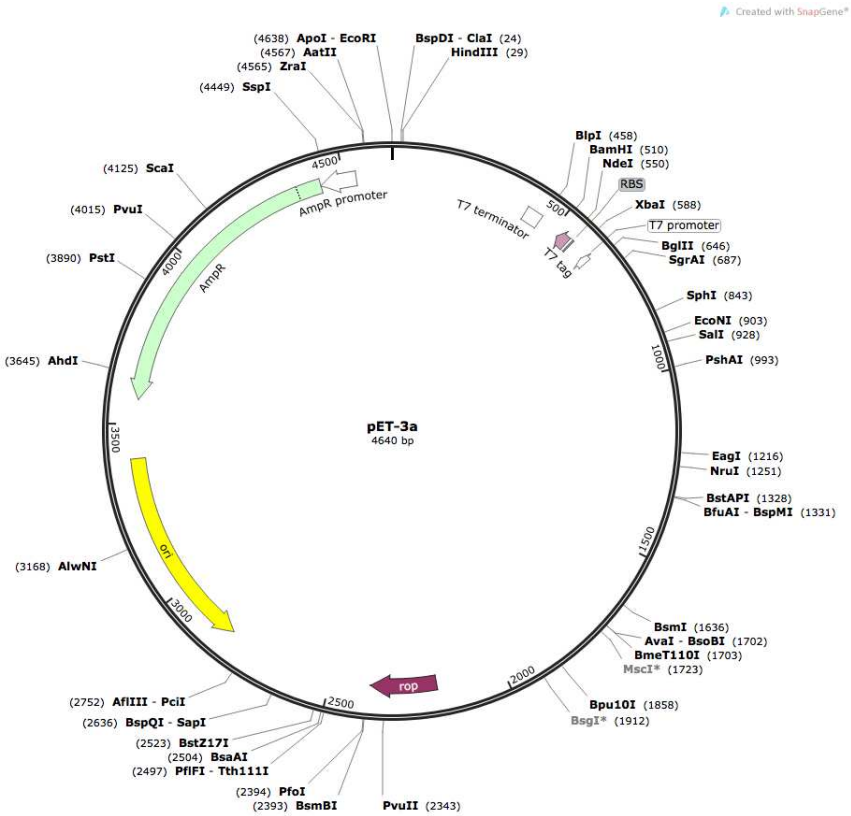


Figure 2.3: pET-3a Vector Map.

2.5.4 pTT5 Mammalian Expression Vector

pTT5 vector used in this study for mammalian expression. This vector is licensed by the Canadian National Research Council (CNRC). pTT5 vector carries G418 resistance gene for selection in the HEK293-6E cell line and ampicillin resistance to assist bacterial selection. This vector furthermore carries an IL-2 signal peptide, a C-MYC tag (EQKLISEEDL) for antibody detection and also a 6× His-tag for purification purposes located at the C-terminus. The vector designed to contain a sequence to act as a very strong promoter, which is cytomegalo virus promoter (CMV). Thus, the expression of the *Echinococcus* gene

is directed by the CMV promoter, which enhances the expression of the IL-2 signal peptide, protein of interest, C-Myc Tag and 6X His-Tag, all in the same reading frame. This is achieved by creation of high level of messenger RNA (mRNA), which can then be translated into protein.

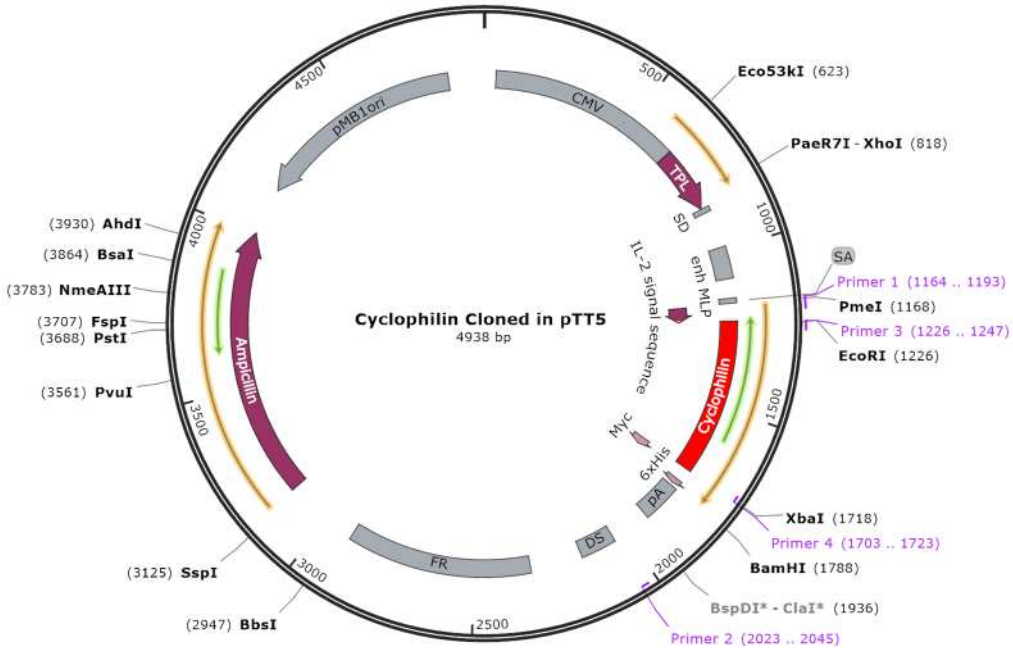


Figure 2.4: pTT5 Vector Map.

pTT5 vector map shows the cloning region which was used for cloning *Echinoccous* gene (Cyclophilin) between the restriction sites EcoRI and XbaI.

Chapter 3: Developing a Recombinant *Echinococcus* Proteins Expression in Wheat Germ Lysate

3.1 Introduction

The first objective of this research was to develop a method to express and purify *Echinococcus* proteins recombinantly. However, as it is known that the expression of recombinant proteins can be difficult or non-efficient due to several issues, such as, low yield of produced protein, deficiency of proteins solubility and refolding from inclusion bodies [89], which consider as a time consuming, as well as the protein may not regain its full function [90], [89]. Thus, in this study, we screened different expression vectors and hosts to find an efficient technique for producing several recombinant *Echinococcus* proteins to be used in this study.

Indeed, it has been reported in the literature of successful proteins expression in cell-free expression system such as wheat germ lysate (WGL) [91]. Wheat germ lysate expression system is considered as the fastest and easiest technique for protein expression in less than two hours and providing quantities of proteins in micrograms [92]. In addition, wheat germ lysate allows producing of various correctly folded proteins in short time [93]. So, using the eukaryotic wheat germ extract for protein production has many advantages which may increase the solubility and correct folding of eukaryotic multi-domain proteins which could be difficult to express in prokaryotic cells. Also, it does support disulphide bond formation which may be important for correct folding [94, 95]. Moreover, wheat germ is a storage form of the plant; therefore, the endogenous mRNA in this form is less than other expression hosts. This will lead to express less endogenous proteins which will help us to recognise the target protein more easily.

The expressed proteins through wheat germ lysate can be analysed using Fluoro Tect™ Green lys tRNA which is a lysine tRNA labelled with a BODIPY®-FL. Since the protein is translated in the presence of a green fluorescent lysine tRNA, the newly made protein will become fluorescent. Because of lysine is a frequent amino acid, therefore most proteins will be labelled.

Our initially chosen strategy therefore was the Wheat Germ Lysate System to express seven *Echinococcus* antigens and allergens.

3.2 Experimental Procedures

3.2.1 Antigen Cloning

Echinococcus antigens were cloned into a suitable vector for cell-free wheat germ lysate expression system which is pF3A WG (BYDV) Flexi Vector (Promega), using the Flexi Vector System (Promega) (See section 2.5.1). To achieve this, several procedures were followed.

3.2.1.1 Primer Design

For each gene, both forward and reverse primers were designed using the Flexi® Vector Systems and the software http://www.promega.com/techserv/tools/FlexiVectorTool. Since Flexi vector has *SgfI* and *PmeI* as restriction sites, these two restriction sites were added to the both primers, forward and reverse, respectively. Hence, the forward primers were structured by adding SgfI restriction site first, then a start codon ATG, as this codon was removed from the gene after signal peptide cleavage at 5' end. Whereas, the reverse primers were constructed by adding a 6X His-tag followed by valine codon for simplification of sub-cloning the target genes into different Flexi Vectors Expression System. Afterwards, stop codon was added to the reverse primer followed by PmeI restriction site at the 3' end. The primers names and sequences are scheduled in the Table 3.1.

Table 3.1: Primers for *Echinococcus* genes amplification to be expressed in cell-free wheat germ lysate.

Forward and reverse indicate sense and antisense primers, respectively. The both restriction sites; *SgfI* and *PmeI* are designated in the primer name and underlined in the oligonucleotide sequence. The red nucleotides refer to His-tag (6x Histidine codon) which was added to the reverse primer.

Gene	Primer's Name	Primer Sequence (5'-3')
EgEF1-a	EgEF1-a Forward_ *SgfI*	CGCGGCGATCGCCATGGGGAAGGAGAAGACGCA
	EgEF1-a Reverse_ *PmeI*	GAGCGTTTAAAC**GTGGTGGTGGTGGTGGTG**TTTT TTGGCCTTGGCGGCCT
EgAg5	EgAg5 Forward_ *SgfI*	TTGTGCGATCGCCATGTTGGAGCTCACTCTCGATC CAGATGAA
	EgAg5 Reverse_ *PmeI*	TTGCGTTTAAAC**GTGGTGGTGGTGGTGGTG**GACT GCGTAGCGGTTGATCCACT
EgAgB2	EgAgB2 Forward_ *SgfI*	GACCGCGATCGCCATGAAAGATGAGCCAAAAGCA CA
	EgAgB2 Reverse_ *PmeI*	GTCGGTTTAAAC**GTGGTGGTGGTGGTGGTG**CTTT GAATCATCATCTTTTTCT
Cyclophilin	Cyclophilin Forward_ *SgfI*	GACCGCGATCGCCATGGGCGTGAAGTGCTTCTTC GACATCT
	Cyclophilin Reverse_ PmeI	GTCGGTTTAAAC**GTGGTGGTGGTGGTGGTG**CAG CTGACCGCAGTCAGTAATCAGTACTTCT
Eg19	Eg19 Forward_ *SgfI*	GACCGCGATCGCCATGGAGGCTGAGGCAAAGTG CCTA
	Eg19 Reverse_ *PmeI*	GTCGGTTTAAAC**GTGGTGGTGGTGGTGGTG**ATCT CGAATACCATGTCCAACTTCTCT
EgTeg	EgTeg Forward_ *SgfI*	CACCGCGATCGCCATGGCTACAGACCCAACCACC ATGTC
	EgTeg Reverse_ *PmeI*	TTGTGTTTAAAC**GTGGTGGTGGTGGTGGTG**GTAG GTAGAGGATTGCCACACAAGA
EgTPx	EgTPx Forward_ *SgfI*	AGGAGCGATCGCCATGGCTGCTGTTGTTGGAAA
	EgTPx Reverse_ *PmeI*	GTCGGTTTAAAC**GTGGTGGTGGTGGTGGTG**CGA GCTCATGAACGACTTCAA

3.2.1.2 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Seven chosen genes representing allergens and antigens in *Echinococcus* were amplified twice from the life stage cDNA library, which was a generous donation by Estela Castillo, University of the Republic-Uruguay, using Red Taq Polymerase Ready Mix (Sigma-Aldrich) first, and then Q5 Polymerase (New England Biolabs) for taking advantage of its proofreading activity (See the details of reactions in General Material and Methods Section 2.3.3). Initially, gradients temperature was run to optimise the annealing temperature for the different primers for each gene. The PCR reactions were then run in 1% agarose gel electrophoresis, using 100bp DNA ladder (New England Biolabs) for reference, to confirm the successful PCR. The PCR products were extracted and purified from the gel using a Wizard SV Gel and PCR Clean-Up System (Promega, UK), as directed by the manufacturer.

3.2.1.3 Restriction Digest of PCR Product and Acceptor Flexi Vector

Following the PCR products extraction from the gel slices, Flexi enzyme blend SgfI and PmeI were used to digest the PCR products and given a purified DNA fragments with restriction sides according to the manufacturer's instructions of Flexi® System (Promega, UK). A digestion reaction was set up as illustrated in the Table 3.2.

Table 3.2: The table below shows the reaction components which were used to cut the PCR product with SgfI and PmeI.

Component	**Volume**
5× Flexi Digest Buffer	4 µl
Purified PCR Product (up to 500 ng)	12µl
Flexi Enzyme Blend (*SgfI* & *PmeI*)	4 µl
Nuclease- Free Water to a Final Volume of	20 µl

The Acceptor Flexi Vector was cut with same enzymes which were used to digest the amplicons DNA, the *SgfI* and *PmeI*. A typical digestion reaction which was used to digest the vector was set up as illustrated in the Table (3.3). Both reactions were incubated at 37˚C for 30 minutes. After the incubation time, the reaction was heated at 65˚C in a water bath for 20 minutes to inactivate the restriction enzyme.

Table 3.3: The table below shows the reaction components to cut the acceptor Flexi Vector with *SgfI* and *PmeI*.

Component	**Volume**
Nuclease- Free Water	12 µl
5× Flexi Digest Buffer	4 µl
Acceptor Flexi Vector (200 ng)	2 µl
Flexi Enzyme Blend (SgfI & PmeI)	2 µl
Final Volume	20 µl

3.2.1.4 Ligation of PCR Product and Acceptor Flexi Vector

The amplicon genes were inserted into Promega pF3A WG (BYDV) Flexi® Vector plasmid. Ligation reactions were implementing for the PCR products using T4 DNA ligase in Flexi ligase buffer according to the manufacturer's protocol of Flexi® System (Promega, UK). A ligation reaction was set up as illustrated in Table 3.4. Finally, the reaction was incubated for 1 hour at room temperature.

Table 3.4: The table below shows the reaction components for ligation the amplicons to the Acceptor Flexi Vector.

Component	Volume
2× Flexi Ligase Buffer	10 µl
Acceptor Flexi Vector (50 ng)	5 µl
PCR Product (Approximately 100 ng)	4 µl
T4 DNA Ligase (HC) (20u/µl)	1 µl
Nuclease- Free Water to a Final Volume of	20 µl

3.2.1.5 Transformation of the ligated Acceptor Flexi Vector into the *E. coli* DH5-alpha Strain

The ligated genes with the Acceptor Flexi Vector were transferred into the competent cells *Escherichia coli* DH5-alpha using the heat shock method. The mixture of cells and ligated plasmids were cooled on ice for 30 minutes then placed in a 42˚C water bath for 90 seconds and placed on ice again for 2 minutes. The culture was incubated in shaking incubator (Barnstead Lab-Line MaxQ 4000) at 37˚C and 250 rpm for 1 hour, after adding 1ml of SOC medium to the mixture. 100µl of the culture was spread on plates containing LB medium agar and ampicillin (100µg/ml). Afterwards, the bacteria were grown overnight upside down in incubator at 37˚C (See General Materials and Methods Section 2.3.7, for more details).

Next day, the plates were checked for bacterial colonies and the 12 colonies were picked for each gene and put into PCR tubes in LB medium containing 100µg/ml of Ampicillin. PCR was set up twice for each gene, using Taq Polymerase Ready Mix enzyme, with 12 PCR tubes each. The first PCR reactions were used SP6 as a forward primer (5'-

ATTTAGGTGACACTATAG-3') and specific reverse primers for each gene. Whereas, in the second PCR reactions SP6 was used as a forward primer and T7 Terminator (T7T) (5'-GCTAGTTATTGCTCAGCGG-3') was used as a reverse primer. 1µl of LB-Amp medium was used as a template. The gel was then run using agarose gel electrophoresis to check size of the PCR products adding 200bp more to the expected size to account for the additional vector derived nucleotides. The colonies which gave positive results with both PCR reactions were picked from the PCR tubes and grown in 15ml of LB-Amp medium overnight using 50ml universal tubes and shaking incubator at 37˚C and 250 rpm, as previously described.

Next day, plasmids were extracted with QIAprep Spin Miniprep Kit, QIAgen (Promega), according to the manufacturer's protocol. To produce glycerol stocks, 500µl of 60 % sterile glycerol was added to 500µl of each overnight culture in LB-Amp medium into a Microcentrifuge tube. The mixture was shaken and stored in freezer at -80˚C.

3.2.2 Coupled in Vitro Transcription-Translation

After amplifying and cloning the *Echinococcus* genes, EgEF1-alfa, EgAg5, Cyclophilin, Eg19, EgTeg and EgTPx, into pF3A WG (BYDV) Flexi vector (Promega, UK), the genes were translated into proteins in TNT® SP6 High-Yield Wheat Germ Lysate Protein Expression System (Promega, UK).

Protein expression was initiated by mixing 3µg of the appropriate pF3A WG (BYDV) construct with 30µl of the TNT SP6 High-Yield Cell-Free Wheat Germ Lysate Extract Master Mix, and complete the final volume to 48 µl with molecular biology grade (nuclease-free) water. 8 µl of this mixture of plasmid and wheat germ lysate was

supplemented with 2 µl of Fluoro Tect Green lys-tRNA in vitro translation labelling system (FluoroTect GreenLys, Promega) for protein expression analysis. Then, both mixtures were incubated for 2 hours at 25°C in a water bath. After incubation, an equal amount of 2x non- reducing sample buffer (NRSB) was added to the mixtures with Fluoro Tect Green lys-tRNA, which were prepared for analysis. Then, the whole mixtures were heated for 2 minutes at 70°C, followed by loading and running the samples in precast Tris-Glycine 4-20% mini gradient gels (BioRad, Uk) for SDS-PAGE. After completion of electrophoresis, the gel was imaged under a laser-based gel scanner (Fuji-LAS 4000, 319 Imaging System) for detection of fluorescence. Molecular weights of the synthesised proteins were assessed using Precision Plus Protein Kaleidoscope ladder (BioRad, UK) which contains fluorescently labelled proteins.

3.3 Results

3.3.1 Antigen Cloning

3.3.1.1 Optimisation of the Annealing Temperature

The annealing temperatures of the PCR for all genes of interest were optimised twice, firstly using the Taq DNA Polymerase Ready Mix, secondly, using Proof-reading Hot Start Q5 DNA Polymerase. The results show that the all gradient annealing temperature from 40 to 64°C worked for three genes out of seven (Ag5, Cyclophilin and Eg19), when Red Taq Polymerase Ready Mix was utilised, see figures (3.1, 3.2 and 3.3). In contrast, all genes, except AgB2, can be amplified using Hot Start Q5 Polymerase at the annealing temperature of 48°C for EgTeg and 64°C for all other genes, EF1-α, Ag5, Cyclophilin, Eg19 and EgTPx (See Table 3.6).

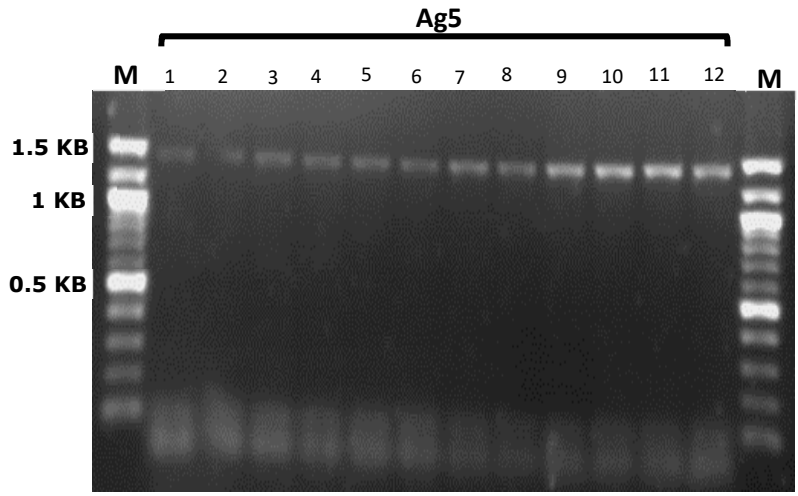


Figure 3.1: Agarose gel electrophoresis – Optimisation of the annealing Temperature for Ag5, Gradient 40-64°C, Using Taq Polymerase Enzyme. The expected size is 1,450 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C. Lane 12= 64°C.

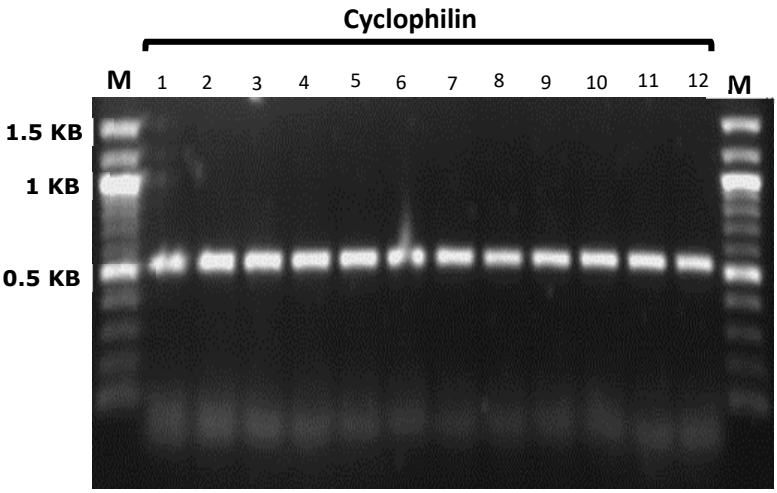


Figure 3.2: Agarose gel electrophoresis - Optimisation of the annealing Temperature for Cyclophilin, Gradient 40 - 64°C, Using Taq Polymerase Enzyme. The expected size is 544 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

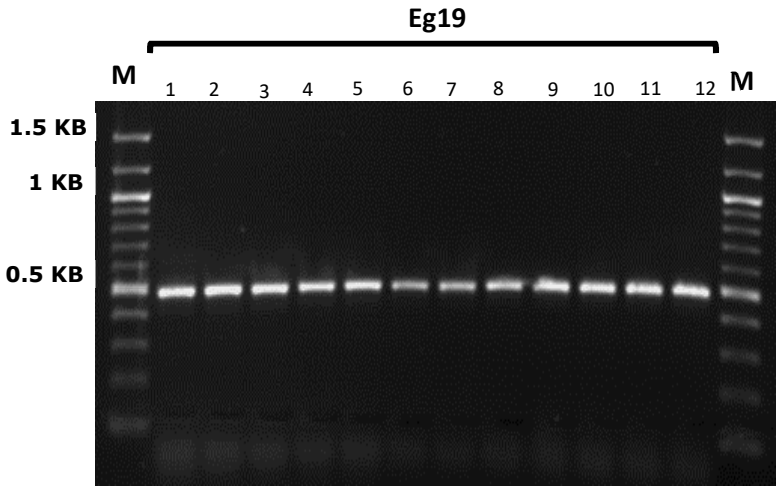


Figure 3.3: Agarose gel electrophoresis - Optimisation of the annealing Temperature for Eg19, Gradient 40-64°C, Using Taq Polymerase Enzyme. The expected size is 502 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

EF1-α gene (1402 bp) gave a positive result at the gradient ranged between 46-64°C, but the optimum annealing temperature was 64°C which gave a strongest band (Figure 3.4).

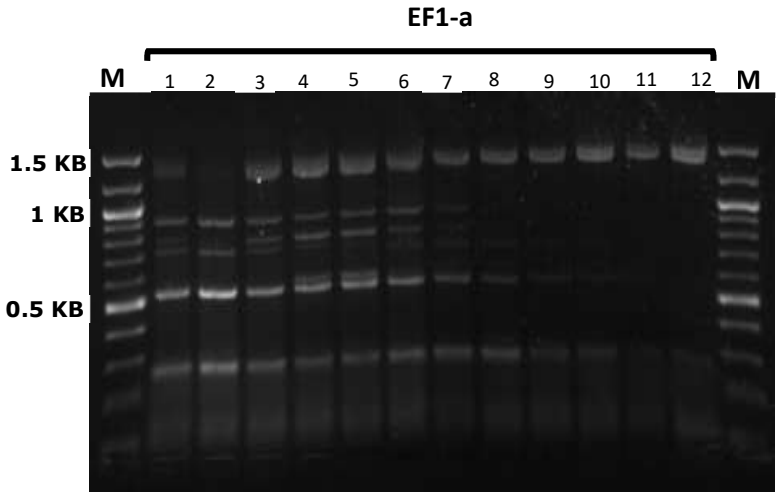


Figure 3.4: Agarose gel electrophoresis - Optimisation of the annealing Temperature for EF1-a, Gradient 40-64°C, Using Q5 Enzyme. The expected size is 1402bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

Regarding to Ag5 gene (1450 bp), gel electrophoresis gave positive results in all gradient temperatures from 40°C to 64°C when Q5 enzyme was used. However, an annealing temperature of 64°C was utilised which showed the strongest band (Figure 3.5).

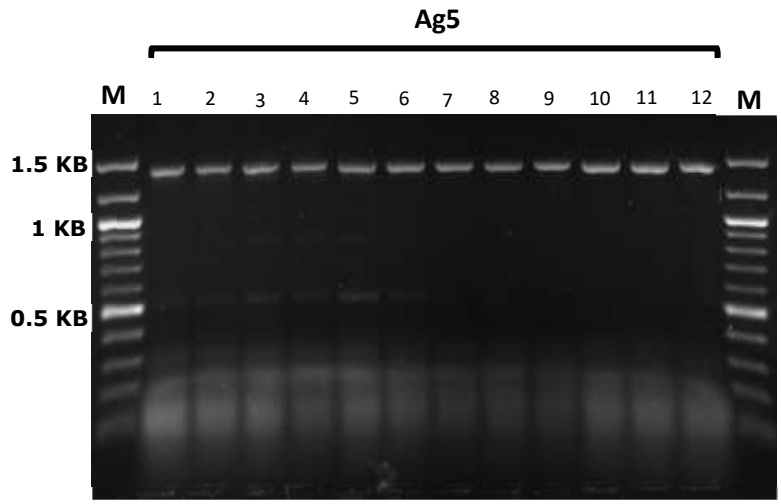


Figure 3.5: Agarose gel electrophoresis – Optimisation of the annealing Temperature for Ag5, Gradient 40 - 64°C, Using Q5 Enzyme. The expected size is 1450 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

AgB2 (271 bp) gave negative results for all gradient temperatures (40-64°C) with the usage of both DNA polymerase enzyme Ready Mix and the proof reading enzyme Q5. Figure (3.6) shows the result of different gradients of annealing temperatures for the AgB2 gene, using Q5 enzyme.

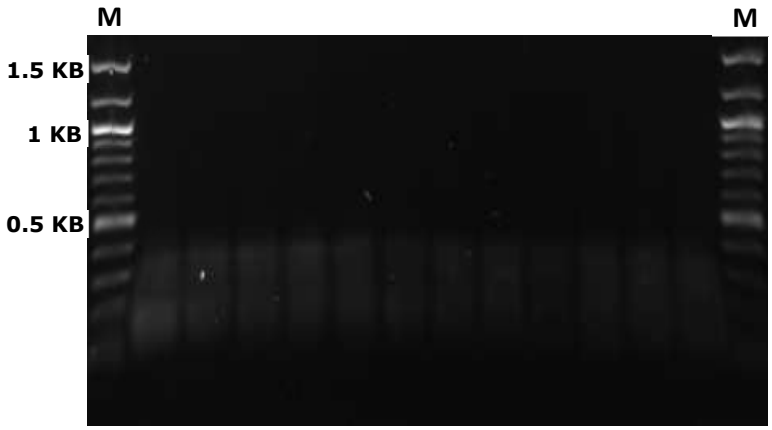


Figure 3.6: Agarose gel electrophoresis – Optimisation of the annealing Temperature for AgB2, Gradient 40 - 64°C, Using Q5 Enzyme. The expected size is 271 bp. M= 100 bp DNA Ladder.

Regarding cyclophilin cDNA (544 bp), the best annealing temperature which gave a strong band in 64°C as shown in figure (3.7).

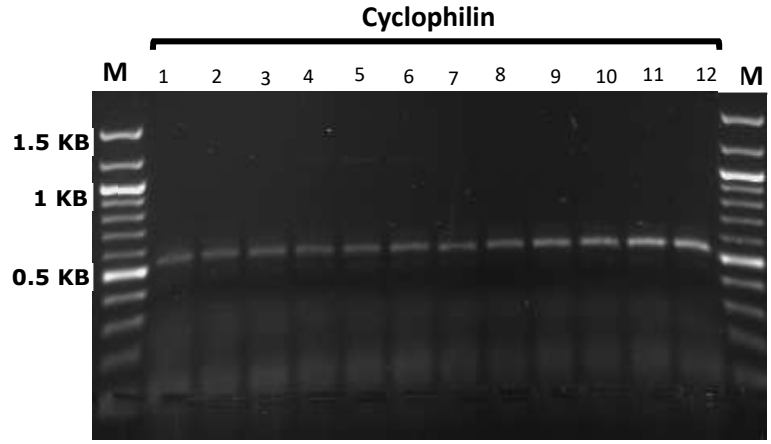


Figure 3.7: Agarose gel electrophoresis - Optimisation of the annealing Temperature for Cyclophilin, Gradient 40 - 64°C, Using Q5 Enzyme. The expected size is 544 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

The result of optimising the annealing temperature for the gene Eg19 (502 bp) shows that the gene can be amplified and give good results in the all gradient temperatures from 40°C to 64°C, but the bands were stronger with the annealing temperature 64°C, when both enzymes were used (Figure 3.8).

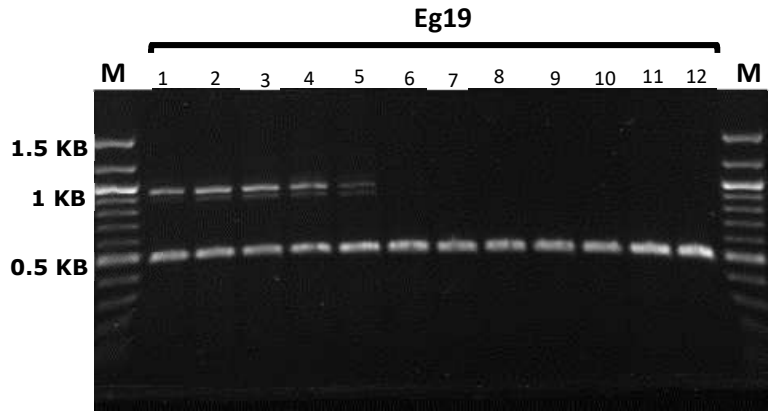


Figure 3.8: Agarose gel electrophoresis – Optimisation of the annealing Temperature for Eg19, Gradient 40-64°C, Using Q5 Enzyme. The expected size is 502 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

The result in figure (3.9) shows that the best annealing temperature for amplifying the gene EgTeg (474 bp), using Q5 polymerase enzyme was in the range between 40°C to 50°C. However, the annealing temperature of 48°C was unequivocal and therefore utilised later for amplification.

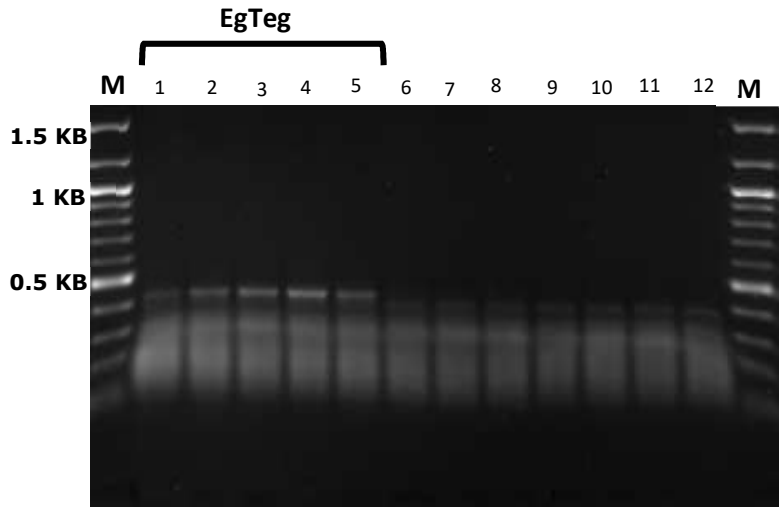


Figure 3.9: Agarose gel electrophoresis – Optimisation of the annealing Temperature for EgTeg, Gradient 40 - 64°C, Using Q5 Enzyme. The expected size is 474 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

In terms of EgTPx gene (637 bp), weak bands can be seen when using the gradient 40-64°C for annealing temperatures with Q5 enzyme (Figure 3.10), while it gave negative results when Ready Mix DNA polymerase enzyme was used.

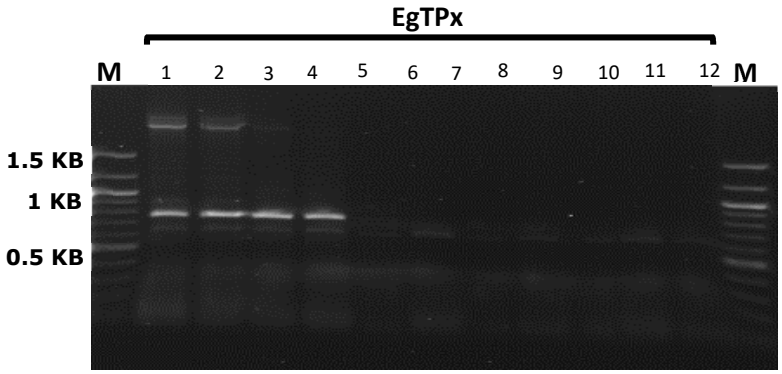


Figure 3.10: Agarose gel electrophoresis - Optimisation of the annealing Temperature for EgTPx, Gradient 40-64°C, Using Q5 Enzyme. The expected size is 637 bp. M= 100 bp DNA Ladder. Lane 1= 40°C, Lane 2= 44°C, Lane 3= 46°C, Lane 4= 48°C, Lane 5= 50°C, Lane 6= 52°C, Lane 7= 54°C, Lane 8= 56°C, Lane 9= 58°C, Lane 10= 60°C, Lane 11= 62°C, Lane 12= 64°C.

Accordingly, the best annealing temperatures for the cDNA encodings are summarised in Table 3.5.

Table 3.5: Optimisation the annealing temperature using conventional PCR conditions.

PCR Enzyme	Gene	Annealing Temperature
Taq Polymerase Ready Mix™	Ag5	64˚C
	EF1-alpha	Negative
	Cyclophilin	64˚C
	EgAgB2	Negative
	Eg19	64˚C
	EgTeg	Negative
	EgTPx	Negative
Proofreading Q5 Master Mix	EF1-alpha	64˚C
	Ag5	64˚C
	Cyclophilin	64˚C
	EgAgB2	Negative
	Eg19	64˚C
	EgTeg	48˚C
	EgTPx	64˚C Very weak

3.3.1.2 Cloning of *Echinococcus* Genes into Expression Vector

The target genes' fragments were amplified from the cDNA library of *Echinococcus* as a first steps for expression of the proteins. Cloning the genes was done in two attempts with different enzymes; firstly, with using the Taq polymerase enzyme (Ready Mix), and in the second attempt, the DNA polymerase with proof reading enzyme Q5 was used to reduce the risk of presenting mutations.

The results show that all genes were successfully amplified as illustrated in the figures (3.11, 3.12, 3.13, 3.14, 3.15, and 3.16) below. The restriction digestion of the PCR products and acceptor Flexi Vectors, to generate appropriate restriction ends, were effectively performed. All genes and plasmids were digested successfully using the Flexi enzyme blend SgfI and PmeI. Ligation was effectively done to join the restriction ends of the genes' fragments and Flexi Vectors together, using T4 DNA ligase enzyme, to insert the DNA fragments into the plasmid.

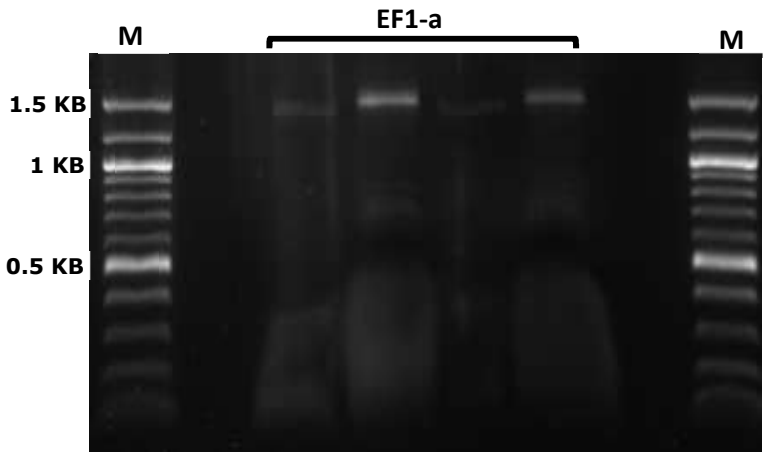


Figure 3.11: Agarose gel electrophoresis – EF1-alpha amplicon by PCR from *Echinococcus*. The bands are in size match the EF1-alpha gene size (1,402 bp). M= 100 bp DNA Ladder.

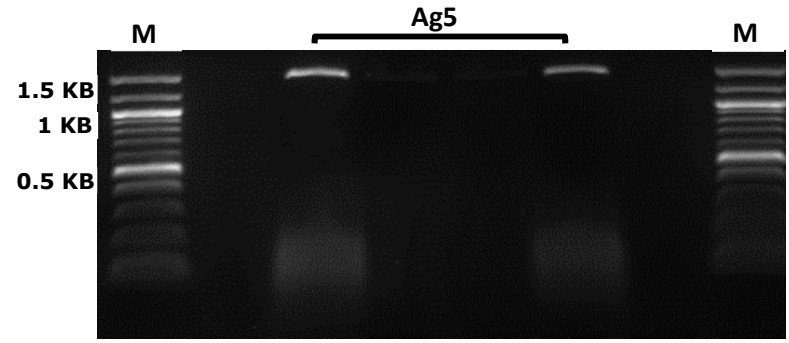


Figure 3.12: Agarose gel electrophoresis – Ag5 amplicon by PCR from *Echinococcus*. The bands are in size around 1500 bp, which close to the Ag5 gene size of 1,450 bp. M= 100 bp DNA Ladder.

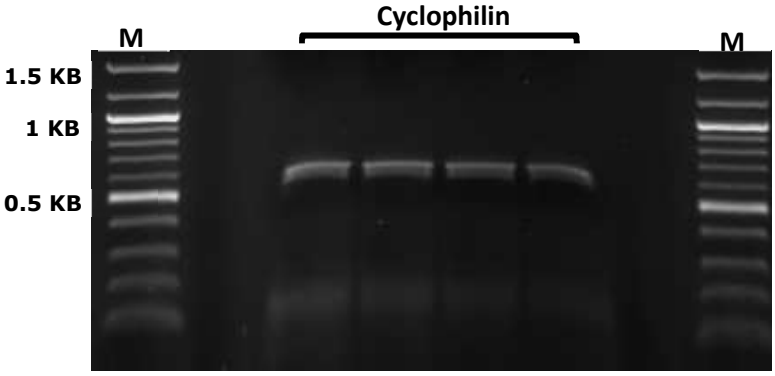


Figure 3.13: Agarose gel electrophoresis – Cyclophilin amplicon by PCR from *Echinococcus*. The bands are in correct size (544 bp). M= 100 bp DNA Ladder.

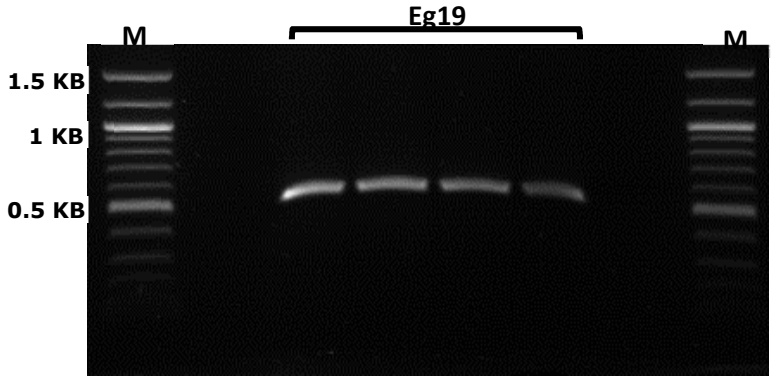


Figure 3.14: Agarose gel electrophoresis – Eg19 amplicon by PCR from *Echinococcus*. The bands are in correct size (502 bp). M= 100 bp DNA Ladder.

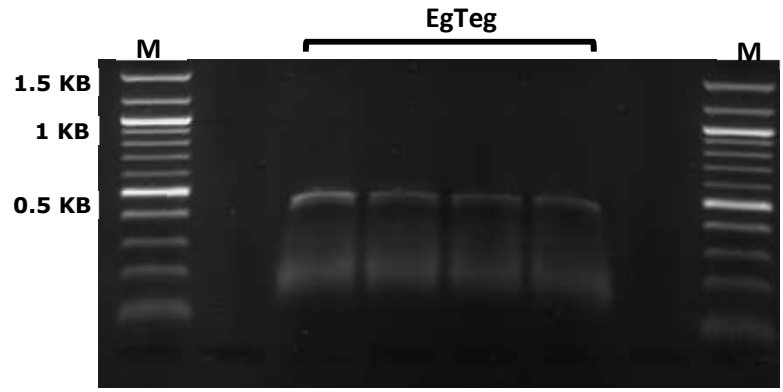


Figure 3.15: Agarose gel electrophoresis – EgTeg amplicon by PCR from *Echinococcus*. The bands are in correct size (474 bp). M= 100 bp DNA Ladder.

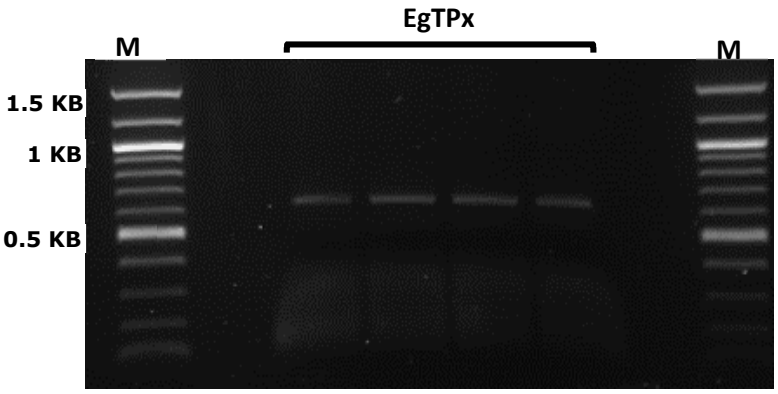


Figure 3.16: Agarose gel electrophoresis – EgTPx amplicon by PCR from *Echinococcus*. The bands are in correct size (637 bp). M= 100 bp DNA Ladder.

3.3.1.3 Colony Screening and Sequencing

PCR colony screening is an important step to verify the successful ligation of target gene and vector and to investigate whether the bacteria *E. coli* DH5-alpha strain uptake the inserted vector or not. To achieve that, single colonies were picked from the overnight growth plates for PCR colony screening using primers presented in Table 3.6 (a and b). These primers were used for amplifying the inserted genes and part of the vector.

For monitoring the PCR products for successful gene insertion, agarose gel electrophoresis was performed. Afterward, positive colonies were grown overnight at 37 °C in 15 ml LB amp medium for plasmid purification and glycerol stock. Consequently, plasmids were extracted using QIAprep Spin Miniprep Kit, QIAgen (Promega, UK), according to the manufacturer's protocol.

Finally, DNA sequences were confirmed for all the clones before in vitro expression. Hence, the multiple cloning site including the inserted gene was sequenced via Source BioScience Company, Nottingham using primers listed in Table (3.6a) for confirmatory sequencing of the plasmids.

Table 3.6: Primers for colony PCR screening and confirmatory sequencing

Forward and reverse indicate sense and antisense primers, respectively. SP6 Forward and T7 Terminator Reverse are universal primer sequences corresponding to the SP6 Promoter and T7 Terminator. [A] Primers used for colony PCR screening. [B] Primer used for confirmatory sequencing.

Table 3.6 a: pF3A WG (BYDV) SP6 forward primer and T7 Terminator reverse for colony PCR screening and confirmatory sequencing which have been used with all the genes of interest.

Construct Name	Primer Name	Sequence (5'-3')
pF3A WG (BYDV)-*Echinococcus* genes	SP6 [23-41 bp] Forward [AB]	ATTTAGGTGACACTATAG
	T7 Terminator [793-811 bp] Reverse [AB]	GCTAGTTATTGCTCAGCGG

Table 3.6 b: *Echinococcus* genes reverse primers for colony PCR screening.

Construct Name	Primer Name	Sequence (5'-3')
pF3A WG (BYDV) – EgEF1-a	EgEF1-a Reverse [A]	GAGCGTTTAAACGTGGTGGTGGTGGTG GTGTTTTTTGGCCTTGGCGGCCT
pF3A WG (BYDV) - EgAg5	EgAg5 Reverse [A]	TTGCGTTTAAACGTGGTGGTGGTGGTGG TGGACTGCGTAGCGGTTGATCCACT
pF3A WG (BYDV) - Cyclophilin	Cyclophilin Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTG GTGCAGCTGACCGCAGTCAGTAATCAGT ACTTCT
pF3A WG (BYDV) – Eg19	Eg19 Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTG GTGATCTCGAATACCATGTCCAACTTCTC T
pF3A WG (BYDV) - EgTeg	EgTeg Reverse [A]	TTGTGTTTAAACGTGGTGGTGGTGGTGG TGGTAGGTAGAGGATTGCCACACAAGA
pF3A WG (BYDV) - EgTPx	EgTPx Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTG GTGCGAGCTCATGAACGACTTCAA

Figures 3.17 to 3.21 below illustrate bands belonging to different colonies inserted with different pF3A-WG-BYDV-EF1-genes ligation. Based on the size of these bands, it can be investigated whether the ligation and transformation steps were effectively done.

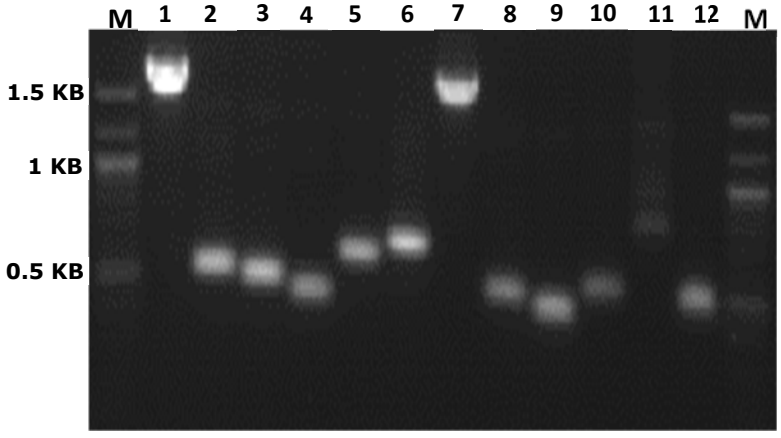


Figure 3.17: Agarose gel electrophoresis – PCR Colony Screen of DH5-alpha transformed with pF3A-WG-BYDV-EF1-alpha ligation mixture. Only two bands in lane 1 & 7 gave a correct size 1578 bp. The sizes include gene, restriction sites and part of the plasmid. Other lanes gave bands with unexpected size. M= 100 bp DNA Ladder.

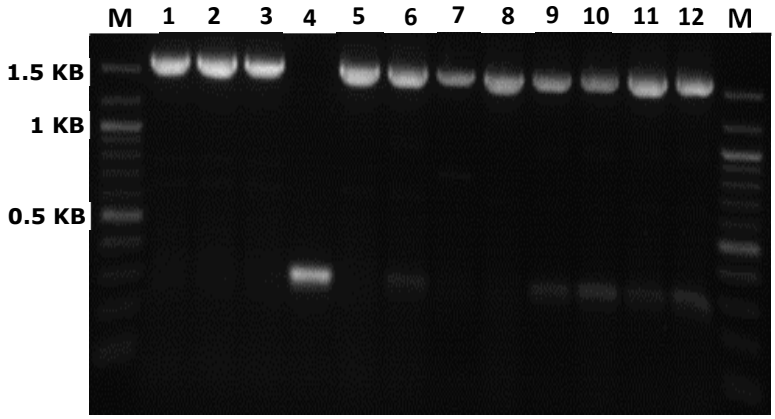


Figure 3.18: Agarose gel electrophoresis – PCR colony screen of DH5-alpha transformed with pF3A-WG-BYDV-Ag5 ligation mixture. The bands of colonies inserted with the ligation mixture plasmid-Ag5 are seen at the correct size 1626 bp, except lane 4. The sizes include gene, restriction sites and part of the plasmid. M= 100 bp DNA Ladder.

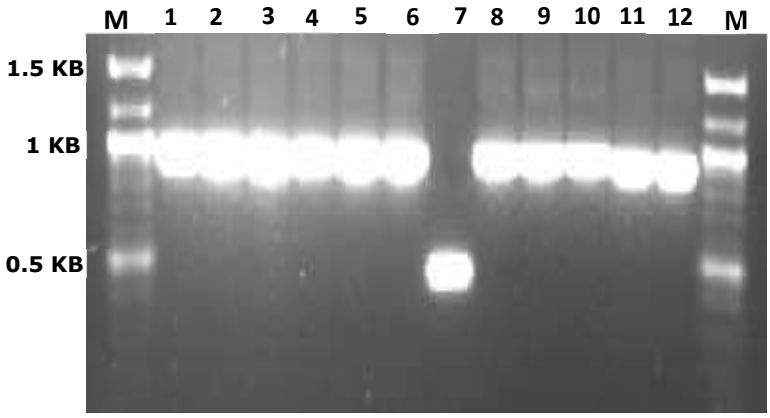


Figure 3.19: Agarose gel electrophoresis – PCR Colony Screen of DH5-alpha transformed with pF3A-WG-BYDV-Cyclophilin ligation mixture. This image shows the plasmids containing recombinant Cyclophilin. All lanes except lane 7 gave bands with correct size 941 bp. M= 100 bp DNA Ladder.

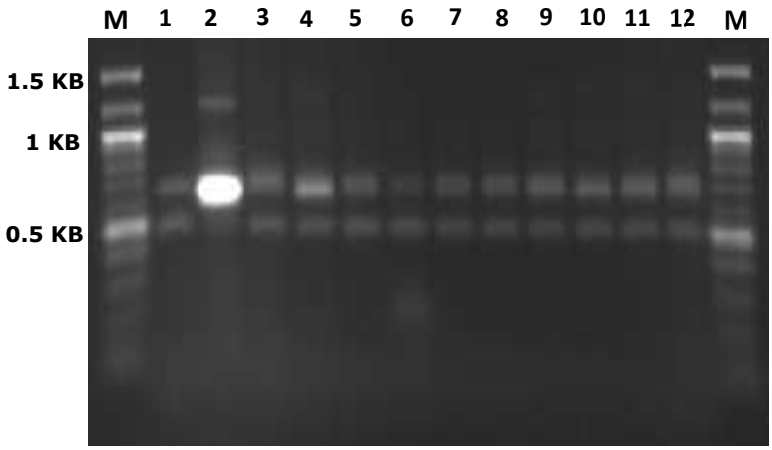


Figure 3.20: Agarose gel electrophoresis – PCR Colony Screen of DH5-alpha transformed with pF3A-WG-BYDV-Eg19 ligation mixture. Only one band in lane 2 gave a strong band with the correct size 678 bp. All other lanes show bands with correct size but the bands are weak. M= 100 bp DNA Ladder.

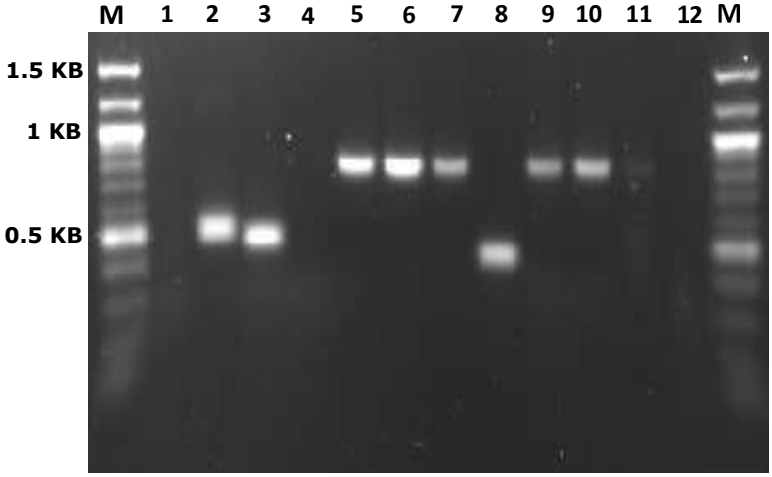


Figure 3.21: Agarose gel electrophoresis – PCR Colony Screen of DH5-alpha transformed with pF3A-WG-BYDV-EgTeg ligation mixture. The band in lane 2 is in the correct size 552 bp. Lanes 3, 5, 6, 7, 8, 9 and 10 gave bands with different size, while lanes 1, 4, 11 and 12 show no bands at all. M= 100 bp DNA Ladder.

Alignment between the cDNA sequences of reference EF1-alpha and the obtained cDNA sequences of the cloned EF1-alpha were produced. The results suggest presence of 17 SNPs in the cloned cDNA, when aligning with the original cDNA of EF1-alpha (Figure 3.22). After aligning the amino acids sequence of the expected EF1-alpha antigen with the cloned EF1-alpha antigen, it was found that all these SNPs were silent and would not have resulted in an alteration of the amino acid sequence. Therefore, the amino acids sequences for both original and deduced EF1-alpha protein are 100% identical, see Figure (3.23).

```
AB306934    ATGGGGAAGGAGAAGACGCACATCAATCTCATTGTTATCGGTCACGTCGATTCTGGTAAA
Obtained    ATGGGGAAGGAGAAGACGCACATCAATCTCATTGTTATTGGTCACGTCGATTCTGGTAAA

Reference   TCGACTTCCACGGGCCATCTTATTTACAAATGTGGTGGGATTGACAAAAGAACTATTGAG
Obtained    TCGACTTCCACAGGCCATCTTATTTACAAATGTGGTGGTATTGACAAAAGAACTATTGAG

Reference   AAATTTGAGAAAGAGGCGGCTGAGATGGGCAAGTCCTCTTTCAAGTACGCGTGGGTGCTT
Obtained    AAATTTGAGAAAGAGGCGGCTGAGATGGGCAAGTCCTCTTTCAAGTACGCGTGGGTGCTT

Reference   GATAAGCTCAAGGCTGAGCGTGAGCGTGGTATCACCATCGACATCGCCCTCTGGAAGTTC
Obtained    GATAAGCTCAAGGCTGAGCGTGAGCGTGGTATCACCATCGACATCGCTCTCTGGAAGTTC

Reference   GAGACTCCGAAGTACCTCGTTACCATCATCGACGCGCCCGGTCATCGTGACTTTATTAAG
Obtained    GAAACTCCGAAGTACCTCGTTACCATCATCGACGCGCCCGGTCATCGTGACTTTATTAAG

Reference   AACATGATTACGGGCACCAGCCAGGCGGACTGCGCAATTCTGGTTGTTGCTGCTGGTACT
Obtained    AACATGATTACGGGCACCAGCCAGGCGGATTGCGCAATTCTGGTTGTTGCTGCTGGTACT

Reference   GGTGAGTTCGAGGCAGGTATTAGTAAGAATGGTCAAACGCGCGAGCATGCGCTTCTCGCC
Obtained    GGTGAGTTCGAGGCAGGTATTAGTAAGAATGGTCAAACGCGCGAGCATGCGCTTCTCGCC

Reference   TTCACTCTGGGTGTGAAGAAGCTCATCATTGCGGTCAACAAAATGGATGCAGTTGATTAC
Obtained    TTCACTCTGGGTGTGAAGAAGCTCATCATTGCGGTCAACAAAATGGATGCAGTTGATTAC

Reference   AGTGAAAAGCGCTTCCAGGAGATTAGTTCCGAAATGAAGGCGTACATTAAGAAGGTGGGA
Obtained    AGTGAAAAGCGCTTCCAGGAGATTAGTTCCGAAATGAAGGCGTACATTAAGAAGGTGGGA

Reference   TACAACCCCGATACTGTAAATATCGTCCCAATCTCCGGTTGGGTCGGCGACAACATGCTG
Obtained    TACAACCCCGATACTGTAAATATCGTCCCAATCTCCGGTTGGGTCGGCGACAACATGCTG

Reference   GAGCCCAGTCCTAACATGCCTTGGTATTAAGGGGCCCACGCTTCTTGCCAGTATCGATCTT
Obtained    GAGCCCAGTCCTAACATGCCTTGGTACAAGGGGCCCACGCTTCTTGCCAGTATCGATCTC

Reference   GTCGAGCCTCCAACTCGTCCCGTGGACAAGCCCCTTCGACTTCCTCTTCAGGATGTGTTT
Obtained    GTCGAGCCTCCAACTCGTCCCGTGGACAAGCCCCTTCGACTTCCTCTTCAGGATGTGTTT

Reference   AAAATTAGCGGTATTGGTACTGTTCCCGTCGGCCGTGTCGAGACTGGCATAATGAAGCCG
Obtained    AAAATTAGCGGTATTGGTACTGTTCCCGTCGGCCGTGTCGAGACTGGCATAATGAAGCCG

Reference   GGTATGATCGTCACATTTGCTCCTGTCGGCATTTCAACCGAAGTGAAATCAATCGAAATG
Obtained    GGTATGATCGTCACATTTGCTCCCGTCGGCATTTCAACCGAAGTGAAATCAATTGAAATG

Reference   CACCATGAAGCCCTATCCGAGGCCGTCCCCGGAGACAACGTTGGCTTCAACGTGAAAAAT
Obtained    CACCATGAAGCCCTCTCCGAGGCCGTCCCCGGAGACAACGTTGGCTTCAACGTGAAAAAT
```

```
Reference     ATCTCTGTAAAGGACGTTCGTCGCGGTAACGTGGCTGGTGACTCAAAGAATCACCCTCCT
Obtained      ATCTCTGTAAAGGACGTTCGTCGCGGTAACGTGGCTGGGGACTCAAAGAATCACCCTCCT

Reference     CGCGAGGCTGCAGAGTTCACCGCCCAAGTTATCGTCCTCAATCATCCTGGCGAGATTGGT
Obtained      CGCGAGGCTGCAGAGTTCACCGCCCAAGTTATCGTCCTCAATCATCCTGGCGAGATTGGT

Reference     GCCGGCTACACTCCCGTTCTGGACTGTCATACTGCTCACATCGCCTGTAAGTTTGCCGAA
Obtained      GCCGGCTACACCCCCGTTCTGGACTGTCATACTGCTCACATCGCCTGTAAGTTTGCCGAA

Reference     CTTAAGGAGAAAATCGATCGCCGTACTGGTCAGGTCAAGGAGACTAACCCTGCGAAGATC
Obtained      CTTAAGGAGAAAATTGATCGTCGTACTGGTCAGGTCAAGGAGACTAACCCTGCGAAGATC

Reference     AAATCCGGTGATGCGGCCATCGTTAGAATGGTGCCCAGCAAACCAATGTGTGTTGAGGTG
Obtained      AAATCCGGTGATGCGGCCATTGTTAGAATGGTGCCCAGCAAACCAATGTGTGTTGAGGTG

Reference     TTCTCGGAGTTCCCGCCTCTGGGTCGCTTTGCTGTTCGTGACATGAGATCGACTGTCGCT
Obtained      TTCTCGGAGTTCCCGCCTCTGGGTCGCTTTGCTGTTCGTGACATGAGATCGACTGTCGCT

Reference     GTAGGCGTGATCAAGGCTGTAACCTTCAAGGATAAGGAAGCCAAGGCCACAAAATCTGCC
Obtained      GTAGGTGTGATCAAGGCTGTAACCTTCAAGGATAAGGAAGCCAAGGCCACAAAATCTGCC

Reference     CAGAAGGCCGCCAAGGCCAAAAAA
Obtained      CAGAAGGCCGCCAAGGCCAAAAAA
```

Figure 3.22: Sequence alignment of native EF1-alpha cDNA with obtaining cDNA sequences of EF1-alpha, using both SP6 and T7R primers for sequencing. The highlighted nucleotides in red refers to the SNPs which can be seen when aligning the recombinant EF1-alpha with the native gene, using Q5 enzyme. The alignment shows presenting numerous SNPs in the obtaining EF1-a cDNA. The characters in red refer to the accession number of the gene.

```
BAF63677      MGKEKTHINLIVIGHVDSGKSTSTGHLIYKCGGIDKRTIEKFEKEAAEMGKSSFKYAWVL
Obtained      MGKEKTHINLIVIGHVDSGKSTSTGHLIYKCGGIDKRTIEKFEKEAAEMGKSSFKYAWVL

Query  61     DKLKAERERGITIDIALWKFETPKYLVTIIDAPGHRDFIKNMITGTSQADCAILVVAAGT
Sbjct  61     DKLKAERERGITIDIALWKFETPKYLVTIIDAPGHRDFIKNMITGTSQADCAILVVAAGT

Query  121    GEFEAGISKNGQTREHALLAFTLGVKKLIIAVNKMDAVDYSEKRFQEISSEMKAYIKKVG
Sbjct  121    GEFEAGISKNGQTREHALLAFTLGVKKLIIAVNKMDAVDYSEKRFQEISSEMKAYIKKVG

Query  181    YNPDTVNIVPISGWVGDNMLEPSPNMPWYKGPTLLASIDLVEPPTRPVDKPLRLPLQDVF
Sbjct  181    YNPDTVNIVPISGWVGDNMLEPSPNMPWYKGPTLLASIDLVEPPTRPVDKPLRLPLQDVF

Query  241    KISGIGTVPVGRVETGIMKPGMIVTFAPVGISTEVKSIEMHHEALSEAVPGDNVGFNVKN
Sbjct  241    KISGIGTVPVGRVETGIMKPGMIVTFAPVGISTEVKSIEMHHEALSEAVPGDNVGFNVKN

Query  301    ISVKDVRRGNVAGDSKNHPPREAAEFTAQVIVLNHPGEIGAGYTPVLDCHTAHIACKFAE
Sbjct  301    ISVKDVRRGNVAGDSKNHPPREAAEFTAQVIVLNHPGEIGAGYTPVLDCHTAHIACKFAE

Query  361    LKEKIDRRTGQVKETNPAKIKSGDAAIVRMVPSKPMCVEVFSEFPPLGRFAVRDMRSTVA
Sbjct  361    LKEKIDRRTGQVKETNPAKIKSGDAAIVRMVPSKPMCVEVFSEFPPLGRFAVRDMRSTVA

Query  421    VGVIKAVTFKDKEAKATKSAQKAAKAKK  448
Sbjct  421    VGVIKAVTFKDKEAKATKSAQKAAKAKK  448
```

Figure 3.23: Alignment the amino acids sequence of the original EF1-alpha and deduced amino acids sequence of EF1-alpha. The alignment shows that both amino acid sequences are identical. The characters in red refer to the accession number of the protein.

Regarding Ag5, after analysing the sequences, 20 apparent SNPs were found in the cloned cDNA (Figure 3.24). However, when the protein sequence of the native protein was aligned with the corresponding predicted protein sequence, it was noticed that there were 7 SNPs in the amino acids of the cloned antigen (Figure 3.25). After aligning the protein sequence of the cloned Ag5, using Taq polymerase with the cloned Ag5 protein, using proof-reading polymerase, it was observed that protein sequences were 100% identical (Figure 3.26).

```
JF970202      TTGGAGCTCACTCTCGATCCAGATGAACTCGTTAAGGCGCAGCGAGAGAGTCACGGTGGC
Obtained      TTGGAGCTCACTCTCGATCCAGATGAACTCGTTAAGGCGCAGCGAGAGAGTCACGGTGGC

Query  61     TTCTACTTTTACGACTCAAATGGCGCAACACTGATGTTTAATCGCAGCCTCTTCGTCTAC
Sbjct  64     TTCTACTTTTACGACTCAAATGGCGCAACACTGATGTTTAATCGCAGCCTCTTCGTCTAC

Query  121    CGCGAGAACATTTACGACGGCTGGTCGCGGTGGAGTGAGTGTTCCCCCCATACCTGCCTC
Sbjct  124    CGCGAGAACGTTTACGACGGCTGGTCGCGGTGGAGTGAGTGTTCCCCCCATACCTGCCTC

Query  181    GAACATCGCTATCGTCGCTGTGTGGACGACTCCTACACTCAACCGGTCAACTACCTCACC
Sbjct  184    GAACATCGCTATCGTCGCTGTGTGGACGACTCCTACACTCAACCGGTCAACTACCTCACC

Query  241    TCCTCTGCCCGCATCTGCCCCTTCAAGTACATTGCAGAGGAGAGACCGTGTGAAGACAAG
Sbjct  244    TCCTCCGCCCGCATCTGCCCCTTCAAGTACATTGCAGAGGAGAGACCGTGTGAAGACAAG

Query  301    TCCAACTGCATCATCAACTCGAAACCGTCAGAAGAACTAACTAGAATGCAAGAAAAGTGC
Sbjct  304    TCCAACTGCATCGTCAACTCGAAACCGTCAGAAGAACTAACTAGAATGCAAGAAAAGTGC

Query  361    GGCATCAGAGGATCGTTTGATAAAAATACTGCAAAGCCCAGCCGTCGAAGATGGAAAGAT
Sbjct  364    GGCATTAGAGGATCGTTTGATAAAAATACTGCAAAGCCCAGCCGCCGAAGATGGAAAGAT

Query  421    ATGGACGATGATGAAGCCGATGACGCTGAGGCCGAAGAACGTGGCGAGTACGAGTCCGAA
Sbjct  424    ATGGACGATGATGAAGCCGATGACGCTGAGGCCGAAGAACGTGGCGAGTACGAGTCCGAA

Query  481    AGGCTACCATATAGCTTAAAGATTCTTGGTGGAAAAAGCGCCAAATCAAAGAGCTGGCCG
Sbjct  484    AGGCTACCATATAGCTTAAAGATTCTTGGTGGAAAAGGCGCCAAATCAAAGAGCTGGCCG

Query  541    TGGCATGTTGGAATCTACAAAGCAGCCAATTACAACGCTTCCGAGGGATTGACTCGCTTG
Sbjct  544    TGGCATGTTGGAATCTACAAAGCAGCCAATTACAACGCTTCCGAGGGATTGACTCGCTTG

Query  601    AAGTCTGAAAACATCATATGCGGGGGCACACTGATTACACCACGATGGGTTCTGACTGCG
Sbjct  604    AGATCTGAAAACATCATATGCGGGGGCACATTGATCACACCACGATGGGTTCTGACTGCG

Query  661    GCGCACTGCTTAAAGCCAATTTTCGGCTCCTCGAATGCCTTGCCTTTTGGCATTCCAGCG
Sbjct  664    GCGCACTGCTTAAAGCCAATTTTCGGCTCCTCGAATGCCTTGCCTTTTGGCATTCCAGCG

Query  721    CCATTGAACACGGACGAGATGAAGCCCATTTTCCTGCTCGTTCGAGCCGGTGACACTGTT
Sbjct  724    CCATTGAACATGGACGAGATGAAGCCCATTTTTTGCTCGTTCGAGCCGGTGACACTGTT

Query  781    CTGGAGGGTACGCGTACTCGAAACGAGCAGGAGAGCGATCATGTGGTCGACCTGGTCGTC
Sbjct  784    CTGGAGGGCACGCGTACTCGAAACGAGCAGGAGAGCGATCATGTGGTCGACCTGGTCGTC

Query  841    ATTCATCCGGATTGGGTTGCCCAACGTGTTGATAGTCCATTCGATGTAGCTCTGTTGAGA
Sbjct  844    ATTCATCCGGATTGGGTTGCCCAACGTGTTGATAGTCCATTCGATGTAGCTCTGTTGAGA
```

```
Query  901   CTCGAGACACCTGTGAACATTGAGAGTGACGGTGCGGGCGTTGCTTGCGTACCCAAAAAT
Sbjct  904   CTCGAGACACCTGTGAACATTGAGAGTGACGGTGCGGGCGTTGCTTGCGTACCCAAAAAT

Query  961   GCAGATGCAACACCGGCGGAGGACGCAGTTTGCTTCTCCGTTGGCTGGGGTGAGAAATCT
Sbjct  964   GCAGATGCAACACCGGCGGAGGACGCAGTTTGCTTCTCCGTTGGCTGGGGTGAGAAATCT

Query  1021  CGACCCATAAGCAAGCCGCGACGCCGCCGTCCTACCTTCTTTAATCCCTTCTTCTGGCCT
Sbjct  1024  CGACCCCTAAGCAAGCCGCGACGCCGCCGTCCCACCTTCTTTAATCCCTTCTTCTGGCCT

Query  1081  TTTGGTCGTTTGTGGGAACGTCGACCACAGCGACCCACTTCCTTGAATGAGATTAGGGTC
Sbjct  1084  TTTGGTCGTTTGTGGGAACGTCGACCACAGCGACCCACTTTCTTGAATGAGATTAGGGTT

Query  1141  TCCATCGACCCACCTGAGAAGTGCTTCCATCATGACGATGAAAATGAGGCCCAAATCTGC
Sbjct  1144  TCCATCGACCCACCTGAGAAGTGCTTCCACCATGACGATGAAAATGAGGCCCAAATCTGC

Query  1201  GCTGGCAGCTCTAATAAGGGCGTTTGCGCAGGCGACACAGGAGGTGGACTCTTCTGCCGA
Sbjct  1204  GCTGGCAGCTCTAATAAGGGCGTTTGCGCAGGCGACACAGGAGGTGGACTCTTCTGCCGA

Query  1261  AATGAGGAGGATGGACGGTGGTACGTGTACGGTGTGATGGGCTCGGGACCCACGCAGTAC
Sbjct  1264  AATGAGGAGGATGGACGGTGGTACGTGTACGGTGTGATGGGCTCGGGACCCACGCAATAC

Query  1321  TGTAAGTCACGTCGTTGGCTCTACAACTCGGTGGGCAGTGTCATCCAGTGGATCAACCGC
Sbjct  1324  TGTAAGTCACGTCGTTGGCTCTACAACTCGGTGGGCAGTGTCATCCAGTGGATCAACCGC

Query  1381  TACGCAGTC  1389
Sbjct  1384  TACGCAGTC  1392
```

Figure 3.24: Alignment the sequences of reference Ag5 cDNA with the obtained sequences of Ag5 recombinant gene, using both SP6 and T7R primers for sequencing. The highlighted nucleotides in red refers to the SNPs which can be seen when aligning the recombinant EF1-alpha with the native gene, using Q5 enzyme. It can be seen 20 SNPs in the cloned cDNA sequence. The characters in red refer to the accession number of the gene.

```
AFI71096     LELTLDPDELVKAQRESHGGFYFYDSNGATLMFNRSLFVYRENIYDGWSRWSECSPHTCL
Obtained     LELTLDPDELVKAQRESHGGFYFYDSNGATLMFNRSLFVYRENVYDGWSRWSECSPHTCL

Query  61    EHRYRRCVDDSYTQPVNYLTSSARICPFKYIAEERPCEDKSNCIINSKPSEELTRMQEKC
Sbjct  62    EHRYRRCVDDSYTQPVNYLTSSARICPFKYIAEERPCEDKSNCIVNSKPSEELTRMQEKC

Query  121   GIRGSFDKNTAKPSRRRWKDMDDDEADDAEAEERGEYESERLPYSLKILGGKSAKSKSWP
Sbjct  122   GIRGSFDKNTAKPSRRRWKDMDDDEADDAEAEERGEYESERLPYSLKILGGKGAKSKSWP

Query  181   WHVGIYKAANYNASEGLTRLKSENIICGGTLITPRWVLTAAHCLKPIFGSSNALPFGIPA
Sbjct  182   WHVGIYKAANYNASEGLTRLRSENIICGGTLITPRWVLTAAHCLKPIFGSSNALPFGIPA

Query  241   PLNTDEMKPIFLLVRAGDTVLEGTRTRNEQESDHVVDLVVIHPDWVAQRVDSPFDVALLR
Sbjct  242   PLNMDEMKPIFLLVRAGDTVLEGTRTRNEQESDHVVDLVVIHPDWVAQRVDSPFDVALLR

Query  301   LETPVNIESDGAGVACVPKNADATPAEDAVCFSVGWGEKSRPISKPRRRRPTFFNPFFWP
Sbjct  302   LETPVNIESDGAGVACVPKNADATPAEDAVCFSVGWGEKSRPLSKPRRRRPTFFNPFFWP

Query  361   FGRLWERRPQRPTSLNEIRVSIDPPEKCFHHDDENEAQICAGSSNKGVCAGDTGGGLFCR
Sbjct  362   FGRLWERRPQRPTFLNEIRVSIDPPEKCFHHDDENEAQICAGSSNKGVCAGDTGGGLFCR

Query  421   NEEDGRWYVYGVMGSGPTQYCKSRRWLYNSVGSVIQWINRYAV  463
Sbjct  422   NEEDGRWYVYGVMGSGPTQYCKSRRWLYNSVGSVIQWINRYAV  464
```

Figure 3.25: Alignment between the amino acids sequence of the original Ag5 and deduced amino acids sequence of Ag5. The highlighted amino acids in red refer to the SNPs which can be seen when aligning the reference Ag5 with the reading frame of deduced Ag5 recombinant protein. The alignment shows 7 SNPs in the amino acids sequence of deduced Ag5 recombinant protein. The characters in red refer to the accession number of the protein.

```
Using Q5     MLELTLDPDELVKAQRESHGGFYFYDSNGATLMFNRSLFVYRENVYDGWSRWSECSPHTC
Using Taq    MLELTLDPDELVKAQRESHGGFYFYDSNGATLMFNRSLFVYRENVYDGWSRWSECSPHTC

Query  61    LEHRYRRCVDDSYTQPVNYLTSSARICPFKYIAEERPCEDKSNCIVNSKPSEELTRMQEK
Sbjct  61    LEHRYRRCVDDSYTQPVNYLTSSARICPFKYIAEERPCEDKSNCIVNSKPSEELTRMQEK

Query  121   CGIRGSFDKNTAKPSRRRWKDMDDDEADDAEAEERGEYESERLPYSLKILGGKGAKSKSW
Sbjct  121   CGIRGSFDKNTAKPSRRRWKDMDDDEADDAEAEERGEYESERLPYSLKILGGKGAKSKSW

Query  181   PWHVGIYKAANYNASEGLTRLRSENIICGGTLITPRWVLTAAHCLKPIFGSSNALPFGIP
Sbjct  181   PWHVGIYKAANYNASEGLTRLRSENIICGGTLITPRWVLTAAHCLKPIFGSSNALPFGIP

Query  241   APLNMDEMKPIFLLVRAGDTVLEGTRTRNEQESDHVVDLVVIHPDWVAQRVDSPFDVALL
Sbjct  241   APLNMDEMKPIFLLVRAGDTVLEGTRTRNEQESDHVVDLVVIHPDWVAQRVDSPFDVALL

Query  301   RLETPVNIESDGAGVACVPKNA
Sbjct  301   RLETPVNIESDGAGVACVPKNA
```

Figure 3.26: Alignment the amino acids sequence of the cloned Ag5 antigen, using Taq polymerase with the amino acids sequence of cloned Ag5 antigen, using proof-reading polymerase. It can be seen that the both protein sequences are 100% identical.

Regarding Cyclophilin, the alignment between the expected and the cloned cDNA sequences identified the presence of 4 SNPs (Figure 3.27). On the other hand, when the cDNA sequence of the recombinant Cyclophilin using proof-reading Q5 polymerase was aligned with the recombinant Cyclophilin which was amplified using Master Mix Taq polymerase, it can be seen that the both cDNA sequences are 100% identical (Figure 3.28). When comparing the reference amino acids with the corresponding cloned amino acids sequences, there were no differences (Figure 3.29). Protein sequences are 100% identical and the SNPs in Figure (3.27) are therefore all silent.

```
AF431734     ATGGGCGTGAAGTGCTTCTTCGACATCTCCATTGGCGGTAAGCCAGCGGGTCGCATCGTC
Cloned       ATGGGCGTGAAGTGCTTCTTCGACATCTCCATTGGCGGTAAGCCAGCGGGTCGCATCGTC

Query  61    TTTGCCCTGTTTGATGACGTCCCAAAGACAGTAGAGAACTTCCGTGCACTGTGCACCGGT
Sbjct  219   TTTGCCCTGTTTGATGACGTCCCAAAGACGGTAGAGAACTTCCGTGCACTGTGCACCGGT

Query  121   GAGAAGGGTTTCGGATACAAGGGCTCGAAGTTTCACCGAATTATTCCCGGTTTTATGTGC
Sbjct  279   GAGAAGGGTTTCGGATACAAAGGCTCGAAGTTTCACCGAATTATTCCCGGTTTTATGTGC

Query  181   CAAGGTGGTGATTTTACTGCCGGGAATGGTACCGGTGGCAAGAGCATATACGGGAGCAAA
Sbjct  339   CAAGGTGGTGATTTTACTGCCGGAAATGGTACCGGTGGCAAGAGCATATACGGGAGCAAA

Query  241   TTTGAGGATGAAAATTTCAATCACAAGCACAGCAAGCCGATGATGCTCTCGATGGCGAAT
Sbjct  399   TTTGAGGATGAAAATTTCAATCACAAGCACAGCAAGCCGATGATGCTCTCGATGGCGAAT
```

```
Query  301  GCGGGTAAGAACACCAATGGCTCGCAATTCTTCATCACTACCGCCGTCACCAGCTGGCTT
Sbjct  459  GCGGGTAAGAACACCAATGGCTCGCAATTCTTCATCACTACCGCCGTCACCAGCTGGCTT

Query  361  GATGGGAAGCATGTTGTCTTTGGTGAGGTAGAAAGTGGCGAAGATGTTGTTAAGGATATG
Sbjct  519  GATGGCAAGCATGTTGTCTTTGGTGAGGTAGAAAGTGGCGAAGATGTTGTTAAGGATATG

Query  421  GAGGCCGTCGGTAGCAGTAGTGGCAAGACTTCCCAAGAAGTACTGATTACTGACTGCGGT
Sbjct  579  GAGGCCGTCGGTAGCAGTAGTGGCAAGACTTCCCAAGAAGTACTGATTACTGACTGCGGT

Query  481  CAGCTG  486
Sbjct  639  CAGCTG  644
```

Figure 3.27: Alignment the sequences of reference Cyclophilin cDNA with the cloned Cyclophilin sequence. It can be seen 4 SNPs in the cloned cDNA sequence. The characters in red refer to the accession number of the gene.

```
Using Q5    ATGGGCGTGAAGTGCTTCTTCGACATCTCCATTGGCGGTAAGCCAGCGGGTCGCATCGTC
Using Taq   ATGGGCGTGAAGTGCTTCTTCGACATCTCCATTGGCGGTAAGCCAGCGGGTCGCATCGTC

Query  61   TTTGCCCTGTTTGATGACGTCCCAAAGACGGTAGAGAACTTCCGTGCACTGTGCACCGGT
Sbjct  61   TTTGCCCTGTTTGATGACGTCCCAAAGACGGTAGAGAACTTCCGTGCACTGTGCACCGGT

Query  121  GAGAAGGGTTTCGGATACAAAGGCTCGAAGTTTCACCGAATTATTCCCGGTTTTATGTGC
Sbjct  121  GAGAAGGGTTTCGGATACAAAGGCTCGAAGTTTCACCGAATTATTCCCGGTTTTATGTGC

Query  181  CAAGGTGGTGATTTTACTGCCGGAAATGGTACCGGTGGCAAGAGCATATACGGGAGCAAA
Sbjct  181  CAAGGTGGTGATTTTACTGCCGGAAATGGTACCGGTGGCAAGAGCATATACGGGAGCAAA

Query  241  TTTGAGGATGAAAATTTCAATCACAAGCACAGCAAGCCGATGATGCTCTCGATGGCGAAT
Sbjct  241  TTTGAGGATGAAAATTTCAATCACAAGCACAGCAAGCCGATGATGCTCTCGATGGCGAAT

Query  301  GCGGGTAAGAACACCAATGGCTCGCAATTCTTCATCACTACCGCCGTCACCAGCTGGCTT
Sbjct  301  GCGGGTAAGAACACCAATGGCTCGCAATTCTTCATCACTACCGCCGTCACCAGCTGGCTT

Query  361  GATGGCAAGCATGTTGTCTTTGGTGAGGTAGAAAGTGGCGAAGATGTTGTTAAGGATATG
Sbjct  361  GATGGCAAGCATGTTGTCTTTGGTGAGGTAGAAAGTGGCGAAGATGTTGTTAAGGATATG

Query  421  GAGGCCGTCGGTAGCAGTAGTGGCAAGACTTCCCAAGAAGTACTGATTACTGACTGCGGT
Sbjct  421  GAGGCCGTCGGTAGCAGTAGTGGCAAGACTTCCCAAGAAGTACTGATTACTGACTGCGGT

Query  481  CAGCTG  486
Sbjct  481  CAGCTG  486
```

Figure 3.28: Alignment the cDNA sequences of the recombinant Cyclophilin, obtained using proof-reading Q5 polymerase, with the recombinant Cyclophilin which was amplified using non-proof reading Taq polymerase. It can be seen that the both cDNA sequences are 100% identical.

```
AAN62875     MGVKCFFDISIGGKPAGRIVFALFDDVPKTVENFRALCTGEKGFGYKGSKFHRIIPGFMC
Obtained     MGVKCFFDISIGGKPAGRIVFALFDDVPKTVENFRALCTGEKGFGYKGSKFHRIIPGFMC

Query  61    QGGDFTAGNGTGGKSIYGSKFEDENFNHKHSKPMMLSMANAGKNTNGSQFFITTAVTSWL
Sbjct  61    QGGDFTAGNGTGGKSIYGSKFEDENFNHKHSKPMMLSMANAGKNTNGSQFFITTAVTSWL

Query  121   DGKHVVFGEVESGEDVVKDMEAVGSSSGKTSQEVLITDCGQL  162
Sbjct  121   DGKHVVFGEVESGEDVVKDMEAVGSSSGKTSQEVLITDCGQL  162
```

Figure 3.29: Alignment the amino acids sequences of native Cyclophilin antigen with the obtaining amino acids sequences of the cloned antigen. Protein sequences are 100% identical. The characters in red refer to the accession number of the protein.

Regarding Eg19, the comparison between cDNA sequences of native and cloned Eg19 showed 6 SNPs in the obtained Eg19, when proof-reading Q5 Polymerase was used (Figure 3.30). It is noteworthy that when the cDNA sequence of recombinant Eg19 using proof-reading Q5 polymerase was aligned with the cDNA sequence of the recombinant Eg19 using non-proof reading Taq polymerase, both cDNA sequences were 100% identical (Figure 3.31). In terms of the amino acids sequences, the result shows that two SNPs in the cDNA were silent, while four resulted in an alteration of four amino acids (Figures 3.32).

```
DQ840455     GAGGCTGAGGCAAAGTGCCTACGTCGACCTCATCAAAGGGTTGTAAAGGAGGGGGAAGTC
Cloned       GAGGCTGAGGCAAAGTGCCTACATCGACCTCATCAAAGGGTTGTAAAGGAGGGGGAAGTC

Query  61    AGTAAAGGAGATGAGGTGGATGGTGAAGATAGAGACGATGAATGTGTTGGCGGAGATGAA
Sbjct  61    AGTAAAGGAGATGAGGTGGATGGTGAAGATAGAGACGATGAATCTGTTGGCGGAGATGAA

Query  121   GGACGGCCCCTACCTCATGAGGTATCCCATGAAGGCAAACAGGAAAGTGAGGATAAGGAT
Sbjct  121   GGGCGGCCCCTACCTCATGAGGTATCCCATGAAGGCAAACAGGAAAGTGAGGATAAGGAT

Query  181   GCCGATAAAATTGCAATTGAAGGTGTTGTCCGCAAGGTAGCTCATTTGGGAACTGGCAAA
Sbjct  181   GCCGATAAAATTGCAATTGAAGGTGTTGTCCGCAAGGTAGCTCATTTGGGAACTGGCAAA

Query  241   AGTCAACATGCTGATGAAAAAGCCCTATTTTatgaagaagaggctgaagatgaaggtgag
Sbjct  241   AGTCAACATGCTGATGAAAAAGCCCTATTTGATGAAGAAGAGGCTGAAGATGAAGATGAG

Query  301   gatgatgaagccgatgaagatgaaagtgatgagatcgaagatgacgatgGCTACTATAAG
Sbjct  301   GATGATGAAGCCGATGAAGATGAAAGTGATGAGATCGAAGATGACGATGGCTACTATAAG

Query  361   ATTGACAAGAAGACCATGGATTCGTTTTGTAATCAAATGAAACGCTATTGGAGAGAAGTT
Sbjct  361   ATTGATAAGAAGACCATGGATTCGTTTTGTAATCAAATGAAACGCTATTGGAGAGAAGTT

Query  421   GGACATGGTATTCGAGAT  438
Sbjct  421   GGACATGGTATTCGAGAT  438
```

Figure 3.30: Alignment the cDNA sequences of native Eg19 with the cloned cDNA, using proof-reading Q5 Polymerase. The highlighted nucleotides in red refer to the SNPs which can be seen when aligning with the native Eg19 gene. It can be seen 6 SNPs in the cloned cDNA sequence. The characters in red refer to the accession number of the gene.

```
Using Q5     ATGGAGGCTGAGGCAAAGTGCCTACATCGACCTCATCAAAGGGTTGTAAAGGAGGGGGAA
Using Taq    ATGGAGGCTGAGGCAAAGTGCCTACATCGACCTCATCAAAGGGTTGTAAAGGAGGGGGAA

Query  61    GTCAGTAAAGGAGATGAGGTGGATGGTGAAGATAGAGACGATGAATCTGTTGGCGGAGAT
Sbjct  61    GTCAGTAAAGGAGATGAGGTGGATGGTGAAGATAGAGACGATGAATCTGTTGGCGGAGAT

Query  121   GAAGGGCGGCCCCTACCTCATGAGGTATCCCATGAAGGCAAACAGGAAAGTGAGGATAAG
Sbjct  121   GAAGGGCGGCCCCTACCTCATGAGGTATCCCATGAAGGCAAACAGGAAAGTGAGGATAAG

Query  181   GATGCCGATAAAATTGCAATTGAAGGTGTTGTCCGCAAGGTAGCTCATTTGGGAACTGGC
Sbjct  181   GATGCCGATAAAATTGCAATTGAAGGTGTTGTCCGCAAGGTAGCTCATTTGGGAACTGGC

Query  241   AAAAGTCAACATGCTGATGAAAAAGCCCTATTtgatgaagaagaggctgaagatgaagat
Sbjct  241   AAAAGTCAACATGCTGATGAAAAAGCCCTATTTGATGAAGAAGAGGCTGAAGATGAAGAT

Query  301   gaggatgatgaagccgatgaagatgaaagtgatgagatcgaagatgacgatgGCTACTAT
Sbjct  301   GAGGATGATGAAGCCGATGAAGATGAAAGTGATGAGATCGAAGATGACGATGGCTACTAT

Query  361   AAGATTGATAAGAAGACCATGGATTCGTTTTGTAATCAAATGAAACGCTATTGGAGAGAA
Sbjct  361   AAGATTGATAAGAAGACCATGGATTCGTTTTGTAATCAAATGAAACGCTATTGGAGAGAA

Query  421   GTTGGACATGGTATTCGAGATcaccaccaccaccaccacGTT  462
Sbjct  421   GTTGGACATGGTATTCGAGATCACCACCACCACCACCACGTT  462
```

Figure 3.31: Alignment the cDNA sequences of the cloned Eg19, obtained using proof-reading Q5 polymerase, with the cloned Eg19 which was amplified using non-proof reading Taq polymerase. It can be seen that the both cDNA sequences are 100% identical.

```
ABI24154     EAEAKCLRRPHQRVVKEGEVSKGDEVDGEDRDDECVGGDEGRPLPHEVSHEGKQESEDKD
Obtained     EAEAKCLHRPHQRVVKEGEVSKGDEVDGEDRDDESVGGDEGRPLPHEVSHEGKQESEDKD

Query  61    ADKIAIEGVVRKVAHLGTGKSQHADEKALFYEEEAEDEGEDDEADEDESDEIEDDDGYYK
Sbjct  61    ADKIAIEGVVRKVAHLGTGKSQHADEKALFDEEEAEDEDEDDEADEDESDEIEDDDGYYK

Query  121   IDKKT  125
Sbjct  121   IDKKT  125
```

Figure 3.32: Alignment of the amino acids sequences of the native and cloned Eg19 antigen. The highlighted amino acids in red refer to the SNPs which can be seen when aligning with the native Eg19 antigen. The characters in red refer to the accession number of the protein.

In terms of EgTPx, the analysis of sequences showed highlighted 11 potential SNPs when the cloned cDNA was aligned with the native EgTPx cDNA (Figure 3.33). The SNPs caused a change in 5 amino acids when comparing with the reference (native) protein (Figure 3.34).

```
EU696759     ATGGCTGCTGTTGTTGGAAAGCTCGCGCCAAGTTTCACGTGCAAGGCTCTTGTCGACGGT
Cloned       ATGGCTGCTGTTGTTGGGAGGCTCGCGCCAAGTTTCACGTGCAAGGCTCTTGTCGACGGT

Query  61    GAACTCAAGGATGTTTCTTTGTCGGACTACCGAGGGAAATATGTGATTCTCTTCTTCTAT
Sbjct  216   GAACTCAAGGATGTTTCTTTGTCGGACTACCGAGGGAAATATGTGATTCTCTTCTTCTAT

Query  121   CCGATGGATTTCACCTTCGTCTGTCCCACTGAGATAATCGCTTTTAACGACCGTGCTGAT
Sbjct  276   CCGATGGATTTCACCTTCGTCTGTCCCACTGAGATAATCGCTTTTAACGACCGTGCTGAT

Query  181   GAGTTTCATCAGCGTGGGTGCCAGCTCCTTGCCTGTTCAACGGATTCCGGCTACTGTCAC
Sbjct  336   GAGTTTCATCAGCGTGAATGCCAGCTCCTTGCCTGTTCAACGGATTCCGGCTACTGTCAC

Query  241   TTGGTGTGGAACAACGTGAGCCGAAAGGAGGGTGGTGTTCAAGGTATGAGGATTCCGATG
Sbjct  396   TTGGCGTGGAATAACGTGAGCCGAAAGGAGGGTGGTGTTCAAGGTATGAGGATTCCGATG

Query  301   CTTGCTGATACCAACCATAAAATCTCACGCGACTACGGCGTACTGATCGAGGATCAAGGC
Sbjct  456   CTTGCCGATACCAACCACAAAATCTCACGCGACTACGGCGTACTGATCGAGGATCAAGGC

Query  361   ATTGCCCTCCGCGGCCTTTTTATCATCGATGACAAGGGAGTTCTGCGTCAAATCACCATC
Sbjct  516   ATTGCCCTCCGCGGCCTTTTTATCATCGACGACAAGGGAGTTCTGCGTCAAATCACCATC

Query  421   AATGATTTGCCCGTCGGCCGCTCTGTGGATGAGGCTCTGCGCCTCTTGGACGCCTTTCAG
Sbjct  576   AATGATTTGCCCGTCGGCCGCTCTGTGGATGAGGCTCTGCGCCTCTTGGACGCCTTTCAG

Query  481   TTCACGGACAAGCATGGTGAGGTTTGCCCAGCAAATTGGCAACCTGGATCAAAAACCTTC
Sbjct  636   TTCACGGACAAGCATGGTGAGGTTTGCCCAGCAAATTGGCACCCTGGATCAAAAGCCTTC

Query  541   AAACCGAGTGCGGGTGATTTGAAGTCGTTCATGAGCTCG  579
Sbjct  696   AAACCGAGTGCGGGTGATTTGAAGTCGTTCATGAGCTCG  734
```

Figure 3.33: Alignment the cDNA sequences of native EgTPx with the cloned cDNA sequences. It can be seen 11 SNPs in the cDNA sequence, when proof-reading enzyme was used for PCR. The characters in red refer to the accession number of the gene.

```
ACD70342     MAAVVGKLAPSFTCKALVDGELKDVSLSDYRGKYVILFFYPMDFTFVCPTEIIAFNDRAD
Cloned       MAAVVGRLAPSFTCKALVDGELKDVSLSDYRGKYVILFFYPMDFTFVCPTEIIAFNDRAD

Query  61    EFHQRGCQLLACSTDSGYCHLVWNNVSRKEGGVQGMRIPMLADTNHKISRDYGVLIEDQG
Sbjct  61    EFHQRECQLLACSTDSGYCHLAWNNVSRKEGGVQGMRIPMLADTNHKISRDYGVLIEDQG

Query  121   IALRGLFIIDDKGVLRQITINDLPVGRSVDEALRLLDAFQFTDKHGEVCPANWQPGSKTF
Sbjct  121   IALRGLFIIDDKGVLRQITINDLPVGRSVDEALRLLDAFQFTDKHGEVCPANWHPGSKAF

Query  181   KPSAGDLKSFMSS  193
Sbjct  181   KPSAGDLKSFMSS  193
```

Figure 3.34: Alignment the amino acids sequences of native EgTPx antigen with the cloned antigen. It can be seen that the both protein sequences are not identical to each other in 5 amino acids. The characters in red refer to the accession number of the protein.

3.3.2 Expression of *Echinococcus* Antigens Using Wheat Germ Lysate Expression System

Almost all (six out of seven) of our selected genes were successfully amplified and cloned into pF3A WG (BYDV) Flexi Vector, with extra six codons encoding histidine (His-tag) at C-terminus to use for purification purposes and for detection of the target proteins using Western Blotting.

After cloning the *Echinococcus* genes into pF3A WG (BYDV) Flexi® Vector, the antigens were expressed through TNT SP6 High-Yield Cell-Free Wheat Germ Lysate Extract Master Mix (WGL). The translated antigens were identified by incorporation of fluorescently tagged lysine (FluoroTect™ Green Lys). It can be seen in the negative control 2, which contains mixture of Wheat Germ Lysate and FluoroTect™ Green Lys only, that the protein expression with Wheat Germ Lysate results in two endogenous fluorescent proteins in the range of 15–17 kDa, see lane two in Figure (3.35). This has the potential to interfere with recognition of the synthesised *Echinococcus* antigens that fall in the same size range. Although the expression of *Echinococcus* antigens through wheat germ lysate was to some extent achieved as there were clear bands at the predicted molecular weight, the SDS-PAGE could not detect two out of five proteins as shown in figure (3.35). This could be due to the fact that these two proteins failed expressing in wheat germ lysate, or the fluorescent bands corresponding to these protein falls in the same range of the wheat germ lysate endogenous proteins size, as the sizes of Cyclophilin and Eg19 are 19 kDa and 17 kDa, respectively, as is the wheat germ lysate endogenous proteins.

Therefore, expression using wheat germ lysate (WGL) indicated that to an extent the expression had been done successfully, but yields were low (Figure 3.35).

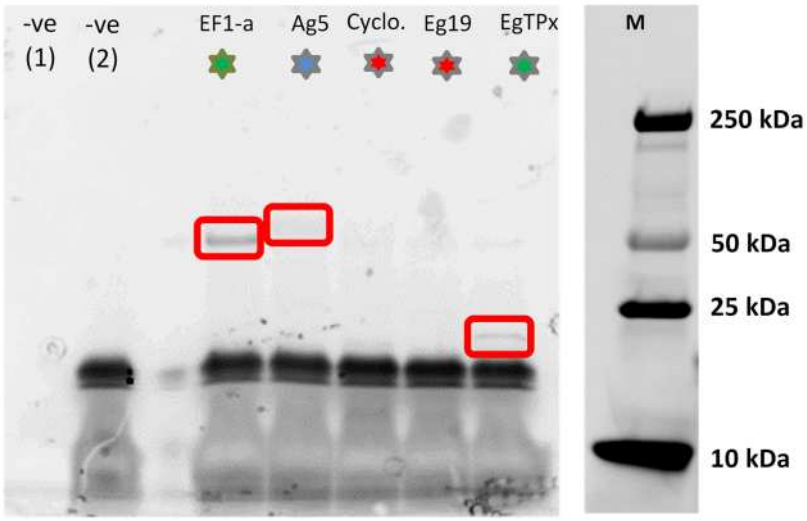


Figure 3.35: SDS-PAGE gel showing *Echinococcus granulosus* antigens expressed in Wheat Germ Lysate. A green star signifies satisfactory protein expression at the predicted molecular weight, a blue star signifies a weak protein expression at the predicted molecular weight, a red star signifies that the band may be obscured by endogenous wheat germ proteins due to the molecular weight of the endogenous protein of the same molecular weight.

Note: The molecular weights of the antigens are EF1-alpha (50.5 kDa), Ag5 (52.3 kDa), Cyclophilin (19 kDa), Eg19 (17 kDa), EgTPx (22 kDa). The abbreviations and symbols represent the following: M – Molecular weight marker; Cyclo.-Cyclophilin; -Ve (1) – Wheat Germ Lysate only; -Ve (2) – Mixture of Wheat Germ Lysate and FluoroTect™ Green Lys.

3.4 Discussion

The first three objectives of this study were performed at the DNA level, which are; firstly, amplifying seven *Echinococcus* genes from the cDNA library of *Echinococcus*, after identifying the optimal annealing temperature for the both primers, forward and reverse, for each gene. Secondly, cloning the correct sizes of the PCR amplicons into an expression vector. Thirdly, confirming the correct sequences of the inserted genes into the expression vector. The fourth objective of this study was at the protein level, which is expression of *Echinococcus* antigens in Cell-Free Wheat Germ Lysate and determination of the sizes of the synthesised proteins by incorporation of fluorescently-tagged lysine. The translated proteins in the presence of fluorescently labelled Lysine were conducted in separate aliquots as this FluoroTect™ Green Lys fluorophore may affect the antigenicity or allergenicity of the expressed proteins.

3.4.1 Antigen Cloning

Amplification and cloning procedures of the *Echinococcus* genes were done, and six out of seven *Echinococcus* genes were effectively amplified from *Echinococcus* cDNA library, after adding six histidine amino acids (His-Tag) to the C-Terminal end of the genes, for purification purpose. In addition, one valine amino acid encoding gene was added to the C-Terminal end as well, for purpose of sub-cloning the insertions into a different Flexi Vector for expression in a different expression system. After amplification, the amplicons were successfully digested and ligated into the expression vector, pF3A WG (BYDV) Flexi Vector, for protein expression in TNT SP6 Cell-Free Wheat Germ Lysate Expression System.

In terms of colony screening, most colonies showed inserts of the appropriate sizes (Figure 3.17, 3.18, 3.19, 3.20 and 3.21), indicating that cDNA had been cloned successfully. Ag5 showed that all lanes, except one, gave bands of colonies inserted with the ligation mixture plasmid-Ag5 are seen in the correct size, when gel electrophoresis was performed (Figure 3.18). This suggests the ligated plasmids were successfully transferred into the bacteria which were loaded in these lanes. However, most of the selected colonies for the other genes, EF1 alpha, Cyclophilin and EgTeg, gave either negative results or unexpected sizes, suggesting that most bacteria failed to take up the recombinant plasmids (Figure 3.17, 3.19 and 3.21). It can be enhanced by increasing the volume of ligation mixture added to the bacteria during transformation. Whereas, absence of bands in some other lanes may suggest colonies were not properly picked before PCR, see Figures (3.21).

Alignment between the cDNA sequences of reference genes and the obtained cDNA sequences of the cloned genes were created for sequences analysis (Figures 3.22, 3.24, 3.27, 3.30 and 3.33). Few SNPs were found in the cloned cDNAs after alignment. However, when the proteins sequences of the native and predicted recombinant protein sequences were aligned with each other, it was noticed that there were identical 100% with each other in terms of EF1-α and Cyclophilin, see Figures 3.23 and 3.29. While, some SNPs were detected in the predicted recombinant Ag5, Eg19 and EgTPx amino acids (Figures 3.25, 3.32 and 3.34).

After aligning the amino acid sequence of the deduced proteins, using Taq polymerase with the deduced proteins, using proof-reading polymerase, it was observed that proteins sequences were 100% homologous (Figures 3.26, 3.28 and 3.31).

With regards to SNPs which have been introduced in the cDNA and amino acids sequences of the obtained cDNAs and predicted proteins, it can be suggested that these SNPs are

either due to cloning process or the use of variance strains of *Echinococcus*. As the whole cloning procedure was repeated twice, once with Taq polymerase and once with proof-reading Q5 polymerase, were giving identical sequences, with regards to all cloned cDNAs, we believe that the strain variation explanation is the most likely one.

3.4.2 Protein Expression in Wheat Germ Lysate Expression System

Recombinant protein expression in *Escherichia coli* is an important tool for protein production. However, deficiency of expression of eukaryotic proteins and insolubility remain major difficulties to the expression in *E. coli*. Therefore, Wheat Germ Lysate Cell-Free technology has been developed for the production of eukaryotic proteins to overcome the insolubility challenges and this may also give satisfied level of protein production. Data show this eukaryotic wheat germ extract expression system has a high achievement proportion. In addition, Wheat Germ Lysate Expression System combines the advantages of eukaryotic and cell-free systems and it enables synthesis of a wide range of proteins for most difficult applications, such as the expression of highly toxic antigens [92, 94-101]. Consequently, cell-free wheat germ lysate expression system alters from a non-competitive to a competitive system in terms of quick, effective and simple technique that can be used to enable producing a wide range of proteins [102, 103]. In addition, wheat germ lysate is a storage form, so it does not have lots of mRNA for translation of the endogenous proteins. Hence, it can be easily detecting the proteins of interest [104]. However, the disadvantages of the expression in wheat germ lysate is the high cost of the kit.

In this study, the target *Echinococcus* genes were expressed in TNT® SP6 High-Yield Wheat Germ Protein Expression System. Fluoro Tect™ Green lysine tRNA was used for detection of the synthesised proteins, which is a Lysine amino acid tRNA labelled with a BODIPY®-

FL. Lysine was used for labelling because it is one of the most frequently amino acids in the proteins.

Although the cloned *Echinococcus* genes were successfully expressed in wheat germ lysate, however when these recombinant proteins were screened through the RS-ATL8 cell line, the cells did not activate (see Chapter 6, figures 6.12, 6.13, 6.14 and 6.15). There is one potential suggestion for the RS-ATL8 cells inactivation which can be explained that the expression of *Echinococcus* proteins in Wheat Germ Lysate gave a low yield which were not efficient for screening through the RS-ATL8 cell line. This could be due to the codons bias for wheat germ expression. Our results were compatible with Endo and Sawasaki (2003) findings, who clarified that the wheat germ lysate produces low yield proteins due to its instability over time [105]. However, Buntbru *et al.* (2014) and Chong (2014) demonstrated that wheat germ lysate could generate high yield of recombinant proteins up to 100 µg/ml [102, 103]. Therefore, there is a second possible explanation why the RS-ATL8 cell line did not activated when stimulating with allergens expressed in wheat germ lysate, is probably because of an issue with the proper folding of the allergens due to disulphide bonds.

Because the production of the recombinant *Echinococcus* proteins in Wheat Germ Lysate was not satisfied to be screened and tested through the RS-ATL8 cell line, we planned switching into a different expression host which was BL21(DE3) *E. coli* for expression of the target proteins.

Chapter 4: Expression of Recombinant *Echinococcus* Proteins in Prokaryotic Hosts

4.1 Introduction

Escherichia coli (E. coli) considers as one of the most commonly used hosts for the expression of heterologous proteins and remains the favoured system for laboratory studies [106]. However, obtaining soluble recombinant proteins in *E. coli* is still the main challenge for structural and functional assessment of the proteins [90, 107].

Over the last few decades, studies present an essential understanding of transcription, translation and protein folding processes in *E. coli*, together with improvement of genetic tools are making *E. coli* more valuable for the production of difficult eukaryotic proteins [107], [90]. Two different strains of *E. coli* expression host; BL21 (DE3) (Invitrogen, UK) and Rosetta 2 (DE3) (Novagen, UK) with two different appropriate expression vectors, which are pF1A T7 Flexi Vector and pET3-a (Novagen), various induction temperatures and time were utilised in this study to recombinantly express soluble and functional *Echinococcus* antigens.

The production of *Echinococcus* genes cloned into pF1A T7 Flexi Vector and pET3-a Vector derived by T7 promoter, the binding site of T7 RNA Polymerase. Therefore, the expression of the proteins can be very effective in hosts that have a source of T7 RNA polymerase, such as *E. coli* BL21 (DE3) and Rosetta 2 (DE3), due to the specificity for the T7 promoter on pF1A T7 Flexi Vector and pET-3a. Hence, DE3 lysogens region in *E. coli* BL21 (DE3) and Rosetta 2 (DE3) makes these strains very effective for bacterial expression [108-110].

The previous chapter described the amplification of several *Echinococcus* genes from *Echinococcus* cDNA library and cloning into pF3A WG (BYDV) Flexi Vector for Wheat Germ Lysate expression. However, despite efficient amplification and cloning, these attempts

did not result in sufficient amount of *Echinococcus* antigens for downstream experiment. In this chapter, we will describe the sub-cloning of these genes from pF3A WG (BYDV) wheat germ lysate vector into pF1A T7 for *E. coli* expression.

In the present study, the production of the recombinant *Echinococcus* proteins (EF1-α, Ag5, Cyclophilin, Eg19, EgTeg and EgTPx) in different bacterial expression hosts was explained in three steps. First, the *Echinococcus* genes were sub-cloned into specific vectors designed for protein synthesis in *Escherichia coli (E. coli)*. *Echinococcus* antigens encoding cDNAs with codon optimizing for bacterial expression were designed for genes synthesis. Secondly, the genes were expressed in different bacterial expression hosts. Finally, the produced proteins were purified using immobilised metal affinity chromatography (IMAC).

4.2 Experimental Procedures

4.2.1 Expression of Cloned *Echinococcus* Genes in BL21 (DE3) Expression System

BL21 (DE3) *E. coli* strain was used as a bacterial expression host to produce recombinant *Echinococcus* antigens. Two different expression plasmids were utilised with BL21 (DE3) strain for bacterial expression, which were pF1A T7 Flexi Vector and pET-3a.

To express *Echinococcus* genes in BL21 (DE3) strain, the six genes (EF1-α, Ag5, Cyclophilin, Eg19, EgTeg and EgTPx) were sub-cloned from a wheat germ expression vector pF3A WG (BYDV) Flexi Vector to a bacterial expression vector pF1A T7 Flexi Vector (Promega, UK), by following the steps below:

4.2.1.1 Restriction Digestion and Ligation

Restriction digestion of the inserted genes with the plasmid pF3A WG (BYDV) was performed by using the enzyme blend SgfI and PmeI, according to the manufacturer's instructions of Flexi® System (Promega, UK). The digestion of the lethal barnase gene from the bacterial plasmid (pF1A T7 Flexi Vector) was done, as well, by using the blend enzyme SgfI and PmeI, as detailed in Section (3.2.1.3) in Chapter (3).

The reactions were run in gel electrophoresis to confirm the excision of the *Echinococcus* genes, this was done in 1% (w/v) Agarose gel (Sigma-Aldrich Co., UK) with 0.5X Tris/EDTA (TBE) buffer (Fisher Bio-Reagent, UK) and 10µl of ethidium bromide (Fisher Scientific, USA) or 10µl Cybr Gold nucleic acid gel stain (Fisher Scientific, USA). The gels were then

imaged in a GeneGenius (Syngene) imaging system. The excised genes and pF1A T7 Flexi®Vector were extracted from the gels using gel extraction kit (Sigma-Aldrich Co., UK) following the manufacturer's protocol.

To join the sticky ends of the extracted genes with the cut bacterial expression vector, 100 ng of each gene was added to 50 ng of the bacterial expression vector pF1A T7, using 1µl of T4 ligase (Promega, UK) in 10µl of 2X Flexi ligase buffer (Promega, UK). The reactions were then incubated for 1 hour at room temperature. 10µl of the ligated plasmid was run in 1% (w/v) gel electrophoresis, as mentioned above, and screened under imaging system to confirm the ligation of the gene with the plasmid.

4.2.1.2 Transformation into the cloning strain

After the sub-cloning, the ligated plasmids were replicated by transformation into chemically competent cells *E. coli* XL10 strain, see section (2.3.7) in Chapter (2) for transformation details. Polymerase chain reaction (PCR) was run using T7 promoter forward primer and genes specific reverse primers, presented in Table 4.1 (a and b), to confirm the ligation of the genes into the pF1A T7 vector. These primers were used for amplifying the inserted genes and part of the vector. The PCR products were then run in 1% (w/v) gel electrophoresis to screen the *Echinococcus* genes insertion.

Afterward, the plasmids were purified and sent to Source BioScience, Nottingham with T7 promoter as a forward primer and a plasmid specific reverse primer T7 terminator for sequencing and confirming the obtained nucleotides sequences before proceeding to bacterial expression (See Table 4.1 a).

Table 4.1: Primers for colony PCR screening and confirmatory sequencing.

Forward and reverse indicate sense and antisense primers, respectively. T7 Promoter Forward and T7 Terminator Reverse are universal primer sequences corresponding to the T7 Promoter and T7 Terminator.

[A] Primers used for colony PCR screening.

[B] Primers used for confirmatory sequencing.

Table 4.1 a: pF1A T7 universal T7 Promoter forward primer and T7 Terminator reverse primer.

Construct Name	Primer Name	Sequence (5'-3')
pF1A T7-*Echinococcus* genes	T7 Promoter [23-41 bp] Forward [AB]	TAATACGACTCACTATAGG
	T7 Terminator [793-811 bp] Reverse [AB]	GCTAGTTATTGCTCAGCGG

Table 4.1 b: *Echinococcus* genes reverse primers for colony PCR screening.

Construct Name	Primer Name	Sequence (5'-3')
pF1A T7 –EgEF1-a	EgEF1-a Reverse [A]	GAGCGTTTAAACGTGGTGGTGGTGGTGGTGTTTTTTGGCCTTGGCGGCCT
pF1A T7-EgAg5	EgAg5 Reverse [A]	TTGCGTTTAAACGTGGTGGTGGTGGTGGTGGACTGCGTAGCGGTTGATCCACT
pF1A T7 - Cyclophilin	Cyclophilin Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTGGTGCAGCTGACCGCAGTCAGTAATCAGTACTTCT
pF1A T7 –Eg19	Eg19 Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTGGTGATCTCGAATACCATGTCCAACTTCTCT
pF1A T7 -EgTeg	EgTeg Reverse [A]	TTGTGTTTAAACGTGGTGGTGGTGGTGGTGGTAGGTAGAGGATTGCCACACAAGA
pF1A T7-EgTPx	EgTPx Reverse [A]	GTCGGTTTAAACGTGGTGGTGGTGGTGGTGCGAGCTCATGAACGACTTCAA

4.2.1.3 Transformation into Expression Strain and Protein Production

For transformation of the purified expression constructs into BL21 (DE3), 2 µl of the ligated plasmid was added to 50 µl of chemically competent cells BL21 (DE3) and placed in ice for 30 minutes. After 30 minutes, the mixture was placed into water bath at 42°C for 10 seconds, and then transferred back into ice for 5 more minutes. The transformed bacteria were then grown in SOC medium for 1 hour, plated onto LB-Amp agar plate (100µg/ml Ampicillin) and grown at 37°C overnight.

Next day, single colonies were picked and grown in 5 ml of LB-Amp medium at 37°C overnight to make a starter culture. Then, the starter cultures were diluted with fresh LB-Amp medium at ratios of 1:200 starter cultures to new medium. After that, the diluted cultures were incubated in a shaking incubator at 37°C and speed of 250 rpm for 3-4 hours. The optical density (OD) of the bacteria was sampled using UV-spectrophotometer to mid log phase at 600 nm (OD^{600}) =0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega) was added to final concentrations of 0.25 mM and 1 mM, for each gene, to induce the bacteria for protein expression [111], after removing 1ml of each culture as a pre-induction control. The cultures were then incubated at 37°C for another 7 hours at 250 rpm. At hourly time points after induction, aliquots of 1 ml were removed from each IPTG concentration culture.

4.2.1.4 Target Protein Verification

To assess the production of the target *Echinococcus* proteins in BL21 (DE3) *E. coli*, the expressed proteins were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie-gel staining and Western Blotting**.** All 1ml samples were centrifuged at 14,000xg for 10 minutes. After that, the supernatant

was discarded and the pellets were re-suspended in 100µl of fresh LB-Amp medium. The re-suspended cells were placed in ice and sonicated in a sonicator for 30 seconds. Afterwards, the cells were placed on liquid nitrogen for 15 minutes and thawed by incubation for 5 minutes at 37°C with shaking (this step was repeated three times) to destroy the bacteria cell wall and release proteins. An equal volume of 2x non-reducing sample buffer (NRSB) was added into each solution. Finally, the solutions were heated for 5 minutes at 100°C to be ready for SDS-PAGE.

After sample preparation, 12 µl of each sample was loaded into precast Tris-Glycine 4-20% TGX mini gels (Bio-Rad, USA). Precision plus protein standards dual colour ladder (Bio-Rad, USA) was loaded into the gel for Coomassie-gel staining and Western C ladder (Bio-Rad, USA) for Western Blotting. The samples were then run in a Mini-PROTEAN™ device (Bio-Rad, USA) at 110mA.

- **Coomassie Stain**

To detect the target proteins, the SDS-PAGE gels were stained with Coomassie stain containing 25% methanol, 10% glacial acetic acid, 65% deionised water and 2 grams of Coomassie powder, for 1 hour at room temperature on a rocking platform. After one hour, the stain was discarded and the gel de-stained with 50ml of de-staining buffer containing 25% methanol, 10% glacial acetic acid and 65% deionised water, for 30 minutes at room temperature on the orbital shaker. Finally, the de-staining buffer was replaced with a fresh de-staining buffer and the gels were shaken on a rocking platform machine overnight at room temperature, until the protein bands determined appropriately.

- **Western Blot**

Western Blot was performed to confirm the position of the bands of the expressed *Echinococcus* antigens. Non-induced transformant bacteria were used as a negative control, while a protein with an approximate molecular weight of 57 kDa containing C-Myc Tag and His-Tag was used as a positive control. After SDS-PAGE, the gel was blotted on a 0.2 µm nitrocellulose membrane (Thermo Fisher, Scientific), using Trans-Blot® Turbo™ Blotting System (Bio-Rad, USA) following the manufacturer's protocol. The membrane was placed into a shaking tray filled with 25ml of 5% Marvel non-fat dried milk dissolved in Tris Buffered Saline (TBS) supplemented with 0.1% Tween-20 (TBS-T) blocking buffer, for 1 hour at room temperature. Mouse Anti-His Tag monoclonal antibody (GE Healthcare, USA) was used as a primary antibody for 6x Histidine immunodetection or Mouse Anti C-Myc:Biotin (AbD Serotec, USA) was used as a primary antibody for C-Myc immunodetection. Both antibodies were diluted in fresh blocking buffer at 1:5000, and placed on the membrane overnight at 4°C, after discarding the old blocking buffer.

Next day, the membrane was washed four times, 15 minutes each, with TBS-T, followed by incubation with Goat Anti-Mouse IgG (whole molecule)-Horseradish peroxidase enzyme (HRP) conjugated antibody (GE Healthcare, USA) (1:5000) as a secondary antibody for 6x Histidine immunodetection. For C-Myc immunodetection, the membrane was incubated with conjugate Streptavidin-HRP (AbD Serotec, USA, also at a 1:5000 dilutions, in fresh TBS-T blocking buffer, for 1 hour at room temperature. The membrane was then washed again four times, 15 minutes each, with TBS-T.

Subsequently, the membrane was exposed to chemiluminescent Western Blot detection substrate (Thermo-Fisher Scientific). Finally, the membrane was imaged using FUJI-LAS 4000 imaging system charge coupled device camera (Fujifilm, Japan) under high binning mode.

4.2.2 Expression of Synthesised *Echinococcus* Genes in BL21 (DE3) Expression System

4.2.2.1 Designing *Echinococcus* Genes for Bacterial Expression

Before synthesis of *Echinococcus* genes by GeneArt, the genes were designed with optimal codons for bacterial expression and 10 codons encoding C-Myc Tag for protein detection, as well as, 6 codons encoding six histidine (His-Tag) for protein purification. In addition, restriction sites of NdeI on 5' end and BamHI on 3' end were added to the genes, see Figure (4.1). The restriction sites were used to join the sticky ends of NdeI and BamHI sites on the pET-3a expression plasmid, generated during digestion. The cDNA sizes and expected protein molecular weights were EF1-a 1,404 bp (51.5 KDa), Ag5 1,452 bp (53.5 KDa) and Cyclophilin 546 bp (20 KDa).

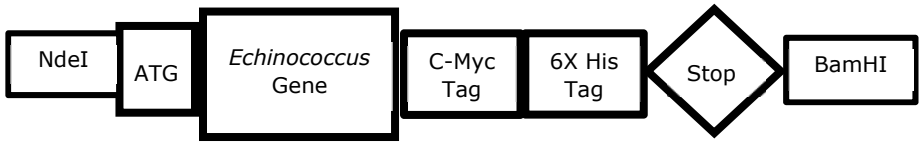


Figure 4.1: Schematic diagram of pET-3a vector showing expression cassette.

Echinococcus genes were cloned into pET-3a using NdeI and BamHI restriction sites. The abbreviations symbolise the following: ATG- Methionine (start codon); C-Myc Tag- Peptide tag for detection of the protein; 6xHis Tag- 6x Histidine peptide tag for protein purification; Stop- Stop codon.

4.2.2.2 Cloning the Synthesised *Echinococcus* Genes into pET-3a Expression Vector

The synthesised *Echinococcus* genes and pET-3a plasmid were digested using the restriction enzymes NdeI and BamHI (both from New England Biolabs) according to the manufacturer's protocol. After that, the genes were ligated to the plasmid using DNA T4 ligase (New England Biolabs), as mentioned previously (Section 4.2.1.1).

The expression region of pET-3a plasmid which is transcribed by T7 RNA polymerase is presented below (see Figure 4.2). Afterward, PCR was run to confirm that the cloning has been done successfully, using primers presented in Table 4.2 (a and b).

The plasmids were then sequenced and analysed for confirmation of nucleotides sequencing using T7 promoter as a forward primer and T7 terminator as a reverse primer, before continuing to proteins expression (See Table 4.2 a).

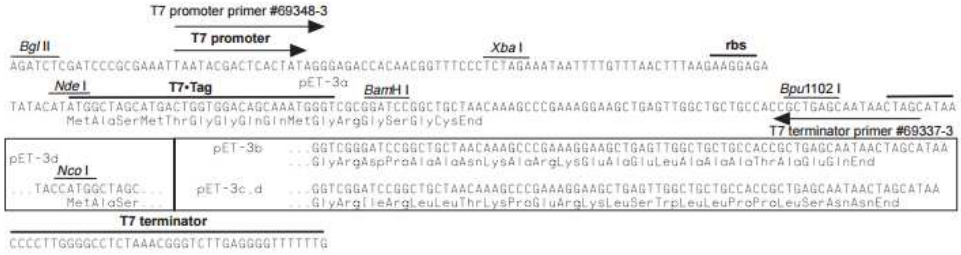


Figure 4.2: pET-3a Cloning/ Expression Region

The map shows T7 promoter and T7 terminator in the multiple cloning site. The map also shows ribosome binding site. So, the restriction sites NdeI and BamHI were used to insert the target gene into the cloning region on the pET-3a vector. Source: Novagen company.

Table 4.2: Primers for colony PCR screening and confirmatory sequencing.

Forward and reverse indicate sense and antisense primers, respectively. T7 Promoter Forward and Reverse are universal primer sequences corresponding to the T7 Promoter and Terminator.

[A] Primers used for colony PCR screening.

[B] Primer used for confirmatory sequencing.

Table 4.2 a: pET-3a universal T7 Promoter forward primer and T7 Terminator reverse for colony PCR screening and confirmatory sequencing which have been used with all the genes of interest.

Construct Name	Primer Name	Sequence (5'-3')
pET-3a - *Echinococcus* genes	T7P Forward [AB]	TAATACGACTCACTATAGG
	T7T Reverse [AB]	GCTAGTTATTGCTCAGCGGT

Table 4.2 b: *Echinococcus* genes reverse primers for colony PCR screening.

Construct Name	Primer Name	Sequence (5'-3')
pET-3a-EgEF1-a	EgEF1-a _ BamHI Reverse [A]	CATTAAGGATCCTTAATGGTGATGATGATGATG
pET-3a-EgAg5	EgAg5 _ BamHI Reverse [A]	CATAAAGGATCCTTAATGATGGTGGTGATGATG
pET-3a-Cyclophilin	Cyclophilin _ BamHI Reverse [A]	TAATAAGGATCCTTAATGGTGATGGTGATGATG

4.2.2.3 *Echinococcus* Protein Production

The ligated plasmid pET-3a with the synthesised genes were then transformed into BL21 (DE3) *E. coli* by adding 2µl of the recombinant plasmid to 50µl of the competent cells. The mixture was kept on ice for 30 minutes and then transferred to water bath for 90 seconds at 42°C, in order to heat shock the bacteria, and again returned back to ice for 2 minutes. The all other processes of transformation, colonies screening, analysing sequences were done as described earlier in section 2.3.7 and 2.3.8. Whereas, the protein expression and verification were performed as explained in section 4.2.1.3 and 4.2.1.4, respectively.
Three various IPTG induction and expression conditions were evaluated:

- Growth at 25 °C after induction with 0.25 or 1.0 mM IPTG for 16 hours.
- Growth at 30 °C after induction with 0.25 or 1.0 mM IPTG for 4 hours.
- Growth at 37 °C after induction with 0.25 or 1.0 mM IPTG for 4 hours.

Pellet and supernatant were taken at each specified time point. Recombinant allergen expression was analysed using SDS-PAGE followed by Coomassie gel staining and Western Blotting, as described in section 4.2.1.4.

4.2.2.4 Purification of *Echinococcus* Proteins Using Immobilised Metal Affinity Chromatography (IMAC)

Immobilised Metal Affinity Chromatography (IMAC) was used to purify the expressed *Echinococcus* allergens EF1-a, Ag5 and Cyclophilin in BL21 (DE3) strain of *E. coli*. This purification technique is used to purify and concentrate His-tagged proteins from mixtures into buffering solutions, and reduce the amount of unwanted materials in the solution. Purification using affinity column was utilised in the current study to purify the allergens.

- **Purification Using Affinity Column**

The His-tagged *Echinococcus* proteins were purified using gravity column chromatography. The filtrated bacterial supernatant was loaded into a Chromatography Column (Bio-Rad, UK), which contained a His-Tag Purification Resin (Nickel-NTA resin) (Roche, UK). EP-1 Econo Pump (Bio-Rad, UK) was utilised to run the solution with a flow rate of 1 ml/min. The loaded column was then washed with washing buffer (300 mM NaCl, 20 mM Tris-Cl, pH 7.6) three times at a flow rate of 2 ml/min. Afterward, the sample was eluted using elution buffer (300 mM NaCl, 20 mM Tris-Cl, 500 mM imidazole, pH 7.6). The eluted fractions were then tested using SDS-PAGE gel and Western Blotting.

4.3 Results

4.3.1 Expression of Cloned *Echinococcus* Genes in BL21 (DE3) Expression System

4.3.1.1 Sub-Cloning of *Echinococcus* Genes into pF1A T7 Flexi Vector

The six *Echinococcus* genes, EF1-α, Ag5, Cyclophilin, Eg19, EgTeg and EgTPx, were successfully excised from the wheat germ lysate vector pF3A WG (BYDV). As well as, the lethal barnase gene (335 bp) was successfully removed from the pF1A T7 acceptor vector, using restriction enzymes SgfI and PmeI to digest the both vectors (Figure 4.3). The *Echinococcus* genes were then effectively sub-cloned into the bacterial expression pF1A T7 vector, using T4 ligase.

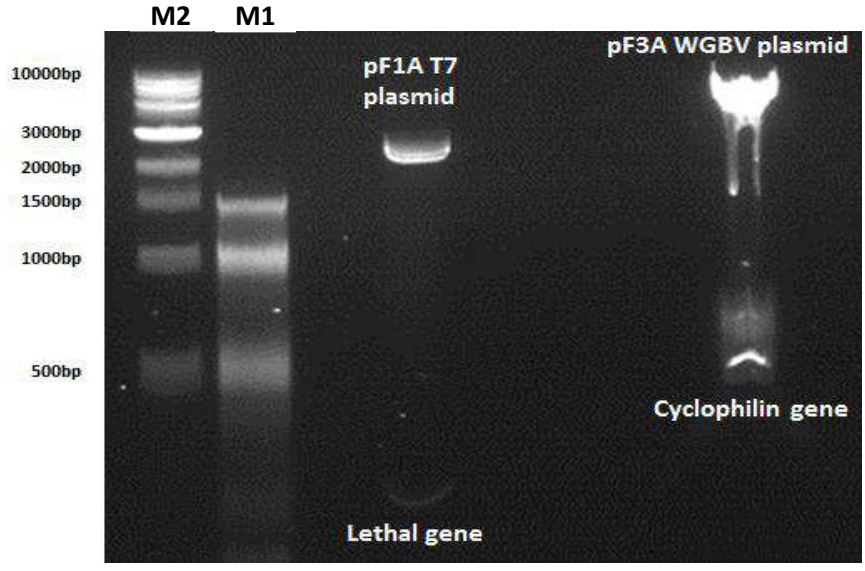


Figure 4.3: Gel electrophoresis of excision of the lethal gene (335bp) from pF1A T7 acceptor plasmid and cyclophilin (503 bp) from pF3A WG (BYDV) donor plasmid using restriction enzymes SgfI and PmeI in agarose gel containing Cybr Gold. M1= 100 bp DNA Ladder, M2= 1Kb DNA Ladder.

4.3.1.2 Transformation of gene-ligated pF1A T7 Vectors into XL10 *E. coli* and Colonies Screening

The recombinant plasmids were successfully transformed into the cloning strain of *E. coli* (XL10). Before PCR amplification for the insert, the optimal annealing temperatures of the T7 promoter primer and the designed gene specific reverse primers were found out for each gene, using the gradient annealing temperature between 45.0°C and 65.0°C, and gels electrophoresis were performed for establishing the optimal annealing temperature (Figure 4.4 – 4.7). Colony screening was performed to confirm plasmid composition. The primers illustrated in Table (4.1) were used to amplify the insert within multiple cloning sites to verify whether the target genes were inserted or not. The results of the colony screening show that the genes were inserted successfully into the pF1A T7 plasmid, see Figures (4.4 – 4.7).



Figure 4.4: Agarose gel electrophoresis – PCR colony screening of XL10 transformed with pF1A T7-EF1-a construct. The required size is (1,465 bp), this include 1,378 bp the size of EF1-a gene and 87 bp from the plasmid. T7 promoter and gene specific reverse primer with the annealing temperature at 56.0^{o}C were used. The image shows colony 5 give a right band size. M1= 100 bp DNA Ladder, M2= 1Kb DNA Ladder.

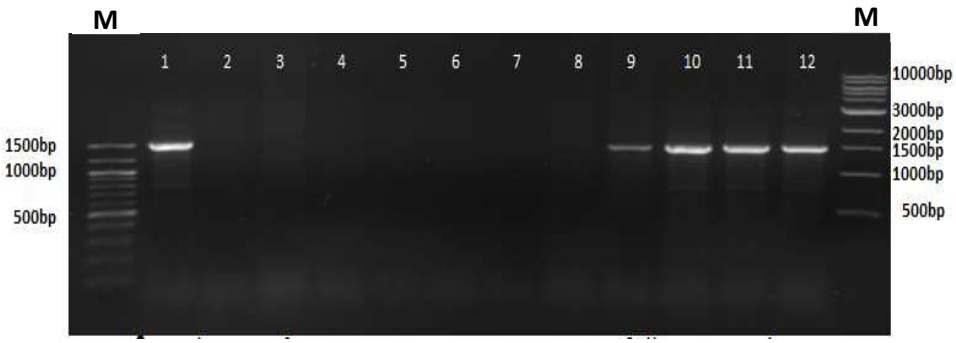


Figure 4.5: Agarose gel electrophoresis – PCR colony screening of XL10 transformed with pF1A T7-Ag5 construct. The required size is (1,513 bp), this include the size of Ag5 gene 1,426 bp and 87 bp from the plasmid. T7 promoter and gene specific reverse primer with the annealing temperature at 46.7^{o}C were used. The image shows colonies 1, 9, 10, 11 and 12 give right bands size. M1= 100 bp DNA Ladder, M2= 1Kb DNA Ladder.

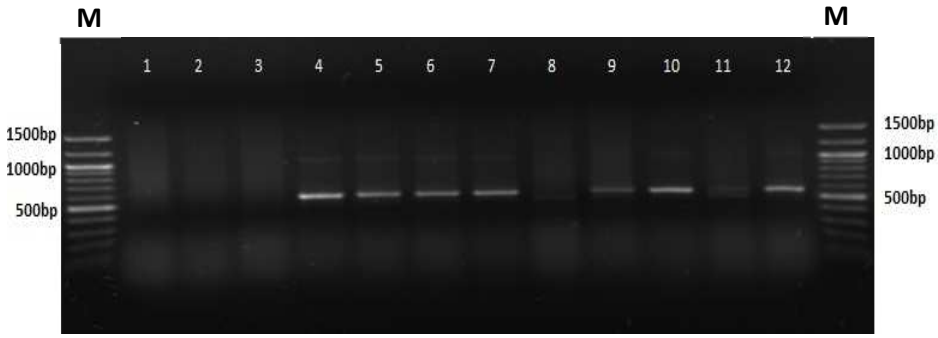


Figure 4.6: Agarose gel electrophoresis – PCR colony screening of XL10 transformed with pF1A T7-Eg19 construct. The required size is (565 bp), this include the size of Eg19 gene 478 bp and 87 bp from the plasmid. T7 promoter and gene specific reverse with the annealing temperature at 56.7^{o}C were used. The image shows colonies 4, 5, 6, 7, 9, 10 and 12 give right bands size. M= 100 bp DNA Ladder.



Figure 4.7: Agarose gel electrophoresis – PCR colony screening of XL10 transformed with pF1A T7-EgTPx construct. The required size is (700 bp), this include the size of EgTPx gene 613 bp and 87 bp from the plasmid. T7 promoter and gene specific reverse with the annealing temperature at 46.7^{o}C were used. The image shows colonies 5, 10 and 12 give right bands size. M1= 100 bp DNA Ladder, M2= 1Kb DNA Ladder.

4.3.1.3 Sequence analysis

The plasmids with inserted parasitic cDNA sequences from positive colonies were purified and sent for sequencing by Source BioScience, Nottingham. The deduced amino acids of the obtained sequences were then blasted with the reference amino acids sequences using protein blast, NCBI database. The results of alignments show that the predicted EF1-α protein is entirely identical to the reference protein sequence from the database (Figure 4.8). However, Ag5 (Figure 4.9), Eg19 (Figure 4.10) and EgTPx (Figure 4.11) deduced amino acid sequences show some differences when matched with the reference sequences from the database, this could be due to the strain variations. Finally, the plasmids which gave correct reading frame were used as expression plasmids for expression their inserts.

```
BAF63677 MGKEKTHINLIVIGHVDSGKSTSTGHLIYKCGGIDKRTIEKFEKEAAEMGKSSFKYAWVL  60
         MGKEKTHINLIVIGHVDSGKSTSTGHLIYKCGGIDKRTIEKFEKEAAEMGKSSFKYAWVL
Deduced  MGKEKTHINLIVIGHVDSGKSTSTGHLIYKCGGIDKRTIEKFEKEAAEMGKSSFKYAWVL  60

         DKLKAERERGITIDIALWKFETPKYLVTIIDAPGHRDFIKNMITGTSQADCAILVVAAGT  120
         DKLKAERERGITIDIALWKFETPKYLVTIIDAPGHRDFIKNMITGTSQADCAILVVAAGT
         DKLKAERERGITIDIALWKFETPKYLVTIIDAPGHRDFIKNMITGTSQADCAILVVAAGT  120

         GEFEAGISKNGQTREHALLAFTLGVKKLIIAVNKMDAVDYSEKRFQEISSEMKAYIKKVG  180
         GEFEAGISKNGQTREHALLAFTLGVKKLIIAVNKMDAVDYSEKRFQEISSEMKAYIKKVG
         GEFEAGISKNGQTREHALLAFTLGVKKLIIAVNKMDAVDYSEKRFQEISSEMKAYIKKVG  180

         YNPDTVNIVPISGWVGDNMLEPSPNMPWYKGPTLLASIDLVEPPTRPVDKPLRLPLQDVF  240
         YNPDTVNIVPISGWVGDNMLEPSPNMPWYKGPTLLASIDLVEPPTRPVDKPLRLPLQDVF
         YNPDTVNIVPISGWVGDNMLEPSPNMPWYKGPTLLASIDLVEPPTRPVDKPLRLPLQDVF  240

         KISGIGTVPVGRVETGIMKPGMIVTFAPVGISTEVKSIEMHHEALSEAVPGDNVGFNVKN  300
         KISGIGTVPVGRVETGIMKPGMIVTFAPVGISTEVKSIEMHHEALSEAVPGDNVGFNVKN
         KISGIGTVPVGRVETGIMKPGMIVTFAPVGISTEVKSIEMHHEALSEAVPGDNVGFNVKN  300

         ISVKDVRRGNVAGDSKNHPPREAAEFTAQVIVLNHPGEIGAGYTPVLDCHTAHIACKFAE  360
         ISVKDVRRGNVAGDSKNHPPREAAEFTAQVIVLNHPGEIGAGYTPVLDCHTAHIACKFAE
         ISVKDVRRGNVAGDSKNHPPREAAEFTAQVIVLNHPGEIGAGYTPVLDCHTAHIACKFAE  360

         LKEKIDRRTGQVKETNPAKIKSGDAAIVRMVPSKPMCVEVFSEFPPLGRFAVRDMRSTVA  420
         LKEKIDRRTGQVKETNPAKIKSGDAAIVRMVPSKPMCVEVFSEFPPLGRFAVRDMRSTVA
         LKEKIDRRTGQVKETNPAKIKSGDAAIVRMVPSKPMCVEVFSEFPPLGRFAVRDMRSTVA  420

         VGVIKAVTFKDKEAKATKSAQKAAKAKK  448
         VGVIKAVTFKDKEAKATKSAQKAAKAKK
         VGVIKAVTFKDKEAKATKSAQKAAKAKK  448
```

Figure 4.8: Alignment of the predicted amino acid sequence of the obtained EF1-α gene (Line 3) with the reference amino acids sequence (Line 1) from NCBI database. The alignment shows that the two sequences are 100% identical. The characters in red refer to the accession number of the protein.



Figure 4.9: Alignment between the deduced amino acid sequence of the obtained Ag5 gene (Line 3) and the reference amino acids sequence (Line 1) from NCBI database, which shows that there are some variations. The characters in red refer to the accession number of the protein.

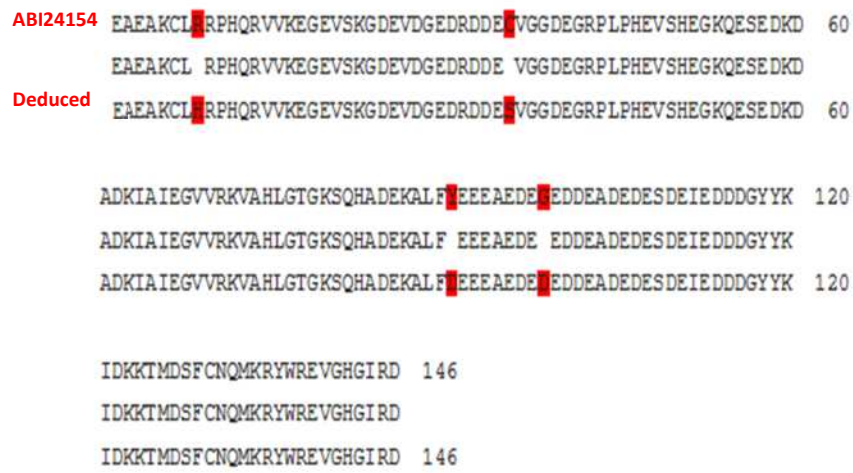


Figure 4.10: Alignment between the deduced amino acid sequence of the obtained Eg19 gene (Line 3) and the reference Eg19 amino acids sequence (Line 1) from NCBI database. The figure shows that there are some variations. The characters in red refer to the accession number of the protein.



Figure 4.11: Alignment between the predicted amino acid sequence of the obtained EgTPx gene (Line 3) and the reference amino acids sequence (Line 1) from NCBI database. The figure shows that there are some variations. The characters in red refer to the accession number of the protein.

4.3.1.4 Transformation of Purified Recombinant Plasmids into BL21 (DE3) Expression Strain and Bacterial Expression

After confirmation of the sequences, the purified inserted plasmids were successfully transformed into the expression strain of *E. coli,* BL21 (DE3). Single colonies were selected to grow in LB-Amp medium for expression of the target genes, using two different IPTG concentrations 0.25mM and 1mM. However, after examining the proteins through Coomassie-stained SDS-PAGE gel and Western Blot, the results showed no any obvious apparent bands indicating over expression of the *Echinococcus* proteins (Figure 4.12 – Figure 4.16).

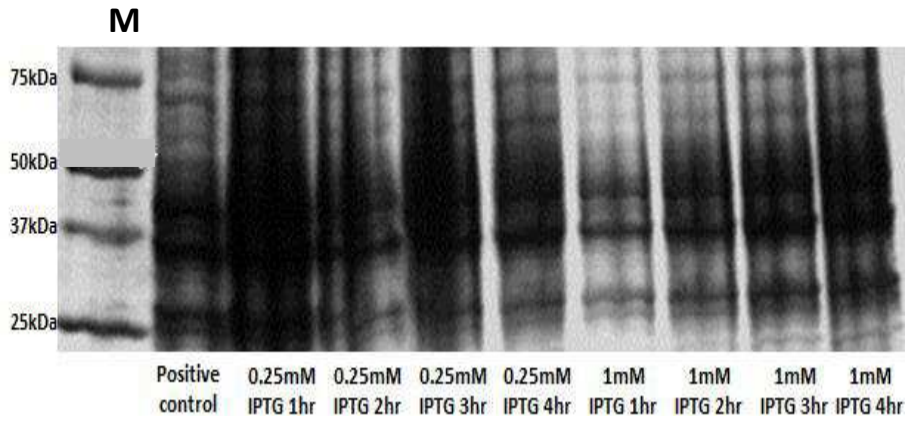


Figure 4.12: SDS-PAGE analysis of EF1-α protein (50.5 kDa) from BL21 (DE3) *E. coli*, using Coomassie staining method. The bacteria were grown at 37°C for 4 hours under two different IPTG concentrations 0.25 mM and 1 mM. The result shows no evident of protein expression. M= Protein Ladder.

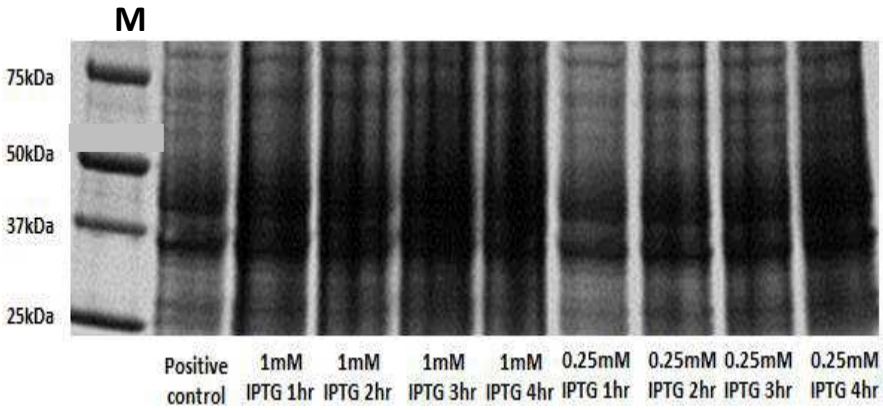


Figure 4.13: SDS-PAGE analysis of Ag5 protein (52.3 kDa) from BL21 (DE3) *E. coli*, using Coomassie staining method. Two different IPTG concentrations were used, 0.25mM and 1mM. The result suggests that the protein was not expressed. M= Protein Ladder.

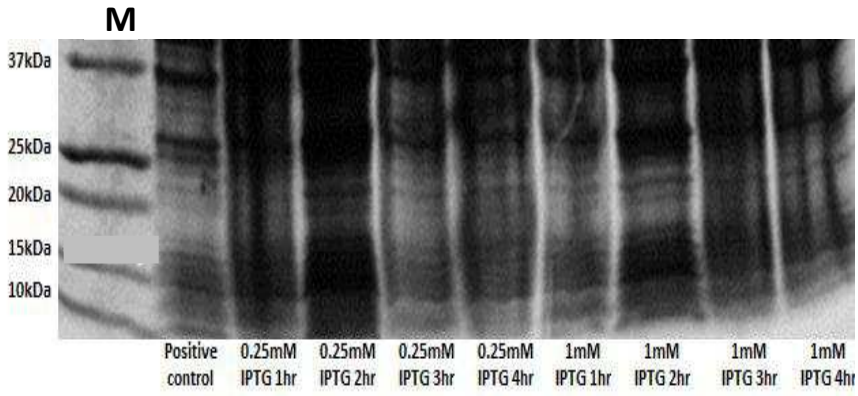


Figure 4.14: SDS-PAGE analysis of Eg19 protein (17 kDa) from BL21 (DE3) *E. coli*, using Coomassie staining method. Two different IPTG concentrations were used, 0.25mM and 1mM. The result shows no evident of protein expression. M = Protein Ladder.

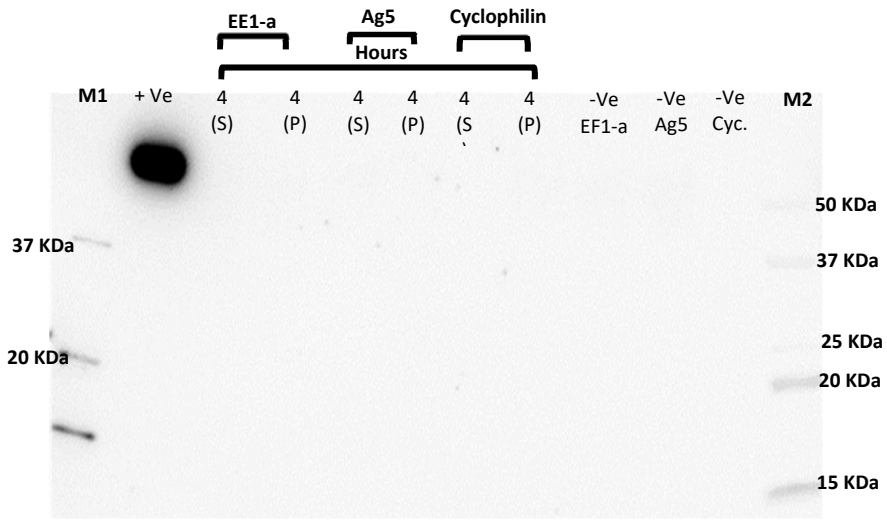


Figure 4.15: Expression of recombinant *Echinococcus* proteins in BL21 (DE3) *E. coli*. Western blot – Chemiluminescent detection of anti His-Tag mouse monoclonal antibody incubated with *Echinococcus* proteins EF1-a (50.5 KDa), Ag5 (52.3 KDa) and Cyclophilin (19 KDa) produced in BL21(DE3) for 16 hours, then were incubated with rabbit antibody (anti-mouse antibody) for 1 hour. The experiment includes un-induced bacteria as a negative control, and a positive control which is an unrelated protein fused with His-Tag (57 kDa). The bacterial lysates were harvested after 4 hours of induction with 0.25mM IPTG at 37°C. The figure shows there is no expression of the proteins. The abbreviations and symbols represent the following: M1 - Western C Protein ladder; M2- Dual Colour Protein Ladder; (S)- supernatant; (P); bacterial pellet.

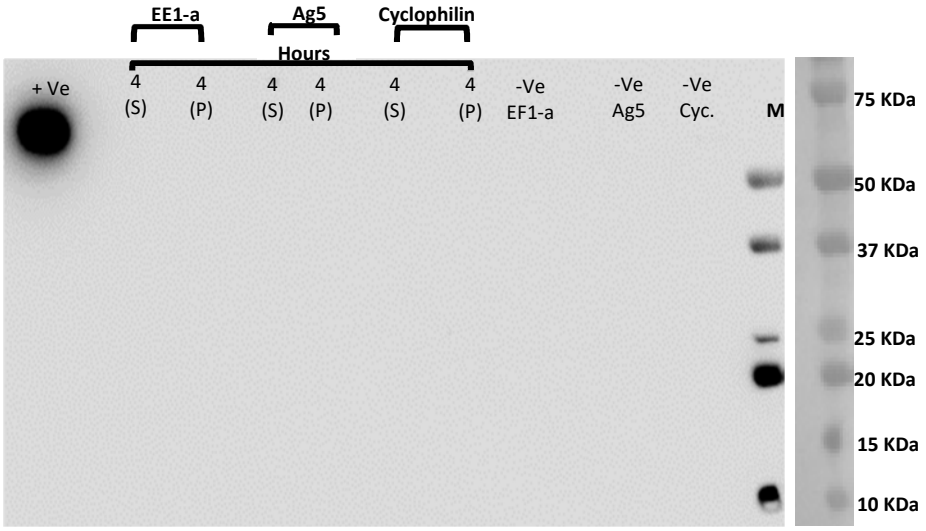


Figure 4.16: Expression of recombinant *Echinococcus* proteins in BL21 (DE3) *E. coli*. Western blot – Chemiluminescent detection of anti His-Tag mouse monoclonal antibody incubated with *Echinococcus* proteins EF1-a (50.5 KDa), Ag5 (52.3 KDa) and Cyclophilin (19 KDa) produced in BL21(DE3) for 16 hours, then were incubated with rabbit antibody (anti-mouse antibody) for 1 hour. The experiment includes un-induced bacteria as a negative control, and an unrelated positive control which is a protein fused with His-Tag (57 kDa). The bacterial lysates were harvested after 4 hours of induction with 1mM IPTG at 37°C. The figure shows there is no expression of the proteins. The abbreviations and symbols represent the following: M- Dual Colour Protein Ladder; (S)- supernatant; (P)- bacterial pellet.

Eukaryotic genes may have some rare codons usage for *E. coli*, and this results in failure in expression of these genes or decrease the protein production efficiency when these proteins express in *E. coli*. Consequently, the codons of the relevant *Echinococcus* genes were analysed using Genscript program and the sequence analysis revealed that there are numerous rare codons for bacterial expression in the expressed genes, as shown in figure (4.17 and 4.18). Nevertheless, it is known that Rosetta 2 (DE3) strain of *E. coli* has the capacity to encode some of these rare codons, as it contains the tRNA for eukaryotic genes rare codons usage for *E. coli*. For this reason, our next step was to express *Echinococcus* proteins in Rosetta 2 (DE3) strain of *E. coli.*

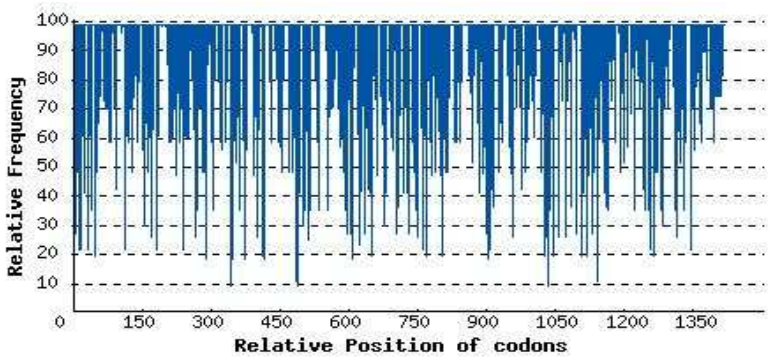


Figure 4.17: The graph shows the rare codons usage for bacterial expression in Ag5. Scale of 100 frequencies refers to high codons usage in bacteria, while 10 frequencies refer to rare codons usage.

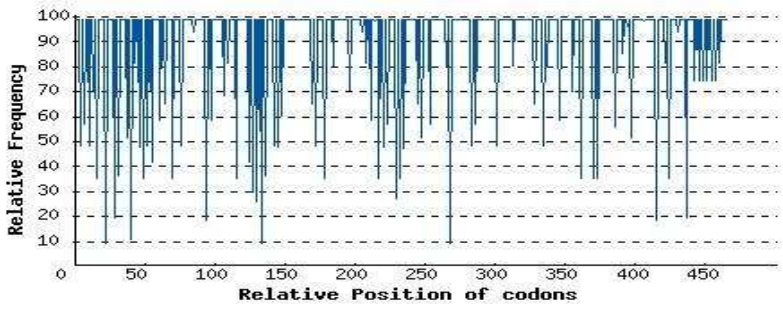


Figure 4.18: The graph shows the rare codons usage for bacterial expression in Eg19. Scale of 100 frequencies refers to high codons usage in bacteria, while 10 frequencies refer to rare codons usage.

4.3.2 Expression of *Echinococcus* Genes in Rosetta 2 (DE3) Expression System

Rosetta 2 (DE3) *E. coli* strain host (Novagen) is derived from BL21 (DE3) strain and has been designed mainly to improve the expression of eukaryotic proteins containing codons rarely used in *E. coli*. Thus, Rosetta 2 (DE3) has tRNAs for seven rare codons (AGG, AGA, AUA, CUA, CCC, GGA and CGG) on a chloramphenicol-resistant encoding plasmid. These tRNA genes are driven by their native promoters [112, 113]. Hence, the *Echinococcus* proteins were expressed in Rosetta 2 (DE3) to overcome the issue of rare codons usage. After successfully transforming the recombinant plasmids into Rosetta 2 (DE3) *E. coli* which was grown in LB- chloramphenicol-ampicillin agar plate (100µg/ml chloramphenicol and 100µg/ml ampicillin), single transformants colonies were selected and analysed as mentioned previously in sections (2.3.7) and (2.3.8). The colonies were then grown in LB- chloramphenicol-ampicillin medium for protein expression using two different IPTG concentrations 0.25mM and 1mM, following the same procedures mentioned previously for BL21 (DE3) expression system, see section (4.2.1.3) and (4.2.1.4). However, the Coomassie staining gels and Western Blot show that the proteins of interest had not been expressed for unknown reason see figures (4.19) and (4.20) for Coomassie Stain, and figure (4.21) for Western Blot.

Therefore, our next step was to synthese *Echinococcus* genes with codons optimised for bacterial expression by GeneArt (Invitrogen, now Thermo-Scientific Fisher).

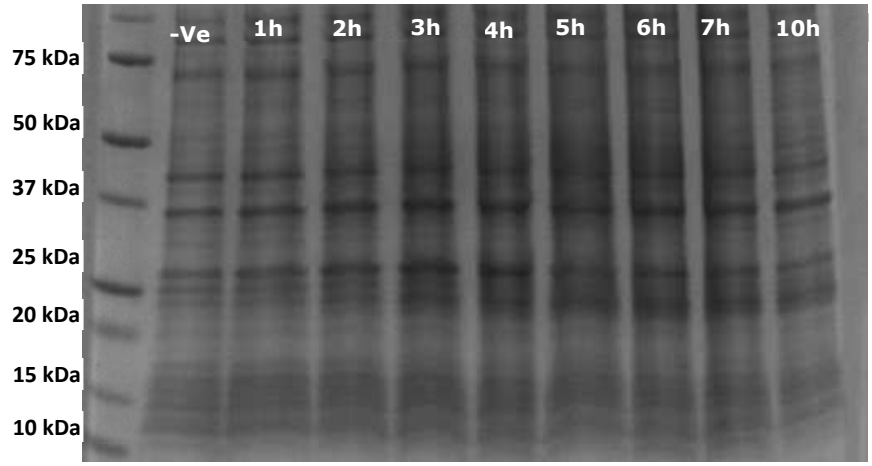


Figure 4.19: SDS-PAGE Gel - Coomassie Stain EF1-a protein was expressed in Rosetta 2 strain in 1x SDS-PAGE non-reducing buffer and denatured at 100^{o}C for 10 minutes. The samples have been taken after 1, 2, 3, 4, 5, 6, 7, 10 hours induction with 1mM IPTG. They were then applied to Mini-Protean® TGX (Bio Rad) prefabricated gels. Negative control was also applied, without IPTG induction. The gels were then run in a Mini-Protean® Tetra System (Bio Rad), using 20mA, for 1hour and then stained by Coomassie stain and imaged in bio imaging system (gene genius).

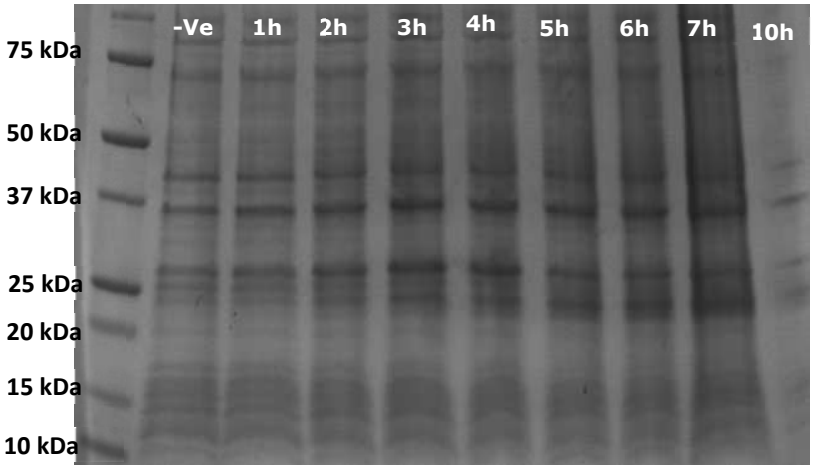


Figure 4.20: SDS-PAGE Gel - Coomassie Stain EF1-a protein was expressed in Rosetta 2 strain in 1x SDS-PAGE non-reducing buffer and denatured at 100^{o}C for 10 minutes. The samples have been taken after 1, 2, 3, 4, 5, 6, 7, 10 hours induction with 0.25mM IPTG. They were then applied to Mini-Protean® TGX (Bio Rad) prefabricated gels. Negative control was also applied, without IPTG induction. The gels were then run in a Mini-Protean® Tetra System (Bio Rad), using 20mA, for 1hour and then stained by Coomassie stain and imaged in bio imaging system (gene genius).

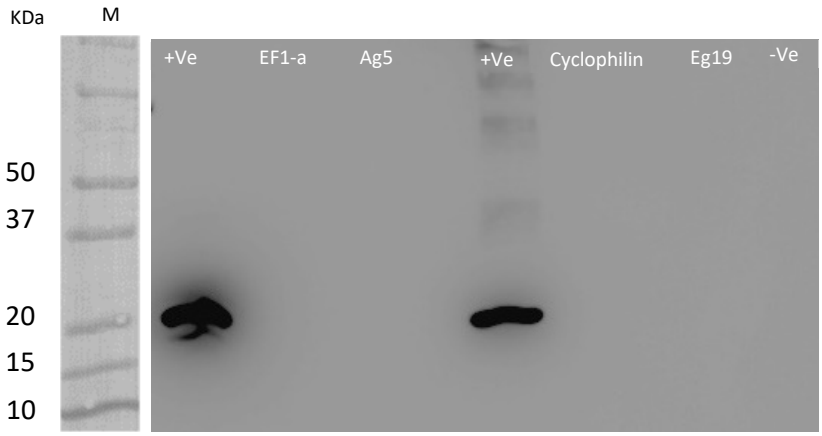


Figure 4.21: Western Blot for *Echinococcus* antigens expressed in BL-21 Rosetta 2 (DE3) strain of *E. coli*. Nitrocellulose membrane was treated with anti-His tag mouse monoclonal antibody, then with rabbit antibody (anti-mouse polyclonal antibody). The image shows that the proteins were not expressed. Membrane was imaged using chemiluminescence and a Fujifilm LAS-4000. The abbreviations and symbols represent the following: M- Dual Colour Ladder (molecular weight marker); +Ve – positive control (20 KDa); -Ve – negative control (non-induced cells transformed with EF1-a ligated plasmid).

4.3.3 BL21 (DE3) Expression System for Synthesised Gene

4.3.3.1 Analysing the Synthesised Genes Sequences

The synthesised *Echinococcus* genes EF1-α, Ag5 and Cyclophilin consist of 1,404 bp (51.5 kDa), 1,452 bp (53.5 kDa) and 549 bp (20 kDa), respectively, including C- Myc Tag and His-Tag on the C-Terminal end, which were added manually for protein detection and purification, respectively. The predicted amino acid sequences of synthesised genes were aligned with the reference amino acid sequences which were taken from NCBI database, and the result shows that they are completely identical (Figure 4.22). However, it could not align the nucleotides sequences for the references with the synthesised genes due to the significant differences between them, although both are giving the same amino acids sequences.

```
AAN62875     MGVKCFFDISIGGKPAGRIVFALFDDVPKTVENFRALCTGEKGFGYKGSKFHRIIPGFMC 60
Synthesised  MGVKCFFDISIGGKPAGRIVFALFDDVPKTVENFRALCTGEKGFGYKGSKFHRIIPGFMC 60

Reference    QGGDFTAGNGTGGKSIYGSKFEDENFNHKHSKPMMLSMANAGKNTNGSQFFITTAVTSWL 120
Synthesised  QGGDFTAGNGTGGKSIYGSKFEDENFNHKHSKPMMLSMANAGKNTNGSQFFITTAVTSWL 120

Reference    DGKHVVFGEVESGEDVVKDMEAVGSSSGKTSQEVLITDCGQL 162
Synthesised  DGKHVVFGEVESGEDVVKDMEAVGSSSGKTSQEVLITDCGQL 162
```

Figure 4.22: Alignment between the deduced amino acids sequence of the synthesised cyclophilin gene with the reference amino acids sequence from NCBI database. The alignment shows that the two sequences are 100% identical. The red characters refer to the accession number of the protein from NCBI Database.

4.3.3.2 Cloning the Synthesised Genes into Expression vector (pET-3a)

The synthesised *Echinococcus* genes were successfully excised from the GeneArt plasmids (pUC-pMA), using the restriction enzymes NdeI and BamHI see Figure (4.23). Figure (4.24) shows that the bands of the synthesised genes EF1-α, Ag5 and Cyclophilin, and pET-3a vector are all in the correct sizes after digestion. The synthesised genes were then sub-cloned efficiently into expression vector (pET-3a) by joining the sticky ends of restriction sites NdeI and BamHI on both insert and vector which were generated during digestion.

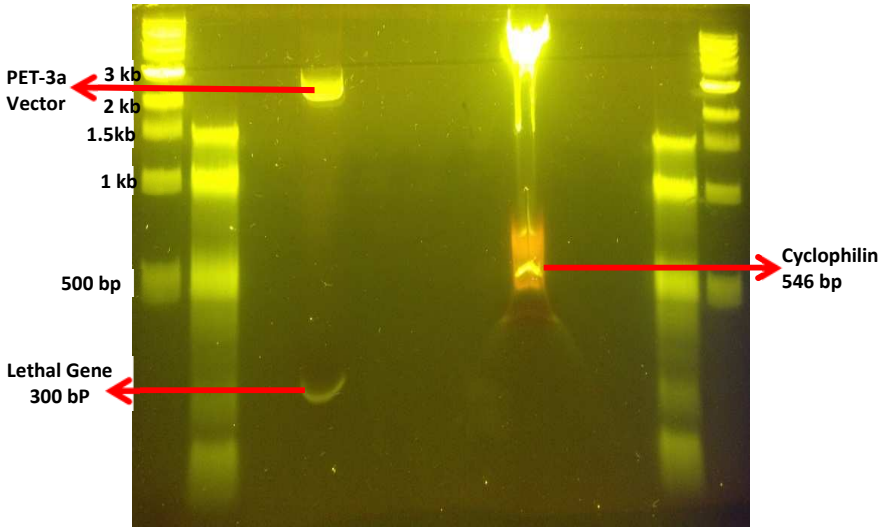


Figure 4.23: The figure shows that the lethal gene (300bp) was digested from PET-3a vector (4,340bp), and the synthesised cyclophilin gene was digested from the vector which is not suitable for bacterial expression.

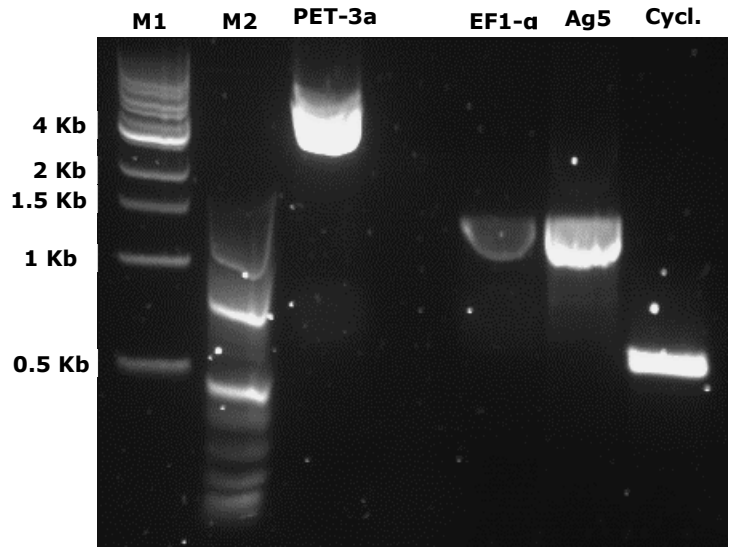


Figure 4.24: Agarose Gel electrophoresis showing pET-3a plasmid and synthesised *Echinococcus spp.* genes.
The figure shows synthesised *Echinococcus spp.* genes after cutting off from pUC-pMA plasmid to confirm the size of the genes and the size of pET-3a plasmid on an agarose gel. The expected sizes of these products are; pET-3a Plasmid 4,600 bp, EF1-a 1,397 bp, Ag5 1,445 bp, Cyclophilin 539 bp. The abbreviations and symbols represent the following: M1= 1KB Molecular DNA ladder, M2= 100 bp Molecular DNA Ladder and Cycl.= Cyclophilin.

4.3.3.3 Transformation into DH5-α Strain and Colony Screening

pET-3a constructs were transformed into DH5-α *E. coli* strain as mentioned in section (2.3.7). To confirm whether the bacteria *E. coli* accepted the inserted vectors or not, the vectors were purified (see section 2.3.8) and sent for sequencing using the universal primers T7 Promoter as a forward, T7 Terminator reverse and genes specific reverses, see Table 4.2 (a and b), for amplifying the cloned synthesised genes and part of the pET-3a vector. The results show that the cloning was achieved successfully and the bacteria were up taken the constructs, see figures (4.25) and (4.26).

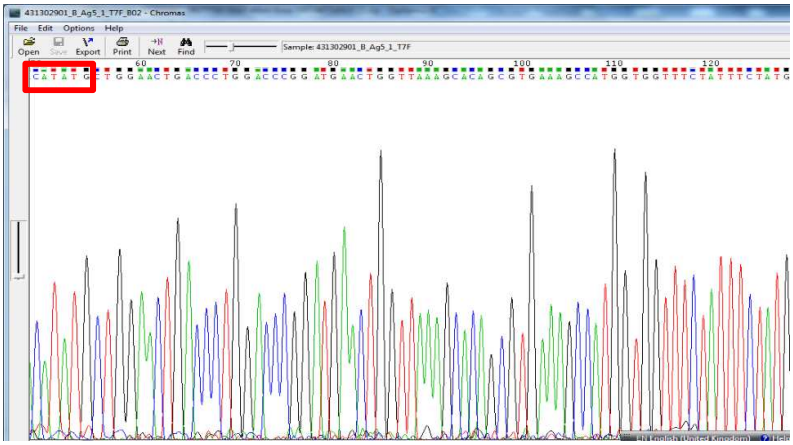


Figure 4.25: Representative DNA sequencing of Ag5 expression construct. Chromatogram trace of the pET-3a_Ag5 construct sequenced after extraction from *E. coli* DH5-a cells. The six nucleotides in the red box represent the NdeI restriction site. To its right is the Ag5 gene fragment.

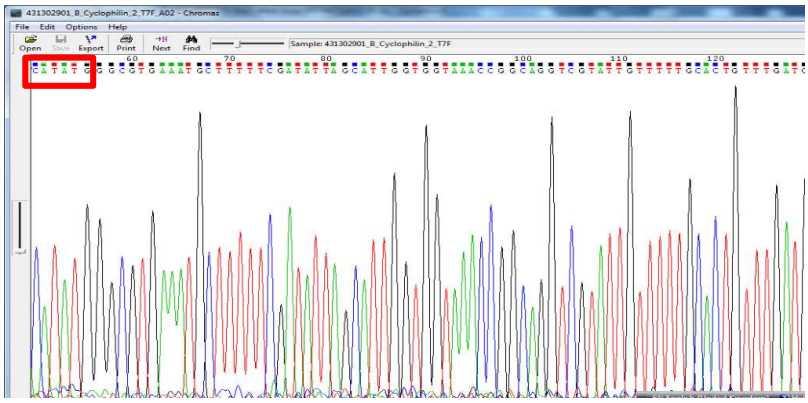


Figure 4.26: Representative DNA sequencing of Cyclophilin expression construct. Chromatogram trace of the pET-3a_Cyclophilin construct sequenced after extraction from *E. coli* DH5-a cells. The six nucleotides in the red box represent the NdeI restriction site. To its right is the Cyclophilin gene fragment.

4.3.3.4 Protein Expression and Verification

The purified cloned vectors with the synthesised *Echinococcus* genes were then transformed into expression host of BL21 (DE3) for producing proteins, see section (4.2.1.3) for details. The protein expression of the synthesised genes was accomplished using different incubation temperatures (25°C, 30°C and 37°C) and longer time of induction with 1mM IPTG up to 16 hours to find the optimum conditions for expression. The synthesised genes expression was then investigated using Western Blotting, which is considered as a sensitive method for protein detection. The proteins bands were expected at 51.5 kDa for EF1-a, 53.5 kDa for Ag5 and 20 kDa for Cyclophilin.

The all three *Echinococcus* proteins were finally expressed at 37°C for up to 4 hours after IPTG induction. However, it was realised that the proteins only can be found in the pellets of the bacteria and it could not be detected in the supernatant, when the samples were analysed using Western Blot, see Figures (4.27), (4.28) and (4.29). This could be due to over-expression and insolubility issue of the target protein in the expression host.

For this reason, the proteins were then expressed at lower incubation temperature of 30°C to avoid the issue of over-expression and producing insoluble proteins. Nonetheless, the proteins were still found in pellets of the bacteria and there were no signs of the proteins in the supernatant and after purification, see Figures (4.28 and 4.29).

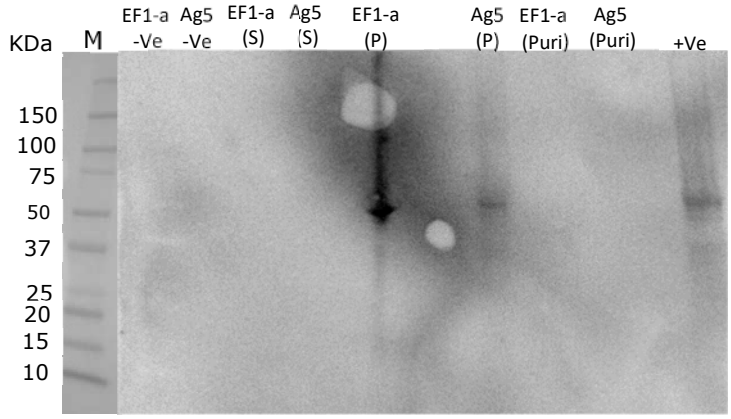


Figure 4.27: Expression of recombinant EF1-α and Ag5 in *E. coli* BL21 (DE3) strain and protein purification using immobilised metal affinity chromatography.

His-Tag immunodetection, using non-reducing sample buffer, showing recombinant EF1-α and Ag5 (The expected sizes are 51.5 KDa and 53.5 KDa, respectively) in the supernatant and the pellet after 4 hours of induction with 1mM IPTG at 37°C and 250 rpm. The abbreviations and symbols represent the following: M- molecular weight marker; +Ve- recombinant BabA protein (57 KDa); -Ve – Un-induced bacteria (bacterial cells before IPTG induction); (S)- Supernatant; (P)- Pellet; (Puri)- Purified Protein.

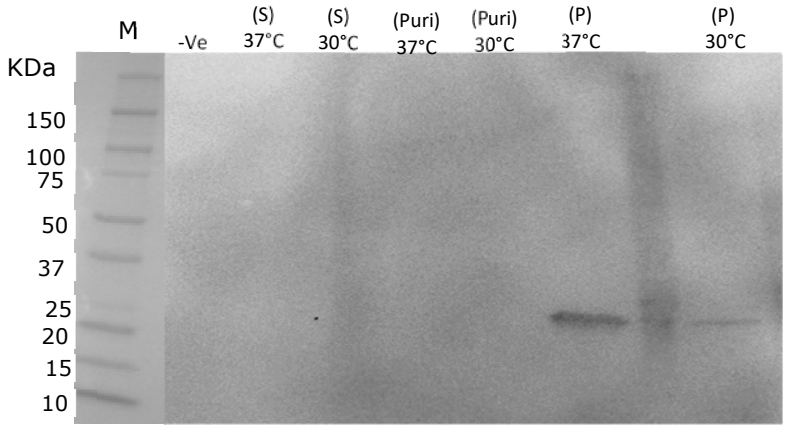


Figure 4.28: Expression of recombinant Cyclophilin in *E. coli* BL21 (DE3) strain and protein purification using immobilised metal affinity chromatography.

His-Tag immunodetection, using non-reducing sample buffer, showing recombinant Cyclophilin (The expected size is 20 KDa) in the supernatant and the pellet after 4 hours of induction with 1mM IPTG at 30°C once, and 37°C once again and 250 rpm. The abbreviations and symbols represent the following: M- molecular weight marker; -Ve – Un-induced bacteria (bacterial cells before IPTG induction); (S)- Supernatant; (P)- Pellet; (Puri)- Purified Protein.

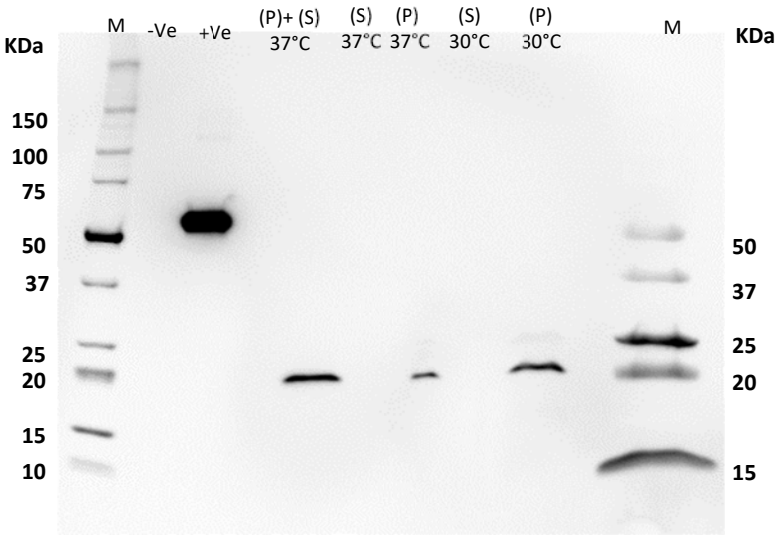


Figure 4.29: Expression of recombinant Cyclophilin in *E. coli* BL21 (DE3) strain. His-Tag immuno-detection, using non-reducing sample buffer, showing recombinant Cyclophilin (The expected size is 20 KDa) in the supernatant and the pellet after 4 hours of induction with 1mM IPTG at 30°C once, and 37°C once again and 250 rpm. The abbreviations and symbols represent the following: M- molecular weight markers; -Ve – Un-induced bacteria (bacterial cells before IPTG induction); +Ve- recombinant BabA protein (57 KDa); (S)- Supernatant; (P)- Pellet.

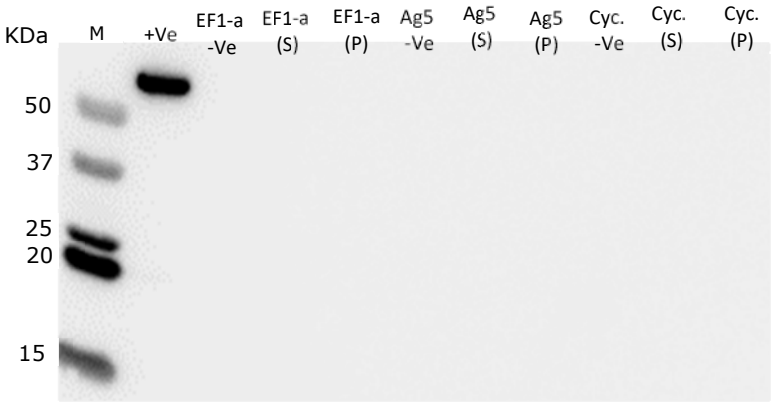


Figure 4.30: Expression of recombinant EF1-α, Ag5 and Cyclophilin in *E. coli* BL-21 (DE3) strain. His-Tag immunodetection, using non-reducing sample buffer, to detect EF1-a, Ag5 and Cyclophilin (The expected sizes are 51.5 KDa, 53.5 KDa and 20 KDa, respectively) in the supernatant and the pellet after 16 hours of induction with 1mM IPTG at 25°C and 250 rpm. The abbreviations and symbols represent the following: M- molecular weight marker; +Ve- recombinant BabA protein (57 KDa); -Ve – Un-induced bacteria (bacterial cells before IPTG induction); (S)- Supernatant; (P)- Pellet.

A lower incubation temperature of 25°C for bacterial expression of *Echinococcus* proteins was utilised to overcome the issue of the over-expression. The samples were kept for Western Blot analysing after 16 hours of induction. However, expression of the proteins at the temperature of 25°C for 16 hours results in no any bands appeared around the required sizes on the membrane when Western Blot was conducted, see Figure (4.30).

Because of the difficulty that we faced to get soluble *Echinococcus* allergens in *E. coli,* to be screened afterwards through the humanised rat basophils leukaemia cell line, therefore our next step was to produce these allergens in eukaryotic cells. This might generate soluble and correctly folded and fully functional proteins.

4.4 Discussion

Escherichia coli (*E. coli*) is an important tool for expression of recombinant protein. *E. coli* use as a cell factory for protein synthesis has become the most popular expression system. Production of heterologous proteins in *E. coli* has several advantages, which are; (i) the host strains can be grown very fast as its doubling time is around 20 minutes [114]; (ii) it is easy to achieve high density of bacterial cell cultures [115, 116]; (iii) the components of rich bacterial culture media are inexpensive and the culture media can be made readily; (iv) the transformation of the DNA construct into *E. coli* is fast and simple [117]; (v) the experiments have shown that large-scale protein production in *E. coli* can give more than 50% of bacterial proteins and around 15% of non-bacterial proteins in a soluble form, which proves the usefulness of the bacterial expression system [118].

4.4.1 Protein Expression in BL21 (DE3)

BL21 (DE3) strain of *E. coli* and its derivatives are considered the most used strains for protein synthesis, as it is important to select a specific strain of *E. coli* containing DE3 region which has T7 RNA Polymerase encoding gene. This gene has been taken from bacteriophage and cloned into a vector to transfer into *E. coli* and generate a modified strain of *E. coli* called BL21 (DE3). T7 RNA Polymerase encoding gene is responsible for T7 RNA Polymerase expression in BL21 (DE3) *E. coli* under the control of *Lac* UV5 promoter. The expression can be enhanced by adding IPTG to the bacterial culture. The production of T7 RNA polymerase in the BL21 (DE3) plays a crucial role of target gene expression, by binding to the T7 Promoter region on vectors, such as pF1A T7 Flexi Vector and pET-3a vector, and drives the expression of the target genes. Thus, DE3 lysogens region in *E. coli* BL21 (DE3) makes this strain very efficient for bacterial expression [108-110, 119-121].

For this reason, BL21 (DE3) *E. coli* strain was used in this study as an expression host for *Echinococcus* proteins production.

4.4.1.1 Sub-Cloning of *Echinococcus* Genes into Bacterial Expression Vector

The Flexi Vector System is designed for expression of the protein in various expression systems by reducing the cloning steps when the target gene is sub-cloned from Flexi vector to another for different purposes. Therefore, the cloned gene into a specific Flexi Vector for a specific expression system can be easily switched into a different Flexi Vector for a different expression system, without requiring amplification and resequencing the gene of interest. Hence, the Flexi Vector system is the best in terms of time and cost savings compared to the other expression vectors [122].

All selected *Echinococcus* genes (EF1-α, Ag5, Cyclophilin, Eg19, EgTeg and EgTPx) were sub-cloned readily from pF3A WG (BYDV) Flexi Vector, the Wheat Germ Lysate expression vector, into pF1A T7 Flexi Vector, the bacterial expression vector. However, after sub-cloning genes into pF1A T7 Flexi Vectors, the constructs were sent for sequencing to verify the cloning.

4.4.1.2 Protein Expression and Verification

After a successfully transformation of the DNA construct into BL21 (DE3) expression host, the bacteria were induced by IPTG for expression of the heterologous genes. However, after protein verification using Coomassie stain and then Western Blot, the results showed that there is no any production of the *Echinococcus* proteins in the bacteria. This might be explained due to the protein toxicity. The issue of protein toxicity appears when

the recombinant protein implements a harmful role in the host. This role interferes with the reproduction of the host cells and the obvious result is slower growth rate, decrease cell density, and finally death [123, 124].

To confirm the potential toxicity of the recombinant protein, the bacterial cells growth was observed before and after induction. Also in a separate flask, an empty-vector bearing BL21 (DE3) cells were grown and the growth rate of these cells was monitored. The obtained measurements showed that the growth rate of the recombinant strain is as high as in an empty-vector bearing strain. Hence, the gene and protein toxicity explanation were excluded.

The second possible explanation might be that there are some RNA structures preventing or inhibiting high levels of translation. It is known that RNA secondary structure could decline protein expression significantly [125], especially when the secondary structure is existing in the translational initiation region of the mRNA. In this case, the translation initiation process becomes more difficult as the ribosome need to overwhelm the barrier of mRNA secondary structure and establish a steady mRNA [126]. However, there is another possible explanation, which could be codon bias or the frequent usage of rare codons for *E. coli* in these genes, and this can affect the effectiveness of heterologous protein expression [113], [127, 128]. Thus, the codons of the *Echinococcus* genes were analysed. It was detected that there are plentiful rare codons for *E. coli* expression host. Because of BL21 (DE3) has no tRNAs to encode these codons, therefore our genes were failing to express in BL21(DE3).

Hence, the *Echinococcus* proteins were then expressed in Rosetta 2 (DE3) strain of *E. coli* as it has been designed to have the tRNAs for seven rare codons for *E. coli* which are AGG, AGA, AUA, CUA, CCC and GGA on an appropriate chloramphenicol-resistant encoding plasmid [112].

4.4.2 Rosetta 2 (DE3) *E. coli* Strain Expression System

Rosetta 2 (DE3) *E. coli* strain is one of the BL21 (DE3) derivatives containing the pRARE plasmid supplying tRNAs for seven rare codons (AGG, AGA, AUA, CUA, CCC, CGG and GGA) on a chloramphenicol-resistant encoding plasmid [112, 113].

The production of eukaryotic proteins in *E. coli* is highly influenced by codon bias. The codons frequencies correlate with the number of their corresponding tRNAs and this differ between different organisms [129, 130]. For instance, eukaryotic cells use the AGG codon for Arginine amino acid, which is rarely used in *E. coli* [127]. Translation of eukaryotic gene with several rare codons in *E. coli* can affect the expression and cause some problems, such as; premature termination of translation or translational discontinuing, frameshifting, and mis-combination of amino acids [131]. For this reason, Rosetta 2 (DE3) strain of *E. coli* was used in this study for expression of *Echinococcus* genes to avoid the rare codon usage issue, as this strain was genetically engineered mainly to overcome the issue of codon bias in *E. coli*. However, the result showed that there is no any evidence of *Echinococcus* gene expression in Rosetta 2 (DE3), for unknown reason. This was totally contradictory with Tegel *et al*. (2010) findings, who had generated positive results and high-throughput of eukaryotic protein expression in Rosetta 2 (DE3) [112]. Also, Kane (1995) who reported that Rosetta 2 (DE3) *E. coli* strain often improves the levels of the heterologous protein expression [113], although we used totally different genes for expression.

The issue of the codon bias can be solved by swapping rare codons in the *Echinococcus* genes with codons more frequently used in *E. coli*. Therefore, our next step was to purchase synthesised *Echinococcus* genes with codons optimisation for bacterial cells.

4.4.3 Expression of Synthesised *Echinococcus* Genes Cloned into pET-3a Plasmid

The effective production of a functional protein in a heterologous host is one of the essential principles of recent studies, as numerous genes fail to express outside their original conditions [132]. Differences in codon usage between various host species are one of the main reasons affecting the level of recombinant protein expression. However, codon optimisation of the heterologous gene can overcome this problem and enhance the expression of the recombinant protein [133]. The rationale behind codon optimisation is to convert the rare codons in the gene of interest to reflect the codon usage in the expression host. The amino acid sequence of the encoded protein should not be modified in the procedure [133-135]. In order to successfully produce eukaryotic *Echinococcus* proteins in bacteria, the eukaryotic genes were optimised to be expressed in bacterial host.

4.4.3.1 Designing *Echinococcus* Genes for Bacterial Expression

In the present study, three *Echinococcus* genes, which are EF1-α, Ag5 and Cyclophilin, were designed with optimal codons and then synthesised in GeneArt Company for expression in *E. coli*. Alignment between the deduced amino acids sequences of synthesised genes were 100% identical with the references amino acids sequences, which were taken from NCBI database (See Figure 4.22). However, there are major differences between the nucleotides sequences of synthesised genes and the references nucleotides sequences, although both are giving the same amino acids sequences.

4.4.3.2 Cloning the Synthesised *Echinococcus* Genes into the Expression Plasmid and Protein Generation

The synthesised *Echinococcus* genes were cloned into the expression plasmid pET-3a, which carries ampicillin resistance marker in its backbone to deter growing of any plasmid-free cell.

Echinococcus genes were expressed in BL21 (DE3) strain of *E. coli* at 37°C, and the results of Western Blot technique clearly determined a successful expression of the heterologous proteins in *E. coli*, after codon optimisation. Our results were compatible with Menzella (2011) findings, who indicated a great enhancement in protein expression in *E. coli* for codon optimised genes [133]. Also, Elena *et al*. (2014) reported a significant improvement in expression level of codon optimised genes expressed in microbial systems [132]. In addition, Welch *et al*. (2009) effectively expressed two of the commercial proteins which are a single chain antibody and DNA polymerase in *E. coli* after synthesising the genes with codon optimisation for bacterial expression [135].

Although, there were a visible band with the correct sizes for the proteins in the bacterial pellets, however we could not detect any proteins in the supernatants and elution fractions for all of the three proteins when instant blue staining and Western Blotting were used for verification. Indeed, when the heterologous gene expresses in *E. coli*, the newly produced polypeptides presence in *E. coli* environment, which is different from their original environment in terms of folding mechanisms. The differences between the original source and expression host environments lead to protein insolubility and aggregation [136-138]. Formation of the protein aggregation called inclusion bodies, and this occurs due to the unbalanced process between protein solubilisation and aggregation [138, 139].

Hammarstrom *et al.* (2002) used fusion tags to enhance the solubility of proteins [140]. Although, it is not clear the reasons behind the fusion tag acting as solubility enhancers [141]. Kapust and Waugh, (1999), also demonstrated that the fusion tag maltose-binding protein (MBP) display great advantages for proteins solubility [142]. Smith and Johnson (1988), found that glutathione S-transferase (GST) fusion tag also has a capability for protein solubility enhancement [143]. However, Esposito and Chatterjee (2006) and Costa *et al*. (2013) identified that the solubility of the desired protein changes after removing these fusion tags [144, 145].

Alternatively, the recombinant proteins can be purified from inclusion bodies and refolded by using chemical chaperones which is urea-denatured. This can increase the yield of soluble protein but cannot help to solubilise all recombinant proteins [89, 146-148].

There is another possibility for protein aggregation and inclusion bodies formation, which is the creation of incorrect disulphide bonds in the newly synthesised recombinant polypeptides that can lead to protein misfolding or incorrect folding [149] .

In addition, high level expression of recombinant proteins results in aggregation of the proteins in inclusion bodies as the mechanisms of protein quality control cannot regulate all the expressed protein in the host cell [138]. Therefore, slowing down the protein production rate overcomes the issue of over-expression of the target proteins in the expression host, also gives time for folding protein properly. The reduction of protein concentration can be achieved by decreasing incubation temperature [150, 151] as the low temperatures play a crucial role to decline aggregation [152-154]. San-Miguel *et al*., 2013 reported that the inclusion bodies formation can be avoided by carrying out the expression at the temperature range 15–25°C [155]. On the other hand, running the

expression at lower temperature may result in slowing down the growth of *E. coli* and reducing the production rates of recombinant protein yields [156, 157] .

To overcome the issue of the over-expression and reducing rate of the newly synthesised recombinant proteins production, the three synthesised *Echinococcus* genes (EF1-α, Ag5 and Cyclophilin) were expressed at the temperature of 30°C. However, the results show that the protein could be only found again in the pellets of the bacteria, with no obvious bands when the supernatant and purified fractions were analysed, see figures (4.28 and 4.29). Therefore, the proteins were then expressed at the temperature of 25°C. Nevertheless, the results of Western Blot show that there is no evident protein production under this condition, see figure (4.30).

In conclusion, although the production of heterologous proteins in *E. coli* has numerous advantages as mentioned previously. The proper folding and solubility of the recombinant proteins remain the major problem.

Hence, our next plan was to express the *Echinococcus* antigens in mammalian cells, using HEK293-6E cell line expression system.

Chapter 5: Expression of Recombinant *Echinococcus* Proteins in Eukaryotic Hosts

5.1 Introduction

Although proteins can be habitually difficult to express outside their original environments and the expression of soluble, correctly folded and functional proteins outside their original contexts can be a major problem, the modern biotechnology studies have focused on the improvement of recombinant protein expression in heterologous hosts and established techniques for production of milligram and even gram amounts of recombinant proteins [127, 158].

The low yield and insolubility of recombinant *Echinococcus* proteins were the main difficulties that we challenged in the optimisation experiments (see Chapter 3 and 4). Selecting the proper expression system to produce sufficient amounts of the soluble recombinant *Echinococcus* proteins is essential to achieve of the aim of this study. For this reason, in this chapter, we will demonstrate the expression of *Echinococcus* antigens and allergens in mammalian expression system.

In the last decade, numerous improvements in mammalian expression system have been achieved. These improvements can be summarised in four elements to produce more soluble, proper folding and functional recombinant proteins, which are: (i) genetically engineering of the mammalian host cells, (ii) backbone designing of the expression vector, (iii) transfection reagents, (iv) the culture growth conditions [159].

In this project, human embryonic kidney 293-6E cells (HEK293-6E cells) were chosen as a mammalian cell line that can express functional recombinant proteins. Also, HEK293 cells express high yields of recombinant proteins and do not demand lots of reagents [160].

The second factor for developing the process of mammalian expression is designing an expression vector with a strong active promotor in the mammalian cells. The human cytomegalovirus promoter (pCMV) is considered a robust promotor, particularly in HEK293 cell line [161].

The third key is related to the effectiveness of the transfection reagent. The cost-effectiveness of transfection reagents is a very important aspect of large scale expression, although numerous efficient commercial transfection reagents are obtainable. There are several criteria that should be taken into consideration regarding the transfection reagent. These criteria are ease of utilising, level of cytotoxicity, and effectiveness in the suspension growth cell culture. Polyethyleneimine (PEI) is one of the most effective transfection vehicles, which assemble these criteria as a cost-efficient, and extremely effective in the transformation of the gene of interest in the host cell with minimum cytotoxicity. The adherent and suspension HEK293-6E can be efficiently transfected with PEI [162, 163].

The fourth element of the expression procedure is the environment of the culture medium. Adapting the HEK293-6E cells to grow in suspension is necessary to allow scaling-up the growth culture easily and quickly rather than restricting to the surface space only as in adherent cells, because the increasing of the cell density results in higher yields. Serum-free medium is necessary to enhance the transfection activity in most transfection reagents [164]. Obtaining growth culture with appropriate conditions for both transfection activity and cell growth is one of the important keys [165].

In this chapter, we aimed to use different approaches for developing optimal expression and purification strategies to produce and purify high yield of recombinant *Echinococcus* antigens.

5.2 Experimental Procedures

5.2.1 Polymerase Chain Reaction Amplification

The seven *Echinococcus* genes, EF1-α, Ag5, AgB2, Cyclophilin, Eg19, EgTeg and EgTPx, were amplified again from *Echinococcus* cDNA library by polymerase chain reaction (PCR) using first Phusion High Fidelity DNA polymerase and then Q5 High Fidelity Hot Star Master Mix DNA polymerase (New England Biolabs, UK), with the restriction sites of EcoRI at the 5' end and XbaI at the 3' end to be cloned into mammalian expression vector pTT5, which was licenced from National Research Council, Canada.

The thermocycling conditions of PCR were performed in three steps using the primers listed in Table 5.1. The thermal cycling conditions were set up as follows: (i) Pre-denaturation step at 98°C for 30 seconds. (ii) 35 cycles of denaturation at 98°C for 30 seconds, annealing temperatures as described in Table 5.2 for 30 seconds, and extension step at 72°C for 30 seconds per kb. (iii) Final extension step at 72°C for 10 minutes.

Agarose gel electrophoresis was performed to confirm successful amplification. Size of PCR products was determined by comparing with the 100bp and 1Kb DNA molecular weight ladders (New England Biolabs).

The Ethidium-bromide stained PCR amplicons were excised from the agarose gel using a sterile scalpel blade under UV illumination, and then extracted and purified using Wizard SV Gel Kit and PCR Clean-Up kit (Promega, UK), according to the manufacturer's protocol. The concentrations and purity of the amplified genes were measured using Nanodrop™ ND-1000 spectrophotometer (Nanodrop Technologies-Thermo Fisher Scientific).

Table 5.1: Primers for *Echinococcus* genes amplification to be expressed in HEK 293-6E cell line.

Both restriction sites; EcoRI and XbaI were designated in the primer name and underlined in the oligonucleotide sequence. Forward and reverse indicate sense and antisense primers, respectively.

Gene	Primer's Name	Primer Sequence (5'-3')
EF1-a	EgEF1-a [1226-1244] Forward_ EcoRI	AAAAAAGAATTCGGGGAAGGAGAAG
	EgEF1-a [2561-2581] Reverse_ XbaI	AAAGCATCTAGACGTTTTTTGGCCTTG
Ag5	EgAg5 [1226-1247] Forward_ EcoRI	AAAAAAGAATTCGTTGGAGCTCACTCTC
	EgAg5 [2611-2629] Reverse_ XbaI	AAAAAATCTAGACGGACTGCGTAGC
AgB2	EgAgB2 Forward_ EcoRI	TCAGCAGAATTCGAAAGATGAGCCAA
	EgAgB2 Reverse_ XbaI	TCAGCATCTAGACGCTTTGAATCATC
Cyclophilin	Cyclophilin [1226-1247 bp] Forward_ EcoRI	AAAAAAGAATTCGGGCGTGAAGTGCTTC
	Cyclophilin [1703-1723 bp] Reverse_ XbaI	AAAAAATCTAGACGCAGCTGACCGCAG
Eg19	Eg19 [1226-1244] Forward_ EcoRI	AAAAAAGAATTCGGAGGCTGAGGCA
	Eg19 [1656-1678] Reverse_ XbaI	TCAGCATCTAGACGATCTCGAATACCATG
EgTeg	EgTeg [1226-1246] Forward_ EcoRI	AAAAAAGAATTCGGCTACAGACCCAAC
	EgTeg [1533-1555] Reverse_ XbaI	AAAGAATCTAGACGGTAGGTAGAGGATTG
EgTPx	EgTPx [1226-1246 bp] Forward_ EcoRI	AAAAAAGAATTCGGCTGCTGTTGTTGG
	EgTPx [1795-1816 bp] Reverse_ XbaI	AAAAAATCTAGACGCGAGCTCATGAACG

Table 5.2: Annealing temperatures of both forward and reverse primers for *Echinococcus* genes.

Gene	Annealing Temperature
EF1-a	62°C
Ag5	
Eg19	
EgTeg	
AgB2	57°C
Cyclophilin	72°C
EgTPx	

5.2.2 Generation of pTT5 and *Echinococcus* Genes Constructs

The amplified *Echinococcus* genes were cloned into pTT5 vectors (National Research Council, Canada), see Figure 2.4 (Chapter 2) and Figure 5.1, after digesting of both PCR products and pTT5 vector with EcoRI and XbaI restriction enzymes (New England Biolabs, UK), according to specific procedures stated by the manufacturer. Both digested *Echinococcus* genes and pTT5 vector were mixed in 3:1 molar ratio (insert to vector), then ligated using T4 DNA ligase kit (New England Biolabs, UK) for one hour at room temperature. After that, the ligated constructs were transformed into the previously prepared competent cells DH5-α strain of *E. coli* using the heat shock technique for plasmid propagation (see Section 2.3.7 in Chapter 2 for details). The transformed bacteria were then grown in LB medium for one hour without adding the selective antibiotic, followed by growing in LB agar containing 100µg/mL ampicillin for selection.

To confirm successful cloning, grown colonies were screened using colony PCR with the primers listed in Table 5.3 and an annealing temperature of 58°C (see Section 2.3.7 in Chapter 2 for more details).

Agarose gel electrophoresis was performed as a first place to prove the insertion of the genes. The colonies, which gave positive results, were grown in LB medium containing 100µg/mL ampicillin and the ligated plasmids were then extracted (See Section 2.3.8 in Chapter 2 for details). Finally, the plasmids were sent to Sanger Sequencing Service (Source Bioscience, Nottingham) for DNA sequencing with the primers detailed in Table 5.3 (a and b). The sequences were then analysed to verify the cloning and to confirm the sequences lack for any point mutation which may have been introduced during PCR amplification.

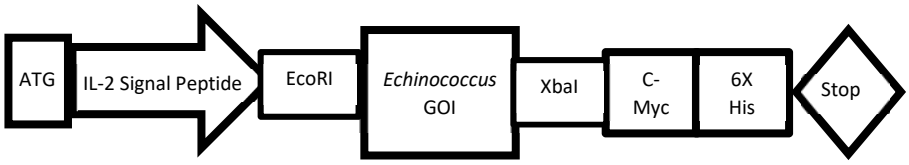


Figure 5.1: Schematic diagram of pTT5 vector showing expression cassette.

Echinococcus genes were cloned into pTT5 using EcoRI and XbaI restriction sites. The abbreviations symbolise the following: ATG- Methionine (start codon); IL-2- Interleukin-2; C-Myc Tag-Peptide tag for detection of the protein; 6xHis Tag- 6 Histidine peptide tag for protein purification; Stop- Stop codon; GOI- gene of interest.

Table 5.3: Primers for colony PCR screening and confirmatory sequencing

Forward and reverse indicate sense and antisense primers, respectively.

[A] Primers used for colony PCR screening. [B] Primer used for confirmatory sequencing.

Table 5.3 a: pTT5 forward primer for colony PCR screening and confirmatory sequencing which has been used with all the genes of interest.

Construct Name	Primer Name	Sequence (5'-3')
PTT5-*Echinococcus* genes	pTT5 [1164-1193 bp] Forward [AB]	TCAGCAGTTTAAACATGTACAGG ATGCAACTCCTG

Table 5.3 b: *Echinococcus* genes forward and reverse primers for colony PCR screening and confirmatory sequencing.

Construct Name	Primer Name	Sequence (5'-3')
PTT5-EF1-a	pTT5 [2881-2903 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	EF1-a [2561-2581 bp] Reverse [A]	AAAGCATCTAGACGTTTTTTGGCCTT G
PTT5-Ag5	pTT5 [2929-2951 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	Ag5 [2611-2629 bp] Reverse [A]	AAAAAATCTAGACGGACTGCGTAGC
PTT5-AgB2	pTT5 [1750-1772 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	AgB2 [1431-1450 bp] Reverse [A]	TCAGCATCTAGACGCTTTGAATCATC
PTT5-Cyclophilin	pTT5 [2023-2045 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	Cyclophilin [1703-1723 bp] Reverse [A]	AAAAAATCTAGACGCAGCTGACCGC AG
PTT5-Eg19	pTT5 [1978-2000 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	Eg19 [1656-1678 bp] Reverse [A]	TCAGCATCTAGACGATCTCGAATACC ATG
PTT5-EgTeg	pTT5 [1855-1877 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA

	EgTeg [1533-1555 bp] Reverse [A]	AAAGAATCTAGACGGTAGGTAGAGG ATTG
PTT5-EgTPx	pTT5 [2116-2138 bp] Reverse [B]	GGTTCAGTTGGCAAGTTGTACCA
	EgTPx [1795-1816 bp] Reverse [A]	AAAAAATCTAGACGCGAGCTCATGA ACG

5.2.3 Genes Expression Technique in HEK293-6E Cell Line

5.2.3.1 HEK293-6E Cells maintenance

A vial of the frozen adherent or suspension HEK293-6E cells were thawed in a water bath at 37°C and then added to 10 ml of F17 medium (Invitrogen) supplemented with 1% of Kolliphor P188 (Sigma-Aldrich, UK), 4mM of L-Glutamine solution (Invitrogen) and 25 µg/ml of G418 (Invitrogen), in a T75 cm^2 canted neck cell culture flask with vent cap (Corning, CellBIND) in terms of the adherent cells, or added to 16 ml of F17 medium in a sterile, disposable, plastic, Erlenmeyer 125 ml shaker flasks (Corning, CellBIND) in terms of suspension cells. The flasks were then incubated in humidified incubator controlled at 37°C with 5% CO_2. The adherent cells were not detached until they reached 80% confluence. At this point, they were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS), pre-warmed at 37°C, and incubated with 2ml of 1x trypsin/EDTA (Sigma Aldrich, UK) for ~3 minutes at 37°C and then the flask was tapped gently, followed by adding 8ml of the F17 medium to the flask. The cells were then transferred into sterile 15ml centrifuge tube (Sarstedt) and centrifuged at 500 xg for 5 minutes to pellet the cells. The supernatant was discarded and the cells were re-suspended with 10ml of F17 medium.

In terms of suspension cells, the cells were not diluted until they reached $1x10^6$ cells/ml (usually taking 3 days), and they were incubated on an orbital shaker plate set at 140 rpm (Corning, CellBIND) in a humidified incubator controlled at 37°C with 5% CO_2.

5.2.3.2 Transfection and Expression Procedure in Adherent HEK293-6E Cell Line

Adherent HEK293-6E cells were transfected with pTT5-*Echinococcus* genes constructs using Polyethylenimine (PEl) transfection reagent (Polysciences) in Nucleon Surface 6-well plate (Thermo-Fisher Scientific). To achieve this, the cells were maintenance and 10µl of the cells were taken to determine cell density and viability, and at the cell density between $1.5\text{-}2.0x10^6$ cells/ml, 1800 µl of the cells were transferred into each well of 6-well plate, and the plate was placed in a humidified incubator controlled at 37°C with 5% CO_2. When the cells reached 80% confluence, the transfected procedure was started by preparing the DNA-PEl complex at a ratio of 1:2 (w/w). Firstly, PEl was prepared to a final concentration of 1mg/ml in high purity water and the pH was adjusted to 7.0, followed by 0.22µm filtration. Then, 672µl of Opti-MEM reduced serum medium (Thermo-Fisher Scientific, UK) was placed into 1.5ml Eppendorf tube for the entire 6-well plate, followed by addition of 28µl of PEI to the medium. The next step was preparing the DNA solution for each well of 6-well plate by placing the corresponding of 2µg of each pTT5 construct to 6 x 1.5 ml tubes, and the volume was topped up to 100µl with Opti-MEM reduced serum medium.

DNA-PEI complex was prepared by adding 100 µl of PEl solution to each DNA solution tube, and the mixtures were vortexed immediately, and incubated for 3 minutes at room temperature. After 3 minutes, the whole amount of 200 µl of each DNA-PEI complex was poured dropwise into each well of 6-well plate and the plate was swirled gently and returned back to the incubator.

pEGFP-Express plasmid (Clontech, USA), which encodes a constitutively express green fluorescent protein, was used to assess the transfection productively. 24 hours' post-transfection, the transfected HEK293-6E cells with pEGFP-Express plasmid were visualised using EVOS fluorescence microscope (Thermo-Fisher Scientific, UK), and 0.5% (50µl/well) of 20% (w/v) Tryptone N1 (TN1) (Organotechnie, Canada) was added to each well of 6-well plate. Finally, 120 hours' post-transfection, the supernatants were collected for investigation of the secreted proteins using SDS-PAGE and Western Blotting (See Section 4.2.1.4 for details), and the pellets were lysed for further analysis, see section (5.2.3.4) for details.

5.2.3.3 Transfection and Expression Procedure in Suspension HEK293-6E Cells Line

The suspension HEK293-6E cells were counted and screened under a microscope using a haemocytometer to determine the density and viability of the cells, respectively. One day before the transfection process, the cells were adjusted to 1 x 10^6 cells/ml with F17 medium in a sterile, disposable, plastic, Erlenmeyer 1 litre shaker flasks (Corning, CellBIND), and placed on an orbital shaker plate set at 140 rpm in humidified incubator controlled at 37°C with 5% CO_2. The next day, transfection solution complex (DNA-PEl) was prepared for 300 ml of the cell growth culture at a ratio of 1:2 (w/w). For DNA solution preparation, a Plasmid Midiprep Pure Yield kit (Promega, UK) was utilised for DNA constructs purification according to the manufacturer protocol, and 1µg of each *Echinococcus* gene construct was used to transfect 1ml of HEK293-6E cells, thus 300µg of each gene construct was added to 15ml of pre-warmed F17 medium in 50 Falcon Tube (Thermo-Fisher Scientific, USA). Separately, 600µg of PEl transfection reagent was added to 15 ml of pre-warmed F17 medium in 50 Falcon Tube. The both solutions were vortexed

with 3 pulses of 1 second each, followed by addition the PEl solution to the DNA solution. The DNA-PEl complex was then incubated for 3 minutes at room temperature and immediately the whole DNA-PEl mixture was added to the cell culture, followed by gently swirling the flask and placing it back on the orbital shaker plate at 140 rpm in the incubator set at 37°C with 5% CO_2. 24 hours' post-transfection, Tryptone N1 (TN1) was added to each transfected HEK293-6E cell culture to have a final concentration of 0.5%. 120 hours' post-transfection, the cells were harvested by centrifugation at 2000xg and 4°C for 10 minutes. The supernatants were collected and filtered using 0.22 µm syringe filters and Protease Inhibitor Mini EDTA-free tablets (Roche, USA) were added to the supernatants according to the manufacturer conditions. The supernatants were then stored at 4°C for testing of the secreted proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting (see section 4.2.1.4). The proteins were then purified using ÄKTA Start Purifier System, see section (5.2.3.5), and stored in - 40°C. Pellets were lysed for further investigation, as described in section (5.2.3.4).

5.2.3.4 Lysis of HEK293-6E Cultured Cells

After collecting the supernatants for analysis, the cells were placed on ice and lysed using Radio immunoprecipitation assay (RIPA) cell lysis buffer (AMERCO) for analysis of non-secreted proteins. This procedure was performed by removing the culture media from the adherent HEK293-6E cells, followed by washing the cells twice with chilled Dulbecco's Phosphate Buffered Saline (DPBS). In terms of suspension HEK293-6E cells, the cells were pelleted by centrifugation at 2500 xg for 5 minutes and the supernatant was discarded, followed by washing the pellet twice with cold DPBS and centrifuging at 2500xg for 5 minutes each time.

After that, 1 ml of cold RIPA Cell Lysis Buffer was added to $5x10^5$ cells. The plate was kept on ice for 15 minutes after adding RIPA, and occasionally swirled. After cell lysis, a cell scraper was used to scrape off the cells, and the cell lysate was passed through the pipette few times for homogenising the lysate. The lysate was then transferred to a cold micro-centrifuge tube and centrifuged at 14,000 xg for 15 minutes at 4°C to separate the supernatant from the cellular debris, followed by transferring the supernatant into a cold micro-centrifuge tube for analysis using SDS-PAGE and Western Blotting.

5.2.3.5 Immobilised Metal Affinity Chromatography (IMAC) Purification Method

Two different variations of Immobilised Metal Affinity Chromatography were used in this study to purify the *Echinococcus* antigens:

- **ÄKTA Start Purifier System using Nickel Resin Column**

The supernatants containing 6xhistidine-tagged *Echinococcus* proteins from the previous steps were purified using immobilised metal affinity chromatography (IMAC). Protein fractions were mixed with filtrated 2x binding buffer (100mM Disodium Phosphate Na_2HPO_4 and 600mM Sodium Chloride NaCl, pH 7.4) at a ratio of 1:1 (v/v), followed by loading onto 1ml nickel-based resin columns (Nickel-NTA resin) (GE Healthcare, USA) and then run at a flow rate of 1 ml/min using ÄKTA start purifier system (GE Healthcare, USA). The proteins were then washed with washing buffer consisting of 50mM sodium phosphate, 300mM NaCl at pH 7.4. The elution step was run with elution buffer consisting of 50 mM sodium phosphate, 300 mM NaCl and 500 mM imidazole with pH 7.4 at the same flow rate. Finally, all elution fractions were loaded onto SDS-PAGE electrophoresis gel and analysed using Coomassie staining and Western Blotting.

- **Magnetic Beads using Cobalt Resin**

The recombinant *Echinococcus* antigens were purified using cobalt Dynabeads His-Tag Isolation & Pulldown kit (Thermo Scientific, UK). Microcentrifuge tube containing Dynabeads was vortex until obtaining a homogenous mixture. The microcentrifuge tube was then placed to an EasySep Magnet (STEMCELL Technologies, Canada) for 5 minutes to separate the beads from the supernatant. After that, the supernatant was discarded, and the filtrated produced protein, which was previously mixed with an equal volume of binding/washing buffer (600 mM NaCl, 100 mM sodium phosphate, 0.02% Tween 20, pH 8.0), was added to the beads. The mixture of the protein and beads was incubated for 10 minutes at room temperature on a roller. After 10 minutes, the mixture was placed in the EasySep Magnet for 5 minutes to remove the supernatant. The Dynabeads were then washed 4 times with 500µl of the washing buffer to remove supernatant, using the EasySep Magnet. After that, *Echinococcus* protein was eluted by mixing the beads with 50µL of elution buffer (600 mM NaCl, 100 mM sodium phosphate, 0.01 % Tween 20, 300 mM imidazole, pH 8.0) and then incubation for 15 minutes at room temperature on the roller. The supernatant was then collected for protein purity analysing, using SDS-PAGE and Western Blot.

5.2.3.6 Protein Quantification

Following the purification, the total concentrations of the proteins were quantified using Pierce BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific, UK) by following the manufacturer's protocol. Finally, the samples containing proteins were loaded onto a Tris-Glycine gel for SDS-PAGE, Coomassie staining and Western Blotting for determining the purity of the proteins.

5.3 Results

5.3.1 Amplification and Cloning of *Echinococcus* Genes into Mammalian Expression Vector

For the generation of *Echinococcus* genes expression constructs, PCR amplification was conducted to amplify genes using primers with EcoRI and XbaI restriction sites listed in table (5.1). An additional nucleotide (G) was added to the forward primers for each gene after the EcoRI restriction site, and two nucleotides (CG) were added to the reverse primers before XbaI to keep the genes in open reading-frame with the signal peptide Interleukin-2 (IL-2), C-Myc and 6x Histidine tags on the pTT5 vector, see Figure (5.1).

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACGAATT
CGGGCGTGAAGTGCTTCTTCGACATCTCCATTGGCGGTAAGCCAGCGGGTCGCATCG
TCTTTGCCCTGTTTGATGACGTCCCAAAGACAGTAGAGAACTTCCGTGCACTGTGCAC
CGGTGAGAAGGGTTTCGGATACAAGGGCTCGAAGTTTCACCGAATTATTCCCGGTTT
TATGTGCCAAGGTGGTGATTTTACTGCCGGGAATGGTACCGGTGGCAAGAGCATATA
CGGGAGCAAATTTGAGGATGAAAATTTCAATCACAAGCACAGCAAGCCGATGATGCT
CTCGATGGCGAATGCGGGTAAGAACACCAATGGCTCGCAATTCTTCATCACTACCGC
CGTCACCAGCTGGCTTGATGGGAAGCATGTTGTCTTTGGTGAGGTAGAAAGTGGCGA
AGATGTTGTTAAGGATATGGAGGCCGTCGGTAGCAGTAGTGGCAAGACTTCCCAAGA
AGTACTGATTACTGACTGCGGTCAGCTGcgTCTAGAACAAAAACTCATCTCAGAAGAG
GATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTGA

Figure 5.1: cDNA sequence for *Echinococcus* EF1-alpha gene. The highlighted nucleotides in dark green refer to the codon encoding Methionine (start codon). The highlighted nucleotides in yellow refer to codons encoding the signal peptide (IL-2). While, the highlighted nucleotides in orange refer to the codon encoding EcoRI restriction site. The highlighted nucleotides in blue refer to the nucleotides, which were added to the primers to keep the gene in opening frame with the IL-2 and tags. The highlighted nucleotides in pink refer to the codon encoding XbaI restriction site. Whereas, the highlighted nucleotides in light grey refer to the codon encoding C-Myc tag. The highlighted nucleotides in light green are on pTT5 plasmid between C-Myc tag and His-tag. While, the highlighted nucleotides in dark grey refer to the codon encoding His-tag. Finally, the highlighted nucleotides in red refer to stop codon.

Gel electrophoresis results confirmed that five out of seven *Echinococcus* genes, Ag5, Cyclophilin, Eg19, EgTeg and EgTPx, were amplified successfully from *Echinococcus* cDNA library using either Phusion High-Fidelity DNA Polymerase or Q5 High-Fidelity DNA Polymerase (New England, Biolab), see figures (5.2), (5.3) and (5.4).

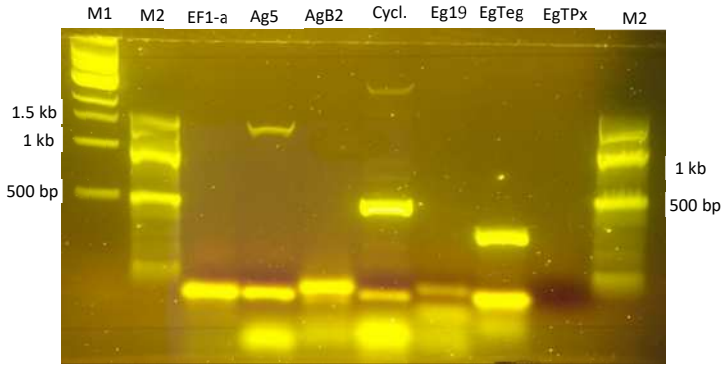


Figure 5.2: Representative amplification of the *Echinococcus* genes
The *Echinococcus* genes, amplified from cDNA library using Phusion DNA polymerase, on 1% agarose gel. The expected sizes of these PCR products, which contains EcoRI and XbaI restriction sites and six nucleotide overhangs at their 5'and 3' ends, are; EF1-a 1,368 bp, Ag5 1,416 bp, AgB2 237 bp, Cyclophilin 510 bp, Eg19 465 bp, EgTeg 342 bp, EgTPx 603 bp. M1= 1kb Molecular DNA ladder, M2= 100 bp Molecular DNA Ladder.

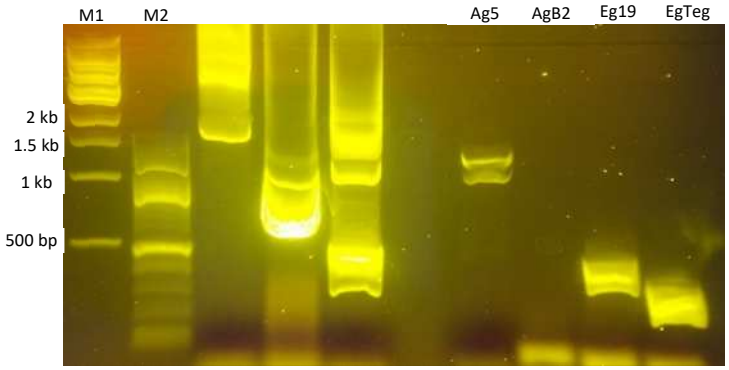


Figure 5.3: Representative amplification of the *Echinococcus* genes
The *Echinococcus* genes, amplified from cDNA library using Phusion DNA polymerase, on 1% agarose gel. The expected sizes of these PCR products, which contains EcoRI and XbaI restriction sites and six nucleotide overhangs at their 5'and 3' ends, are; Ag5 1,416 bp, AgB2 237 bp, Eg19 465 bp, EgTeg 342 bp. M1= 1kb Molecular DNA ladder, M2= 100 bp Molecular DNA Ladder.

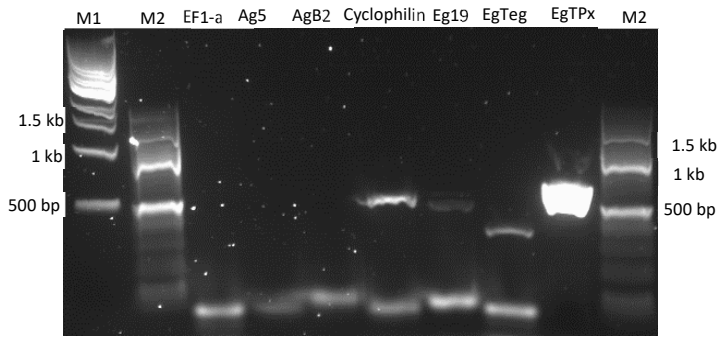


Figure 5.4: Representative amplification of the *Echinococcus* genes
The *Echinococcus* genes, amplified from cDNA library using Q5 DNA polymerase, on an agarose gel. The expected sizes of these PCR products, which contains EcoRI and XbaI restriction sites and six nucleotide overhangs at their 5'and 3' ends, are; EF1-a 1,368 bp, Ag5 1,416 bp, AgB2 237 bp, Cyclophilin 510 bp, Eg19 465 bp, EgTeg 342 bp, EgTPx 603 bp. M1= 1kb Molecular DNA ladder, M2= 100 bp Molecular DNA Ladder.

Following the amplification procedure, the PCR products along with pTT5 plasmid were digested through EcoRI and XbaI restriction enzymes and run on 1% agarose gel, see figure (5.5). The digested products were then extracted from the gel and clean up, see section (2.3.5) in general materials and methods. The extracted genes were then ligated with the digested vector according to the protocol detailed in section (4.2.1.1) in Chapter (4). The cloned genes into the expression vector were transformed into DH5-α *E. coli* strain for plasmid propagation, see section (2.3.7). Colonies were then picked randomly and screened through PCR, using the primers illustrated in Table (5.3 a and b), for confirmation of the gene insertion, see figures (5.6) and (5.7), followed by growing the colonies with positive results in LB medium for plasmid propagation and purification. After PCR colony screening, the cloned genes were sent along with their corresponding primers (See Table 5.3 a and b) for DNA sequencing to confirm the successful cloning of the *Echinococcus* genes into mammalian expression vector pTT5, see Figures (5.8 – 5.12).

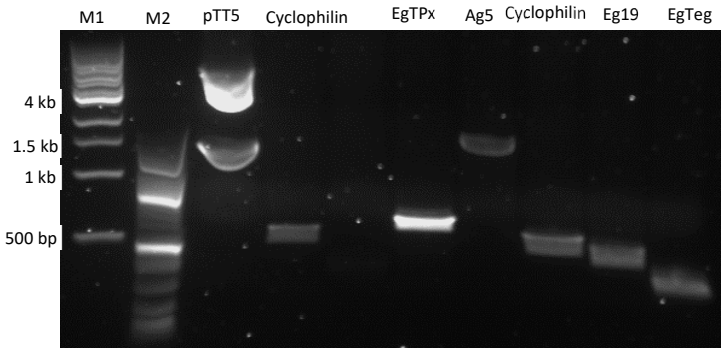


Figure 5.5: Restriction digestion of pTT5 plasmid and *Echinococcus* genes using EcoRI and XbaI restriction enzymes.

The *Echinococcus* genes and pTT5 plasmid on 1% agarose gel. The expected sizes of these digested products are; PTT5 Plasmid 4,446 bp (excluding the fragment between EcoRI & XbaI), Ag5 1,398 bp, Cyclophilin 492 bp, Eg19 447 bp, EgTeg 324 bp, EgTPx 585 bp. M1= 1kb Molecular DNA ladder, M2= 100 bp Molecular DNA Ladder.

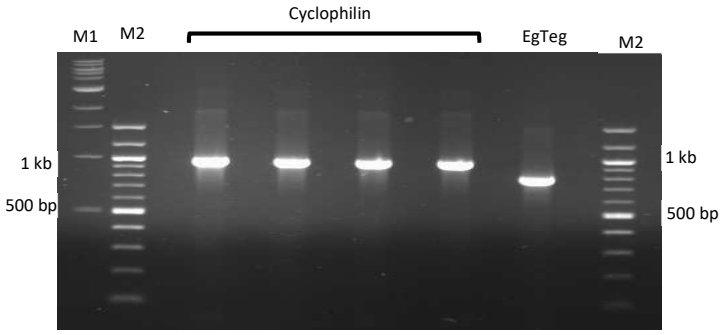


Figure 5.6: Representative verification of Cyclophilin and EgTeg insertion into an expression vector (pTT5).

Agarose gel of PCR-amplified multiple cloning sites from separate bacterial colonies hosting either Cyclophilin or EgTeg into pTT5 expression vector. The expected size of the successful Cyclophilin and EgTeg insertion into pTT5 results in sizes of 981 bp and 813 bp, respectively. The sizes include overhangs, restriction sites, signal peptide and part of the plasmid. M1= 1kb molecular DNA ladder, M2= 100 bp molecular DNA Ladder.

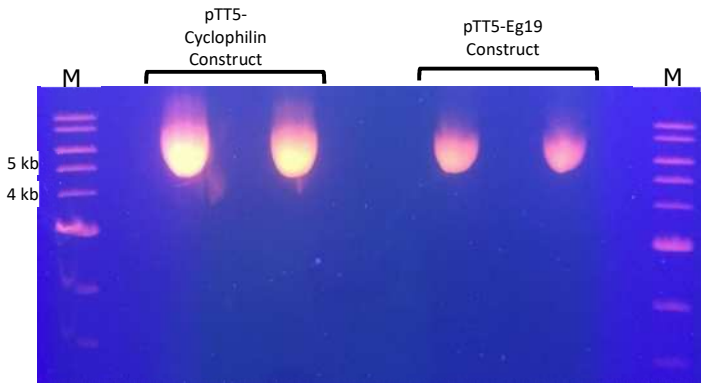


Figure 5.7: Representative verification of Cyclophilin and Eg19 insertion into an expression vector (pTT5).
Agarose gel of cyclophilin-pTT5 and Eg19-pTT5 constructs. The expected size of the successful Cyclophilin and Eg19 insertion into pTT5 results in sizes of 4938 bp and 4893 bp, respectively. M= 1kb molecular DNA ladder.

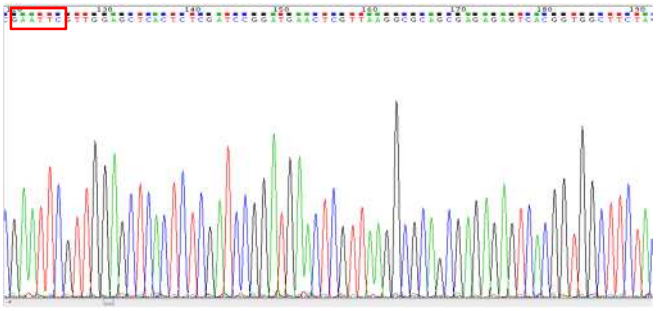


Figure 5.8: Representative DNA sequencing of Ag5 expression construct.
Chromatogram trace of the pTT5_Ag5 construct sequenced after extraction from *E. coli* DH5-α cells. The six nucleotides in the red box represent the EcoRI restriction site. To its right is the Ag5 gene fragment.

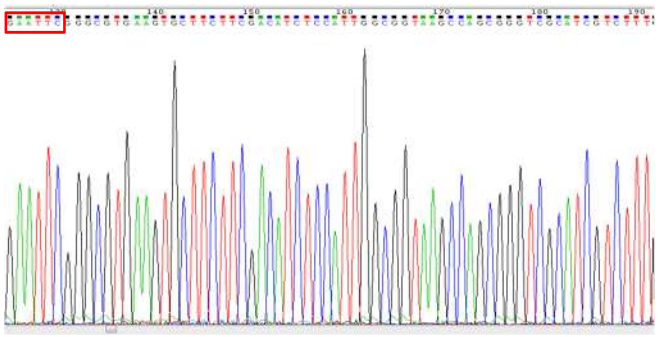


Figure 5.9: Representative DNA sequencing of Cyclophilin expression construct.
Chromatogram trace of the pTT5_Cyclophilin construct sequenced after extraction from *E. coli* DH5-α cells. The six nucleotides in the red box represent the EcoRI restriction site. To its right is the Cyclophilin gene fragment.

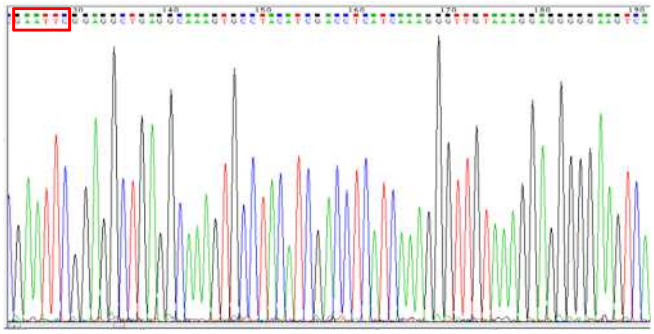


Figure 5.10: Representative DNA sequencing of Eg19 expression construct.

Chromatogram trace of the pTT5_Eg19 construct sequenced after extraction from *E. coli* DH5-α cells. The six nucleotides in the red box represent the EcoRI restriction site. To its right is the Eg19 gene fragment.

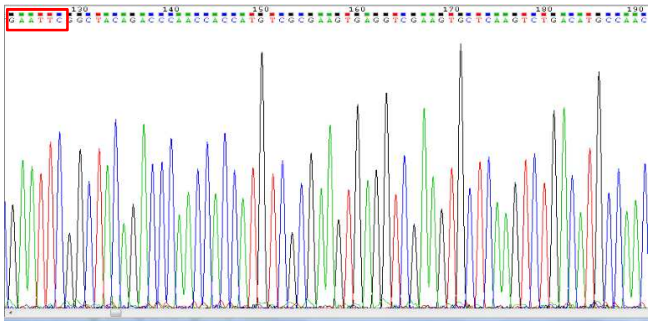


Figure 5.11: Representative DNA sequencing of EgTeg expression construct.

Chromatogram trace of the pTT5_EgTeg construct sequenced after extraction from *E. coli* DH5-α cells. The six nucleotides in the red box represent the EcoRI restriction site. To its right is the EgTeg gene fragment.

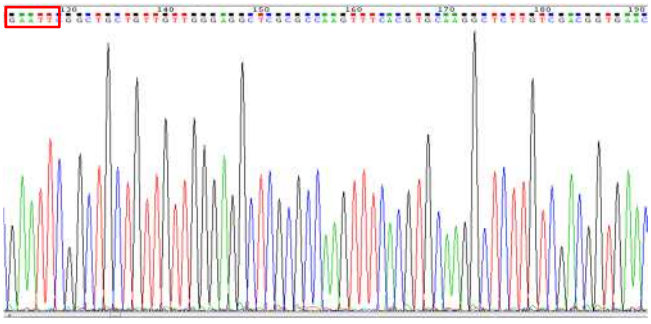


Figure 5.12: Representative DNA sequencing of EgTPx expression construct.

Chromatogram trace of the pTT5_EgTPx construct sequenced after extraction from *E. coli* DH5-α cells. The six nucleotides in the red box represent the EcoRI restriction site. To its right is the EgTPx gene fragment.

5.3.2 Recombinant Expression in HEK293-6E Cell Line

Expression of *Echinococcus* recombinant proteins was tried using a human embryonic kidney 293-6E (HEK293-6E) cell line from pTT5 construct containing *Echinococcus* cDNAs generated as illustrated in experimental procedures Section 5.2.2. The cDNAs were inserted in pTT5 and fused in open reading frame with the nucleotides encoding the human interleukin 2 (IL-2) signal peptide for secretion of produced recombinant proteins, C-Myc and 6xHistidine polypeptide tags (See Figure 5.1). Thus, pTT5 expression constructs were used to express Myc-tagged and 6xHis-tagged *Echinococcus* proteins in eukaryotic cells. The expression constructs were then transiently transfected into HEK293-6E cells using PEl transfection reagent.

5.3.2.1 Recombinant Expression in Adherent HEK293-6E Cell Line

The first mammalian expression strategy was attempted utilising adherent HEK293-6E cell line. The cells were transfected with pTT5 constructs containing different *Echinococcus* cDNAs using PEl at 1:2 ratios (w/w) of DNA to PEl.

To assess the transfection efficiency of PEl, pEGFP-Express plasmid encoding a green fluorescent protein was utilised in parallel. The cells were assessed for transfection achievement 24 hours' post-transfection using fluorescence microscopy, and approximately 60% of the cells were transfected with pEGFP-Express plasmid using PEl transfection reagent, see Figure 5.13.

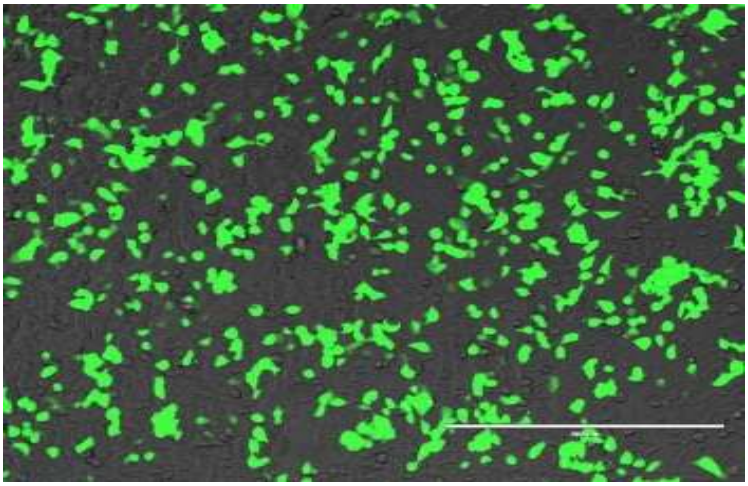


Figure 5.13: Fluorescence microscopy image which was taken 24 hours' post-transfection of HEK293-6E cell line with pEGFP-Express plasmid using PEI transfection reagent at a ratio of 1:2 (w/w) (plasmid to PEI). The figure was taken as an overlay section and it convincingly shows that the transfection efficiency is high.

To evaluate the secretion of *Echinococcus* recombinant proteins, Ag5 (54 KDa), Cyclophilin (20.5 KDa), Eg19 (19 KDa), EgTeg (14 KDa) and EgTPx (24 KDa), the supernatants of the five different transfected adherent HEK293-6E cells with *Echinococcus* genes were collected in 2, 3, 4 and 5 days' post-transfection, and each solution was mixed with an equal volume of 2X non-reducing sample buffer (NRSB) or reducing sample buffers (RSB) in separate tubes. The solutions were then boiled at 100°C for 5 minutes, to be used for SDS-PAGE and Western Blotting using antibodies against C-Myc tag once and His-tag once again as described in Chapter (4), section (4.2.1.4).

As shown in Figures (5.14), (5.15) and (5.16), Cyclophilin, Eg19 and EgTeg could be successfully expressed in HEK293-6E cells. However, it has to be noted that the match between the cDNAs size of Cyclophilin, Eg19 and EgTeg and their corresponding proteins size were imperfect. Although Cyclophilin, Eg19 and EgTeg molecular weights calculated of 20.5 KDa, 19 KDa and 14 KDa, respectively, they appeared to migrate at approximately 25 kDa, 30 KDa and 20 KDa, respectively, after expression in HEK293-6E cells and even

under reducing conditions. It has not been identified yet whether this is the effect of the post-translational modifications or not. However, under non-reducing conditions, the apparent molecular weights of these proteins were 60 KDa and 120 KDa for Eg19; 25 KDa for Cyclophilin and 20 KDa for EgTeg, see Table (5.4). Whereas, the results of Western Blot showed no protein expression for Ag5 (54 KDa) and EgTPx (24 KDa), see Figures (5.15) and (5.17).

The cell pellets of the five transfected adherent HEK293-6E cells group containing *Echinococcus* cDNAs expression constructs were lysed 5 days after transfection, as described in Section 5.2.3.4, and the cell lysates were also screened for detection of non-secreted recombinant proteins, although the all *Echinococcus* cDNAs were engineered to be expressed in the HEK293-6E cells and then secreted in the cell supernatant due to adding the signal peptides IL-2. However, this experiment was performed to verified the efficiency of the IL-2 signal peptide. The results show that there is no any evident of remaining expressed *Echinococcus* proteins in the cells when the cell lysate samples were tested under the reducing condition using Western Blotting, see Figures (5.15), (5.16) and (5.17).

Table 5.4:- Molecular weights of recombinant *Echinococcus* proteins expressed in HEK293-6E cell line. RSB- reducing sample buffer. NRSB- non-reducing sample buffer.

Echinococcus Proteins	Putative Molecular Weight	Obtained Molecular Weight with RSB	Obtained Molecular Weight with NRSB
Ag5	54 KDa	Negative	Negative
Cyclophilin	20.5 KDa	25 KDa	25 KDa
Eg19	19 KDa	30 KDa	60 KDa 120 KDa
EgTeg	14 KDa	20 KDa	20 KDa
EgTPx	24 KDa	Negative	Negative

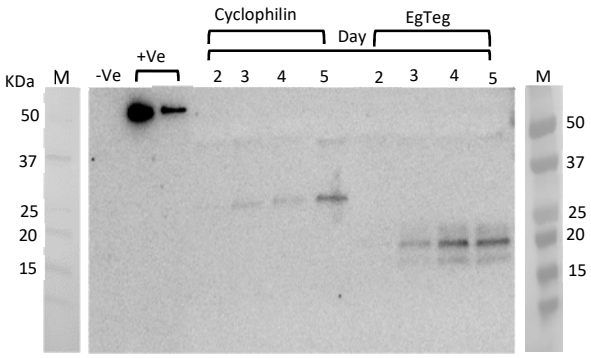


Figure 5.14: Time course evaluation of expression of recombinant Cyclophilin and EgTeg in adherent HEK293-6E Cells.

C-Myc immunodetection, under reducing condition to detect Cyclophilin and EgTeg in the supernatant 2, 3, 4 and 5 days' post-transfection. Cyclophilin showed bands at 25 KDa and EgTeg showed bands at 20 KDa. M- molecular weight markers; +Ve- Myc-tagged protein (57 KDa) as a positive control; -Ve – non-transfected cells as a negative control.

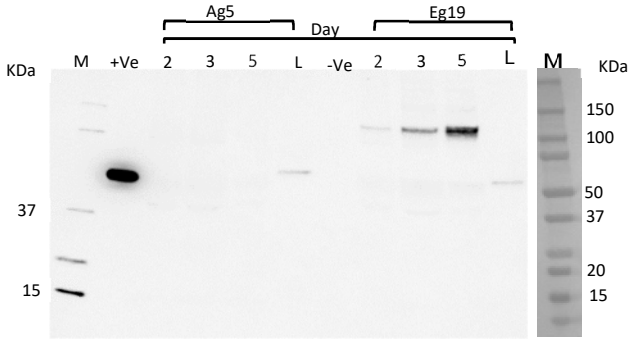


Figure 5.15: Time course analysis of expression of recombinant Ag5 and Eg19 in adherent HEK293-6E Cells.

C-Myc immunodetection using Western Blot, under non-reducing condition for detection of secreted and non-secreted recombinant Ag5 and Eg19 localisation in the supernatant 2, 3 and 5 days' post-transfection and cell pellet analysis. The expected size of Ag5 is 54 KDa. Eg19 showed bands at 120 KDa. M- molecular weight markers; +Ve- Myc-tagged protein (57 KDa) as a positive control; -Ve – non-transfected cells as a negative control; L- cell lysate.

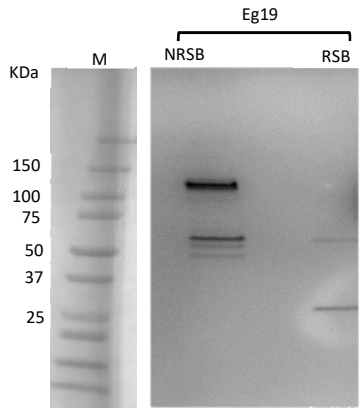


Figure 5.16: Expression of recombinant Eg19 in adherent HEK293-6E Cells.
C-Myc immunodetection, under reducing and non-reducing conditions, to detect recombinant Eg19 in the supernatant 5 days' post-transfection. Eg19 showed bands at a molecular weight of 30 KDa under reducing condition; 60 KDa and 120 KDa under non-reducing condition. M- molecular weight marker; RSB- reducing sample buffer; NRSB- non-reducing sample buffer.

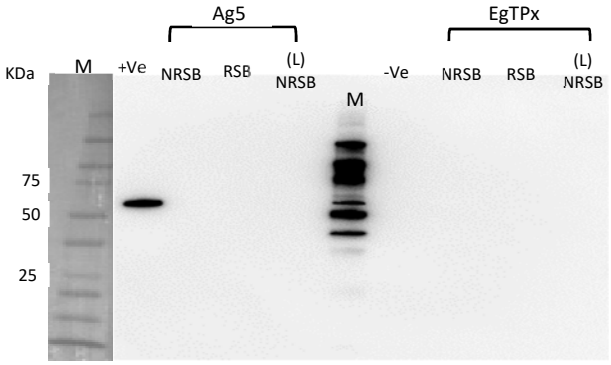


Figure 5.17: Expression of recombinant Ag5 and EgTPx in adherent HEK293-6E cells.
His-Tag immunodetection using Western Blot, under reducing and non-reducing conditions, to detect Ag5 and EgTPx (The expected sizes are 54 KDa and 24 KDa, respectively) in the supernatant and cells lysate 5 days' post-transfection. M- molecular weight markers; +Ve- His-tagged protein (57 KDa) as a positive control; -Ve – non-transfected cells as a negative control; RSB- reducing sample buffer; NRSB- non-reducing sample buffer; L-cell lysate.

5.3.2.2 Recombinant Expression in Suspension Growing HEK293-6E Cell Line

HEK293-6E cells grown in suspension were used as a second mammalian expression strategy to express *Echinococcus* recombinant proteins. The suspension cells were grown in F17 medium in sterile, disposable, plastic, Erlenmeyer 1 litre shaker flasks (Corning, CellBIND) as described above in experimental procedures, see Section 5.2.3.1 for more details. The flasks were then incubated in humidified incubator controlled at 37°C with 5% CO_2 on an orbital shaker plate set at 140 rpm. The suspension HEK293-6E cells were initially transfected with pEGFP-Express plasmid encoding green fluorescent protein using PEl transfection reagent at a ratio of 1:2 (w/w) (DNA to PEl) to evaluate the transfection efficacy. The transfected suspension cells were screened using fluorescence microscopy 24 hours' post-transfection and the outcome of the transfection was positive, as nearly 60% of the cells were transfected with pEGFP Express plasmid, see Figure (5.18). The suspension HEK293-6E cells were then transfected with pTT5-*Echinococcus* cDNA constructs using PEl transfection reagent at the same ratio of 1:2 (w/w) (DNA to PEl) to transiently express *Echinococcus* proteins.

The supernatants of transfected suspension HEK293-6E cells with pTT5-*Echinococcus* cDNA constructs were harvested 5 days' post-transfection and analysed using C-Myc or His-tag immunodetection method by Western Blotting. Regarding Cyclophilin and Eg19, the data confirmed again the presence of bands at the molecular weight of 25 KDa and 30 KDa, respectively, under reducing conditions. Under non-reducing conditions, the results of Eg19 demonstrated two main bands of 60 KDa and 120 KDa, see Table 5.4, Figures (5.19) and (5.20).

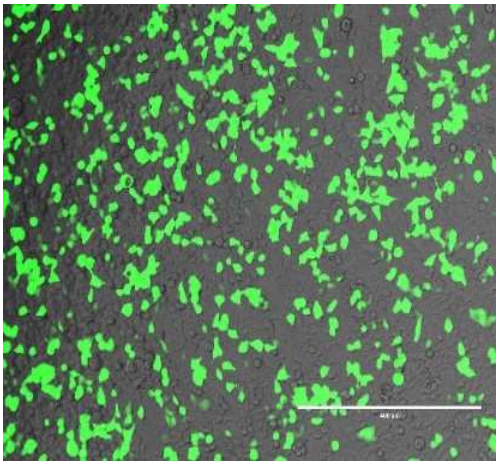


Figure 5.18: Fluorescence microscopy image which was taken 24 hours' post-transfection of suspension HEK 293-6E cell line with pEGFP-Express plasmid using PEI transfection reagent at a ratio of 1:2 (w/w) (plasmid to PEI). The figure was taken as an overlay section and it convincingly shows that the transfection efficiency is high.

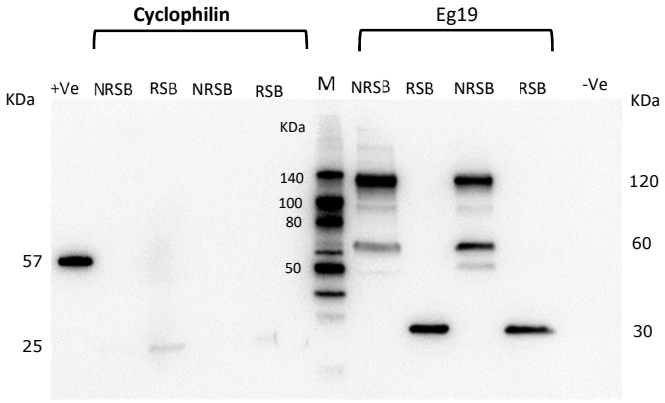


Figure 5.19: Expression of recombinant Cyclophilin and Eg19 from transient transfection of suspension HEK293-6E cells.
Western Blot analysis using anti-C-Myc Tag antibody to detect Eg19 and Cyclophilin. Eg19 showed a molecular weight of 30 KDa using reducing sample Buffer (RSB); and molecular weight of 60 KDa and 120 KDa using non-reducing sample buffer (NRSB). Cyclophilin showed a molecular weight of 25 KDa under both reducing and non-reducing conditions. M- molecular weight marker; +Ve- Myc-tagged protein (57 KDa) as a positive control; -Ve – non-transfected cells as a negative control.

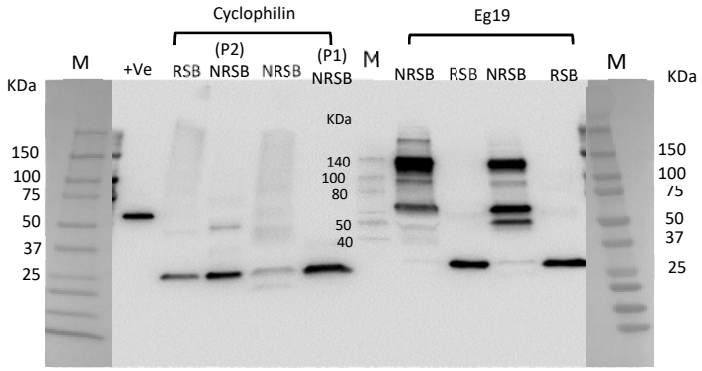


Figure 5.20: Western Blot analysis of transient expression of Cyclophilin and Eg19 proteins in suspension HEK293-6E cells and purification using Magnetic dynabeads and ÄKTA Start System.

Supernatant aliquots of secreted Cyclophilin and Eg19 were collected 5 days' post-transfection and analysed by C-Myc immunodetection using non-reducing sample buffer (NRSB) and reducing sample buffer (RSB). Cyclophilin showed bands at 25 KDa under both reducing and non-reducing conditions. Eg19 showed bands at 30 KDa under reducing condition and 60 KDa and 120 KDa under non-reducing condition. M- molecular weight markers; +Ve- C-Myc-Tagged protein (57 KDa) as a positive control; P1- purified protein using cobalt Dynabeads; P2- purified protein using ÄKTA Start System through nickel-based resin.

5.3.2.3 IMAC Purification of Recombinant Proteins Using Magnetic Dynabeads

After the expression of *Echinococcus* proteins in HEK293-6E cells, the supernatants containing secreted recombinant proteins were collected and firstly concentrated using ultrafiltration Vivaspin MW 10kDa Centrifugal Concentrator columns (GE Healthcare, USA), following the manufacturer instructions. The concentrated produced proteins were then purified using cobalt resin of Dynabeads His-Tag Isolation & Pulldown kit (Thermo Scientific, UK), see section (5.2.3.5) for details. The purified proteins were initially tested using SDS-PAGE gel stained with Coomassie blue for purity evaluation, see figure (5.21). Following the Coomassie staining evaluation, Western Blot was utilised, and the results of Western Blotting visibly demonstrated bands of purified Cyclophilin at the molecular weight of 25 KDa under both reducing and non-reducing conditions. Whereas, Eg19

presented bands at 30 KDa under reducing condition, and 60 KDa and 120 KDa under non-reducing condition, see Figures (5.20), (5.22) and (5.23).

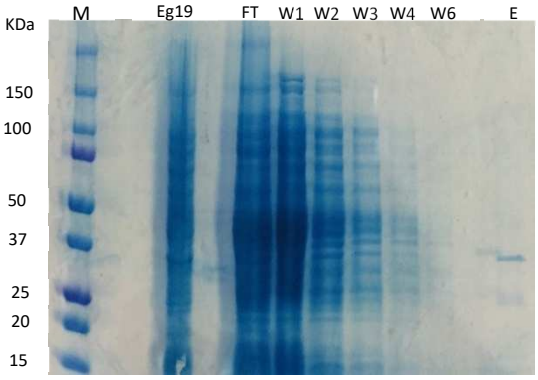


Figure 5.21: Expression of recombinant Eg19 in suspension HEK 293-6E cells and purification using Magnetic Dynabeads.
SDS-PAGE stained with Coomassie blue showing expressed and purified Eg19 using cobalt dynabeads. Eg19 introduced a band at 30 KDa. The supernatant aliquote was collected in 5 days post-transfection. M- molecular weight marker; W(n)- washing fractions; FT- Flow through; E- Elution fraction.

5.3.2.4 Large-Scale Purification of Recombinant Proteins Using IMAC by ÄKTA Start System

After the expression analysis and optimisation procedures, a large scale of HEK293-6E cells transfection and purification of the recombinant proteins were attempted. To reach these objectives, the *Echinococcus* cDNA constructs were transformed into previously prepared chemically competent DH5-α *E. coli* strain cells and grown in 100ml of LB medium for propagation of the expression constructs, followed by purification of the *Echinococcus* cDNA constructs using Plasmid Midiprep Pure Yield kit (Promega, UK) to be utilised for transfection of 300 ml volume of HEK293-6E cells, see Section (5.2.3.3) for details. The supernatants containing secreted *Echinococcus* recombinant proteins were harvested, and the proteins were purified via their 6xHis-tags by immobilised metal affinity chromatography (IMAC) through 1ml nickel-based resin (Nickel-NTA resin) columns using ÄKTA Start Purifier System. The proteins were then concentrated using ultrafiltration Vivaspin MW 10kDa Centrifugal Concentrator columns.

The elution and flow throw fractions of purified Cyclophilin and Eg19 were collected and tested by SDS-PAGE stained with Silver-staining and Western Blot analysis. The results clearly present a successful purification of Cyclophilin and Eg19 proteins in the elution fractions through nickel-based resin column using ÄKTA Start Purifier System, as confirmed by presence of bands at the molecular weight of 25 KDa and 30 KDa, respectively, under reducing condition (see Figures 5.20, 5.22 - 5.24). Under non-reducing condition, Eg19 shows two bands at 60 KDa and 120 KDa (see Figures 5.20, 5.22 - 5.24). However, the data shows obvious contaminants in the elution fractions using nickel-based resin by ÄKTA Start Purifier System comparing to the Magnetic Dynabeads, see Figure (5.20 and 5.24). The results prove that there is no evidence of losing the proteins in the flow through by using ÄKTA System.

After the purification process, the eluted fractions of purified recombinant *Echinococcus* proteins were quantified by utilising BCA Assay kit. The results identified that the quantity of Cyclophilin and Eg19 were 1.60 mg/ml and 2.45 mg/ml, respectively.

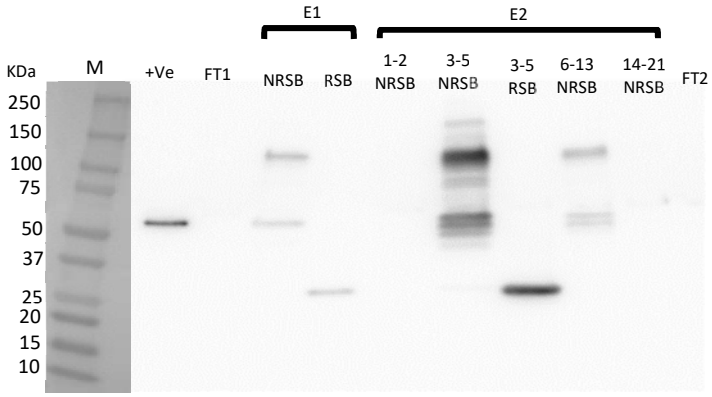


Figure 5.22: IMAC purification of recombinant Eg19 using Magnetic Dynabeads and ÄKTA Start System.

His-Tag immunodetection to detect the purified Eg19 using cobalt Dynabeads and nickel-based resin by ÄKTA Start System under both reducing and non-reducing conditions. Recombinant Eg19 shows bands at 30 KDa using reducing sample buffer (RSB), and with non-reducing sample buffer (NRSB) shows bands at 60 KDa and 120 KDa. E1- purified elution fractions using cobalt Dynabeads; E2- purified elution fractions using nickel resin by ÄKTA Start System; FT1-flow through from Magnetic Dynabeads method; FT2-flow through from ÄKTA Start System; M- molecular weight marker; +Ve- 6xHis-tagged protein (60 KDa) as a positive control.

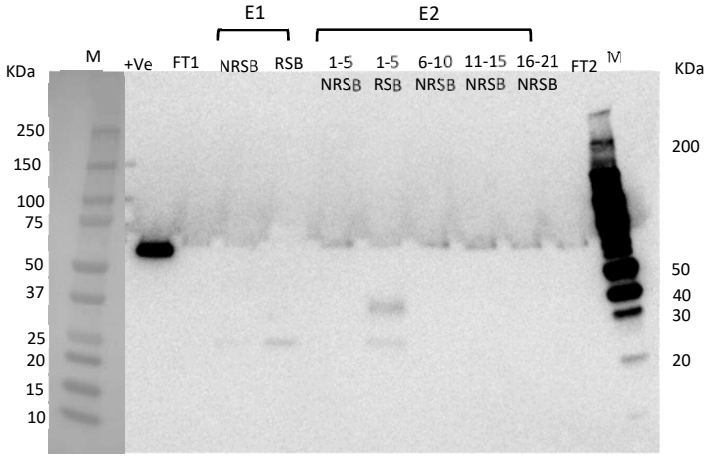


Figure 5.23: IMAC purification of recombinant Cyclophilin using Magnetic Dynabeads and ÄKTA Start System.

C-Myc immunodetection by Western Blot to detect the purified Cyclophilin using cobalt Dynabeads and nickel resin ÄKTA Start System under both reducing and non-reducing conditions. Recombinant Cyclophilin shows bands at 25 KDa under both reducing and non-reducing conditions. E1- purified elution fractions by using cobalt Dynabeads; E2- purified elution fractions using nickel-based resin by ÄKTA Start System; FT1-flow through from Magnetic Dynabeads method; FT2-flow through from ÄKTA Start System; M- molecular weight marker; +Ve- Myc-tagged protein (57 KDa) as a positive control; RSB- reducing sample buffer; NRSB- non-reducing sample buffer.

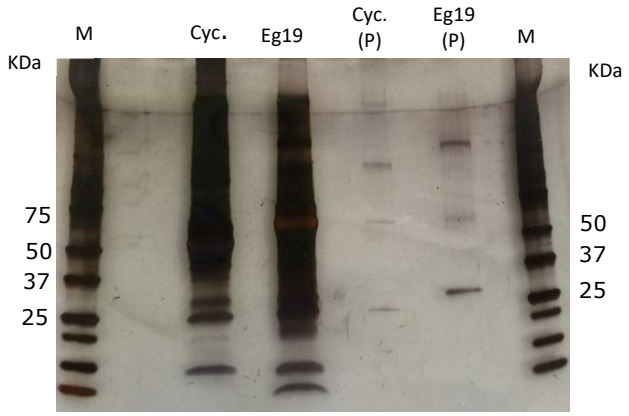


Figure 5.24: Expression of recombinant Cyclophilin and Eg19 in HEK293-6E cells and purification using IMAC by ÄKTA Start System. Silver-staining showing purified Cyclophilin and Eg19 using nickel-based resin columns. Cyclophilin and Eg19 presented bands at 25 KDa and 30 KDa, respectively, under reducing condition. The symbol represent the following: M– molecular weight marker; Cyc.- Cyclophilin; P- purified protein.

5.4 Discussion

To overcome the challenges of low yield and insoluble recombinant *Echinococcus* proteins that we faced during the expression, in the previous chapters, we decided to use an alternative strategy for expressing these proteins. Thus, the aim of the work in this chapter was achieving a successful expression and purification of seven recombinant *Echinococcus* antigens and allergens (EF1-a, Ag5, AgB2, Cyclophilin, Eg19, EgTeg and EgTPx) in a soluble form. To achieve this aim, a new expression and purification approach was applied to this study and careful optimisation protocols were required for expression and purification processes. Few different steps were involved in the optimisation procedures. Firstly, PCR conditions were optimised to amplify *Echinococcus* cDNAs by using two different polymerase enzymes. Secondly, optimal ligation protocol of the PCR products and expression vector (pTT5) was required for successful cloning by using different ratios of PCR products to vector and using various incubation time. Thirdly, optimal conditions for proteins synthesis is necessary after confirming the sequences of the cloned cDNAs, such as finding the optimal time for collecting the supernatant. Finally, the successful approach identified during expression screening was scaled up for proteins purification and the appropriate purification system was chosen to achieve highly pure protein yield.

HEK293-6E cell line was chosen as an expression system that can produce functional and soluble recombinant proteins. HEK293-6E expression system enables a high yield production of recombinant proteins [160]. In this study, two expression platforms were tried with this cell line, which were adherent and suspension grown HEK293-6E cells. Following the cloning procedure, pTT5-*Echinococcus* cDNA constructs were transiently transfected into HEK293-6E cells using a commercial transfection vehicle Polyethyleneimine (PEI).

There are two different isoforms of PEI which are linear and branched that can be used as a gene delivery vector [166]. Few published papers verified the distinction between the two isoforms of Polyethyleneimine in terms of transfection capacity, and it had been demonstrated that linear PEI has better transfection effectiveness than the branched PEI [167-170]. Hence, in this study we utilised the 25 kDa linear PEI as transfection vehicle. Based on some published studies, the ratio of 1:2 (w/w) of DNA construct to PEl was used as an optimal ratio for transient transfection of both adherent and suspension growing HEK293-6E cells [171-173]. The pEGFP-Express plasmid was regarded as an indicator of successful gene delivery. The transfection was then evaluated by fluorescence microscopy (Figure 5.13 and 5.18), and approximately 60% of the transfected adherent and suspension growing HEK293-6E cells showed green fluorescent protein.

Two different proceedings were accomplished after assessing the transfection efficiency using pEGFP-Express plasmid, which were; (i) small-scale expression screening, (ii) time course evaluation for protein harvesting (Figures 5.14 and 5.15).

In the present study, the commercial antibodies for the poly-His and C-Myc tags were used, separately, for recombinant proteins detection. The results of Western Blot demonstrated that the 6xHis and C-Myc tagged recombinant Cyclophilin, Eg19 and EgTeg proteins could be successfully expressed and secreted into the cell supernatant (Figures 5.14, 5.15, 5.16, 5.19 and 5.20). It must be noted that these three expressed proteins migrated at significant higher molecular weights than their predicted sizes. Hence, Cyclophilin, Eg19 and EgTeg, despite their calculated molecular weights at 20.5 kDa, 19 KDa and 14 KDa, respectively, drifted at 25 kDa, 30 kDa and 20 kDa, respectively, after production of these proteins in HEK293-6E cell line and tested under reducing condition. There is a main possibility for the migration of the proteins' molecular weights, which can be the result of post-translational modifications, such as glycosylation and

phosphorylation. Interestingly, this migration action was continuously observed in SDS-PAGE and Western Blot analysis, although the experiments were repeated three times, independently. Our results are comparable with Ricardo *et al.* (2008) findings, who expressed EmSmad family proteins of *Echinococcus multilocularis* in HEK293 cells and they found that EmSmadA, EmSmadB, EmSmadC and EmSmadD proteins were phosphorylated by human bone morphogenetic protein (BMP) and/or transforming growth factor- β (TGF-β) receptors, and the molecular weight of, for instance, EmSmadC protein migrates from 37 KDa to 50 KDa, after expression [174].

On the other hand, Amelie *et al.* (2012) expressed 12 different proteins in HEK293 cells. After SDS-PAGE and Western Blot monitoring, they realised that the proteins migrated significant higher than the expected molecular weights. The mass spectrometry analysis showed that, for instance, protein 5 has been glycosylated in 3 different N-linked sites, and the protein's molecular weight migrated from 20 KDa to 40 KDa. After the protein was treated with de-glycosylating enzyme to remove glycosylation, it migrated at a lower molecular weight at the predicted size of 20 KDa [175].

Hence, we analysed the two recombinant proteins, Cyclophilin and Eg19, for potential N- and O-linked glycosylation sites using NetNGlyc 1.0 and NetOGlyc 4.0 servers. We found zero N-linked glycosylation sites in Eg19, but three predicted O-linked glycosylation sites in the positions of 41, 69 and 101 amino acids. While in Cyclophilin, two potential N-linked glycosylation sites were detected in the positions of 88 and 125 amino acids, and one potential O-linked glycosylation site in the position of 195 amino acids. Consequently, we believe that the sizes migration of these two proteins can be due to the proteins glycosylation.

Under non-reducing condition, the Western Blot results for Eg19 showed several bands with different molecular weights. However, the strongest bands were at the molecular

weight of 60 and 120 KDa. The potential explanation is disulphide bond formation in the protein due to the presence of four cysteines residues, leading to dimeric and tetrameric structures of the protein. These disulphide bridge were reduced by using the reducing sample buffer, therefore, the SDS-PAGE results of Eg19 demonstrated only one band at 30 KDa, which could be the monomer structure of the protein, with the difference accounted for by e.g. glycosylation. Our findings were comparable with Delunardo *et al.* in 2010 who were the first group who successfully expressed a 19 KDa antigen for *Echinococcus granulosus* and gave it the name Eg19. Delunardo and co-authors revealed several bands of Eg19 in 12% SDS-PAGE ranging between 19 and 100 kDa [176].

The expression of Ag5 and EgTPx in the supernatant of adherent HEK293 cells was also monitored on Western Blot using anti-Myc and anti-His tags antibodies (Figures 5.15 and 5.16). The Western Blot results of secreted Ag5 and EgTPx proteins show that these two proteins were not secreted in HEK293-6E cell supernatant. The possible suggestion is ineffectual secretion of the proteins, as the proteins were expressed but not secreted into the cell supernatant. Consequently, the cell lysates production of the transfected cells with both gene constructs were analysed (Figures 5.15 and 5.17), however, the Western Blot outcomes did not show any retention of Ag5 and EgTPx proteins in the cell lysates. Hence, there are two other potential reasons for this result. Although the ratio of DNA construct to PEl transfection reagent was optimised, the transfection was not efficient enough. The second possible explanation is that although Western Blotting is considered as a sensitive method for protein detection, however the secreted proteins was limited to the available superficial space of the adherent cells, thus the yield was very low and could not be detected.

Time course expression analysis was performed to demonstrate the optimal time of harvesting the secreted proteins for obtaining high protein yield level and avoiding

proteins degradation. The Western Blot analysis shows that 120 hours' post-transfection was an optimal time for harvesting the supernatant containing secreted proteins, see Figures (5.14) and (5.15).

The encouraging results accomplished from the optimisation steps and expression screening of Cyclophilin and Eg19 drove us to produce both proteins in large scale and purify them through their 6xHistidine-tags using Magnetic Dynabeads and ÄKTA Start System.

In the present work, His-tag was used for protein purification by immobilized metal affinity chromatography (IMAC) with two metal ions, nickel and cobalt. The recombinant *Echinococcus* proteins were purified through both cobalt and nickel resin columns using Magnetic Dynabeads and ÄKTA Start Purifier System, respectively (Figures 5.20 - 5.24). Both cobalt and nickel display affinity for poly-histidine tagged proteins. However, based on SDS-PAGE results and Western Blot findings, cobalt Dynabeads presented better purification for the proteins than nickel-based resin, as few contaminants after elution were found with the purified recombinant proteins when nickel resin was used, see Figures 5.21 and 5.24 for SDS-PAGE results, and Figures 5.20, 5.22 and 5.23 for Western Blot. The potential explanation is the cobalt may have a weaker interaction, consequently it has a higher specificity. Our outcomes were compatible with Chaga *et al.* (1999) results who found that cobalt introduced a better specificity than the nickel for His-tagged protein purification [177].

Generally, the yield of purified recombinant proteins was satisfactory to carry out the downstream experiments.

Chapter 6: Use of RS-ATL8 Reporter System for Screening of *Echinococcus* Antigens

6.1 Introduction

The interaction between allergens and specific IgE (sIgE) leads to the cross-linking of sIgE bound to the high-affinity receptor FcεRI on mast cells and basophils and results in fast degranulation and release of synthesised mediators, such as histamine, and this event considers as the centre of the allergic symptoms [178]. Consequently, the measurement of allergen-specific IgE levels in the blood of allergic individuals is at the heart of allergy diagnosis.

Although serological assays are available, cellular tests have also been commonly used in the diagnosis of allergy, since Blackley discovered the skin prick test in 1873 to diagnose the allergy in the individuals who are allergic to environmental allergens. Cellular diagnostic technology for the valuation of cell sensitisation has been raised by various studies, such as the use of humanised rat basophilic leukaemia (RBL) cell lines activated with the serum of individual allergic patient and suspected allergen. Allergen-specific IgE can be tested using this technology without requiring expensive and specific equipment [179]. It is possible but still needs of further experiments to verify that the humanised RBL cellular technology will be able to ultimately achieve the diagnostic of clinical symptomatic of allergy rather than measuring the sensitisation of sIgE levels only, consequently reducing the chance of false positive or false negative results. However, due to a set of limitations, these cells are not routinely used for diagnostic purposes. Overcoming these limitations could ultimately result in developing a robust and sensitive

reporter system, which could lead to establishing a new technique to be used for clinical allergic diagnosis [180]. Like human mast cells and basophils, RBL cells possess a functional FcεRI with four endogenous chains, α, β, and two γ; however, the rat FcεRI-α chain does not bind human IgE [39, 181]. For this reason, RBL cells are not appropriate for screening human sera. Hence, several studies have been conducted to generate stable transfectants of RBL which express the human IgE FcεRI chains [182].

Nakamura *et al*. (2010) [82] did an intensive study to overcome the limitations of using the RBL cells. They established a new assay called EXILE (IgE cross-linking (x) Induced Luciferase Expression) for measuring activation of the cells and calcium influx rather than mediator release (Figure 6.1). As a starting point, Nakamura used the stably transfected RBL SX-38 cell line, created by Wiegand *et al.* (1996) [84], which had been stably transfected with human FcεRI receptor α, β, and γ chains. Nakamura transfected SX-38 cells with a plasmid encoding a nuclear factor of activated T-cells (NFAT) regulated firefly-luciferase reporter gene, as an indicator for activation the cells in the presence of allergen-specific IgE and cognate allergen. The cells were then called RS-ATL8, and are considered an advanced development of RBL SX-38 transfectant. The principle underlying the NFAT reporter is as follows: Hutchinson and McCloskey (1995) [183] demonstrated that the cross-linking of FcεRI receptors resulted in translocation of NFAT from the cytosol to the nucleus. NFAT translocation is a result of activating the calcium channels and influx of Ca^{2+} from the extracellular environment into the cell cytosol. The influx of calcium into the cell leads to activating calmodulin-dependent enzyme, followed by activation of calcineurin and multiple de-phosphorylation of NFAT [184]. The de-phosphorylation of 13 phosphorylation sites on NFAT leads to unmasking of a previously hidden nuclear localization sequence (NLS) and masking of a nuclear export signal (NES) [185]. NFAT nuclear translocation leads to binding of the NFAT to specific promoters, and this causes induction of the firefly luciferase reporter gene to produce luciferase, that works as a

catalyst enzyme and participates in the production of luciferin leading to light emission, which can be detected using suitable chemiluminescent substrate and scanned by a luminometer. The light emission is an indicator of sIgE and allergen presence (Figure 6.2). The use of this reporter gene, which based on NFAT-dependent production of the luciferase enzyme and detection of RBL activation, has significant advantages over the traditional tests. For example, Nakamura *et al.* (2010) demonstrated that the humanised RBL cell line showed very high sensitivity comparing to the traditional beta-hexosaminidase assay when testing allergens using some specific human sera based on NFAT luciferase assay [82]. This work paved the way for the use of humanised RBL cell line in detection of sIgE in human sera as a diagnostic tool.

It was reported that some of *Echinococcus* proteins have a strong relationship with increasing the level of IgE and triggering allergy, and this could result in a risk of anaphylactic shock in patients with echinococcosis [7, 8].

Although serological tests are available, using rat basophilic leukaemia (RBL) cell line with the stable expression of human FcεRI (HsFcεRI) receptor has made possibility for detection of parasite-specific IgE. Hence, the humanised RS-ATL8 system has the potential to become a new powerful tool and a novel diagnostic approach, which could be applied in the medical setting for diagnosis of parasitic helminths diseases, such as echinococcosis.

In this study, we aimed to test the use of RS-ATL8 reporter system for detection of the specific IgE against *Echinococcus spp.* that binds to the parasite's allergens and can activate the reporter system. The recombinant expression of *Echinococcus* Cyclophilin and Eg19 and sera of echinococcosis infected patients were used in this work to assess the use of RS-ATL8 cell line.

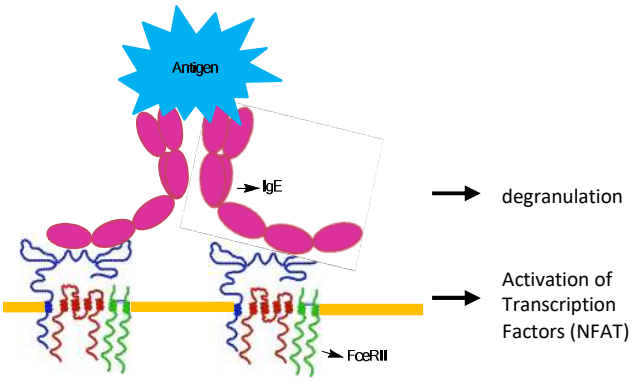


Figure 6.1: Schematic diagram illustrating the mechanism and the events occurring after cross-linking of IgE FcεRI receptors, by allergen to allergen-specific IgE. (Source: adapted from Franco H. Falcone).

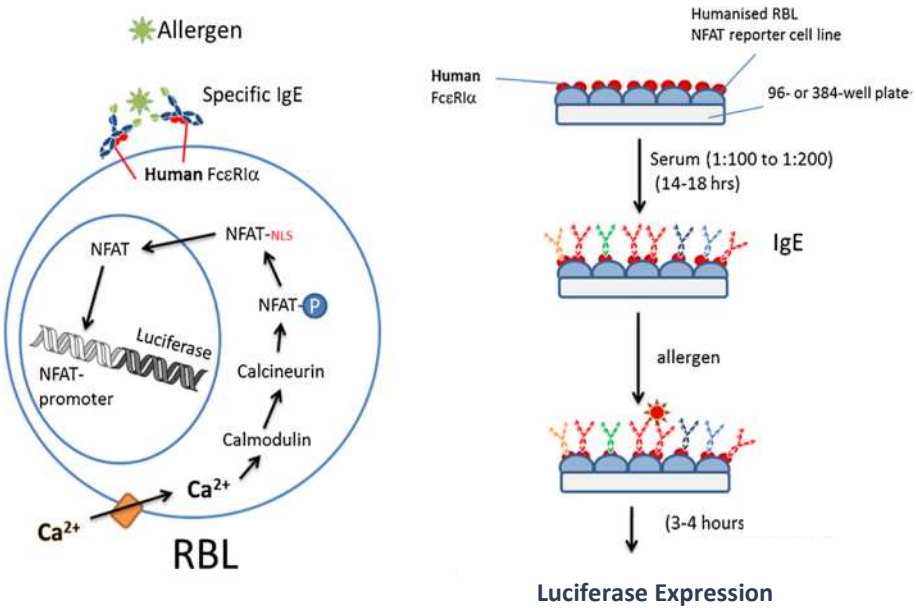


Figure 6.2: Schematic diagram showing the mechanisms underlying the use of the humanized RS-ATL8 reporter system for detection of sIgE in patients' sera.
RS-ATL8 cells are incubated with sera of the echinococcosis patients containing sIgE (sensitisation step) overnight. The next day, the sensitised cells are stimulated with the recombinant *Echinococcus* antigens. In the presence of cognate sIgE, the allergens will cross-link the FcεRI receptors (stimulation step). This event will ultimately result in activating the Ca^{+2} to influx through specific channels into the cells and followed by nuclear translocation of NFAT, which activates the firefly-luciferase reporter gene to express luciferase. Luciferase activity can then be measured using chemiluminescence assay (Image source: Franco H. Falcone).

6.2 Experimental Procedures

6.2.1 Cell Culture and Routine Passaging

Humanised rat basophilic leukaemia cell line (RS-ATL8 cell line) was kindly donated by Ryosuke Nakamura (National Institute of Health Sciences, Tokyo, Japan) and the cell line was covered by a material transfer agreement (MTA). Sterile procedures, inside class II microbiological safety cabinets, were used to perform all cell culture work. RS-ATL8 cells were grown as adherent monolayers in tissue culture-treated 75cm^2 flasks, with 0.2µm vent caps (Corning, UK). Each flask contained 10ml of Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated Foetal Bovine Serum (FBS) (GIBCO), 1% (w/v) of Penicillin (100µg/ ml), 100µg/ ml Streptomycin and 1% (2mM) of L-Glutamine (2µg/ ml) (Sigma-Aldrich Co. Ltd., UK), at 37˚C in a humidified atmosphere and 5% CO_2. Serial passaging was performed at least twice a week, at ratio of 1/10, when the cells were 80-100% confluent by washing the cells twice with 10ml Dulbecco's Phosphate Buffered Saline (DPBS), without Ca^{+2}/Mg^{+2} (Lonza, Switzerland), and detached by trypsinization with 2ml of Trypsin Ethylene Diamine Tetraacetic Acid (EDTA) (Sigma-Aldrich) for approximately 10 minutes. Finally, the desired eukaryotic selection antibiotic was added to preserve transgene expression in stably transfected cell lines, which was 1 mg/ml of G418 sulphate, for selection of HsFcεRI expression (Fisher, UK), and 600 µg/ml Hygromycin, for selection of luciferase expression (Invitrogen, UK). The flask was placed into the humidified incubator at 37˚C and 5% CO_2 until used. White flat-bottomed, untreated 96-well plates (NUNC™ Nunclon Flat-bottomed, Thermo Fisher Scientific) were used to measure luminescence. 15ml and 50ml sterile centrifuge tubes (Corning, UK) were used. Cells were produced as stocks by freezing aliquots in Cryogenic vials (Nunc, Denmark) at 6x10^6 cells in 1ml of 10% (v/v) dimethyl sulphoxide (DMSO) in medium for each vial, using a Nalgene® Mr. Frosty™ Cryo 1°C Freezing Container (Nalgene, USA),

before transferring the vials to the vapour phase of a cell bank containing liquid nitrogen for long-term storage.

6.2.2 Cell Counting

Cells were counted using an improved Neubauer haemocytometer. For viability assessment, 0.4% of Trypan blue (Sigma Aldrich) was employed, mixed 1:1 with the cell suspension. The cell concentration was adjusted to $1x10^6$ cells/ml.

6.2.3 RS-ATL8 Assay

6.2.3.1 Cell Sensitisation

To test the sera from the echinococcosis infected patients for the presence of parasite-specific IgE, the RS-ATL8 reporter system was sensitised with the serum of the infected patient containing IgE. The sensitisation step is necessary to upgrade IgE HsFcεRI receptors and increase their density on the cells surface to allow the allergen-specific IgE (sIgE) binding to the HsFcεRI [186]. Sensitisation needs at least 10 hours for human IgE receptors to reach its stable phase of upregulation [187].

RS-ATL8 cells were harvested from flasks, upon reaching 85-100% confluency. Then, the cells counted using an improved Neubauer haemocytometer, followed by centrifugation at 300 xg for 5 minutes. The cells were then resuspended in an appropriate volume of MEM to achieve a concentration of $1x10^6$ cells/ml. The viability of the cells was assessed by staining the cells with trypan blue. A viability of 95% or more was deemed appropriate for the experiments. After adjusting the cell concentration, some of the cells were sensitised using 1µg/ml purified human IgE (AbD Serotec, UK) or human serum diluted

1:50 (Figure 6.3). 150µl ($15x10^4$ cells) of unsensitised or sensitised cells were seeded into each well of a clear-bottom tissue-culture treated 96-wells plate (NUNC™ Nucleon Delta surface Flat bottomed, Thermo Fisher Scientific) as required. The plates were placed overnight in a cell culture incubator for 16 hours at 37˚C and 5% CO_2.

6.2.3.2 Cell Stimulation

The stimulation step is essential for detecting the presence of parasite-specific IgE in the sera of individual infected patients by cross-linking the IgE HsFcεRI receptors with cognate allergen, this leads to activate the cell and luciferase expression, followed by light emission once the cell will be lysed and the luciferase incubates with luciferase reagents (Figure 6.3).

In the next day, the cells condition was checked under the microscope, followed by removing the media from the wells, and 50µL of stimuli (allergen) in Minimum Essential Medium (MEM) were added to the wells, and the plate was placed in a cell culture incubator for four hours. The first 3 wells, containing 50µL MEM each were used as blanks. As positive controls, cells were sensitised with 1µg/ml purified human IgE (Polyclonal IgE produce from human serum, house made by Helen who was Dr Marcos Alcocer student), and stimulated with 1µg/ml IgG goat anti-human IgE (Vector Labs, UK). Another group sensitised with purified human IgE but was not stimulated in the next day, as a negative control.

6.2.3.3 Luminescence Measurement

Four hours after stimulation, 50µl of luciferase substrate solution containing cell lysis reagent, Promega ONE-Glo™ System, was added to the cells in all wells containing

supernatants or MEM (blanks), and the contents (100µl) transferred to a new clear-bottomed 96 well white plate (NUNC™ Nunclon Flat-bottomed, Thermo Fisher Scientific). The luminescence of the cells was read 30 minutes after the addition of Promega ONE-glo™ reagent by unification time of 1000ms using I-control™ software on TECAN® infinite 200 luminometer to detect the light emission (Figure 6.3).

6.2.4 Statistical Analysis of Luminometer Reading

All experiments were carried out in triplicate and were repeated at least three times. Standard deviations were used as error bars on all graphs. One-way ANOVA and post-hoc Multiple comparisons was utilised to measure statistical significance of the results at probabilities ****p<0.0001, *** p<0.001, **p<0. 01 and *p<0. 05, using Graph Pad Prism 7 Software.

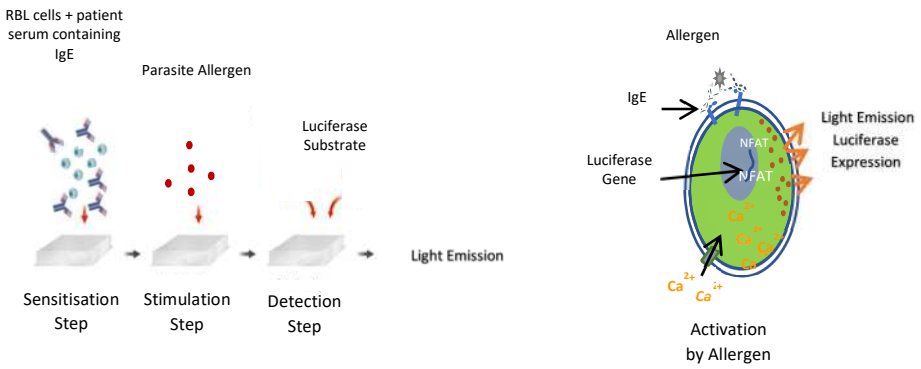


Figure 6.3: Schematic diagram illustrating RS-ATL8 Reporter System for detection of parasite - specific IgE in the sera of echinococcosis patients.
RS-ATL-8 cells were incubated with sera containing sIgE (sensitisation step), and in the next day the cells were stimulated with the recombinant *Echinococcus* allergens. The allergen crosslinks the FcεRI receptors in the presence of parasitic allergen-specific IgE (stimulation step). This events resulted in activating the Ca^{+2} to influx through their channels into the cells and followed by nuclear translocation of NFAT, which activated the firefly-luciferase reporter gene to express luciferase and leading to light emission.

6.3 Results

6.3.1 Optimisation of the RS-ATL8 Reporter System

6.3.1.1 Assessment of RS-ATL8 Reporter System Using Par j2

As stated earlier, the RS-ATL8 cells are RBL-SX38 cells stably transfected with the human IgE FcεRI receptor α/β/γ subunits and NFAT responsive luciferase reporter gene, so it can be used to test the presence of allergen-specific IgE in human sera. The sensitisation of the RS-ATL8 cells with pooled serum of *Parietaria judaica* (pollen) allergic patients (kindly donated by Adriano Mari, Latina, Italy), and the high affinity receptor HsFcεRI of IgE cross-linking induced luciferase expression, after stimulating the cells with anti-IgE and Par j2 allergen, were analysed with a luminometer.

The assay was performed, as follows: The RS-ATL8 cells were harvested and adjusted, as described in section 6.2.3.1, to $1x10^6$ cells/ml. RS-ATL8 cells were then seeded into a clear-bottom 96-wells plate (NUNC™ Nucleon Delta surface Flat bottomed, Thermo Fisher Scientific) at $15x10^4$ cells/150µl/well), and sensitised with 1µg/ml of polyclonal human IgE (AbD Serotec, UK) (first group), as a positive control group, RS-ATL8 cells sensitised with human serum contains IgE, from pollen allergic patients, diluted 1:50 (second group). Finally, adding 150µl of MEM to the $15x10^4$ cells/well of RS-ATL8 cells as a negative control group. The 96-wells plate was incubated overnight for 16 hours at 37˚C and 5% CO_2 to upregulate the HsFcεRI receptor.

The next day, after overnight incubation, the plate was checked under a microscope for absence of contamination and a healthy phenotype (as judged by fibroblast-like shape and attachment). Then, the supernatant was removed from the adhered cells. After that,

the cells were stimulated by adding 50µl (1µg/ml) of anti-human IgE (IgG Goat, anti-human IgE, Vector Labs, UK) to the first three wells (anti-human IgE was diluted in MEM), and adding 50µl (100 pg/ml) of stimuli Par j2 allergen to the second three wells, for assessing the system to analyse the allergenicity. Finally, adding 50µl of MEM to the last three wells as a negative control group. The 96-well plate was then incubated for a further 4 hours at 37˚C and 5% CO_2.

After stimulation, 50µl of luciferase substrate solution containing cell lysis reagent, Promega ONE-Glo™, was added to the cells, as described in section 6.2.3.3. The luminescence of the cells was read by unification time of 1000ms using I-control™ software on TECAN® infinite 200 luminometer to detect the light emission.

As expected, treatment with pure human IgE or sera from pollen allergic patients, followed by stimulation with 1 µg/ml anti-human IgE or with 100 pg/ml of recombinant Par j2, respectively, induced luciferase production (Figure 6.4).

The luciferase expression in RS-ATL8 cells was strongly decreased in the treatment with serum containing IgE or with pure human IgE, but un-stimulated (Figure 6.4). The same lack of luciferase expression was seen in the treatments which were un-sensitised but stimulated with 1 µg/ml anti-IgE or 100 pg/ml Par j2 allergen (Figure 6.4).

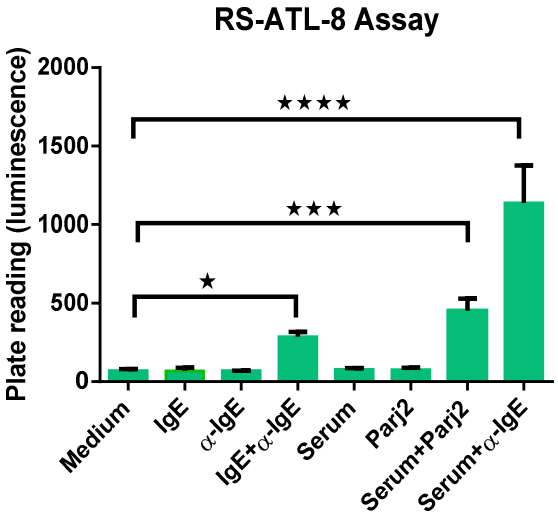


Figure 6.4: - Luminescence data from the RS-ATL8 cells.

NFAT luciferase production was induced in RS-ATL8 cells by sensitising the cells for 16 hours with human IgE (1µg/ml; 50µl/well) and stimulating for 4 hours with 1µg/ml anti-human IgE (α-IgE) or with 100pg/ml Par j2. One-way ANOVA and post-hoc Multiple comparisons was used to calculate the significance ****p<0.0001, ***p<0.001, *p<0.05.

6.3.1.2 Serum Complement Inactivation and Preserving IgE from Heat-Denaturation

Human sera can be toxic to the RBL cells due to the presence of cytotoxic factors, such as complement proteins. Hence, this experiment was performed to pre-treat the serum from cytotoxic proteins, which can then increase the cell activation after allergen stimulation. Serum complement can be inactivated by heating at 56°C for 1h. However, this heating also leads to denaturation of the IgE molecule and loss of its ability to bind the high affinity receptor. For this reason, we used 2M of glucose to preserve IgE from heat denaturation, as stated by Binaghi and Demeulemester (1983) [188].

The experiment was performed as follows: The 15µl deionised water or 15µl of 4M glucose were mixed separately with 15µl of human serum containing 1µg/ml IgE, (dilution 1:2). The mixture of 30µl deionised water and serum; and the mixture of 30µl glucose and serum was pipetted into four different Microcentrifuge tubes, 15µl each tube. One tube of each mixture was incubated in water bath at 56˚C for 1h. While, the other tube of each mixture was stored at room temperature for 1h. Then, 4µl of untreated serum was added to 196µl of RS-ATL8 cells (1:50 dilution), as a negative control. The plate was incubated overnight at 37˚C and 5% CO_2 for 16 hours to upregulate the human IgE FcεRI receptor and to confirm whether the IgEs have been protected from the heat effects or not.

The next day, the mixture of the previous day was removed and replaced with 50µl stimuli, 3 wells of each condition were replaced with 50µl/1µg/ml of anti-human IgE and 3 wells of each condition were replaced with 100pg/ml Par j2.

After addition of stimuli, the 96-well plate was incubated for a further 4 hours at 37˚C and 5% CO_2. After incubation, 50µl of Luciferase substrate was added to the reaction mixtures and the mixtures transferred into 96-well clear bottomed white plate. Finally, the light emission was read using luminometer.

The results show that the production of NFAT luciferase expression still was high in the RS-ATL8 cells, which were sensitised with heated IgE supplemented with glucose (Figures 6.5 and 6.6). On the other hand, it can be noticed that the NFAT luciferase expression was strongly decreased in the cells which were sensitised with heated IgE and water, without glucose (Figures 6.5 and 6.6).

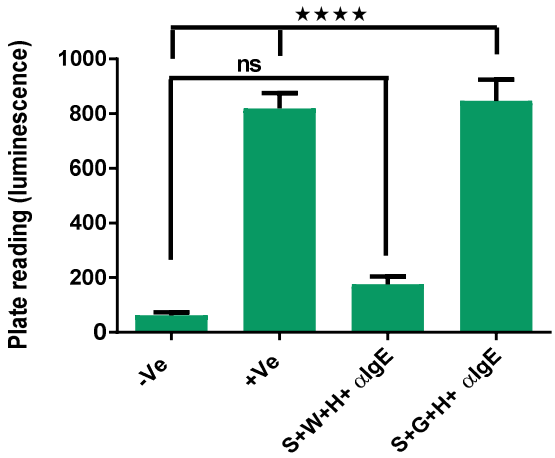


Figure 6.5: Luminescence data demonstrating, protection of IgE from heat denaturation by adding 2M glucose. NFAT luciferase production was induced in RS-ATL8 cells sensitised for 16 hours with the heated mixture of human serum containing IgE diluted 1:50 (50µl/well) and 2M glucose. RS-ATL8 cells were sensitised again with the heated mixture of human serum containing IgE and water, as a positive control (1). The experiment included a negative control group (-Ve) (unsensitised and unstimulated cells). Then, the cells were sensitised with 1µg/ml pure IgE and stimulated for 4 hours with anti-human IgE (α-IgE) 1µg/ml, as a positive control (2). S- Serum; W- water; H- heated; G- glucose. One-way ANOVA and post-hoc Multiple comparisons was used to calculate the significance ****p<0.0001, *** p<0.001, **p<0. 01.

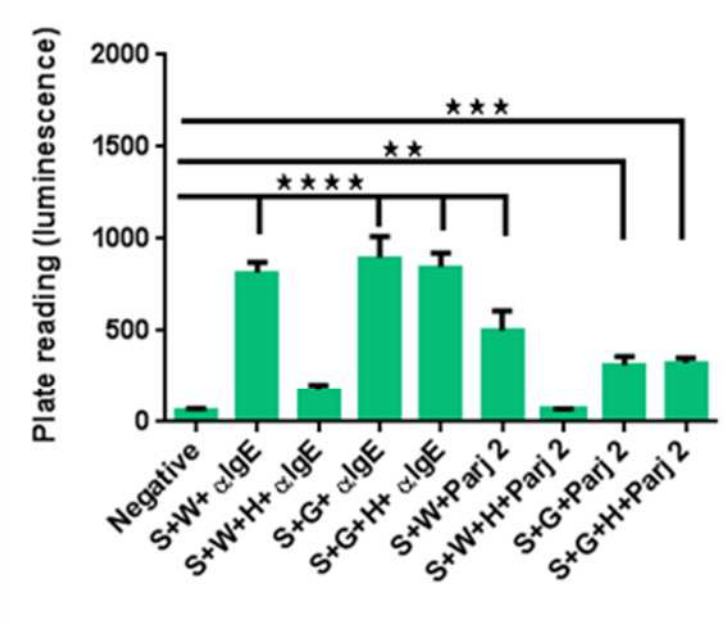


Figure 6.6: Luminescence data demonstrating, protection of IgE from heat denaturation by adding 2M glucose. NFAT luciferase production was induced in RS-ATL8 cells which were sensitised for 16 hours with the heated mixture of human serum containing IgE diluted 1:50 (50µl/well) and 2M glucose. RS-ATL8 cells were sensitised again with the heated mixture of human serum containing IgE and water, as a positive control. The experiment included the negative control group (unsensitised and unstimulated cells). Then, the cells were stimulated for 4 hours with anti-human IgE (α-IgE) 1µg/ml once and with Parj2 (100 pg/ml) once. S- serum; W- water; H- heated; G- glucose.

One-way ANOVA and post-hoc Multiple comparisons was used to calculate the significance $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$.

6.3.1.3 Effect of different concentrations of glucose on IgE and cells

This experiment was conducted to find the optimal concentration of the glucose to be added to the mixture of cells and serum without affecting cells viability. Different glucose concentrations (25, 50, 100, 200, 400 mM) were tested to identify the concentration which gives higher luciferase production.

The RS-ATL8 cells were sensitised with the mixture of 1µg/ml pure human IgE and different concentrations of glucose for 16 hours and stimulated with 1µg/ml anti-human IgE for 4 hours. Then, the luciferase production was measured using the RS-ATL8 assay procedures explained in section 6.2.3.3. Figure (6.7) illustrates that the concentrations of glucose ranged between 25-200mM had no negative effect on the luciferase expression. The same figure clearly shows that 400mM glucose has a negative effect on the cells, which can be seen by a significantly decreased production of luciferase.

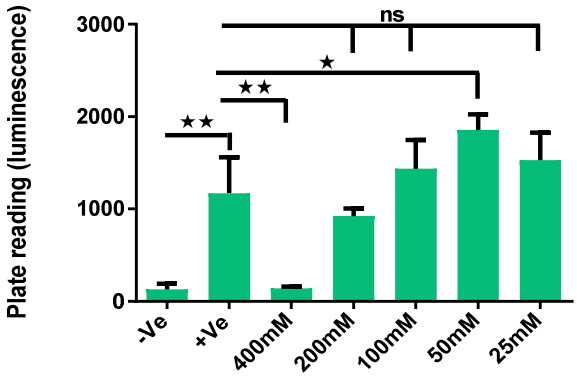


Figure 6.7: Luminescence data from the optimisation of different concentrations of glucose and their effect on the sensitisation of the RS-ATL8 cells. NFAT luciferase production was induced in RS-ATL8 cells which were sensitised for 16 hours with the mixture of 1µg/ml pure human IgE and different concentrations of 2M glucose, as treated groups, and stimulated with1µg/ml human anti-IgE. The experiment included a positive control (without glucose), and negative control group (unsensitised and unstimulated cells). Then, the cells were stimulated for 4 hours with anti-human IgE (1µg/ml). One-way ANOVA and post-hoc Multiple comparisons was used to calculate the significance $^{**}p<0.01$ and $^{*}p<0.05$.

However, Nakamura *et al.* (2010) [82] reported that sensitising the RBL cells with sera diluted 1:100 preserve the cell viability from the cytotoxic factors. We, therefore, diluted the human sera for cell sensitisation instead of heating the sera in the presence of glucose.

6.3.1.4 Testing Different Seeding Densities of RS-ATL8 Cells for the Assay

It is important to find the optimal cell density for using the assay. Therefore, four different densities of RS-ATL8 cells, 50,000, 100,000, 150,000 and 200,000 cells/well (using 96-well plate), were seeded and sensitised each with 50µl of purified human IgE, diluted 1:50 (1µg/ml) (Polyclonal IgE produce from human serum, house made by Helen who was Dr Marcos Alcocer student) for 16 hours, followed by stimulating the cells with 1µg/ml of anti-human IgE, for 4 hours to degranulate the cells.

As shown in Figure 6.8, luciferase expression in RS-ATL8 cells was strongly decreased in the un-sensitised treatments (negative control groups). As expected, lower luciferase expression was seen in the treatment of 50,000 cells/ well sensitised with pure human IgE (Figure 6.8). On the other hand, there was a significant increase in cells' activation in the treatment with 150,000 cells/well and 200,000 cells/well, sensitised with purified IgE. From this, a cell density of 150,000 cells/well appeared to give the best results.

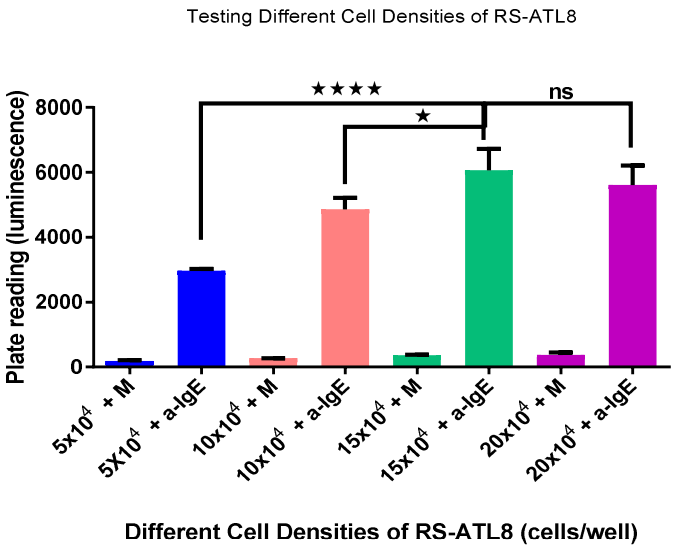


Figure 6.8: Luminescence data from the RS-ATL8 cells testing different cell densities of RS-ATL8.

NFAT luciferase production was induced in different RS-ATL8 cells densities by sensitising the cells for 16 hours with 1µg/ml purified IgE (50µl/well) and stimulating for 4 hours with either 1µg/ml anti-human IgE (a-IgE) or medium (M).

6.3.1.5 Testing Different Time-points for Luciferase Expression

RS-ATL8 cells were seeded into a clear-bottom 96-wells plate (NUNC™ Nucleon Delta surface Flat bottomed) at $15x10^4$ cells/150µl/well) in nine groups (three wells for each group). The cells were then sensitised with 50µl purified human IgE, diluted 1:50 (Polyclonal IgE produce from human serum, house made by Helen who was Dr Marcos Alcocer student). A separate group was done as a negative control group by adding 50µl of MEM to the $15x10^4$ cells/well of RS-ATL8 cells. The 96-wells plate was incubated overnight, as mentioned previously.

After overnight incubation, the supernatant was removed from the adhered cells, followed by adding MEM to the first group as time zero, see figure 6.9. In contrast, the other eight groups were stimulated by adding 50µl (1µg/ml) of anti-human IgE (IgG Goat, anti-human IgE) (anti-human IgE was diluted in MEM) (Figure 6.9). Finally, adding 50µl of MEM to the negative control group, non-sensitised cells (data not shown). The 96-well plate was then incubated at 37˚C and 5% CO_2. The eight stimulated groups were incubated for; 1, 2, 3, 4, 5, 6, 7 or 8 hours, respectively.

At the end of stimulation each time point, 50µl of luciferase substrate solution containing cell lysis reagent, was added to the cells and the luminescence of the cells was read, as recited before.

The results show that 3 and 4 hours' stimulation give a highest NFAT luciferase generation in RS-ATL8 cells, see figure 6.9.

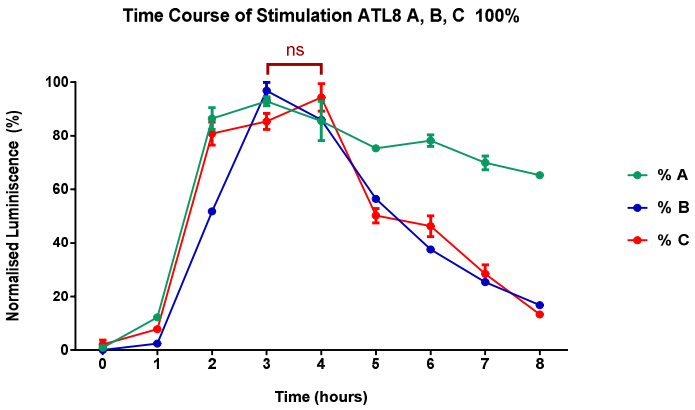


Figure 6.9: - Time-course of NFAT luciferase production in RS-ATL8 cells sensitised for 16 hours with purified human IgE 1µg/ml (50µl/well) and stimulated with 1µg/ml anti-human IgE. One-way ANOVA and post-hoc Multiple comparisons was used to calculate the significance ****p<0.0001, ***p<0.001, *p<0.05. A, B and C indicate to a triplicate experiment.

6.3.1.6 Lectins can induce NFAT translocation in RS-ATL8 cells

There are some publications identified that lectins have the capability to bind the IgE and activate the basophils [189]. Hence, lectins can be used as an additional positive control, or instead of the anti-human IgE for cross-link the IgE receptors. The reason bind using lectins as a positive control is: (i) lectins are more cheaper than the anti-human IgE, (ii) also lectins can bind to the IgE receptors independently and activate the mast cells and basophalis without the presence of IgE [189].

In this experiment, therefore, three lectins Concanavalin A (Con A) from *Canavalia ensiformis*, *Phaseolus vulgaris* erythroagglutinin (PHA-E) and *Sambucus nigra* agglutinin (SNA) were used instead of anti-human IgE to activate the cells and produce NFAT luciferase. It is important to know that NFAT luciferase expression does not have a commensurate relation with the quantity of allergens. Hence, different concentrations of these three lectins were used to find the optimal concentration of each type of lectins for stimulating the RS-ATL8 cells.

The experiment was designed into 12 conditions. 50µl of human pure IgE (1µg/ml) and 150µl of RS-ATL8 cells ($15x10^4$ cells) were applied to each well. After supplying each well with the IgE and cells, the plate was incubated overnight at 37˚C and 5% CO_2 for 16 hours to upregulate the FcεRI receptor. Next day, the lectins were serially diluted into different concentrations (10µM, 1µM, 0.1µM, and 0.01µM). The mixture of the previous day was removed and replaced with 50µl of stimuli (each 4 wells were replaced with the different concentrations of Con A, PHA-E and SNA lectins, separately). Four hours later, the cells were lysed by adding luciferase substrate and the light emission was detected using luminometer, as stated in section 6.2.3.3.

Figure (6.10) illustrates that luciferase generation was at the highest level with Con A at the concentration of 0.1 µM. SNA gave also some response in the concentration of 1µM and 10µM (Figure 6.10). However, the result shows that there is a lack of luciferase production using PHA-E with all concentrations.

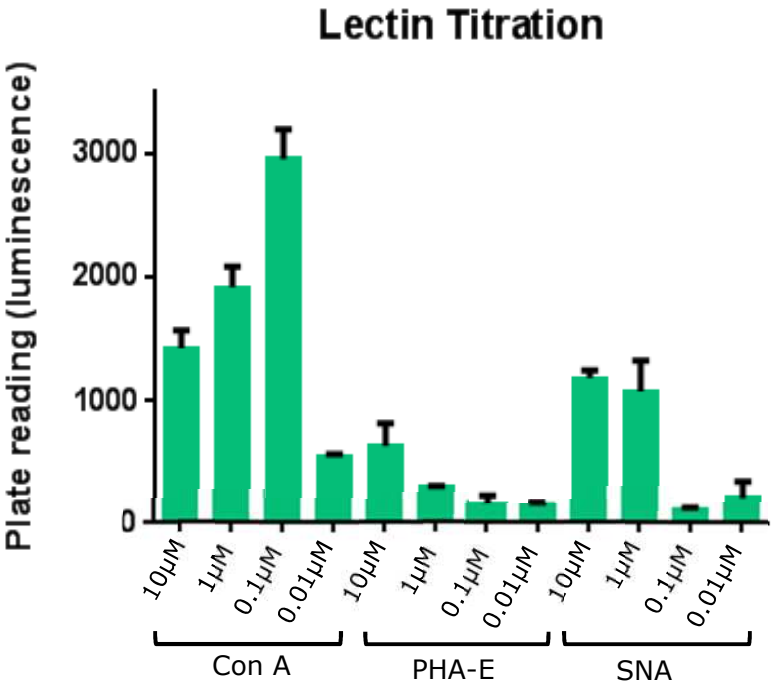


Figure 6.10: Luminescence data from the optimisation of the different concentrations of Con A, PHA and SNA lectins which were used as stimuli of the RS-ATL8 cells. Optimal concentration of different lectins for RS-ATL8 cells stimulation was determined by sensitising the cells for 16 hours with pure human IgE (1µg/ml), then the cells were stimulated for 4 hours with different concentrations of Con A, PHA-E and SNA lectins, serially diluted to concentration of 10µM, 1µM, 0.1µM and 0.01µM.

6.3.1.7 Screening of Echinococcosis Patients' Sera

Different Echinococcosis patients' sera were screened using RS-ATL8 reporter system to find out the sera with highest IgE level. The results show that the (83062) serum gives the maximum activation for RS-ATL8 cells, see in figure 6.10, and it was significantly higher than the group which was sensitised with pure human IgE and stimulated with human anti-IgE. Whereas, (85461) serum gave very low response when it was screened through RS-ATL8 reporter system, see figure (6.11).

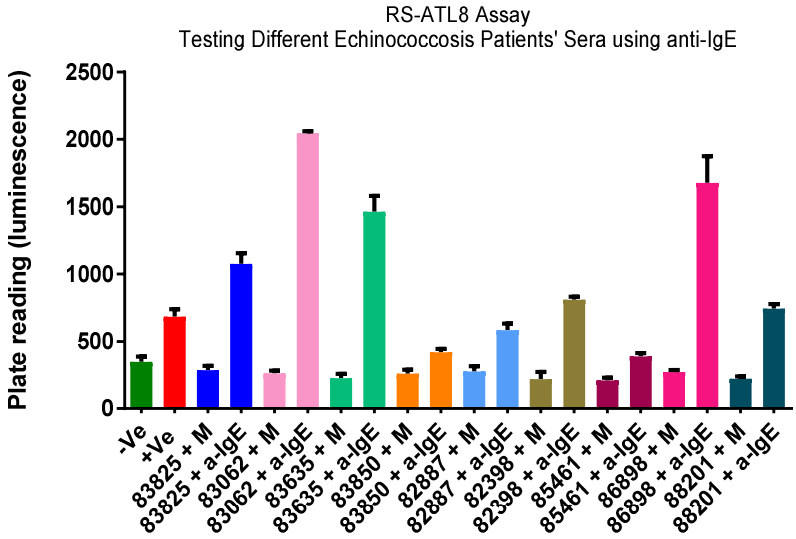


Figure 6.11: Comparison between different echinococcosis patients' sera, based on NFAT luciferase production in RS-ATL8 cells by sensitising the cells for 16 hours with diluted 1:50 (50µl/well) sera of different echinococcosis patients (83825, 83062, 83635, 83850, 82887, 82398, 85461, 86898 and 88201) containing parasite-specific IgE, and 4 hours' stimulation with 1µg/ml anti-human IgE (a-IgE). -Ve_ refers to a negative control group (un-sensitised and un-stimulated cells). +Ve_ refers to a positive control group (sensitised with 1µg/ml pure human IgE and stimulated with 1µg/ml anti-human IgE). M_ refer to the medium.

6.3.1.8 Finding the Optimal *Echinococcus* Antigens Dilution and Testing Through RS-ATL8 Reporter System

The sensitisation of the RS-ATL8 cells with the sera of *Echinococcus spp.* patients (kindly donated by Professor Peter Kern, University of Ulm, Germany. The ethical committee of the University of Ulm approved the use of these sera for the development of serological diagnostic techniques), and the IgE high affinity receptor FcεRI cross-linking induced luciferase expression, after stimulating the cells with allergens or anti-IgE as a positive control, were analysed with a luminometer base on chemiluminescence.

Finding the optimal concentration or dilution of the allergen to perform the RS-ATL8 reporter assay is important to obtain a maximum luciferase production, although the optimal concentration of allergen will vary between allergens.

The assay was performed as follows: RS-ATL8 cells were seeded into 96-wells plate at $15x10^4$ cells/150µl/well, in several different groups, and sensitised with diluted 1:50 of different echinococcosis patients' sera, which gave high RS-ATL8 cell activation in the previous experiment, see section 6.3.1.1 for details of sensitisation step. As a positive control group (1), RS-ATL8 cells sensitised with 1µg/ml purified human IgE. Finally, adding 150µl of MEM to the $15x10^4$ cells/well of RS-ATL8 cells, as a negative control group (1) (un-sensitised). Then, the 96-wells plate was incubated overnight for 16 hours at 37˚C and 5% CO_2.

Next day, groups of the cells were stimulated by adding serial dilutions of different *Echinococcus* allergens, previously expressed in wheat germ lysate; 1:100, 1:300, 1:500, 1:700, 1:1000, 1:2500, 1:5000 and 1:10000, see section 6.3.1.1 for details of stimulation step. Whereas, second groups were stimulated with the same serial dilutions of wheat germ lysate as positive control groups (3) which were corresponded the first groups, to assess the effect of WGL itself on cell activation.

On the other hand, 50µl (1µg/ml) of anti-human IgE (IgG Goat, anti-human IgE) was added to the positive control groups (1), which was cells sensitised with purified human IgE; and positive control groups (2), which was the cells sensitised with the echinococcosis patients' serum containing IgE (anti-human IgE was diluted in MEM). A positive control (2) was included to investigate whether the echinococcosis patients possess IgE specific for *Echinococcus* allergens only or to some other environmental allergens.

Finally, 50µl of MEM, instead of stimuli, was added to the negative control group (1) (non-sensitised and non-stimulated cells) and negative control group (2) (the sensitised cells with echinococcosis patients' sera but un-stimulated), to evaluate whether the IgE molecule can cross-link the FcεRI receptors without the presence of stimuli. The cells were then incubated for a further four hours at 37˚C and 5% CO_2, followed by measuring the luminescence production of the cells as mentioned before in section 6.2.3.3.

The results show that the activation of RS-ATL8 cells was very low using all serial dilutions of different *Echinococcus* allergens for stimulation, see figure (6.12) until figure (6.15) (some data only shown, as all sera showed a similar response). This occurred with all tested echinococcosis patients' sera, which had been tested in the previous experiment and showed high luciferase production when stimulated with anti-human IgE, except (86292) serum which gave no response with anti-human IgE (blue bar).

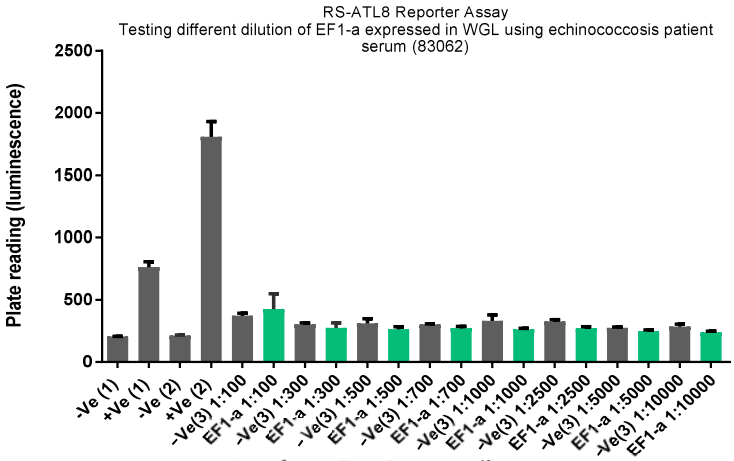


Figure 6.12: Luminescence data from the RS-ATL8 cells.

Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with echinococcosis patient's serum (83062), and then stimulated for 4 hours with serial dilutions of *Echinococcus* EF1-α allergen; and with NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (83062), and then stimulated with the same serial dilutions of wheat germ lysate (positive control group 3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulated with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated.

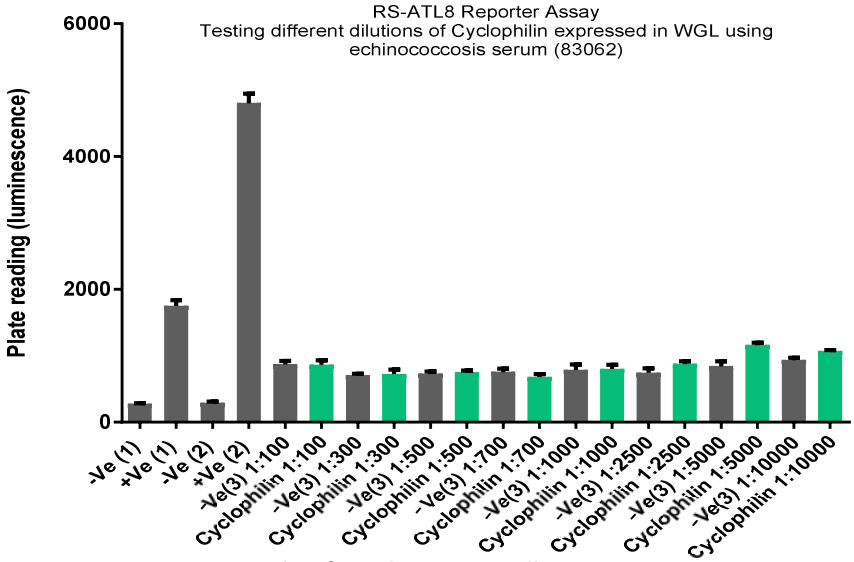


Figure 6.13: Luminescence data from the RS-ATL8 cells.

Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with *Echinococcosis* patient's serum (83062), and then stimulated for 4 hours with serial dilutions of *Echinococcus* Cyclophilin allergen; and NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (83062), and then stimulated with the same serial dilutions of wheat germ lysate (positive control group 3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulating with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated.

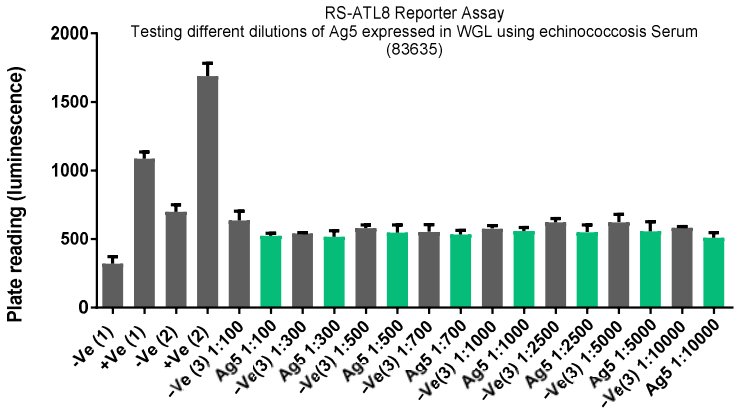


Figure 6.14: Luminescence data from the RS-ATL8 cells.

Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with *Echinococcosis* patient's serum (83635), and then stimulated for 4 hours with serial dilutions of *Echinococcus* Ag5 allergen; and NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (83635), and then stimulated with the same serial dilutions of wheat germ lysate (positive control group 3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulating with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated.

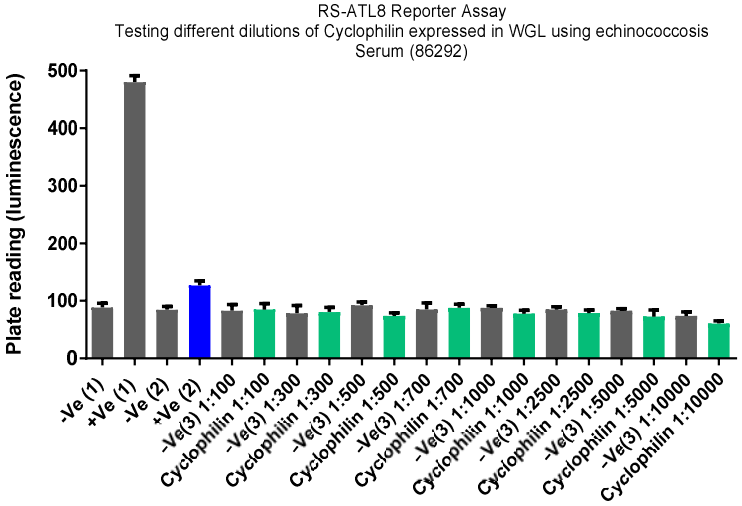


Figure 6.15: Luminescence data from the RS-ATL8 cells.

Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with *Echinococcosis* patient's serum (86292), and then stimulated for 4 hours with serial dilutions of *Echinococcus* Cyclophilin allergen; and NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (86292), and then stimulated with the same serial dilutions of wheat germ lysate (positive control group 3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulating with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated.

One of the possible explanations for the low production of NFAT luciferase in RS-ATL8 cells when the *Echinococcus* antigens were tested through the cells was the low yield of the antigens which were expressed in wheat germ lysate. Thus, the *Echinococcus* antigens were then expressed again, however, in HEK293-6E cells to produce more protein to be suitable for testing through the reporter assay.

Following the synthesis of *Echinococcus* antigens in the mammalian cell line, the antigens were screened through RS-ATL8 reporter system using echinococcosis patients' sera to evaluate the reporter system to be used as a diagnostic tool for echinococcosis.

The experiments were conducted by following the same procedures mentioned previously and the antigens were diluted into serial dilutions, 1:10, 1:50, 1:100, 1:300, 1:500, 1:700, 1:1000, 1:2500 and 1:5000, to be used as stimuli for activating the cells. On the other hand, same serial dilutions of the non-transfected HEK293-6E cells supernatant were used as positive control groups (3) which were corresponded to the first groups, to rule out an effect of the supernatant of the non-transfected cells on cell activation.

The result shows that the NFAT luciferase generation was significantly increased in the RS-ATL8 cells which were sensitised with echinococcosis patient serum and stimulated with *Echinococcus* allergen Cyclophilin, see figure (6.16). However, the NFAT luciferase production dramatically decreased in the RS-ATL8 cells which were sensitised with the same echinococcosis patient serum and stimulated with *Echinococcus* antigen Eg19, see figure (6.17).

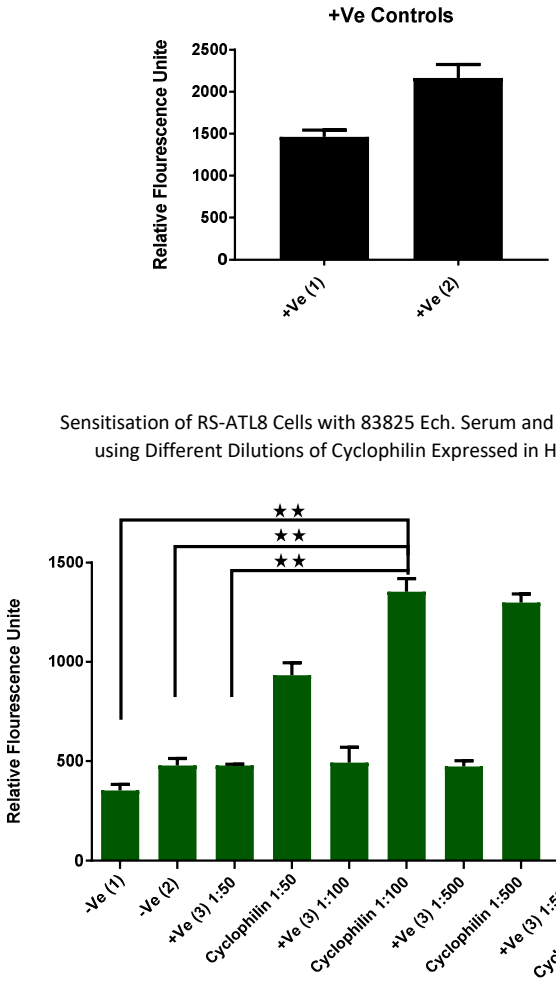
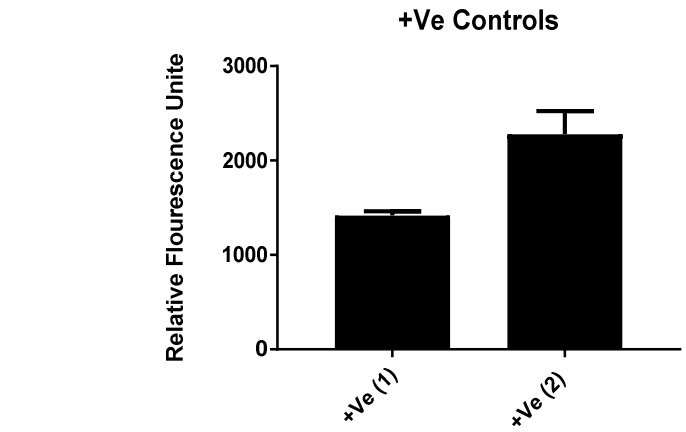


Figure 6.16: Luminescence data from the RS-ATL8 cells.

Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with *Echinococcosis* patient's serum (83825), and then stimulated for 4 hours with serial dilutions of *Echinococcus* Cyclophilin allergen; and NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (83825), and then stimulated with the same serial dilutions of non-transfected HEK293-6E cells supernatant as a positive control group (3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulating with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated. 1:50=32µg/ml; 1:100=16µg/ml; 1:500= 3.2µg/ml; 1:5000= 320ng/ml.



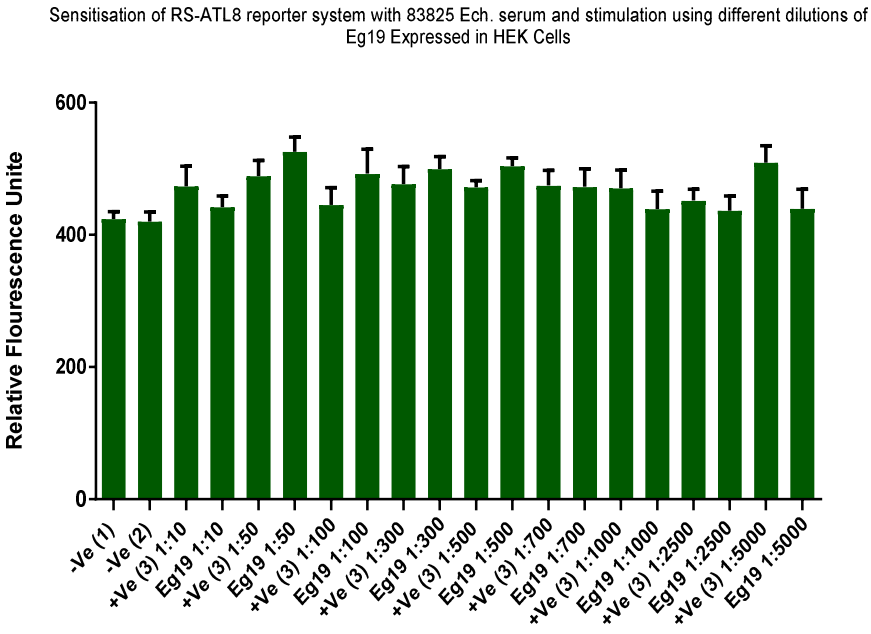


Figure 6.17: Luminescence data from the RS-ATL8 cells.
Side by side comparison between NFAT luciferase production induced in RS-ATL8 cells which were sensitised for 16 hours with *Echinococcosis* patient's serum (83825), and then stimulated for 4 hours with serial dilutions of *Echinococcus* Eg19 antigen; and NFAT luciferase production induced in RS-ATL8 cells were sensitised with *Echinococcus* patient's serum (83825), and then stimulated with the same serial dilutions of non-transfected HEK293-6E cells supernatant as a positive control group (3). The experiment includes: positive control (1), which was sensitised cells with pure human IgE and stimulated with anti-human IgE; positive control (2) which was sensitised cells with diluted 1:50 of echinococcosis patient's serum containing parasite-specific IgE and stimulating with 1µg/ml anti-human IgE; negative control (1) un-sensitised and un-stimulated cells; negative control (2) sensitised cells with patient serum and un-stimulated. 1:10 = 245µg/ml; 1:50 = 49µg/ml; 1:100 = 24.5µg/ml; 1:300 = 8.2µg/ml; 1:500 = 4.9µg/ml; 1:700 = 3.5µg/ml; 1:1000 = 2.45µg/ml; 1:2500 = 980ng/ml; 1:5000 = 490ng/ml.

6.4 Discussion

In this study, RS-ATL8 reporter system, which is a rat basophilic leukaemia (RBL) cell line, was used as a new method for diagnosis of *Echinococcus* infection. These RBL cells, as mentioned previously, express the α/β/γ subunits chain of human high affinity IgE HsFcεRI receptors allowing the human IgE molecules in the sera of *Echinococcus* infected patients binding to their receptors on the RBL cells surface. The binding of parasitic allergen to at least two IgE molecules results in cross-linking of IgE FcεRI receptors. This leads to induce firefly luciferase gene reporter system to generate luciferase, followed by light emission once the cell will be lysed and the luciferase incubates with luciferase substrate. Hence, this system can be used to test the presence of parasite-specific IgE in the sera of infected individuals, as RS-ATL8 system so far has only been used for assessment of sIgE in allergy [82, 83, 190]. So, we are the first to assess RS-ATL8 NFAT reporter system to be used for detection of sIgE in parasitic infection.

Echinococcus has a tight link with allergy because this parasite has ability to induce the immunological system in the human host to elevate the IgE level, as well as, its potential causing clinical anaphylactic reactions in infected patients [8].

It has been stated that the *Echinococcus* antigens, EF1-a, EgAg5, AgB and Cyclophilin, play a crucial role for inducing IgE and triggering allergy in the infected patients. This can be very serious and may cause anaphylactic shock to the patients when the cyst ruptures either spontaneously or during the operation due to spreading the cystic fluid and protoscoleces when attempting to remove the cyst [7, 8]. Thus, in the present study, several *Echinococcus spp.* antigens and allergens were chosen for investigation into their diagnostic value. These included EF1-α, EgAg5, AgB2, Cyclophilin, Eg19, EgTeg, and EgTPx. Having a successful achievement of the recombinant expression of *Echinococcus* Cylophilin and Eg19, the final objective of this study focused on assessing the RS-ATL8

reporter system to be used as a new diagnostic tool for echinococcosis. However, it is important to find the optimal conditions for using the assay. Therefore, the reporter system has been optimised in several different aspects before being evaluated for diagnostic value.

The RS-ATL8 system was firstly tested with *Parietaria judaica* (Par j2) allergen using sera from allergic individuals to check whether the system works or not, which will use later as a new method to diagnose echinococcosis. The results of this experiment clearly demonstrated that the treatment with polyclonal human IgE or sera of the allergic individuals with Par j2, followed by stimulation with 1µg/ml anti-human IgE or with 100pg/ml of recombinant Par j2, respectively, induced the expression of luciferase (Figure 6.4). This shows that RS-ATL8 system, as described by Nakamura *et al.* (2010), can be successfully used to measure IgE-dependent cross-linking events, whether induced by anti-IgE antibodies or by the more natural situation of allergens [82].

The luciferase production in the cells was significantly declined in the treatment with serum containing IgE or with pure human IgE, but un-stimulated (Figure 6.4). The same lack of luciferase expression was seen in the treatments which were un-sensitised, but stimulated with anti-human IgE or Par j2 allergen (Figure 6.4). This appears to contradict Nakamura *et al.* (2010) who found that luciferase expression can be produced, spontaneously, in high levels even in cells which were sensitised with IgE but un-stimulated. This can be explained by the extremely high concentration of IgE (12700 IU/ml=28 µg/ml), which could be reduced by dilution. Our data show that this is not the case with lower serum concentrations used in our assay [82].

As mentioned previously, the RS-ATL8 cells can be degranulated after activation. This can be achieved using human sera from allergic patients which contain IgE for sensitising the cells, and then stimulating the cells using cognate allergens or anti-human IgE. However,

higher concentrations of human serum can be cytotoxic to the rat cells due to presence of cytotoxic proteins, such as serum complement. The pre-treatment of the sera may allow to utilise higher sera concentrations and achieving more sufficient cell sensitisation by upregulating the human high affinity IgE receptor. Thus, inactivated serum complement is required to preserve viability of the RS-ATL8 cells.

Based on standard protocols, serum complement is inactivated by heating at 56°C for 1h. However, the same treatment affects IgE negatively by irreversibly denaturing the IgE molecule, abrogating its ability to bind to its high affinity FcεRI receptor [188, 191].

Binaghi and Demeulemester (1983) [188] reported that IgE can be preserved from heat denaturation, when heating the serum at 56°C for 1h, by using 2M glucose or 1M $MgSO_4$. They showed that 50-100% of the IgE can be protected.

We therefore sought to confirm Binaghi's and co-author results and to determine the best condition for heat-inactivating cytotoxic factors in the serum such as complement, and sensitising the RS-ATL8 cells, while at the same time, using 2M glucose to protect IgE from heat denaturation. Our results, figures (6.5) and (6.6), confirm that the heating in the presence of glucose can preserve IgE molecule from heat denaturation during inactivating serum complement.

An experiment was performed in our lab indicated that using a high concentration of un-cytotoxic serum (1:10 dilution) with a high concentration of glucose (≥200mM) without heating led to a strong reduction of luciferase expression. Interestingly, when using the same concentrations of serum and glucose with heating, the reduction in luciferase expression was much smaller. This suggested that glucose itself may have been a toxic effect on the cells. Hence, we analysed the direct effect of different concentrations of glucose on the cells ability to induce luciferase production upon appropriate stimulation. The results are shown in Figure 6.7.

Then, the luciferase expression was measured using the RS-ATL8 assay procedures. Figure (6.7) shows that concentrations at or below 200 mM of glucose had no effect on the production of luciferase. The same figure clearly illustrates that 400mM glucose has a toxic effect on the cells, therefore the expression of luciferase reduced significantly. It may suggest that the high concentration of glucose cause dehydration to the cells, which led to the death.

However, Nakamura *et al*. (2010) suggested diluting sera 1:100 avoid the risk of cytotoxicity while preserving high sensitivity [82]. Hence for our next experiments, we diluted the sera, instead of heating in the presence of glucose, for RS-ATL8 cells sensitisation to avert the effect of cytotoxic factors.

It is important to find the optimal cell density for using RS-ATL8 assay. For this reason, four different densities of RS-ATL8 cells, 50,000, 100,000, 150,000 and 200,000 cells/well were tested in this study to verify the optimal concentration of the RS-ATL8 cells which gives a high signal and luciferase expression. As expected, treatment with anti-human IgE (Figure 6.8) induces the expression of luciferase enzyme. This result illustrates that RS-ATL8 cells, as described by Nakamura *et al.* (2010), can be effectively used to measure IgE-dependent cross-linking events [82].

The luciferase production in RS-ATL8 cells was strongly decreased in the un-sensitised treatments, which we used as negative control groups, see figure 6.8. Also, the luciferase production was weak in the treatment with 50,000 cells/well sensitised with pure human IgE (Figure 6.8) and stimulated with anti-human IgE, as the cells density was low and there were not enough cells to produce a high level of luciferase. On the other hand, there was a significant increase in luciferase generation in the treatment with both 150,000 and 200,000 cells/well, sensitising with purified IgE and stimulated with anti-human IgE, see

Figure 6.8. Hence, a cell density of 150,000 cells/well was used for the downstream experiments.

Evaluating an optimal stimulation time for RS-ATL8 cells has a crucial role for producing a highest luciferase expression. The incubation time reflects the amount of time needed for mRNA transcription and translation into luciferase. Nakamura *et al.* (2010) [82] reported that the RS-ATL8 cells can be highly NFAT luciferase expression after stimulating the cells for 3 hours at 37˚C and 5% CO_2. We, therefore, sought to confirm Nakamura's results and to determine the best condition for activation the cells. For this reason, the experiment of testing different time-points for luciferase expression was conducted using nine different conditions. The results confirm that the highest luciferase production in RS-ATL8 cells was in 3 and 4 hours' post-stimulation, see figure 6.9. This was almost correspondent with Nakamura *et al.* (2010) [82] study, who revealed that 3 hours incubation is the optimal time to stimulate the cells.

Lectins are carbohydrates-binding proteins and can be found in animals, bacteria and plants, such as beans and nuts. Concanavalin A (Con A) which is derived from *Canavalia ensiformis*, *Phaseolus vulgaris* erythroagglutinin (PHA-E) and *Sambucus nigra* agglutinin (SNA) are examples of dietary lectins. Haas *et al.* (1999) [189] reported that lectins have the capability to cross-link FcεRI receptors bound IgE on basophils and mast cells, leading to secretion of IL-4 and IL-13, and release of histamine. IgE is a heavily glycosylated protein with up to 15% of its molecular weight accounted by carbohydrates [192]. Thus, lectin with the appropriate specificity can bind IgE by these glycoligands.

In the present study, Con A, PHA-E and SNA were used as stimuli instead of anti-human IgE or as an additional positive control. The reasons behind using lectins as a positive control are; firstly, lectins have the ability to cross-link the FcεRI receptors indirectly via IgE molecules. In addition, lectins have the capacity cross-linking the FcεRI receptors

directly and activate the cells, therefore this gives an indicator whether the system is working or not. Secondly, it is cheaper than the anti-human IgE.

The capability of these lectins to cross-link the high affinity IgE receptor directly, or via bound IgE, was measured through NFAT luciferase production. Because of the activation via the high affinity IgE receptor is a bell-shaped, as increasing the concentration of stimulus leads to move from the sub-optimal stimulation range, followed by an optimal range (the top of the curve). If the concentration of activating agent is further increased above optimal, then this leads to supra-stimulatory range, in which inhibitory signalling is switched on: rather than kinases, the phosphatases activate (e.g. SHIP-1), which inhibits basophil responses. Therefore, lectins were serially diluted into different concentrations, to determine the optimal conditions for stimulation of the RS-ATL8 cells and dose response.

As illustrated in Figure (6.10), a maximal release of luciferase was found with Con A at the concentration of 0.1µM. Thus, Con A used at 0.1µM was the optimal condition for stimulating RS-ATL8 cells. Figure (6.10) also shows that PHA-E gave a negative response. Our data correlate with Shibasaki *et al.* (1992) results, who measured lectin dependent on the releasing of histamine from human basophils and mast cells [193]. In contrast, the result is contradictory with Haas *et al.* (1999) finding that low of histamine was released from the basophils and mast cells when stimulated with PHA-E lectin [189].

SNA gave an intermediate response in the concentration of 1µM and 10µM. These results are comparable with the results of Haas *et al.* (1999) who stated that SNA stimulation resulted in the release of IL-4 and IL-13 (Figure 6.10) [189].

Comparing to the first RS-ATL8 assay (Figure 6.4) when anti-IgE was used as a stimulus, it can be noticed that the level of luciferase production with using of 0.1µM Con A was significantly higher than those obtained by stimulation with anti-IgE. These results

correspond with Haas *et al.* (1999) and Shibasaki *et al.* (1992) studies [189, 193]. The probable explanation for that is the lectins might cross-link directly with FcεRI receptors of IgE due to the components of lectins and IgE bound receptor which are both glycoprotein, and bind to the IgE as well, according to Siraganian and Siragania (1975) [194], and Shibasaki *et al.* (1992) [193].

RS-ATL8 reporter system was used for screening different Echinococcosis patients' sera, and then choosing the sera with highest level of total IgE antibodies which gave a highest response to polyclonal anti-human IgE, for investigation of *Echinococcus* allergens. Moreover, RS-ATL8 reporter system was used to find the optimal dilution of *Echinococcus* infected patients' sera which give maximum activation of the cells.

The results show, as illustrated in figure (6.11), that the (83062) serum gives the maximum RS-ATL8 cells' activation, even considerably higher than the positive control which was cells sensitised with purified human IgE and stimulated with human anti-IgE.

This could be explained that the patient has been infected with highly virulent strain of *Echinocossus*, or the patient could be allergic to some environmental allergens in addition to the infection with echinococcosis, so the extremely high level of IgE might be due to more than one case. However, RS-ATL8 assay might prove whether the IgE molecules in the patient serum are only specific to *Echinococcus* allergens or some other allergens as well. This could be done by using the *Echinococcus* allergens for IgE receptors cross-linking inducing luciferase expression (EXILE). These results correspond to what Nakamura *et al.* (2012) verified when they found that EXILE assay could be used to diagnose patients with egg white specific-IgE clear systematic reactions [83].

On the other hand, the RS-ATL8 cells gave very low response when they sensitised with (85461) serum (Figure 6.11). This could be explained that the serum has been stored for long-term and the IgE molecules might be degraded during this period, although the

serum was preserved in -80°C freezer. However, this contrary with Henderson *et al*., (1998) outcomes, who found that long-term storage of IgE molecules does not affect its efficiency [195]. Therefore, the other possible explanation is the patient could be cured from the disease when serum sample was taken. This could be more logical suggestion as in 2002 Rigano *et al.* conducted a study measuring the level of specific IgE for *Echinococcus* allergen in patients with acute infection, stable-disease patients and recovered patients. They found that the IgE expression was significantly reduced in patients with stable disease and cured individuals with chemotherapy treatment [196].

In this study, several *Echinococcus spp.* antigens were chosen for investigation into diagnostic value. These antigens are; EF1-α, EgAg5, AgB2, Cyclophilin, Eg19, EgTeg, and EgTPx. The proteins have been expressed, after cloned into pF3A WG (BYDV) Flexi® Vector, through cell-free wheat germ lysate (WGL). Subsequently, they have been tested using RS-ATL8 reporter cell line based on parasite-specific IgE cross-linking inducing luciferase expression (EXILE). It is important to take into consideration that the luciferase generation does not have a commensurate relation with the quantity of allergen [82, 91]. Therefore, finding the optimal concentration or dilution of the allergen adding to the assay is valuable, although the optimal concentration of allergen will vary from allergen and serum to another [44].

Our results show that the activation of RS-ATL8 cells was very low using all serial dilutions of different *Echinococcus* antigens for stimulation, see figure (6.12) until figure (6.15) (some data only shown, as all sera showed a similar response). This occurred with all seven different echinococcosis patients' sera, which had given high luciferase expression when stimulated with anti-human IgE in the experiment of screening different echinococcosis patients' sera, thus could be assumed to contain sufficient IgE, at least for polyclonal activation purposes, except (86292) serum gives very poor to no response with polyclonal anti-human IgE stimulation (blue bar), this could be due to the cytotoxicity of

serum at 1:50. This result was comparable to Taudou *et al.* (1993) [197] and Takagi *et al.* (2003) [198] data who humanised RBL-2H3 cells with IgE FcεRI-alpha chain receptor and used the cell line to test the dust mite allergic patients' sera depending on calcium influx rather than mediator release. They stimulated the cells with dust mite allergen Der f1 following the cells sensitisation with the dust mite allergic patients' sera containing IgE. However, Taudou *et al.* and Takagi *et al.* did not report a successful cell activation as they stated a high toxicity of some sera when used in high concentrations to sensitise the cells. Regarding to the RS-ATL8 cells sensitised with *Echinococcus* infected individuals' sera and stimulated with either wheat germ lysate (WGL) alone as a positive control group (3) or with *Echinococcus* antigens mixed with WGL, the little NFAT luciferase expression was due to WGL itself which could be suggested that WGL has some non-specific interference with the signal transduction of FcεRI, consequently, activate the RS-ATL8 cells to express luciferase. This was reported by Nagata and Burger (1974) who revealed that WGL has some lectin which agglutinated and cause the IgE receptors cross-linking [199]. Moreover, Haas H. *et al.* (1999) proved that WGL's lectin has carbohydrate side chains which could bind and cross-link the IgE receptors and lead to activate the basophils [189].

In contrast with McCloskey who conducted a study, in 1993, measuring the activation of RBL cells, depending on IgE receptors cross-linking, using wheat germ lysate. McCloskey found that WGL has a crucial role to inhibit the RBL cells activation and decrease the high affinity FcεRI signal transduction. This can be happening as the WGL engages directly and independently with the IgE high affinity receptors and occupy the IgE molecules places, consequently this will prevent allergens binding and cross-link the IgE receptors [200]. However, Wan *et al.* (2014) demonstrated the optimal dilution of wheat germ lysate for stimulation the RBL cells is 1000-fold dilution to achieve the allergen concentration of 100pg/ml. Thus, stimulate the cells with WGL diluted 1:1000 or higher will not decrease the signal transduction of IgE receptors and inhibit the activation of the cells [91].

One possible explanations for low luciferase expression level in the cells stimulated with *Echinococcus* antigens, after sensitising with *Echinococcus* infected patients' sera containing IgE, could be that the concentrations of the allergens which have been expressed through wheat germ lysate and used for the assay were too low and could therefore not induce the RS-ATL8 cells to generate luciferase, and this could be due to the codons bias for wheat germ expression, although our previous findings would appear to rule out this explanation, as previous experiments demonstrated that 100pg/ml of allergen was optimal for detection, and 1pg/ml was still sufficient to obtain measurable activation of the RS-ATL8 cells [91]. Thus, the second potential explanation of low activation of the RS-ATL8 cells is due to issues with proper folding of the proteins due e.g. to disulphide bonds when they expressed in WGL. For this reason, the *Echinococcus* antigens were synthesised again however in mammalian HEK293-6E cell line expression system and this may produce higher yield with proper folding of the antigens to be tested using the RS-ATL8 reporter system.

Following the production of *Echinococcus* antigens in the HEK293-6E cell line, see chapter (5), the next step was to test the ability of the RS-ATL8 reporter system sensitised with sera from *Echinococcus* infected individuals to report IgE-dependent activation events when stimulated with *Echinococcus* Cyclophilin and Eg19 expressed in HEK293-6E cells.

The antigens were diluted into serial dilutions and screened through the RS-ATL8 reporter system again. Figure 6.16 confirms that stimulation of RS-ATL8 cells, sensitised with a serum from echinococcosis patient, with 1:100 (16µg/ml) and 1:500 (3.2µg/ml) dilutions of Cyclophilin results in obvious elevation of NFAT luciferase generation, in contrast to stimulate the cells with different dilutions of Eg19, which does not show any cell activation, see figure 6.17.

This was reported by Wan *et al*., in 2014, who conducted a study testing different of *Schistosoma mansoni* allergens such as SmTAL1, SmTAL2 and IPSE/alpha-1, expressed in wheat germ lysate and *E. coli*, using RS-ATL8 reporter cell line to report the cross-linking of the IgE receptor. Wan and co-authors found that the RS-ATL8 cells can be activated and produce high level of luciferase by stimulating the cells with 1:1000 dilution of *S. mansoni* allergens [91].

Nevertheless, the explanation behind low level of NFAT luciferase production in RS-ATL8 cells stimulated with *Echinococcus* Eg19 could be due to the non-allergenicity of Eg19 and, therefore, could not bind to IgE and cross-link the IgE receptor and consequently could not activate the cells to generate luciferase.

Negative control group (2), which is sensitised RS-ATL8 cells with echinococcosis patients' sera without stimulation, was included in the all experiments to evaluate whether the IgE molecule can cross-link the FcεRI receptors without the presence of stimuli or not. Our results show that there was no noticeable luciferase generation in RS-ATL8 cells sensitised with 1:50 dilution of *Echinococcus* infected patients' sera without stimulating with either *Echinococcus* allergen or anti-human IgE. This result was contradicted to Dibbern and co-authors outcomes, in 2003, who assessed the ability of humanised RBL SX-38 cell line to detect the sIgE in the sera of 18 highly peanut allergic patients. They found that the RBL cells were spontaneous degranulated due to the sensitisation with sera alone [201]. Nakamura and co-workers in (2010) [82], also, stated that the RS-ATL8 cells can be induced by sensitising with sera containing high level of IgE even without stimulating with an allergen. However, this can be eliminated by diluting the high IgE concentrated sera before sensitising the cells.

Taken together, the RS-ATL8 reporter system offers a robust method for testing the potential allergen of *Echinococcus* or any other helminthic parasite which can trigger the allergy in the infected patients and can be used as a novel diagnostic tool for

echinococcosis. The novelty of the system lies in the fast and robust reporter assay which allows detection of parasite-specific IgE-dependent activation events within three to four hours in a high-throughput format.

Chapter 7: General Conclusion

This study was inspired by the idea of developing a cellular technique which is suitable for medium to high throughput screening of *Echinococcus spp.* allergens to be used as a novel diagnostic tool for echinococcosis and able to overcome the limitations of current echinococcosis diagnostic methods.

Although serological tests are available, this has not helped to achieve an effective and acceptable method to diagnose this disease in the early stage of infection, before forming cysts in the body organs. Also, these available serological tests lack of specificity due to the cross-reactivity at the species level and with other helminthic parasites. Using stable transfection of rat basophilic leukaemia (RBL) cell line with the human FcεRI has made possibility for detection of *Echinococcus*-specific IgE in the early stage of infection, since it has been reported that the human basophils become sensitised to parasitic secretions already 4 weeks after a single low dose of hookworm infection [202], so it may be possible to detect infection relatively early, and this technique may give better specificity and higher sensitivity. Hence, the humanised RS-ATL8 system was applied in this research to assess the use of this cellular reporter system for diagnosis of echinococcosis.

We believe that our cellular reporter assay can be more sensitive and more specific than the available serological assays if better allergens were chosen that are unique to the parasite to be diagnosed. Thus, only the allergen-specific IgE from serum binds to the cells, and any other immunoglobulins directed against all the other antigens are washed away, and cannot cause false positive signals. So this limits the likelihood of false results and can give a better specificity. Regarding sensitivity, the reporter cell line acts as a signal amplifier due to the signalling cascade, and the luciferase measurement is also very sensitive.

In the present study before evaluating the use of RS-ATL8 reporter system for diagnostic purposes, several *Echinococcus* antigens (EF1-α, Ag5, Cyclophilin, AgB2, Eg19, EgTeg and EgTPx), which have a potential leading to inflammatory developments in the infected individuals, were attempted to express in different expression systems and screen through the cellular assay. First of all, recombinant expression was attempted in wheat germ lysate, as it is known that protein synthesis using cell-free wheat germ lysate is a quick and effective method for recombinant protein expression [102, 103]. However, when the expressed *Echinococcus* proteins in WGL were tested through the RS-ATL8 cell line, the cells did not activate when these allergens were added to the sensitised cells with echinococcosis human patients' sera. This was either due to the low yield of the expressed proteins which was deemed insufficient for testing through the RS-ATL8 cell line, or due to issues with the proper folding of the proteins when they expressed in WGL. For this reason, our next step was to generate the all selected *Echinococcus* antigens in different bacterial expression platforms, after a successful amplification from cDNA of *Echinococcus* library and incorporation of peptide tags (poly-histidine and c-Myc). The tagged recombinant *Echinococcus* antigens were finally expressed at 37°C for up to 4 hours' post-induction, after series of optimisation procedures. However, the Western Blot results, using commercial antibodies for the tags, showed that the produced proteins were in insoluble form.

Hence, our following stride was to synthesise these antigens in a mammalian expression system, HEK293-6E cell line. Constructs for the production of His-tagged and Myc-tagged *Echinococcus* antigens under control of the cytomegalovirus promoter in mammalian cells were made using plasmid pTT5. We have demonstrated a successful approach for generating recombinant *Echinococcus* Cyclophilin, EgTeg and Eg19 (see Chapter 5). Although, the SDS-PAGE stained with Coomassie Blue, Silver Stain and Western Blot results clearly show a successful expression and purification of Cyclophilin, EgTeg and

Eg19 proteins using HEK293-6E cell line, the bands were always present at molecular weights that are higher than predicted, this was most probably due to the post-translational modifications, such as protein glycosylation, as two potential N-linked glycosylation sites with one potential O-linked glycosylation site were detected in Cyclophilin. Whereas, three predicted O-linked glycosylation sites were found in Eg19.

Following the recombinant expression of *Echinococcus* antigens in HEK293-6E cell line, Cyclophilin and Eg19 were used as a candidate antigen for cell-based diagnosis of echinococcosis. The RS-ATL8 reporter assay was firstly optimised in several different aspects before evaluating for diagnosis purpose. The end results regarding the cytotoxic effect on the RS-ATL-8 cells due to using sera containing complement factor are either to heat the sera at 56°C for 1 hour in the presence of glucose or by diluting the sera 1:50 or 1:100 dilutions. However, glucose itself at the concentrations of 200mM and higher has a cytotoxic effect on the cells. In the present study, we used diluted sera for cell sensitisation instead of the heating in the presence of glucose to avoid the cytotoxic effect of the complement factor. Our optimisation experiments demonstrated that Concanavalin A at the concentration of 0.1 µM can be used instead of anti-human IgE to stimulate the RS-ATL-8 cells, as a positive control, due to its capability to independently cross-link the IgE bound receptors. Testing different time-points for luciferase expression experiment outcomes identified that 3 to 4 hours' post-stimulation is the best time for producing a highest level of NFAT luciferase. Also, the cell density of $15x10^4$ cells/well was the optimal for generation a maximum NFAT luciferase generation.

Our final step was to test the successfully expressed recombinant *Echinococcus* antigens for their ability to activate the RS-ATL8 reporter system. The recombinant Cyclophilin and Eg19 were used for the investigation into their diagnostic value (Chapter 6). Our findings clearly demonstrate high level of NFAT luciferase expression in RS-ATL8 cells stimulated with recombinant Cyclophilin, after sensitisation of the cells with the sera of *Echinococcus*

infected patients. Assuming that activation does not occur with sera from non-infected individuals, this suggest that the RS-ATL8 cellular assay (NFAT luciferase reporter system) has the ability to detect the *Echinococcus*-specific IgE in the sera of infected individuals, after screening the parasitic allergen and reporting the cross-linking of the IgE receptor. In this study, we therefore demonstrated as proof-of-principle the suitability of RS-ATL8 reporter system as a novel technique for diagnosis of echinococcosis, although this needs to be confirmed by further testing of *Echinococcus* infected patients' sera, other parasitic infected patients' sera and non-infected control sera to determine the specificity and sensitivity of this novel method. It is also important screening more *Echinococcus* allergens, which could not be performed in this study due to the time constraints. We hope that with the choice of an appropriate allergen we may be able to generate a more specific diagnostic test and it can perhaps discriminate between cystic and alveolar echinococcosis. Hence, the humanised RS-ATL8 system has the potential to become a new powerful and robust tool and a new diagnostic application for echinococcosis in the medical setting.

Chapter 8: Future Work

This research aimed to develop a novel diagnostic strategy for diagnosis of early stages of echinococcosis before forming cysts in the body organs of infected patients. Overall, this work paved the way for expressing more *Echinococcus spp.* allergen candidates to be tested through RS-ATL8 reporter system and to confirm the suitability of our reporter system to be used as a new diagnostic tool for the disease. In addition, assessing the use of RS-ATL-8 assay for screening more sera of *Echinococcus* infected individuals with cure, acute and chronic infection, using the expressed allergens to judge the capacity of our cellular system to distinguish between these three cases. It would also be important to evaluate whether the RS-ATL-8 reporter cell line is able to discriminate between the two species of *Echinococcus*; *granulosus* and *multilocularis*.

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