PROJECT TOPIC:

DEVELOPMENT OF SEROLOGICAL ASSAYS FOR NOVEL RHABDOVIRUSES RECENTLY DISCOVERED IN NORTHERN UGANDA

COURSE: DISIGNING A RESEARCH PROJECT

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CHAPTER 0NE

1.1 HYPOTHESIS;

Novel and emerging Rhabdoviruses such as the Le Dante virus and Adumi virus may contribute to acute febrile illness in patients in Northern Uganda.

1.2 OBECTIVES;

To develop serological assays for novel Rhabdoviruses recently discovered in Northern Uganda.

1.3 AIMS;

- Develop and improve existing EIA based serological assays for Le Dante and Adumi virus to be used for sero-surveillance in Ugandan populations for pathogen exposure.

1.4 ETHICAL CONSENTS;

Patient samples to be tested in serological assays were collected from convalescent patients at least 21 days after acute illness. All patients had been consented. Samples were analysed under protocols approved by the Uganda Virus Research Institute ethics committee.

1.5 STRATEGY FOR THE DISSEMINATION OF RESULTS/PUBLIC ENGAGEMENT;

There are many strategies for the dissemination of results.

For the purpose of this project, a poster can be presented at the RSTMH meeting. Subsequently, results from this project will contribute to a report on acute febrile illness in Uganda. Information on methodology used in the project can be disseminated to the various health sectors/ research institute in and outside Uganda where these viruses were found for further assay development and its use in regular diagnostics. The research will also be presented at

various seminars, both locally in Uganda and internationally to raise scientific as well as public awareness through open talks.

1.6 POTENTIAL IMPACT OF THE PROJECT;

The assay has potential for use in screening populations in Uganda and elsewhere in Africa and can be used as a diagnostic tool. The discovery/ re-emerging viruses and the extent of exposure to these potential pathogens is relevant to the understanding that there are several pathogens that remain undiagnosed by current diagnostic methods, and it will buttress the relevance of using high-throughput assays like Next Generation Sequencing and the development of better ones. This particular project will help develop novel serological assays that will be useful for immunological testing of these novel viruses. This will help create awareness as to the impact of certain illnesses and the importance of clinical/ research intervention, it will increase the consciousness of the masses and even clinical health care on performing more deep diagnosis on clinical samples. This research may also lead to a call to pay more attention than before on infections that are not currently considered threatening; because these infections are often neglected, or regular drugs are prescribed for treatment, clinical testing of patient samples should be practiced in local health sectors for more efficient diagnosis and treatment. Public engagement aims to achieve the aforementioned goals.

CHAPTER TWO

2.1 SUMMARY OF PROPOSED RESEARCH PROJECT

Preliminary data from a study of acute febrile illness (AFI) in undiagnosed patients in Uganda carried out by researchers from the CVR and the Uganda Virus Research Institute showed that approximately 15% of AFI in Uganda is caused by arboviral infections. In addition to well described viruses such as *Chikungunya*, the study revealed the contribution of novel and emerging viruses, including two viruses within the Rhabdoviridae; the *Le Dante* virus, and a novel Rhabdovirus given the name *Adumi* virus. The RNA sequences of both viruses were detected from acute patient samples using metagenomic high-throughput sequencing (HTS), a technique not yet routinely employed for diagnostic purposes. We propose to develop an ELISA for two Rhabdoviruses of interest (*Le Dante* and *Adumi*) found in this AFI study. There are currently no existing assays for these viruses that can be used to investigate the seroprevalence of these viruses in the population and this project will add an important tool into the study of arboviral exposure in Uganda. Methods to be employed will involve expression of Rhabdoviral proteins (matrix and glycoprotein) in a bacterial expression system followed by purification of the proteins and then use as antigen in ELISA to test convalescent and control sera.

2.2 SUMMARY OF PROPOSED RESEARCH PROJECT IN LAY LANGUAGE

Preliminary data from a study of illness characterised by rapid onset of fever with symptoms such as chills, headache, muscle and joint pains (acute febrile illness; AFI) in undiagnosed patients in Uganda carried out by researchers from the CVR (Centre for Virus Research) and the Uganda Virus Research Institute has shown that approximately 15% of AFI in Uganda is

caused by arboviral infections (infections transmitted by arthropod vectors). The study revealed that well described viruses such as *Chikungunya* (an arbovirus), as well as emerging and novel viruses like *Le Dante* and *Adumi* virus found in the Rhabdoviridae family of viruses, contribute to AFI in these patients. The RNA sequences of both viruses were detected by subjecting acute blood samples from patients with AFI to metagenomic high-throughput sequencing. Our aim is to develop and improve existing Enzyme Immunoassay (EIA) for *Le Dante* and *Adumi* virus that have been discovered in this study. There are currently no existing assays for these viruses that can be used to investigate their seroprevalence in the population and this project will add an important tool into the study of arboviral exposure in Uganda. The ELISA is a technique that works on the principle of detecting antibodies against a pathogen of interest in a patient sample using an antigen from the particular pathogen. ELISA will aid our knowledge of the immunity of the patients, give us insight into past and recent infections and eventually aid epidemiological studies that will measure the exposure rate of Ugandan populations to these viruses. Methods to be employed will involve expression of viral proteins (matrix and glycoprotein) in a bacterial expression system followed by purification of the proteins and then use as antigen in ELISA to test convalescent and control sera.

CHAPTER THREE

LITERATURE REVIEW

3.1 INTRODUCTION

AFI is commonly found in health care settings and can be caused by many agents, it is highly presumed to originate from diverse infectious sources (Eskerud, 1992: Chiu, 2013) like bacteria, parasites, viruses. AFI is characterised by the sudden onset of fever, chills, muscle or joint pain. Investigations of blood from patients revealed that symptoms may have diverse causes, and many remain as yet unknown. The present study as focussed on viruses as the causative agent of AFI.

The discovery of viruses and the infections they cause began in the 19th century from experiments with filters that had small pores and could trap bacteria. The filtered sap from infected tobacco plants infected healthy plants in the experiment that Dmitry Ivanovsky carried out in 1892 (Iwanowski, 1892). The filtered sap that was infectious to the healthy tobacco plants was called a virus by Martinus Beijerinck and this discovery pioneered the field of virology. Following this discovery, bacteriophages were discovered and characterised, and by the 20th century, many viruses had been discovered.

Viruses are classified into multiple families and one diverse group is the family *Rhabdoviridae*, order; *Mononegavirales*. Members of this family are arguably one of the leading causes of AFI in patients (Walker *et al*., 2018, Kuzmin *et al*.,2009). This family of viruses are the most ecologically diverse families of RNA viruses having over 160 isolated species (Walker *et al*., 2000). The natural hosts for these viruses are vertebrates (humans and mammals), invertebrates and plants (Tang and Chiu, 2010). There are eleven genera of *Rhabdoviruses* and four unassigned species (Dietzgen *et al*., 2012: Adams and Cartstens, 2012: Adam *et al*., 2013). These unclassified viruses make up the dimarhabdovirus supergroup (Fu, 2005). However,

according to Walker *et al*., 2018, *Rhabdoviridae* consists 18 genera and one unassigned species (*Moussa* virus). Though *Rhabdoviruses* are ubiquitous and exhibit a wide host range, only a few (less than ten) are pathogenic to humans (Fu, 2005). Two common human *Rhabdoviruses* that have been identified to cause acute encephalitis syndromes or vesicular stomatitis and flu-like syndromes respectively are *Rabies virus* (leads to more than 25,000 deaths of humans yearly) and *vesicular stomatitis indiana virus* (Gurav *et al*., 2010, Warrell and Warrell, 2004, Rodriguez, 2002). The diseases caused by these human *Rhabdoviruses* present clinical manifestations that begin with rapid onset of fever, body aches and chills, symptoms that are similar to AFI thus it is important that rhabdoviral screening should be carried on any AFI patient for early diagnosis and treatment.

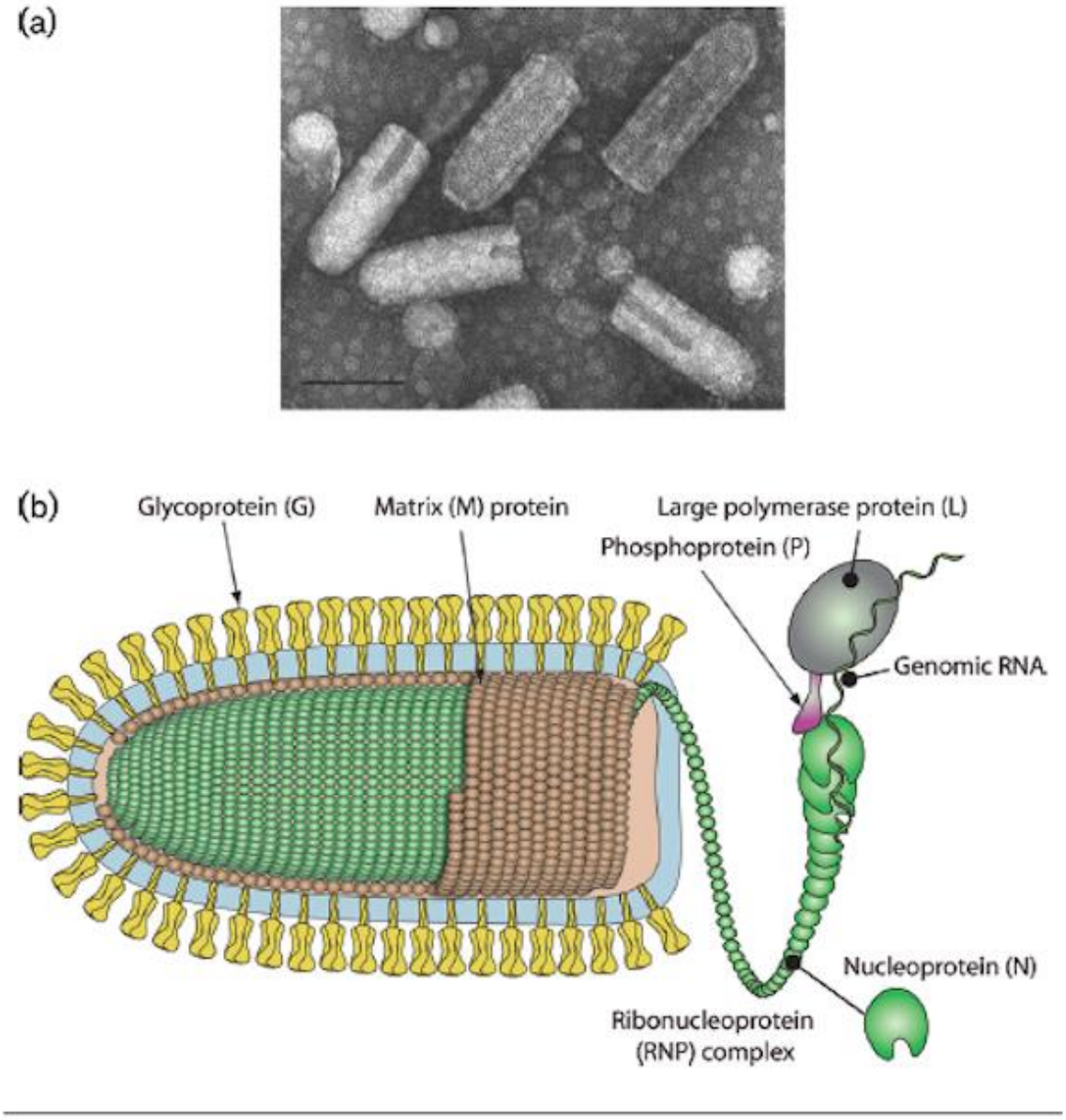

Figure 1: (a) Negative-contrast electron micrograph of vesicular stomatitis Indiana virus particles. The bar represents 100 nm (b) Schematic illustration of a rhabdovirus virion and ribonucleocapsid structure. Unravelling of the RNP is illustrative to show its association with Large protein and Phosphoprotein. Referenced from; Walker et al., 2018.

3.2 GENOME ORGANISATION OF RHABDOVIRUSES

The RNA genome of *Rhabdovirus* comprises five structural proteins in the order; 3'-nucleoprotein (N), polymerase-associated phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large or RNA-dependent RNA polymerase (L)- 5' (Walker *et al*., 2000: Rose and Whitt, 2000).

Each of the aforementioned viral protein have different roles in a viral particle. The nucleoprotein (N) is the foremost component of the nucleocapsid. It aids the encapsidation of the genomic RNA into an RNase-resistant core which is the template for transcription and replication (Banerjee and Chattopadhyay, 1990: Wunner 1991: Yang *et al*., 1998). The phosphoprotein (P) builds up the viral polymerase, it binds to N and confers the specificity of N encapsidation of genomic RNA (Banerjee *et al*., 1989: Yang *et al*., 1998). The matrix protein (M) aids the budding and assembly of virion through its binding to the nucleocapsid and the glycoproteins cytoplasmic domain (Walker *et al*., 2000: Wunner, 1991). The glycoprotein (G) makes up the peplomers or spike of the virus envelope being the only surface protein for *Rhabdoviruses* (Rose and Whitt, 2000), it aids viral binding to host receptors for infection to occur (Dietzschold *et al*., 1996: Walker *et al*., 2000). Large protein (L) is responsible for copying the N-RNA template to produce mRNA, or complete antigenomic and genomic RNA (Baltimore *et al*., 1970: Emerson and Yu, 1975), mRNA capping, methylation of 5' cap structures and polyadenylation (Rose and Whitt, 2000).

Rhabdoviruses are enveloped and the nucleocapsid are helical in shape, hence the typical bullet or rodlike shape of virus particles (Brown *et al*., 1979: Meier *et al*., 1984).

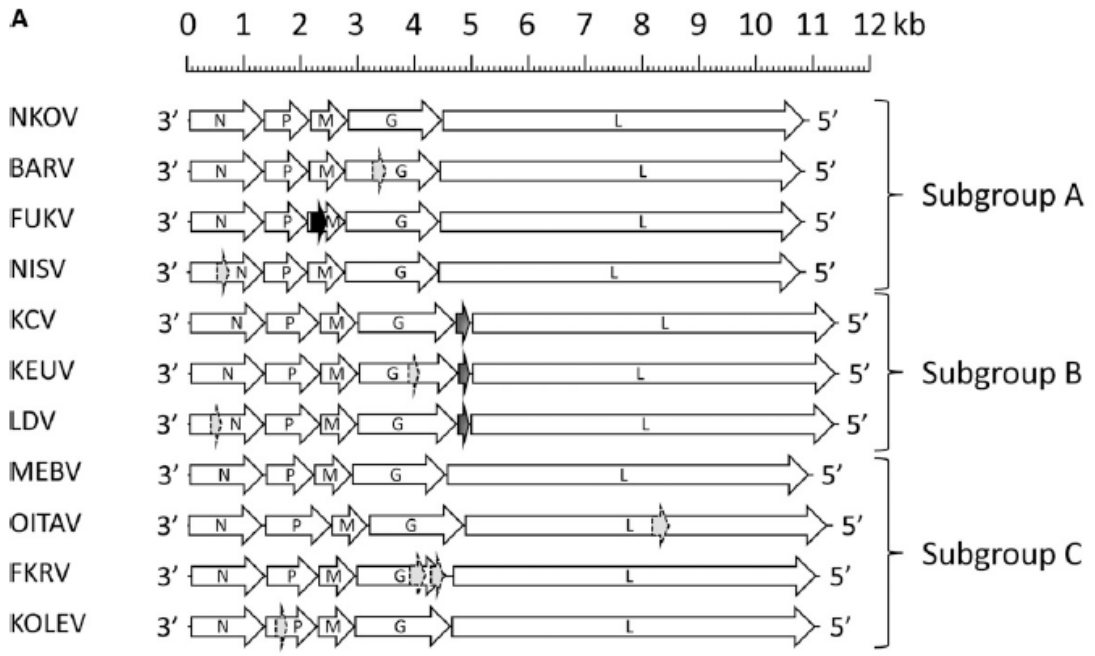

Figure 2: This is a schematic representation of *ledantevirus* genome organisations showing the locations of all ORFs >180 nt, with dark gray denoting U1 ORFs, black *Fukuoka* virus (FUKV) Mx ORF, and pale gray with dashed outline other possible ORFs. Referenced from; Blasdell *et al*., 2015.

3.3 REPLICATION OF RHABDOVIRUS

The replication of *Rhabdoviruses* takes place in the cytoplasm subsequent to receptor-mediated endocytosis of the viral G protein to the host receptor. Major transcription is initiated from the incoming (-) RNP complex by RNA-dependent RNA polymerase. Transcription occurs from 3’ to 5’ by means of gene start and end sequences which produces capped and polyadenylated mRNAs. The RNA-dependent RNA polymerase initiates replication from a single promoter at the 3’ end thereby generating a (+) RNP. This is how nascent (-) RNPs are produced and they are assembled with M and G proteins into enveloped virions. Budding of individual virions may take place at either the plasma membrane or internal membranes, there are also some plant Rhabdoviruses that replicate in the nucleus (Walker *et al*., 2018).

3.4 LE DANTE AND ADUMI VIRUS

In the study of the blood samples collected from acute febrile patients in Northern Uganda, serum samples were subjected to high metagenomic high-throughput sequencing which revealed the presence of different organisms of which key interest was on two Rhabdoviruses namely; *Le dante* virus and the novel virus, *Adumi*.

The *Le Dante* Rhabdovirus was first identified and isolated in 1965 from a 10- year old girl who presented with acute febrile illness symptoms and signs of hepatosplenomegaly. The virus was isolated at the Le Dante University Hospital in Dakar, Senegal (Karabatsos, 1985). Another case of *Le Dante* virus antibody was reported in 1969 from a 47- year old man in Wales. This individual at the time had reported fever, headache and delirium after being bitten by an insect while uploading peanuts from a ship from Nigeria (Woodruff *et al*., 1977). In these two human cases, there has however been no strong correlation to prove the clear causal relationship of LDV with hepatosplenomegaly and Parkinson's disease which the girl and the man developed respectively.

Though there are limited studies of LDV, based on the second incident of the virus by an insect bite, speculation on the routes of transmission for LDV can be said to include insects thus indicating that LDV is an arboviral disease that infects mammals (Walker *et al*., 2018), it may as well include bats. Irrespective of the clinical significance and ecological association with resilient vectors such as insects, there is very little characterization of *Le Dante* virus. *Adumi* virus on the other hand has only been identified in the course of this project, there is therefore no existing literature on the virus. The major information about *Adumi* virus from high genomic sequencing is that it is a *Rhabdovirus* and its closest relatives are viruses found in mosquitoes. The discovery of LDV and *Adumi* virus in Uganda suggests that additional *Rhabdoviruses* of clinical and public health importance likely await identification.

3.5 HIGH METAGENOMIC SEQUENCING AND THE DISCOVERY OF VIRUSES

Sequencing of a viral genome aids better understanding of a virus genetic makeup. Deep or next-generation sequencing (NGS) is a relatively new and emerging method for the surveillance and discovery of pathogens in clinical samples (Tang and Chiu, 2010: Koboldt *et al*., 2013). It is used to sequence genomes of viral particles.

This technique has been used to assemble the 2009 pandemic influenza H1N1 virus genome from a single patient's nasal swab without the use of a reference sequence (Greninger *et al*., 2010), other viruses that have been discovered with NGS are *Arenaviruses* (Briese *et al*., 2009), *Phleboviruses* (McMullan *et al*., 2012), *Coronaviruses* (Van Boheemen *et al*., 2012), the *Rhabdovirus*; *Bas-Congo virus* (Grard *et al*., 2012) as well as *Adumi* virus.

The main aim of this project is to develop serological assays for the *Le Dante* and *Adumi* viruses that were discovered in clinical samples of patients with acute febrile illness. There are different types of assays however, the ELISA is our most preferred choice for its high sensitivity and broad application. The use of serologic assays for clinical samples will help us establish the immune status following the viral infection, that the discovered viruses were the cause of the AFI, that we have actually discovered a human viral pathogen and that it is highly important to employ more sophisticated diagnostic tools to screen clinical samples in advent of uncertain cause of an illness.

3.6 USE OF ELISA TO DETECT ANTIBODIES TO INFECTIOUS AGENTS

Serology as the name implies, is the study of serum or bodily fluids, it is a diagnostic study that aims at identifying antibodies in serum (Ryan and Ray, 2004) or other clinical samples. Serology is also used to identify antigens in clinical samples using immunochemical techniques. When the body is attacked by an antigen, antibodies are formed in response to this

antigens that may be an infection caused by a microorganism (Washington, 1996), a foreign protein or self-proteins in case of autoimmune disease.

Serological methods or assays as outlined in table 1 are the diagnostic techniques that are used to identify antibodies and antigens in clinical samples (serum, tissues, semen or saliva). Antigenic determinant or epitope is the site where an immunoglobulin binds. Not all antigens immunogenic.

The ELISA tests should be improved with respect to the major antigenic components of *Rhabdoviruses* for example glycoprotein; this rhabdoviral structural protein initiates the infectious cycle of the virus by identifying receptors at the host surface, following virion endocytosis it mediates the fusion between the viral and endosomal host membranes. There is evidence for the interaction of the G and M protein together (Mebatsion *et al.*, 1999). Thus, the G and M proteins are the widely accepted Rhabdoviral antigen to cross react with patient serum for antibodies detection.

Neutralizing antibodies have been discovered to target the G ectodomain (Flamand *et al.*, 1993: Lefrancois and Lyles, 1983: Prehaud *et al.*, 1988: Seif *et al.*, 1985: Vandepol *et al.*, 1986).

Many expression systems aimed at producing recombinant proteins have been developed and examples are; bacterial (Escherichia coli), yeast (Pichia pastoris), baculovirus-infected insect cells, mammalian cells and cell-free systems (Yokoyama, 2003).

Serological diagnosis is useful tool to know the immunity of an individual, it gives information about a recent and past infection and can be used for epidemiological studies.

Serological assays as shown in the table above have different sensitivities. As shown in table 1, ELISA and IFA are the most sensitive primary serological assays with sensitivity levels of 0.0005 and 0.005ug/mL respectively. Immunofluorescence assay is used when solid samples such as a skin biopsy is involved whereas in this case the clinical sample used is serum thus ELISA will be the preferred serological assay.

SEROLOGICAL DIAGNOSIS	SENSITIVITY OF DIFFERENT SEROLOGICAL TECHNIQUES USED FOR ANTIBODY DETECTION (ug/mL)	
METHODS USED	METHOD	SENSITIVITY (ug/mL)
• Precipitation • Nephelometry • Agglutination • Neutralisation • Complement fixation • Immunofluorescence • Radio immunoassay • Enzyme immunoassay • Western blot • Recombination immunoblots • Viral haemagglutination inhibition	Gel diffusion	30
	Ring precipitation	18
	Bacterial agglutination	0.05
	Complement fixation	0.05
	Passive haemagglutination	0.01
	Haemagglutination inhibition	0.005
	Immunofluorescence	0.005
	ELISA	0.0005
	Bacteria neutralisation	0.00005

Table 1: Shows the different serological methods and their sensitivity in ug/mL. Referenced from;(https://www.rcpa.edu.au/getattachment/8dbcb7b8-6364-41a8-800f-f0129b5cd7c7/Ross-Whybin-Fundamentals-of-Serology.aspx).

CHAPTER FOUR

WORK PLAN (METHODS AND PROCEDURES USED)

4.1 CLONING FOR PROTEIN EXPRESSION

The G (Glycoprotein) and M (Matrix) protein were chosen to be the preferred antigens for the development of our assay. There is evidence that G and M protein work hand in hand. More so, these proteins are the major antigenic proteins used in most rhabdoviral research and they are the most likely proteins detected by the immune system.

The coding sequence for this protein is obtained from NGS and the Glycoprotein and Matrix protein gene is amplified using a PCR. Following the PCR, gel electrophoresis is used to extract DNA that are ligated into the plasmid vector using restriction enzyme sites introduced using specifically designed oligonucleotides (DNA samples are sterilised by washing the DNA precipitates with ethanol and then dissolving them in sterile buffer solutions at physiological pH). During the cloning process we use an expression vector called the pET-52b(+) vector where our genes of interest are inserted. Afterwards, the expression vector; pET-52b(+) is induced into competent bacteria cells (E.coli) and a mild heat shock is introduced to open pores in the cells thus allowing for entry of the plasmid. This construct will be sequenced to confirm the correct sequence of the gene of interest and then introduced into E. coli to produce proteins that the plasmid codes for.

4.2 PROPERTIES OF THE EXPRESSION VECTOR

As shown in Figure 3 below, our expression vector pET-52b(+) is designed with the pBR322 ori that initiates the replication process. It carries two tags known as the N-terminal Strep-tag and a C-terminal His-tag coding sequence for the detection (tags flank the ends of the protein) and purification of our genes of interest. In between these tags are the human rhinovirus (HRV)

3C protease and thrombin recognition site attached to the vector for the purpose of cleaving off the tags if necessary (The protease is highly specific for cleavage of the sequence LEVLFQ↓GP, it is also active at low temperatures. Next to the multiple cloning region is a thrombin recognition site that allows proteolytic removal of the fused peptide from the expressed target protein). At the multiple cloning site, we will use the Not 1 and Kpn 1 restriction sites. These sites are absent in the DNA sequence of our genes of interest. The two epitope tags will aid dual detection, purification and isolation of full length proteins. The selection marker AmpR, T7 promoter (for transcription) and the T7 terminator is also present. The Lac operator serves as a binding site for the lac repressor (encoded by the lacl gene), and functions to further repress T7 RNA polymerase-induced basal transcription of our gene of interest.

4.3 BACTERIA EXPRESSION AND PURIFICATION

In the cells, there is a high yield of desired product as the E. coli molecular machinery replicate the plasmid DNA.

The selection marker is a gene that confers resistance to an antibiotic; ampicillin. With the aid of this marker, only bacteria that contain the ampicillin-resistance gene will grow. Protein production is induced with IPTG and bacteria are grown to specific OD before being lysed to extract protein on a column with affinity for the 6xHis or Step tags. Combination of the two tags flanking the N- and the C-terminal and the capacity to cleave off guarantees the recovery of a highly purified protein of interest. Affinity-tag purification is our preferred method for purifying our recombinant proteins. Extracted proteins are run through a nickel column that has affinity for His-tag. Desired proteins have the 6xHis- and Strep-tag thus proteins without these tags are washed off since they would not be retained in the column during the purification process. A western blot is carried out to determine the purity of the proteins.

4.4 WESTERN BLOTTING

Recombinant proteins extracted are analysed on SDS-PAGE to determine the presence of our desired proteins in the extract. It is used for the qualitative detection of G and M protein, the relative size and abundance as well as post-translational modifications of the proteins.

4.4.1 PROCEDURE

Gel electrophoresis using polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS) is used to separate proteins by their molecular weight and electric charge. Following this process, there is a transfer of the proteins to a membrane so that they would be accessible to antibody detection. The membrane used is made of nitrocellulose or polyvinylidene difluoride. An electro-blotting process that uses electric current is employed to pull the proteins from the gel to the membrane.

Total protein staining is the next step, it entails visualisation of the protein in the membrane to check its uniformity and normalize it by staining. Non-specific binding of the protein to antibodies is prevented by blocking process. The membrane is placed in a dilute solution of protein and the protein in this solution then binds to membrane where there are no target proteins. Afterwards, the membrane is probed for the G and M protein with a primary antibody against the 6xHis tag with incubation under gentle agitation. PBS wash buffer is used to wash off unbound primary antibodies the membrane. A fluorescently labelled secondary antibody against the host species of the primary antibody is introduced into the membrane. The fluorescence of this antibody bound to the membrane is measured and analysed.

4.5 ELISA

The enzyme linked immunosorbent assay is the final analytical procedure we would carried out. It uses antibodies (serum of patients) and colour change to identify the G and M protein. A colour change signifies the presence of antibodies against infection from the antigens, meaning that these proteins are instrumental to the AFI in the patients. Both the direct and indirect ELISA test will be employed.

THE PROCEDURE FOR THIS EXPERIMENT IS AS FOLLOWS

4.5.1.1 DIRECT ELISA

The His-tag containing the proteins is our antigen and it is added to each well in the microtiter plate. The primary antibody (serum sample) followed by an anti-human IgG secondary antibody with an attached enzyme is added to the plate thus binding to the antigens through the primary antibody. Afterwards, a substrate that triggers colour change that is measurable is added. Intermittent washing with buffer solution is ensured.

4.5.1.2 INDIRECT ELISA

A capture antibody known as an anti-His-tag antibody is added to the plate, this binds all His-tagged antigen in the sample while unbound antigens are washed off. The primary antibody (serum sample) followed by an anti-human IgG secondary antibody with an attached enzyme is added to the plate thus binding to the antigens through the primary antibody. This triggers colour change and a colorimeter is used to measure.

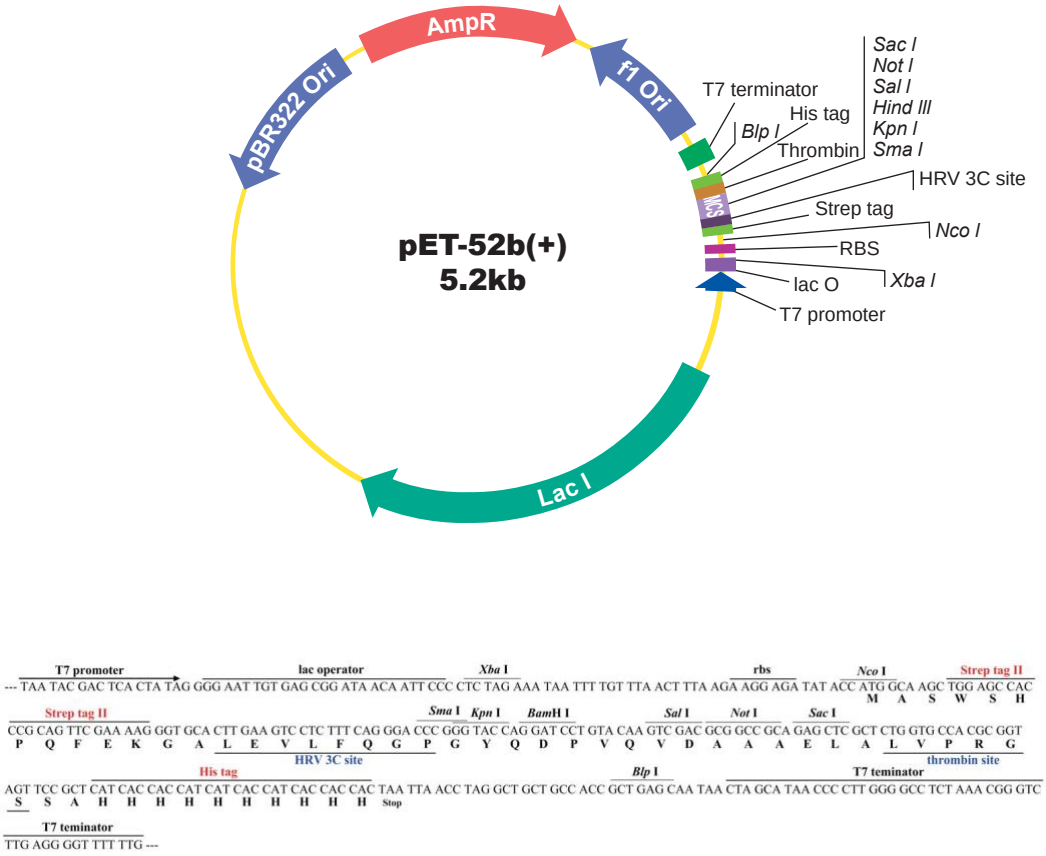

Figure 3: Schematic diagram of an expression vector.

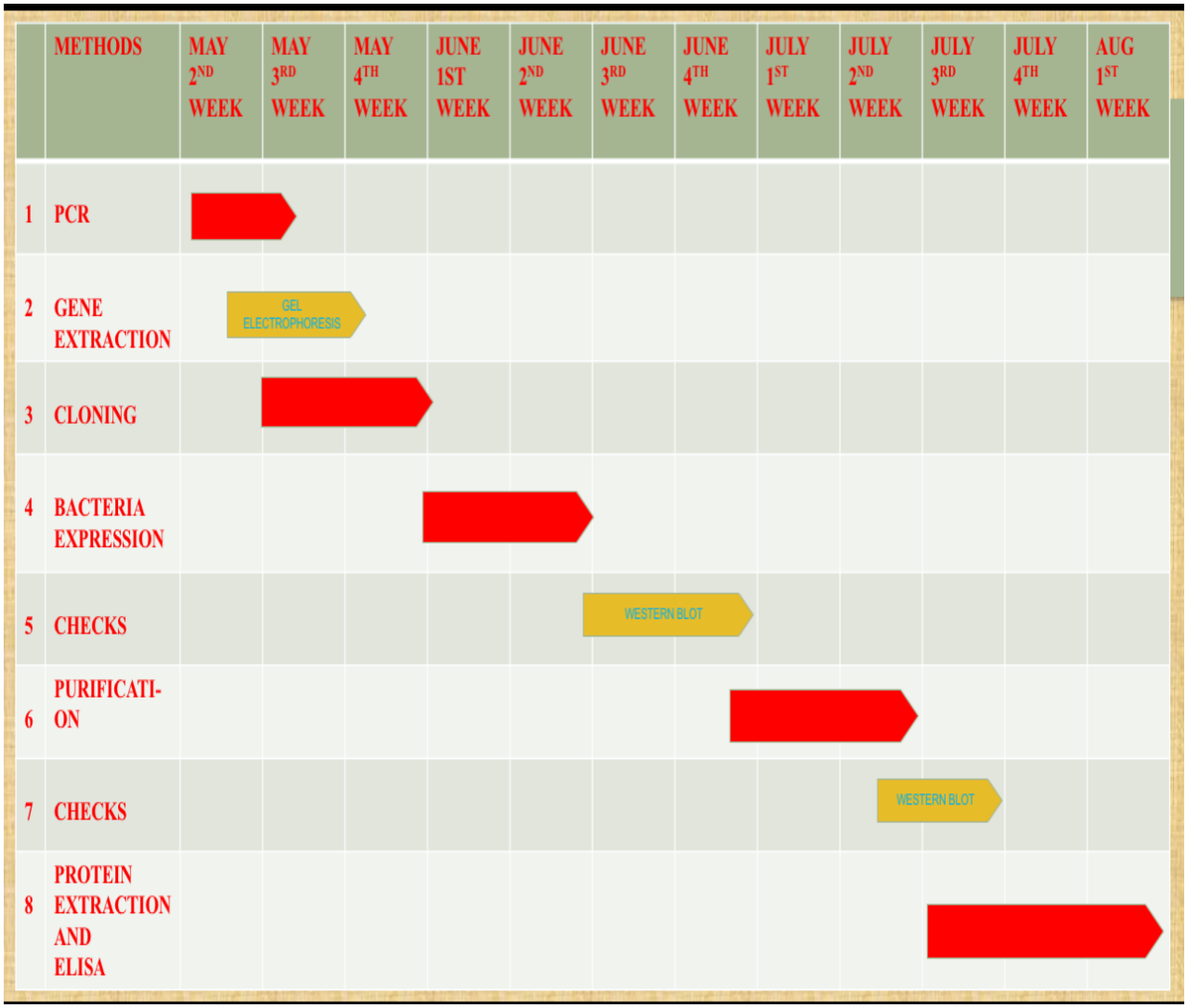

Table 1: Gantt chart showing work plan of my project.

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