Novel IgG and IgY Sandwich Immunoassays for Rapid and Low-Cost Ebola Virus Detection

by

Bharti Singh

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ABSTRACT

Ebola Virus Disease (EVD) is a major public health concern with a high mortality rate in infected individuals. Overlapping symptoms of EVD with other diseases and lack of sensitive diagnostics increases mortality. Thus, there is an urgent need to develop a rapid, reliable, inexpensive and sensitive diagnostic to control the spread and manage the disease at the initial stages. Therefore, early detection of VP40 in 7 days is required to increase survivability. VP40 is the abundant protein consisting of 40% of total protein in Ebola virus. Our research aims to develop immunoassay (DAS-ELISA) based on IgY, biotin labelled monoclonal antibody (bmAb) and bispecific antibodies (bsAb) for the detection of VP40 protein in in-vitro test with lowered limit of detection (LOD) than prevailing assays. The VP40 protein was expressed in *E.coli* and purified using immobilized metal ion chromatography. The recombinant VP40 (rVP40) was used to immunize chickens for the development of specific IgY antibodies. Anti-VP40 hybridoma cells were cultured and second generation bispecific antibodies were developed by quadroma technology. Standardized combinations of IgY, bmAb and bsAb has been used to optimize Ebola detection assay and LOD was calculated for each DAS-ELISA format. VP40 protein expression and purity was confirmed by western blot. The yield of purified rVP40 obtained was 12 mg/l of bacterial culture. Three Abs (IgY, mAbs and bsAb) were produced in sterile conditions. Purity and specificity of the antibodies was confirmed by SDS-PAGE and western blot. The different parameters of DAS-ELISA was optimized (i) DAS-ELISA-mAb-IgY : capture antibody as mAb (cAb) 4μg/ml, detecting antibody (dAb) IgY 8 μg/ml, the optimized dilution of anti-chicken IgY at 1:10,000; (ii) DAS-ELISA-mAb-bmAb: cAb 4 μg/ml, bmAb 31.25 ng/ml, the optimized dilution of streptavidin-HRP at 1:10,000; (iii) DAS-ELISA-mAb-bsAb: cAb 2 μg/ml and bsAb 14 ng/ml. The LOD of VP40 antigen in PBS for each format was (i) 33 ng/ml, (ii) 23 ng/ml, and

(iii) 9.72 ng/ml. In DAS-ELISA-mAb-bmAb, the LOD for VP40 protein spiked in serum samples of human was found to be 71.25 ng/ml whereas for DAS-ELISA-mAb-bsAb it was 6.28 ng/ml when the serum sample was two-fold diluted. DAS-ELISA-mAb-bsAb showed higher sensitivity (10 times) to detect VP40 in spiked human serum samples compared to DAS-ELISA-mAb-bmAb. A student t-test was performed for the statistical analysis.

In conclusion, the formats of DAS-ELISA developed have specificity and sensitivity to detect VP40 protein in nanogram levels. DAS-ELISA-mAb-bsAb showed the highest sensitivity among the three different formats. The developed assays has the potential to be used in clinical settings for screening suspected individuals in the early stage of the infection.

PREFACE

The work described in this thesis was presented and accepted in several Conferences. Recombinant VP40 protein expressing Ebola virus antigen was prepared by Biwen Xu (University of Alberta), and anti-VP40 hybridoma cells were gifted by Dr. Ayato Takada (University of Hokkaido, Japan).

Conference Presentations

1. Bharti Singh, Advaita Ganguly, Hoon Sunwoo. Expression of Ebola Virus VP40 Protein for Potential Diagnostic Applications. At the Campus Alberta Students Conference on Health (CASCH) – Banff, Alberta, 2015.

2. Bharti Singh, Advaita Ganguly, Hoon Sunwoo. Expression of Ebola VP40 Protein and Characterization of Novel Antibodies for Rapid Ebola Diagnostic Assay Development. At University Of Alberta, Faculty of Pharmacy and Pharmaceutical Sciences Research day – Edmonton, Alberta - 2015

3. Bharti Singh, Advaita Ganguly, Hoon Sunwoo. Heterosandwich Immunoassay for the detection of Ebola Viral antigen VP40. At the Molecular Medicine Tri-Conference - San Francisco, California, 2016.

Manuscripts

Enhanced Diagnostic Sensitivity to detect Ebola VP40 antigen using IgG and IgY based Sandwich Immunoassay (manuscript in preparation)

Development of a single-step two-site immunoassay using a novel bispecific immunoprobe for rapid and sensitive detection of Ebola VP40 antigen (manuscript in preparation)

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-Bharti Singh

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List of abbreviations and units

µg	Microgram
µg/ml	Microgram/milliliter
Ab	Antibody
ADE	Antibody dependent enhancement
Ag	Antigen
β –ME	β-mercaptoethanol
BEBOV	Bundibugyo Ebola virus
BSA	Bovine serum albumin
bsAb	Bispecific monoclonal antibody
BSL	Biosafety level
bmAb	Biotin labelled mAb
CAb	Capture antibody
CCAW	Canadian council of animal welfare
CDC	Centre for Disease Control and Prevention
cell/ml	Cell per milliliter
CIEBOV	Cote d'Ivoire Ebola virus
DAb	Detection antibody
DAS-ELISA	Double antibody sandwich- enzyme linked immunosorbent assay
DBSA	Dialyzed bovine serum albumin
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
EBOV	Ebola virus
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EHF	Ebola hemorrhagic fever
ELISA	Enzyme linked immunosorbent assay
Env	Envelope
EVD	Ebola virus disease

FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFU	Focus forming units
FITC	Fluorescein isothiocyanate
GAM-HRPO	Goat anti-mouse IgG conjugated to horseradish peroxidase
GP	Glycoprotein
H	hour
HRP	Horseradish peroxidase
IgG	Immunoglobulin class G
IgY	Immunoglobulin class Y [chicken]
IFA	Immunofluorescence Assay
IMAC	Immobilized Metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
L	RNA-dependent RNA-polymerase
LLOV	Lloviu virus
LOB	Limit of blank
LOD	Limit of detection
mAb	Monoclonal antibody
m-APBA	m-Amino phenyl boronic acid agarose
MARSA	Monoclonal affinity reagent sandwich assay
MARV	Marburg virus
MIT	Massachusetts Institute of Technology
mg	Milligram
min	Minute
ml	Milliliter
ng	Nanogram
ng/ml	Nanogram/milliliter
NGS	Next generation sequencing
NHS-Biotin	biotinyl-N-hydroxysuccinimide ester

Ni-NTA	Nickel nitrilotriacetic acid
NP	Nucleoprotein
OD	Optical density
pAb	Polyclonal antibody
PBS	Phosphate saline buffer
PBST	Phosphate saline buffer containing 0.05% tween-20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PET	Positron emission tomography
PFU	Plaque forming units
PFU/ml	Plaque forming units/milliliter
POC	Point of care
PSG	Penicillin-streptomycin-glutamine
RAVV	Ravn virus
REBOV	Reston Ebola virus
RNA	Ribonucleic acid
RPM	Revolution per minute
RT	Room temperature
RT-LAMP	Reverse transcription–loop-mediated isothermal amplification
RT- PCR	Reverse transcriptase polymerase chain reaction
SARS	Severe acute respiratory syndrome
SD	Standard deviation
sdAbs	Single domain antibodies
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEBOV	Sudan Ebola virus
S/N	Signal/Noise ratio
SPECT	Single photon emission computed tomography
St-HRP	Streptavidin tagged horseradish peroxidase
TAFV	Tai forest Ebola virus
TMB	3,3', 5,5'- Tetramethylbenzidine
TRITC	Tetramethylrhodamine isothiocyanate

USA	United States of America
VP	Virion protein
WHO	World Health Organization
WSF	Water soluble fraction
ZEBOV	Zaire Ebola virus

Chapter 1: Literature Review - Current and future diagnostic tests for Ebola virus disease

1.1 Introduction

Ebola virus disease (EVD), also known as Ebola haemorrhagic fever, is caused by the Ebola virus (EBOV), a filovirus containing a non-segmented, filamentous, negative-sense, and single-stranded RNA genome (19 kb). The *Filoviridae* family members are among the most lethal human viral pathogens in the world. There are three filovirus genera: Marburgvirus, Cuevavirus, and EBOV. Marburgvirus species consist of Marburg virus (MARV) and Ravn virus (RAVV). Cuevavirus includes a single species, Lloviu virus (LLOV). Ebola virus members consist of Zaire EBOV (ZEBOV), Bundibugyo EBOV (BEBOV), Sudan EBOV (SEBOV), Tai forest EBOV (TAFV-formally known as CIEBOV or Côte d'Ivoire EBOV) and Reston EBOV (REBOV) (Kuhn et al., 2014; Negredo et al., 2011), all named after their countries of origin, except Reston, which was from the Philippines (Barrette et al., 2009) (Table 1.1). Among the different EBOV species, ZEBOV is the most virulent pathogen, resulting in high mortality rates of 85-95% in infected populations (McCormick et al., 1983; Tracey, 2013). Among the eight filovirus species, six (ZEBOV, BEBOV, SEBOV, TAFV, MARV, and RAVV) are known to cause disease in humans (Messaoudi et al., 2015). There are no drug and vaccine available in the market for the treatment and prevention of EVD although they are in the developmental stage. According to the report of World Health Organization (WHO) in 2014, Greater than 2,400 people have died from EVD in recent months and approximately 4,700 people have been infected (Davies, 2014). Once the person is infected with EVD then there are very fewer chances of survival and also the disease can be transmitted through the body fluids of EVD patient to a healthy person. The nosocomial transmission among health care workers and EVD patients represents potential risk for outbreak amplification. These findings highlight the great importance of infection control in dedicated isolation facilities as well as in treatment centers

where undifferentiated patients with fever and nonspecific symptoms were admitted (Bah et al., 2015).

To manage the spread of EVD there are diagnostic methods available that are based on viral RNA detection by PCR (Towner et al., 2004), antibody detection assays (Ksiazek et al.,1999a), or antigen detection assays (Niikura et al., 2001)—all of which are expensive, time consuming, and requiring a sophisticated infrastructure (Rougeron et al., 2015).

This review focuses on the latest epidemiology, immunology, pathology, and diagnostic tests available, as well as the improvements to current diagnostics necessary to curb the spread of EBOV filoviruses.

Table 1.1: Classification of Ebola virus

GROUP	Group V ((-)(ssRNA)	
ORDER	Mononegavirales	
FAMILY	Filoviridae	
GENUS	Ebolavirus	
SPECIES	Zaire	Democratic Republic of Congo
	Sudan	Sudan
	Bundibugyo	Bundibugyo, Uganda
	Tai forest	Cote d'Ivoire
	Reston	Philippines

1.2 Epidemiology of Ebola virus

The first documented outbreak of the EBOV occurred in 1976 at Yambuku Mission Hospital, Northern Zaire, and caused 280 deaths. This outbreak of ZEBOV (WHO, 1978) was followed by another outbreak in Sudan by EBOV subtype SEBOV. EBOV outbreaks continued to occur,

affecting mostly Sub-Saharan African countries (Zaire, Sudan, Côte d'Ivoire, Republic of Congo, Gabon, and Uganda). In these early cases, mortality rates varied according to age and sex; for children it was 44%, whereas for teenagers and adults it was 39% and 56%, respectively. In the male population, mortality was 56%, and in the female population, it was 48% (Smith, 1978).

After an 18 year "silent phase," the EVD re-emerged in 1995 in northern Gabon and the Democratic Republic of Congo (Bwaka et al., 1999). Since then, four EBOV outbreaks occurred in Gabon, the Republic of Congo, the Democratic Republic of Congo, and Sudan, from 2001 to 2005. In 2007, a new virus subtype, BEBOV (Bundibugyo Ebola Virus), was isolated from 149 infected people in Uganda and resulted in 37 deaths. (Towner et al., 2008; WHO, 2008). In 2012 and 2013, outbreaks of the Sudan EBOV in Uganda affected 17 people, resulting in 7 deaths (CDC, 2016). EBOV cases peaked from November 2015 to May 2016, when there were 28,616 cases of infection and 11,310 deaths reported worldwide (WHO, 2016a).

The outbreak of 2014, the largest single outbreak of EVD in history, was widely spread with higher fatality rates in West African countries. As of September 14, 2014, there were 4,507 confirmed and probable cases and 2,296 deaths reported in five African countries (Sierra Leone, Guinea, Liberia, Nigeria, and Senegal) (Drazen et al., 2015; WHO, 2014). The breakdown of the 2014 EVD-confirmed cases and deaths was as follows: 3,814 cases with 2,544 deaths in Guinea; 10,678 with 4,810 deaths in Liberia; and 14,124 with 3,956 deaths in Sierra Leone (CDC, 2014) (Table 1.2, (WHO, 2016b)).

Although EVD is prevalent in African countries, it is also being spread to developed countries, due to emigration and travel. In September 2014, the Centers for Disease Control confirmed a case of Ebola in the United States in a person who traveled to Dallas, Texas from

Liberia. On October 23rd, 2014, the New York City Department of Health and Mental Hygiene reported a case in which a medical aid worker was infected with Ebola when he returned from Guinea after treating Ebola patients (CDC, 2014). On January 9th, 2015, six people from West African countries affected by Ebola were granted visas for Canada. Upon arrival, they were kept under surveillance for 21 days, as the incubation period of EBOV is 2-21 days (Levitz, 2015).

1.3 Disease onset and mortality by age group

The most recent EVD epidemic in Africa has caused ailments and death in various age groups, ranging from newborn children to older populations. In January 2015, 79% of diagnosed patients were 16 years of age or older in West African countries. The time of onset of symptoms varies with different age groups. Children less than 1 year of age first exhibit the symptoms of disease on day 6, whereas the 10-15 year age group show disease onset on day 9. However, the fatality rate in children less than one year of age was 90%, whereas it was 50% in 10-15 years of age group. The older population (+45 years) showed a 70% death rate. When the statistical analysis was done for EVD among hospitalized children and adolescents in northern Uganda, it was observed that 90 out of the 218 confirmed EVD cases by national laboratory were children and adolescents with a case fatality rate of 40%. The mean age observed was 8.2 years ± SD 5.6 with a range of 16.99 years (Bah et al., 2015; Mupere et al., 2001; WHO, 2015). In 2014, WHO team notified that the majority of EVD patients in Guinea outbreak are 16 to 45 years of age (49.9% male), and the case-fatality rate was 70.8% among the individuals with known clinical outcome of infection. The period of infection, signs, symptoms, and incubation period (12 days) is very much comparable to the previously reported outbreaks of EVD (WHO, 2014).

Table 1.2: Statistics of Ebola disease in population.

Country	Case definition	Cumulative cases	Cumulative deaths
Guinea	Confirmed	3,351	2,083
	Probable	453	453
	Total	3,804	2,536
Liberia	Confirmed	3,151	‡
	Probable	1,879	‡
	Suspected	5,636	‡
	Total	10,666	4,806
Sierra Leone	Confirmed	8,704	3,589
	Probable	287	208
	Suspected	5,131	158
	Total	14,122	3,955
Total	Confirmed	15,221	‡
	Probable	2,622	‡
	Suspected	10,767	‡
	Total	28,610	11,308

1.4 Transmission

EBOV needs a host cell to replicate and augment virulence. The virus is thought to exist in a reservoir host, with bats, especially the *Epomops franqueti, Myonycteris torquata, and Hypsignathus monstrous* species of fruit bats, thought to carry and spread EBOV. The transmission of EBOV from natural reservoir to humans is not very clear and under study. Although, it is believed that EBOV infection in humans directly from fruit bats is possible because these animals are eaten and consumed by local people living in the outbreak regions. (Eric M et al., 2005; Pourrut et al., 2007; Pourrut et al., 2009). The transmission of the virus

among humans is through direct contact with body fluids (saliva, urine, blood, or semen) or organ transplantation from an EVD-infected individual (F. Feldmann et al., 2013; Francesconi et al., 2003), and the primary routes of EBOV entry are via the conjunctiva of the eye, mucous membranes, and skin lesions (Feldmann et al., 2003). EVD transmission may also occur when a person comes in contact with objects such as needles contaminated with the fluids of EVD infected patients (Roels et al., 1999). Thus, medical staffs are at high risk of contracting EBOV if proper hygiene and safety procedures are not followed.

1.5 Genome organization

EBOV has an RNA genome of 19 kb. The EBOV RNA itself is not infectious, as verified by fluorescent focus-forming assay (Regnery et al., 1980). Gene order is conserved in all filoviruses as: 3' - leader - NP - VP35 -VP40 - sGP/GP - VP30 - VP24 - L - trailer - 5' (H. Feldmann et al., 1992; A. Sanchez et al., 1993). The untranscribed regions (leader and trailer) are partially complementary to each other, a common feature among the order *Mononegavirales* (H. Feldmann et al., 1999). These untranscribed regions contain the signals necessary for replication, transcription, and packaging of the viral RNA genome, as well as replication of the antigenomic viral RNA (Muhlberger et al., 1999). Start and stop signals for transcription are conserved in each gene among the *Filoviridae* family, having the conserved consensus sequence of 3'-CUNCNUNUAAUU-5' and 3'-UAAUUCUUUUU-5' (H. Feldmann et al., 1999; Sanchez et al., 1993). Intergenic regions have overlapping start and stop signals and separate the genes (Sanchez et al., 1993). Among filoviruses, the unique feature of EBOV is that the fourth gene encodes a minimum of two proteins; the second protein is expressed after the addition of adenosine at a stretch of seven adenosines into the mRNA (Sanchez et al., 1996; Volchkov et al., 1995).

1.6 Structure of EBOV

The EBOV members of filoviridae family are approximately 80 nm in diameter and up to 14,000 nm in length (H.Feldmann, 2005). The structure of EBOV consists of an envelope, viral matrix, and nucleocapsid proteins (Figure 1.1). The envelope part contains transmembrane protein - glycoprotein (GP), the viral matrix contains the VP40 and VP24 proteins, and the nucleocapsid complex contains proteins such as nucleoprotein (NP), the RNA-dependent RNA-polymerase (L), the polymerase cofactor VP35 and the transcription activator VP30 (Sanchez et al., 1993).

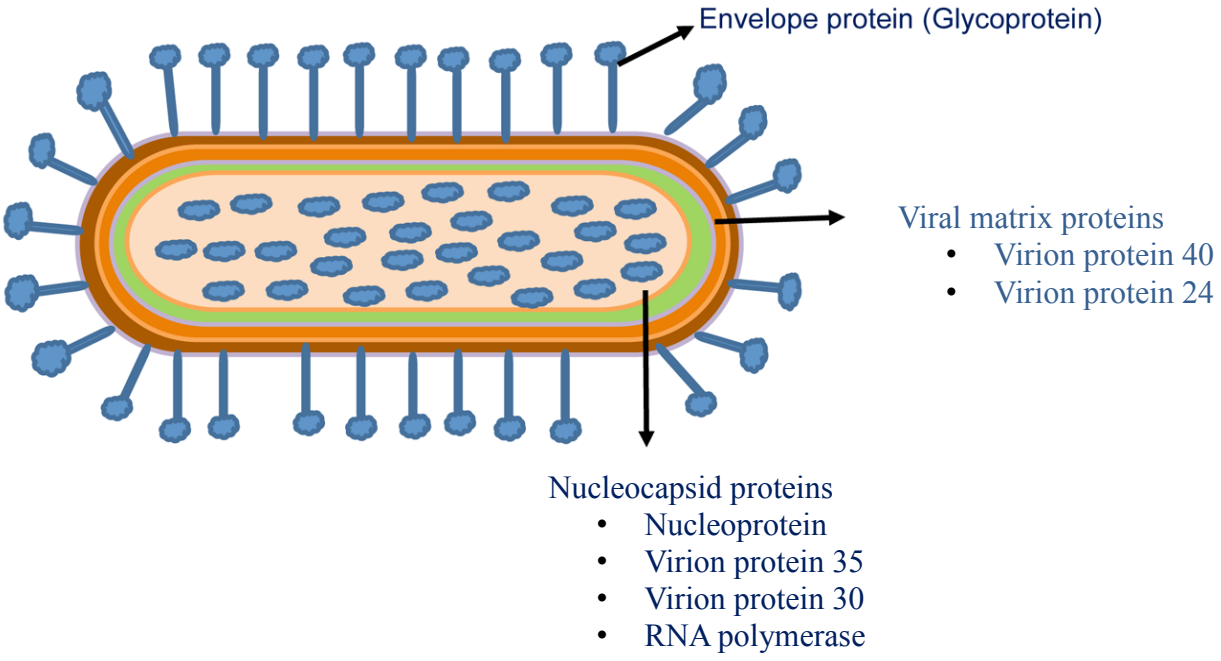


Figure 1.1: Structure of EBOV showing seven structural proteins of EBOV including Envelope protein (Glycoprotein), Viral matrix protein (Virion protein 40, Virion protein 24) and Nucleocapsid proteins (Nucleoprotein,Virion Protein 35,Virion Protein 30, RNA Polymerase).

1.7 Envelope Protein

1.7.1 Glycoprotein (GP)

Viral GP is 681 amino acids, 450 kDa structural transmembrane protein. The GP contributes to the pathogenesis of Ebola virus by causing cytotoxicity in cells and damaging endothelial cells (Yang et al., 2000). GP has a trimeric crystal structure and consists of a highly glycosylated region known as the mucin domain, which induces cell cytotoxicity in infected host cells (Dolnik et al., 2004; Jeffers et al., 2002). The GP forms spike-like structures on the viral surface and helps in attachment and entry of the virus (Gallaher, 1996; Lee et al., 2009). The GP is expressed in large amounts in infected cells and also circulates in the blood, making it an attractive target for Ebola antigen screening procedures, as it can be screened from the blood of infected patients (Lucht et al., 2004).

1.8 Viral Matrix Proteins

1.8.1 Virion protein 40 (VP40)

The 37 kDa VP40 is a 326 amino acid, peripheral matrix protein. This protein is transported to the plasma membrane by exploiting the retrograde late endosomal pathway (Kolesnikova et al., 2004). It may occur in hexamer (Ruigrok et al., 2000; Scianimanico et al., 2000) and octamer (Gomis et al., 2003; Timmins et al., 2003) forms in infected cells, binding to the inner surface of the plasma membrane and helping to form new virus particles (Licata et al., 2004). Abundantly present in the cytoplasm of infected cells (Soni et al., 2014), VP40 supports in membrane association, capsid assembly, and budding of the virus. Due to its abundance in the infected host cells, it is a good candidate for developing antigen detection assays.

1.8.2 Virion protein 24 (VP24)

VP24 is a matrix protein that is 251 amino acids long, with a molecular weight of 28 kDa (Han et al., 2005). It remains in inclusion bodies in infected cells, interacting with NP (Bamberg et al., 2005) for its localization, and helping in the packaging of the viral RNA genome. As well, VP24 has been reported to interfere with the interferon signaling pathway (Reid et al., 2006). VP24 is a minor matrix protein, and it is associated with lipid membranes (Han et al., 2003). Therefore, the protein is not a suitable diagnostic target.

1.9 Nucleocapsid Proteins

1.9.1 Nucleoprotein (NP)

Nucleoprotein (NP) is 739 amino acid long (83.3 kDa), sialylated, O-glycosylated structural protein (Huang et al., 2002). Along with RNA-dependent RNA polymerase and the viral RNA, NP forms a nucleoprotein complex (Kiley et al., 1980), helping in the transcription and replication of RNA. The NP has ten linear B-cell epitopes, ten antigenic sites, and five surface accessible epitopes, predicted as a conserved region among EBOV species. Among these regions, GEQYQQLR has been reported to have immunogenic and antigenic properties, making NP a suitable target for antigen detection and treatment of EVD (Ali et al., 2015).

1.9.2 Virion protein 35 (VP35)

VP35 consists of approximately 340 amino acids and its molecular weight is 37 kDa. Containing a C-terminal dsRNA binding domain and acting as a cofactor of RNA-dependent RNA polymerase (Becker et al., 1998), VP35 plays important roles in transcription, replication, and assembly of the virus. It also has been reported to mask viral RNA from host innate immune system (Bale et al., 2012).

1.9.3 Virion protein 30 (VP30)

VP30 consists of approximately 288 amino acids and its molecular weight is 33 kDa. It is a part of the viral RNA transcription complex, facilitating mRNA transcription. VP30 contains a zinc finger between amino acids 68 and 95. These zinc finger amino acids are necessary for transcription (Modrof et al., 2003). In inclusion bodies, VP30 becomes phosphorylated, and in the cytoplasm of infected cells, it takes a non-phosphorylated form. Phosphorylation of VP30 inhibits transcription of the Ebola virus gene (Hartlieb et al., 2003).

1.9.4 Viral polymerase (L protein)

The L protein consists of 2,212 amino acids and its molecular weight is 253 kDa. It acts as an RNA-dependent RNA polymerase and has three domains: the RNA binding element, a phosphodiester bond formation domain, and a purine ribonucleotide triphosphate-binding domain (Muhlberger et al., 1992). This is among the most conserved proteins in the order *Mononegavirales* and helps in transcription as well as replication.

1.10 Pathophysiology of Ebola Virus

The Ebola virus enters the host cell through the endocytic pathway (Geisbert et al., 2003) (Figure 1.2). EBOV infects endothelial cells, hepatic liver cells, dendritic cells, monocytes, and macrophages. Initially, the attachment of the virus VP35 to dendritic cells leads to the absence of an adaptive immune response (Jin et al., 2010). Then, the replication of EBOV in monocytes and dendritic cells causes cell damage, which leads to the release of cytokines and nitric oxide that contribute to fever and the body's inflammatory response (Baize et al., 2002; Sanchez, et al.,

2004). Lymphocytes are depleted during EBOV infection and do not allow the host body to mount an adaptive immune response.

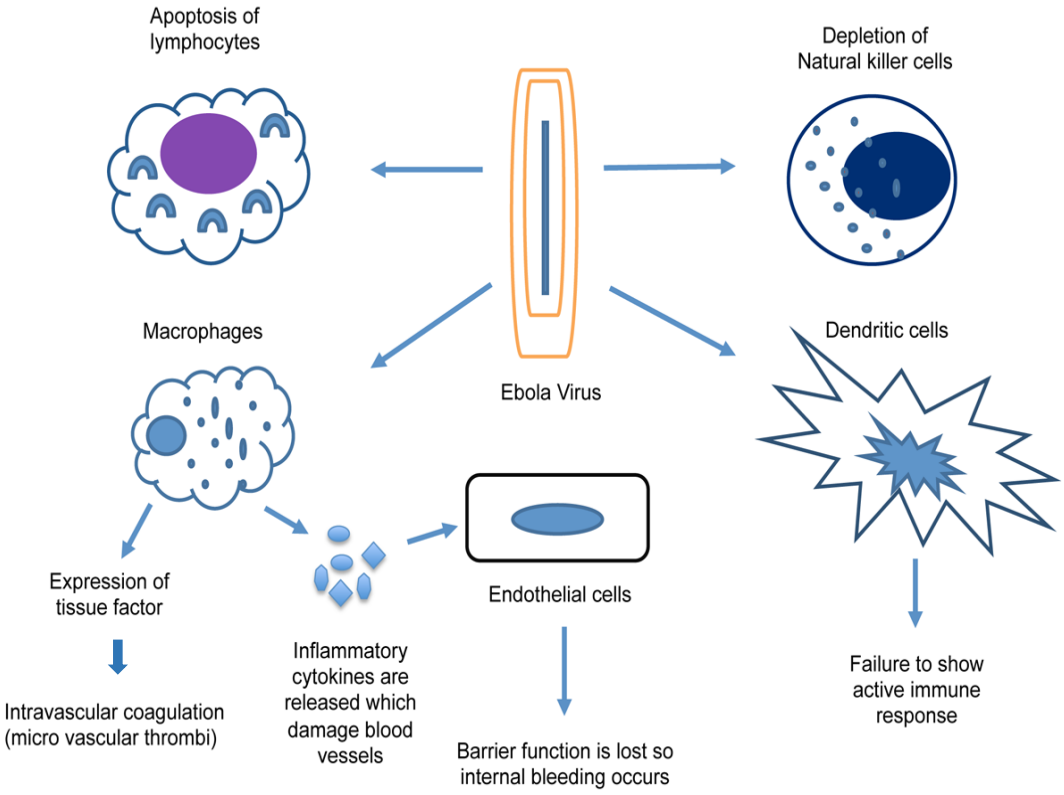


Figure 1.2: Pathophysiology of Ebola Infection: Immune cells affected by Ebola virus (lymphocytes, natural killer cells, dendritic cells and macrophages) upon entering into host cell.

The structural proteins also play a role in disease pathogenesis. The VP40 protein helps in the transport of the virus to the plasma membrane of the host cell. The plasma membrane has lipid rafts, which are the sites of budding and virus assembly (Bavari et al., 2002). The RNA-

dependent RNA polymerase helps form the transcription and replication complex, which transcribe viral genes and make multiple copies of the Ebola RNA genome, resulting in new virion assembly occurs after replication.

1.11 Clinical symptoms of Ebola infection

The first stage of clinical symptoms, known as early stage EVD (within 7 days), includes fever, diarrhea, appetite loss and vomiting. The next stage, known as late-stage EVD, is characterized by internal and external bleeding (e.g. blood in the stools and gums), vomiting, chest pain, muscle pain, low white blood cell and platelets count (Chertow et al., 2014). In severe cases, there is bleeding in the impaired liver and kidney functions with possibilities of multi-organ failure, ultimately resulting in death (Chertow et al., 2014; WHO, 2016a). During the outbreak it was observed that there were individuals who were in direct contact with EVD patients but never showed or developed any symptom. Therefore, it was concluded that EBOV infections can also be asymptomatic in some cases or only manifest at later stages of the disease. The reason behind this is that asymptomatic individuals have a strong inflammatory response characterized by higher concentrations of circulating cytokines and chemokines (Baxter et al., 2000; Leroy et al., 2000). Some symptoms of EVD, such as fever and vomiting, match symptoms of other tropical diseases, such as cholera but diarrhea remains for longer in EVD cases (7 days). Pregnant women, elderly and children (below 5 years of age) are the most vulnerable patient populations (Chertow et al., 2014). This overlapping of symptoms makes a differential diagnosis of EVD very challenging. However, early (within 3 days post-infection) detection of EVD is critical, as the majority of patients succumb within 7-10 days of the appearance of clinical symptoms (Towner et al., 2004).

1.12 Current Ebola diagnosis techniques

There are several methods currently available for the diagnosis of EBOV infection, but all have limitations, warranting more research in this area. Due to a relatively short incubation period of the infection and non-specific symptoms, a diagnosis should be fast and accurate. An ideal Ebola virus diagnostic method must satisfy the globally recommended features of an ideal diagnostic test, which follows the ASSURED principles. The acronym stands for A- affordable, S- specificity, S-sensitivity, U- User friendly, R- robust, E- equipment free and D- delivered to those in need (Mabey et al., 2004). Currently, EVD can be detected by EBOV isolation from patient specimens, viral antigens (NP, GP, and VP40), and viral nucleic acid.

1.13 Virus Isolation methods

Evaluating EBOV isolated from biopsied tissues or specimens collected from patients requires a highly sophisticated laboratory and technically trained staff. Ebola virus particles can be isolated from biopsied tissues (e.g. Liver) under an electron microscope (Ellis et al., 1979). Handling EBOV requires biosafety level 4 (BSL-4) experimental facilities, which are only available in a few countries (Table 1.3): Canada, Germany, Europe, the UK, and the USA (Duc, 2011). Even with all BSL-4 facilities and trained staff, screening analyses for virion are risky and time-consuming.

Table 1.3: Biosafety Level 4 Laboratories

Name of Institute	Place
National Institute of Virology	Pune, India
Centre for cellular and Molecular Biology	Hyderabad, India.
National Microbiology Laboratory	Manitoba, Canada
Wuhan Institute of Virology of the Chinese Academy of Sciences	Wuhan, China.
European Union Mobile Laboratory Consortium	Paris, France
National Institute for Medical Research	London, U.K
National Institute of Health	Maryland, U.S.A
National High Security Laboratory	Melbourne, Australia
Wuhan Institute of Virology of the Chinese Academy of Sciences	Hubei, China
Jean Merieux BSL-4 Laboratory	Rhone-Alpes, France
Robert Koch Institute	Berlin, Germany
Bernhard Nocht Institute for Tropical Medicine	Hamburg, Germany
Philipps University of Marburg	Marburg, Germany
National Center for Epidemiology	Budapest, Hungary
High Security Animal Disease Laboratory	Bhopal, India
National Institute for Infectious Disease	Tokyo, Japan
National Institute for Communicable Disease	Johannesburg, South Africa
Public Health Agency of Sweden	Solna, Sweden
Institute of Medical Virology	Zurich, Switzerland
Health Protection Agency's Centre for Infections	Colindale, United Kingdom
Centers for Disease Control and Prevention	Georgia, United States
National Institute of Allergy and Infectious Diseases	Montana, United States
Texas Biomedical Research Institute	Texas, United States
Kenya Medical Research Institute	Nairobi, Kenya
Uganda Virology Research Institute	Entebbe, Uganda
WHO Collaborating Centre for arboviruses and viral hemorrhagic fevers	Dakar, Senegal
International Centre for Medical Research in Franceville	Franceville, Gabon

1.14 Biosensors

Researchers have developed an EBOV-detecting biosensor that consists of arrays of plasmonic nanoholes, each having a diameter of about 200 to 350 nm. These arrays are metallic films and transmit light at particular wavelengths. When a solution containing live virus sample (from blood or serum) binds to the metallic surface of sensor, the effective refractive index changes and there is a shift in the resonance frequency of the transmitted light. The magnitude of the shift is detectable by the naked eye, indicating the presence and concentration of EBOV (Yanik et al., 2010) at a detection limit of 10^5 PFU/ml. Although the biosensor method has a high signal throughput, it needs a proper alignment of light coupling with the bio detection volume and it has the drawback of non-specific binding, resulting in background shifting at lower analyte concentration (Yanik et al., 2010). Therefore, these sensors are not suitable for point-of-care detection.

1.15 Single particle interferometric reflectance imaging sensor

Recently, a new detection assay, known as "Single Particle Interferometric Reflectance Imaging Sensor" was developed (Daaboul et al., 2014). In this method, the virus particles are captured by a silicon nanoparticle. A digital detection sensor enables counting of single virus particles. The imaging of the virus helps in identifying the virus based on its genome length. Discrimination by size of the imaged nanoparticles (i.e. virions) allows differentiation between modified viruses having different genome lengths and enables a reduction in the counting of nonspecifically bound particles to obtain the limit of detection of 5 x 10^3 PFU/ml in blood and serum, all within 2 hours (Daaboul et al., 2014).

1.16 Nucleic acid detection methods

1.16.1 RT-PCR

EBOV can be detected by the presence of viral nucleic acid in biopsy or blood specimens. This is commonly accomplished by reverse transcription-PCR (RT-PCR) assay, an extremely sensitive technique that unfortunately also yields false-negative and false-positive results (Drosten et al., 2002). Care must be exercised to transport the samples to prevent denaturation of viral RNA, and a high level of proficiency is required to perform RT-PCR. Sanchez et. al. developed RT-PCR, which has showed increased sensitivity and lowered the assay time period as compared to conventional RT-PCR (Sanchez et al., 1999). In this RT-PCR, the cDNA synthesis and PCR amplification occurs in a single tube. This single tube RT-PCR has been used to detect ZEBOV and REBOV in tissues and body fluids obtained from Ebola infected patients. In this study, the author's did the molecular characterization of EBOV and detected EVD by RT-PCR assay (Sanchez et al., 1999). In a study by Gibb et al., one tube RT-PCR was developed for EVD diagnosis; Specifically, GP was detected in genetic material; one primer set was used with two differentially labelled fluorescent probes to identify ZEBOV and SEBOV (Gibb et al., 2001). The study's authors used EBOGP-1D primer to calculate the limit of detection (LOD). LOD observed for ZEBOV RNA was 10 femtogram (fg) and for virus it was 8 PFU, whereas for SEBOV RNA it was 100 fg and for virus it was 3 PFU. This assay was unique in its ability to simultaneously detect and distinguish between ZEBOV and SEBOV (Gibb et al., 2001). Drosten and coworkers developed another one-step real-time reverse transcription (RT)-PCR (which consumes less time than traditional RT-PCR) (Drosten et al., 2002). This assay uses the Platinum Taq polymerase enzyme mixture and products are analysed in real time on a light cycler instrument using Sybr green dye intercalation (Drosten et al., 2002). These assays are validated analytically using *in vitro*-transcribed standard RNA as a positive control. The probability of detecting virus genome equivalents per ml of plasma was greater than 95%, and

the regression analysis ranged from 1,545 to 2,835 viral genome equivalents/ml of serum i.e. 8-16 RNA copies per assay (Drosten, et al., 2002). Therefore, the assay is suitable for the detection and quantification of viral RNA in serum samples of EVD patients

Another real-time RT-PCR-based method was developed to detect NP mRNA. This assay can detect as few as 10^2 copies per microliter and has no cross-reactivity with other filoviridae viruses (Y. Liu et al., 2012). Using Taqman polymerase RT-PCR with enp T-F, enp T-R, enp-F and enp-R primers, the NP gene of the ZEBOV virus was amplified. This RT-PCR method, compared with standard ones, could detect up to a range of 10^9 RNA copies per milliliter. This assay was assessed for rapid detection of ZEBOV as well as MARV. The quantification value of this viral RNA was 10^4 to 10^{10} copy numbers per reaction for ZEBOV and 10^3 to 10^9 for MARV. Due to the reproducibility of this technique, it was deemed reliable. The viral genomic number calculated by this method was 4 logs higher than a simple plaque titration method (Huang et al., 2012).

McKinney *et. al.* developed an improved procedure for extracting RNA (by proteinase K digestion for 24 hours) from formalin-fixed, paraffin-embedded (FFPE) tissues followed by its detection by TaqMan RT-PCR (McKinney et al., 2009). The tissues infected with BSL-4 biological agents had to be fixed in 10% neutral-buffered formalin for 30 days before proceeding to molecular pathology studies. Besides this lengthy timeframe, formalin fixation is thought to cause irreversible modifications to the RNA. When the formalin-fixed tissues were used in RT-PCR assays, the detection limit for viral genome was reduced by 2 $\log_{10}$ times compared to fresh tissues (McKinney et al., 2009).

One technique frequently used for the detection of EBOV RNA is the RealStar® Filovirus Screen RT-PCR Kit 1.0 from altona Diagnostics, targeting the L gene. During the 2014 Ebola outbreak, capillary blood samples were collected from finger pricks in order to detect EBOV using this kit. The total number of samples collected were 120 from 53 patients admitted to the Ebola Treatment Centre in Guéckédou, Guinea, and RealStar® RT-PCR was used to examine these specimens. The results obtained from capillary blood samples were correlated with the samples taken by venipuncture. As compared to venous blood samples, capillary blood samples showed a sensitivity of 86.8%. It was observed that if swabs of capillary blood samples are taken, then there were chances that RNA may get degraded, resulting in lower sensitivity (Strecker et al., 2015).

1.16.2 LAMP assay

Kurosaki *et. al* developed a reverse transcription–loop-mediated isothermal amplification (RT-LAMP) test to detect ZEBOV by targeting the viral genomic trailer region, since it is highly conserved among different strains of ZEBOV (Kurosaki et al., 2007). In this trailer region, six sites were selected for binding of primers. The primers used were FIP, BIP, F3 and B3. In 26 min, RT-LAMP detected 20 copies of ZEBOV RNA. The detection limit for real-time monitoring was 10^{-3} FFU (focus forming unit) of the cell culture propagated virus. This assay is sensitive and specific and can detect 6.2×10^{6} to 2.2×10^{2} RNA copies per reaction. The limitation of this test is that it is unable to diagnose the disease with oral samples, such as saliva (Kurosaki et al., 2007).

1.16.3 DNA fluorescence method

In 2005, Li *et al* introduced a gene-guided approach to detect Ebola DNA, using DNA-based fluorescence nano-barcodes (Li et al., 2005). These DNA-based, fluorescence-intensity-coded nano-barcodes, contains a built-in code and a probe for molecular recognition. This unique feature of nano-barcodes allows the detection of a pathogen's genetic material using fluorescence microscopy, flow cytometry, and dot blotting. The limit of detection was found to be $6.2x10^{-16}$ moles within 30 seconds for Ebola DNA (Li et al., 2005). This approach is rapid and sensitive, but not a good point-of-care diagnostic method, as it uses expensive equipment and infrastructure, and requires highly skilled personnel.

1.17 Immunoassays: Detecting viral antigens

During initial infection with EBOV, patients have high amounts of viral antigens circulating in their blood streams and excreted in urine, so antigen detection procedures have become a crucial focus for early diagnosis. Formenty *et al*. in recent study demonstrated the suitability of oral fluid samples for the detection of EVD, as the RT-PCR results were positive for the oral fluid specimens obtained from EVD patients (Formenty et al., 2006). Till now there is no study that has evaluated the use of oral fluid samples to diagnose EVD patients in Congo (Formenty et al., 2006). Viral antigen can be detected in body fluids beginning at 3-6 days post infection. Over the course of infection, antigen titer may either decrease to negligible (i.e. for most survivors) or increase until death. Several EBOV antigen detection kits based on different antigens of the Ebola virus have been developed with varying specificity and sensitivity.

VP40 is abundantly present in the virus-infected cells and hence is a good target for diagnosis of EVD (Elliott et al., 1985). In VP40-based sensitive sandwich ELISAs, the limit of detection of VP40 was 2 ng (Kallstrom et al., 2005). A recently developed ReEBOV rapid

diagnostic was used to screen 28 patients at the point-of-care level and analyzed 45 patient blood samples in the laboratory. ReEBOV is a simple dipstick test and doesn't require electricity. This assay showed that the patients who were positive in real time RT-PCR assay (RealStar Filovirus Screen RT-PCR kit 1·0; altona Diagnostics GmbH, Hamburg, Germany) were positive with this test as well. This ReEBOV diagnostic kit showed excellent specificity (92.2%) and sensitivity (100%) in both point-of-care and laboratory settings, when testing patients wtih high viral load. The assay showed 100% sensitivity and 92% specificity to detect EVD in people with a mild infection at the initial stage (Broadhurst et al., 2015).

NP is considered a good diagnostic target as it is also present in infected persons in early phase of infection. Niikura *et. al.* developed monoclonal antibodies (mAbs) that detected as little as 33 ng/well of recombinant EBOV NP in an antigen capture ELISA (Niikura et al., 2001). Ikegami *et. al.* developed mAbs (Res2-6C8 and Res2-1D8) against REBOV NP. These antibodies showed high specificity and sensitivity for REBOV in macaques and did not react with the NP antigen of ZEBOV and SEBOV (Ikegami et al., 2003). The antigen capture ELISA developed using Res2-6C8 and Res2-1D8 mAbs can be a promising tool for the diagnosis of REBOV infection, especially in monkey quarantine and field studies. REBOV has not caused infection in humans, therefore this testing system can be useful for detection of REBOV infection in non-human primates (Ikegami et al., 2003).

The polyclonal antibodies (pAbs) and mAbs produced against soluble EBOV GP protein have revealed information about the diversity of this envelope protein and helped develop reagents that can be used in faster and accurate diagnostics. The N-terminal, C-terminal, and mid-GP protein of SEBOV have been used to generate the pAbs. The C-terminus pAbs can

detect GP protein of ZEBOV as well as SEBOV, whereas antibodies produced from central region or N-terminus of GP can detect only SEBOV (Yu et al., 2006).Three regions of GP protein were used to raise mAbs 15H10, 17A3, and 6D11. The 15H10 recognizes human EBOV GPs of three species (SEBOV, ZEBOV, and TAFV) and non-human primates EBOV GPs of REBOV; 17A3 recognises human and non-human primate EBOV GPs of SEBOV and ZEBOV; and 6D11 recognizes EBOV GPs of SEBOV only in humans and non-human primates. These mAbs and pAbs have been used in ELISA, surface plasmon resonance, and quartz crystal microbalance immuno-sensor. In surface plasmon resonance, EBOV mAbs were immobilized on a sensor chip (CM5) by using standard amine coupling chemistry and EBOV GPs were injected to anti-EBOV mAb immobilized surface. The quartz crystal microbalance immuno-sensor comprises of gold electrode and antibody capture agent (Protein A, Protein G or Protein L). The steps in assembly of the sensor involved the cleaning of gold electrode with piranha (3:1 concentrated sulfuric acid; 30% hydrogen peroxide). The quartz crystal was placed in the flow cell, washed (50 mM phosphate buffer) and brought to resonant frequency of 5 MHz. Capture agent (concentration range of 2–5 μM) diluted in acetic acid (100 mM, pH 4.5) or in 50/50 acetic acid/phosphate buffer was introduced to the sensor until binding saturation was reached for the detection of GP protein (Yu, et al., 2006). Lucht *et. al.* Generated GP-specific mAbs 3B11 and 1G12. 3B11 was specific to ZEBOV whereas 1G12 was specific to TAFV (Lucht, et al., 2004). These mAbs were used in a sandwich ELISA, in which MAb 3B11 was used as capture and 1G12 as the detection antibody at an optimized concentration of 5 μg/ml and 20 μg/ml, respectively. The limit of detection observed for GP viral antigen was 10^3 PFU/ml in serum (Lucht et al., 2004).

MIT Researchers have developed a “Lab on a Chip” that can detect EVD in 10 minutes (Yen et al., 2015). This silver nanoparticle-coated paper-based strip is a type of lateral flow immunochromatographic assay. The patient’s blood serum flows along the paper strip where immobilized anti-GP antibodies bind to the GP viral protein. A positive test result (the formation of Ebola antigen-Ab complexes) is visualized as a red band on the strip. The limit of detection for ZEBOV GP antigen for this “Lab on a Chip” procedure is 150 ng/ml. The advantages of this testing procedure is that it is extremely rapid detection method (10 min) with no requirement for electricity. It can detect other viruses in the blood sample simultaneously. The limitation of this multiplex approach is that can show false positive, non-specific binding, and crossover results (Yen et al., 2015) Hence, it is not a reliable diagnostic technique.

Lucht et. al. has also developed an immunofiltration-based antigen detection assay that can detect VP40 protein of EBOV in urine in about 30 minutes. In this assay one mAb binds to the matrix of column to immobilize EBOV VP40 and the second biotin labelled mAb used for detection of the bound viral antigen. In spiked urine samples, the VP40 antigen from ZEBOV was detected at a limit of 1.25×10^4 PFU/ml. This new immunofiltration assay could provide a novel platform for future studies of EVD outbreaks. Although it is less sensitive than RT-PCR analysis, but showed comparable sensitivity to that of the widely used antigen-detection ELISA (Lucht et al., 2007).

1.17.1 Immunochromatographic strip

An immunochromatographic “Nanozyme” strip, embedded with ferric oxide magnetic nanoparticles, can detect EBOV GP with as low as 1 ng/ml of antigen (Duan et al., 2015). The diagnosis of EBOV infection can be made in just 30 min using this strip. This Nanozyme-strip

have intrinsic peroxidase-like activity that catalyzes the peroxidase substrate and produce a color reaction. In this study, metal nanoparticle were used as a nanozyme probe. After labelling with anti-EBOV antibody, this probe was able to identify, separate, and visualize EBOV on the nanozyme strip. low cost, and reusable are two advantages that makes this strip a suitable candidate for biomedical detection. Unfortunately, this strip has not been tested for ZEBOV, the species that is the major cause of outbreaks and leads to a significant number of deaths. This strip is less sensitive than RT-PCR that means it cannot diagnose EVD immediately after the appearance of symptoms and 15% false positive rate also limits its application (Duan et al., 2015).

1.17.2 Microfluidic chip

In 2014, Miethe *et.al.* devised a rapid detection test for VP40 antigen. They performed the assay in a microfluidic chip for immune filtration analysis (Miethe et al., 2015). The device has anti VP40-polyHRP40 stored as dry pellet in the reservoir. The sample injection port goes to a series of four immunofiltration frits. The liquid enters from the bottom and flows through the frits. This liquid leaves through the top of frits and is brought back through microfluidic to the bottom of the chip so as to enter the next frit. This arrangement allows the identification of VP40 with a positive and a negative control. The fiber optic setup was used for the photometric read-out in transmission mode. The polyethylene filter exhibits a pronounced light scattering and the optical densities were determined following Beer Lambert law. All these test components detect the antigen. The detection limit for recombinant VP40 was 8 ng/ml. In serum and blood samples containing viral culture material the limit of detection was found to be 2.2×10^2 PFU/ml. The

time taken by the microfluidic chip to detect the antigen was 15 minutes (Miethe et al., 2015). The drawback is that it needs skilled personnel to read the results.

1.17.3 MARSA (monoclonal affinity reagent sandwich assay)

Monoclonal affinity reagent sandwich assay (MARSA) uses one recombinant antibody clone as both the capture as well as the tracer to detect the EBOV NP. The Ab consists of a single domain targeting the conserved domains of NP protein. The polyvalent nature of NP enables the formation of a sensitive sandwich immunoassay (Sherwood et al., 2013).

Changula *et al.* developed mAbs to the ZEBOV NP and identified NP's conserved and antigenic regions (Changula et al., 2013). These mAbs were then divided into seven groups according to the specificity and cross-reactivity profiles to other species in the EBOV. The binding sites of the mAbs were mapped to seven antigenic regions in the C-terminal half of the NP including two highly conserved regions among all species of EBOV by using synthetic peptides corresponding to the EBOV NP sequence. These techniques provide information about antigenic sites that can help in forming other monoclonal affinity reagent driven antigen sandwich assays for the *Ebolavirus* genus and in the development of viral antigen detection assays, such as an immunochromatography-based rapid diagnosis (Changula et al., 2013).

1.18 Comparison diagnosis of EVD by RT-PCR and antigen capture ELISA diagnostics.

The EVD outbreak occurred in the Gulu district of northern Uganda, with secondary transmission to other districts. National Institute for Virology in Johannesburg, South Africa did the pre-liminary diagnosis of SEBOV and a temporary diagnostic laboratory was established in the Gulu district. The laboratory used a combination of antigen capture and reverse transcription-

PCR (RT-PCR) to detect SEBOV infection in suspected patients. These assays (RT-PCR and antigen-capture) proved very efficient for diagnosing EVD in patient serum, plasma, and whole blood. In total 49 serum samples that showed positive results by antigen capture ELISA, only 30 samples tested positive by RT-PCR. All positive samples tested by RT-PCR also showed positive results tested by antigen-capture ELISA. Therefore, this study concluded that RT-PCR is a useful assay, but should always be used with a reliable test, such as antigen-capture ELISA (Towner et al., 2004).

1.19 Immunoassays: Detecting viral antibodies

The presence of serum antibodies against a pathogen is considered as the primary assay used for diagnosis. However, in EVD, specific antibodies are generally only detectable in the late phase of infection or sometimes may be undetectable. For example, IgG and IgM antibodies against EBOV are observed in individuals after 8-10 days post-infection or in individuals who have recovered from the virus infection (Ksiazek et al., 1999a). IgM antibodies are identified in the first week after onset of symptoms, reaching peak levels in the third week (18 days) after symptom onset and disappearing after six weeks of infection. IgM screening assays cannot detect IgM antibodies after 60 days of virus infection. Furthermore, IgM antibodies are found in only half of the patients at the time of their death (Ksiazek et al., 1999a).

IgG antibodies appear between 8-10 days after the appearance of IgM antibody in Ebola infection and can be seen for up to ten years (Ksiazek et al.,1999). In similar cases, however, IgG antibodies were not detected regardless of infection phase. The sample used for Ab detection plays an important role. Formenty *et. al.* collected samples from patients between 10-24 days post-EBOV infection and reported positive results for anti-Ebola IgG antibodies from serum

samples but negative for oral samples (Formenty et al., 2006). In another study, recombinant-expressed NP and GP proteins positively reacted in ELISA with IgG antibodies present in human convalescent sera only (Prehaud et al., 1998). Ikegami et. al. used an immunofluorescence assay (IFA) to detect serum antibodies with recombinant NP (Ikegami et al., 2002). The results of their studies showed that the assay was more sensitive for the detection of IgG antibodies in serum samples of monkeys infected with REBOV than ZEBOV. This IFA method could be useful in seroepidemiological studies of REBOV infected monkeys, but not for human diagnostics. Collectively, these studies demonstrate that an IgG-based assay is not a good option for early stage diagnosis of EVD.

On a positive note, a baculovirus-expressed NP antigen used to detect IgG antibodies in an ELISA assay showed positive results for IgG detection in sera of patients, with 93% sensitivity (Saijo et al., 2001). The authors of this study identified that the C-terminal region of NP was highly antigenic, which suggests that these antigenic regions could be used for detection of EVD.

In another study, histidine-tagged recombinant GP of five different EBOV species and one MARV species were used as antigens for the detection of filovirus species-specific antibodies (Nakayama et al., 2010). The authors developed an IgG antibody detection ELISA by collecting antisera from immunized mice, EBOV infected humans, and non-human primates. The anti-EBOV-GP MAb ZGP42/3.7 and anti-MARV-GP MAb AGP127-8 were used to determine the sensitivity of ELISA. The researchers observed that ZGP42/3.7 reacted with the recombinant GP of all the EBOV species, whereas AGP127-8 MAb reacted only with recombinant GP of MARV. The limit of detection for specific antibodies (IgG) observed using GP antigen in ELISA

was approximately 0.1 μg/ml. This study helped in distinguishing the serotypes of filovirus species, as GP is species specific protein due to the greater genetic variability with this protein (Nakayama et al., 2010).

In Yambio Payam, Sudan, a study of 36 patients with probable EVD yielded 13 EVD-confirmed patients, with 8 having both IgG and IgM against Ebola virus and 4 having IgM antibodies only (Onyango et al., 2007). These 12 patients had complete disease remission. Another patient whose blood sample was collected after two days of illness which was confirmed by RT-PCR did not show antibodies against the virus. The EVD patient died after 10 days of illness. The author's observed that blood samples collected after two days of illness showed a positive result for EBOV using RT-PCR but not for IgM/IgG antibody ELISA. The study concluded that antibodies were not present in the initial phase, whereas they were present after patient recovery. Once again, this study shows that the diagnosis of EVD with antibody-based ELISA is not a good option for early screening (Onyango et al., 2007).

1.19.1 Synthetic gene network

Pardee *et al.* developed a paper-based synthetic gene network to detect and screen for EBOV using engineered gene circuits and visual transduction (colorimetric outputs of circuits present in gene network for detection by eye (Pardee et al., 2014). The synthetic gene network is freeze-dried onto paper by freeze drying. These synthetic gene circuits are composed of a sensor element and a transducer which regulates a measureable output. These engineered logic has sequence-specific sensing of nucleic acids and small-molecule recognition. The glucose sensors as well as strain-specific sensors help in in-vitro diagnosis of virus. This approach can detect anti-Ebola antibodies in a cost-effective, rapid, and sensitive manner. The limitation of this

approach is that it cannot be used for primary disease diagnosis. Earlier cell-based synthetic networks have also used for detection of EVD. However, their application is restricted to the laboratory due to the biosafety concerns (Pardee et al., 2014).

1.20 Limitations of current diagnostics

We have discussed many different Ebola diagnostic tests available, based on various technologies and viral components. All of these screening procedures have limitations, some of which were discussed (Table 1.4).

Virus isolation and identification by electron microscope is 100% accurate, but requirea an expensive, well-equipped BSL-4 laboratory and presents a high risk to handlers of patient specimens containing live virus. Despite being a useful tool for diagnosis of EBOV, RT-PCR based methods are time-consuming (greater than 48 hour turn-around) and can yield either false positive or negative results (Towner et al., 2004). Additionally, PCR-based techniques might not detect newly emerged or vastly divergent strains of an infectious virus. The example of this is the current description of a new strain of Ebola that was not recognised in initial PCR-based diagnostics (Towner et al., 2008).

Most antigen-based ELISAs detect Ebola viral antigens such as GP, NP, or VP40 with such low sensitivity and specificity that the diagnostic test results need to be correlated with other epidemiological and clinical parameters of Ebola infection before concluding Ebola infection in tested patients. For tissue samples, the sensitivity and specificity of antigen-based ELISA were promising. Sensitivity tested in animals infected with the EBOV showed 98% in the liver and 93% in the spleen (Ksiazek et al., 1992). However, it takes five hours to show the

results. Developed by Corgenix, USA and now available on the market, ReEbOV antigen rapid test kits do not require a power source and are easy to perform, making this an ideal point-of-care test. Although less accurate, they can detect 92% of patients infected with EVD.

Immunological assays can detect Ebola antigen-specific IgM and IgG antibodies, but these methods are not useful at the time of initial infection, since these antibodies surface 10-20 days post-infection and remain in the patient's bloodstream for up to 2 years after being infected, due to the development of a specific immune response (Ksiazek et al., 1999a).

Table 1.4 - Advantages and Disadvantages of Diagnostics available.

Diagnostic Tests	Target	Advantages	Limitation
Polymerase Chain Reaction (PCR)	Nucleic acid of virus	• Rapid and sensitive • Quantification of RNA molecule (10^4 to 10^{10} per reaction)	Require special equipment (Huang et al.2012)
DNA-based fluorescence nanobarcodes methodology	Nucleic acid of virus	• Multiplexed approach for detection. • Detection limit is 620 attomole.	Require skilled personnel.
ELISA (antigen detection)	Viral antigen	• Rapid and sensitive. • 30 ng/well of recombinant NP antigen could be detected (Niikura, et al.2001)	Sometimes provide false results.
Immunohistochemistry	Viral antigen	• It's a qualitative imaging method.	Time required.
Fluorescence assay	Viral antigen	• Rapid technique. • Qualitative analysis.	Interpretation of results requires skilled personnel.
ELISA (antibody based)	Virus specific antibodies	• Sensitive and specific technique. • Can detect upto 20 ng of EBOV Zaire GP protein.	Time required. Primary disease diagnosis is not possible.

Indirect Immunofluorescence assay	Virus specific antibodies.	• Easy to perform • Qualitative imaging method	Non-specific technique. Interpretation of results is difficult
Immuno-blot assay	Virus specific antibodies	• Specific technique. • Easy to perform	Interpretation of results is difficult sometimes.
Biosensors	Virus detection	• Based on antibody based specific detection. • Rapid and sensitive • Limit of detection is 0.005 PFU/ml	Based on antibody detection results, so cannot be used for primary disease diagnosis. As antibodies are generated in patient body on 7^{th} day and remains up to 3 months.
Electron Microscopy	Viral particles detection	• Immunostaining method used. Morphology can be seen. • Qualitative analysis.	Insensitive technique. Needs special equipment.
Immunochromatographic strip	Glycoprotein antigen	• Sensitive method. • Limit of detection 1 ng/ml of GP antigen.	It is not tested for ZEBOV.
Next generation sequencing	Viral genetic material	• Sequence large amount of genetic material. • Remarkable depth of covering sequence of genetic material	Do not identify sequence of interest and sequence all the host genetic material present.
LAMP assay	Virus	• Limit of detection was 10^{-3} FFU.	Unable to detect virus in oral samples.
Immunofluorescence assay	NP antigen	• Sensitive for the serum samples infected with REBOV and ZEBOV.	Detect antibodies to EBOV so cannot be used for initial screening of EVD

1.21 New tools for Ebola diagnosis

1.21.1 Single domain antibodies

Single domain antibodies (sdAbs) are the small (14 kDa) fragments, which are thermostable in nature and have a good shelf life (Pain et al., 2015). They have the capability to refold and bind to antigen once denatured and can be produced with different specificities. These features make sdAbs ideal a for Ebola diagnostic development.In contrast, mAb production is time-

consuming, and under extreme environmental/storage conditions (e.g. temperatures >65 C), the Ab heavy and light chains unfold and aggregate irreversibly (Pain et al., 2015).

Shonda *et. al.* developed a single molecule array (Simoa) in which sdAbs are chemically attached to a paramagnetic surface for the detection of ricin (Gaylord et al., 2015), a potent ribosome-inactivating protein that can inactivate 1500 ribosomes/min and cause cellular necrosis, followed by death of the person exposed to this toxin. Since there is no treatment for ricin poisoning, a very sensitive detection assay is required. The Simoa method detected 1pg/ml of ricin in a buffer, urine, and serum samples. This approach showed its applicability for detecting ricin in environmental, food, and clinical specimens (Gaylord et al., 2015). sdAbs have been used to detect the presence of amyloid bodies in the brains of Alzheimer's patients (Pain et al., 2015). These FITC-labelled sdAbs bind to intraneuronal amyloid body peptides and stain extracellular plaques. These results show that sdAbs are very useful in the diagnosis of diseases and should be considered for developing Ebola-specific assays (Pain et al., 2015).

sdAbs have various advantages over traditional technologies such as Immunotherapy (Siontorou, 2013). They have high binding affinity and specificity to the target of interest. These can easily tagged with fluorescent proteins to produce color for detection of a particular moiety (Siontorou, 2013). The shelf life of sdAbs maintains their integrity of antigen binding even after storage for a few months at 4^0C and for several months at -20^{o}C. sdAbs can be produced economically in microbial expression systems within a short period of time and they are less immunogenic in nature and have an efficient refolding capacity. All these features make sdAbs good candidates for future Ebola diagnostic assays (Siontorou, 2013).

1.21.2 IgY-based ELISA

Another immune-diagnostic system, which is simple, reliable, sensitive, rapid, and robust and has the potential to detect disease at an earlier stage, relies on polyclonal, chicken-produced IgY. In chickens, the IgG from serum is transferred to eggs in the form IgY. When hens are immunized, high titers can be maintained up to 720 days (Sunwoo et al., 1996). IgY antibodies can be obtained noninvasively from eggs in large quantities within a short period of time. Studies show that a laying hen can produce antibodies 18 times higher than the production of antibodies in other lab animals (e.g. rabbit and mouse) (Schade et al., 1996). Specifically, a laying hen can produce 2 g of antibody every month (Akita et al., 1992). These antibodies are stable under different conditions of pH, pressure, acidity, and alkalinity and also in the presence of proteolytic enzymes, trypsin and chymotrypsin (Shimizu et al., 1992). Additionally, IgY does not cross react with mammalian immune components (WBC, lymphocytes, natural killer cells) (Schade et al., 1996).

IgY is already being used for diagnosis of some infectious diseases, such as Dengue, hepatitis, and SARS, binding viral antigen with high affinity and no cross-reactivity with other viral proteins (Palaniyappan et al., 2012). IgY antibodies, obtained from NS1 protein-(of Dengue type 2 virus)-immunised chickens, were used with a potentiometric immunosensor containing a gold electrode immobilised with anti-NS1 IgY antibodies. This IgY-immunosensor provided an efficient measurement of NS1 protein (Alessandro et al., 2009). IgY also proved to be useful in detecting hepatitis A virus (Silva et al., 2012). An immuno-enzymatic assay using IgY conjugated with horseradish peroxidase (HRP) as detector antibody was used to detect anti-hepatitis antibody levels. This test showed a sensitivity of 95% and specificity of 98.8%, which shows that competitive immunoassay using IgY can substitute for other tests like ELISA that use mammalian antibodies (Silva et al., 2012).

An immunomagnetic bead ELISA has been developed, where IgY Abs coated with magnetic beads are used as capture antibodies to detect clonorchiasis caused by the liver fluke, Clonorchis sinensis (Nie et al., 2014). HRP-IgG is used as a detector antibody. The results of this IgY immunoassay show a sensitivity of 93.3% when there is a heavy parasite load and 75% when there is light infection. In this assay, cross-reactivity is observed with other parasitic worms: 6.7% with Schistosoma japonicum and 10% with the nematode causing paragonimiasis. The findings suggest that IgY-IMB-ELISA is relatively sensitive and specific for the detection of clonorchiasis (Nie et al., 2014). A sandwich ELISA has been developed for the detection of excretory-secretory antigens of *Trichinella spiralis,* which causes trichinellosis (Liu et al., 2013). IgY is used as capture antibody and IgM mAb against excretory-secretory antigen is used as detector antibody and HRP-conjugated sheep anti-mouse IgM is used as secondary antibody; the limit of detection for this assay is 1 ng/ml. The sandwich ELISA was sensitive and proved to be successful in early detection of disease (Liu et al., 2013).

Sandwich ELISA using IgY has also been used to detect prostate–specific antigen, which is a marker for prostate cancer, with a detection limit of 50 pg/ml, and the reactivity profile of IgY was comparable to that of mouse monoclonal IgG antibodies. This study showed that IgY can be used in the diagnosis of prostate cancer and can replace mammalian antibodies (IgG) that are difficult to produce and costly (Slowik et al., 2014).

1.21.3 Bispecific antibodies

Bispecific antibodies (BsAb) are a second generation mAb, characterized by dual functions: a BsAb is a single molecule with two different binding sites specific for two different moieties (Suresh et al., 1986). One paratope is specific for the disease-related antigen or protein, whereas

the other paratope is specific for the assay detection enzyme. In ELISA, the use of BsAb provides accurate and reproducible results, with a reduced probability of false positive results (Milstein et al., 1983). Since BsAb has an intrinsic binding site for the detection enzyme,it avoids the step of chemical conjugation, which can be responsible for the loss of activity of the antibody or the protein, aggregation, and formation of unwanted complexes (Kreutz et al., 1997). Therefore, the use of BsAb is advantageous as they are inexpensive and can augment the level of ELISA detection by their specificity and sensitivity. With BsAbs, viral samples can be analysed faster and less expensively than traditional ELISA protocols, as there is only one step of adding reagents rather than adding several reagents (Byrne et al., 2013). Bispecific antibodies have better binding affinity to their target antigen compared to mAbs, and can be better candidates for molecular targeting and imaging, as they have high specificity (Luo et al., 2014).

We know that it is imperative to screen for EVD accurately at early stages of infection, as with time, it becomes severe and incurable, leading to higher mortality rates. Therefore, BsAbs can play a significant role in diagnostic assay development to help to easily and quickly identify the disease at an early stage.

1.22 Rationale

EVD is endemic in tropical regions, with occasional deadly outbreaks worldwide. Due to the increasing incidence, lack of treatment, and high mortality of Ebola virus infection, there is an urgent need to develop rapid, simple, accurate, inexpensive, and effective diagnosis methods. The disease has to be tracked and monitored at an early stage so that it could be prevented from spreading. There are many diagnostic tests available such as RT-PCR, antigen-based test kits, and ELISA kits but none of them meet all relevant criteria to be an ideal point-of-care diagnostic

test. Therefore, diagnostic methods based on emerging technologies, such as a sandwich assay based on mAb, BsAb and/or IgY could be developed to detect antigen levels seen in patient body fluid samples for screening applications in hospital settings. In addition to ELISA assays, other easily administered tests such as immunoswabs, Nanochip- or other immunohistochemistry-based tests should be developed to detect EVD in the field.

1.23 Hypothesis

We hypothesize that Double Antibody Sandwich Immunoassay (DAS-ELISA) based on IgY, mAb, and/or bsAb can detect nanogram levels of antigen (VP40) in PBS and spiked human serum samples. The assay can be used at clinical settings to monitor suspected individuals at the early stage of 7 days of infection.

1.24 Objectives

The objectives of this study are (1) To produce VP40 Protein by the recombinant technology, (2) To produce three different types of antibodies: IgY, mAb, and bsAb, (3) to optimize three different formats of DAS-ELISA, and (4) to determine LOD of VP40 in each DAS-ELISA format in PBS and human serum samples

1.25 Specific objectives

1.25.1 Production of VP40 Protein by the recombinant technology.

- Optimization of nucleotide sequence.
- Expression of VP40 protein in E.coli
- Purification of VP40 protein by immobilised metal ion chromatography.
- Yield, SDS-PAGE and Western blot analysis of VP40 protein.

1.25.2 Production of three different types of antibodies:

IgY

- Chicken Immunisation with recombinant VP40 protein

• Isolation and purification of VP40 specific IgY from egg yolk

• Titer of IgY (Indirect ELISA) at different weeks

• Western Blot and cross reactivity analysis of IgY

• Calculation of Yield of IgY

Monoclonal antibodies (mAbs)

• Cell culture of anti-VP40 hybridoma cells and collection of supernatant

• Purification of VP40/16-2 clone by Protein-G affinity chromatography

• SDS-PAGE and western blot analysis of mAbs

• Yield of mAb obtained

Bispecific antibodies (BsAb)

• Bispecific fusion of two different hybridoma cells

• FACS sorting of double positive cells

• Re-cloning of bispecific cells

• Purification of bispecific cells by protein G column and then m-APBA column

• Calculation of Yield of bsAb

1.25.3 Optimization of three different formats of DAS-ELISA

DAS-ELISA-mAb-IgY

- Optimization of capture antibody (mAb)
- Optimization of detecting antibody (IgY)
- Optimization of secondary antibody (anti-chicken IgY)

DAS-ELISA-mAb-bmAb

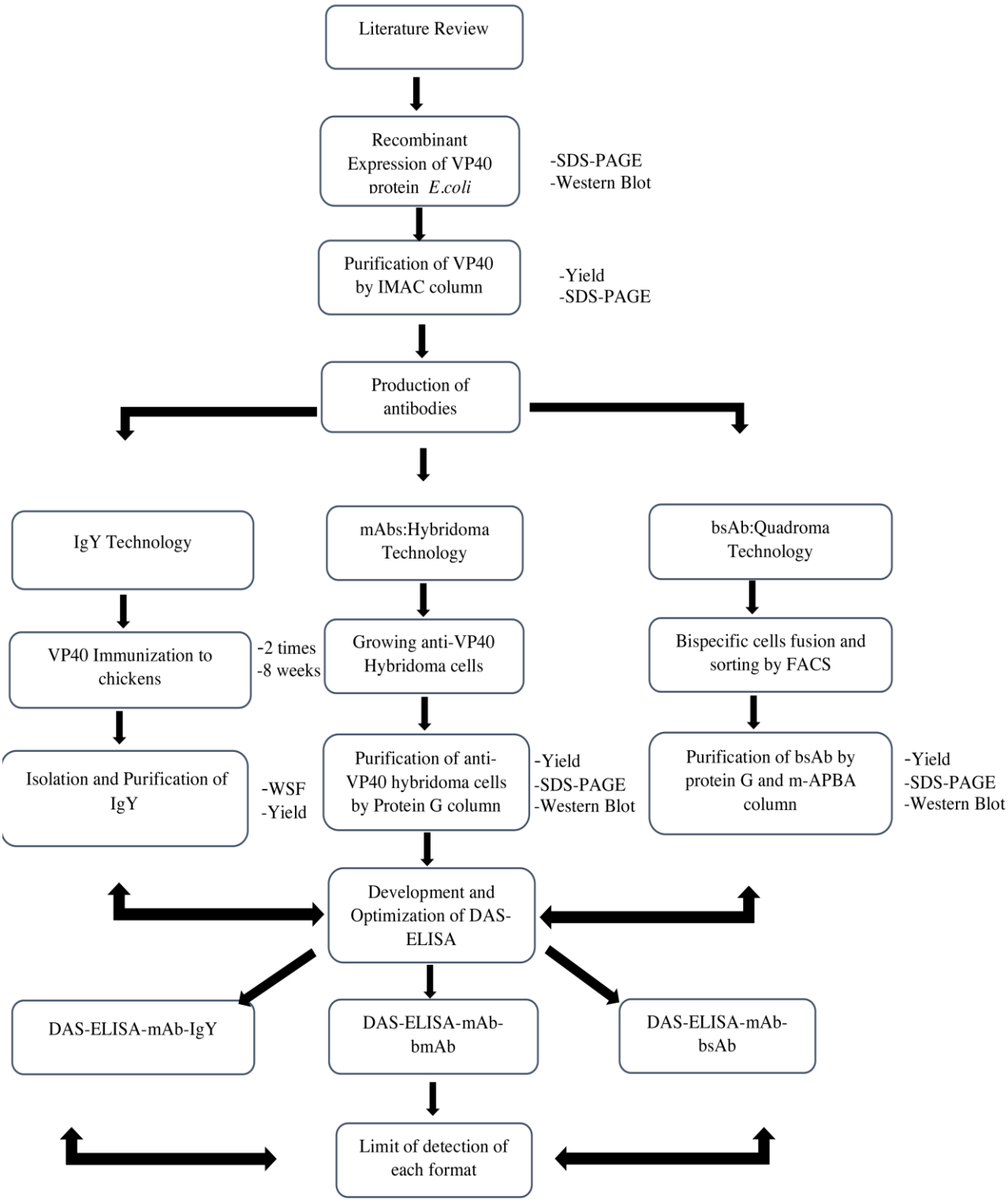
- Optimization of capture antibody (mAb)
- Optimization of detecting antibody (biotin labelled mAb - bmAb)
- Optimization of conjugate (Steptavidin-HRP)

DAS-ELISA-mAb-bsAb

- Optimization of capture antibody (mAb)
- Optimization of detecting antibody (bsAb)

1.25.4 Determination of limit of detection (LOD) of VP40 antigen in each of three DAS-ELISA format in PBS and human serum samples

1.26 Study plan



Chapter 2: Development of sensitive double antibody sandwich-ELISA to detect Ebola VP40 antigen using IgG and IgY

2.1 Introduction

Ebola Virus Disease (EVD) is a highly contagious and rapidly spreading disease that has caused repeated epidemics and high mortalities over the last 40 years (Dhama et al., 2015). The Ebola virus (EBOV) was first recognized in 1976 after its outbreaks in African countries such as Sudan and the Democratic Republic of Congo (WHO, 1976). Since then, EBOV infection has been reported in other countries, such as England in 1976 (Emond et al., 1977), Switzerland in 1994 (Van, 2000), South Africa in 1996 (CDC, 2016a), and the United States in 2015 (CDC, 2016b). The latest epidemic of 2014 led to about 12,000 deaths, and as of April 2016, 28,652 cases and 11,325 fatalities have been reported worldwide (CDC, 2016c).

Although the World Health Organization monitors EVD in endemic areas, this task proves difficult due to inadequate hygiene conditions in endemic areas, noncompliance issues, and movement of infected people. In addition, the symptoms of EVD include vomiting, bloody stool, fever and appetite loss (Bausch et al., 2006), which may be confused with other diseases such as malaria, typhoid, and cholera. Thus, any measures (e.g. quarantining infected individuals) taken to prevent outbreaks of EBOV beyond the susceptible regions have achieved limited success.

It is very challenging to detect EVD, due to its short incubation period and the severity and nature of the viral infection process. There are five EBOV species found globally: *Ebola Zaire* (ZEBOV), *Ebola Sudan* (SEBOV), *Ebola Bundibugyo* (BEBOV), Ebola *Cote d'lvore* (CIEBOV), and *Ebola Reston* (REBOV) (Wozniak et al., 2015). EBOV is animal-borne but needs a host cell to survive and augment virulence. Bats, especially the *Epomops franqueti, Myonycteris torquata,* and *Hypsignathus monstrous* species of fruit bats, are thought to carry and spread EBOV (Leroy et al., 2005; Pourrut et al., 2007). EBOV is a filamentous and negative-stranded RNA virus encoding its structural proteins, including its envelope glycoprotein (GP),

viral matrix proteins (VP40 and VP24), and nucleocapsid complex proteins, nucleoprotein (NP), RNA-dependent RNA-polymerase (L), polymerase cofactor (VP35) and transcription activator (VP30) (Elliott et al., 1985). Nearly all of the EBOV-encoded proteins have been targeted for viral detection purposes.

Suspected cases of EVD are currently diagnosed by isolating virus and using detection techniques such as: RNA detection by PCR, Ebola-specific IgM/IgG antibody detection by enzyme-linked immunosorbent assay (ELISA), or Ebola antigen detection by ELISA of patients' sera (Towner et al., 2004). Unfortunately, these techniques bear the limitations of being time-intensive and requiring specialized lab equipment. EBOV isolation for virus confirmation is costly and requires biosafety level 4 laboratories. PCR-based detection systems are the most sensitive techniques but are also costly, require special equipment, and are time-intensive. These assays detect EBOV nucleic acids and the test requires collection of blood via venipuncture, safe transport of blood from the point of collection to laboratory, nucleic acid purification, and EBOV nucleic acid testing. These steps delay molecular testing and require a high level of laboratory biosafety, technically-trained staff, and sophisticated testing equipment (Schieffelin et al., 2014). ELISA, using recombinant NP protein for the detection of EBOV-specific antibodies, has a reasonable assay detection limit of 0.1 μg/ml (Huang et al., 2014), but this is only attained 4 to 6 days (anti-EBOV IgM detection) and 7 to 16 days (specific IgG detection) after the onset of visible symptoms (Ksiazek et al., 1999a). Therefore, IgG- and IgM-based detection systems are not suitable for effective primary disease diagnosis and management. Various EBOV antigen detection ELISA assays (Schieffelin et al., 2016) are available, including one that detects NP antigen with a detection limit of 0.3 μg/ml (Niikura et al., 2001). In 2015, using the concept of antigen detection, MIT researchers developed a "lab on a chip". This paper-based strip is a type

of lateral flow immunochromatographic assay. The limit of detection for ZEBOV GP antigen for this "lab on chip" procedure was still only 0.15 µg/ml (Yen et al., 2015). Despite the multitude of existing assays, sensitivity remains too low, timeliness insufficient, and implementation costs are too high. Thus, there is a dire need to improve the sensitivity and affordability of the detection assay to detect EVD in suspected individuals in clinical settings.

In our study, we developed a double antibody sandwich-ELISA (DAS-ELISA) using a mouse monoclonal Ab (mAb) as a capture antibody (cAb) and either the first-of-its-kind anti-VP40 chicken IgY or a biotinylated mAb (bmAb) as a detecting antibody (dAb) to detect VP40 antigen in phosphate buffer saline and spiked human serum samples. We targeted the biomarker VP40 as the detection antigen. VP40 is highly circulated during the early stages of infection (2-7 days) and has been reported to be detectable in EBOV infected patient body fluids, such as blood, urine, and saliva (Feldmann et al., 2011). We believe the immediate detection after infection onset of the VP40 protein using our DAS-ELISA system may prove to be the best way to diagnose the Ebola virus disease.

2.2 Materials and methods

2.2.1 Materials and reagents

Cell culture media RPMI 1640, fetal bovine serum (FBS) and penicillin-streptomycin-glutamine (PSG) were purchased from Gibco (New York, USA); goat anti-mouse IgG conjugated to horseradish peroxidase (GAM-HRP) was purchased from Sigma-Aldrich (Darmstadt, Germany). Acrylamide:bisacrylamide (40%), pre-stained low range protein molecular weight markers and protein assay reagents were purchased from Bio-Rad Laboratories Ltd. (Ontario, Canada). For western blots, hybond-ECL nitrocellulose membranes were obtained from Amersham Biosciences (Freiburg, Germany) and reagents were purchased from Amersham

Pharmacia Biotech (Quebec, Canada). Bovine serum albumin, rabbit anti-chicken IgY-horseradish peroxidase (HRP), 2-2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), and other general reagents were purchased from Sigma (St. Louis, MO). Microtiter 96-well plates were purchased from Costar Inc. (Cambridge, USA) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was purchased from KPL Inc. (Frederick, USA). The ELISA Vmax kinetic microplate reader was obtained from Molecular Devices Corp. (Sunnyvale, USA).

2.2.2 Production of recombinant VP40 protein

2.2.2.1 Protein expression

The *Escherichia coli* system was exploited to express full-length EBOV-VP40 (Das, 2009). The EBOV-VP40 nucleotide sequence was optimized to avoid codon bias (Biobasic Inc, Ontario, Canada). The optimized VP40 gene was amplified by PCR and digested with NdeI and EcoRI followed by gel purification and ligation. The ligated gene was transformed in *E. coli* Rosetta (DE3) cultures (Das, 2009). The gene was cloned in correct reading frame into the pET22b vector containing a His6 tag at the C-terminal end for higher expression within inclusion bodies of *E.coli*. The identification of the expressed VP40 gene was confirmed by nucleotide sequence analysis, and restriction digestion mapping and SDS-PAGE (Bio-Rad, USA). A single bacterial colony was inoculated in 10 ml 2x YT medium (16 g/l Tryptone,10 g/l Yeast Extract, 5.0 g/l NaCl) and grown overnight at 37°C in a shaker. The overnight culture was diluted (1:100) in 2x YT medium and grown at 37°C until an $OD_{600\ nm}$ of 0.7–0.9 was reached. VP40 expression was induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside) and the bacterial culture was further incubated for 18 hours with vigorous shaking (250 rpm) at 37°C. The culture was harvested by centrifugation at 5,000 × g for 20 min at 4°C. The pellet was collected after centrifugation to determine total cell protein (TCP) from induced and uninduced culture.

2.2.2.2 Protein purification

The VP40 protein obtained from inclusion bodies of *E.coli* was purified by immobilized metal–affinity chromatography (IMAC) chromatography. The inclusion bodies were solubilized in a denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 8.0) for 1 hour at room temperature (RT) with gentle shaking. Solubilized, denatured recombinant VP40 (rVP40) protein were separated from insoluble material by centrifugation at 15,000 × g for 30 min at 4°C. An immobilized metal-affinity chromatography (IMAC) based separation system was prepared by loading 1 ml of Ni–NTA agarose on a column and left to bind for 2 hours. The column was equilibrated with 5-bed volumes of lysis buffer (6M guanidine, 100 mM NaH_2PO_4, 10 mM Tris.Cl, pH 8.0). Denatured soluble protein was loaded on the column and the column was washed initially with 5-bed volumes of washing buffer (8M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3). Bound protein was eluted with gradient elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 4.5) and 30 mM to 250 mM imidazole. The eluates collected were purified rVP40 protein. This purified rVP40 was used for chicken immunization and also as an antigen for the development of heterosandwich (DAS-ELISA-mAb-IgY) and homosandwich (DAS-ELISA-mAb-bmAb) assays.

2.2.3 Production of IgY

2.2.3.1 Chicken immunization

Hens were immunized with rVP40 antigen according to standardized protocol following animal ethics guidelines (CCAW, 2016). The rVP40 antigen (500 µg/ml) was emulsified with an equal volume of Freund's incomplete adjuvant suspended in a phosphate buffer saline (PBS) pH 7.3. Two 23 week old single comb white leghorn chickens were injected intramuscularly

with the antigen (0.25 ml) in four different places. After two weeks, a booster dose of 0.25 ml VP40 antigen (500 μg/ml) was given. The eggs were collected daily and stored at 4°C until further processing.

2.2.3.2 Purification of anti-EBOV VP40 IgY polyclonal antibody

The water dilution method (Sunwoo et al., 2002) was used to obtain a water-soluble fraction (WSF) from the egg yolk containing specific IgY. In brief, the egg yolk was isolated from the egg white and mixed with eight volumes of cold distilled water (acidified with 0.1M HCl). Acidified cold distilled water (pH 2.0) was slowly added to make the final dilution of 1:10. The pH was adjusted to the 5.0 - 5.2 range. The diluted egg yolk with acidic water was incubated at 4°C for 12 hours. After centrifugation at 3500 × g for 20 min at 4°C, the WSF was obtained and saturated with 60% ammonium sulfate. IgY was purified by Sephacryl S-300 gel chromatography (GE Healthcare, UK). The yield was measured by Bradford assay (see below) to quantify the amount of purified IgY obtained by gel chromatography (Bradford, 1976). The IgY obtained in the same manner from non-immunized eggs was used as a negative control.

2.2.3.3 Weekly titer check of specific IgY by indirect ELISA

The titer for specific IgY was measured weekly by indirect ELISA according to established procedure (Sunwoo et al., 2002). Briefly, rVP40, 100 μl (10 μg/ml) in carbonate-bicarbonate buffer (0.01 M, pH 9.6), was immobilized on 96-well plates and incubated at 4°C overnight. Plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T) then blocked at 37°C for 2 hours with 200 μl per well of 5% skim milk solution (wt/vol) in PBS-T. Specific IgY from 2 to 7 weeks post-antigen challenge and nonspecific IgY as a control at different

dilutions (1,600, 3,200, 6,400, 12,800, 25,600 and 51,200) in PBS-T were added to each well in triplicate (100 µl /well) and incubated at 37°C for 1 hour. Plates were washed again with PBS-T, and secondary antibody (1: 5,000 Rabbit anti-chicken IgY-HRP) was added. After incubation at 37°C for 1 hour, plates were washed and TMB (3,3', 5,5'-Tetramethylbenzidine) substrate was added. Plates were kept on a shaker for 15 minutes, and then stop solution (0.5M sulfuric acid) was added. Absorbance was measured at 450 nm using an ELISA Vmax kinetic microplate reader.

2.2.4 Production of mAb

2.2.4.1 Production of anti-VP40 monoclonal antibody (mAb)

Anti-VP40 hybridoma cell lines (VP40/3-1, VP40/12-2, VP40/16-2, VP40/21-6, VP40/24-6, VP40/25-2, VP40/26-3, VP40/29-3, and VP40/5-3-2) were a kind gift from Dr. Ayato Takada, Division of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University, Japan. The hybridoma cells were cultured in 75 mm^3 flasks (approximately 1×10^5 cells/ml). When the cell density reached $1–3 \times 10^6$cells/ml, cells were split and expanded in large 175 mm^3 flasks (New York, USA). The mAb-containing supernatant was harvested from log growth phase cells and stored at −20°C (Sunwoo et al., 2013).

2.2.4.2 Screening of VP40 mAbs by indirect ELISA

Hybridoma culture supernatants were assayed for binding to rVP40 antigen. 96 well microtiter plates (each well coated with 100µl of purified rVP40 (10 µg/ml) in PBS) were incubated overnight at 4°C, then blocked with 5% skim milk (blocking buffer) for 2 hours at 37°C. After washing the plates 3 times with PBS-T, 100 µl of 1:10 diluted mAb-producing

hybridoma culture supernatant from each of the nine anti-VP40 clones (VP40/3-1, VP40/12-2, VP40/16-2, VP40/21-6, VP40/24-6, VP40/25-2, VP40/26-3, VP40/29-3, VP40/5-3-2) was added to separate wells. The plates were incubated for 1 hour at 37°C.After washing with PBS-T 3 times, 100 µl of secondary antibody (goat anti-mouse IgG-HRP) diluted (1:10,000) in blocking buffer was added and incubated for 1 hour at 37°C. The plates were again washed 3 times with PBS-T and TMB substrate was added. After 15 min, absorbance was measured at 650 nm using a Vmax ELISA plate reader (Ganguly et al., 2013).

2.2.4.3 Purification of anti-VP40 mAbs

Crude hybridoma supernatants were loaded onto a protein-G affinity chromatography column (MilliporeSigma, Germany) equilibrated with PBS at pH 7.4 (Huse et al., 2002). The bound immunoglobulins (mAbs) of VP40/16-2 clones were eluted with 0.1M glycine, pH 2.8 and the eluents were neutralized with 1M Tris pH 9. The pooled eluates were dialyzed in dialysis tubing with a wall thickness 20 µm (25 mm width, 15.9 mm diameter) (Thermofischer, Massachusetts, USA) overnight at 4°C with three changes of PBS at pH 7.2 (Ganguly et al., 2015).

2.2.4.4 Subclass determination of mAbs

The subclass of VP40/16-2 mAb was determined using the isostrip mouse monoclonal antibody isotyping kit (MilliporeSigma, Germany). In this procedure, a development tube containing latex beads bearing anti-mouse kappa and anti-mouse lambda antibodies reacts with mouse mAb regardless of its isotype. The isotyping strip bears immobilized bands of goat anti-mouse antibodies corresponding to each of the common mouse antibody isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA) and kappa (k) and lambda (ʎ) light chains. The strip also bears a

positive control band, which indicates that the antibody-coated beads traveled up the strip. Our mAb was screened for isotype by diluting the antibody sample in 1X phosphate buffer saline (PBS), pH 7.2-7.6 (1 µg/ml). The freshly diluted sample of mAb (150 µl) was added to the development tube, and the strip inserted. The mAbs complexes with the antibody coated latex beads. When the isotyping strip was placed in the development tube, this complex flowed up the strip via capillary action until it was bound by the immobilized goat anti-mouse antibody specific for the monoclonal's isotype and by the immobilized antibody specific for the monoclonal's light chain. In approximately 10 minutes, latex beads aggregated as blue bands in the two subsections corresponding to the antibody's isotype and the nature of its chain. The antibody of interest appeared as a clear, dominant band on this sensitive strip (Sigma-Aldrich).

2.2.4.5 Biotinylation of anti-Ebola VP40 mAbs

Biotinylation of VP40/16-2 mAb was performed by long arm biotinyl-N-hydroxysuccinimide ester (NHS-Biotin). 1 mg of protein-G purified VP40 mAbs in PBS, pH 7.4 was added to a long chain biotin volume equal to 10% of the total amount of immunoglobulin solution of (22 mg/ml) and incubated at room temperature (RT) for 4 hours. The solution was dialyzed in a slide-A-lyzer (Thermofischer Scientific, USA) against PBS, pH 7.4 containing 0.1% sodium azide overnight at 4°C. After dialysis, the bmAb was stored at -20°C (Sigma-Aldrich).

2.2.5 Analytical methods

2.2.5.1 SDS-PAGE and western blot analysis of rVP40

The Bio-Rad Mini-Protean III apparatus was used for electrophoretic separation of rVP40 by SDS-urea electrophoresis, according to the method of Laemmli (Laemmli, 1970). The rVP40 protein samples were prepared with four volumes of sample solution in 8M urea and one volume of sample buffer (pH 6.8, 0.0625M Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue and applied in equal amounts (1mg). Separation was performed at 80 V for 2 hours, in precooled running buffer (pH 8.3, 0.025M Tris, 0.192M glycine, 0.1% SDS), using a 12% polyacrylamide separating gel, and then electroblotted onto a hybond ECL nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS-T for 1 hour, washed 4 times with PBS-T, and incubated for 1 hour with anti-VP40 IgY (1μg/mL) and anti-VP40 mAb (VP40/16-2). After washing 4 times with PBS-T, the nitrocellulose membrane was incubated with rabbit anti-chicken IgY-HRP and goat anti-mouse IgG-HRP for 1 hour. All incubations were carried out at RT. Finally, the membrane was washed with PBS-T four times, and electro-chemiluminescence detection was performed to visualize the specific binding.

2.2.5.2 Cross-reactivity of antibodies (IgY and mAb)

ELISA and Western blot analyses were performed to determine the cross-reactivity of our IgY and mAb to other viral proteins, such as Ebola GP, SARS-NP, and Dengue NS1, as described above.

2.2.5.3 Bradford protein assay

The Bio-Rad protein assays based on the method of Bradford were performed using bovine serum albumin (BSA) (2 mg/ml of protein) as a standard. The purified mAbs, IgY and rVP40 protein (diluted 1:10 in PBS) and two-fold serial dilutions of the BSA in PBS (1 to 0.05 mg/ml) were analyzed on a microtiter plate. Absorbance at 595 nm after 10 min reaction was measured using an ELISA Vmax kinetic microplate reader. The protein concentration was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples (Bradford, 1976).

2.2.6 ELISA system

2.2.6.1 DAS-ELISA-mAb-IgY and bmAb

Microtiter plates were coated overnight at 4°C with 100 µl per well of various concentrations of VP40 mAb, ranging from 500 to 12000 ng/ml of coating buffer in triplicate. Plate wells were blocked with 200 µl of 5% skim milk at 37°C for 2 hours. One hundred microliters of rVP40 antigen, dilutions ranging from 16000 to 1.95 ng/ml, were added and incubated for 1 hour.

For the IgY anti-rVP40 system, IgY was added as the detection antibody (ranging from 0 to 16 µg/ml). After incubation for 1 hour, rabbit anti-chicken IgY-HRP at different dilution ranges of 1:4,000 to 1:48,000 was added, and the plate was further incubated for 1 hour at 37°C. For the bmAb-anti rVP40 system, bmAb was used as a detection antibody (ranging from 16,000 to 1.95 ng/ml). After incubation for 1 hour, streptavidin-HRP conjugate at different dilution ranges of 1:4,000 to 1:48,000 was added, and the plate was further incubated for 1 hour at 37°C. In

both cases, the plates were washed 3 times with PBS-T after each of the steps mentioned above. Lastly, TMB was added for color development and read at 650 nm using a microplate reader.

2.2.7 Limit of detection of rVP40 antigen in PBS by sandwich ELISA

The sandwich assay was carried out with Ebola anti-VP40 mAb as the capture antibody. In DAS-ELISA-mAb-IgY, 8 µg/ml of IgY as a detecting antibody and 1:10,000 anti-chicken IgY as a secondary antibody were used. In DAS-ELISA-mAb-bmAb, 31.25 ng/ml of bmAb as a detecting antibody and 1:10,000 streptavidin-HRP conjugate was used. The microtiter plate was immobilized with 100 µl of purified VP40 mAb (4 µg/ml) in 0.01M carbonate/bicarbonate buffer at 4°C overnight. Nonspecific binding sites were blocked with 200 µl of 5% skim milk for 2 hours. Different concentrations of rVP40 antigen ranging from 16,000 ng/ml to 0.95 ng/ml were used and then the plates were incubated at 37°C for 1 hour. After washing, 100 µl of detecting IgY antibody (8 ug/ml) or bmAb (31.25 ng/ml) was added to each well and incubated at 37°C for 1 hour. The secondary antibody on top of IgY (anti-chicken IgY-HRP at 1:10,000) and conjugate on top of bmAb (streptavidin-HRP at 1:10,000) was added and incubated at 37°C for 1 hour. Finally, TMB substrate was added (Blake et al., 2001). Absorbance was measured after 15 min using an ELISA Vmax kinetic microplate reader. All incubations were done at 37°C, except when coating the plate with mAb. Washing was done 5 times with PBS-T after each step. The wells without VP40 antigen were used as negative control. Concentrations of other reagents (cAb, dAb and anti-chicken IgY-HRP or streptavidin-HRP) were according to optimized standards (as per section 2.6). These assays were performed at least four times, in triplicate, for each dilution.

2.2.8 Limit of detection of rVP40 antigen in spiked serum samples by sandwich ELISA

Ebola rVP40 antigen was used to spike serum samples of humans and the sandwich assay was carried out with Ebola anti-VP40 mAb (VP40/16-2) as the capture antibody. Human serum samples were purchased from Sigma (Cat# H4522). In DAS-ELISA-mAb-bmAb, 31.25 ng/ml of bmAb as a detecting antibody and 1:10,000 streptavidin-HRP conjugate were used. The microtiter plate was immobilized with 100 µl of purified VP40/16-2 mAb (4 µg/ml) in 0.01M carbonate/bicarbonate buffer at 4 °C overnight. Nonspecific binding sites were blocked with 200 µl of 5% skim milk for 2 hours. Different concentrations (ranging from 16,000 ng/ml to 0.95 ng/ml) of rVP40 antigen in human serum samples were used and then the plates were incubated at 37°C for 1 hour. After washing, 100 µl of detecting bmAb (31.25 ng/ml) were added to each well and incubated at 37°C for 1 hour. The conjugate on top of bmAb (streptavidin-HRP at 1:10,000) was added and incubated at 37°C for 1 hour. Finally, TMB substrate was added. Absorbance was measured after 15 min using an ELISA Vmax kinetic microplate reader. All incubations were done at 37°C except coating the plate with mAb. Washing was done 5 times with PBS-T after each step. The wells without rVP40 antigen were used as negative control. Concentrations of other reagents (cAb, dAb and streptavidin-HRP) were according to optimized standards. These assays were performed at least four times, in triplicate, for each dilution.

Limit of detection (LOD) was determined by the formula, LOD = LOB + 1.645(SD low concentration sample), where LOB = mean blank + 1.645(SD blank). Limit of blank (LOB) is considered to be the highest apparent analyte concentration that can be found when replicates of a blank sample containing no analyte are tested, whereas LOD is the lowest analyte concentration that can be distinguished from the LOB and at that concentration, detection is

feasible. LOD is measured by using both the measured LOB and test replicates of a sample known to contain a low concentration of the analyte (Armbruster et al., 2008).

2.2.9 Statistical analyses

The Student t-test (one-tailed t-test) was used to analyze the results.

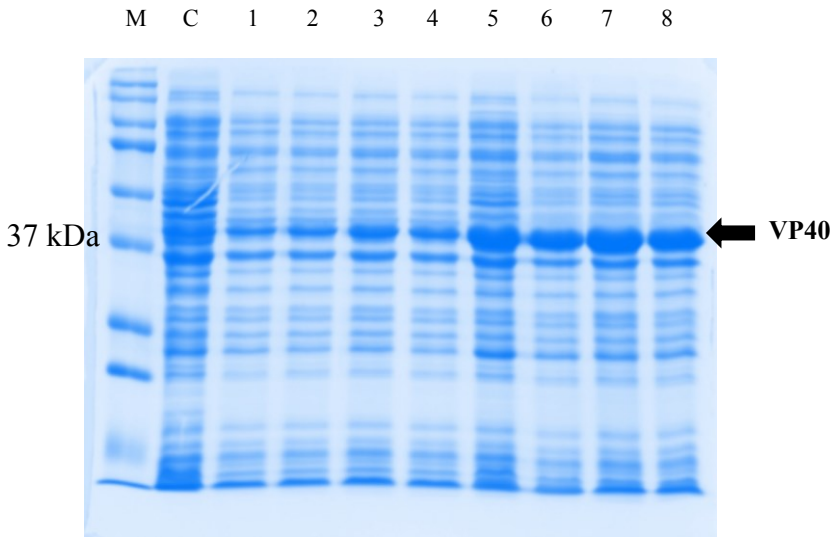
2.3 Results

2.3.1 Production of VP40

The full-length viral matrix gene VP40 was cloned into plasmid pET22b and used to transform *Rosetta (E.coli DE3)*. Figure 2.1A illustrates the expression of rVP40 protein separated on SDS-PAGE. Lanes 1 to 4 show weak bands of VP40 protein at 37 kDa after 2 hours of IPTG induction, whereas thick and intense bands of VP40 protein at 37 kDa were observed after 18 hours of induction. This indicates the use of IPTG inducer increased yield of rVP40 during the *E.coli* protein expression. The optimal conditions for VP40 protein expression were 37°C temperature and 18 hours induction time.

The expressed rVP40 protein was purified by IMAC. The yield of eluted fractions of rVP40 was 12 mg/l of bacterial culture by Bradford protein assay. Western blot analysis using anti-his-tagged Ab was performed to confirm that the expressed protein was rVP40 (Figure 2.1B).

(A)



(B)

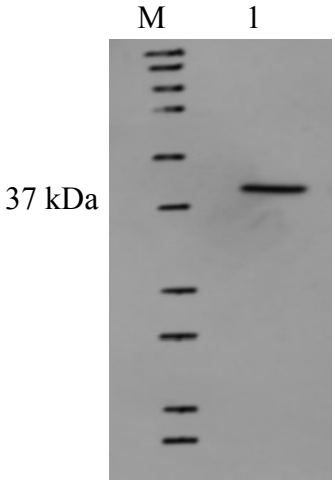


Figure 2.1 (A) SDS PAGE of VP40 expression in E.coli system, Lane M: Molecular marker; Lane C: control of uninduced VP40; Lane 1-4 : VP40 at 2 hours of induction ; lane 5-8 : VP40 at 18 hours of induction. **(B)** Western Blot of VP40 with his tag antibodies. Lane M: Molecular marker; Lane 1: Purified Ebola recombinant VP40 protein.

2.3.2 Production of IgY

Eggs collected from chickens immunized with Ebola rVP40 protein were used to purify anti-VP40 IgY antibodies. The yield of the purified IgY was measured to be 720 µg/yolk by Bradford protein assay. We performed indirect ELISA with different dilutions of IgY antibody (1:1,600, 1:3,200, 1:6,400, 1:1,2800, 1:25,600, 1:51,200) in PBS-T to measure titers of IgY during the immunization period (Figure 2.2) and found high titers at up to 1:6,400 dilution of IgY.

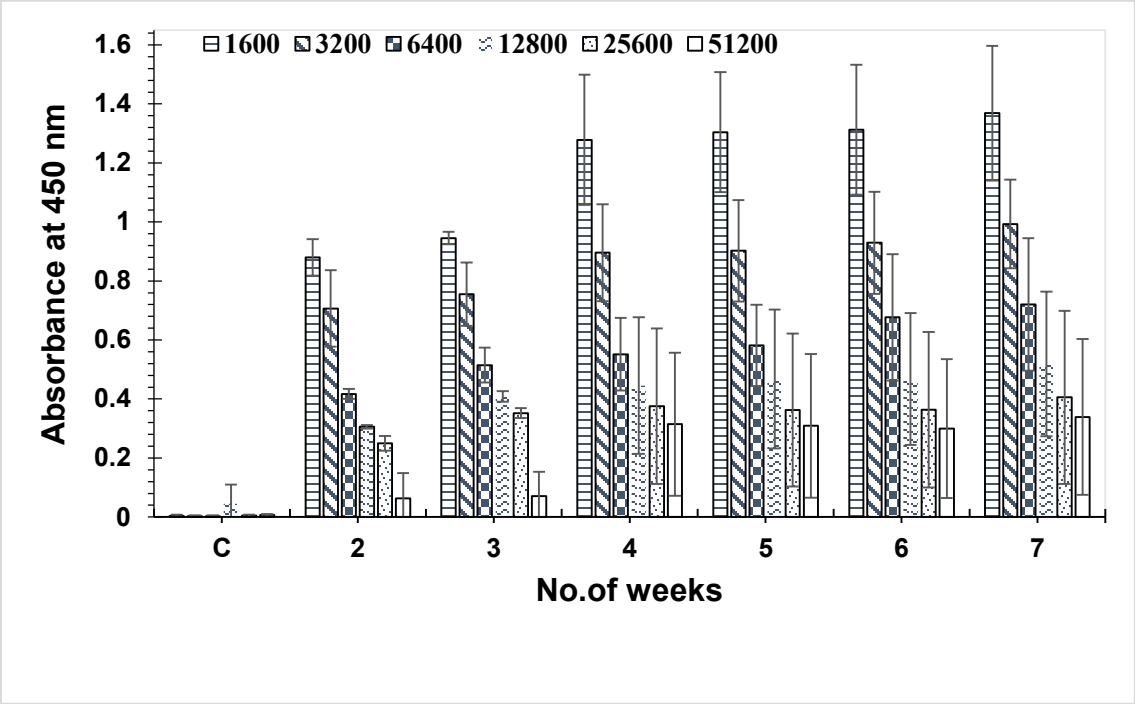


Figure 2.2: IgY titer to VP40 at different dilutions during the immunization period. The specific activity of IgY in egg yolk of chickens immunised with Ebola VP40 antigen at different weeks. The extent of IgY activity in dilutions (1,600, 3,200, 6,400, 12,800, 25,600, and 51,200) was measured by ELISA with the use of VP40 antigen, C is negative control of non-specific IgY to VP40 and absorbance was measured at 450 nm. X- axis numbers represent the number of weeks. Standard deviation was shown by vertical bars.

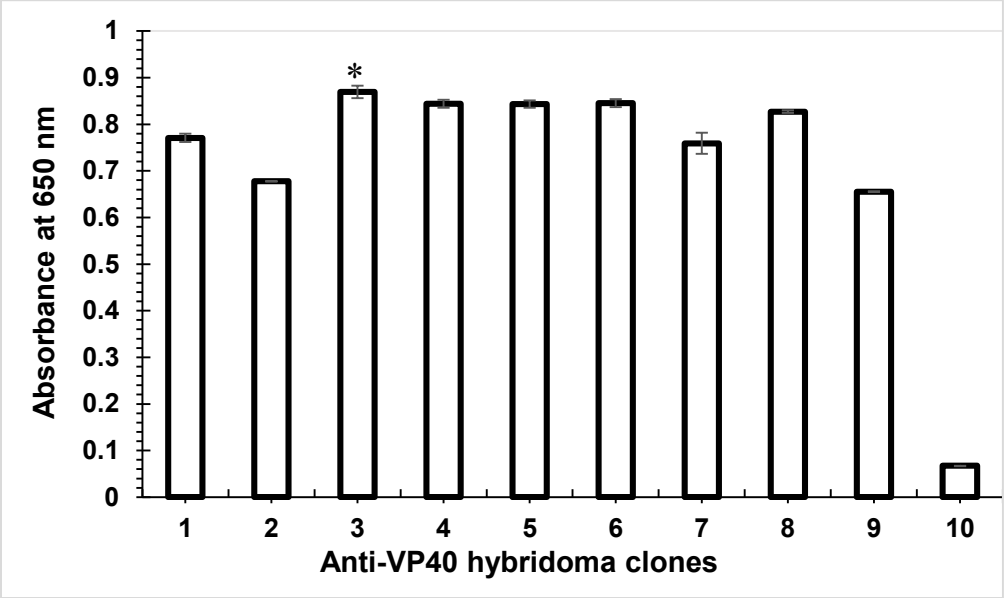
2.3.3 Production and characterization of mAb

Nine hybridoma clones (VP40/3-1, VP40/12-2, VP40/16-2, VP40/21-6, VP40/24-6, VP40/25-2, VP40/26-3, VP40/29-3, and VP40/5-3-2) were screened to check supernatant titer and binding affinity with rVP40 protein. We found that all the anti-VP40 hybridoma clones produced mAbs, but the VP40/16-2 hybridoma cell culture supernatant showed the highest anti-rVP40 titer of the nine different VP40 clones (Figure 2.3A). Based on the results, the best clone VP40/16-2 was selected and cultured (Figure 2.3B).

The VP40/16-2 clone yielded 10 mg of mAb/l of hybridoma cell culture supernatant after protein G affinity chromatography, as measured by the Bradford protein assay. SDS-PAGE, under reducing conditions, of column-purified supernatant showed a heavy chain band at 50 kDa and light chain band at 28 kDa, and no other band of any other protein, confirming the purity of our anti-VP40 mAb (Data not shown).

Using an isotyping strip available commercially, we confirmed that the heavy chain of the anti-VP40 mAb (from VP40/16-2) was of the IgG1 type, and the light chain was kappa (k) (Figure 2.4).

(A)



(B)

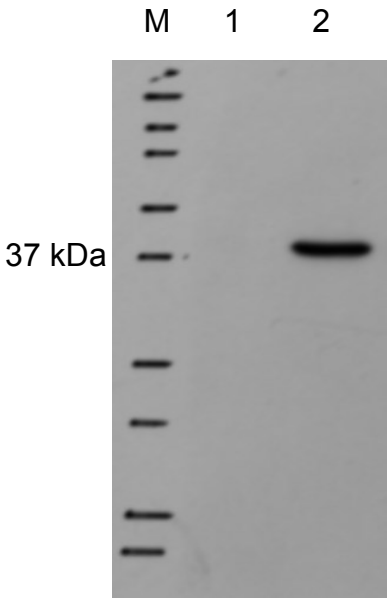


Figure 2.3 (A): Screening of anti-VP40 hybridoma clones to measure antibody titer (1-ZVP40/3-1, 2-ZVP40/12-2, 3-ZVP40/16-2, 4-ZVP40/21-6, 5-ZVP40/24-6, 6-ZVP40/25-2, 7-ZVP40/26-3, 8-ZVP40/29-3, 9-ZVP40/5-3-2, 10- ZGP133/3-16)* : Highest OD titer clone selected for further studies. GP133/3-16 was used as negative control. Standard deviation is shown in vertical bars. **(B)** Western Blot of VP40 probed with anti-Ebola VP40/16-2 mAb. Lane M : Molecular marker ; Lane 1: Ebola GP133/3-16 protein, Lane 2 : Ebola rVP40 protein.

(A) **(B)**

Figure 2.4: Isotype determination of mAbs. **(A)** Isotype strip showing the IgG1 isotype of heavy chain. **(B)** Isotype strip showing the kappa isotype of light chain

2.3.4 Biotinylation of VP40/16-2

We confirmed successful biotinylation of the VP40/16-2 mAb to form bmAb by dot blot assay. A blue dot was observed on the nitrocellulose membrane containing the biotin labelled protein, whereas no dot was observed on the membrane with unlabelled VP40/16-2 mAb (Figure 2.5), since conjugate streptavidin-HRP can only bind to the biotin labeled VP40/16-2 mAb but not to the unlabelled one.

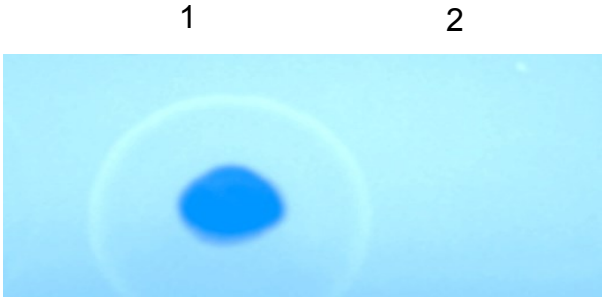


Figure 2.5: Dot Blot Assay showing biotinylation of mAb. 1: labelled mAb ; 2: Unlabelled mAb

2.3.5 Cross-reactivity of IgY and mAb

We performed western blot analysis to measure the specificity of our IgY. Anti-VP40 IgY purified from chicken egg yolks at 7 weeks of immunization showed a 37-kDa rVP40 protein (Figure 2.6A). Figure 2.6B shows that there was no cross-reactivity of anti-rVP40 IgY antibody or mAb with other viral proteins, such as SARS-NP, Dengue NS1, and Ebola GP. These results indicate the specificity of IgY to the 37 kDa rVP40 antigen.

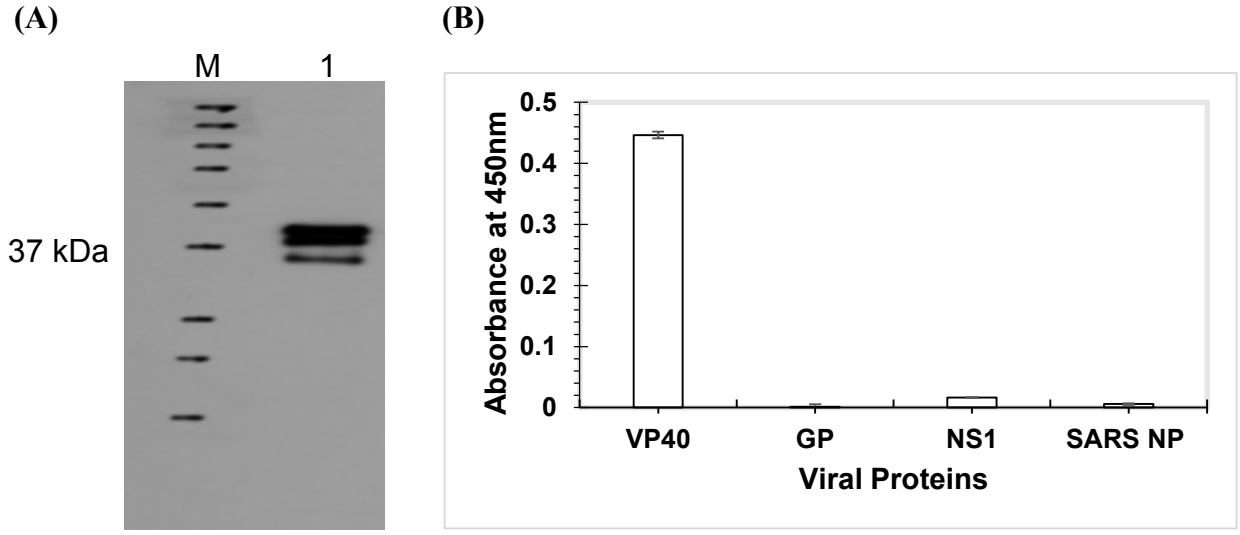


Figure 2.6: (A) Western Blot of IgY to rVP40 : Lane M : Molecular marker ; Lane 1 : Ebola recombinant VP40 protein. **(B)** ELISA : Specificity of IgY to rVP40 and cross reactivity of IgY to other viral proteins (VP40-Ebola virion matrix protein ; GP- Ebola glycoprotein ; NS1-Dengue non-structural protein 1 ; SARSNP- Severe acute respiratory syndrome nucleoprotein. Standard deviation is shown in vertical bars.

2.3.6 ELISA system optimization

mAb as the capture antibody and IgY or bmAb (VP40/16-2) as the detecting antibody were used to develop the heterosandwich DAS-ELISA-mAb-IgY and homosandwich DAS-ELISA-mAb-bmAb assay. Different concentrations of capture mAb (VP40/16-2), anti-VP40 IgY, bmAb, anti-chicken IgY and strep-HRP were used to select the optimum concentration for our DAS-ELISA systems.

2.3.6.1 DAS-ELISA-mAb-IgY

Capture antibody (VP40/16-2 mAb) was optimal at a concentration of 4,000 ng/ml ($P < 0.05$) (testing ranges of 500 to 12,000 ng/ml) with 5 µg/ml of rVP40, 10 µg/ml of detecting antibody (IgY) and 1:5000 dilution of secondary antibody (anti-chicken IgY) (Figure 2.7A). When we varied the concentration of detecting antibody (anti-VP40 IgY) from 0-16 µg/ml and maintained the same concentration and dilution of rVP40 and anti-chicken IgY, we found the optimal concentration of anti-VP40 IgY was 8 µg/ml ($P <0.05$)(Figure 2.7B) using the optimized concentration of capture mAb of 4000 ng/ml.

We created a dilution series (4,000 x – 48,000 x) of rabbit anti-chicken IgY antibody conjugated with HRP and used it to determined its optimal dilution as 1:10,000 ($P <0.05$); capture mAb was 4,000 ng/ml, detection antibody (anti-VP40 IgY) was 8 µg/ml and rVP40 was 5 µg/ml (Figure 2.7C).

2.3.6.2 DAS-ELISA-mAb-bmAb

Using the capture antibody VP40/16-2 mAb, we found that the optimal concentration was 4,000 ng/ml ($P<0.05$) (Figure 2.8A) after assessing different ranges of 500 to 12,000 ng/ml, and maintaining 5 µg/ml of rVP40, 4 µg/ml of bmAb (VP40/16-2) and 1:5,000 dilution of the streptavidin-HRP conjugate.

Varying the concentration of bmAb antibody (VP40/16-2) from 16000 – 1.95 ng/ml allowed us to determine the optimal concentration of bmAb to be 31.25 ng/ml ($P<0.05$), while maintaining the capture mAb constant at 4,000 ng/ml (Figure 2.8B); this was assayed with 5 µg/ml of rVP40 and 1:5,000 dilution of strep-HRP. The optimized concentration of bmAb was determined as 31.25 ng/ml

We created a dilution series (4,000x – 48,000x) of streptavidin conjugated with HRP and used it to determine its optimal dilution as 1:10,000 ($P<0.05$) (Figure 2.8C); capture mAb was 4000 ng/ml, detection antibody (anti-VP40 bmAb) was 31.25 ng/ml and rVP40 was 5 µg/ml.

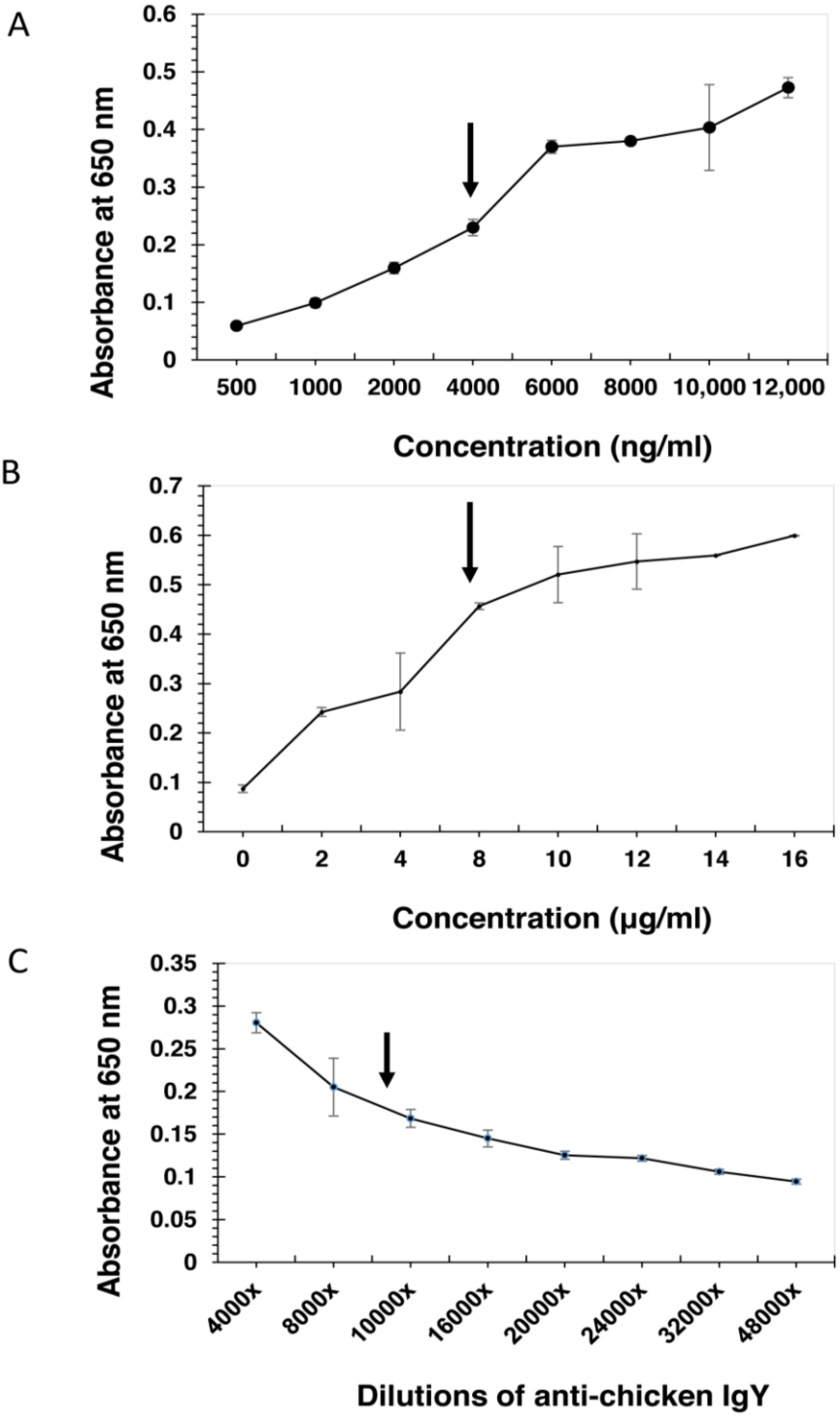


Figure 2.7: Development of DAS-ELISA-mAb-IgY to optimize the concentration of three different antibodies. Arrows indicate the optimum concentration of antibodies. Standard deviation is shown in vertical bars. **(A)** Optimization of capture antibody. **(B)** Optimization of detecting antibody. **(C)** Optimization of secondary antibody (Rabbit anti-chicken IgY).

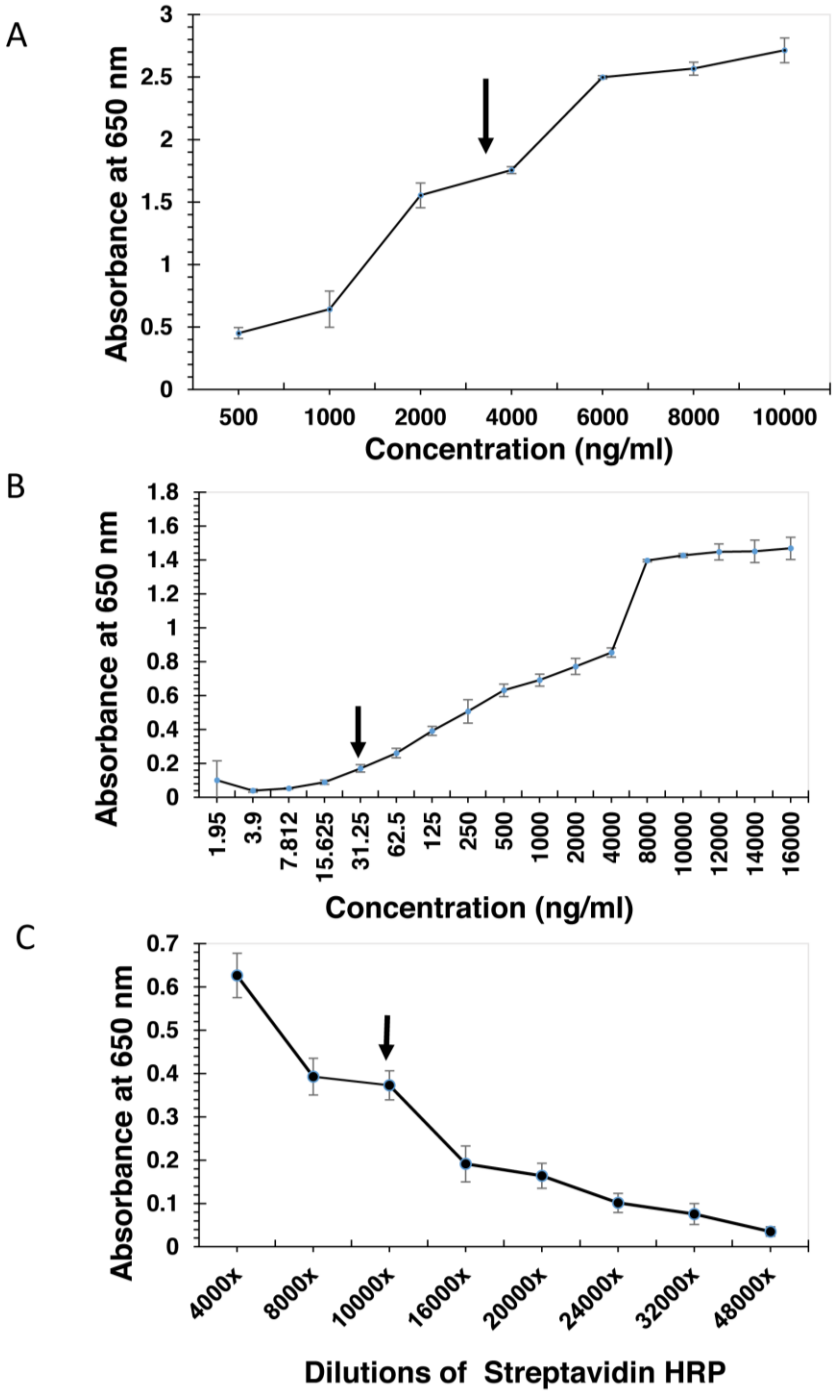


Figure 2.8: Development of DAS-ELISA-mAb-bmAb to optimize the concentration of two different antibodies and conjugate. Arrows indicate the optimum concentration of antibodies. Standard deviation is shown in vertical bars. (**A)** Optimization of capture antibody. (**B)** Optimization of detecting antibody. (**C)** Optimization of conjugate (Streptavidin-HRP).

2.3.7 Limit of detection (LOD)

2.3.7.1 DAS-ELISA-mAb-IgY with rVP40 in PBS

In heterosandwich DAS-ELISA-mAb-IgY, Figure 2.9 illustrates that when the optimized monoclonal cAb (4,000 ng/ml), dAb (8 µg/ml) and anti-chicken IgY concentration (1:10,000) were used to quantitate rVP40 antigen in a serial dilution from 16,000 to 0.95 ng/ml, then the immunoassay can detect as little as 33 ng/ml of rVP40 antigen.

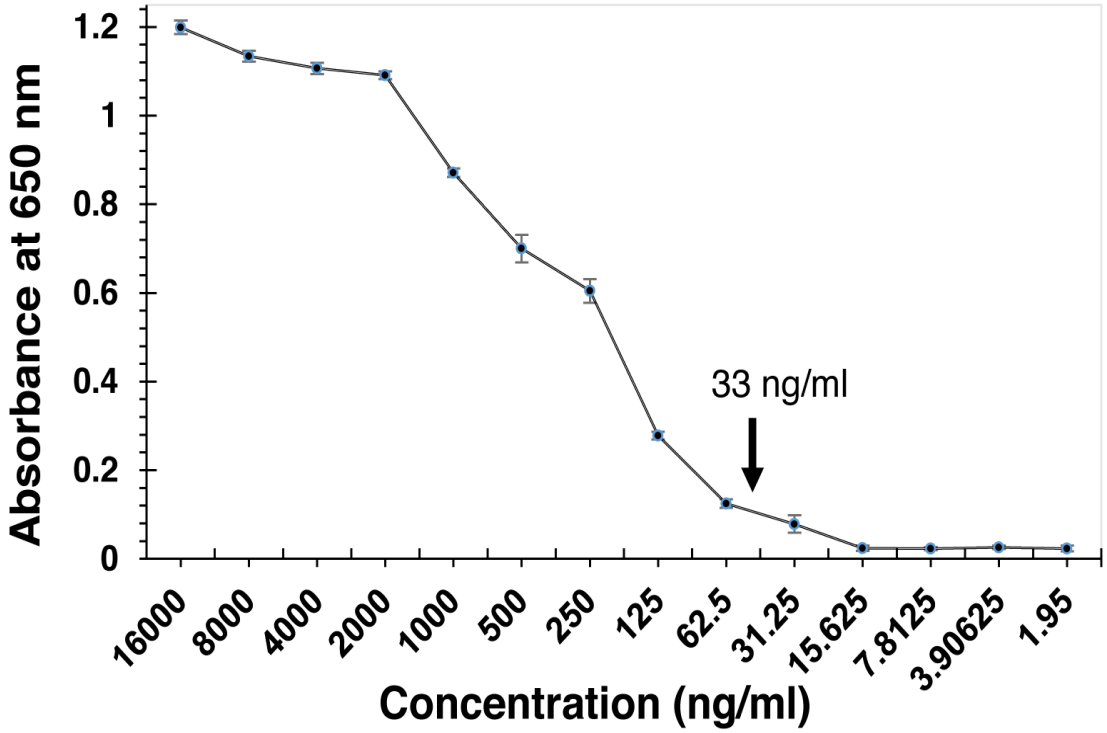


Figure 2.9: Limit of detection of rVP40 in PBS for DAS-ELISA-mAb-IgY. Arrow shows the limit of detection. Standard deviation is shown in vertical bars.

2.3.7.2 DAS-ELISA-mAb-bmAb with rVP40 in PBS

In homosandwich DAS-ELISA-mAb-bmAb, Figure 2.10 illustrates that when the optimized monoclonal cAb (4,000 ng/ml), dAb (31.25 ng/ml) and strep-HRP conjugate concentration (1:10,000) were used to quantitate rVP40 antigen in a serial dilution from 16,000 to 0.95 ng/ml, then the immunoassay bottom limit of detection is 23 ng/ml of rVP40 antigen.

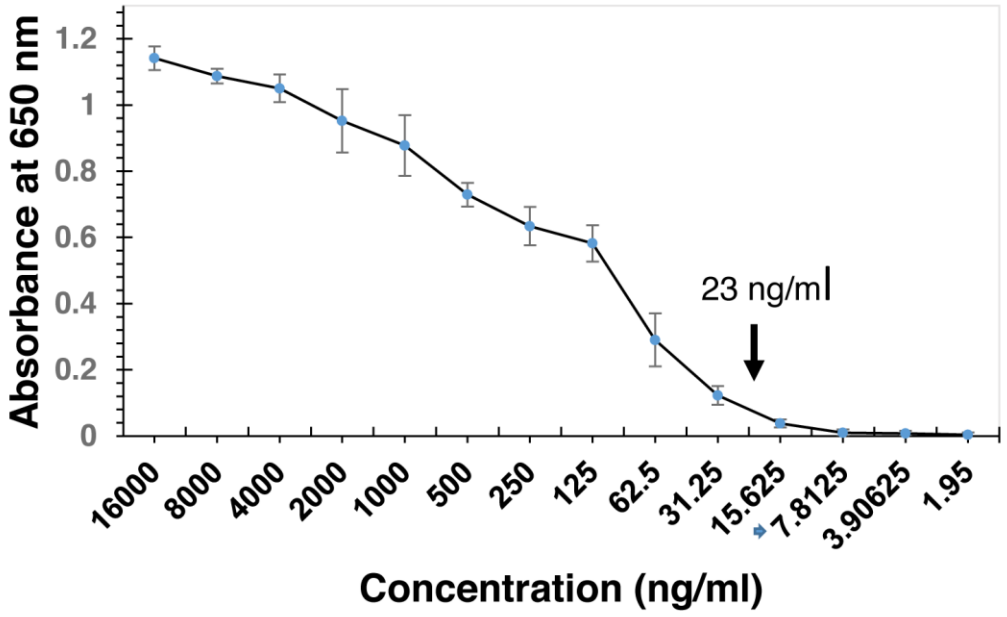


Figure 2.10: Limit of Detection of VP40 in PBS for DAS-ELISA-mAb-bmAb. Arrow shows the limit of detection. Standard deviation is shown in vertical bars.

2.3.8 DAS-ELISA-mAb-bmAb with rVP40 antigen spiked in serum samples of humans.

In homosandwich DAS-ELISA-mAb-bmAb, Figure 2.11 illustrates that when the optimized monoclonal cAb (4,000 ng/ml), dAb (31.25 ng/ml) and strep-HRP conjugate concentration (1:10,000) were used to quantitate rVP40 antigen in serum samples at a range from 16,000 to 0.95 ng/ml, then the immunoassay can detect a minimum of 71.25 ng/ml of rVP40 antigen.

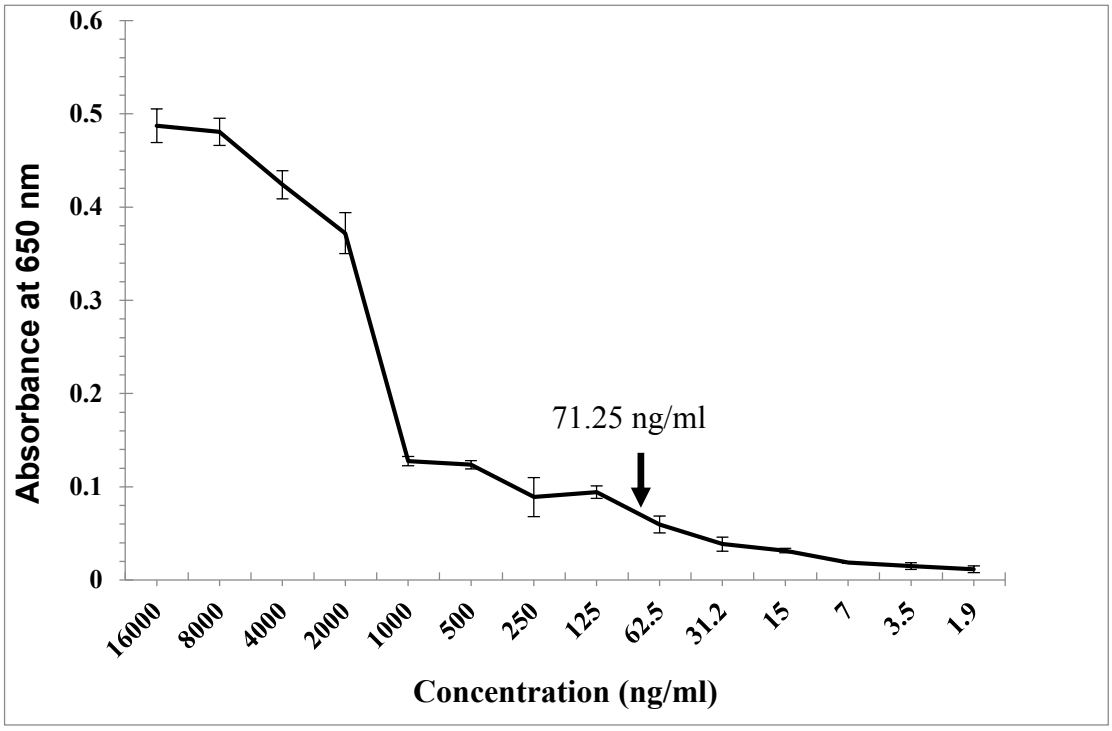


Figure 2.11: Limit of Detection of VP40 in spiked serum samples of humans for DAS-ELISA-mAb-bmAb. Arrow shows the limit of detection. Standard deviation is shown in vertical bars.

2.4 Discussion

There have been concerted global efforts to develop sensitive diagnostics for safe and accurate assessment of Ebola in suspected patients (Olupot, 2015). Current diagnostics virus isolation, viral RNA detection by PCR, (CDC, 1996) or antigen and antibody detection ELISA assays (Ksiazek et al., 1999a; Saijo et al., 2006) all have drawbacks. Virus isolation is very expensive and time intensive (Webb, 1978). Immunofluorescence staining can rapidly detect Ebola virus, but the interpretation of results is difficult (Rollin et al., 1990) and requires a large number of virus cells in the test sample and the use of costly equipment i.e. a cell sorter or

fluorescent microscope. Real-time RT-PCR (Drosten et al., 2002; Weidmann et al., 2004) and reverse-transcription loop mediated isothermal amplification (RT-LAMP) PCR can detect viral RNA (Kurosaki et al., 2007). Both PCR methods are sensitive, but prone to giving false positives due to cross-contamination issues (Rougeron et al., 2015; Towner et al., 2004) and are not appropriate for use in endemic areas. RT-PCR can, conversely, yield false negatives and those patients may be assessed as healthy and released from hospitals (Nouvellet et al., 2015; Qureshi et al., 2015), allowing further spread of EVD. The ELISA system, using microtiter 96-well plates and allowing visual evaluation of the reaction, is fast and does not require expensive equipment, making it acceptable for initial screening of a substantial number of samples. In a direct comparison of RT-PCR with ELISA, Filo A and B primers targeted L region in RT-PCR (Sanchez et al., 1999) of 49 samples that tested positive by ELISA, only 30 samples tested positive with the Filo A and B primer set. None of the samples that tested positive by RT-PCR were antigen negative (Towner et al., 2004). This showed that antigen capture ELISA is a reliable assay. Several attempts have been made to develop an antibody-detection ELISA that detects recombinant nucleoprotein (NP) and glycoprotein (GP)-specific IgG and IgM antibodies in infected individuals (Prehaud et al., 1998). Unfortunately, these immunoassays are based on IgG/IgM detection, and IgG antibodies remain in the patient up to 2 years (Gupta et al., 2001), therefore IgM/IgG detection assays are not good options for early detection of Ebola. Antigen detection via radioimmunoassay is a possibility, however the complicating factors of reagent longevity, storage, and disposal make it unsuitable for use in developing nations (Posner et al., 1982)

When considering public health measures to control EBOV outbreaks, it is important to detect the severity of infection during the initial period of clinical symptoms. This occurs when

viral proteins are being shed in great quantities. The Ebola structural protein VP40 is the dominant viral protein, at 3600 molecules per virion and comprising 37.7% of the whole protein mass (Elliott et al., 1985). VP40 antigen can be detected circulating in blood and serum from the second day after symptom onset (Rowe et al., 1999), and its levels rise until patient death (Baize et al., 1999). VP40 is therefore an ideal diagnostic biomarker for screening EBOV infection, and antigen detection ELISA could be the best way to detect EVD at the initial stages.

To address current Ebola diagnostic limitations, we developed an ultrasensitive heterosandwich DAS-ELISA-mAb-IgY and a homosandwich DAS-ELISA-mAb-bmAb to screen potential EVD in the early phase of infection (within 7 days). Our DAS-ELISA-mAb-IgY combines the use of murine hybridoma-derived mAb and chicken IgY for precise detection of rVP40 antigen. The detection limit observed for this assay was 33 ng/ml or 3 ng per well, which is more sensitive than existing antigen based diagnostic assays. For example, Kziasek and coworkers developed a sandwich ELISA with monoclonal antibody as capture antibody and polyclonal antibody as detecting antibody with a detection limit of 10^2 PFU/ml (Ksiazek et al., 1992). Lucht's group used a capture antibody concentration of 10 μg/ml (Lucht et al., 2003), whereas for the DAS-ELISA-mAb-IgY and DAS-ELISA-mAb-bmAb, the concentration of capture antibody used was 4 μg/ml. Niikura and coworkers developed an assay to detect NP antigen with a sensitivity of 0.3 μg/ml, which is almost 10 times less sensitive than our IgY and bmAb based immunoassay (Niikura et al., 2001).

This is the first report of a use of novel, specific chicken IgY in EVD screening. We chose chickens as the model to develop highly specific diagnostic reagents against mammalian proteins since it is well known that the greater the evolutionary distance, the better the immune

response (Dasilva et al., 2010). IgY has been successfully used for the development of antibodies against viral antigens (Piela et al., 1984) and in various diagnostic applications, such as detecting *E. coli* O157 (Sunwoo et al., 2006), Salmonella (Terzolo et al., 1998), and SARS (Palaniyappan et al., 2012). The polyclonal Abs produced in chickens offer unique advantages over those developed in rodents and mammalian species. Large amounts of IgY can be produced in less time and at a lower cost, which is a significant advantage when compared with mammalian serum antibody production (Schade et al., 1994). IgY is also stable under different conditions of heat, acidity (>pH 3.5), pressure, and alkalinity (Shimizu et al., 1992). The capacity to produce large quantities of antigen-specific IgY with fairly good stability makes it a viable option for use in diagnostics against different viral antigens (Erhard et al., 2000).

When we immunized our laboratory chickens with rVP40 antigen, specific anti-VP40 IgY were transferred from the maternal chicken serum to egg yolk. Our titer measurements indicate that the primary immune response of chicken B-cells rapidly increased after booster immunization, with the formation of memory B cells clones, the peak was at 7 weeks. It is worth noting that specific IgY antibodies only make up 2% of the egg yolk, with the remaining constituents being α and β-livetins, lipoproteins, and fatty acid molecules, which could potentially lower assay sensitivity (Fischer et al., 1996). However, our protocol involved thoroughly isolating and separating these fats from proteins, rendering purified egg yolk IgY.

For DAS-ELISA-mAb-bmAb, we used VP40/16-2 mAb as capture antibody and the same mAb labeled with biotin for detection. The detection limit observed for rVP40 in PBS was 23 ng/ml whereas using human sera spiked with rVP40 in a dilution range of 16,000 to 1.95 yielded a detection limit of 71.25 ng/ml. The higher detection limit in serum-spiked samples as

compared to rVP40 in PBS may have been due to the pH difference or an interaction between the analyte of interest and the matrix. We used biotin labeled mAb for detection as it enhances the detection signal after binding with multiple streptavidin molecules with high affinity (Lectez et al., 2014). Biotin strep-HRP is very specific and shows high binding at the site where the complex has formed with the antigen of interest (Mustafaoglu et al., 2015). After optimizations, our assay turns out to be highly efficient, with as little as 4 µg/ml capture antibody and 31.2 ng/ml detecting antibody able to detect Ebola rVP40 antigen, far better than any existing antigen based ELISA assay.

We realize that sometimes a biotinylated mAb based assay might show a slightly higher background because of the random conjugation process involved in labeling. This could result in an altered ratio between the mAb and biotin. The arbitrary conjugation process could also lead to the partial blocking of the antigen binding site, as the conjugation of biotin to the site is variable (Huang et al., 2010). Also, using monoclonal antibodies as detection antibodies may lower sensitivity due to epitope masking. This issue can be overcome by using IgY, as it is a polyclonal antibody and can bind to multiple epitopes of VP40. In the point of care setting, use of a polyclonal antibody for detection could be of great importance.

The goal of this research was to overcome the shortcomings of the existing assays and develop a rapid, sensitive, and economical assay for the screening of suspected Ebola patients. The detecting antibody IgY used in this study along with mAb against rVP40 antigen showed a sensitivity of 3.3 ng (absolute concentration), whereas with bmAb the sensitivity observed was 2.3 ng (absolute concentration). Our assays have the potential to detect even trace amounts of antigen in infected individuals. Additionally, our diagnostic assays are extremely low cost. The

approximate cost analysis shows that one ELISA test will cost around $5, but if we will make point of care swab using these antibodies then it will cost approximately $1, which is really less and affordable. Although our ELISA assays demand a few washing (fill and aspirate) and incubation steps, we presume frontline health care aides can perform them with minimal training. Our assays could be very beneficial in underdeveloped and developing parts of the world where lack of sophisticated and costly technology can be a constraint to diagnosis of acute infections like Ebola.

In the future, we will use simulated spiked samples or biological samples such as human saliva from EBOV-infected patients to further validate our DAS-ELISA assays and explore different platforms to determine the best suitable to be used at point of care sites. This will help establish the sensitivity more accurately, as it can mimic the actual EVD conditions. Compared to assessing serum from EBOV patients, saliva samples will provide an advantage of non-invasive collection of clinical samples, simultaneously reducing screening time.

Chapter 3: Development of a single-step two-site immunoassay using a novel bispecific immunoprobe for rapid and sensitive detection of Ebola VP40 antigen

3.1 Introduction

Ebola virus (EBOV), a member of the family Filoviridae is among the most fatal pathogens. This virus infects humans as well as nonhuman primates, inducing severe hemorrhagic fever, with mortality rates as high as 90 % in humans (Kiley et al., 1982; Mahanty et al., 2004). In March 2014, the World Health Organization (WHO) was notified of an outbreak of *Zaire ebolavirus* in an isolated area in Guinea. The outbreak rapidly spread to the neighbouring countries of Liberia and Sierra Leone (WHO, 2015). This outbreak is the largest ever Ebola virus disease (EVD) epidemic, with more than 27,000 cases, and close to 12,000 fatalities in West Africa (Krahling et al., 2016).

Ebola viruses comprises of a single-stranded RNA genome encoding seven viral proteins, nucleoprotein (NP), glycoprotein (GP), polymerase (L), VP24, VP30, VP35, and VP40. Several methods for detecting Ebola infection have been developed that are useful in clinical laboratory settings (Strong, 2006). Serologic assays that detect host antibodies generated against the virus, antigen detection to detect Ebola viral proteins, and analyzing viral RNA sequences are the broadly used methods for clinical diagnosis. Specific viral antibodies can have a prolonged existence following Ebola infection. However, the variable onset of antibody responses during acute illness makes serology minimally useful as a diagnostic tool in the acute setting. Conversely, antigen detection and molecular tests have proven very effective for acute diagnosis as virus levels in the blood typically raise to high levels within the first few days of symptoms (Towner et al., 2004). The incubation period following EBOV infection typically ranges between 3 and 13 days, but may be as long as 21 days (Broadhurst et al., 2015; Schieffelin et al., 2014). No tests have yet demonstrated the ability to reliably detect EBOV prior to the onset of symptoms.

Although recognizing that rapid and accurate diagnostics are crucial to successful containment of an Ebola outbreak, determining the best strategy and the impact of rapid diagnostic assays is not straightforward. Poor diagnostic specificity risks patients without Ebola infection being admitted to Ebola treatment units (ETUs) where they potentially acquire infection, and use a bed that could otherwise be used by a patient who does have EVD. Conversely, poor diagnostic sensitivity can lead to infected individuals being discharged back to the community or sent to non-Ebola-specific wards, with the consequent risk of onward transmission (Nouvellet et al., 2015).

In resource constrained countries, affordable methodologies for the detection of disease biomarkers at low or trace concentrations can potentially improve the standard of living. However, prevailing strategies for ultrasensitive detection often require sophisticated equipments that may not be available in laboratories with fewer resources and highly skilled personnel (Delarica et al., 2012; Nam et al., 2003). The Ebola VP40 protein being the most abundant protein in virus particles (Elliott et al., 1985) plays an important role in viral particle formation (Noda et al., 2002; Shahhosseini et al., 2007). VP40 is also known for self-assembly of the viral proteins to non-pathogenic filamentous virus like particles, which are highly immunogenic and mimic the morphology and antigenicity to the native virus (Bavari et al., 2002; Noad et al., 2003; Wahl et al., 2005). VP40 potentially serves as a valuable target for diagnostic detection assays due to high viral load in infected individuals in early stages of infection (Ksiazek et al., 1999a; Ksiazek et al., 1999b; Lucht et al., 2003).

Bispecific antibodies (bsAbs) are uniquely engineered antibodies consisting of two different binding sites (paratopes) in a single antibody molecule in comparison with the monoclonal antibodies that have two congruous binding sites (Milstein et al., 1983; Suresh et al., 1986). This

bifuctional design allowed us to develop a bsAb with one site capable of binding Ebola VP40 and the other an enzymatic marker viz. peroxidase in this study. This immunoprobe with intrinsic enzyme marker binding capability can be used directly as a tracer in immunoassays (Archana, 2011; Kreutz et al., 1997; Nolan et al., 1992) approaching the theoretical limit of the specific activity, with every bsAb molecule uniformly bound to the enzyme marker (Malabadi et al., 2012). Antibodies are extremely versatile and are incorporated into a variety of different immunodiagnostic assay platforms such as: microtiter plate assay, swab, strip, filter disk, and 'spinning disc type' assays. bsAbs are attractive in such assays because they simplify the detection steps (Byrne et al., 2013).

This new immunoprobe can be used to develop new generation of assays for clinical laboratories, hospitals, point of care applications and can be adapted to screening devices for physicians' offices as well as health care workers on screening sites.

3.2 Material and methods

3.2.1 Materials and reagents

Cell culture media RPMI 1640 and Penicillin-streptomycin-glutamine (PSG) were purchased from Gibco (New York, USA). Fetal bovine serum (FBS) was purchased from PAA laboratories (Pasching, Austria). Goat anti-mouse IgG conjugated to horseradish peroxidase (GAM-HRP), bovine serum albumin (BSA), polyethylene glycol (PEG) 1300–1600, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), HRP Type IV, Protein G-agarose, *m*-amino phenyl boronic acid (*m*-APBA) agarose, and long chain sulfosuccinimidyl NHS biotin were purchased from Sigma Chemicals (St. Louis, USA). Streptavidin tagged HRP (Strep-HRP) was purchased from BD Biosciences (California, USA). Tetramethylbenzidine (TMB) was purchased from BioFx Laboratory (North Carolina, USA). For Western blots, hybond-ECL

nitrocellulose membranes were procured from Amersham Biosciences, Germany and the blot detection system was procured from GE Healthcare (USA). Nylon fibre swabs were bought from Micro Rheologics (Brescia, Italy). Non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR (Ontario, Canada). Fluorescence activated cell sorter, FACSAria (BD Biosciences, USA), was accessed from the Department of Medical, Microbiology and Immunology, University of Alberta. For protein purification, we used a Biologic Duoflow system (Bio-Rad, USA) and the ELISA absorbance was taken using Versa max microplate reader (Molecular Devices, USA).

3.2.2 Prokaryotic expression and purification of recombinant Ebola VP40 protein

The full length Ebola VP40 protein of makona strain was expressed in the *E.coli* system and purified as previously described (Ganguly et al., 2013). Briefly, the recombinant plasmid containing the codon optimized gene encoding the VP40 protein was transformed into *Escherichia coli BL21* (DE3). Subsequently expression of recombinant protein was induced at 37°C for 6 hour with 1.0mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant protein was dissolved in 8M urea and purified using a high-affinity nickel-nitrilotriaceticacid (Ni-NTA) resin column according to the manufacturer's instructions. The purified protein was analyzed by SDS-PAGE (Laemmli, 1970) and Western blot (Towbin et al., 1979) and its concentration determined using a Bradford Protein Assay (Bradford, 1976). The purified protein was refolded in renaturation buffer. The refolded VP40 was used as the coating antigen for the diagnostic assay developed in this work.

3.2.3 Cell lines for quadroma development

The anti-HRP YP4 is a well-characterized rat hybridoma that was earlier screened for drug resistance to 8-azaguanine, making it sensitive to aminopterine in HAT medium. This cell line

(YP4) along with anti-VP40 Ebola mAb was chosen for developing quadromas (hybridoma × hybridoma) (Ganguly et al., 2015; Suresh et al., 1986). YP4 secretes (IgG2a) monospecific anti-HRP antibodies and was obtained from the late Dr. C. Milstein, Medical Research Council for Molecular Biology, Cambridge, United Kingdom. The cell line producing VP40 was obtained from Dr. Ayato Takada, University of Hokkaido, Japan

3.2.4 Development of anti-Ebola VP40/anti-HRP quadroma cell line

The production of anti-Ebola VP40/anti-HRP quadroma cell line involved maintaining the two hybridoma cell lines (Mouse anti-VP40/16-2 and rat anti-HRP YP4 hybridomas) in logarithmic growth phase containing RPMI (Gibco, New York, USA) medium with 5% FBS (PAA cell culture company, Ontario, Canada) at 37°C supplemented with 5% CO_2. A stock solution of tetramethylrhodamine isothiocyanate (TRITC, 0.5 mg/ml) or fluorescein isothiocyanate (FITC, 0.5 mg/ml), (Sigma, St. Louis, USA) was diluted in 1:5 ratios to be used as the working solution. The following steps as reported earlier were then followed for successful completion of a quadroma fusion (Ganguly et al., 2015; Sunwoo et al., 2013). Briefly 2×10^7 cells/ml of anti-VP40 hybridomas and YP4 hybridomas were separately resuspended in RPMI pH 7.4 and 6.8, respectively. Anti-VP40 hybridomas were then labelled with TRITC (Sigma, St. Louis, USA) (red fluorescence) and YP4 cells were labelled with FITC (Sigma, St. Louis, USA) (green fluorescence). Following 30 min incubation at 37 °C in a 5% CO_2 incubator, the cell suspensions were washed and mixed in a 50 ml tube and centrifuged at 459 × *g* for 7 min. Two milliliter of PEG (Sigma, St. Louis, USA) was added to the cell pellet slowly over a period of 2 min, with gentle mixing. Following addition of PEG (Sigma, St. Louis, USA), the cell suspension was incubated at 37°C for 3 min. Twenty milliliter of serum-free RPMI medium was added to dilute the toxic effects of PEG (Sigma, St. Louis, USA). Flow cytometry (FACSAria,

Becton Dickinson) with an argon ion 488 nm air cooled laser (Department of Medical Microbiology and Immunology, University of Alberta) was used to sort cells with dual fluorescence and seeded at 1 cell/well in 96 well sterile culture plates with RPMI (Gibco, New York, USA) containing 20% FBS (PAA cell culture company, Ontario, Canada). The plates were incubated at 37°C with 5% CO_2. The clones were screened twice by bridge ELISA (ELISA plates were purchased from Nunc International Maxisorp, New York, USA). The best clones were subject to recloning to select strong positive and highly specific quadroma secreting bsAbs against Ebola VP40.

3.2.5 Bridge ELISA screening of anti-Ebola VP40/anti-HRP quadromas secreting bsAbs

Bridge ELISA was used for all the screening procedures after 10-12 days of quadroma fusion. This involved coating the 96-well ELISA plate with 100 µl of 5 µg/ml Ebola VP40 antigen as solid phase. The microtiter plate was then incubated at 4°C overnight, washed with PBS-T (phosphate buffer saline, pH 7.4 with 0.05% Tween-20) 3 times and blocked with 3% bovine serum albumin (BSA) in PBS-T for 2 hours at 37°C. After washing, 100 µl of the serially diluted (neat, 1:10, 1:100, 1:1,000) quadroma supernatant was added to the plate and further incubated for 1 hour at 37°C. A 100 µl of 10 µg/ml HRP was then added to the microtiter plate followed by another washing step. Finally, 100 µl of TMB substrate was added to the plate. The positive quadromas were then selected after 15 min of color development and the plate was read at 650 nm on V_{max} ELISA plate reader. RPMI media and BSA were used as blanks. Negative controls were wells without VP40 antigen and HRP.

3.2.6 Purification of anti-Ebola VP40/anti-HRP bsAb

The purification of the bsAb was done by using m-aminophenylboronic acid agarose column (m-APBA) as described (Bhatnagar et al., 2008; Sarkar et al., 2012). Briefly the APBA column

was saturated with HRP to capture the bsAb along with the monospecific anti-HRP mAb eliminating the monospecific anti-VP40 species. Following a short wash procedure, the bound antibodies were eluted with PBS containing 0.1M sorbitol. The pooled elutes were finally dialyzed with PBS (Gibco Inc., USA) at pH 7.2. Protein estimation was done by Bradford assay (Bradford, 1976).

3.2.7 Purification of Ebola mAb

The purification and characterization of the anti-Ebola VP40/16-2 mAb' was performed by affinity chromatography according to the published protocol (Das, et al., 2005; Ganguly, et al., 2015). This purified mAb antibody was subsequently used in the diagnostic assay, as the capture antibody.

3.2.8 Assay optimization

The different attributes of the assay such as concentrations of capture antibody (cAb), and detection antibody (dAb), were optimized independently. Optimization of the assay parameters for the assay design is discussed in the next section.

3.2.8.1 Optimization of capture antibody concentration

Purified VP40 mAb was used as cAb in the assay. A microtitre plate was coated overnight at 4°C. Different concentrations of VP40 mAb (100 µl) ranging from 500-12,000 ng/ml were used in triplicates. The unbound binding sites on the plate were blocked with 200 µl of 3% BSA in PBS at 37°C for 3 hours. 100 µl of 5 µg/ml recombinant VP40 antigen was added and incubated for 2 hours, and subsequently 4 µg/ml of bispecific antibody (dAb) was added and incubated for 1 hour. The plate was washed 5 times with PBS-T after each of the steps described above. Finally, TMB was added and color development was measured at 650 nm using the microplate

reader. The mean of three readings for each concentration was plotted against the corresponding concentration using Microsoft Excel.

3.2.8.2 Optimization of detection antibody concentration

The bsAb was used as the detection antibody. A fixed concentration of cAb (2 µg/ml) was used to coat a microtiter plate and varied concentrations of dAb ranging from 0.25 to 60 ng/ml were used. The assay protocol and the concentration of the other parameters were same as described in the earlier section and the data were similarly analyzed.

3.2.9 Sandwich ELISA using bsAb

Except specifically mentioned, all incubation steps were performed at 37°C for 1 hour. Washing 5 times was conducted by PBS-T between each step. Plates were coated with 100 µl of purified anti-Ebola in 100mM carbonate buffer (pH 9.6). Non-specific sites were blocked with 200 µl of blocking buffer (3% (w/v) BSA in PBS-T) at 37°C for 1 hour. A volume of 100 µl of Ebola VP40 antigen (serial dilution in 1% (w/v) BSA in PBS-T) was added to the wells, which was then followed by 100 µl of bsAb-HRP complex. The plates were washed 5 times and TMB substrate was added for color development and absorbance was measured at 650 nm after 5 min incubation using an ELISA plate reader (Ganguly et al., 2013; Sunwoo et al., 2013).

3.2.10 Evaluation of assay with human sera

Clinical samples from Ebola infected patients being not available, we simulated the antigen with human serum samples purchased from Sigma (Catalog no. H4522). Ebola VP40 antigen was spiked in the samples in different concentrations. Non-spiked samples were used as control. 96 well plates were coated overnight at 4°C with anti-Ebola VP40/16-2 mAb diluted to 2 µg/ml in 0.1M bicarbonate buffer (pH 9.6). The plates were then washed with PBS and blocked with 3%

BSA for 2 hour at 37°C. The plates were washed again with PBS-T. 100 µl of the samples were added to the wells and incubated for 2 hour at 37°C. After washing the plates again with PBS-T, bsAb was added as the detecting antibody for 30 min at 37°C. The plates were washed again with PBS-T and TMB substrate was added. Absorbance was measured at 650 nm. The assay was performed in triplicates.

3.2.11 Statistical analyses

The student's *t*-test (one-tailed *t*-test) was used to characterize the significant difference between the control (zero antigen) and samples.

3.3 Results

3.3.1 Expression and purification of recombinant Ebola VP40 protein

The purified VP40 was analyzed by SDS-PAGE (Refer Figure 2.1A). The specificity was determined by Western blotting, which demonstrated that VP40/16-2 mAb binds strongly to the VP40 antigen (Refer Figure 2.1B). No cross-reactivity of mAb with other viral recombinant proteins including nucleocapsid protein (NP) of Severe acute respiratory syndrome, NS1 envelope derived protein from Dengue virus or GP from Ebola virus was observed.

3.3.2 Quadroma development

The dual labelled fused cells were sorted by FACS and the fusion efficiency was found to be 16% (Figure 3.1). The positive cells were screened by bridge ELISA and were repeatedly cloned by the limiting dilution method to increase the cloning efficiency. Cloning efficiency was calculated after each re-cloning by using established methods (Kammila et al., 2008; Sarkar et al., 2012). An increase in activity and cloning efficiency was observed with each subsequent re-

cloning. After 5 rounds of re-cloning, a robust quadroma clone (having cloning efficiency higher than 90%) secreting bsAb was isolated and expanded.

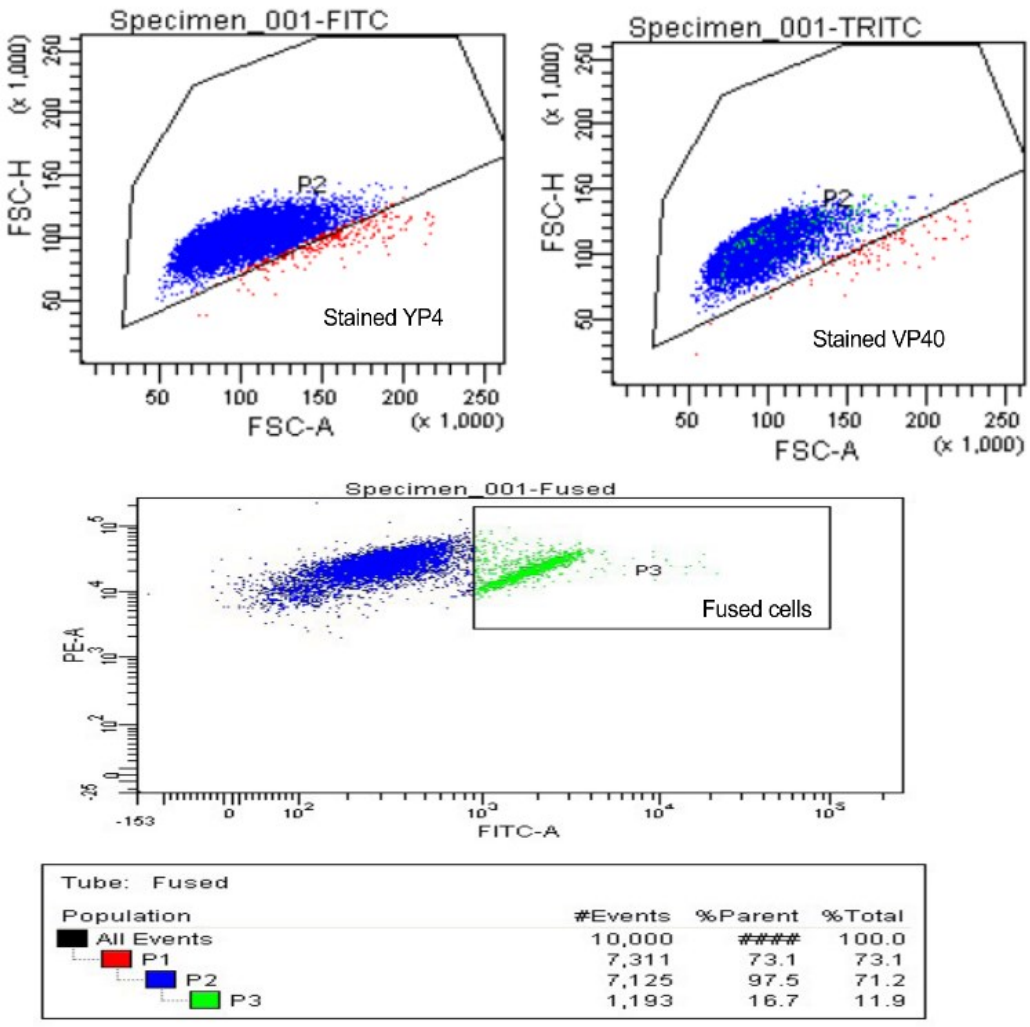


Figure 3.1: Fluorescence activated cell sorting of bispecific cells : Specimen-001 FITC are YP4 cells stained with fluorescein isothiocyanate dye (FITC) and specimen-001 TRITC are VP40/16-2 cells stained with tetramethylrhodamine dye. Specimen-001 fused shows the fusion of YP4 and VP40/16-2 cells in P3 region. The table shows that P3 region has 16.7% of fused cells obtained from parent cells.

3.3.3 Purification of bsAb

As discussed earlier the two-stage purification of bsAb was performed according to our established procedure (Bhatnagar et al., 2008; Ganguly et al., 2014) (Figure 3.2). The purified fraction from the initial step comprised of an assortment of three antibodies, two parent mAbs (VP40/16-2 and YP4) and bsAb, all of them being the IgG isotype. A second stage of purification was performed using m-amino phenyl boronic acid (m-APBA) agarose to remove the VP40 16-2 mAb as its presence could impede the sensitivity of the assay. The bsAb obtained from m-APBA column was complexed to the reporter molecule HRP via the HRP-binding paratope. After two stages of purification an increased bsAb activity was observed in the ELISA activity assay. The extent of purification was confirmed by SDS-PAGE, three distinct bands were observed: heavy chain (~50 kDa) and light chain (~25 kDa) of IgG antibody, and HRP (~44 kDa) (Figure 3.3).

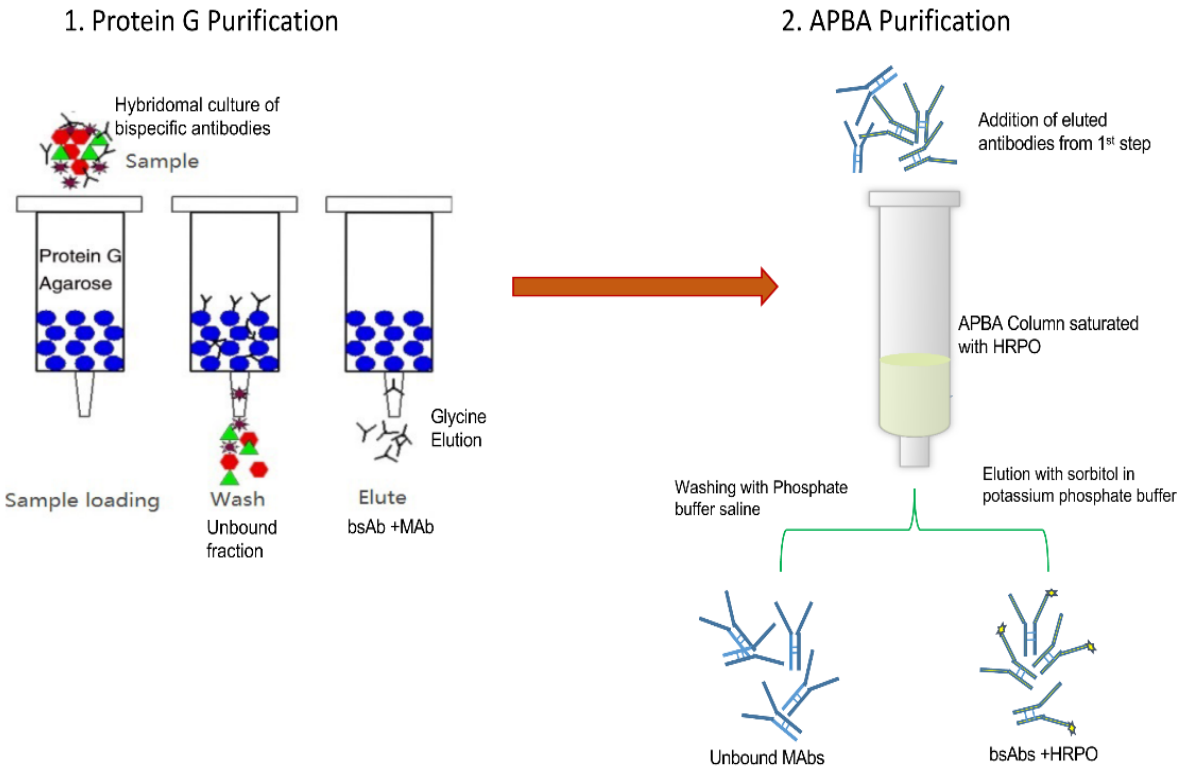


Figure 3.2: Two step purification of bispecific antibodies. Step 1: Purification of bispecific hybridomal culture with Protein G column to obtain antibodies; Step 2: Purification of antibodies obtained from step 1 with APBA column saturated with HRP to obtain bispecific antibodies tagged with HRP.

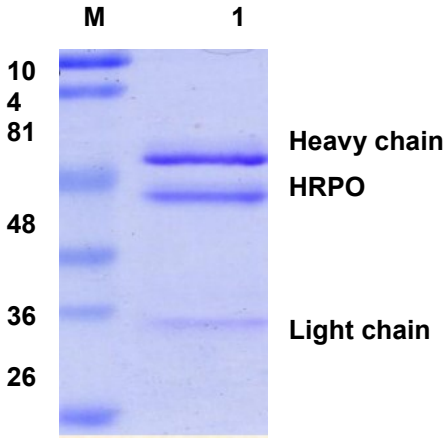


Figure 3.3: SDS-PAGE in denaturing condition showing bands of bispecific antibody. Lane M : Molecular marker ; Lane 1 : bispecific antibody (VP40/16-2 x YP4).

3.3.4 Optimization of assay parameters

After obtaining the purified anti Ebola mAb and bsAb, vital parameters of the assay were optimized by sandwich ELISA. Two different sets of assays with different concentrations of cAb and dAb were performed in triplicate and repeated twice independently for precision assessment. In each optimization assay, only one parameter was varied while the others were kept constant. The mean of three absorbance values was plotted against the respective concentration using standard procedures. Each parameter of the assay showed an initial steady increase in absorbance value with increasing concentration ultimately saturating becoming parallel to the concentration axis. The optimal concentration for cAb was found to be 2 μg/ml ($P<0.05$) (Figure 3.4A) and 14 ng/ml for dAb ($P<0.05$) (Figure 3.4B).

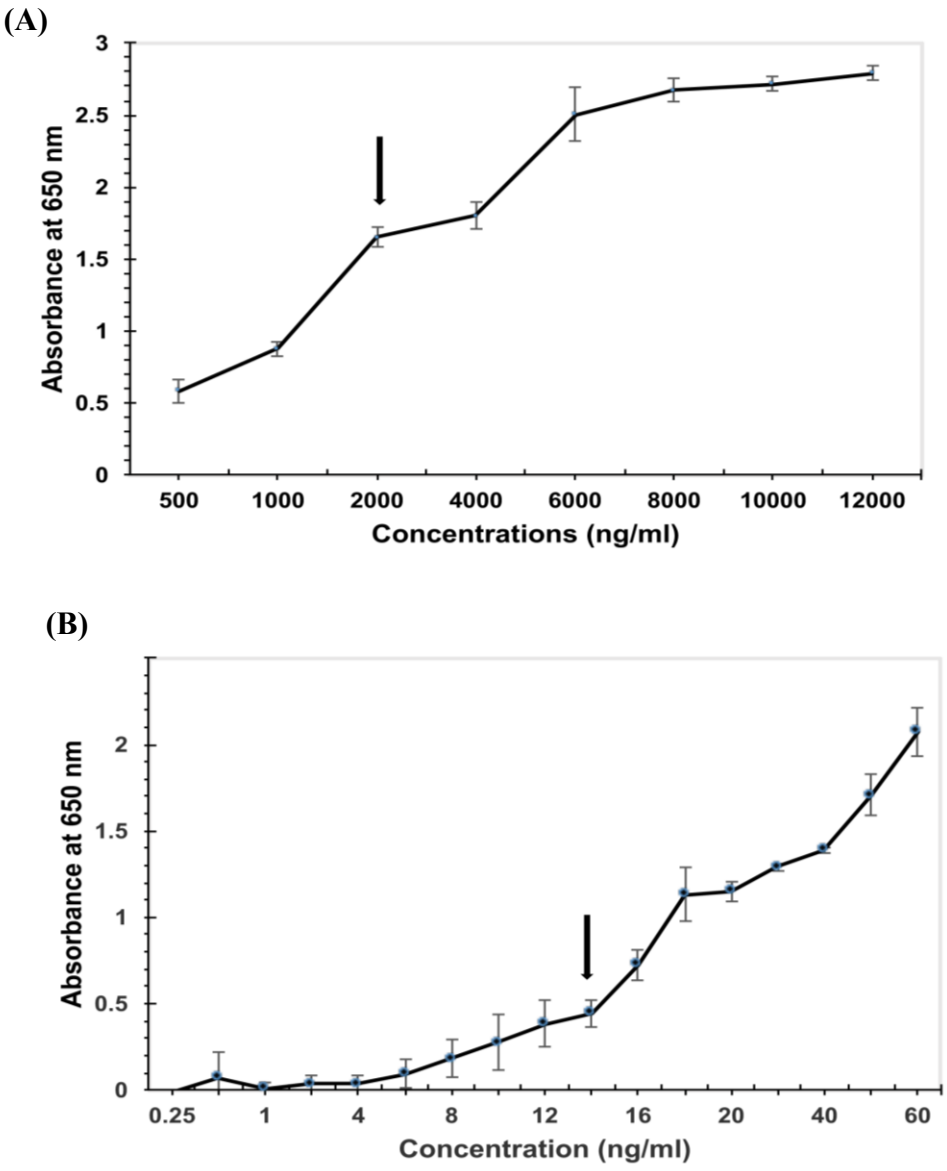


Figure 3.4: Development of DAS-ELISA-MAb-bsAb to optimize the concentration of two different antibodies. Arrows indicate the optimized concentration. Standard deviation is shown in vertical bars. **(A)** Optimization of capture antibody (mAb). **(B)** Optimization of detection antibody (bsAb).

3.3.5 Diagnostic analysis of sandwich ELISA with bsAb

The sandwich ELISA for detection of the Ebola VP40 antigen using anti-Ebola VP40/16-2 mAb as a coating antibody and novel bsAb-HRP as a detection antibody is illustrated in Figure 3.5. The limit of detection was found to be 9.72 ng/ml (Figure 3.6).

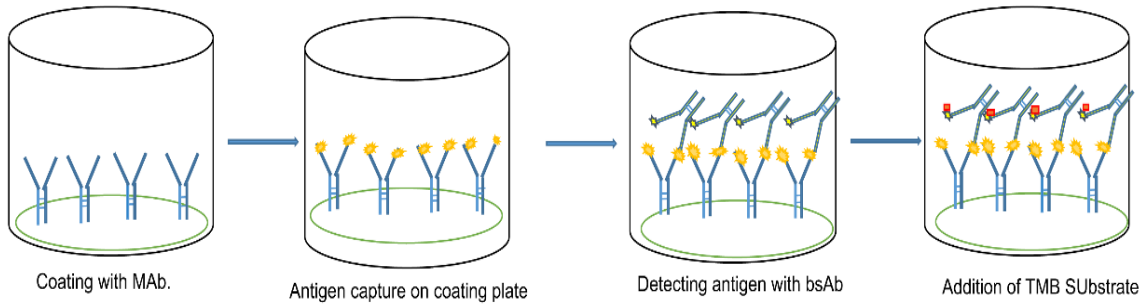


Figure 3.5: Sandwich ELISA with monoclonal antibody (VP40/16-2) as capture antibody and bispecific antibodies as detecting antibody.

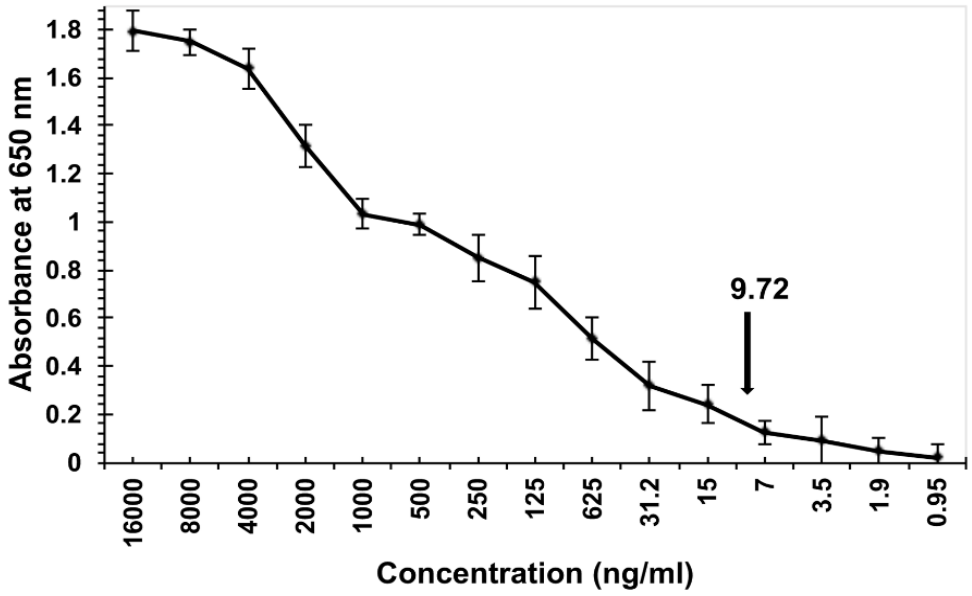


Figure 3.6: Limit of detection of VP40 in PBS for DAS-ELISA-mAb-bsAb. Arrow shows the limit of detection. Standard deviation is shown in vertical bars.

3.3.6 Simulation of diagnostic assay with human clinical sera

The assay was able to successfully detect the rVP40 antigen spiked in human sera in the nanogram range. Control sample that were not spiked with the recombinant antigen gave negative result. We diluted the sera 2-fold to eliminate the hindering effect of matrix and endogenous proteins. The sensitivity of the assay corresponded to approximately the similar range and limit of detection was found to be 6.28 ng/ml (Figure 3.7).

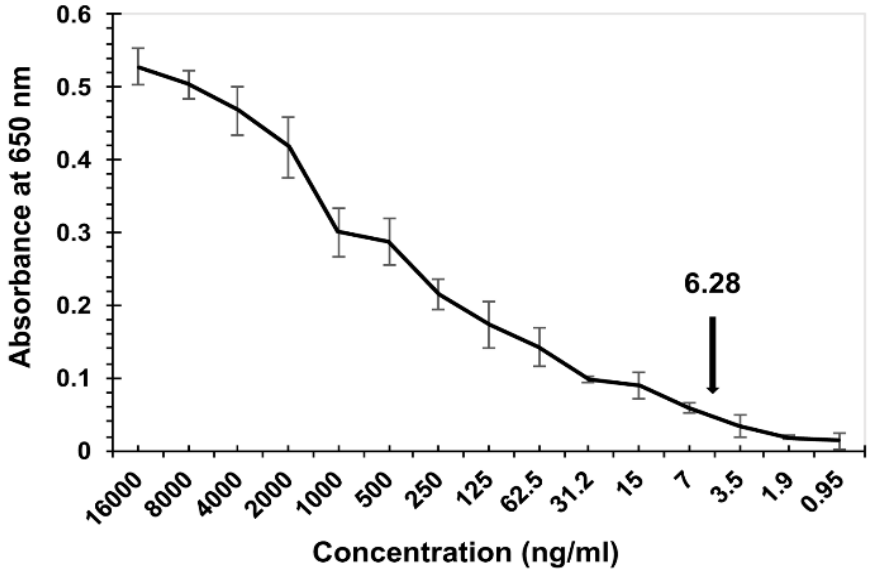


Figure 3.7: Limit of detection of VP40 in spiked serum samples for DAS-ELISA-mAb-bsAb. Arrow shows the limit of detection. Standard deviation is shown in vertical bars.

3.4 Discussion

The Ebola endemic of 2014 had devastating effect in West Africa where most countries have limited economic arsenal to combat and manage such an outbreak effectively (Yoshida et al., 2016). Affordable diagnostics for the detection of disease biomarkers at low concentrations can greatly improve disease management and arrest spreading of infection across. However, current strategies for Ebola detection such as PCR, virus isolation, antibody detection assays (Boisen et

al., 2015; Lucht et al., 2007) have either specificity issues or require sophisticated infrastructure that may not be available in laboratories with modest resources. Here, we tried to address this problem by developing a bispecific probe based assay that enables ultrasensitive detection of Ebola VP40 antigen which constitutes nearly 38% of the Ebola viral structure thereby a suitable diagnostic marker (Elliott et al., 1985; Shahhosseini et al., 2007).

We have shown the detection of Ebola VP40 antigen by designing a new antibody entity with higher sensitivity and reduced cost that provides assay output in much less time compared to conventional ELISA. Potent detection agents, no-frills methodology and ultrasensitive detection of VP40 could be highly advantageous for diagnosing Ebola infection in developing countries. The total assay time is much less in this format as the number of steps reduced from a conventional monoclonal antibody based sandwich immunoassay. The detection limit in spiked human sera with 2-fold dilution was 6.28 ng/ml. This is more sensitive than existing assays by multiple times.

Assays involving monoclonal and polyclonal antibodies necessitate a secondary antibody enzyme conjugate, which is usually done by chemical conjugation and lacks consistent batch to batch results (Cao et al., 1998). The purified bsAb is already saturated with HRP thereby reducing additional steps such as secondary antibody addition and associated washing. This significantly cuts the time taken to perform the assay.

Enzymes labelled antibodies are routinely incorporated in biochemical and immunological assays where the enzyme is covalently linked to the antibody moiety (Hermanson, 1996). Chemical cross-linking can be inconsistent and therefore inactivation of either the enzyme or the antibody is a possibility (Kricka, 1994; Milstein et al., 1983). Inappropriate cross-linking could also cause nonspecific binding, which would enhance assay background (Kreutz et al., 1997).

Specific activity of bsAb is theoretically considered one in which every molecule is uniformly associated to a signal moiety (Tang et al., 2004). Based on this rationale, the quadroma derived designer bsAb that has one antigen-binding site capable of binding to VP40 and the other capable of binding to an enzymatic marker (HRP). Assessing other combinations to generate unique bispecific probes would be beneficial for rapid screening.

Future studies would be focused on improving the assay by integrating to better user-friendly platforms for point of care analysis of samples for better management of disease outbreak. The next stages of research would be adapting our single site ELISA detection to a visual diagnostic, which would be apt for laboratories and health care centers with limited resources. The study demonstrated here could potentially be adapted for the detection of other Ebola biomarkers as well and helps mitigate diagnostic shortcomings in case of a future outbreak.

CHAPTER 4: Conclusions

Ebola Virus Disease (EVD) is a major public health concern with a high mortality rate in infected individuals. The outbreaks of Ebola have been widespread due to the unavailability of rapid, sensitive, specific and affordable diagnostics. Overlapping symptoms of other diseases such as malaria and cholera make it difficult to diagnose. For clinical management, outbreak investigation and proper surveillance, EVD requires a detection system, which should be fast, sensitive, specific, efficient, durable, user friendly with in-country staff and affordable.

Filoviruses are among the most lethal human viral pathogens. There are three genera of filovirus: Marburgvirus, Cuevavirus and Ebolavirus (EBOV). The structure of EBOV contains an envelope (GP), viral matrix (VP24 and VP40) and nucleocapsid proteins (NP, VP35 and VP30). The virion matrix VP40, a structural protein of Ebola virus, is a suitable target for developing diagnostics of Ebola infection. This protein is present in large amounts in the blood during the acute phase of the disease (within 7 days). Studies have confirmed that VP40, GP and NP structural proteins are suitable biomarkers for detection of Ebola viral infection. Screening Ebola virus antigens would be more beneficial for developing early accelerated diagnostics. In our current research, we hypothesized that Double Antibody Sandwich Immunoassay (DAS-ELISA) based on IgY, biotin labeled monoclonal antibody (bmAb), and bispecific antibody (bsAb) can detect nanogram levels of antigen (VP40) in sera of humans (within 7 days) who may be infected with EVD.

The objectives of this study were (1) to produce VP40 Protein by the recombinant technology, (2) to produce three different types of antibodies: IgY, mAb, and bsAb, (3) to optimize three different formats of DAS-ELISA, and (4) to determine LOD of VP40 in each DAS-ELISA format in PBS and human sera.

In the first objective, we efficiently expressed codon optimized recombinant VP40 in *E. coli* to be used as a model antigen in the development of the assays. The purity and expression of rVP40 was analyzed and confirmed by SDS-PAGE and western blot analyses. The expression of rVP40 protein checked by SDS-PAGE. Expressed rVP40 protein thin bands at 37 kDa was observed after 2 hours of IPTG induction, whereas thick and intense bands of VP40 protein at 37 kDa were observed after 18 hours of induction. This indicates the use of IPTG inducer increased yield of rVP40 during the *E.coli* protein expression. The optimal conditions for VP40 protein expression were 37°C temperature and 18 hour induction time. The expressed rVP40 protein was purified by IMAC. The yield of recombinant protein purified by metal ion affinity column was 12 mg/l of bacterial culture by Bradford protein assay.

In the second objective three different antibodies including IgY, mAb and bsAb were produced. Firstly, to produce IgY antibodies from the egg yolk, the recombinant VP40 was immunized into chickens to produce specific IgY. The yield of specific IgY is about 720 µg per egg yolk,The high titers of IgY was obtained from immunized chickens. The indirect ELISA was performed with different dilutions of IgY antibody such as 1:1600, 1:3200, 1:6400, 1:12,800, 1:25,600, 1:51,200 in PBS-T to measure titers of IgY during the immunization period. It was observed that even 1:6400 dilution of IgY antibody showed high titer. The indirect ELISA results confirmed that the IgY titer was increased with booster immunization and peaked at 7 weeks. Specificity and cross reactivity studies were also performed to validate the robustness of the assay. Secondly, to produce mAb nine different anti-VP40 hybridoma cell lines (VP40/3-1, VP40/12-2, VP40/16-2, VP40/21-6, VP40/24-6, VP40/25-2, VP40/26-3, VP40/29-3, VP40/5-3-2) were cultured and harvested followed by the screening of all nine different clones of VP40 by indirect ELISA. The results showed that the VP40/16-2 clone has a high yield of mAbs.

VP40/16-2 was further cultured and purified by Protein G affinity chromatography. SDS-PAGE, under reducing conditions, of column-purified supernatant showed a heavy chain band at 50 kDa and light chain band at 28 kDa, and no other band of any other protein, confirming the purity of our anti-VP40 mAb. The yield of mAb obtained from VP40/16-2 clone after purification was 10 mg/l of hybridoma cell culture supernatant as measured by the Bradford protein assay. The isotyping results of the mAbs showed that the mAbs heavy chain was of the IgG1 type, whereas the light chain was kappa (k), indicating that the mAb is of IgG1 subclass in IgG type antibodies isotype. Lastly, to produce bsAb, the fusion of anti-VP40 hybridoma cell line clone (VP40/16-2) and anti-HRPO hybridoma cell line (YP4) was done by quadroma technology. The percentage of fused double positive cells obtained by FACS sorting was 16%. The positive cells after fusion were screened by bridge ELISA and were repeatedly cloned by the limiting dilution method to increase the cloning efficiency. After each subsequent cloning, an increase in activity and cloning efficiency was observed. After 4-5 rounds of re-cloning, a robust quadroma clone (having cloning efficiency higher than 90%) secreting bsAb was isolated and expanded. The two-stage purification of bsAb was performed, quadromas were purified by protein G column followed by APBA column. The yield of purified bsAb obtained after purification was approximately 7.5 mg/l of culture supernatant. After purification of bsAb, its activity was observed in the ELISA assay. The extent of purification was confirmed by SDS-PAGE; three distinct bands were observed: heavy chain (~50 kDa) and light chain (~25 kDa) of IgG antibody, and HRPO (~44 kDa).

In the third objective three different formats of DAS-ELISA were optimized: (i) We first designed a quantitative heterosandwich DAS-ELISA-mAb-IgY for Ebola VP40 detection, using a combination of VP40 specific chicken IgY (detecting antibody) and VP40/16-2 monoclonal

antibody (capture antibody), the optimized concentration of capture antibody as mAb (McAb) was 4000ng/ml in the range of 500 to 12000 ng/ml , detecting antibody (dAb) IgY was 8 µg/ml in the range of 0-16 µg/ml and the optimized dilution of anti-chicken IgY at 1:10,000 in the dilution range of 4,000 to 48,000.(ii) The second format of immunoassay was DAS-ELISA-mAb-bmAb, it involved the VP40/16-2 monoclonal antibody both as a capture and detecting antibody. The detecting antibody was biotin labeled and streptavidin-HRPO was used as conjugate. The optimized concentration of McAb was 4000ng/ml in the range of 500 to 12000 ng/ml, bmAb was 31.25 ng/ml in the range of 16000 to 1.95 ng/ml and the optimized dilution of streptavidin-HRP at 1:10,000 in the dilution range of 4,000 to 48,000 (iii) The third format of immunoassay was DAS-ELISA-mAb-bsAb, based on VP40/16-2 mAb as a capture antibody and the bsAb as a detection antibody. The optimized concentration of McAb was 2 µg/ml in the dilutions of 500 to 12000 ng/ml and bsAb was 14 ng/ml in the dilutions of 0.5 to 60 ng/ml.

In the fourth objective the limit of detection (LOD) was determined for each assay. (i) The LOD of DAS-ELISA-mAb-IgY assay could detect viral antigen as low as 3.3 ng in PBS. The assay employed chicken IgY antibodies for the first time for Ebola detection. (ii) The LOD of homosandwich DAS-ELISA-mAb-bmAb assay was 23 ng/ml. The assay can potentially detect 2.3 ng of viral antigen in PBS and 71.25 ng/ml of spiked VP40 in human sera. The use of biotin labeled mAb is considered a better option as streptavidin binds to Biotin with high affinity. The rapid non-covalent interaction and greater specificity are some of the other characteristics which make biotinylated molecules important in immunodiagnostics. (iii) The LOD of DAS-ELISA-mAb-bsAb assay was 9.72 ng/ml recombinant VP40 or approximately 1 ng in PBS and 6.28 ng/ml of spiked VP40 in human sera at two fold serial dilution, thereby enhancing the sensitivity 2-3 fold over the other two formats DAS-ELISA-mAb-IgY and DAS-ELISA-mAb-bmAb

developed. An important feature of the assay is the reduced number of steps, especially the addition of secondary antibody or conjugate as one arm of bi-specific antibodies was tagged with HRPO. This leads to obtaining results faster and also fewer steps signify less reagents used and less man hours, resulting in less background, high sensitivity and lower cost of diagnosis.

In summary, we proved the hypotheses proposed in the thesis and completed all the objectives designed. Optimization of these new reagents allows us to develop easy to use Ebola diagnostics like the DAS-ELISA-mAb-bsAb and economical DAS-ELISA-mAb-IgY assay for the diagnosis of the Ebola virus structural protein VP40 in PBS and human sera samples. We developed the second generation, self-assembling bsAb with two different binding specificities. This bsAb based ELISA was found to be highly sensitive diagnostics for the detection of Ebola VP40 antigen in comparison to the homosandwich mAb (biotin-labeled) based format. Another immunoassay involving chicken IgY as a less costly diagnostic tool with good sensitivity was also successfully developed as a supplementary diagnostic to detect Ebola infection. There are many advantages of producing IgY from chicken eggs, primarily the procedure being non-invasive in nature and does not involve euthanizing the animal. Secondly, the cost of production is very low compared to rabbit and goat polyclonal antibodies. These immunoassays developed with IgY as a detecting antibody could be potent and sensitive methods of diagnosing suspected individuals and screening patients in clinical settings, potentiating on-site diagnostic assay. The assay protocol described in this study can be used as a platform for developing point of care diagnostic assay.

In conclusion, DAS-ELISA-mAb-bsAb showed highest sensitivity and lowest LOD for VP40 antigen of 9.72 ng/ml in PBS and 6.28 ng/ml in spiked human sera (two fold serial dilution) among three different formats. The assay developed is far more than sensitive than the exisiting

ones. Lucht's group developed ELISA assay and used a capture antibody concentration of 10 µg/ml, whereas in our assays DAS-ELISA-mAb-IgY and DAS-ELISA-mAb-bmAb, the concentration of capture antibody used was 4 µg/ml. Niikura and coworkers developed another ELISA assay to detect nucleoprotein antigen with a sensitivity of 0.3 µg/ml, which is almost 10 times less sensitive than our DAS-ELISA assays. The designed DAS-ELISA assay has all the potential to serve as an ideal detection for Ebola virus disease, as the assay is affordable, sensitive, specific, user-friendly, rapid and robust and equipment-free. These parameters, identified by the acronym ASSURED characterize this as an ideal diagnosis test.

Limitations of study

In my opinion, the limitation of the assay could be the qualitative visual analysis. It can show inter-observer variations, which can influence the sensitivity. To minimize the inter-observer variability, the result of the assay should always be compared with a robust negative control and ELISA results should always be confirmed with quantitative analysis.

The first format DAS-ELISA-mAb-IgY showed good sensitivity. Polyclonal IgY did not showed cross reaction with other viral proteins in western blot whereas with rVP40 protein it showed three bands (Figure 2.6 A). This may be due to the binding of polyclonal IgY with other fragments or linker protein of IgY. Therefore, the specificity of IgY should be validated accurately.

The DAS-ELISA-mAb-bmAb based assay might show a slightly higher background because of the random conjugation process involved in labeling of mAb. This can lead to the altered ratio of the mAb and biotin. As the conjugation of biotin to the site is variable, the arbitrary conjugation process can lead to the partial blocking of the antigen-binding site.

The use of same monoclonal antibody as capture and detection in DAS-ELISA-mAb-bmAb format may decrease the sensitivity due to the masking of epitope. This issue can be overcome by other format of DAS-ELISA-mAb-IgY, as it is a polyclonal antibody and can bind to multiple epitopes of VP40.

The structural study on the recombinant protein should be done to know more about the antigenic sites of the rVP40 protein. It will help us in developing a sensitive and more accurate diagnostic assay.

The third format DAS-ELISA-mAb-bsAb could be a costly choice of diagnostic assay as it involves the production of bsAb (fusion of two mAbs) which is not very cost effective process.

Future directions

Future directions that can be suggested from this study are as follows:

1. The assay has to be evaluated with various human biological samples (Blood, Urine and Saliva).Urine sample collection will be a good option as it is non-invasive. The sensitivity with each biological sample has to be evaluated and compared so as to know which sample is best for diagnosis of EVD at early stage.
2. Development of other novel platforms for rapid and point of care detection for Ebola disease by targeting specific antigens (VP40) such as chip based assay, oral immunoswab assay.
3. More study on the structure of rVP40 protein has to be done, to know more about the antigenic sites. The Native SDS-PAGE should be run to know about the refolding characteristics.
4. DAS-ELISA-mAb-IgY assay format results targeting VP40 antigen could be used to make point of care immunoswab assay and the sensitivity with oral samples can be tested.

5. Other antigens of EBOV such as glycoprotein and nucleoprotein can be tested and compared to VP40 antigen for developing better and more sensitive diagnostic assay for the early detection of EVD.

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