University of Kent

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Sero-prevalence of Ebola and Lassa virus in the Republic of Guinea, Macenta Prefecture.

by

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Abstract

Following the 2013–2016 West Africa *Ebolavirus* (EBOV) outbreak, a broad range of scientific research has been conducted to understand and prevent future epidemics. Our current study, supported by the United States Food and Drug Administration (FDA), uses sero-epidemiology to look at the footprint incidence of EBOV and Lassa virus (LASV) in the prefecture of Macenta located in forested Guinea. Our study assesses seroprevalence of these zoonotic viruses in bushmeat hunters and their household members. These groups of people are at risk of exposure to multiple viral zoonotic infections via constant contact with wildlife, either by hunting, trapping or butchering. In order to investigate potential historical EBOV and/or Lassa virus LASV infections, serum samples were collected from villages that were affected or unaffected by the 2013-2016 EBOV outbreak in Guinea. We performed Enzyme-Linked Immunosorbent Assay (ELISA), western blot analysis and flow cytometry for the detection of EBOV glycoprotein (GP) and LASV nucleoprotein (NP) antigens specific immunoglobulin G. A number of positive samples were detected for both pathogens, suggesting that these two pathogens are circulating in Guinea and may cause mild or asymptomatic infection in a proportion of cases. Importantly, this study suggests EBOV may have been circulating in Guinea before the 2013-2016 outbreak.

Hypothesis of the Thesis

The primary purpose of this study is to assess to what extent *Ebolavirus* has been circulating within the forested region of Guinea prior to the 2013-2016 Ebola virus disease (EVD) outbreak in West Africa. Due to the nature of their work, bushmeat hunters and their household family members are deemed to be at high risk of *Ebolavirus* contamination and spill over. We then established a cohort of bushmeat hunters and their immediate families and collected serum samples from volunteers. We looked for the presence of anti-*Ebolavirus* antibodies to determine previous exposure to the virus. Our cohort includes bushmeat hunters from villages affected by the 2013-16 *Ebolavirus* outbreak and villages that were not affected. Therefore, we hypothesized that a number of samples from previously non exposed villages to EVD will contain anti-*Ebolavirus* antibodies, suggesting that the virus has been circulating or still circulate in the zone prior to the past Ebola virus outbreak.

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Abbreviation list

1-ethyl-3-[3-dimethlyaminopropyl] carbodiimide-HCl	EDC
3,3′,5,5′-Tetramethylbenzidine	TMB
Antigen presenting cells	APCs
Bernard Notch Institute of Tropical Medicine	BNITM
Center of Disease Prevention	CDC
Centre d'Excellence de Formation et Recherche sur le Paludisme et les maladies Prioritaires en Guinée.	CEFORPAG
Cytotoxic T lymphocytes	CTLs
Democratic Republic of Congo	DRC
Dendritic cells	DC
Dibasic sodium phosphate	DSP
Ebola treatment center	CTE
Ebola virus	EBOV
Ebola virus disease	EVD
Electron microscopy	EM
Enhanced chemiluminescence	ECL
Enzyme Immuno-Assay	EIA
Enzyme linked immunosorbent assay	ELISA
Food and Drug Administration	FDA
GB virus C	GBV-C
Glycoprotein	GP
Guanario virus	GTOV
Human cytomegalovirus	HCMV
Human immunodeficiency virus	HIV
Indirect fluorescent antibody	IFA
Interferon	IFN
International committee on taxonomy of viruses	ICTV
Junin virus	JUNV
Lassa haemorrhagic fever	LHF
Lassa virus	LASV
Late endosome	LE
LIoviu virus	LLOV
lower limit of detection	LLD
Lymphocytic choriomeningitis	LCMV
lymphocytic choriomeningitis virus	LCMV
Machupo virus	MACV
Macrophages	MP
Microbiological Research Establishments	MRE
Monobasic sodium phosphate	MSP
Monoclonal antibodies	mAbs
Mopeia virus	MOPV
Multiple organ failure	MOF
National Health Service	NHS
National security agency system for disease surveillance	ANSS
Natural killer	NK
Neutralising antibodies	Nabs
Non-human primates	NHPs

Normal goat serum	NGS
Nucleoprotein	NP
Peripheral blood mononuclear cells	PBMCs
Phosphate Buffered Saline	PBS
Pichinide virus	PICV
Pirital virus	PIRV
p-Nitro phenyl phosphate	p-NPP
Polyvinylidene difluoride	PVDF
Prefectural Health Direction	DPS
Reston ebolavirus	RESTV
Reverse transcription polymerase chain reaction	RT-PCR
Ribonucleoprotein	RNP
Sabea virus	SABV
Single-stranded RNA genome	ssRNA
Size exclusion chromatography	SEC
Sudan ebolavirus	SUDV
T cell receptor	TCR
T regulatory	Treg
Tacaribe virus	TCRV
Upper limit of quantification	ULQ
Vesicular stomatitis virus	VSV
Viral haemorrhagic fever	VHF
Viral ribonucleoprotein	vRNP
Virion proteins	VP
Virus-like particles	VLPs
Whitewater arroyo virus	WWAV
World health organization	WHO
Yambuku Mission Hospital	YMH
Zika virus	ZIKV

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DECLARATION OF AUTHORSHIP

I, JOSEPH AKOI BORE

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.
SERO-PREVALENCE OF EBOLA AND LASSA VIRUS IN THE REPUBLIC OF GUINEA, MACENTA PREFECTURE

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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Date: 28 JANUARY 2020

Chapter 1: GENERAL INTRODUCTION

1.1 Ebola virus

Over the last 50 years, there has been a large increase in the occurrence of new diseases that affect humans. Many of these emerging infectious diseases are zoonotic in nature, in that they are transmitted from animal to human. Increased contact between humans and wildlife through activities such as agriculture and hunting provide an opportunity for these pathogens to spill-over and cause disease in the human population. *Ebolavirus* (EBOV) are one such zoonotic pathogen, causing high rates of mortality and social unrest.

1.1.1 Discovery of Ebola virus

EBOV was first discovered in 1976 in Central Africa near the river Ebola in Bomba zone at Yambuku village[1], the virus was named after the Ebola river which means "Clear" in the local language. Initially, this previously unknown haemorrhagic fever outbreak was thought to have started in South-Sudan and then later expanded to the Northern region of the Democratic Republic of Congo (DRC), formally Zaire[1]. It is reported that a 42-year old patient was admitted to Yambuku Mission Hospital (YMH) with an unknown haemorrhagic fever and was treated for malaria[1]. Later, several patients from the zone treated in YMH with non-sterilized materials, such as syringes, presented with similar symptoms. A team from Kinshasa and the Belgian institute for tropical medicine was sent to Yambuku to collect samples which were then sent to laboratories with high containment facilities. Those Microbiological Research Establishments (MRE) included that of Porton Down in England and the Center for Disease Prevention and control (CDC) at Atlanta, Georgia for analysis[1]. The unknown virus was grown in animals (New borne mice, weaning mice and Guinea pig) and into cell lines (Vero cells and patient liver). Marburg-like virus particles were identified by negative contrast electron microscopy (EM)[1]. Moreover, serological testing was performed by Patricia Webb (CDC) using Yambuku convalescent serum for this new circulating virus[1]. Reports indicate that there were 318 cases with 280 deaths during the Yambuku outbreak, giving a case fatality rate of almost 90 %, this species of EBOV was subsequently named *Zaire ebolavirus*[2].

1.1.2 Notable historical outbreaks

There have been 27 notable Ebola virus disease (EVD) outbreaks to date which have, in the most part, been restricted to countries around the Congo basin: Gabon, South-Sudan, Uganda, Angola, DRC, and Congo-Brazzaville (**Table 1**). Notable Outbreaks of EVD, leading to newly identified species, have occurred in Sudan (*Sudan ebolavirus*), Uganda (*Bundibugyo ebolavirus*) and Ivory Coast (*Tai Forest ebolavirus*).

The first haemorrhagic fever outbreak in Sudan occurred during 1976 in Nzara and Maridi (Southern Sudan)[3]. The disease was first identified among farmers working in the cotton industry of Nzara. It was described as a severe illness with bleeding complications and simultaneously spread between Nzara and Maridi located about 250 km away[4]. The World Health Organization (WHO) was alerted and specimens were collected from infected patients for viral isolations and identification via histopathology and electron microscopy analysis at CDC (Atlanta), the MRE of Porton Down (England) and in Bernhard Nocht Institute for Tropical Medicine (Germany)[4]. The isolated virus was named Sudan *Ebolavirus* (SUDV) and the case fatality rate was recorded as approximately about 53 %[5].

Furthermore, in 1994 another new ebolavirus species was identified in the Ivory Coast within the national Tai Forest park, the virus was detected amongst chimpanzees and troops near the Liberian border[5]. In the Tai Forest park, several cases of chimpanzee death were reported during the same period. To investigate these chimpanzee deaths, three scientific ethologists conducted a necropsy on one chimpanzee corpse found in the forest, this chimpanzee was subsequently tested positive for EBOV via indirect fluorescent antibody (IFA) test and enzyme linked immunosorbent assay (ELISA) at the Pasteur Institute in France. The newly discovered haemorrhagic fever virus was named the Tai Forest virus (TAFV). Later, a Swiss ethologist actively working on infected chimpanzees in the Tai Forest park was infected when performing a necropsy on another dead chimpanzee corpse. She was rigorously treated with fluid and electrolyte replacement therapy and fully recovered 6 weeks later with no secondary transmission to humans[5].

During April 1995, a laboratory staff member of Kikwit hospital in the DRC was admitted to the viral haemorrhagic fever (VHF) unit[6]. As he underwent treatment, the entire caring medical staff presented with similar symptoms. Blood samples from acute patients were collected and analysed by ELISA for EBOV antigen or antibody at CDC in a high containment biosafety environment[6]. Additionally, reverse transcription polymerase chain reaction (RT-PCR) test was performed to detect viral RNA and additional investigations revealed that the new *Zaire ebolavirus* was closely related to that

of EBOV, identified in 1976[6]. The newly discovered virus was reported to have infected 317 people amongst which 245 succumbed to disease giving a case fatality rate of 79 %[7].

Reston ebolavirus is the first ebolavirus species reported outside Africa. Notably, Reston virus (RESTV) was identified between 1989 and 1996 during a haemorrhagic fever outbreak in Cynomolgus macaques exported from Philippines and China to a non-human primate facility in Reston, Virginia in the United States[10]. An ELISA test performed on 179 blood samples to assess the prevalence of RESTV amongst animal handlers indicated a seroconversion rate of 3.4%[8]. Furthermore, an animal handler who cut himself while performing a necropsy on an infected macaque liver failed to become sick[8]. Though the virus is known to cause severe haemorrhagic fever in non-human primates, experiments indicate that RESTV does not cause any illness in pigs and human[9]. It was then concluded that RESTV is asymptomatic in humans.

In 2007, haemorrhagic fever was notified in Bundibugyo district in Western Uganda[10]. Samples were collected from 29 patients and tested negative for known filoviruses (EBOV and Marburg viruses). In addition, a newly developed metagenomic pyro-sequencing method was performed using traditional primer approaches[10]. Acquisition of these sequences revealed that it was in fact an EBOV that was circulating in Bundibugyo district. The identified virus was different to the four EBOV species previously discovered[10]. The newly discovered virus was then named after the place of its discovery: Bundibugyo virus (BDBV)[10]. Reports finally indicate that the Bundibugyo outbreak resulted in 149 suspected cases with 37 deaths giving 25% case fatality rate[10].

In 2002, a new *ebolavirus* specie (*LIoviu Cuevavirus*) with one single virus type, LIoviu virus (LLOV) was discovered in Cueva del LIoviu (Asturias) in Spain[11]. The virus was identified from Schreiber long-fingered bats (*M. schreibersii*). A mysterious discovery of several corpses of *M. Schreiber* bats and giant mouse (*M. myotis*) found in a cave and suggested to have succumbed to an unknown disease lead to serious scientific search which resulted in LLOV discovery only in bats. Additionally, a new *ebolavirus*, Bombali virus (BOMV) has recently been discovered in Sierra Leone in free-tailed bats (*Chaerephon pumilus)*[12]. Presently, no study has documented both LLOV and BOMV infection disease in human or NHPs.

The West African Ebola virus disease (EVD) epidemic was known as the biggest, largest and deadliest ever since the discovery of the virus with 28,616 confirmed cases amongst which 11,310 deaths occurred[13]. The outbreak greatly impacted public health systems in the three main affected countries (Guinea, Liberia and Sierra Leone)[14]. Currently, 2,236 people have succumbed to an on-going EBOV outbreak in

the DRC (North Kivu and Ituri provinces)[15]. With 3,409 confirmed cases since August 2018, the outbreak is known as the second biggest epidemic of the virus on record.

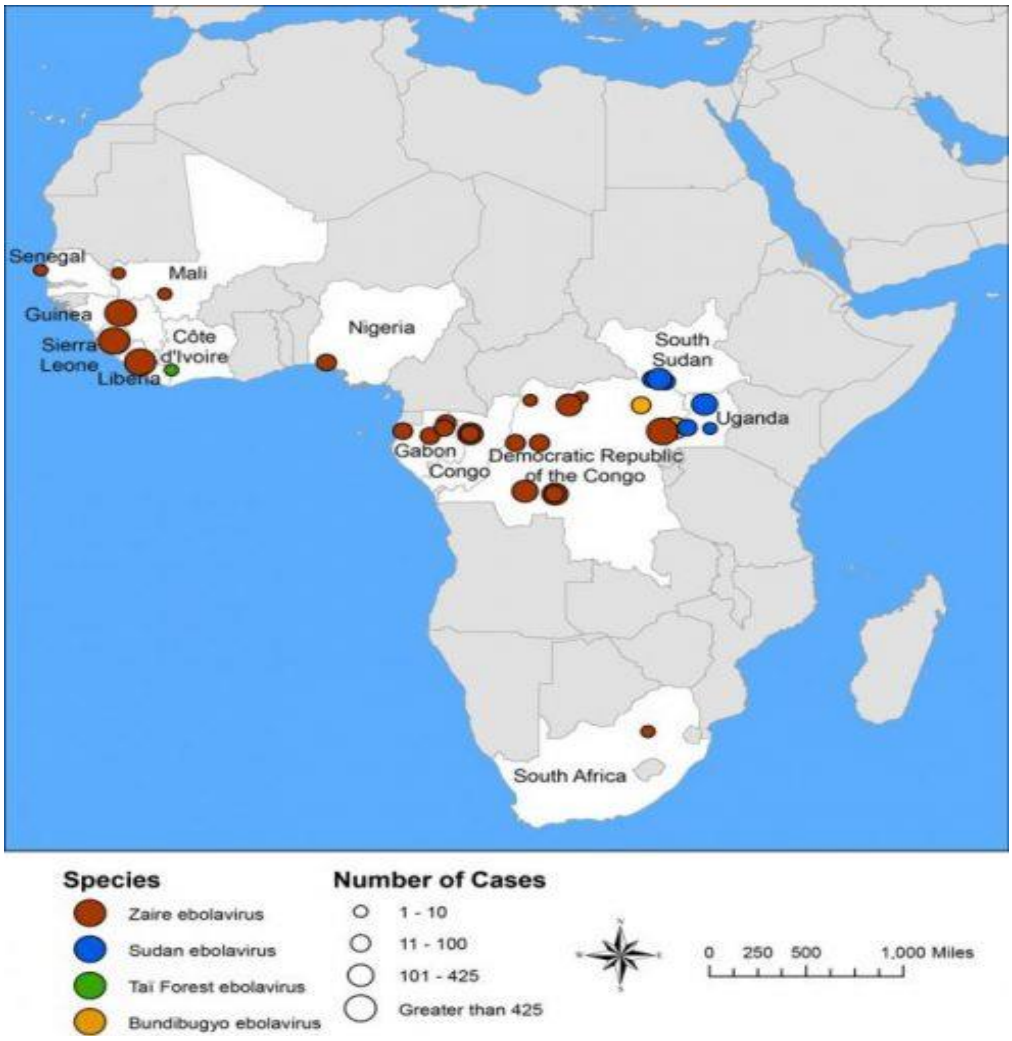
Table 1: Ebolavirus outbreaks in Africa since 1976

Location of outbreak of the *Ebolavirus* and total laboratory confirmed cases.

Year	Country	Virus	Cases	Deaths
1976	DRC	*Zaire ebolavirus*	318	280
1976	Sudan	*Sudan ebolavirus*	284	151
1977	DRC	*Zaire ebolavirus*	1	1
1979	Sudan	*Sudan ebolavirus*	34	22
1994	Gabon	*Zaire ebolavirus*	52	31
1994	C. d'ivoire	*Tai forest ebolavirus*	1	0
1995	DRC	*Zaire ebolavirus*	315	254
1996	Gabon	*Zaire ebolavirus*	31	21
1996	Gabon	*Zaire ebolavirus*	60	45
1996	South Africa	*Zaire ebolavirus*	1	1
2000	Uganda	*Sudan ebolavirus*	425	224
2001	Gabon	*Zaire ebolavirus*	65	53
2001	DRC	*Zaire ebolavirus*	59	44
2003	DRC	*Zaire ebolavirus*	143	128
2003	DRC	*Zaire ebolavirus*	35	29
2004	Sudan	*Sudan ebolavirus*	17	7
2005	DRC	*Zaire ebolavirus*	12	10
2007	DRC	*Zaire ebolavirus*	264	187
2007	Uganda	*Bundibugyo ebolavirus*	149	37
2008	DRC	*Zaire ebolavirus*	32	14
2011	Uganda	*Sudan ebolavirus*	1	1
2012	Uganda	*Sudan ebolavirus*	24	17
2012	Uganda	*Sudan ebolavirus*	7	4
2012	DRC	*Bundibugyo ebolavirus*	57	29
2014	DRC	*Zaire ebolavirus*	66	49
2014-16	West Africa	*Zaire ebolavirus*	28,616	11,310

Finally, it is worth noting that there have been previous suspected EVD outbreaks in Guinea. Between 1982-1983, an unknown hemorrhagic fever disease was reported amongst populations in the Madina Ula, district of Kindia region (Republic of Guinea)[188]. A serological survey conducted amongst convalescent people in the affected area (Madina Ula) revealed 19 % sero-positivity to EBOV. Although, the study revealed 8 % EBOV antibody detection amongst the healthy population and 3 cases of exposure to Lassa virus (LASV). The serological testing used ELISA assays probing all EBOV and LASV antigens. In addition, a recent study on probable existence

of EBOV in Guinea prior to the 2013-2016 outbreak has been published[16]. In this study, blood samples were broadly collected in Guinea in 2012 as part of a demographic and health survey program. The program was led by the national institute of statistics. Those collected samples were tested on Luminex-based assay technology and revealed the presence of EBOV-specific antibodies in one specimen. The identified case was known to be from the forested region of Guinea (Nzerekore), but it was uncertain whether the individual had traveled from a known EBOV endemic area such as the DRC.



https://www.cdc.gov/vhf/ebola/history/distribution-map.html, accessed 28 JAN 2020

Figure 1: WHO map of *ebolavirus* outbreaks in Africa since 1976.

Four *ebolavirus* species have been simultaneously circulating in Africa. The most frequent is the *Zaire ebolavirus* followed by *Sudan ebolavirus*. *Tai forest ebolavirus* had occurred once in Ivory Coast (1994) whereas the *Bundibugyo ebolavirus* had appeared twice in Uganda (2007) and in the DRC (2012).

1.1.3 Ebola; Species and phylogenetics

Filoviruses are members of the Mononegavirales order and the Filoviridae family which is composed of three genera: *Cuevavirus*, *Ebolavirus* and *Marburgvirus*[17] (**Table 2**). The international committee on taxonomy of viruses (ICTV) has named Filoviridae after its morphological structure which is filamentous (Filum) and the virus family (viridae)[18]. It follows that Filoviruses are known to appear in multiple shapes which are either long or short filaments. Those Filaments (particles) are sometimes branched, circular or presented in a ''6'' or ''U'' form and extend from 650 to 14,000 nanometres in length, 80 nanometres in diameter. Virions are enveloped in a lipid membrane and their surface covered with a single glycoprotein that is dispersed in spikes[14]. In addition, the filoviruses genome is approximately 18 to 19 kb long and encodes for seven proteins including nucleoprotein (NP), glycoprotein (GP), virion proteins (VP) 24 , VP30, VP35, VP40 and the large (L) protein[19]. Both ebolavirus and marburgvirus are morphologically filamentous particles and have a negative-sense single-stranded RNA (ssRNA) genome[13,14].

To date, no case fatalities in humans have been reported for Cuevavirus. The type virus of the *Cuevavirus* genus, LIoviu virus (LLOV) was initially discovered in Cueva del LIoviu (Asturias), Spain in 2002[11,20]. The virus was identified from Schreiber long-fingered bats (*Miniopterus schreibersii*). This finding was a result of a mysterious discovery of several corpses of Schreiber's bats and giant mice (*Myotis myotis*) in a cave and suggested to have succumbed to an unknown disease. The newly discovered virus was named LLOV after the Spanish region where it was discovered (Cueva mains cave). Unfortunately, LLOV viral RNA was only found in bats but not in the mice. Additionally, another study from a deceased bat in Spain not only identified LLOV but also revealed that LLOV expresses seven structural proteins (NP, VP35, VP40, GP, VP30, VP24, and L-protein) from six genes in contrast to other Filoviruses which encode seven genes[17].

Table 2: Filoviridae; Genus and Species

Genus name	Species name
Cuevavirus	*LIoviu cuevavirus*
Ebolavirus	*Bombali Ebolavirus*
	Bundibugyo Ebolavirus
	Reston Ebolavirus
	Sudan Ebolavirus
	Tai Forest Ebolavirus
	Zaire Ebolavirus
Marburgvirus	*Marburg Marburgvirus*
	Ravn Marburgvirus

1.1.4 Ebola; Genome structure and organization

The EBOV genome is about 18–19 kb in length and encodes for seven proteins: NP, GP, RNA-dependent RNA polymerase (L-protein), and structural proteins VP24, VP30, VP35 and VP40, as well as the GP splice variants: secreted GP (sGP) and small soluble GP (ssGP)[21] (

Figure 2).

Experiments have demonstrated that the GP is the only protein located on the EBOV surface[22]. It follows that the GP protein contributes to viral pathogenicity and is paramount to EBOV infection. The ebolavirus NP is the largest gene within these non-segmented negative stranded viruses and plays a central role in viral replication[23]. NP protein in association with VP35, VP40 and L-protein are part of the ribonucleoprotein complex responsible for virus genome transcription and replication. In addition, VP24 is thought to be involved in nucleocapsid formation[24]. On the other hand, yang *et al* demonstrated that NP, VP35 and VP24 are absolutely essential and sufficient for Ebola virus genome nucleocapsid assembly[24].

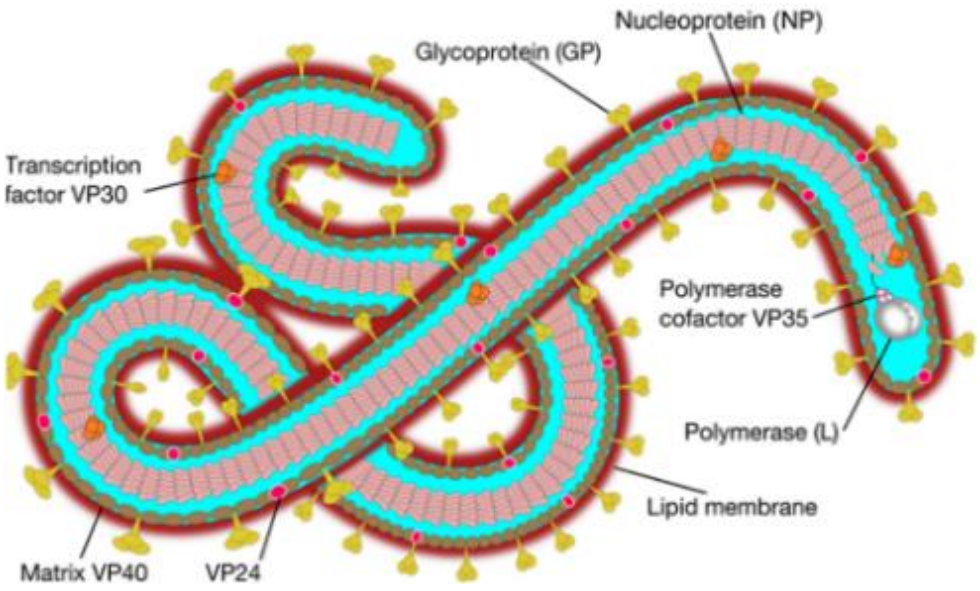


Figure 2: Filoviridae (virion)

Filoviridae virions are filamentous and in the case of EBOV they are ~970 nm long and about 80 nm in diameter. Image taken from https://viralzone.expasy.org/23?outline=all_by_species

1.1.5 Ebola; Replication

Replication of EBOV takes place into the host cell cytoplasm. Virus entry will start when the virion becomes attached to cell surface via specific GP receptors such as TIM-1 on the cell membrane.

Though EBOV encodes for several proteins; only the GP protein determines virus tropism and interaction with target cells such as monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, hepatocytes, and adrenal cortical cells[25]. Notably, GP protein is made up of two subunits including GP1 which is the receptors binding subunit and GP2 the fusion subunit[26]. It has been demonstrated that virus binding efficiency is enhanced by the activity of acid sphingomyelinase (aSMase) in association with the plasma membrane sphingomyelin of the host cell[27]. Upon virion attachment to host membrane, several cellular factors including DC-SIGN and L-SIGN are implicated in enhanced virus entry into host cell via receptor mediated endocytosis mechanisms[28]. Following its entry into the cell, the virion particles enter into endosomes where GP1 is cleaved in the presence of low-pH-dependent cysteine cathepsins B and L. The processed GP1 interacts with the late endosomal/lysosomal protein Neimann-Pick C1 (NPC-1)[29]. This interaction results in GP2-dependent fusion of the viral envelope with the endosomal limiting membrane of host cell. In contrast, host cell deficiency in NPC-1 proteins result in poor binding of the virus which consequently may prevent EBOV infection in some patients. Furthermore, the virion fusion also requires Two-Pore Channel 2 (TPC2) activity and the role of TPC2 in viral entry is not, to date, clearly specified[30]. In the final stages of virus infection, the viral nucleocapsid is released into the host cell cytoplasm which is then followed by the viral genome transcription and translation[31]. Finally, virus particles are assembled to form new infectious viruses which are released via budding into host organs. The viral progeny then spread to a variety of target cells resulting in general infection which consequently leads to the multiple organ failure (MOF) observed in EVD.

EBOV genome transcription starts with the binding of the polymerase complex to a single binding site located within the leader region of the genome[32]. The EBOV RNA genome, which is dependent on the transcription activator VP30, synthesis seven monocistronic mRNAs (encapsidated) that are capped and polyadenylated with precised length determined by highly conserved start and stop signals. Individual genes are then sequentially transcribed into viral proteins along the RNA template following 3’ to 5’ order using the host cell’s ribosomes and tRNA molecules. EBOV genome replication is performed via three proteins including NP, VP35 and L and starts when

nucleoprotein is sufficient to encapsidate neo-synthetized antigenomes and genomes[33]. As viral protein levels rise, a switch occurs from translation to replication. This phenomenon uses the negative-sense genomic RNA as a template. Further, a complementary +ssRNA is synthesized and used as a template for the synthesis of new genomic (-) ssRNA quickly encapsidated[32]. The newly formed nucleocapsids and envelope proteins associated with the host cell's plasma membrane are released via budding. Finally, multiple virus release lead to host cell destruction (lysis) which consequently lead to cell death.

1.1.6 Ebola; Disease and immune response

Reports from previous filovirus outbreaks have demonstrated high mortality rates ranging between 30 to 90 % in human and non-human primates according to the virus subtype[9]. EVD starts with high fever >39°C, serious headache and multiple symptoms can occur, including abdominal pain, nausea, sore throat, vomiting which becomes bloody at the last stage of the disease, and diarrhoea which is responsible for severe dehydration. Haemorrhagic manifestations are the last signs and occur when there is multiple organ failure (MOF) due to high levels of viral spread throughout the organs [41]. Infected subjects encounter loss of appetite and disorientation which can often end with unconsciousness and death [10,41]. The initial cells that succumb to EBOV infection are myeloid cells (macrophages and dendritic cells), followed by hepatocytes and vascular endothelial cells. Early infection of mononuclear and dendritic cells that are involved in the immune response are thought to contribute to immune suppression. As the disease progresses, parenchyma cells, like hepatocytes and adrenal cortical cells are infected, thereby affecting the function of liver and kidneys [41]. A massive release of inflammatory mediators increases vascular permeability leading to shock and disseminated intravascular coagulation which leads to coagulopathy and bleeding[1]. Finally, the virus may persist for some days in blood, vomit, sperm or sliver, though the virus is fragile and is rapidly inactivated when exposed to chlorine disinfection, heat, direct sunlight or UV light, soaps and detergents [10].

Although the human immune system is made up of several physical, chemical, and cellular components, it is generally divided into two major groups, the innate and adaptive systems. Both systems contribute to protect individuals from a wild range of pathogens. As the innate system provides a constant level of physical protection; the adaptive system recognises and provides specific protection against microorganisms and provides long-lasting immunological memory. Interestingly, both systems work

closely together to protect the body against either new invasive or previously encountered microorganism [34].

EBOV is one of the most lethal pathogens and to date the interaction of EBOV with the human immune system remains incompletely understood[35,36]. In January 2017, the first report was published to show T-cell and B-cell dynamics and antigen-specific T-cell response in EVD survivors receiving only supportive care with no experimental drugs[37]. The data combined with other available reports revealed a vigorous specific humoral response following EBOV infection and the genesis of neutralizing antibodies that could persist weeks after recovery[38,39]. Severe disease following EBOV infection is probably related to an early activation of T-cell responses followed by the production of high levels of inflammatory cytokines known as “cytokine storm” which contribute to coagulopathy, oedema and multiple organ failure [45]. Importantly, Agrati *et al* demonstrated that EBOV infection induces sustained $CD8^+$ T-cell activation which further leads to the overall decline of functional lymphocytes[45]. Understanding the contribution of human specific immune response to EBOV infection and the effective protection or pathogenic responses will effectively improve the development of therapeutics and vaccines.

1.1.6.1 Innate immune response to Ebola virus

In general, the innate immune system comprises intact skin and mucous membranes secretions (enzymes and acids), which essentially protect the body from infection. Moreover, an antiviral family of molecules identified as interferons (IFN-α, IFN-ß, IFN-y) provides an important additional line of innate defence to microorganism invasion[43]. Interferons molecules are infected cells products (cytokines) which enhance host protective defences to eradicate evading pathogens[43]. They activate host immune cells (macrophages and natural killer cells), up-regulate the expression of major histocompatibility complex (MHC) for antigen presentation and increase certain symptoms of infection such as fever and muscle pain. Consequently, viral replication is inhibited in host cells resulting in host protection from viral infection. When physical defence barriers are breached, microorganisms are targeted by phagocytic cells including polymorphonuclear cells (Neutrophils, Eosinophils, and Basophils). Firstly, microorganisms are grasped and engulfed by those cells (phagosome), then fused with lysosomes which finally release toxic enzymes to destroy them (phagolysosome)[43]. And then inflammatory cytokines are produced to protect neighbouring cells.

Lue *et al*. conducted a transcriptomic analysis to determine the host immune response to EBOV using messenger RNA expression patterns and this indicated that

natural killer (NK) cell populations are abundant in EVD survival patients as opposed to fatal cases[35]. These experiments were carried out on blood samples from Guinea EVD infected patients of the 2013-2016 West Africa outbreak. In the event of EVD infection, the lack of dendritic cell activation leads to a poor immune response mediated by NK, T and B cells[40]. As a result, invasive virus outgrowth and spread in host organs occurs. Therefore, early activation of the immune responses at all levels is critical to limit virus growth and prevent a fatal outcome. NK cells actively respond to viral infection by secretion of high levels of cytokine and chemokines such as perforins and granzymes which play a key role in infected cell destruction[41]. The deficiency of NK cells or NK cell-mediated lysis due to removal of the inhibitory signals leads to host vulnerability to EBOV infection which consequently contributes to an EVD fatal outcome[49]. Nadia *et al*. investigated EBOV infection in humans and showed that the innate immune response is associated with a “storm of cytokines” linked to hypersecretion of numerous pro-inflammatory cytokines including (IL-1ß; IL-1RA; IL-6; IL-8; IL-15; IL-16), chemokines and growth factors (MIP-1α; MIP-1ß; MCP-1; M-CSF; IP-10; GRD-α and octavin)[42]. The investigation finally indicates no increase of antiviral IFN-α but a massive decrease of peripheral CD4 and CD8 positive lymphocytes and established that the Fas/FasL-mediated apoptosis could probably be related to an immunosuppression of circulating cytokines produced by T lymphocytes.

1.1.6.2 Adaptive response to Ebola virus

It is known that the basic components of the adaptive immune system are lymphocytes[43]. These cells originate from stem cells in the bone marrow and are sub-classified into three groups; lymphocytes T, lymphocytes B and natural killer cells according to their origin of maturation. B cells are matured in the bone marrow and are responsible for antibody production whereas T cells complete their maturation in the thymus and have antiviral, antifungal, and immunoregulatory functions[43].

1.1.6.3 B cells responses to Ebola virus

A study conducted by Wu *et al* to determine the nature of the immune response to EBOV[43] established that the rapid depletion of lymphocytes by apoptosis is a prominent feature of the disease, resulting in the impairment of the host immune response. Though the role of antibodies in viral clearance is not fully understood, however some findings indicate that antibodies seem to appear as early as day-5 after symptoms onset, then rise to peak after two weeks. In the event of recovery, antibody titre declines slowly over years[44]. The features of human antibody response to ebolavirus

GP remain unknown since only a few quantities of monoclonal antibodies (mAbs) to GP have been isolated from EVD survivors[45,46]. B cell response to EBOV GP was studied by cloning an extensive panel of mAbs to GP harvested from EVD survivor's peripheral B cells following the 2014 EBOV outbreak in the DRC[54]. This study showed that the B cell response to EBOV is characterised by a broad diversity of clones primarily targeted three non-overlapping antigenic sites: the glycan cap, the GP1/GP2 interface and the stalk region inclusive of the HR2 helices. Also, the study demonstrated that a substantial fraction of the mAbs cloned from GP-specific B cells show neutralizing activity; therefore, it indicated that neutralising antibodies (Nabs) could develop during EBOV infection. Furthermore, experimental research aiming to develop a protective monotherapy against lethal ebolavirus infections established that B cell memory specific to EBOV could persist for more than a decade after recovery[55].

1.1.6.4 T cells responses to Ebola virus

An experimental study using a mouse model infected with lethal EBOV demonstrated that mice presenting deficient in $CD8^+$ T-cell were overwhelmed whereas, mice deficient in B cells or $CD4^+$ T cells cleared the infection and survived[47]. The study then established that $CD8^+$ T cells play an important role of protecting the host against acute disease. $CD8^+$ T cells initially clear the virus while $CD4^+$ T cells were associated with antibody mediated protection. Immunophenotyping analysis of acute EVD patient samples indicated that the levels of CTLA-4, a negative regulator of T cell activity, were significantly higher in $CD8^+$ T-cells from fatal EVD cases compared to EVD survivors[47]. Thus CTLA-4 may play an important role in T cell inhibition and in immune homeostasis in the context of EVD; however, data to examine the cellular responses in EVD supported that a strong antigen-specific T cell response could develop during an EBOV infection and demonstrated that EBOV nucleoprotein is largely targeted by T cells during human infection[46]. Furthermore, Julie *et al* conducted another experiment on mice to demonstrate cytotoxic T lymphocytes (CTLs) mediated protection to EBOV[44]. The study not only reported that CTLs-mediated contribute to protect against EBOV but also comment on the need to evaluate viral proteins for protective cellular immune responses in the development of effective vaccine strategies. Therefore, to develop an optimal and efficient EBOV vaccine, researchers will have to induce a combination of protective humoral and protective cellular responses to simultaneously clear extracellular virus and virus-infected cells.

1.1.6.5 B and T cells dynamics

Despite being the strong line of the cellular immune response to a range of invasive microorganisms; data on lymphocyte dynamics and the antigen specificity of T cells in *Ebolavirus* infection are little known. As a result, T and B cells dynamics remain incompletely understood.

In the context of EVD, available human data is lacking but includes a longitudinal report[45]. In 2014, Emory University in USA carried out a study focusing on B and T cell activation exploiting blood sample from four EVD patients using phenotypic markers[46]. The study described the presence of robust B and T cell responses and established the first kinetic data on the activation status of human B and T cell population responses in the acute phase of EBOV infection. Another interesting study on an EVD survivor who, under medical care, cleared the infection without experimental drug assistance showed neutralizing antibodies that persisted almost 5 weeks after recovery[47]. Additionally, EBOV specific T cells measured in 3 EVD survivors observed consistent IFNy responses to nucleoprotein and multiple responses to GP[48]. The study additionally reported the presence of consistently high T cell activation and a strong IFNy response to GP. Furthermore, *in vitro* experiments on EBOV infection of dendritic cells carried out by Mahanty *et al* indicated that T and B cell function could be slightly impaired[49]. Baise *et al* have demonstrated a decrease in mRNA levels of IFNy, Interleukin 4 (IL-4) and Interleukin 2 (IL-2) and have closely evoked a lack of anti-viral immune response at the infection time[50]. Furthermore, Corti *et al* using infected human PBMCs provided direct evidence that EBOV infection causes dramatic cell apoptosis in lymphocytes mainly in $CD4^+$ and $CD8^+$ T cells[46]. The published data indicated that 30-40% of the $CD4^+$ and $CD8^+$ T-cells were dead on day 8 following infection.

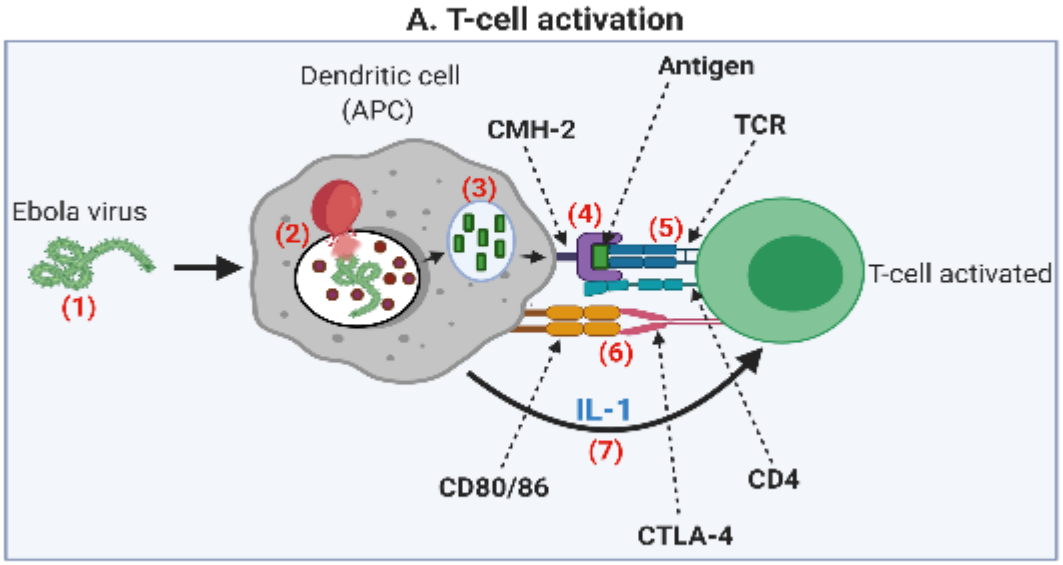
While $CD4^+$ T cells are required for IgM to IgG switching and $CD8^+$ T cells are involved in EBOV infection control, cytokines play a fundamental role in communication allowing the immune system and host tissue cells to exchange information and to recruit macrophages and T cells at the site of infection[43]. The apoptosis of these cells leads to host inability to control virus levels in an ongoing infection. Consequently, the virus could rapidly spread in the host organism and causes severe damage which may lead to a fatal outcome[59].

Conclusion from the above reports indirectly suggest that EBOV causes massive death of human $CD4^+$ and $CD8^+$ T-cells delaying an anti-EBOV IgG response in infected patients.

1.1.7 Host immune response and outcome to survival

Findings from 2013-2016 EVD survivor samples are providing a deep understanding on several aspects of EBOV infection and the human immune response. Though Albumin and fibrinogen proteins are known to be associated with a significant liver pathology; and recent data has reported that during an acute phase, fatal cases show strong upregulation of interferon signalling, albumin and fibrinogen genes compared to non-fatal individuals[35]. These studies further identified a small panel of responding genes that might intervene as strong predictors of patient outcome, independent of autonomous of viral load. Some studies have previously indicated that IL-6 signalling has been linked with fatal outcome to EBOV infection[57,42]. In 2010, the largest study ever done on blood samples from 50 volunteers from EVD survivors revealed that fatal outcome is mainly associated with an excessive innate immune response. This include the hypersecretion of multiple pro-inflammation cytokines and growth factors followed by a massive inhibition of adaptive immunity but no increase of IFN-α in patients[57]. A published study has shown that PD-1 ligation inhibits T-cell activation only upon T cell receptor (TCR) engagement and interferes with signalling molecules like phosphatidylinositol-4,5-bisphosphate 3-kinase and Ras, which are important for T-cell proliferation, cytokine secretion and metabolism[51]. The study indicates that T-cell exhaustion is mainly characterized by persistent infection. However, long-term exposure to viral antigen significantly causes T-cell reprogramming leading to high PD-1 expression and T-cell exhaustion[52]. Almost all evading pathogens have successfully developed mechanisms to evade or supress host protective immune response.

1. Normal cellular immune response to Ebola virus



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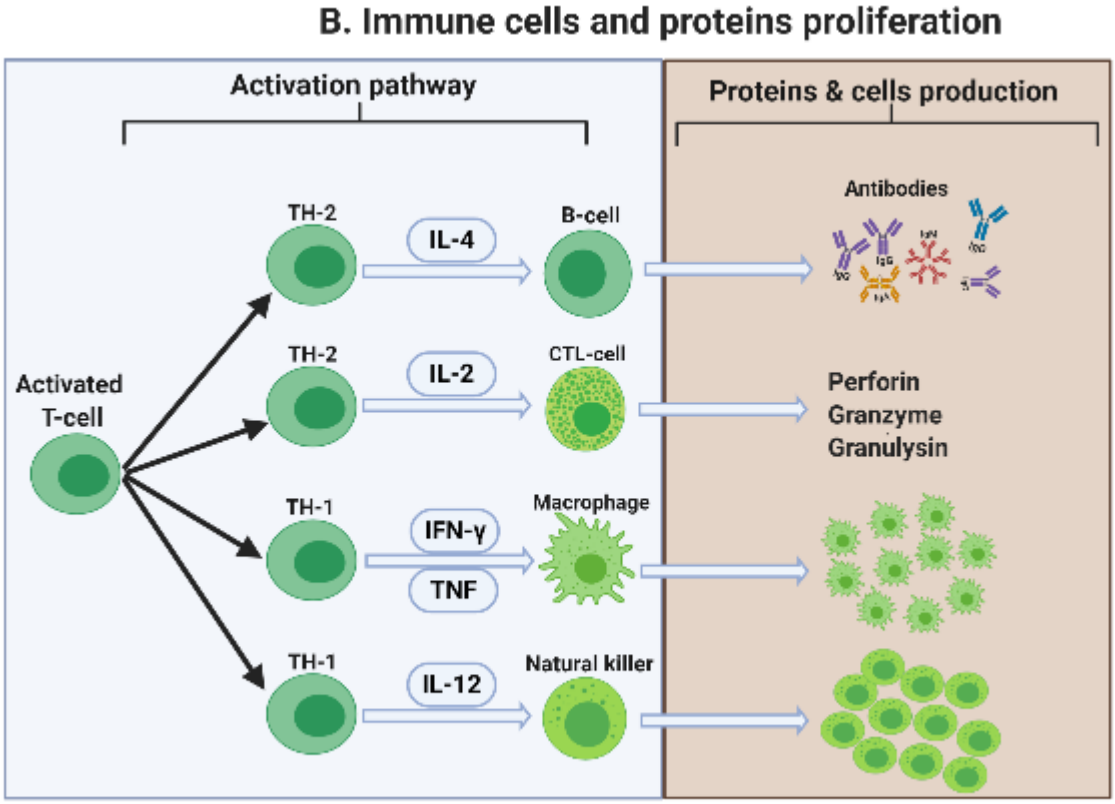

This figure used images that were created with Biorender.com and are permissible to use.

Figure 3: Normal cellular immune response to Ebola virus.

(**A**) **T-cell activation phase**: (1) *ebolavirus* attaches and is engulfed into host cell via GP1/2 receptors. (2) Lysosomes fuse with late endosome, release enzymes to degrade the virus. (3) *ebolavirus* is dissected into particles. (4) Viral particle (antigen) is presented on the antigen

presenting cell (APC) cell surface via complex major histocompatibility-2 (CMH-2). (5) T-cell binds to APC on CMH-2 receptor via T cell receptor (TCR). This binding is reinforced by CD4 proteins. (6) CD80/86 binds to CTLA-4 and consequently, (7) APC releases interleukin 1 (IL-1) to activate T cell. **(B) Immune cells activation**: Activated cell ($CD4^+$) activates TH-1 and TH-2. Activated T cells (TH-1 and TH-2) produces cytokines (ILs, IFNs, TNF, and growth factors). IL-2 stimulates cytotoxic T lymphocytes (CTL) which produces enzymes for virus lysis. IFN-ɣ stimulates phagocyte macrophages which engulf degraded cells. IL-12 stimulates natural killer cells (NK). IL-4 activates B cells which produce specific EBOV antibodies.

2. Inhibition of cellular immune response to Ebola virus

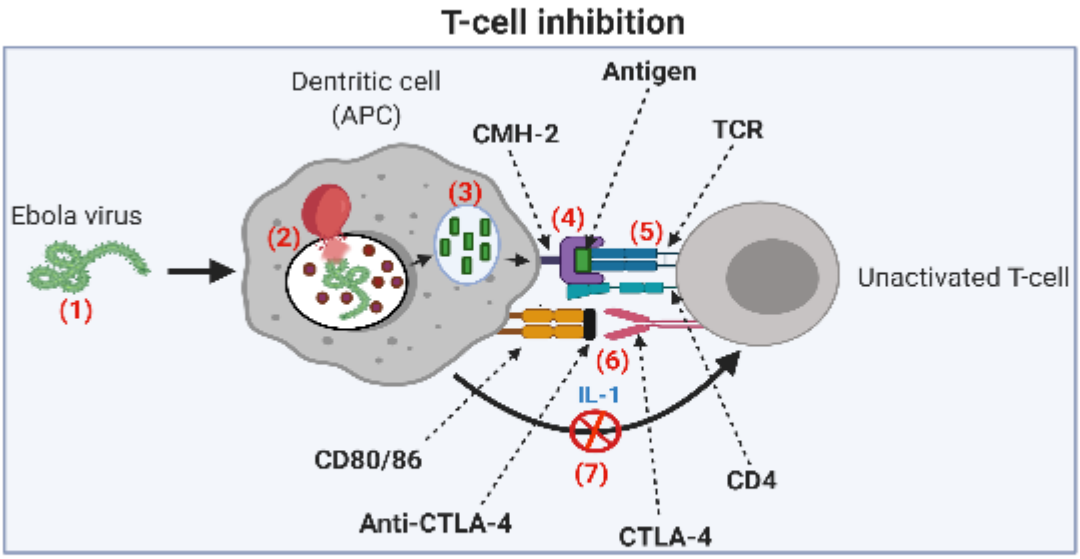

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Figure 4: Inhibition of cellular immune response to Ebola virus.

(1)ebolavirus attaches and is engulfed into host cell via GP1/2 receptors. (2) Lysosomes fuse with late endosome, release enzymes to degrade the virus. (3) *ebolavirus* is dissected into particles. (4) Viral particle (antigen) is presented on the antigen presenting cell (APC) cell surface via complex major histocompatibility-2 (CMH-2). (5) T-cell binds to APC on CMH-2 receptor via T cell receptor (TCR). This binding is reinforced by CD4 proteins. (6) Anti-CTLA-4 binds to CD80/86 binds to prevent CTLA-4 binding. (7) Consequently, Interleukin-1 (IL-1) production is inhibited leading to T cell inactivation. As a result, the cellular immune response is damped.

Anja *et al* evaluated the phenotype of antigen presenting cells (APCs) in the peripheral blood of Guinea EVD patients [53]. The experiment indicated that there are established roles for T regulatory (Treg) cells and the immunosuppressive cytokines IL-10 and TGF-ß in limiting anti-pathogen effector T-cell responses. Therefore, severe disease in humans affected by EBOV is a result of an important transformation of the immune system. Not only did the study identify classical CD14, intermediate CD14/ CD16, non-classical CD16 monocytes, activated HLA-DR neutrophils and DCS but it also observed a reduction of CD1c DC monocytes and a significant increase of CD16 monocytes. Finally, the study reported that there is a significant reduction in circulating monocytes which is associated with EBOV severity whereas; a robust activation of CD16 monocytes (APC) is correlated with a survival outcome[63].

John *et al* indicated that in general EVD fatal outcome is associated with clinical factors such as fever, weakness, dizziness, diarrhoea, high level of blood urea nitrogen, aspartate aminotransferase and creatinine[54]. The study established a positive correlation between the viral load and the fatal outcome of the disease. It shows that, patient presenting with fewer than 100,000 EBOV copies per/ml in serum on RT-PCR testing had 33% chance of surviving to disease, whereas, those with more than 10 million EBOV copies per/ml had 94% of succumbing to disease (P=0.003).

1.1.8 Ebola; Reservoir and Transmission

Bats appear to be the most likely *Ebolavirus* reservoir (**Figure 5**); however, there has been difficulty in isolating virus or recovering a complete genome which has frustrated efforts to conclusively link EBOV to any host species. However, EBOV RNA has been isolated in a few specimens of captured fruit bats (*Epomops frangueti, Hypsignatus monstrosus, and Myonycteris torquata*) in Gabon, suggesting that these may be a natural reservoir of the EBOV[55,56]. Moreover, reports have indicated that at least 8 historical outbreaks in Africa have occurred through zoonotic transmission via direct contact with infected wild animals, which seem to be mainly fruit bats and carcasses of potential infected non-human primates[57,58,59]. Between 2002 and 2003, Bermejo *et al* observed the Lossi sanctuary in northwest of the DRC and found a large number of unexplained deaths amongst gorillas and chimpanzee populations[60]. To understand this, twelve carcasses were assayed for EBOV-specific antibodies and nine were found positive. In the same period, the virus spread from north to south DRC and infected another troop of gorillas killing 91 amongst 95 individuals. Further investigations to understand non-human primate fatality and EBOV infection were conducted by Rouquet *et al* including both bordering countries, the Republics of Gabon

and Congo[61]. The investigation identified 98 carcasses of wild animals including great apes amongst which 10 gorillas (10.2%), 3 chimpanzees (3.06%) and 1 duiker (1.02%) were tested positive for EBOV specific antibodies. With regards to these findings, it is likely that the high fatality rates (75-95%) observed in non-human primates exposed to EBOV infection exclude them as a host reservoir.

A new ebolavirus, Bombali virus (BOMV), has recently been discovered in Sierra Leone in free-tailed bats (*Chaerephon pumilus)*[12]. In addition, the first evidence of any bat carrying EBOV was recently cited in Liberia in a greater long-fingered bat (*Miniopterus inflatus*)[62]. Studies to isolate LLOV virus in culture and determine whether the virus can persist in animals, primarily *Miniopterus schreibers* bats failed[17]. As a result, LLOV viral pathogenicity and virulence against infection in bats remains unknown. On the other hand, the high case fatality observed in bats suggest that there could be another host reservoir for LLOV virus rather than *Miniopterus schreibers*. Nevertheless, researchers have found EBOV IgG in 3 African bat species (*Epomops frangueti, Hypsignatus monstrosus and Myonycteris torquata*) in central Africa, Gabon and the DRC[30]. In West Africa, the first evidence of any bat species carrying about one-fifth of EBOV genome was recently found in Liberia. Unfortunately, researchers could not isolate the virus itself. The viral genome was found in a greater long-fingered bat[38]. In general, the maintenance and inter-species transmission systems remain unknown. So, finding more ebolavirus EBOV species will help to understand what makes some pathogenic and others non-pathogenic and provide useful information about how to protect population from pathogenic strains. It will also help prove whether bats are actually a reservoir.

Ebola virus transmission and spillover route from wildlife to human population.

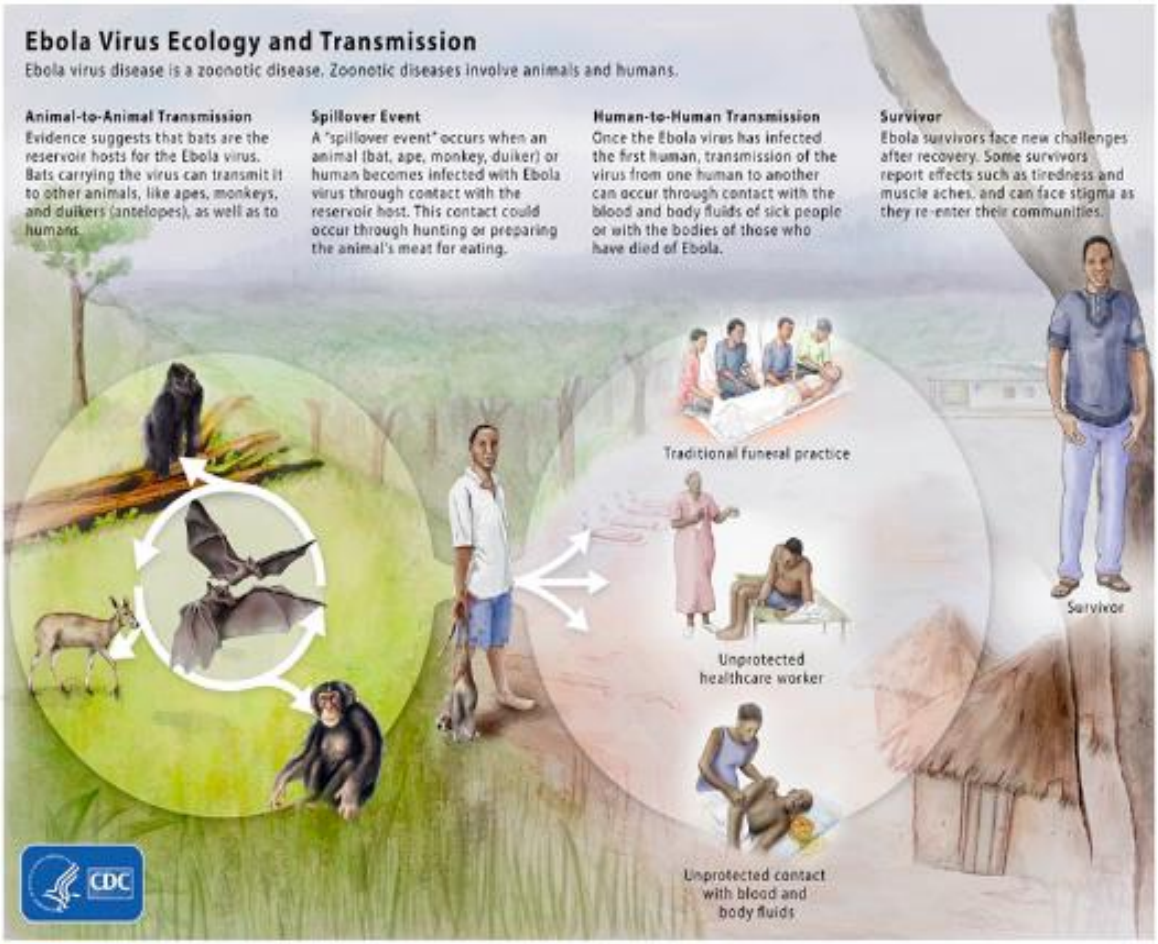


Figure 5: Schematic representation of EBOV transmission.
(1) Ebola virus circulates in wildlife with an unidentified reservoir. (2) First contamination (index case) occur from animal to human through infected animal manipulation (hunting and butchering) or consumption. (3) Disease spread in community (primary and secondary infection) from human-to-human contact via bodily fluids. Highly exposed peoples are household family members and unprotected healthcare workers. (4) EVD survivors encounter long effect of the disease such as tiredness, sight problem, kidneys failure and social problems as stigma in their community. Image from https://www.cdc.gov/vhf/ebola/resources/virus-ecology.html.

1.1.8.1 Transmission

Using caves for shelter, collecting fruit bats guano to fertilize cultural fields, consuming bushmeat and fruit semi-eaten by animals are major routes of human exposure to zoonotic infections. Understanding how emerging viruses are transmitted to humans is essential to identify and prevent practices that promote transmission. To date, there is little data with regards to human-wildlife interaction. For the last three years, the food and agriculture organisation (FAO) of the United Nation has focused on the importance of qualitative analysis of the bushmeat sector for food security in many African countries including Guinea[63]. Unfortunately, findings on community behaviour have highlighted the fact that practices vary widely across cultures and regions and that information remains insufficient to prevent emerging diseases in specific regions and populations.

Following infection of the index case, human-to-human transmission follows when caring for infected patients via feeding, cleaning and often in African cultures preparation of bodies for funeral practices. Traditional burial rituals (especially in Africa) consist of washing and touching the body (even sleeping beside) in order to prepare and accompany the dead to his/her ancestors. People touching, washing, dressing and sleeping beside the corpse are at high risk of exposure. This was likely a major route of transmission during the West Africa outbreak.

Studies performed with Guinean survivors of the 2013-2016 EBOV epidemic established that EBOV viral RNA can be found in semen up to 13 months following clinical recovery[64]. This indicates that the persistence of ebolavirus genome in semen may contribute to disease spread, as for that of Nzerekore (Guinea) with sexual transmission from an EBOV male survivor[65]. It follows that the major risk factors for disease transmission and amplification in communities is likely via household family members, healthcare workers, laboratory scientists, travelers from affected areas and, relatives. Moreover, re-using medical items without proper sterilization, consulting with infected patients, attending health facilities system without gloves and proper hand disinfection constitute the main source of healthcare worker transmission[66]. Notably, during the 2013-2016 EVD outbreak in West Africa, a total of 881 cases were notified amongst healthcare workers leading to 513 deaths in the three main affected countries (Guinea, Sierra Leone, Liberia)[67]. Thus, the use of personal protective equipment (PPE) including clothing, helmets, goggles and other garments is necessary to protect healthcare workers from infections[68]. The PPE constitutes a barrier between the wearer's body and the work environment which then reduce exposure to hazards. Unfortunately, this equipment is not always available in underdeveloped countries resulting in healthcare workers exposure to numerous infections.

1.1.9 Age factor in Ebola virus infection outcome

Though several studies have indicated that the case-fatality rate in EBOV infection disease is dominated by different factors including viral genotype, host immune response, and bed side care, we found few studies regarding the role of age in EVD survival.

Available records from previous Ebola outbreaks in Africa and notifications on some data indicate a lower case-fatality rate in persons under 16 years of age compared to elderly people[69]. Previous findings have noted that children are relatively protected against EBOV infection due to limited exposure to EVD patients or due to prohibition of funeral activities preparation compared to adults[70]. Nevertheless, regarding household contacts, the risk of virus exposure in both adults and children are relatively equal[66,71]. In Africa, elderly people have an important position in societies, which consequently put them on the frontline to EBOV either with sick persons or dead bodies. Caring of EVD patients and attending funeral activities is significantly higher in the elderly age group[67,72]. As a result, old age remains the most exposed group to EBOV infection. This fact has been demonstrated by Dowell e*t al* after the 1995 Kikwit EBOV outbreak. The study indicated that only 27 over 315 EVD patients were under 17 years old and reported an increase risk of household exposure in adults[67].

1.1.10 Co-infection and clinical outcome of EVD

Co-infections are common amongst EVD patients, and it is relatively unknown if they are harmful or beneficial. Malaria *(Plasmodium falciparum)* co-infection has been associated with a significant increase in fatal outcome with 2.5-fold odds of death in EVD [68]. Additionally, a systematic search on EBOV patient samples to understand the outcome of EVD associated with *P. falciparum* load indicated that 87% of patients with broad range load (highest reads mapping) of *P. falciparum* passed away, however this was similar to that seen amongst survivors[73]. This finding suggests a non-additional effect of *P. falciparum* on the response during EVD. Furthermore, another study aiming to show the correlation on the abundance of *P. falciparum* gene transcripts associated with the coagulation pathway was conducted on 23 Guinean EVD survivors by Liux *et al* [35]. In the report, the study indicates an increase of transcripts in patients tested positive for *P. falciparum* compared to those tested negatives for *P. falciparum* and showed the effectiveness of associated antimalarial therapy in EBOV treatment. The study also demonstrated through deep sequencing the presence of viral nucleic acids, bacteria, fungal, and parasitic organisms in many patient blood samples. Those opportunistic microorganisms may be responsible for severe bacteria

sepsis, pneumonia or bacteraemia leading to host death. Results from oral swab samples collected from EVD patients indicated the presence of bacteria such as *Neisseria meningitidis* and fungi which largely contribute to weakened host immune system [74].

GB virus C (GBV-C), known as human pergivirus, was associated with EBOV during the West Africa 2013-2016 outbreak. GBV-C is an immunomodulatory virus which attenuates the pathogenesis of HIV. Subsequently, GBV-C co-infection with EBOV show no significant survival or fatality outcome in EVD patients examined in Sierra Leone[75]. Finally, understanding co-infection in EVD may lead to new treatments and better understanding of immune interactions.

1.1.11 Ebola; Diagnosis, prophylaxis and vaccines

Early detection of the disease, good supportive care and a strong adaptive immune response will help patients recover from the disease[66]. Rapid diagnostics is one of the most important aspects in preventing an EVD fatal outcome. Disease confirmation is done in laboratories through detection of the causative viral RNA genome via RT-PCR. ELISA techniques are used for antibody detection and virus isolation is performed by cell culture[76].

Selective viral inhibitors or neutralizing antibodies, such as ZMapp, can retard or block viral entry into host cell[81]. An identified molecule of benzylpiperazine adamantane diamide-derived compound is capable of inhibiting EBOV infection and is currently being investigated[75]. The experiment reported that the inhibitors target is the virus endosomal membrane protein which blocks interactions with NPC1, which plays an important role in viral membrane fusion. In Guinea, during the 2013-16 outbreak Sissoko *et al* performed an experimental treatment with the viral replication inhibitor, the Favipiravir (clinical trials.gov. NCT02329054). Unfortunately, the trial ended with nuanced conclusions. No conclusion on the efficacy of the drug was established; however, it generated understandings about a quick set up and run of Ebola trial in EVD foyer [77]. Additionally, other experiments have established that a combination of humanized monoclonal antibody (ZMapp vaccine) has given promising result in NHPs[79].

Since the 1980s, EBOV vaccines development has been a global health concern. The first EBOV vaccine produced was an inactivated virus vaccine[43]. Since, multiple vaccine platforms have been developed such as: conventional ebolavirus vaccines, ebola virus-like particles (VLPs), non-replicating vaccine vectors and, replicating-competent vaccine vectors[52]. Presently, the increasing number of ebolavirus vaccines in development are most commonly base on GP, NP and VP40 viral proteins. These vaccine platforms have demonstrated successful results in protecting NHPs against

lethal infection and were advanced to clinical trials in humans[52]. Notably, rAd26-EBOV, chAd3-EBOV, rVSV-EBOV and MVA-BN filo vaccines are the most promising vaccine candidates for EBOV infection[83]. ChAd3-EBOV, known as cAd3-EBOZ derived from chimpanzee adenovirus type 3 and expresses ZEBOV and SUDV GP, was formally used in trial in the US and Malian adults volunteers for its safety, tolerability and immunogenicity[87, 84]. The experiment documented that 1 x 10^{11} pfu of ChAd3-EBOV is well tolerated and could boost an antibody titer that could protect against wild-type EBOV infection. Finally, the experiment suggested that ChAd3-EBOV is effective to terminate Ebola virus transmission and consequently protect household family members and relatives. Currently, on-going clinical trials involving chAd3-EBOV and rVSV-EBOV are in progress but not yet published[83]. Trials feature the United Kingdom, the United States of America, Switzerland and some African countries. On the other hand, an FDA approved Ebola Zaire vaccine ERVEBO (rVSV ZEBOV) has been used in the current DRC outbreak since December 2019[80].

The licensed vaccine ERVEBO is an attenuated vesicular stomatitis virus with its host glycoprotein replaced by an EBOV GP gene. To date, rVSV-based vector is being used as a promising live virus vaccine candidate for a panel of viruses including influenza virus, human immunodeficiency virus, and bovine viral diarrhea virus[88,89,90]. It is then obvious to conclude that in an outbreak event, ring vaccination should inclusively include local medical staff, family members and close contact relatives as the most appropriate strategy to go forward infection eradication.

1.2 The West African EVD Outbreak of 2013-2016

The West Africa EVD epidemic has been the largest to date resulting in numerous deaths and social unrest[13,91]. The outbreak is thought to have originated during December 2013 in a small village called Meliandou in Gueckedou (Republic of Guinea) and was confirmed in March 2014 (Figure 6).

The index case was reported to be a 2-year-old boy and was related to a zoonotic transmission, probably, from bats[92]. In the beginning, the unknown disease was considered as a mysterious event. It quickly spread to the nearest prefecture Macenta and eventually reached the capital city, Conakry. First samples were collected from deceased patients in Macenta and sent to Europe where it was confirmed to be EBOV pathogen. Unfortunately, the epidemic spread to the neighboring countries Liberia and Sierra Leone via land borders. In a short period of time, the disease was notified in other countries such as Mali, Senegal and Nigeria. Several efforts were made by partners (international organisations) and national teams to contain the epidemic. The

last WHO report on June 10, 2016 revealed 28,616 confirmed cases amongst which 11,310 deaths occurred[13]. The report stated that the West Africa ebolavirus outbreak was the longest, largest, deadliest and the most complex ebolavirus outbreak since the discovery of the virus in 1976. Previous EVD outbreaks in Africa have been linked to Zaire, Uganda or Sudan EBOV strains and the 2013-16 West Africa epidemic was thought to be from the Zaire Ebolavirus (EBOV-Mayinga) strain[14]. But the strain was later named Makona (EBOV/Mak) after the Makona river bordering the three main affected West African countries. The Makona river originates from Guinea (Makona means *bitter* in the local language of the Toma tribe), passes through Liberia and Sierra Leone and ends in the Atlantic Ocean. The EBOV/Mak strain encountered different mutations (lineages) in Guinea (GN1, GN2, GN3, GN4), Sierra Leone (SL1, SL2, SL3) and Liberia. These lineages were all linked to the initial lineage first notified in Guinea[93]. Jeffrey *et al* sequenced Liberian EBOV/Mak positive samples and observed 23 nonsynonymous mutations and 1 nonsense mutation[94]. The study finally reported that at least 33 viral mutations occurred during the West Africa ebolavirus outbreak. In addition, EBOV GP mutant GP-A82V was found to be located at the NPC1 binding interface and could possibly be responsible for high infectivity in human cells[95].

Marzi, *et al* investigated the similarity between the two strains of EBOV, Mayinga and Makona[96]. Both strains were inoculated into Cynomolgus macaque models to compare their pathogenic effects and it was reported that EBOV/Mak infection disease progressed slower than EBOV-Mayinga and the experiments showed a high case fatality rate in EBOV/Mayinga (≈90%) compared to EBOV/Mak (≈50%). In contrast, major release of IFNγ was notified in EBOV/Mak individuals compared to EBOV/Mayinga subjects.

In Guinea, by the end of September 2016 there were 3,358 confirmed cases and 2,088 deaths amongst which 115 were healthcare workers. In the Macenta prefecture, located in Forested region of Guinea, a total of 714 confirmed cases were reported with 467 deaths[97]. Unfortunately, there are no publications on two cases of suicide likely due to both sexual dysfunction and stigmatization. Investigations on the long-term effects currently seen amongst EVD survivors will bring more understanding about the disease in Gueckedou. Common side effects include ocular pain, photophobia, renal and sexual disfunction, amenorrhea and trouble of vision. Nevertheless, ebolavirus persistence was studied in some Guinean male survivors following the outbreak[64]. RT-PCR was performed every three months to determine the presence of viral RNA in sperm and the study report established that viral RNA is detect-

able in the sperm at least 13 months after recovery. Although, a similar study performed on MARV survivors indicated that MARV RNA could persist 3 months after the onset of the disease[66]. Therefore, survivors from both ebolavirus and marburgvirus should be provided and instructed to use condoms during the convalescence period in order to avoid sexual transmission. The last case of the West Africa ebolavirus disease outbreak was known to be from a sexual transmission. It was notified in Guinea after 3 community deaths in Nzerekore region at Koropara subprefecture and later spread in Liberia. The virus might have persisted in seminal fluid for about 531 days after recovery and was transmitted in February 2016 via sexual intercourse[65].

The starting point of the West Africa EVD outbreak

Figure 6: Meliandou Village

Meliandou village shown by the red dot is in West Africa in the Republic of Guinea. Lat/Long 8°37'20'' N10°03'51.1''W. From https://probaway.files.wordpress.com/2014/10/meliandou_map.jpg . Meliandou village, the starting point of the West African 2013-2016 EVD huge outbreak. The village is about 8 km from Gueckedou prefecture. Borrowed from https://probaway.files.wordpress.com/2014/10/meliandou_village.jpg

1.2.1 **West African Outbreak management**

The 2013-16 ebolavirus outbreak in West Africa illustrates the potential for epidemics to emerge from a single zoonotic transmission. In the three affected countries, local government authorities and international partners effectively acted to halt the epidemic. The outbreak started with early mistakes including poor dissemination of information about the disease from inexperienced persons (e.g. media), political mistrust of local governments and opposition parties[98]. Other elements of the disease spread could be associated to poor healthcare systems, bad infrastructures and untrained healthcare workers in affected countries. These factors seriously contributed to the spread of the disease. While local government thought about population manipulation by opposition parties; opposition parties accused local government of inventing new strategies to avoid presidential elections planned in the year 2015 in Guinea and Sierra Leone. Political considerations, local government's unclear position, and population mistrust lead to hamper international efforts. Moreover, the spread of the disease was also thought to be planned by leaders of the three affected countries Guinea, Liberia, and Sierra Leone to raise money from worldwide organizations. As misunderstandings took place, the disease kept spreading rapidly to become an uncontrollable crisis of international level. The large population base, growing trade business between countries via land borders and health system inexperience regarding the disease all contributed to the spread of EVD in West Africa.

The causative agent of the outbreak was simultaneously identified to be *ebolavirus* (EBOV Zaire strain) at P4 laboratories respectively in Lyon (France) and in BNI (Germany) by polymerase chain reaction (PCR)[99]. On March 23, 2014 WHO declared the epidemic as an international concern[91,92]. It followed that local governments, partners, and NGOs actively promoted information about the disease via illustrations, fact sheets, videos and, posters. Although, healthcare systems improved through construction of treatment centres, isolation systems, rapid diagnostic (RT-PCR and sequencing) and staff training. Additionally, local communities successfully got involved via multiple outreach on securing funerals and burials and contact tracing. International interventions include clinical trials (JIKI, ZMAP)[77]; experimental treatment (interferon)[100]. These combined efforts all helped with EVD eradication in West Africa.

1.2.2 Social impact of the West Africa EVD

The impact of EVD on public health systems, economic and social life in Guinea, Liberia and Sierra Leone was seismic. It brought fear and paranoia to the population. People (workers or traders) were immobilized or moved from affected areas to unaffected areas to avoid transmission. Schools, training centres, enterprises and businesses temporarily or totally closed causing loss of jobs and education. Fear and stigmatization toward survivors of the disease was common. As a result, some cultures were broken, extended family meetings avoided, and ceremonies restricted. Discharged patients (survivors) and family members were banned from attending public assemblies. In some places, this exclusion provoked mental problems in some individuals leading to suicide as for that of Gueckedou (no reported).

High infection rates amongst close contacts, relatives and healthcare workers, significant case fatalities in patients attending healthcare facilities, and finally a lack of curative treatment contributed to increase fear and distress in the population. These effects acted to considerably reduce health facilities attendance in affected areas. Fear, mystery, paranoia and unfamiliarity related to the disease often provoked population movement and, consequently, medical staff were reluctant to assist patients or return to regular duties in healthcare facilities[14].

Furthermore, emotion and ignorance lead local people to perceive that healthcare centres and partners including NGOs and international organizations were responsible for spreading the virus. As a result, common tropical curable diseases such as malaria were not treated resulting in more deaths than the filovirus itself. Field intervention groups from national authorities and partners added to community fear and distress when wearing full personal protective equipment (PPE) and while carrying patients or disinfecting zones. Also, previous outbreaks have come to prove that multiple and diverse media attention including bad reporting from unexperienced and uninformed persons about the disease contributed to fear and distress. This resulted in resistance due to population mistrust and made it difficult to control and sustain the epidemic. Finally, stigmatization behind EVD left affected individuals (widows and orphans) homeless and abandoned after recovery.

1.3 Sub symptomatic infections

Sub-symptomatic and asymptomatic infections are defined as infections with complete absence of clinical symptoms whereas, pauci-symptomatic is an infection with minimal symptoms[101]. Detecting asymptomatic infection early is important to prevent disease and infection spread. Asymptomatic persons can be a carrier of the disease and constitute high risk to vulnerable people, therefore, asymptomatic persons should be diagnosed and treated so that future medical problems and infection transmission can be avoided.

In general, asymptomatic infections are often discovered when subjects undertake medical tests either for research proposes or in hospital admittance conditions associated with another disease occurrence. One of the major problems with asymptomatic infections such as HIV/AIDS and cytomegalovirus is a weakening of the immune system or damage to tissues and organs[101]. Consequently, people become vulnerable and susceptible to opportunist co-infections. For example, human immunodeficiency virus (HIV) causes severe systematic T cell (CD4) destruction which reduces cell-mediated immunity[102]. Therefore, a wide range of opportunistic infections and cancers can occur followed by tissues damages (e.g. brain) and systemic organ damage (e.g. chronic cardiovascular, hepatic). Notably, asymptomatic infections are silent therefore, people are unaware about serious medical problems. In a recent study published on zika virus (ZIKV) it was reported that 3.2% of asymptomatic ZIKV infections were found among pregnant women (n=1116) during 2016 in Cajamarca (Peru-South America)[103]. Another prospective study carried out in India reported that the incidence rate of asymptomatic leishmaniasis cases progressing to symptomatic cases in humans was about 3.3%[104]. In addition, Human cytomegalovirus (HCMV) is known to be one of the most common asymptomatic infections around the world[105,106]. The HCMV is known as a major cause of childbirth defects. HCMV is also noted as the responsible agent for permanent neurodevelopmental disability in children which leads to intracranial abnormalities including calcifications and polymicrogyria in infants with congenital cytomegalovirus infection[107]. Finally, findings on HCMV infections have established that the infection could become life-threating and causes immune system failure, systemic organs injuries and possibly inhibition of cell death over time, leading to various cancers[106,107].

Asymptomatic infection should be also considered in the context of Ebola virus infections as a public health concern and symptomless people constitute potential reservoirs in infection transmission in communities.

1.3.1 Potential reason for sub -symptomatic infection

As previously described, filoviruses (e.g. Marburg and Ebola virus) are potentially dangerous and cause severe haemorrhagic fever disease in human and non-human primates (NHPs). Research on filovirus asymptomatic infections in humans could help to understand occurrence and contribute to control the spread of disease.

When exposed to a pathogenic agent, there are two possibilities. Either the infection progresses toward disease with apparent signs and symptoms or toward cure with no symptoms. In the event of the asymptomatic situation, the infection progresses without symptoms therefore, a pathogenic agent could be more easily transmitted as the infected person who is still mobile and has no knowledge of their infectious status.

1.3.2 Historical Filovirus sub symptomatic infection

Since its discovery almost 40 years ago in Germany in the city of Marburg and Yugoslavia in 1967, MARV has been associated with several haemorrhagic fever outbreaks in humans and non-human primates with a high rate of mortality up to 90%[108]. Studies in Central Africa (Gabon and Republic of Congo) indicate a fruit bats, *Rousettus aegyptiacus*, to be the potential reservoir of the Marburg virus[109]. Specific viral RNA and IgG antibody were identified in those bats' species with no apparent signs or symptoms. unfortunately, there are few available publications regarding Marburg virus asymptomatic disease infection.

In 1999-2000, the first non-imported MARV outbreak occurred in the DRC in Durba region at Watsa Town[110]. Following the outbreak, a sero-survey study to identify possible asymptomatic patients among household contacts was conducted in Watsa subdistrict. A total number of 124 direct contacts of 73 affected households participated in the survey. Blood samples were collected, and anti-MARV IgG detection was performed by ELISA. Two volunteers known to have presented symptoms but were not laboratory tested were reported to be seropositive. Unfortunately, no serologic evidence for asymptomatic or mild MARV infection was reported. Furthermore, in 2003 in the DRC, another survey to assess risk factors for Marburg haemorrhagic fever revealed 2% of seropositivity for Marburg immunoglobulin G antibody[111]. To conclude, asymptomatic Marburg virus infection is not well understood due to a limited number of available documentations compared to ebolavirus infection.

With regards to EBOV, it is one of the most virulent pathogens in the *Filoviridae* family with a fatal outcome from 7 days onset of the disease[73,112]. However, minor cases of EVD may not be recognized if there are no associated symptoms[50]. Infected subjects

possibly remain sub or asymptomatic due to a low viral infectious dose or a strong antibody response to the virus.

Work on 24 close contacts of EBOV RT-PCR positive subjects in Gabon following the 1996 EBOV outbreak revealed 11 asymptomatic cases (45.83 %)[50]. In this study, blood samples were collected 4 times within a month from exposed individuals to EBOV infected people. Both asymptomatic individuals (close contacts) and symptomatic people (EVD confirmed) were included in the study. ELISA and Western blot tests were successively performed for EBOV antigen specific antibodies (IgM and IgG) detection. Additional investigation included the determination of different cytokines and chemokines. Extracted viral RNA from individuals' peripheral blood mononuclear cells (PBMCs) were amplified and sequenced. Interestingly, asymptomatic individuals 45.83% (11 of 24 asymptomatic people) show both IgM and IgG responses to EBOV antigen. In addition, Western blot test indicated that IgG response were oriented to VP40 and to the NP. In contrast, VP24 and the GP showed no nucleotide differences regarding both symptomatic and asymptomatic individuals. Notably, the search demonstrated the presence of strong inflammatory response characterized by massive circulation of cytokines and chemokines in asymptomatic individuals. Finally, these results strongly demonstrated the evidence of possible asymptomatic occurrence of EBOV infection in individuals which might not necessarily be related to viral mutations.

Mulangu *et al* investigated EBOV infection in a large population base (n=3415) in the DRC and found that 11% showed anti-EBOV IgG[113]. The study revealed high seropositivity in males aged over 15 and provides strong evidence that unrecognized EBOV infection has been circulating in the population. In addition, Mulangu *et al* studied the seroprevalence of EBOV in Efe pygmies in DRC to determine the possible circulation of the virus within this population[114]. In total, 300 participants (aged over 10 years) volunteered for the study. The Efe population is known to be highly exposed to EBOV through their daily activities such as bushmeat hunting and manipulation, entry into caves generally hosted by fruit bats, and contact with diverse wildlife. Collected samples were assayed by ELISA for IgG to EBOV. Efe pygmies were found to be highly exposed to EBOV with 18.7% seropositivity with no link to a particular activity or contact with wildlife. This seroprevalence to EBOV was known to be the highest ever reported. It is unfortunate that the nature of this exposure was not established. Moreover, in 1983, a published serological and epidemiological survey in Cameroon, Central Africa among healthy rain forest farmers (n=1517) showed a positive rate of 9.7% to EBOV[115]. The study included volunteers from five regions and blood samples were tested using indirect immunofluorescence assay. With regards

to the data produced, it was confirmed that EBOV has been circulating in those regions of Cameroon with mild or no clinical cases mainly amongst young adults fully involved in bushmeat hunting and manipulation.

Furthermore, Leroy *et al* conducted a serological survey to determine ebolavirus prevalence in nonhuman primates troops (chimpanzees) in Cameroon, Gabon, and DRC[116]. The study involved 790 NHPs from 20 different species. Samples were assayed by ELISA to determine EBOV specific IgG antibodies. The data indicated 12.9% of seropositivity to anti-EBOV in wild-born chimpanzees and that EBOV-specific IgG was detected in five species of NHPs including Drills, Baboon, Mandrill, and Cercopithecus as well. Finally, the report established that EBOV broadly circulates in Central Africa and that the virus was present in some places prior to first outbreak in human. Also, it demonstrated the possible occurrence of asymptomatic EBOV infection in NHPs

These findings demonstrate that ebolaviruses have been circulating in human and non- human primate in multiple locations throughout Africa. In particular, the detection of EBOV-specific antibody in NHPs might lead to more investigations to define the virus reservoir probably in NHPs. Considering these results, it follows that EBOV outbreaks may occur anywhere in Africa. So, action should be taken regarding population awareness on EBOV to prevent future outbreaks.

1.3.3 Case studies on Ebola virus asymptomatic transmission

Understanding the context of asymptomatic transmission is important to contain and prevent outbreaks. Results from previous studies in the context of *ebolavirus* as reviewed by Heeney 2015[117] demonstrate that more work is needed to understand the triggers that result in virus persistence and re-emergence. Work performed by Sissoko *et al*. reported that a breast-fed female infant of 9 months died after succumbing to EVD, probably infected by her asymptomatic mother[118]. Guinean authorities were first notified of the case in the fall of 2015 and no epidemiological link could be established. Investigations show that both the mother and the father did not develop detectable symptoms of EVD, and RT-PCR tests indicates no viral RNA in their blood samples. However, further analysis of bodily fluids revealed EBOV RNA in both the mother's breast milk and the father's seminal fluid. Molecular epidemiological tracing indicates that the virus from the child and the mother belonged to the Sierra-Leone 3 (SL-3) lineage and clustered to the large Conakry-Dubreka circulating sub-lineage strain. It was then concluded that the virus might have circulated from the mother to the child via breast feeding. Further, sequence analysis of mother and father RNAs

suggested that the virus from the mother came from the father without classical symptoms. Another breast-feeding infection was notified in Guinea by Nordenstedt *et al*[119] , in this study a certain pregnant woman who formally cared to her EVD suffering mother (unfortunately the mother died) gave birth to twins and a few days following the birth, one of the twins passed away presenting with general symptoms of EVD. Unfortunately, no PCR test was performed prior to the burial. Afterwards, the mother was suspected to have EVD-like symptoms such as fever and headache. She was then admitted to an EVD treatment center with her second baby and tested positive to EBOV using RT-PCR test. The breast feeding was immediately stopped and fortunately, the baby stayed EBOV negative and was released after two PCR negative results. In addition, another study was performed in Guinea on two cases of infant transmission through breast feeding, where mothers were tested positive to EBOV by RT-PCR[120]. In the first case, the mother (approximatively 30 years) and child (six months infant) were both admitted to the Ebola treatment center (ETC) at Gueckedou (Guinea). The mother's blood, urine and breast milk were all tested on RT-PCR and only the urine test showed positivity to EBOV. As the mother's breast milk was tested negative breastfeeding continued. Fortunately, the mother recovered and was released from the ETC after two RT-PCR negative tests on her blood. On the other hand, the child's blood was tested positive to EBOV and he later passed away. In the second case, a woman of about 20 years was admitted to the ETC a week after childbirth. She was blood tested positive to EBOV on RT-PCR. The breast feeding was immediately stopped, and the child separated. Unfortunately, no RT-PCR test was performed on her breast milk. On the other hand, EBOV RT-PCR was negative on the child's blood after a sudden onset of fever. Fortunately, the mother recovered and was released after two RT-PCR negatives on her blood and happily the child never developed EVD. The study concluded that when caring for mother-child pairs in the event of EVD, potential attention of testing bodily fluids such as breast milk and urine in addition to blood test should be considered due to the possibility of prolonged EBOV shedding in those fluids.

Furthermore, the transmission of EBOV via breast feeding was also commented upon when sequencing samples from both mother and child found positive to EBOV in Sierra Leone[121]. Unfortunately, transmission via breast milk was uncertain as no blood testing was earlier performed for the mother and both mother and child were close contacts to an EVD confirmed patient. The fact that the mother (who was asymptomatic) had her breast milk tested twice positive to EBOV and that the child (who was symptomatic) was actively breastfed suggested that the child might have been infected by the mother through breastfeeding. These findings conclude that EBOV can persist in breast milk and possible transmission may occur via breast feeding. Further

investigations should be undertaken to determine the duration of the virus in breast milk.

In Guinea, Daouda *et al* conducted a longitudinal study on men identified as EVD survivors. The study enrolled 26 volunteers to evaluate the dynamic of EBOV persistence and clearance in seminal fluid[64]. Samples were collected every 3 to 6 weeks and tested for EBOV RNA via RT-PCR until two negative results were simultaneously confirmed. The study revealed that ebolavirus RNA could persist in seminal fluid for up to 13 months after being discharged. In addition, an example of possible sexual transmission was reported in the past West Africa EBOV outbreak in Guinea. The case was notified and reported to WHO following three epidemiological linked community deaths[65]. The new cluster started at Nzerekore region in Koropara sub-prefecture and later spread to Liberia. Reports concluded that the virus could have persisted in seminal fluid for approximately 470 days following discharged from the treatment center and might be sexually transmitted during February 2016. In total, eight community deaths occurred, and the case fatality ratio was reported as 80%. Although, the Sierra Leone health ministry and partners, including WHO and CDC, conducted another study amongst EBOV disease male survivors of Sierra Leone[122]. The survey generated a cohort of 220 volunteers to investigate the duration of EBOV in seminal fluid. Samples were tested by RT-PCR for several months following discharged from the treatment centre. The study established that the shortest time to carry EBOV RNA in seminal fluid was approximately 100 days (3.3 months) and the longest time was about 500 days (16.7 months). In addition, Prof. Kader and his team conducted a cross sectional study on sex practices and awareness of EBOV disease among male survivors and their partners in Guinea (Coyah prefecture)[123]. The survey included a total of 234 survivors, 256 sexual partners and 65 control group individuals. It was concluded with strong recommendation that: ‘’*sexual health interventions, including counselling services in the presence of sexual partners, become an integral component of survivors post discharged follow-up to minimise probable sexual transmission due to viral persistence in the seminal fluid* ‘’*.*

1.3.4 Pros and cons of seroprevalence studies

Seroprevalence studies are often used to determine prior exposure to a pathogen. Detecting asymptomatic infection via molecular diagnostics such as RT-PCR gives us definitive proof that an individual has a viral infection. However, seroprevalence studies conducted using ELISA based methodology, will give a retrospective view on prior exposure to a pathogen. This exposure may be recent or years ago, therefore, even when combined with subjective health questionnaires seroprevalence studies might not be fully reliable in ascertaining the provenance of asymptomatic infection. However, they may contribute to information on disease burden in a community and help to identify, support, and prevent infection spread in healthcare settings[124]. Additionally, there is a fact that wild antibodies might cross react with the protein of interest and this would generate false positive data.

In conclusion, it is probable that EVD may, in some cases, has resulted in sub clinical or asymptomatic infection, and both low infection dose and strong innate immunity could have successfully helped patients to remain disease free. Our study aims to determine the circulation of ebolaviruses in the forested region of Guinea among highly exposed individuals (bushmeat hunters and their household members). These people are in daily contact with wildlife animals including several kinds of fruit bats considered as potential virus reservoirs.

1.4 Lassa virus; Introduction

Lassa virus (LASV) is an *Arenaviridae* family member which represents a large group of viruses that cause persistent infections in human and NHPs (**Table 3**)[125]. Infections are usually asymptomatic and transmission to human or NHPs occurs through contact with the virus reservoir. The *Arenaviridae* family is divided into three genera (*Mammarenavirus, Reptarenavirus and Hartmanivirus*) and is composed of two major subgroups including Old World viruses (Lassa and Lujo virus) and New World viruses (Junín, Machupo, Guanarito, Sabia and Chapare virus)[126,127].

For the last 30 years, LASV hemorrhagic fever has been broadly studied. Nevertheless, new discoveries in viral pathogenesis, and advanced diagnostic techniques have recently progressed[128].

To conclude, it is wildly known that Lassa virus transmission occurs from rodent to human and the disease spreads in community from human to human via close contact with bodily fluids. LASV is an emerging virus which needs to be handled in biosafety level 4 containment.

Table 3: Arenaviridae family; Genus, viruses

Family	Genus	Viruses
Arenaviridae	*Mammarenavirus*	Old World Arenaviruses Lassa virus (LASV) Lymphocytic choriomeningitis (LCMV) Mopeia virus (MOPV) New World Arenaviruses Guanario virus (GTOV) Junin virus (JUNV) Machupo virus (MACV) Pichinide virus (PICV) Pirital virus (PIRV) Sabea virus (SABV) Tacaribe virus (TCRV) Whitewater arroyo virus (WWAV)

1.4.1 Discovery of Lassa virus

LASV disease was first identified in 1969 in Nigeria at Lassa village, hence its name[129]. The disease was notified in a nurse working at Jos missionary hospital in Nigeria[130]. Later, the virus was isolated from the affected nurse who subsequently survived with thorough medical support in the United States. Lassa fever is an animal-borne or zoonotic acute viral disease endemic in West Africa particularly in Nigeria, Sierra Leone, Liberia and Guinea[131,132]. In contrast, the disease seems to be mild or undiagnosed in neighboring countries Cote d'ivoire, Ghana, Togo and Benin. However, the presence of the virus reservoir, the Natal multimammate mouse (*Mastomys natalensis*) and a few isolated cases indicate the circulation of the virus in those countries[92]. The virus is responsible for about 100,000 to 300,000 human infections a year with approximately 5000 annual deaths[91]. To date, the only virus reservoir known is *M. natalensis* which is largely distributed in West African countries.

In Nigeria, Lassa fever is endemic. The disease is confirmed in about 6% of febrile adults and 3.5% of febrile children when admitted to hospitals[133]. Recently, Nigeria health authorities have reported a case fatality rate of 24 - 33% in tertiary hospitals. In contrast, despite the seriousness of the disease, it is reported that Lassa fever control in Nigeria has been widely neglected[131]. Though several efforts have been deployed at all levels including international partners and local government; however, Lassa fever remains a major public health concern in the country. From January to March 2018, Nigeria experienced its largest outbreak with a total of 1,893 cases reported amongst which 423 were laboratory confirmed with 106 deaths (case fatality rate 25.1%)[94].

In Guinea, Lassa fever is infrequently observed. The last confirmed case occurred in September 2018 in Mamou. The subject was known to have originated from the Southern region of Guinea at Kissidougou town. Fortunately, early diagnostic and rapid intervention through contact tracing and follow up successfully contained the disease[134]. Moreover, past sero-surveillance studies on Lassa fever disease, conducted in Guinea, established population exposure to the virus[135,136]. Bausch *et al* investigated the epidemiology and clinical presentation of the Lassa fever in Guinea in 2001. Lassa fever suspected cases were tested using an ELISA platform for antibody detection and the analysis indicates 7% of positivity out of 311 samples tested[137]. Additionally, a total of 1616 small rodents (956 *Mastomys* sp.) captured in 444 households in the savanna, mixed savanna-forest and forest region of Guinea were studied for Lassa virus antibody and antigen detection[135]. The study results respectively show 11% and 5% of antibody and antigen detection in *M. natalensis* rodents. Moreover, in 2006, Koivogui *et al* found up to 55 % seropositivity to LASV

amongst murid rodents population of 13 genera from 8 different localities in Guinea[132]. Another study to estimate the prevalence of Lassa IgG in human population of rural area of Guinea including Gueckedou, Lola and Yomou (Southern region) indicates 12.9% positivity in humans with no prior report of Lassa fever disease[136].

1.4.2 Lassa; Species and Phylogeny

LASV is an RNA virus of the *Arenaviridae* family, it has a negative-sense, single stranded RNA viral genome [128,129]. The viral RNA is enveloped, bi-segmented and possess both large and small genome section. Formally, four strains of LASV lineages were identified amongst which three were identified in Nigeria (GA391, AV and LP) and one in Sierra Leone, Liberia and Guinea [138]. Presently, based on the geographical limitations and the new virus strain discovery, LASV strains have been classified into six lineage groups [139]. Lineage I identified in 1969 mainly occurs in North-eastern Nigeria; Lineage II was isolated in South-eastern and South-central of Nigeria (Irrua, Ekpoma, Abakatti, Aba); Lineage III strains are found in North-central of Nigeria; lineage IV was isolated in Sierra Leone, Liberia and Guinea; Lineage V is from Mali, Ghana and Cote D'ivoire and Lineage VI was found in Togo in 2016. Notably, few genetic diversities result in lineages diversification. Especially, antigenic differences have been noted between Nigeria strains and mano river union strains[140,138]. Although, a single strain of LASV found in Sierra Leone and Liberia was genetically different from two strains found in Guinea regarding RNA fingerprinting comparison[140]. In addition, remarkable sequence dissimilarity in NP region was noted between Nigerian strains and the Sierra Leone Josiah strain and between two Nigerian strains[141].

1.4.3 Lassa; Genome structure and organization

LASV belongs to the Old-World complex of the *Arenavidae* family. The virus genome contains two RNA segments (large and small) which encode for two proteins in opposite directions resulting in four proteins in total. The large segment (L RNA, 7 kb in length) encodes for the small matrix protein Z and the RNA polymerase L (200 kDa)[142]. The small segment (S RNA, 3.4 kb in length) encodes for nucleoprotein NP and the surface glycoprotein precursor GP[143]. The coding strategy is named ambisense and the genome replication and transcription require the action of NP and L protein[144,145]. Based on the genome structure, the RNA-dependent RNA polymerase (RdRp) is located in the central region of L protein[146]. And it mediates the synthesis of two RNA species including mRNA and antigenomic RNA which represent the full

copy of the genome. Findings in *Arenaviridae* family established that the L protein encounters two binding sites of the Z protein respectively in the N terminus and in the RdRp[147]. Finally, regarding its large size and key role in viral replication and transcription, it has been indicated that L protein comprises four domains or regions[148].

Laboratory scientists in Germany isolated and sequenced LASV from the first exposed subject outside Africa, in a German funeral home worker[149]. The virus was cultured in Vero E6 cells and viral RNA was extracted using a RNeasy Qiagen minit kit. The isolated viral RNA library was sequenced on the Illumina MiSeq platform using v3 chemistry and paired-end sequencing (2x300 cycles) aligning the sequence reads to LASV L and S segments. The results show a coverage of 165x (median 145 x) for the L-segment and 225 x (median 185 x) for the S-segment).

Lassa virus

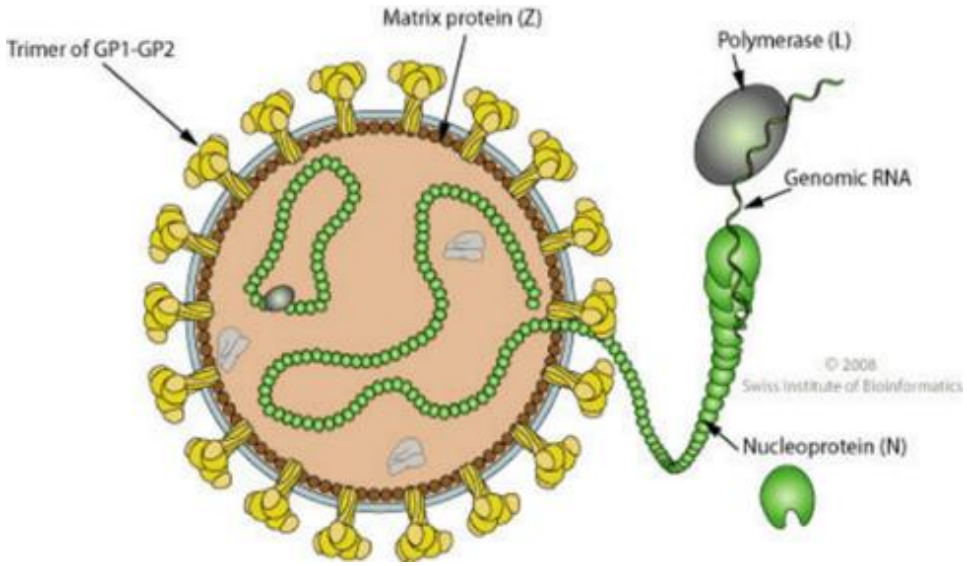


Figure 7: Lassa Virus structure

Lassa virus possess a negative strand bisegmented RNA genome. It encodes four viral proteins including GP/NP (S segment) and Z/L (L segment). Z protein shown as a small ring plays a central role in viral assembly and budding. The membrane glycoprotein GP-C is cleaved into two subunits including GP-1 which interacts with cellular receptor α-dystroglycan and GP-2 which is used for viral fusion to host membrane. Image from https://www.healthfitnessresource.com/lassa-fever-symptoms-prevention-and-treatment-by-who/

1.4.4 Lassa; Replication

Like in most common viral infections, virus attachment and entry into host cell cytoplasm is the determinant of potential disease occurrence and viral replication cycle. It follows that Lassa virus transmission occurs when the virus is transmitted from the rodent *M. natalensis* to human. Lassa virus attaches and enters host cells via receptor-mediated endocytosis. Unfortunately, to date, the real endocytosis pathway is not clearly defined. Nevertheless, the cellular entry is known to be highly sensitive to the decrease of cholesterol in host cell membrane[150]. Dystroglycan protein is expressed in most cells for tissues maturation. This protein is later divided into beta-Dystroglycan and alpha-Dystroglycan which is used by LASV receptors for cell entry[150]. Genome replication and transcription will then start in the host cytoplasm where fusion occurs at low PH. When the virus enters host cell through α-Dystroglycan mediated endocytosis, viral ribonucleoprotein (RNP) complex is released into the cytosol and the viral genome RNA is released. These phenomena occur as both S and L RNA segments produce simultaneously the antigenomic S and L RNAs. Both genomic RNA and antigenomic RNAs are actively involved in the transcription and translation process and serve as template for viral mRNA transcription[151]. It follows that new viral proteins are transcribed, and viral particles are assembled forming new LASV progeny. Finally, the matrix Z protein associated with endosomal sorting complex required for protein transport are crucial for virion budding and release from the host cell[151].

Upon host infection, LASV targets tissues and organs starting with mucosa, intestine, lungs, urinary system and later the virus progresses into the vascular system[150]. Like filoviruses, LASV mainly targets cells of the immune system including antigen-presenting cells (dendritic cells) and endothelial cells [49].

Lassa virus replication

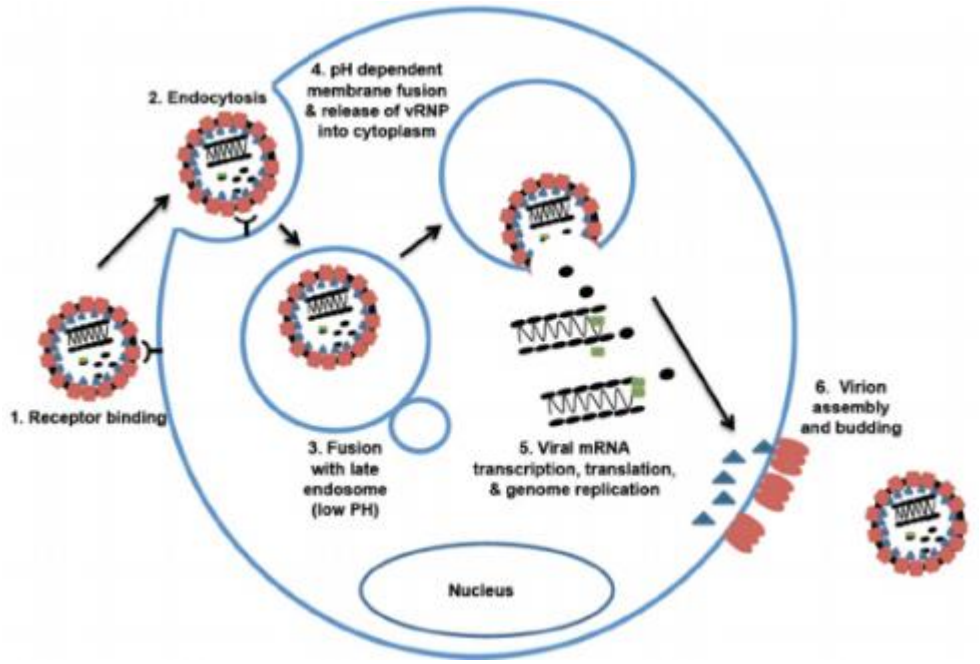


Figure 8: Schematic of Lassa virus life cycle.

(**1**) LASV virus is attached to host membrane via its GP proteins (GP-1 and GP-2) and host membrane receptors (α-dystroglycan or TfR). (**2**) The virus enters the host cytoplasm by endocytosis in late endosome (LE). (**3**) Following its entry, a subsequent fusion mechanism occurs with low pH. (**4**) Viral ribonucleoprotein (vRNP) is released and the genome is transcribed and replicated. (5) Newly translated viral particles are assembled to form new virion which is then transported to the membrane for budding. (6) Finally, new virus progeny is released from the host cell surface. Image from https://www.researchgate.net/figure/Arenavirus-life-cycle-1-Cellular-entry-is-mediated-by-different-cellular-receptors-a_fig3_277413510

1.4.5 Lassa; Transmission

The multimammate mouse also known as African rat has been identified as the reservoir or host[92]. The first evidence of LASV in West African mouse specie, the *M. natalensis*, was demonstrated by Monath *et al* in Sierra Leone in 1974[152]. LASV was isolated from mice sharing rooms with Lassa fever patients in an on-going LASV outbreak in Sierra Leone. Later, another investigation to indicated the possible similarity regarding LASV reservoir between *M. natalensis* in Sierra Leone compared to those in Nigeria was performed by Wulff et *al*. in 1975[153]. During the investigation, blood, tissues and urine from 8 different rodents' species broadly collected in Nigeria in Benue-plateau (North-eastern) were cultured in African green monkey kidney cell lines (Vero E6 cell) for LASV isolation. Of 151 rodents tested, the study reported LASV isolation from 8 wild rodents including *M. natalensis* (5), *R. rattus* (2) and *M. minutoides* (1). Unfortunately, no virus was isolated from rodents' urine. Recently, LASV was isolated from two new rodent species including *H. pamfi* (forest dwelling rat) and *M. erythroleucus* [154]. Those rodents were collected in Nigeria and Guinea. Strains from Nigeria were sequenced using next generation sequencing (NGS) and strains from Guinea used Sanger sequencing[154].

Notably, rodents largely circulate in savannas and forests of African countries including West, Central and East Africa. Multimammate rodents are found in occupied houses and are consumed in some places. Consequently, infected rodents (virus carriers) spread or shed the virus through urine and faeces for a long period of time and human-rodent contact is a source infection. So, human infection occurs when consuming contaminated food or infected rodent meat or when manipulating infected materials. Person-to-person transmission can occur from direct contact (blood, urine, excretion, tissue) of infected subjects or through indirect contact when manipulating contaminated materials (e.g. medical equipment or domestic materials)[91,92,93]. Also, a possible airborne transmission mode has been commented upon by Goeijenbier *et al* and Stephenson *et al* [101,155]. The reports indicate that airborne transmission can occur through inhalation of infected tiny particles in air (aerosol). Healthcare workers are at high risk of exposure therefore protective equipment should be used prior to sample handling or when caring for infected patients. It is also reported that Lassa virus may persist in urine for nine weeks and in semen for 3 months after recovery[97,99].

Lassa virus transmission and spread in population

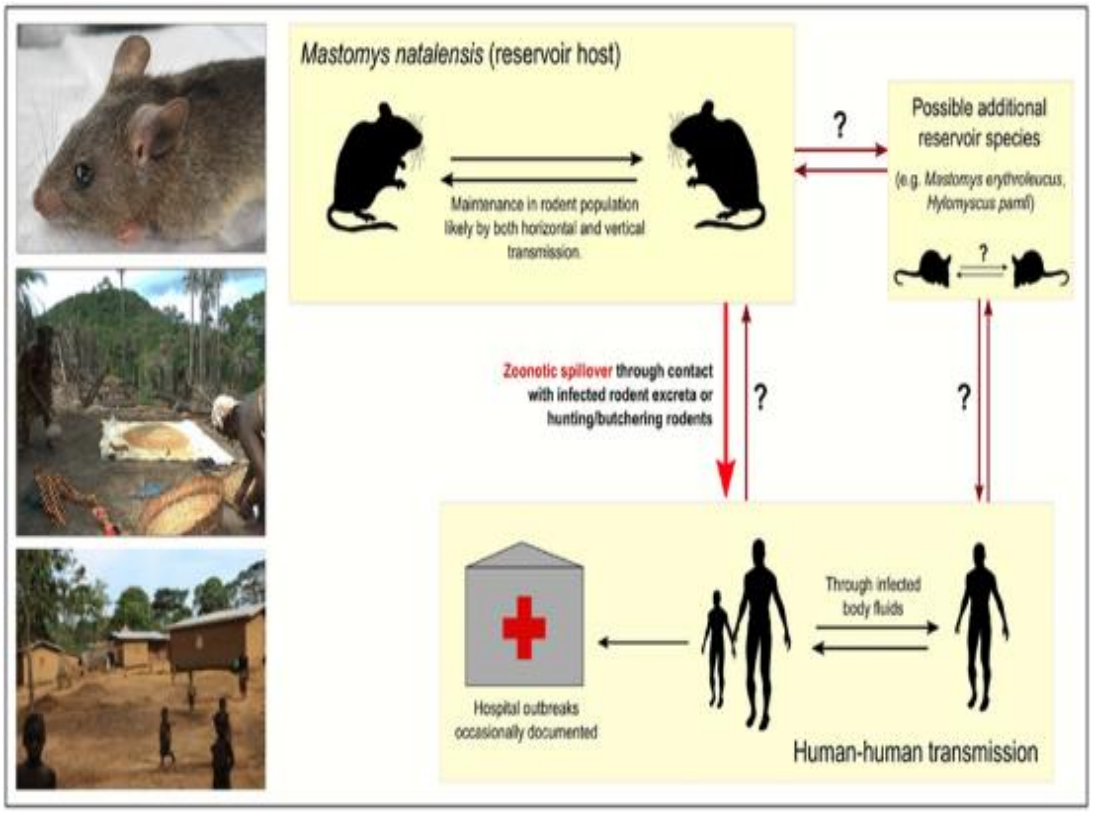


Figure 9: Schematic of Lassa virus transmission.

LASV circulates in host reservoir (*M. natalensis*) population and primary infection occurs from infected host to human through contact with infected fluids or materials (zoonotic transmission). Lassa virus infection disease spill over in community from human to human contact via infected bodily fluids and in several cases, Lassa fever disease is mild or asymptomatic and can be detected only through laboratory diagnosis such as IFA, ELISA or PCR assays. Image from https://www.researchgate.net/figure/transmission-dynamics-of-lassa-virus-in-rodents-and-humans_fig2_319099964

1.4.6 Lassa; Immune response

LASV is an Old World Arenavirus which is responsible for haemorrhagic fever infection disease in Human and NHPs. Lassa fever disease occurs sporadically in some West African countries and is endemic in Nigeria with multiple reported outbreaks since the discovery of the virus[156]. The incubation period is about 21 days with non-specific clinical manifestations. The major symptoms are common to that off other tropical diseases (malaria, typhoid fever and influenza) and include fever, fatigue, haemorrhaging, gastro-intestinal symptoms, respiratory and neurological symptoms[157].

Following LASV infection, the host immune system quickly responds first non-specifically then specifically as the virus induces effective immunity. However, fatal outcome of the disease might be associated with defective immunity or immunodepression[158]. Notably, LASV first infected cells are dendritic cells (DCs), macrophages (MPs) and antigen presenting cells (APCs) in which the initial viral replication cycle occurs[49]. Following infection of APCs, the virus will spread to the mucosal tissues and skin, secondary lymphoid organs and liver, hepatocytes, fibroblasts and endothelial cells are targets for viral replication. Normally, DC and MP cells activate the innate immune response through the induction of inflammatory mediators and subsequently initiate adaptive and cellular response to control viral infection[159]. Conversely, LASV-infected DC cells do not produce inflammatory cytokines (crucial to induce adaptive immunity) nor express activation molecules at their surface[160]. In fact, LASV infection fails to maturate DCs, consequently, the absence of DC activation and maturation in LASV infection might be associated with the immunosuppression observed in severe infection. In addition, it is reported that LASV-MP infected cells are not activated which extremely favours viral spread as otherwise the activated MP increase viral clearance[161,162].

Furthermore, the inhibition of innate immunity caused by LASV NP protein has been documented[163,164]. These experiments demonstrated that Arenaviruses' NPs, except the Tacaride virus, inhibit the production of type I IFN, IRF3 activation and nuclear translocation in response to viral infection. These attributions depend on the presence of a dsRNA-specific to the 3'-5' exonuclease in the C-terminal part of the viral protein[163,164]. Flatz *et al* demonstrated a two-faced role for T cell responses encountered in LASV infection[165]. Both normal laboratory mice and genetically engineered mice (HHD mice) with a humanized repertoire of T cells were used as animal models. While inoculated with live LASV, normal laboratory mice show resistance to the virus while HHD mice succumbed. Furthermore, HHD mice, containing humanised T cells that were experimentally removed could resist LASV infection. The study reported that

HHD mice with T cell depletion survived LASV infection whereas, HHD mice with no T cell depletion succumbed LASV infection. It established that the absence of extensive monocyte/macrophage activation in T cell-depleted mice contribute to deleterious innate inflammatory reaction. Finally, the study concluded that host immune defences against LASV either defeat the virus or act as essential facilitator for disease maintenance and evolution[165].

Specific immune response in LASV infection is mediated through early IgM antibody production. However, Johnson *et al*. measured the level of LASV antibodies in hospitalized patients and found that LASV disease outcome was associated with viremia but not with the development of antibodies[166]. Alternatively, the study reported that death possibly occurs before the usual time of IgG antibody production.

1.4.7 Lassa; Clinical symptoms and treatment

The incubation period for LASV is about 21 days[91]. In about 80% of people, Lassa fever is mild or asymptomatic and is left generally undiagnosed[99]. Mild symptoms are usually fever, tiredness, weakness and headache. However, in about 20%, Lassa fever symptoms can be severe including; respiratory distress, chest pain, abdominal pain, vomiting and bleeding (gums, eyes, nose)[94]. Death generally occurs 10-14 days post onset of disease and is associated with multiple organ failure. In some cases, neurological problems have been notified as palpitation, loss of hearing and encephalitis[131]. Despite LASV infection prevalence in most African countries, it is reported that only 1% of Lassa fever infections result in death[156]. Although, the mortality rate in patients hospitalized from Lassa fever infection disease is reported to be approximatively 15-20%[167]. Additionally, foetal death, miscarriage and, long term complications which result in deafness have been described in some cases[157,168]. Due to the number of febrile illnesses (e.g. malaria, typhoid fever), LASV disease often goes undiagnosed. To date, a wide range of laboratory tests are available for Lassa fever infection disease detection. These tests include RT-PCR[169], ELISA and plaque neutralization assays, immunofluorescence and cell culture [170].

For decades, Lassa fever has been successfully treated with Ribavirin an antiviral drug. It is reported that the main action of ribavirin is to prevent host cells from dying in reducing the inflammatory response[171]. However, various mode of action of ribavirin have been evaluated such as direct inhibition of viral RNA-dependent RNA polymerases and host inosine monophosphate dehydrogenase, modulation of the host immune response, inhibition of viral capping enzymes and lethal mutagenesis[171,172].

Nowadays, both favipiravir and ribavirin have been under preclinical trials and favipiravir has been indicated as the best therapeutic approach in LASV infection diseases[173]. Nevertheless, early diagnose, effective supportive care and detecting associated co-infections contribute to patient recovery. There is no approved treatment for LASV infection: however, human monoclonal antibodies have been recently experimented in Guinea pigs and NHPs models[174,175]. Reports established that when the treatment is initiated at advanced stages of the disease, there was a possibility to rescue 100% of NHPs challenged with live LASV.

To date, there is no available licensed vaccine for Lassa hemorrhagic fever. However, multiple vaccine candidates in development are showing efficacy in animal models at the preclinical trial stage. Those vaccine candidates are non-replication component vaccines such as alphavirus replicons, whole inactivated LASV and DNA vaccines[176,177,178]. There are multiple animal models for vaccine experimentations, such as Guinea pig, mice, cynomolgus monkeys, macaques; however, NHPs are indicated as the gold standard presenting numerous conditions similar to that of humans[173,179]. It follows that the selection of animal model is related to the ability of inducing strong cell-mediated cross-protective immunity against different LASV lineages.

To conclude, LASV disease requires deep investigation (i) to elucidate the pathogenic mechanisms leading to disease fatal outcome and (ii) to understand the potential cause of LASV asymptomatic infection shown in some countries as Guinea despite its high prevalence rate.

1.5 Study aim and objectives

The primary purpose of this study is to assess the level of sub symptomatic EBOV infection in communities at high risk of exposure in the forested region of Guinea. Those exposed people within the community are mainly bushmeat hunters and their household family members, located in remote places, close to the nature and deep within the forest. Volunteer recruitment was conducted in year 2017 and a total of 517 serum samples were collected and thoroughly assayed on ELISA (using simultaneously Casein and ChonBlock blocking buffers), Western blot analysis and flow cytometry test for EBOV GP antibodies detection. Alternatively, an ELISA assay was performed for LASV NP detection. Our study intends to (i) demonstrate the circulation of EBOV in the forested region of Guinea prior to the 2013-2016 EVD outbreak in Guinea; (ii) assess the degree of sub symptomatic, mild or unrecognized EBOV infection disease in the Macenta prefecture, (iii) asses the incidence of LASV infection in Macenta prefecture and, (iv) help to understand and identify practices that promote the circulation and transmission of zoonotic pathogens.

Regarding the growing importance of zoonosis and their impact on public health, surveillance of emerging diseases is essential to avoid serious epidemic outbreaks in communities. This study will help to understand and probably prevent the risk of contamination and disease spill over via community behaviour with regards to bushmeat hunting, manipulation and consumption.

Finally, this seroprevalence study attempts to develop and strengthen Guinean capacities for scientific research. The existing network between European and African researchers of this study is based on more than a decade of collaborative projects in Africa led by Professor Günther of Bernard Nocht Institute of Tropical Medicine (BNITM) and supported by a grant from food and drug administration program (FDA) of the United States awarded to Professor Miles Carroll of Public Health England (PHE) to support research in Guinea.

Chapter 2: MATERIALS AND METHODS

2.1 Sample collection

Human peripheral blood samples were collected every week at the nearest available health centre. Sampling sites were selected based on available facilities, travelling distance for volunteers and accessibility by motorbike. A small volume of blood (approx. 5 ml) was collected into BD vacutainer® blood collection tubes (red cap. REF: 367895) by experienced, local, phlebotomists. Volunteers were compensated for their loss of time and any inconveniences experienced during sample collection according to Guinean ethics scales.

Blood was collected into 5 ml of vacutainer blood collection tubes. These tubes contained no anticoagulant and therefore blood could clot within two hours at room temperature. Samples were transported to the field laboratory in a cool box at 4 ºC. In the lab, samples were centrifuged at 2,000 x g for 10 minutes at room temperature. Serum was then aliquoted into 2 ml microtube RT-PCR tubes (Starsted, REF 72.694.406) in an air purifying class II microbiological safety cabinet (Envair Ltd, England). Serum aliquots were labelled and stored at -20°C and later shipped on dry ice by an International Airline Transport Association-compliant safety shipper (World Courier) to the United Kingdom. Finally, samples were processed at PHE Porton Down laboratories and at University of Marburg.

This study was ethically approved by the board of UK research ethics council as well as the national research for health ethic committee of Guinea (permit Nº012/CENRS/2017; Appendix 1).

2.2 Enzyme-Linked Immunosorbent Assays (ELISA)

Enzyme Linked Immunosorbent Assays (ELISA) were used to measure EBOV- and LASV-specific antibodies in serum samples.

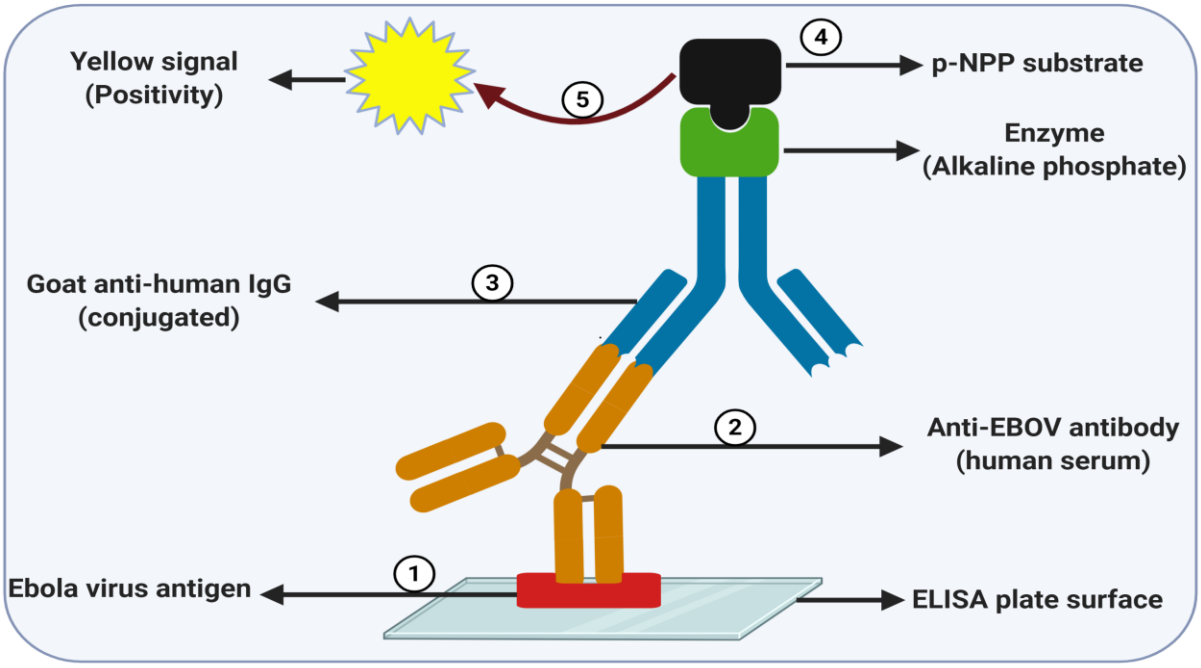
2.2.1 Indirect ELISA assay procedure

Indirect ELISA detects an analyte by way of using a secondary antibody. The specific antigen of interest is immobilized (coated) onto an appropriate ELISA plate. Then primary antibody (usually patient specimen) is added to the plate. The secondary antibody (enzyme-conjugated) is added and specifically binds to the preformed complex antigen-antibody on the plate. Finally, chemical substrate is added which initiates a colour change that is read at various optical densities by an ELISA plate reader (

Figure 10). Indirect ELISA is more sensitive than other platform as the secondary antibody can amplify the signal[190].

ELISA assay procedure for EBOV GP antibody detection

A. Assay description



This figure used images that were created with Biorender.com and are permissible to use.

B. ELISA plate showing response to EBOV GP

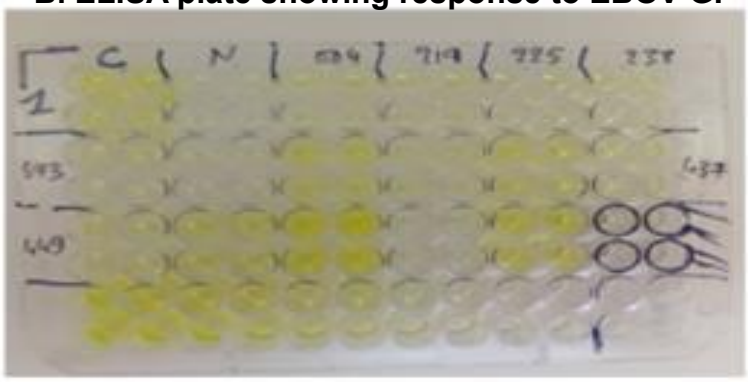


Figure 10: Description of ELISA assay procedure (Indirect ELISA)

A)Detection of Anti-EBOV GP antibody in human blood sample using Goat anti-human IgG alkaline phosphatase conjugated in the presence of p-NPP substrate. (1) EBOV GP tetramer antigen is coated into ELISA NUNC high binding plate. (2) Volunteer's serum, probably carrying anti-EBOV antibody (primary antibody) is added to the plate which may bind to the EBOV GP antigen coated. (3) Goat anti-human IgG (secondary antibody) conjugated with alkaline phosphatase is added and binds to the primary antibody. (4) P-NPP substrate is added and interact with the enzyme giving colour change into the plate. (5) Yellow signal equates to positive and indicates the possible exposure to EBOV. However, this should be measured on ELISA reader and subsequent OD value is analysed and compared to controls for validation.

2.2.2 Ebola virus Glycoprotein detection

Using participant serum samples, the detection of antibodies specific to EBOV (Makona) GP was achieved by coating Nunc high binding plate-Sigma 96-well plates (Sigma, Lot. Number M9410-1CS) with 50 µl of recombinant protein at a final concentration of 1 µg/ml, diluted in Phosphate Buffered Saline (PBS) (PHE media. Lot number 1755496). The EBOV GP was purchased from the Jenner Institute at Oxford University and is a trimerized Zaire strain (amino acids 1-649, GenBank protein AHX24649.1). The protein contains a c-tag EPEA (glutamic acid-proline-glutamic acid-alanine) and was expressed in HEK293E cells. Cell culture supernatant was harvested presumably four days after transfection and concentrated using a Pellicon 3 Tangential flow filtration system (Millipore, Herts, UK). Protein purification was achieved using an AKTA pure system (GE Healthcare, Bucks, UK) with a C-tag affinity capture set up (Thermo Fisher Scientific, UK) along with a polishing size exclusion chromatography (SEC), using a SepFast GF-HS-L 26/1000 column (Biotolomics, Durham, UK). SEC peak fractions corresponding to the trimeric EBOV GP were pooled and the molecular weight further confirmed by blue native PAGE (Thermo fisher scientific, UK). The purified protein was quantified by spectrophotometry (Nanodrop. Thermo Fisher) aliquoted and stored at -80˚C until use.

Coated ELISA plates were incubated overnight, in the fridge, at 4 ˚C. The following day plates were washed six times in PBS/0.005% tween (PHE media. Lot number P7949) using the AquaMax 4000 with 96-well plate wash head (Molecular Devices). Plates were then blocked using 200 µl of casein (Thermo Scientific. Lot number 37528) or ChonBlock (Chondrex. Inc. Redmond. WA. Cat. Number 9068) and incubated at room temperature for one hour to block any nonspecific active sites. While this was occurring, samples were prepared in 96-well “Dummy” plates (Nunc low binding plates. Cat. Number 611F96) as illustrated in **Figure 11**. Samples were diluted either 1:100 or 1:500 using either casein or ChonBlock. To quantify results in international units per millilitre (IU/ml) a standard curve was used. This curve contained pooled plasma from three EVD survivors of the 2013-2016 West Africa outbreak and was quantified in house against the WHO international standard 15/262 [180]. Additionally, to map the consistency of results, an internal QC which consisted, again of three pooled EVD survivor plasma samples was acquired on every plate at a dilution of 1:500. Finally, negative plasma from non-infected West African nationals was also acquired on every plate.

Following incubation with either casein or ChonBlock ELISA plates were washed six times and 50 µl of sample transferred from the “Dummy” plate to the test plate. Samples were then incubated for a further two hours at room temperature. Secondary

Goat anti-human Fc, alkaline phosphatase (AP) conjugated (Cat. Number A3187, Sigma) antibody was prepared at a dilution of 1:1000 in either casein or ChonBlock. Following their incubation, plates containing test sample were washed six times and 50 µl diluted secondary antibody was added to each well. Samples were incubated for one hour at room temperature and while this was occurring the developmental substrate was made by mixing one 4-Nitrophenyl Phosphate, 20 mg (Cat. Number N2765. Sigma) into 16 ml of distilled water and then adding 4 ml of 5X diethanolamine (Cat. Number 34064. Pierce) to give a 1x solution. Substrate buffer was kept at room temperature in the dark until use. Samples were finally washed a further six times before adding 100 µl of substrate buffer. Samples were left to develop in the dark for 20 minutes before being analysed on a SpectraMax M3 spectrophotometer (Molecular Devices) with Softmax™7.0 software program. Plates were read at 405 nm and data was exported in excel format before being graphed in either Excel (Microsoft) or GraphPad™ V8 software.

	1	2	3	4	5	6	7	8	9	10	11	12
A	QC		NEG		Sample 1		Sample 2		Sample 3		Sample 4	
B												
C	Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		Sample 10	
D												
E	Sample 11		Sample 12		Sample 13		Sample 14		Sample 15		Sample 16	
F												
G	SD 01	SD 02	SD 03	SD 04	SD 05	SD 06	SD 07	SD 08	SD 09	SD 10	Blank	
H												

Figure 11: Representation of ELISA plate layout.

In total 16 samples were assayed per plate including controls. NEG, Blank and samples were duplicated each respectively 1/100 and 1/500 (e.g. A3/A4 and B3/B4 for NEG; A5/A6 and B5/B6 for sample 1). (2) QC was duplicated respectively as 1/400 (top rows: A1/A2) and 1/800 (bottom rows: B1/B2). (3) Standard was serially diluted 10 times staring 1/100, 1/200, 1/400 to 1/51200. Dilution was done in two rows (e.g. G1/H1 for 1/100 dilution and G2/H2 for 1/200).

2.2.3 Lassa virus IgG detection

This assay was performed using the BLACKBOX® LASV IgG kit from BNITM. The Kit was used as per the manufacturer's instructions. In addition to negative and positive controls, blank and background controls were added in order to monitor non-specific binding of proteins to reagent contaminants or to the ELISA plate. The BLACKBOX® LASV IgG ELISA kit is designed for qualitative serological detection of acute or past LASV infection using conjugate IgG (biotinylated recombinant LASV antigen).

All reagents were at room temperature for 20 minutes before use. Frozen samples were thawed and vortexed prior to processing. Samples, as well as positive and negative controls were diluted 1:50 in sample dilution buffer. Pre-coated LASV IgG micro well plates were removed from the alumina pouch and were manually washed three times with 300 µl of washing buffer after which 25 µl of IgG LASV antigen (conjugate IgG biotinylated recombinant LASV antigen) was added to each well. Additionally, 25 µl of pre-diluted sample was added to each well as shown on the plate layout (**Figure 12**).

Samples were incubated for 24h at 2 – 8 ºC in a wet chamber, this was made with wet paper towels inserted into a small closing box. This keeps the micro well plates from drying and prevents IgG LASV antigen and antibodies denaturation. Following this incubation plates were washed five times with 400 µl of washing buffer before adding 50 µl of diluted HRP-streptavidin at a final concentration of a 1 µl/10 ml in conjugate dilution buffer into wells. Plates were covered with adhesive plastic and incubate for one hour at 2-8 ºC. Following this incubation period, plates were washed three times as described above and 100 µl of substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was added to each wells. Plates were incubated for 10 minutes in the dark at room temperature. Finally, stop solution was added (100 µl) before being analysed on a 'SpectraMax M3 spectrophotometer (Molecular Devices) using Soft MaxTM 7.0 software. Plates were read at two wavelengths, 450 and 620 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG ctrl	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B		Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	POS ctrl	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	Sample 1	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample 89
E	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Blank ctrl
F	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	
G	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	BG ctrl
H	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	

Figure 12: Representation of ELISA plate layout for LASV IgG detection.

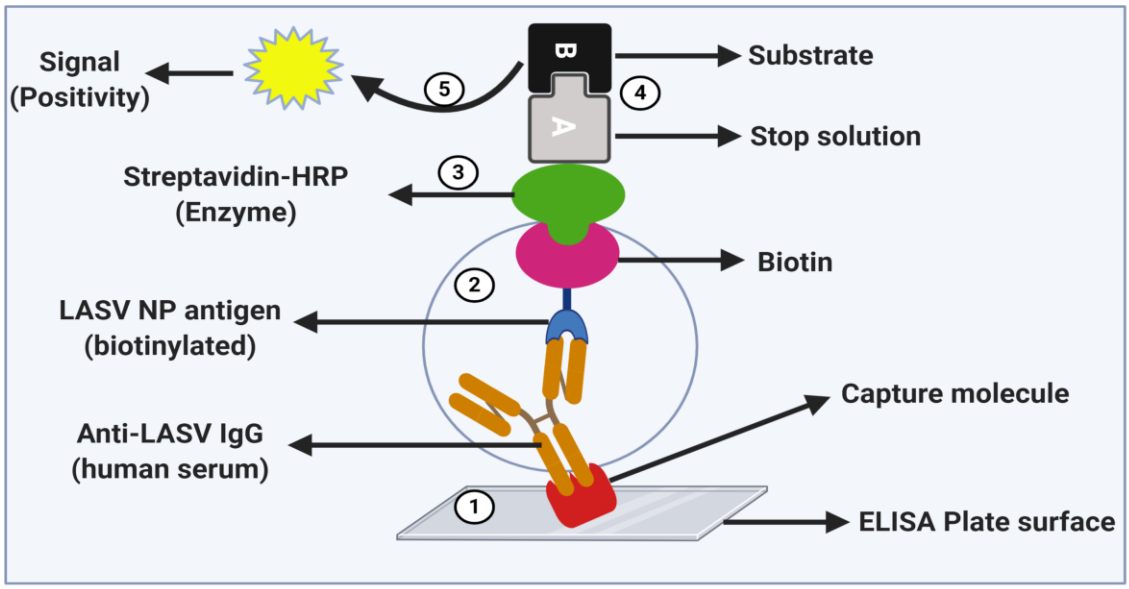
In total 89 samples were assayed per plate with four controls. NEG, Blank (BL) and background (BG) were duplicated. NEG and positive controls were provided with the kit. Blank and background noises controls were added on the bench to monitor, non-specific bindings of proteins to reagent contaminants or to the plate. No standard control was provided in the kit.

2.2.4 Lassa virus IgG detection ELISA assay procedure.

To evaluate anti-LASV IgG in our samples, we performed ELISA technique. The Blackbox LASV IgG ELISA kit is provided with a plate coated with an undisclosed protein that binds immune complexes (LASV antigen and anti-LASV IgG). The kit includes positive and negative controls. In addition, two controls blank and background noises were added to monitor non-specific bindings. In the blank wells, no serum was added in order to measure detection antibody (secondary antibody) interaction with ELISA plate plastic surface. Although, background wells were LASV antigen free so that to measure specimen wild proteins binding to ELISA plate. Further, test serum is incubated with LASV protein to form immune complex. Then sample is incubated with ELISA plate. Afterward, subsequent protein labelled with enzyme (e.g. streptavidin-HRP) is added to the plate and binds to a biotinylated site of the LASV protein. Finally, the reaction is visualized when applying substrate and stop solution (**Figure 13**).

ELISA assay procedure for LASV IgG antibody detection

A. ELISA procedures



This figure used images that were created with Biorender.com and are permissible to use.

B. ELISA plate showing Lassa positives

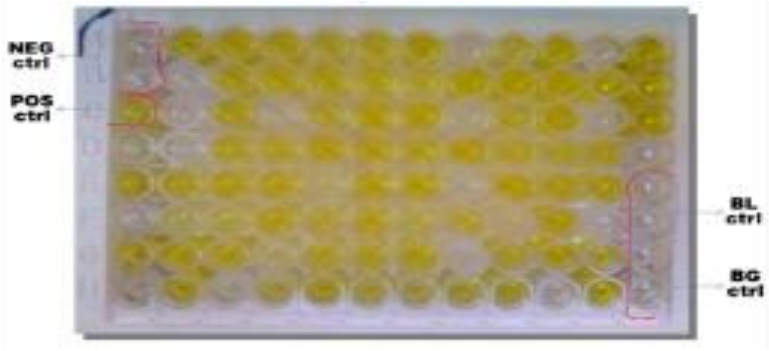


Figure 13: Competitive ELISA assay for LASV detection.

A) (1) ELISA plate is provided with capture molecule that binds immune complexes. (2) Participant serum (anti-LASV IgG) and LASV antigen (Biotinylated) are incubated in vitro to form immune complex (antibody-antigen) which is then added to the ELISA plate. (3) Streptavidin-HRP labelled is added and binds to the immune complex. (4) Stop solution and substrate are simultaneously added for antibody detection. (5) Yellow signal equates assay positivity. **B**) Schematic representation of performed ELISA plate for the detection of anti-LASV IgG antibody. Four controls are used including NEG and POS provided with the kit; Background (BG) and blank (BL) are controls made on bench. BG wells are with no LASV antigen addition to monitor serum proteins binding to ELISA plate surface and BL wells are with no anti-LASV IgG (serum) addition to monitor LASV antigen interaction with ELISA plate surface. Yellow signals indicate sample positivity.

2.3 Ebola virus protein detection by western blot

EBOV proteins including GP (Oxford university. In house), NP (Gentaur. REF. MBS1206629) and VP40 were diluted in Laemmli lysis buffer (Sodium lauryl sarcosine. Cat. Number: 38733. Sigma). Mixtures were then heated at 95 ºC for 10 minutes to denature proteins. Denatured samples (10 µl) were then loaded onto 4-12 % Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (REF: NW04122BOX. Lot: 19022611. Life technology). Protein standard-Magic Mark XP (Lot: Lc5602. Thermo Fisher) and Protein standard Pre-stained (Lot: 10640504. Thermo Fisher) ladders were used. Both proteins are thawed, gently vortexed, mixed at equal volume and 10 µl is loaded into the SDS-PAGE gel. Gel electrophoresis was run using XCell sureLock™ chambers (Invitrogen) using MOPS-SDS (REF: NP0001. Lot: 1935953. Thermo Fisher) with the addition of an antioxidant (N, N-dimethyl form amide, sodium bisulfite) (REF:NP0005. Lot: 1909040. Thermo Fisher) as a running buffer. Samples were run using a power pac™ Bio-RAD machine at 200 volts for 50 minutes.

Following separation by electrophoresis, proteins were transferred from the gel to polyvinylidene (PVDF. Cat. Number: LC2002. Invitrogen) membrane using iBlot transfer stack (REF: IB401001. Lot: 120319-01. Thermo Fisher) and iBlot machine (iBlot™. CEPR portable. Lot: 52701. Invitrogen). Following protein transfer, the PVDF membrane was blocked for two hours in a 15 ml tube on a roller (HR-120. Max 120g, Mini10 mg. REF 1370045) in 5 % non-fat milk (REF: A0830.0500. VWR), subsequently, the samples were stored overnight at 4˚C.

The following day PVDF membranes were washed five times with 0.05 % PBS Tween-20 solution. Then, membranes were incubated with 2.5 ml test serum sample at 1:100 in non-fat milk or EBOV specific antibodies as rabbit anti-EBOV GP 100 µl (REF: 0301-012. Gentaur); rabbit anti-EBOV NP 100 µl (REF: 0301-015. Gentaur) and rabbit anti-EBOV VP40 100 µl (REF: 0301-010. Gentaur). Samples were incubated for 2 hours at room temperature on a rolling platform, after which, they were washed five times and incubated with 1:1000 secondary anti human or rabbit IgG-HRP (REF: A2290-1ML. Sigma). Samples were again incubated 1-2 hours before being washed a final time and developed using enhanced chemiluminescence (ECL) prime western blotting system (REF: RPN2232. Sigma) solution. PVDF membranes were developed by adding 2.5 ml of equivalent volume of ECL solution A and B and incubating membranes for 30 second in dark place before reading the membranes on a ChemiDoc™ XRS+ imaging system (Bio-Rad). Images were obtained every 30 seconds over a five minutes period and selected images were transformed and saved as TIFF files.

Western blot assay procedure for EBOV GP, NP and VP40 proteins detection

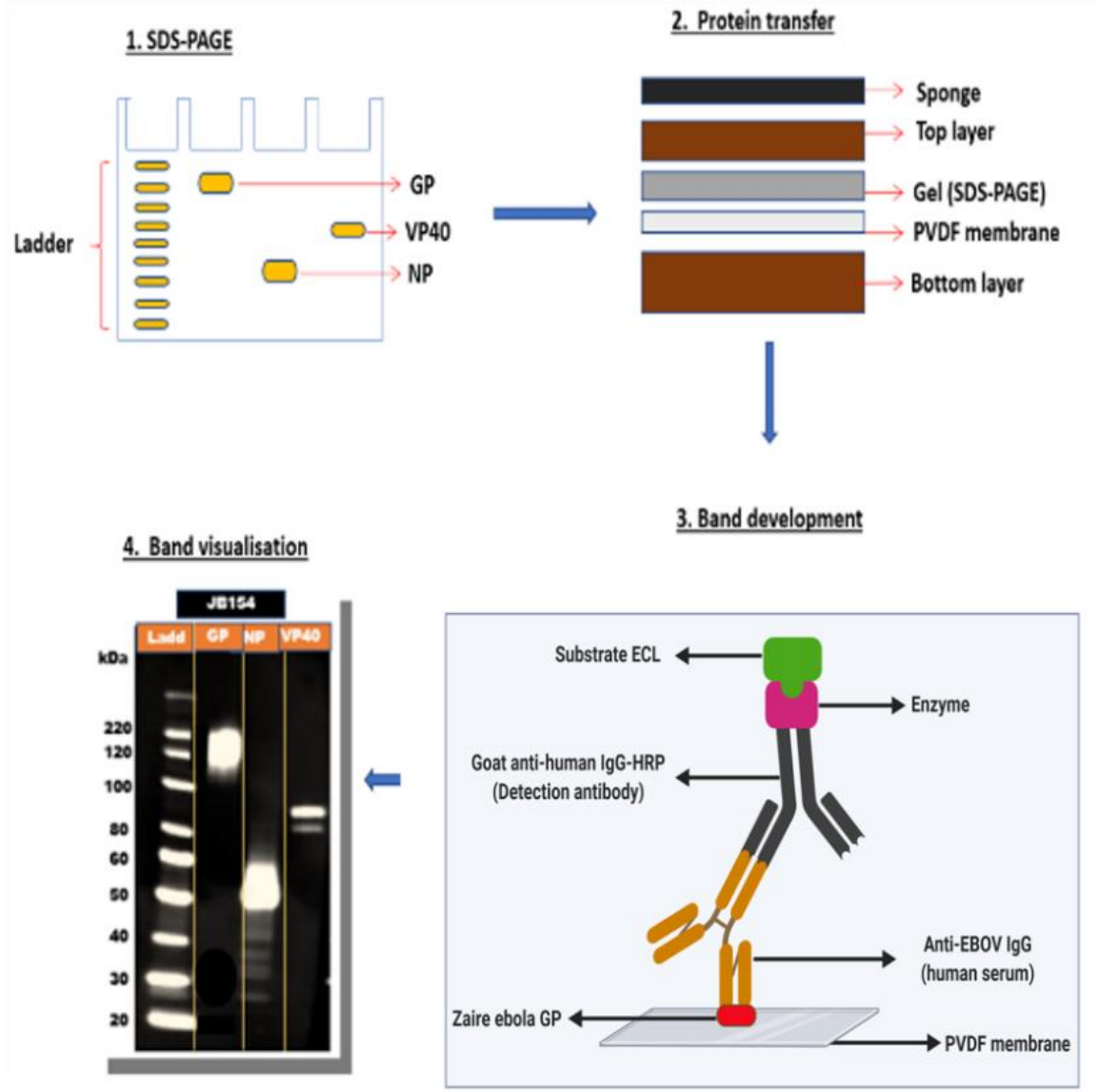


Figure 14: Western blot assay description.

(**1**) Separation of EBOV GP, NP and VP40 proteins on SDS-PAGE gel electrophoreses. (**2**) Protein transferred from the gel to PVDF membrane. (**3**) Protein detection using primary human antibodies and goat anti-human IgG-HRP, visualized by ECL (sigma). (**4**) Protein band visualization using luminescence and the BIO-RAD ChemiDoc XRS+ imaging system.

2.4 Anti-EBOV IgG subclass detection

This assay aimed to determine the contribution of various IgG isotypes to any determined EBOV antibody response. First, EBOV protein (Zaire GP tetramer. Oxford) was conjugated to the surface of carboxyl beads (Lot: CMPAK-4068-6K. Spherotech). These beads (10^7/ml) are magnetic and come as a set of six peaks, with each peak having a distinct intensity of APC fluorescence. Beads were initially sonicated for 20 seconds and removal of supernatant achieved using a magnetic stand (Easy Eights™ EasySep™ Magnet. Stemcell technologies. Lot: 18103). Beads were then washed twice using in house washing buffer (PBS, 0.1 % BSA and 0.5 % Triton X100). and 200 µl of activation buffer was added. Activation buffer consisted of monobasic sodium phosphate (MSP) (Lot: 10751135. Fisher Scientific) with dibasic sodium phosphate (DSP) (Lot: S0876-100G. Scientific Laboratory Supply); *N-hydroxysulfosuccinimide* (Sulfo-NHS) to 6.25 mg/ml (Lot: 24510. Life Technology) and *1-ethyl-3-[3-dimethlyaminopropyl] carbodiimide-HCl* (EDC) to 6.25 mg/ml (Lot: 22980. Life Technologies).

Samples were incubated for 20 minutes and then washed three time with MES buffer. Next, beads were incubated with either 40 µl of EBOV GP (EBOV tetramer 0.35mg/ml. Oxford); 40 µl of recombinant EBOV NP, partial mammalian cell derived (0.15 mg/ml; 0.35 ml. Lot: G251468. Gentaur); 40 µl of VP40 (REF: 0301-010. Gentaur) and 200 µl of MES. Samples were incubated for 2h in the dark at room temperature on a rotating mixer (Lot: 130-090-753. MACS, Miltenyi Biotech). Following the incubation period, beads were washed twice with blocking buffer (PBS, 0.1 % BSA, 0.05 % Sodium Azide and 0.02 % Tween) and re-suspended in storage buffer (PBS, 0.1 % BSA and 0.05 % sodium Azide). Samples were then stable for up to six months at 4˚C.

For IgG isotype detection all reagents were at room temperature before use. Beads (20 beads/ µl), conjugated to the EBOV protein of choice were diluted into assay buffer (1xPBS and 0.1% bovine serum albumin) to obtain approximatively 1000 beads per tube. 10 µl of test serum (volunteers' samples) was then added to 100 µl of prepared conjugated beads. Samples were incubated for one hour at room temperature in the dark on a rotating mixer. Beads were then washed once with assay buffer and 100 µl of diluted secondary antibody (human IgG1 Isotype control recombinant antibody; 1.0 µg/ml. Lot:403504. Bio legend) was added. Samples were incubated for one hour at room temperature on the rotating mixer. Finally, beads were washed twice with assay wash, re-suspend in storage buffer and analyzed on a BD Acuri™ C6 plus flow cytometer (BD Biosciences). Following acquisition, samples were analyzed using FlowJo™ V10 (Becton, Dickinson) and GraphPad V8 software.

Multiplex bead assay procedure for EBOV IgG subclasses detection

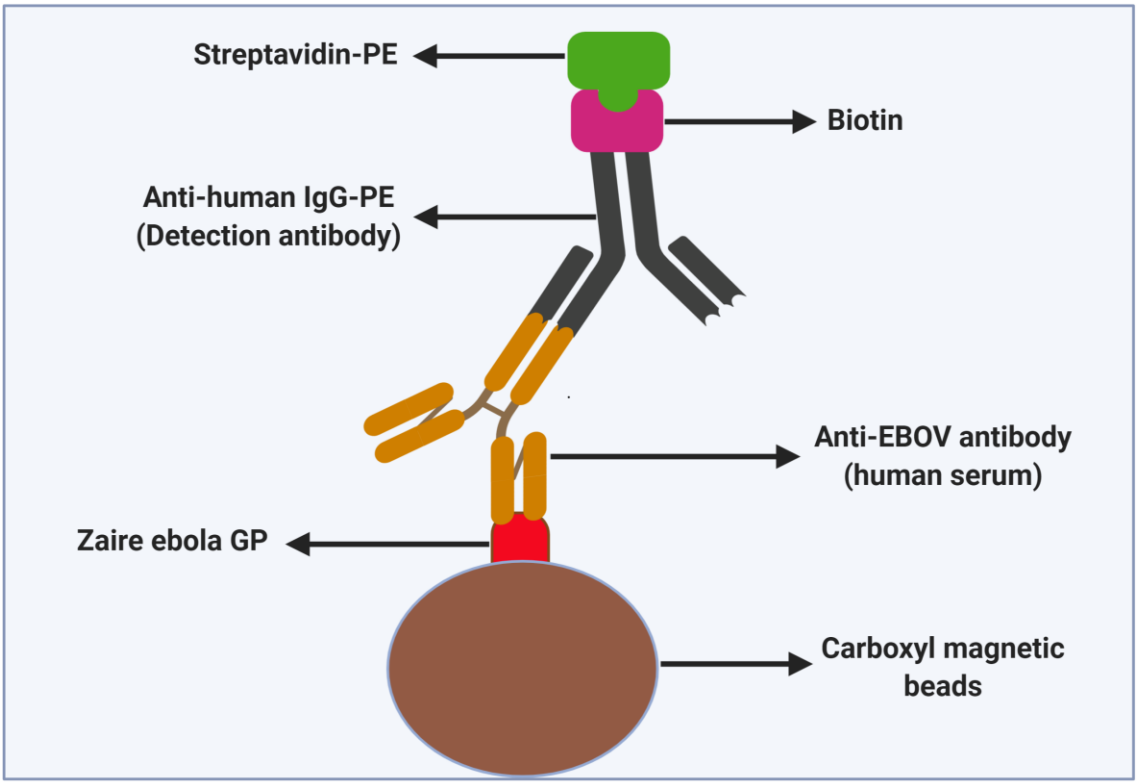


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Figure 15: Multiplex beads assay description.

Antigen binds to Luminex beads in vitro. Human serum possibly carrying anti-EBOV antibody (primary antibody) is added and the antibody binds to the antigen (EBOV GP) coated on the beads. Finally, Anti-human IgG-PE (detection antibody) biotinylated is added and binds to the anti-EBOV IgG. The mixture is read on flow cytometer instrument.

Chapter 3: SAMPLE COLLECTION RESULTS

3.1 Study framework

This sero-prevalence study was carried out in the Republic of Guinea, Western Africa (southwest, midway between the equator and the Tropic of Cancer, Lat: 9.749615°. Long: -13.456806°). The country covers an area of 245,857 km^2 and is composed of both plains and mountains reaching in some places to an altitude of over 1500 m. It is bordered by the Atlantic Ocean, Sierra Leone, Liberia, Côte d'Ivoire, Mali, Senegal and Guinea Bissau[181]. With regards to the diversity of ecological conditions, Guinea is divided in to four regions including lower Guinea, middle Guinea, upper Guinea and Forested Guinea. To date, it is reported that the country features 260 vertebrate mammals species, 518 birds species, 140 reptiles species and 31 viruses discovered[181]. This study targeted groups of people living in remote villages, close to nature, deep in the forest and near large rivers. We intend to investigate the circulation of EBOV in the local population prior to the 2013-2016 EVD outbreak in Guinea and therefore we focused on remote villages that had never previously reported case of EVD. Our cohort primarily includes bush meat hunters, their household family members and people involved in both bushmeat manipulation and consumption. This population is known to be at high risk of exposure to viruses that coexist with forest animals (zoonotic diseases). We performed serological testing on human samples to determine the presence of anti-EBOV antibodies. Immunity to LASV was also studied as both Ebola and Lassa viruses are responsible for severe haemorrhagic fever in humans and NHPs.

Our study focused on the region of Macenta (**Figure 16**) which has a young and uneducated population with an approximative age of 35. In this area, population main occupations are farming and hunting. Bushmeat consumption results in daily contact with wildlife either through trapping, hunting or butchering and these are thought to be the major routes of viral transmission. This study attempts to contribute to the development of the Country's early awareness system for emerging disease containment. It is part of the One Health approach for disease surveillance, prevention, control and eradication established in country after the 2013-2016 EBOV outbreak[182]. The One Health approach is an interdisciplinary and multi-sectoral framework including the Ministry of Health, the Ministry of Agriculture, Livestock and Fisheries. This multi-sectoral collaboration highlights the public health importance of endemic and epidemic zoonoses management. It provides capacity building programs, disease surveillance, workforce development, research, coordination and investigation of an outbreak response. Its implementation considers actors and communities concerns to assess the risks of human exposure to filoviruses in order to prevent and control emerging and re-emerging viral diseases within the population. Results from the study

can be used to strengthen the national security agency system for disease surveillance (ANSS) and that of other West African countries in general.

This chapter describes volunteer and study area selection. It also indicates population interest and involvement in the study regarding different levels of population occupations.

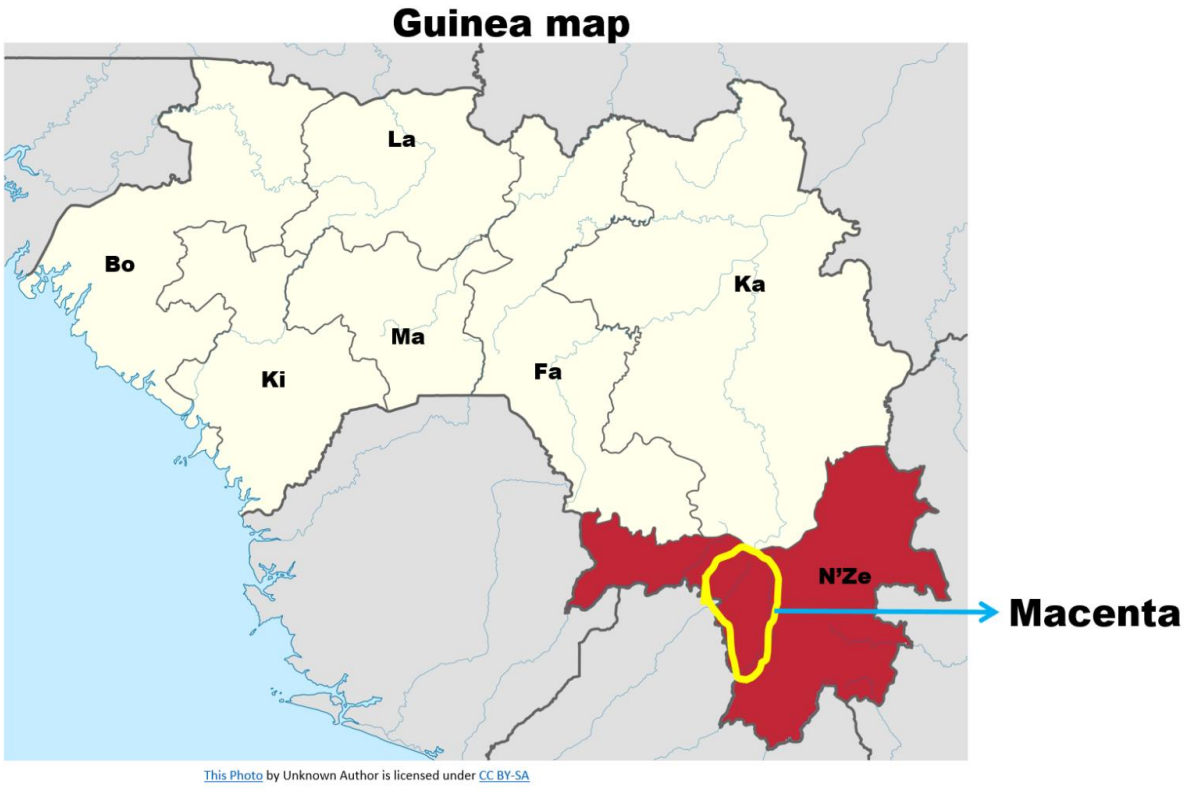


Figure 16: Representation of Macenta prefecture map

Map of the Republic of Guinea. It comprises 7 administrative regions including Kindia (Ki), Boke (Bo), Labe (La), Mamou (Ma), Faranah (Fa), Kankan (Ka) and N'zerekore (N'Ze). Red colour indicates the forested region of Guinea with Nzerekore as the regional capital city. Yellow circle indicates Macenta prefecture where the study samples were collected.

3.2 Wildlife

Wildlife Protection Code and Hunting Regulations have been in place since February 1990 by ordinance Nº007/PRG/SGG/90 and reviewed on December 1997 under law L/97/038/AN[181]. Although the code defines hunting areas these are often violated, and poachers are taken to the law court of the prefecture. In 2013, the national office of water and forest management censured 2,990 gun-hunters in Macenta prefecture. Approximatively 31 viruses were discovered and classified in Guinea over more than a decade[181]. Recently, Piet et al. discovered a novel arterivirus in African giants Shrew (Olivier's Shrew virus 1, A) in Meliandou village at Gueckedou[183]. Additionally, the country's biodiversity is almost identical from one region to another with minor differences in seasonal period. In general, all kinds of animal species including vertebrate mammals, birds and reptiles are subject to hunting and consumption without distinction.

3.3 Study zone selection

The forested region of Guinea was selected for this study due to the fact that the West African outbreak of 2013-2014 originated within the region[59]. Also, the region compared to others, encounters greater diversity of wildlife (fauna) which may be reservoirs for several types of viruses including filoviruses and Arenaviruses. Population occupations such as farming, and hunting constitute the main route of exposure to zoonotic infection in the zone likened to other regions in Guinea with less exposure to wildlife.

Our study zone Macenta prefecture is in the forested region of Guinea and covers an area of 7046 km^2 with an estimated population of 278,456 inhabitants (**Table 4**)[97]. It comprises 15 sub-prefectures and 314 villages with an approximative density of 41 inhabitants per km^2. The residential population in the urban and rural areas are approximately 65,755 and 212,701 inhabitants respectively. Macenta is characterized by a significant rain forest with an abundant hydrographic network made up of rivers, flows and streams (e.g. Makona, Loffa, Diani). Its biodiversity is almost identical from one side to another but differs slightly from other regions. The general population of Macenta is young with the average age ranged between 25 to 45 years and life expectancy at around 55 years for men and 65 years for women[97].

Within the Macenta region we were able to work with a number of sub-prefectures to establish a sample cohort which consisted of 44 villages and 517 participants.

Table 4: Population of Macenta prefecture.

Macenta is composed of 14 sub-prefectures, 314 villages and Macenta town centre. A sub-prefecture may count more than 50 villages. In total, 7 sub-prefectures were selected for the study. An approximate number for the population (male and female) from each sub-prefecture including its surrounding villages as for 2014 census can be seen[97].

Prefecture	Male	Female	Total
Balizia	7101	7591	14692
Binikala*	8574	9113	17687
Bofossou*	6600	7203	13803
Daro	6969	7576	14545
Fassankoni*	6415	7477	13892
Kouankan	14500	15725	30225
Koyama*	11623	13389	25012
Macenta centre**	32014	33741	65755
Zebela	7842	8426	16268
Oremai*	4261	4741	9002
Panziazou	3933	4507	8440
Semgbedou	6510	7254	13764
Seredou*	9651	10598	20249
Vasseredou	4114	4489	8603
Watanka*	2938	3581	6519
Total	133045	145411	278456

* Study areas
**Urban area not included in the study

3.3.1 Study villages selection

The selection of villages for this study was based primarily on their location and their exposure to EVD during the 2013-2016 outbreak (**Figure 17**). Selected villages were close to nature, deep in the forest and near large rivers. This was so we could involve people at high risk of exposure to zoonotic disease as their daily activities involved contact with wildlife through subsistence activities such as farming and hunting. Some villages on the main national road were included as they constitute the main route of disease spread from remote places to agglomerate cities. An established trade occurs once a week in those villages where sellers and buyers meet and exchange products as fruits and meat (fresh or smoked). Although, some of the villages were selected as positive controls as they had experienced cases of EVD during 2013-2016.

Myself, a Guinean national, originates from Macenta prefecture which is occupied by the Toma tribe. This made interactions with the local people possible and was instrumental in our ability to set up cohorts within these rural villages. Although, the presence of ethics approval document (**Annex 1**) signed at the back by the highest administrative authorities such as the governor of Nzerekore and the prefect of Macenta enhanced population certainty about the study. However, minor resistances were raised mainly with regard to the past EVD and its consequences in the zone.

Map to locate sample collection villages

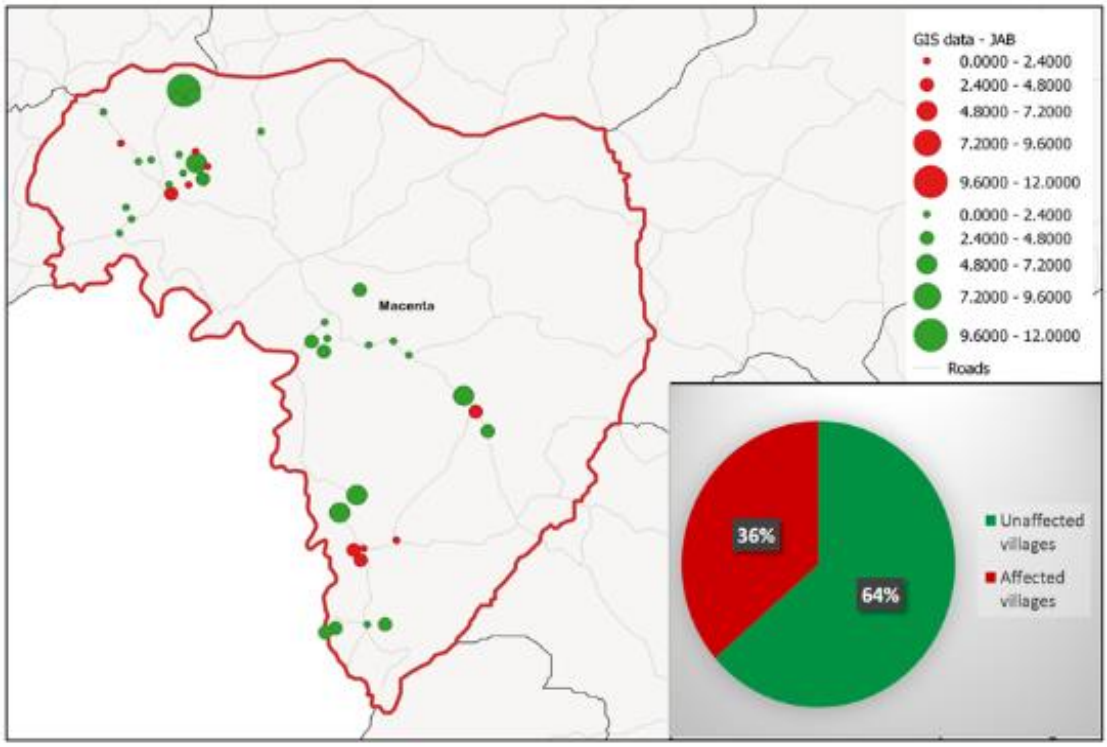


Figure 17: Representation of the sample collection sites

Map showing the location and whether the village had previously experienced EVD. Of 314 villages in Macenta, 44 participated to the study amongst which 16 villages, red dots, reported EVD case (affected) and 28 villages, green dots, reported no EVD case (unaffected) during the 2013-2016 outbreak. Dots sizes are related to the number of samples collected in each village ranging between 2 to 29 with an average of 11.75.

3.3.2 Volunteers recruitment

Volunteers were recruited upon agreement. First, administrative authorities in the village were notified about the study and mass sensitization was organised to communicate the project. Participants were then selected with the support of both the chief of the village and the chief of hunters. Regarding the number of samples to be collected for the study (primarily 400), the number of participants per village was limited in order to cover a large area of the study zone. Notably, villages are divided into families which may count up to 7 in some places. Families are divided into households and at least two households were selected per family within the village. Furthermore, an individual interview was organised for participants and details were assembled using a data collection sheet (Questionnaire, **Appendix 2**). Volunteers under 16 of age, pregnant women, travellers and/or visitors, and those suffering from chronic diseases were excluded. Finally, consent forms were signed prior to sample collection **(Appendix 3)**.

The study participants were empirically classified in to three age groups including young (16-24), adult (25-55) and elderly (>55) (**Table 5**). Both males and females were involved and volunteers under 15 of age were excluded for ethical considerations. High participation rate was seen in the middle age group ranging between 25 to 55 (n=374). This group is the most involved in farming and hunting activities. Also, it is composed of vigorous and healthy people and encounters high number of people within the population as the life expectancy is at 55 years old. In contrast, less participation was seen in the old age group. This might be related to the exclusion of several participants with chronic diseases and the lower numbers of elderly people found in villages due to the early death of men.

Table 5: Breakdown of study cohort with regards to age and sex.

Three age groups were considered and both male and female participated. Volunteers under 16 of age were not eligible. The adult age group (25-55) shows high participation rate while the old age group shows less participation.

Age group	Male	Female	Total
16 – 24	30	56	86
25 – 55	211	163	374
>55	51	6	57
Total	292	225	517

3.3.2.1 Occupation of volunteers

Within the villages, farming (agriculture) remains the primary occupation. However, people are involved in other activities such as carpentry, masonry, blacksmith, merchant (small shop and bushmeat traders) and healthcare worker (alternative and modern medicine practitioners). Hunting is not considered as a professional activity but rather as a subsistence activity which is authorised for people aged over 20 years[184]. Our study not only considered hunters and close contacts but also volunteers practising other activities and involved in bushmeat manipulation and consumption. Healthcare workers were included due to their involvement in caring for people presenting with any infectious disease.

Table 6: Occupational data for study cohort participants.

Hunters are found only amongst the male group. Household family members include wives, children, grand-parents and other relatives. Traders mainly include women and the workman group which is composed of young males.

Occupation	Male	Female
Hunters	242	0
Household member	29	211
Healthcare worker	23	11
Workman	26	0
Traders	1	15

3.3.3 Participant outreach

Volunteer recruitment and sample collection was conducted within 11 months inclusively from February to December 2017. Out of 314 villages in Macenta prefecture, 47 (14.96%) villages were contacted and 44 participated in the study. Villagers were informed about the study through mass communication and volunteers were individually interviewed for their consent. Though the study was largely accepted we did encounter minor resistances in some places which seemed to be related to the past EVD outbreak fear and trauma. It followed that due to fear, three villages refused to participate in the study. Entirely, women from another selected village didn't show up for sample collection, likely due to pre-existing fears surrounding the context of the study. As a result, amongst 47 villages contacted, participants from 44 villages agreed to the study (**Figure 18**).

Samples were collected in sixteen villages known to have had previous exposure to EVD (affected villages) and in twenty-eight villages with no previous reports of EVD exposure (unaffected villages), as reported in the national database regarding the 2013-2016 EVD management in Guinea[97]. This detailed data set is available in the Macenta Prefectural Health Direction (DPS) office. As the 2013-16 EVD outbreak progressed in Guinea, national health authorities were notified of any suspected cases in villages by healthcare agents broadly distributed and provided with mobile phones and motorbikes. Suspected people were then transported to the nearest ETC for EVD confirmation via RT-PCR testing. Data about EBOV presence in villages are available at the DPS in Guinea but unfortunately not published. We were able to visit such data sets at Macenta DPS to classify our selected villages as either affected or non-affected. More importantly, healthcare agents in those villages were involved in our study and mostly helped to confirm the accuracy of the data we collected from the DPS. In addition, Macenta population is about 300,000 habitants and the 44 villages selected for our study encounter approximatively 106,164 habitants which represent 38.52% of the general population of Macenta.

The highest participation rate was seen amongst the 25-55 year olds with (374 of 517), followed by 16-24 years old. Within the sample collection period, 212 households of bushmeat hunters were reached. Subsequently, with regards to community social life in the zone, each hunter possesses at least two wives, 5 children and two close relatives per household. So, we are confident that we have reached ~ 2,120 people (≈10 peoples per household). This indicates how many people are at risk and how fast viral infection could spread when first contamination spillover event occurs[185].

During both mass communication and interview stages, a number of questions were raised. Community as well as volunteers were eager; 1) to know what the benefit of the study for volunteers and the entire village in the future would be, (2) to understand why samples were collected from healthy peoples instead of diseased peoples, (3) to be notified of any other disease and provide medication in the event of samples being found positive. In response to these questions, we were able to explain how risky the population is with regards to zoonotic disease exposure through their daily activities. Mainly, these activities are bushmeat hunting, manipulation and consumption. Importantly, we emphasised on the fact that wildlife carrion found in the bush should be avoided for consumption as no one knows what they died off. This constitutes the riskiest exposure to viral transmission and spread. We also explained how possibly a disease could spill over in the entire community (village) through only one first expo-

sure (index case). Further, we highlighted the fact that the study would help to understand virus circulation in the zone and possibly prevent future outbreak occurrence. Finally, we suggested that in the event of any outbreak occurrence, there may be a possible shipment of drugs or available vaccines for population wellbeing. However, we strictly emphasised on the fact that samples were being tested to identify any exposure to filoviruses prior to the past EBOV epidemic.

Participants outreach flow chart

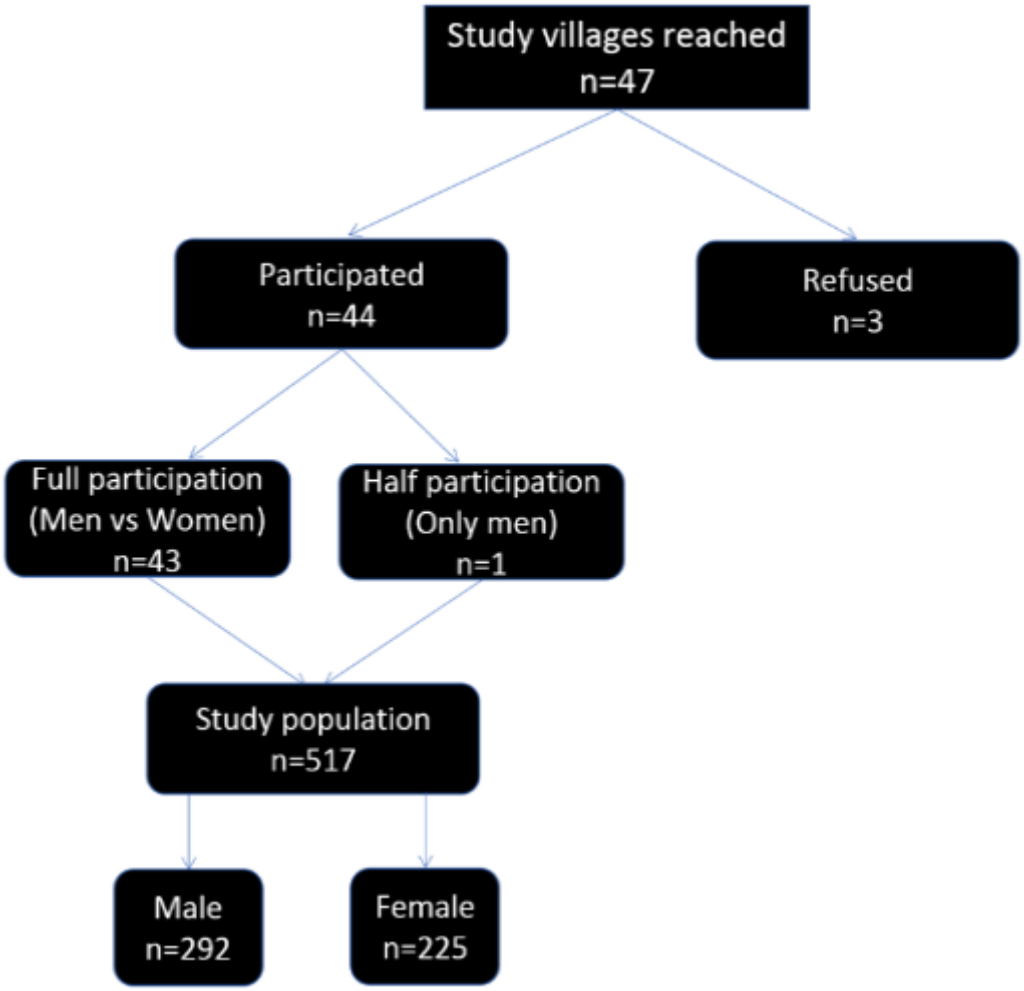


Figure 18: Flow chart of study villages and population participation.

In total 47 villages were reached and sensitized amongst which 43 villages entirely participated, one village showed half participation (only men) and 3 villages disagreed to participating in the study

3.4 Participant questionnaire data

Sample collection took place within 11 months from February to December 2017. Eligible participants were enrolled and sampled upon understanding and self-commitment to the study. An individual consent form was signed and basic available data on volunteer's age, weight, occupation, and health history were recorded prior to sample collection.

In general, hunters use both traps and guns. However, following the government policies, it is forbidden for people under 20 of age to utilise guns[184]. So, for people under 20 of age, traps and trained dogs are essentially used to hunt small animals such as rodents, rats, reptiles and birds. Hunted animals are usually sold to bushmeat traders who sell to the population in small pieces either fresh or smoked. When interviewed, participants reported having experienced fever at least three times in the past 12 months. Unfortunately, the only disease they had thought of was Malaria as no medical consultation or diagnostic was performed. In fact, alternative medicine practitioners are the most consulted in these remote places. Nevertheless, only 2 people reported to have experienced haemorrhaging which unfortunately remained undiagnosed.

On the other hand, women are not involved in hunting activities; however, manipulating and butchering of hunted animals for food preparation constitutes their daily duties. Few women reported fishing activities which is done on small rivers with a particular circular net made in circle shape. Finally, no data was recorded for excluded participants presenting chronic diseases and the number of EVD infected people per village, as this was very controversial.

3.5 Chapter discussion and conclusion

Samples were collected in selected areas which included affected and unaffected villages from the 2013-2016 EVD outbreak in Guinea. Study village selection was primary based on the accessibility and then the possible report of any EVD infection. The aim was to investigate the potential exposure of different villages to EBOV and LASV. Unfortunately, due to the limited number of samples designed for the study and population consent we could not bleed the same number of participants in all villages and this may bias results with regards to social groups involved.

EBOV and LASV are thought to be zoonotic diseases and therefore, remote villages were regarded as potential source of previous infection.

There were various limitations to this study. Following the 2013-2016 EVD outbreak in Guinea, the fear and trauma in the community that usually follows[1], the very limited access to some places and, minor resistances in the population, we could not perform sample collection in some potential places that were of study interest.

To conclude, our sero-prevalence study on EBOV and LASV attempts to understand pre-existing exposure of the population to these viruses. It also intends to understand the route of infection transmission (wildlife to human) and epidemics spread (infection site to cities) in the population. Alternatively, research on other existing emerging disease pathogens using our collected samples could bring more understanding regarding known or unknown zoonotic diseases transmission in the region.

Chapter 4: RESULTS, PREVIOUS EXPOSURE TO EBOLA VIRUS

4.1 Enzyme linked immunosorbent assay to detect Ebola glycoprotein

The ELISA platform was developed and described in 1971 by two Swedish scientists Peter Pearlman and Eva Engvall[186]. To date, ELISA, also known as EIA (Enzyme Immuno-Assay) is wildly regarded as the most reliable immunoassay of antigen-antibody detection[187]. It is used to detect and quantify substances such as peptides, proteins and hormones for diagnosis and scientific research[188,189]. The test utilises a reporter protein, typically an antibody, that is conjugated to an enzyme which changes colour upon reaction with a specific chemical substrate. The nature of the colour change depends on the enzyme-substrate reaction. For instance, the p-Nitro phenyl phosphate (p-NPP) turns yellow in contact with alkaline phosphatase and the streptavidin-HRP (horse radish peroxidase) turns blue in the presence of TMB substrate. Though several types of ELISA assay have been developed over time, we performed indirect ELISA, as described in the material and methods, with the p-NPP for anti-EBOV GP antibody detection. For the test sensitivity and specificity, blocking reagents are used to interfere with a wide range of proteins in samples which may cause false positives when binding to the secondary antibody. Casein, ChonBlock and non-fat milk have been demonstrated in interacting with several types of proteins and subsequently prevent false positives in ELISA assay[188].

4.1.1 Detection of anti-EBOV glycoprotein antibodies using Casein as a blocking reagent

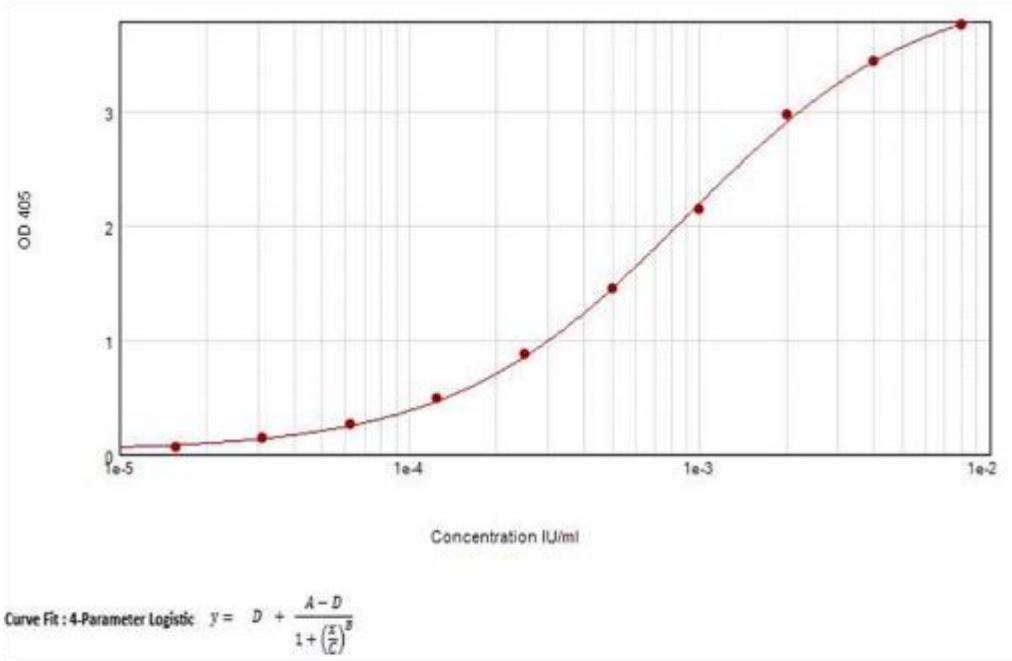
517 serum samples were collected from volunteers in 44 villages across Macenta prefecture. We initially looked at the ability of these serum samples to bind the EBOV GP using casein as a blocking reagent. Casein is a milk-derived substance composed of proteins and amino-acid[190]. It was discovered to be an effective agent to block non-specific protein binding in ELISA assay procedures. It has been used as a blocking and sample dilution buffer to enhance the ability to block or prevent nonspecific bindings. Unfortunately, a significant amount of bacterial debris present in milk including different bacterial antigens such as teichoic acids still cross-react and obstruct the assay leading to false positives while using Casein reagent.

We first sought to determine the range within our standard curve within which we would interpret sample OD values, **Figure 19** shows an example of the standard curve with known EVD survivors' samples. We decided to interpret sample OD values that fell between the upper asymptote for parameter A and the lower asymptote of parameter D. The upper asymptote of parameter A represents the lower limit of detection (LLD) indicating the lowest concentration to be detected. It is the concentration level that can be different from a blank at 99% confidence level. It means that if the

sample and blank are tested several times, likely, the resulting brackets would approximatively be the same. Alternatively, the lower asymptote of parameter D is the upper limit of quantification (ULQ) which indicates the highest concentration to be detected. If values fell above or below these parameters, they would be classed as being beyond the limit of quantification and the corresponding IU/ml value to either the upper asymptote of A or the lower asymptote of D would be reported. Precisely, samples values above ULD are diluted and tested within the calibration range.

ELISA assay standard curve

A



B

R^2 = 1.000 Chi-Squared = 0.007

Parameter	Estimated Value	Std. Error	Confidence Interval
A	0.031	0.030	[-0.079, 0.140] i
B	1.080	0.043	[0.920, 1.240]
C	8.93e-4	3.61e-5	[7.60e-4, 0.001]
D	4.126	0.069	ii [3.868, 4.384]

Figure 19 : EBOV GP ELISA Standard curve

A) Example of standard curve. Non-linear regression curve of 4 parameters (A, B, C and D), samples were serially diluted 1:2 with a starting dilution of either 1:100 or 1:500. **B**) We set a cut off to accept or reject values, we rejected values that fell beyond the upper confidence interval of parameter A or lower confidence interval of parameter D (red boxes), these values were determined by SoftMax™ software.

Following sample acquisition, we next looked to determine a cut off value which would distinguish positive and negative. To do this we used a known EBOV negative sample which consisted of pooled plasma from three native West African volunteers. This known negative was acquired on each ELISA plate that was assayed. To determine our positive and negative cut off we took the mean of all the negative plasma results plus 5 standard deviations from the mean and this gave us a cut-off of 0.0304 IU/ml. In addition to acquiring a negative sample on each plate we acquired a quality control which consisted of pooled plasma from three known EVD survivors and **Figure 20** shows the variation in this QC from assay to assay.

ELISA assay controls and cut-off results

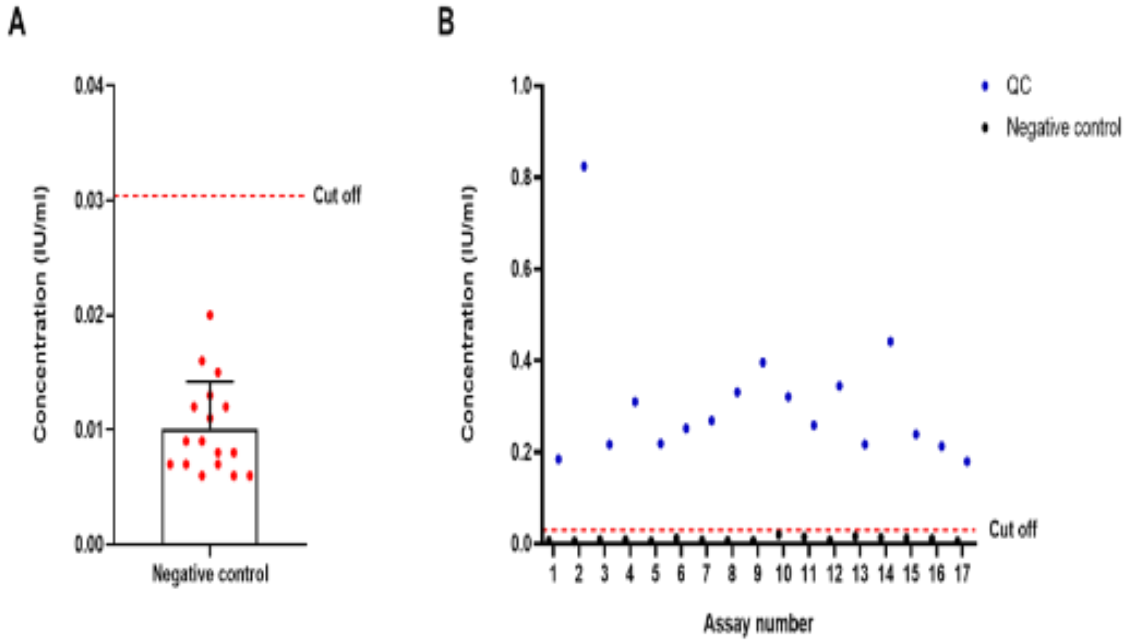


Figure 20: EBOV GP ELISA negative control and QC

A) Negative cut off is determined by taking the mean result of negatives plus 5*STD of the mean. This was so we could resolve positive from negative cases as stringently as possible. **B**) Variation in the QC by assay number. QC values above cut off indicate assay validation in daily basis. Non validated assays indicate QC value under the cut off and subsequently re-peated.

Once we were happy with our assay platform and how we would interpret the results we acquired and analysed our unknown participant serum samples. Results in **Figure 21** show that 119 participants had an IgG response that was above our determined cut off of 0.0304 IU/ml suggesting that they may have had prior exposure to EBOV. When these results were broken down by affected and non-affected villages, there was no significant difference in terms of the magnitude in the response reported or the proportion of villages affected. Again, when these results were broken down by sex there was no significant difference in the magnitude of response, or the proportion of each sex affected.

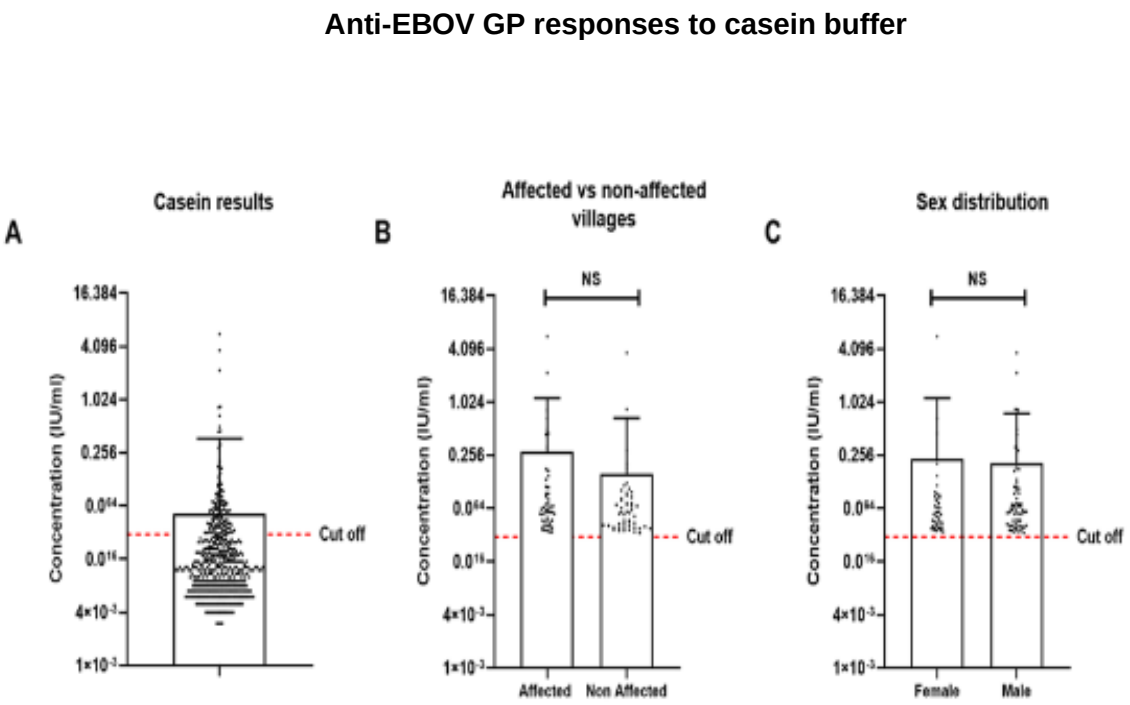


Figure 21 : ELISA anti-EBOV GP detection results using Casein reagent

A)All casein results. The OD values above the cut-off indicate the presence of anti-EBOV GP in participant's sample. In contrast, OD values under the cut-off indicate no response. **B**) Positive results by village. No major difference is observed regarding villages status either previously exposed to EVD (affected) or not (non-affected). Both show similar responses to anti-EBOV antibody. **C**) Positive results by sex. Although, male and female show almost an equal signal of responses to anti-EBOV antibody. It follows that when exposed, both sex groups are likely subject of infection with no particular limitation. Test Mann Whitney U P=>0.05.

We next looked to determine if there was a significant correlation between age group and anti-EBOV GP IgG titre, as previous reports have indicated that anti-EBOV antibody decrease over time[115,191]. It follows that people exposed about 40 years ago might have shown weak signal and consequently would have been below the defined cut off. However, as the search could not determine anti-EBOV antibody duration, **Error! Reference source not found.Figure 22** shows that there is no correlation in terms of age and the magnitude of the IgG response.

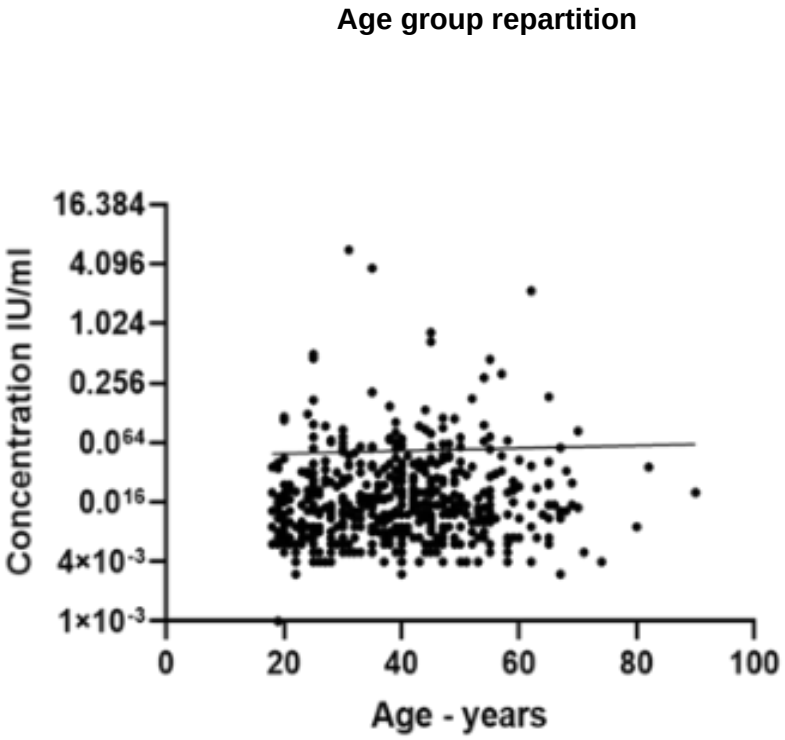


Figure 22: Anti-EBOV GP detection result regarding age group

XY graph of age and IU/ml Linear regression used. This figure shows no significant correlation P=>0.05 regarding age group response to anti-EBOV antibody.

Some of the villages (16 of 44) had previously experienced EVD during the 2013-2016 outbreak. Of these 16 villages we found 13 that showed at least one positive result, whereas 16 out of 28 previously non-affected villages showed an antibody response using our ELISA platform (**Figure 23**).

Villages that responded to anti-EBOV GP detection on Casein

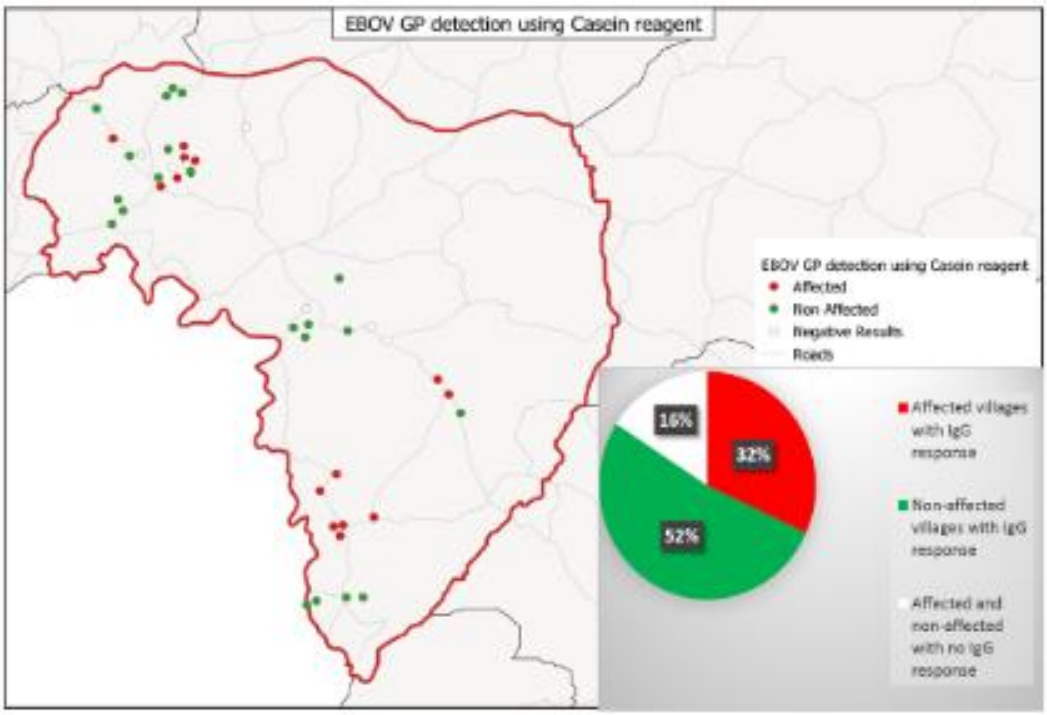


Figure 23: Map of positive cases on casein.

Regarding both affected and non-affected villages, there is no fundamental difference of anti-EBOV IgG response. EBOV IgG response in previous affected villages is shown in red and green indicates EBOV IgG response in non-affected villages. White color indicates both affected and non-affected villages with no EBOV IgG response.

We next looked at the EBOV anti-GP antibody prevalence using casein reagent and participants' occupations (**Figure 24**). With regards to volunteers occupations, hunters (first contact to wildlife) and household family members (close contacts) showed high level of anti-EBOV antibody response. Notably, this might be related to the number of samples collected within these groups. Healthcare workers, workmen and traders showed less participation due to their limit in number in villages as the main activities are farming and hunting.

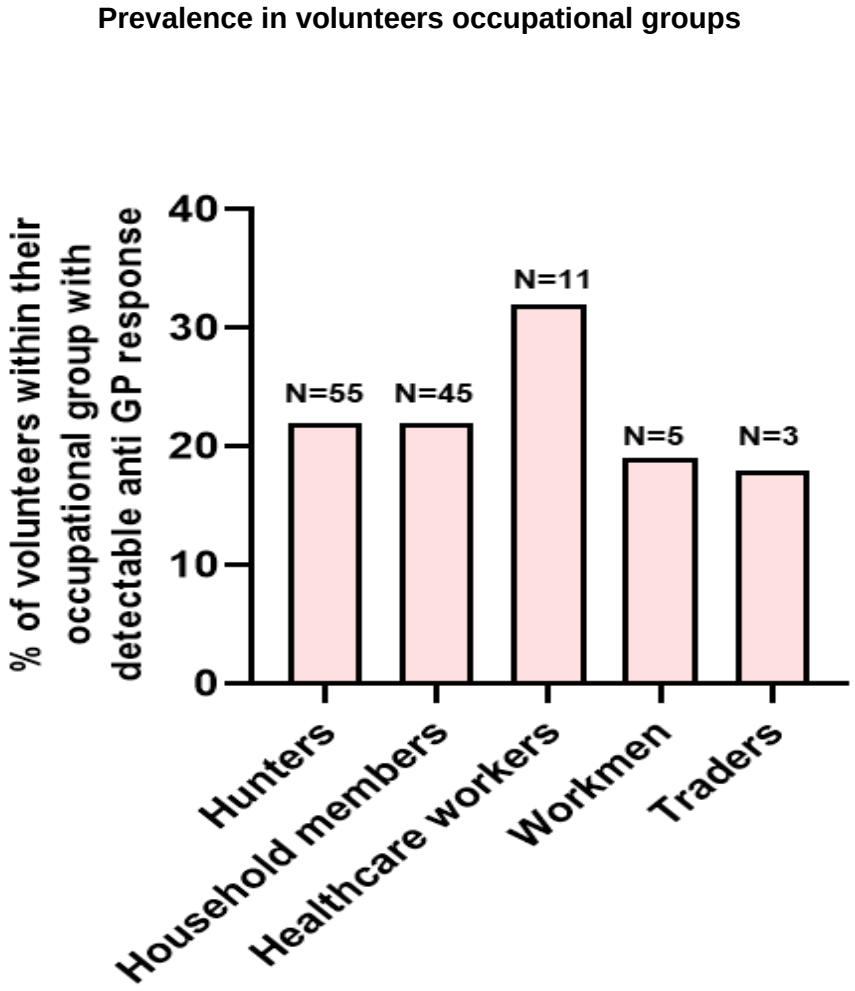


Figure 24: Anti-EBOV GP detection using casein reagent.

High prevalence rate found in Healthcare workers, hunters and household family members as expected. Others occupational groups show exposure and these results relate to the number of volunteers in each group.

4.1.2 Detection of anti-EBOV glycoprotein antibodies using ChonBlock™ as a blocking reagent.

Previous work has considered using various blocking buffers including Tween-20, BSA, normal goat serum (NGS), Casein and ChonBlock. Results indicated that ChonBlock is more effective in reducing several types of non-specific protein interactions[188,192]. These experiments demonstrate that the blocking effect of 0.1% of ChonBlock is equivalent to 4% of BSA and 0.5% of normal goat serum blocking respectively. Additionally, it proved that ChonBlock buffer can be used to block several types of proteins in human and animal specimens. ChonBlock then reduces background noise and other false positive results involved in ELISA assays. Finally, the study established that ChonBlock buffer prevent hydrophobic binding of immunoglobulins to plastic surface and non-specific protein-protein interaction between antigens and immunoglobulins[192]. This reagent has helped to reduce false positive results in both diagnostic of infectious diseases and in the scientific research fields. Therefore, we next assayed those samples that were found to be positive by Casein again for anti-EBOV GP antibodies but this time, we blocked our plates using ChonBlock. From **Figure 25** it can be seen that 67 out of 119 samples were found to be above the cut off of 0.0319 IU/ml, which was calculated based on Chonblock blocked negative sample.

Anti-EBOV GP responses to Chonblock buffer

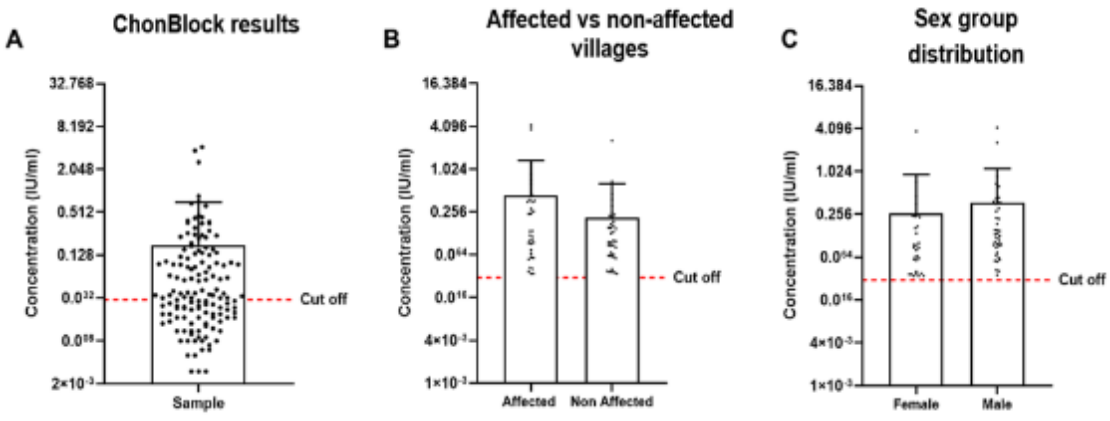


Figure 25: Anti-EBOV antibody detection using ChonBlock reagent

A) Magnitude of IgG response amongst ChonBlock positive samples. The cut off was considered by adding the mean of negative results to 5*STD of the mean negative result. OD values above the cut off indicate anti-EBOV antibody response. Subsequently, OD values under the cut off indicate no response to anti-EBOV IgG. B) Magnitude of IgG response within affected vs non-affected villages with no significant differences. C) Magnitude of IgG response in male's vs females was comparable. Mann-Whitney test used, P=>0.05 for all tests.

In terms of the magnitude of anti-EBOV IgG response, there were again no significant differences between affected or non-affected areas or with sex group (**Figure 26**).

Villages that responded to anti-EBOV GP detection on Chonblock

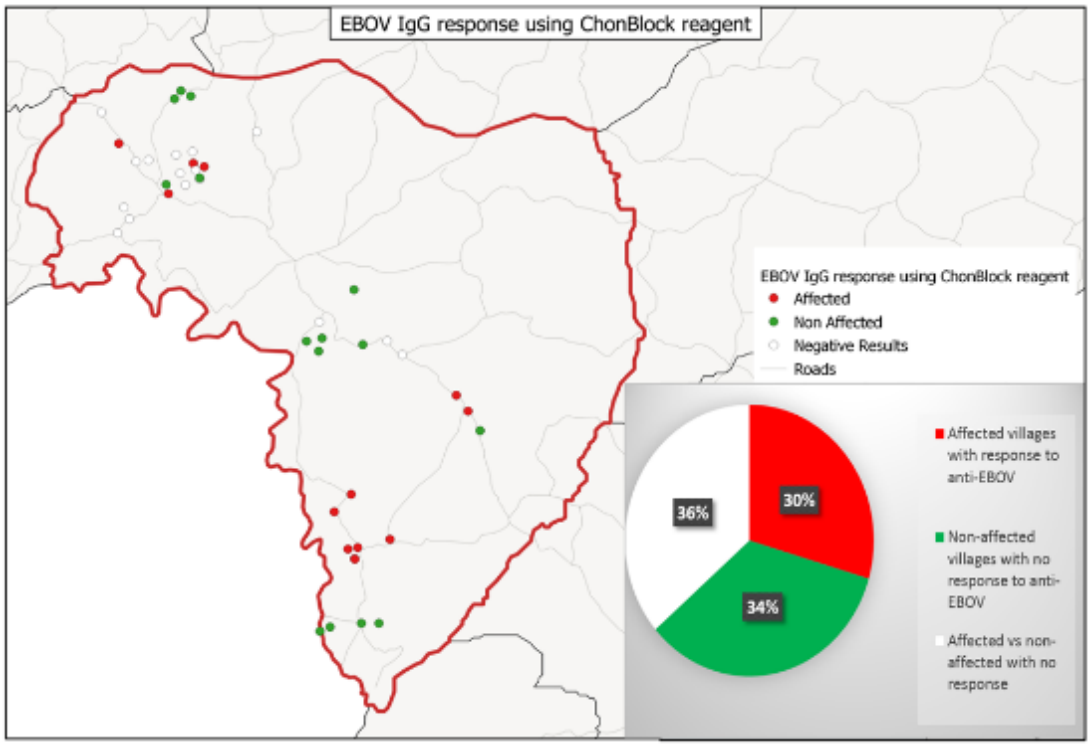


Figure 26: Anti-EBOV IgG response in study villages by ChonBlock reagent

GIS map of affected and non-affected villages that showed IgG response to ChonBlock reagent or not. EBOV IgG antibody was broadly detected in the study zone. Red color indicates EBOV IgG response in previous affected villages and green indicates EBOV IgG response in non-affected villages. White color indicates both affected and non-affected villages with no EBOV IgG response.

4.2 Detection of anti-EBOV antibodies by Western blot

We next looked to confirm our EBOV ELISA findings using an additional assay platform. Western blot is an effective method used to detect and characterize specific proteins of interest in a complex mixture of proteins[156]. It is wildly used in research to identify specific proteins of interest regarding their molecular weight in a complex mixture of proteins extracted from cells or tissues[154]. Proteins are run on an SDS-PAGE gel and separated according to their sizes and molecular weight. Proteins are then transferred to a PVDF or nitrocellulose membrane and enzyme conjugated antibodies are added to the membrane, bands are visualized by addition of an appropriate substrate and samples are acquired using Bio-RAD molecular imager (CHemi-Doc^{TM}XRS+ Imaging system) (**Figure 14**). We used the Western blot technique to confirm our positive samples from ChonBlock ELISA results. We determined previous exposure to EBOV and the generation of antibodies reacting to EBOV GP, NP and/or VP40. Notably, the VP40 matrix protein of EBOV is abundantly expressed in viral infection[193]. VP40 along with GP and VP24 are membrane associated proteins and contribute to viral replication. On the other hand, NP associates with VP30, VP35 and L proteins to form the virus nucleocapsid and plays an important role in genome transcription and replication[23]. It follows that targeting anti-NP and anti-VP40 antibodies as well as anti-GP antibodies would bring more accuracy in anti-EBOV antibody detection in our Western blot assay. As these three proteins constitute major viral antigens, Hafmann *et al* performed a serological analysis targeting those proteins in EVD survivors and their close contacts in Sierra Leone[194]. A commercial ELISA kit was used for protein detection and report indicated 97.7% of EVD survivors have antibody levels against GP, NP and VP40 proteins either singularly or coupled (GP/NP, GP/VP40, or NP/VP40) or tripled (GP/NP/VP40).

The 67 samples previously tested positive to EBOV GP by ELISA assay (ChonBlock reagent) were tested by Western blot. Before acquiring data on volunteer samples, we looked to confirm the specificity of our secondary detection antibodies. In **Figure 27**, it can be seen that monoclonal antibodies specific to GP, NP or VP40 showed specificity and did not cross react with other EBOV proteins. GP is visualised at 100 kDa, NP at approx. 40 kDa and VP40 at approx. 60kDa.

Western blot assay validation results

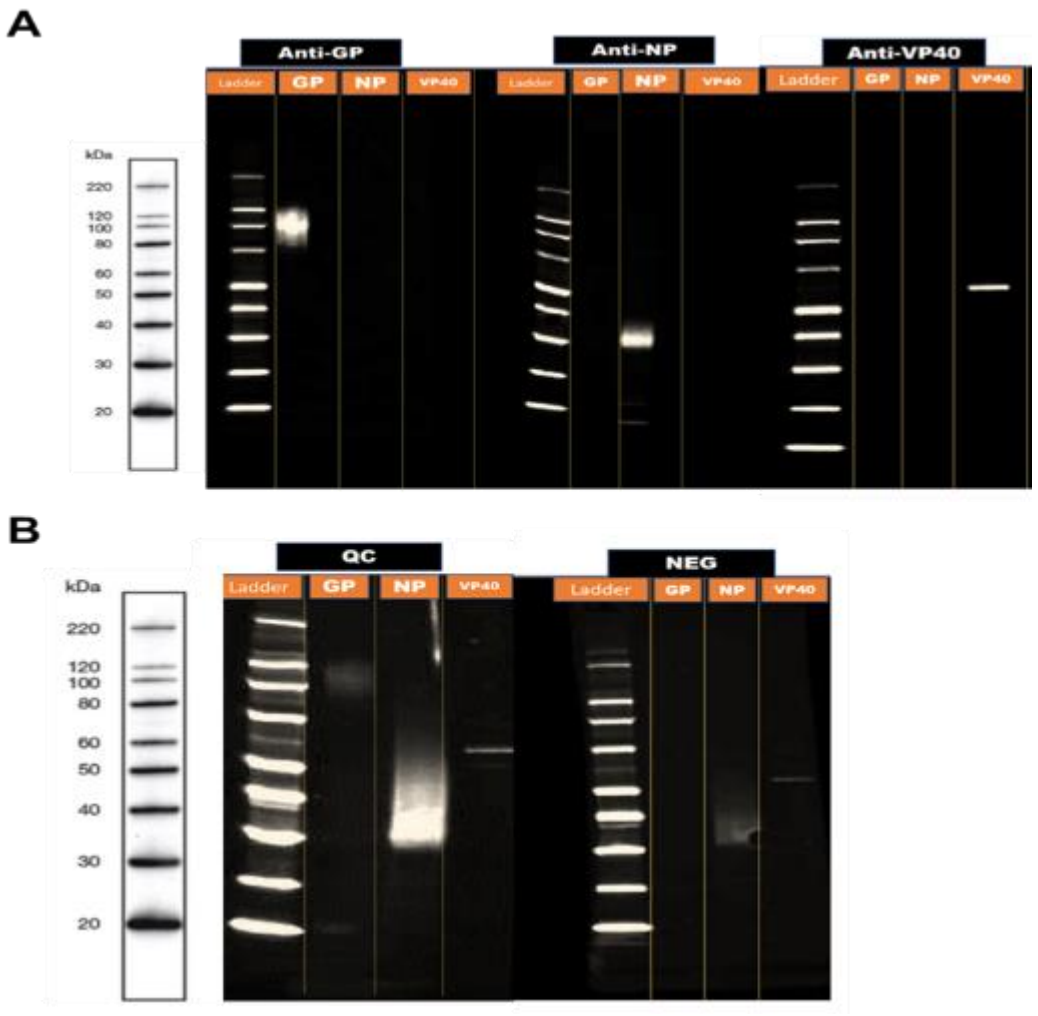


Figure 27: Specificity of EBOV monoclonal antibodies.

A) Control antibodies to show specificity in interacting with selected antibody in EVD survivor's serum. B) Show QC and NEG controls results for assay validation. Purified EBOV GP, NP and VP40 proteins were subjected to SDS-PAGE and Western blotting. Blots were probed with monoclonal antibodies specific to the indicated EBOV proteins as well as with a QC standard from survivor serum and a Negative control from uninfected serum. No cross reactivity between antibodies was observed.

In addition to testing the specificity of our monoclonal antibodies, **Figure 27** also shows that our QC plasma has a detectable GP, NP and VP40 response whereas negative control plasma does not. Notably, the QC plasma is a pool of three EVD survivors' serum whereas the negative plasma is a pool of three non EVD subjects. We next analysed our participant samples that showed a positive result by ELISA and it can be seen in **Figure 28** that 11 samples out of the 67 tested clearly showed a response to one or more EBOV proteins. When the samples' ELISA value (by ChonBlock) are highlighted it can be seen that they are not all the maximum responders and that they cover a broad range of values. With regards to Western blot method specificity and sensitivity to detect and characterize specific protein/antibodies of interest[195]; it is evident that those 11 samples were from people who experienced EBOV infection in our study cohort.

Anti-EBOV GP responses on Western blot

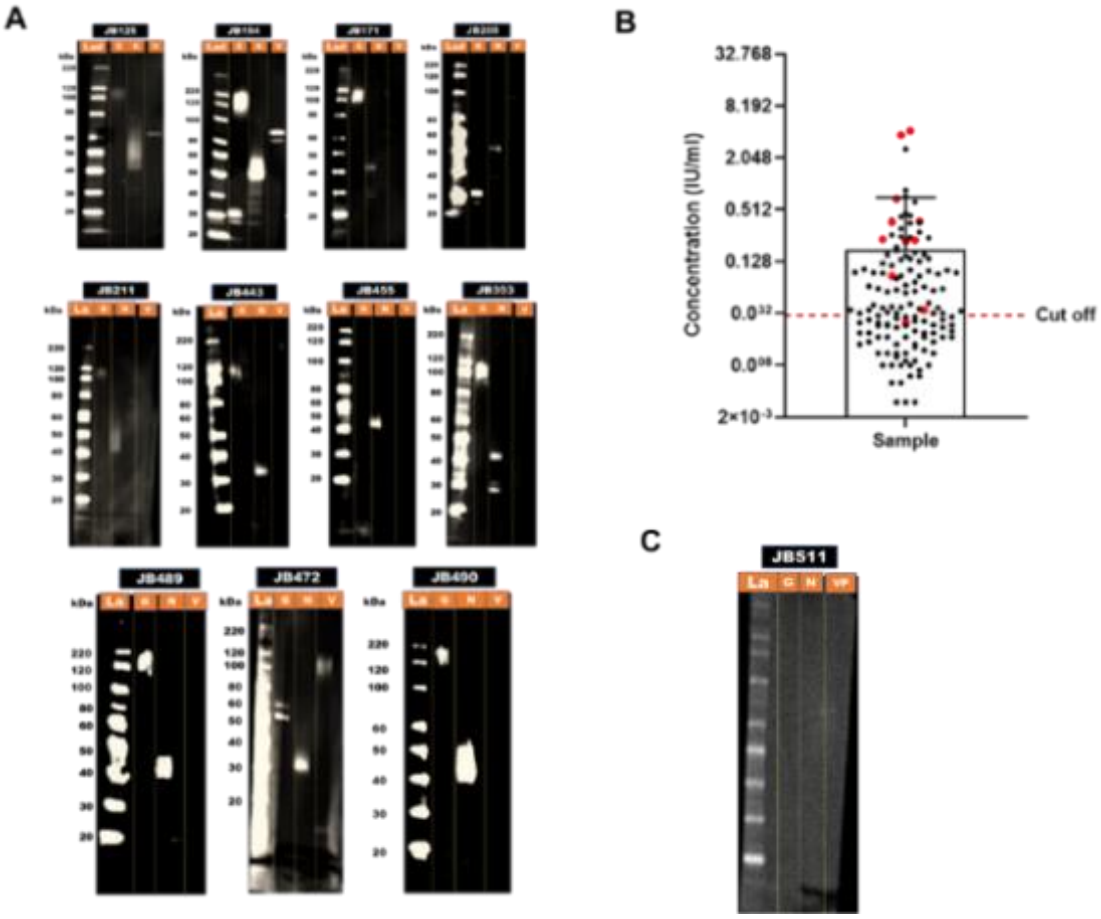


Figure 28: EBOV GP protein detection by western blot

A)Western blot of those deemed to show a response. **B)** red dots indicate the ChonBlock™ ELISA result from the Western blots in A. **C)** Representative **e**xample of Weston blot membrane returning a negative result.

Of 44 villages included in the study, 11 show antibody detection through Western blot assay amongst which five villages have reported EVD (affected) and five villages have never reported EVD (non-affected) during the 2013-2016 outbreak. **Table 7** gives breakdown of these participants location, age and sex group. It can be seen that they originate from 5 affected and 5 non-affected villages and that there were 7 females and 4 males.

Table 7: Table showing the 11 participants with detectable western blot bands to at Least the GP.

Sample ID	Sex	Age	Village	Village status	ChonBlock™ ELISA
JB455	M	45	Iyezou	Affected	4.211
JB472	F	45	Fassankoni	Affected	3.742
JB353	M	45	Namouyapoulou	Unaffected	0.683
JB205	M	57	Vassaizeazou	Affected	0.376
JB443	M	25	Zenie	Affected	0.366
JB489	F	39	Sassaguizezou	Unaffected	0.232
JB490	M	60	Sassaguizezou	Unaffected	0.227
JB171	M	57	Dohomai	Unaffected	0.221
JB211	M	70	Baizea	Unaffected	0.087
JB125	F	40	Sovaou	Unaffected	0.035
JB154	F	39	Bofossou	Affected	0.025

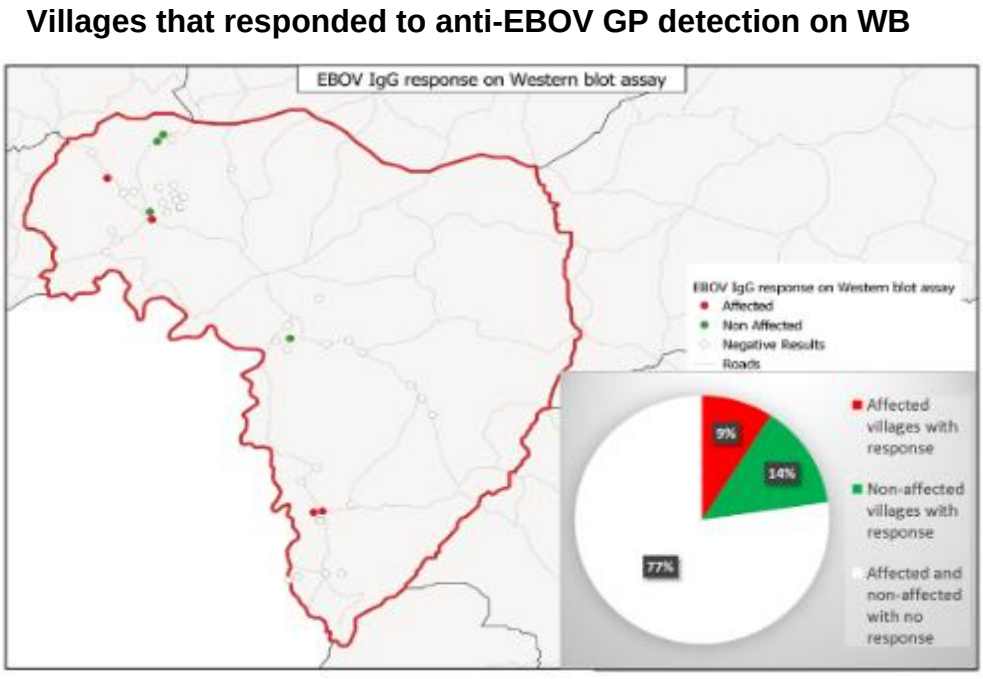


Figure 29: Anti-EBOV IgG response in study villages by Western blot

GIS map of affected and non-affected villages that showed and IgG response to *Zaire ebolavirus* GP by Western blot test. Both affected and non-affected villages show anti-EBOV response with no major differences.

Interestingly, as it can be seen in **Figure 288** not all samples were positive for all EBOV proteins. We reported on the 11 participants that were positive to EBOV GP; however, there were 33 participants that were positive to NP and 18 positive to VP40. Those that were positive for EBOV GP were not necessarily positive for other EBOV proteins, in fact, only three participant samples were found to show activity against all three proteins.

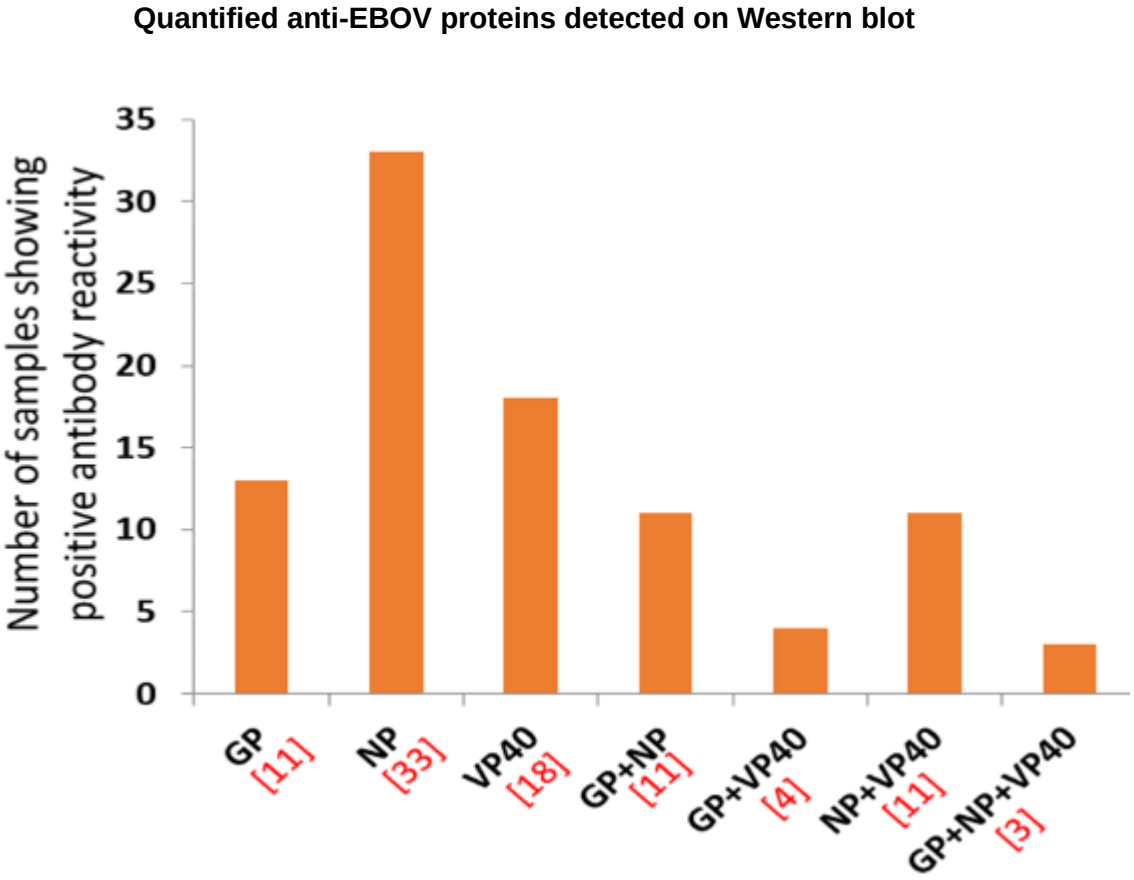


Figure 30 : Ebola virus proteins detection.

Bars and numbers in red indicate the number of participants showing anti-EBOV antibody responses by Western blot to the indicated EBOV proteins.

Our data suggests that 2.12% (11 of 517) of participants show an anti-EBOV GP response, which is contradictory to our ELISA findings. The ability of ELISA assay to effectively block different types of non-specific reactions is poorly understood with regards to numerous proteins in specimen and the high binding affinity of proteins to plastic surfaces[187,196]. Consequently, a supplementary assay, Western blotting, which is identified for its high sensitivity and specificity at a molecular level of protein detection has been developed[195]. This assay was then performed subsequent to ELISA assay positivity.

Regarding sample collection areas, anti-EBOV IgG detection was nearly similar in unaffected villages 21.42% (6 of 28) and affected villages 25.00% (4 of 16) with regards to the number of samples collected per zone. Western blot data taken on its own would suggest that 22.72% (10 of 44) show antibody response to EBOV. Considering antibody response via ELISA assays using both Casein and ChonBlock reagents and Western blot analysis, our findings established evidence of either mild or asymptomatic EBOV infection occurring in several villages.

4.3 Multiplex beads assay data for Ebola virus proteins detection

ELISA and Western blotting have been the most reliable assay for protein detection and quantification. However, they are not well equipped to measure simultaneously several analytes in a single reaction[197]. Multiplex beads array assays have been recently developed to measure proteins and other analytes in both research and clinical laboratories[198]. The potential benefit of the assay is its ability to evaluate multiple proteins or analytes in a small volume of biological material. The assay results are often read on flow cytometry instruments (e.g. BD AccuriTM C6 Plus). Flow cytometry instruments detect, measure and analyse thousands of cell populations or particles per second[199]. Cells or particles are labelled with fluorescent markers and injected into a flow cytometry instrument. When passing through a laser beam, light scatter identifies a target population by their phenotypic markers and the data produced is computerised. Our in house beads array assay is a multiplex technology (immunoassay) which determines simultaneously immunoglobulin isotype to various proteins in human specimens. The assay uses magnetic beads to capture and measure multiple proteins or analytes of interest in a single sample at the same time.

4.3.1 Multiplex beads assay results

The 67 samples which show antibody response on ELISA and were tested on Western blot were re-tested using our multiplex bead array assay. This assay detects proteins and analytes in a small volume of patient's specimen. First, carboxyl magnetic beads (color coded) were coated with EBOV GP tetramer (0.35mg/ml). Volunteer serum samples and coated beads were mixed, and existing anti-EBOV antibodies could then bind to the EBOV GP antigen. Further, the detection antibody (anti-human IgG-PE, 100 mg/ml) conjugated with biotin was added and specifically binds to the anti-EBOV antibody. Later, streptavidin-PE substrate was added which subsequently binds to biotin on the anti-human IgG-PE detection antibody. Finally, the mixture is read on a dual-laser flow cytometry-based instrument, BD Acuri C6 Plus flow cytometer. While passing through the laser, beads are classified regarding the analyte detected (side scatter) and the magnitude of PE-derived signal (forward scatter)[198]. (**Figure 15**).

4.3.2 Detection of Anti-GP IgG isotype

We next look to determine the isotypes involved in suspected EVD survivor samples. Work performed by Davis *et al* on the human B cell response to EBOV infection looked at the dynamic changes in IgG subclass of EBOV antibodies after infection[200]. The data established that when EVD patient is discharged IgG1 is high and decreases slowly over time, IgG2 remains undetectable, IgG3 is high and decreases rapidly, IgG4 is undetectable but appears later about 1-2 years post-infection. The study has then suggested that the long-term IgG response switches to IgG4 over time.

To determine the specificity of the assay we conjugated our recombinant EBOV GP to carboxyl magnetic beads. To check the specificity of these conjugations we used the monoclonal antibody KZ52 as well as EVD survivor QC and negative control plasma. KZ52 is an antibody derived from EVD survivor (Kikwit, 1995) which neutralizes EBOV when specifically bound to viral glycoprotein (GP1/GP2)[201]. It is likely that KZ52 requires both GP1 attachment subunit and GP2 pre-fusion conformation subunit for proper binding to viral surface[202,201]. This enhances KZ52s specificity to recognize and bind to anti-EBOV antibody and subsequently make it an antibody of interest for virus neutralisation [202].

It can be seen from **Figure 31** that negative plasma shows a background fluorescence between 10^2 - 10^3 mean fluorescent intensity (MFI) whereas both QC plasma and KZ52 show a strong positive signal between 10^4 - 10^5 MFI, whereas in all cases the isotype control (grey) shows a base level of 10^2 MFI.

Multiplex beads array assay controls

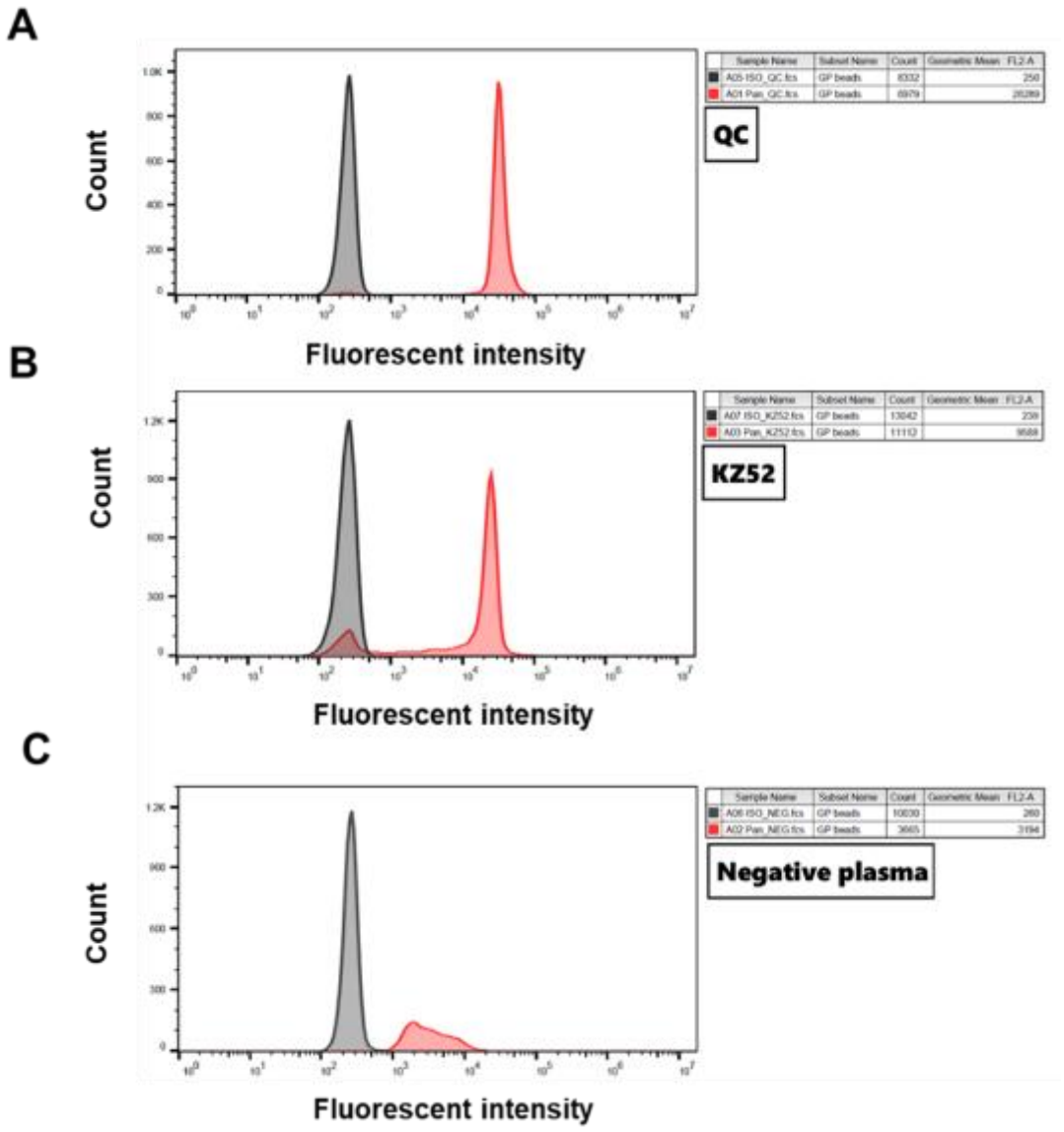


Figure 31: Bead isotype array set up

A)QC plasma, **B)** KZ52 antibody, **C)** Negative plasma. Representative flow cytometry of plasma control (QC) and antibody (KZ52) showing strong anti-IgG isotypes response (red peak, A and B while negative control plasma with no prior exposure to EBOV show no anti-IgG isotype response.

Once we were convinced that the beads had GP, NP or VP40 bound to their surface and that they showed specificity, we next looked to run participant samples using this assay format. We acquired data on 67 samples that we have previously shown by Western blot and/or ELISA to have had prior exposure to EBOV. Results in **Figure 32** suggest that not all samples show robust anti-GP IgG responses, four of the total anti-GP IgG responses appear to be the same level as the negative control whereas the remaining samples that showed high IgG levels to EBOV GP showed mixed responses to EBOV NP and VP40.

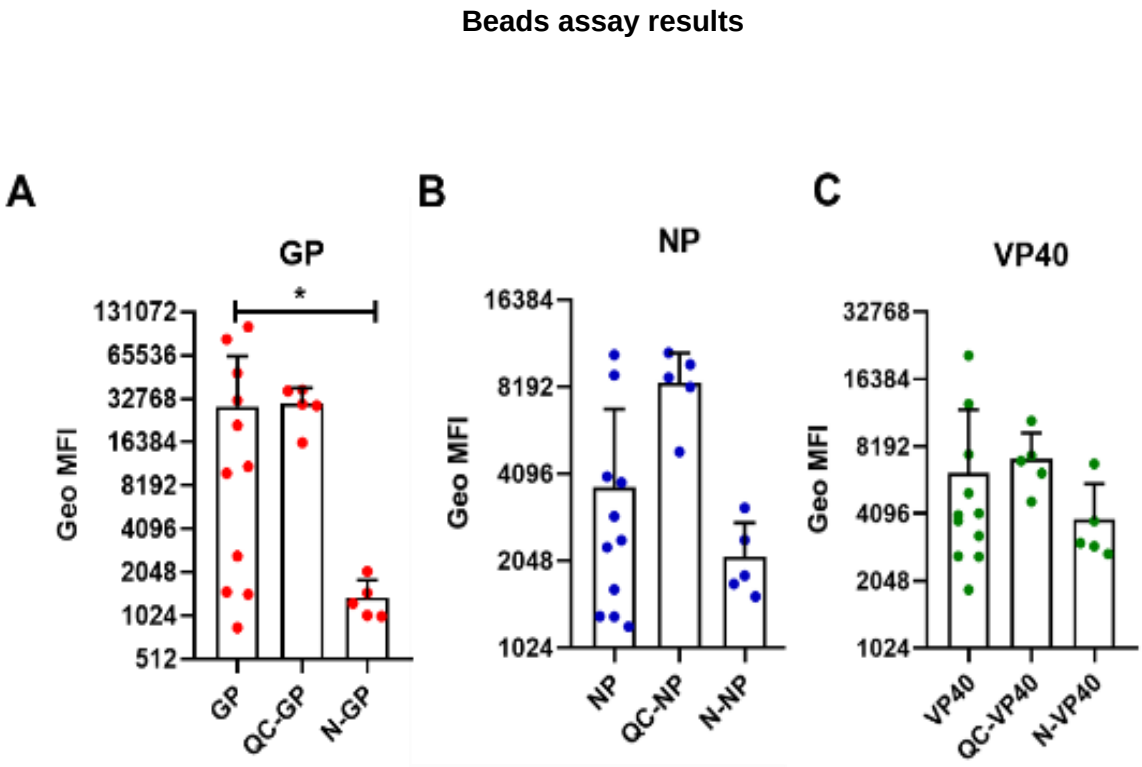


Figure 32: EBOV anti-IgG response

A) GP total IgG. **B)** NP total IgG. **C)** VP40 total IgG. APC beads were conjugated with target EBOV protein then incubated with EVD survivor plasma, QC or negative control plasma (N). Samples were washed and acquired on a flow cytometer, data reported shows the individual results and the mean MFI and SEM. Mann-Whitney was used to test for significance P=<0.05. NP and VP40 vs their negative counterparts did not reach significance P=>0.05.

In addition to obtaining multiplexed data on the total IgG response we were also able to probe the IgG isotypes that make up the total IgG response to EBOV GP. In **Figure 33**, it can be seen that IgG1 is the dominant isotype amongst our sero-positive participants and that responses to IgG2-4 were mixed. Again, there were four participants that showed no or minimal response in our assay with regards to IgG1 subclass detection.

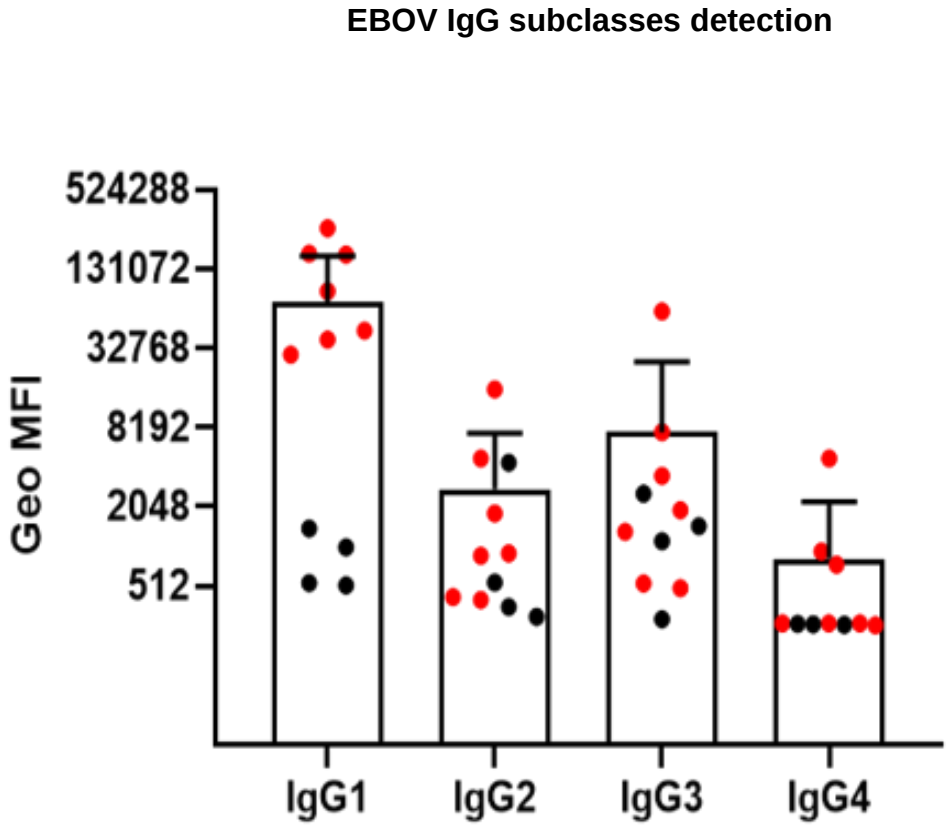


Figure 33: GP beads, IgG subclass binding.

Breakdown of the EVD survivor IgG sub type response to EBOV GP. APC beads were conjugated with EBOV GP protein then incubated with EVD survivor plasma. Samples were washed and acquired on a flow cytometer, data reported shows the individual results and the mean MFI and SEM. No data from negative control plasma was collected for IgG subclass analysis.

4.4 Chapter discussion

This study is the first to investigate anti-ebolavirus IgG responses in the population of the forested region of Guinea, Macenta zone. Limitations to this study could include: a short life expectancy in the population (mainly amongst men)[97], anti-ebolavirus IgG decreases over time and the increase of wildlife disappearance due to human behaviour which result in climate change[202,203]. Previous reports have established EBOV neutralising capacity up to 14 years after infection[202]. However, antibodies may cross react in ELISA assay as demonstrate by Eric Nakayama et al. while investigating ELISA for the detection of filovirus species-specific antibodies[186]. The study used His-tagged secreted forms of the transmembrane glycoproteins (GPs) of five *ebolavirus* species and one *marburg*virus specie as antigen. Report indicated notable cross reactivity in human and NHPs serums whereas a little cross reactivity was observed in mouse sera both previously exposed to EBOV or MARV. Our study volunteers over 55 (n=4) who show anti-EBOV antibody response may have been exposed years ago. It is unfortunate that we could not determine the duration of the exposure through antibody characterisation. Initial experiments using Casein provided the greatest number of seropositive cases, however additional experiments using a more reliable blocking buffer, ChonBlock™, reduced this number from 119 to 67. Though ChonBlock is more accurate than the Casein reagent the stringent assay cut off used (5*STD) might have also contributed to decrease the number of positive cases.

Both EVD affected, and unaffected villages showed evidence of EBOV anti-GP antibody detection without clustering at any particular location. In fact, the shortest distance between two villages of the study is about 5 km. This could possibly enhance the dynamic of wildlife colonies circulation in the zone and infection spill over in communities. Considering that there are no ecological differences between zones, it is suggestive that an unknown EBOV or related filovirus reservoir might have been circulating in the zone for decades.

Importantly, it is hard to determine whether the positive cases seen in the affected villages resulted from sub symptomatic or mild EVD infection of the 2013-2016 outbreak or it is an evidence of unknown EBOV late transmission in the region.

Sex group distribution shows no significant differences of seropositive; which was unexpected since males are known to be the first line of exposure through hunting activities. However, women are exposed via domestic activities such as butchering, skinning, smoking meat, eating, cooking and can be bitten, scratched or exposed when, manipulating or picking up dead animals' carcasses. An infection may also occur when cutting themselves with knife or an infected animal bone.

There is some discrepancy between experimental approaches, and it follows that developing an acceptable, sensitive and specific assay for antibody characterisation would aid in accurate identification of historical infections. In addition, the discovery of a potential EBOV reservoir will help to prevent community exposure and reduce the likelihood of future EVD and related outbreaks.

Our Western blot and Multiplex bead assay data suggest that those that are positive for antibody to EBOV GP are not necessarily positive for antibodies to NP or VP40. Moreover, comparing ELISA assay results to Western blot, it is evident that in an ELISA the protein is not denatured whereas it is during SDS-PAGE for Western blot. So, if human antibodies are best at detecting native proteins, they would work much better in ELISA than Western Blot. Western Blot data is great for confirming, but the ELISA data is likely more accurate in terms of actual numbers of seropositivity detection within population. However, Mulangu *et al* conducted a serosurvey study to detect anti-EBOV antibodies in human using a full-length of recombinant NP (r-NP) through ELISA assay[204]. The selection of viral NP detection was due to its abundancy in viral particle and most importantly the level of host antibody response to this protein. The study was performed in Sankuru district in the DRC amongst healthy population (n=3415) to assess animal exposures and people behaviour with regards to EBOV antibody prevalence. The data reported 11% of population exposure to EBOV. Alternatively, we found that a large number of our participant samples were positive to EBOV NP and moderately for VP40 proteins. Therefore, we would suggest that future seroprevalence studies look at multiple EBOV targets to minimise the chance that we are seeing cross reactivity from other circulating pathogens.

In conclusion, our findings indicate the possible circulation of EBOV or a related filovirus with mild or asymptomatic unreported symptoms in the Forested region of Guinea. Unfortunately, there have been no available data regarding EVD surveillance in the zone due to lack of diagnostic facilities before the 2013-2016 outbreak. Currently, following the West African EVD outbreak which primarily started in the zone, a haemorrhagic fever diagnostic laboratory (EMLab) has been established in the forested region of Guinea for infectious disease surveillance. The EMLab analysis platform include EBOV, MARV, LASV, yellow fever and dengue. Additionally, similar seroprevalence study performed in Cameroon established the presence of anti-EBOV antibodies among healthy farmers with no prior EVD reported[116]. From our findings, it is unfortunate that we could not determine whether these detected antibodies relate to the past 2013-2016 EVD outbreak in the zone or from an unknown late transmission. Since the level of anti-EBOV antibody in both affected and unaffected villages is consistent and considering that the ecological system is broadly identical in the

zone, we suggest that the virus reservoir might have been subject of hunting in the zone for decades.

More investigations are needed to identify the EBOV reservoir and further studies to better understand *Ebolavirus* infection and circulation in the forested region of Guinea. Those studies should be conducted with no restriction regarding volunteers' age group, occupations and locations. The recent discovery of BOMV in Sierra Leone[12] indicate the possible circulation of unknown *ebolavirus* species that may cross-react while using ELISA for the detection of anti-EBOV antibodies in human.

Chapter 5: RESULTS, LASSA VIRUS IGG ELISA DATA

5.1 Introduction

In order to determine the prevalence of LASV in Macenta prefecture, we performed ELISA to identify the presence of LASV antibodies in our study samples. We used the Blackbox LASV IgG ELISA' kit from BNITM in Germany. The kit is designed for qualitative serological detection of acute or past LASV infection. The kit includes positive and negative controls, blanks and background levels/noises were added to monitor non-specific bindings. In the blank wells, no serum was added in order to measure detection antibody (secondary antibody) interaction with ELISA plate plastic surface. Although, background wells were LASV antigen free in order to measure specimen's wild proteins binding to ELISA plate.

LASV antibody prevalence varies significantly between studies. In 2009, a broad search on LASV sero-survey conducted in Guinea including the main geographic regions of Guinea established high prevalence rate of 25-55% in the Forested and savannah regions of Guinea[135]. This high prevalence in forested region might be associated with the presence and consumption of rodents which are largely distributed in communities including the *M. natalensis* a known reservoir of LASV. In contrast, the lower prevalence of 7 %, was found in the middle and lower regions of Guinea. These regions encounter less contact with rodents as consumption is forbidden for religious proposes. However, infections in those regions are probably associated with food or materials contaminated by infected rodents' fluids and feces. Another study performed in Guinea at Gueckedou, Lola and Yomou prefectures indicates 12.9% of sero-positivity to LASV in rural areas and 10% in urban areas[136]. Further, LeCompte *et al* performed RT-PCR in rodents in Guinea (n=1482) and reported a prevalence rate of 1.2 % for LASV. The study established that *M. natalensis* might be the only LASV reservoir in Guinea[132]. Notably, one sporadic LASV infection disease case was notified in Mamou in January 29, 2019[134]. Active contacts tracing and follow up likely indicated no further contamination within close relatives and healthcare workers.

5.1.1 LASV seropositive rate in Macenta zone

In total, 517 blood samples were collected from EVD reported and unreported areas. Samples were tested for anti-LASV IgG and **Figure 34** shows that out of the 517 people tested 302 were found to be positive for LASV antibodies. As stated in the protocol, the assay cut-off was calculated using the following formula: Sample result (index value) = OD450-OD620(sample)/OD (cut-off) where the cut off value is calculated: Cut off = OD (neg average) + 0.150, Negative and positive controls were pro-

vided with the kit. Positive samples (Index value ≥ 1.00) suggest past or current infection with LASV and negative samples (Index value ≤ 0.90) indicate no anti-LASV antibody was detected. However, as the antibody is detectable only after two weeks of LASV infection, additional testing or clinical findings should be considered[135].

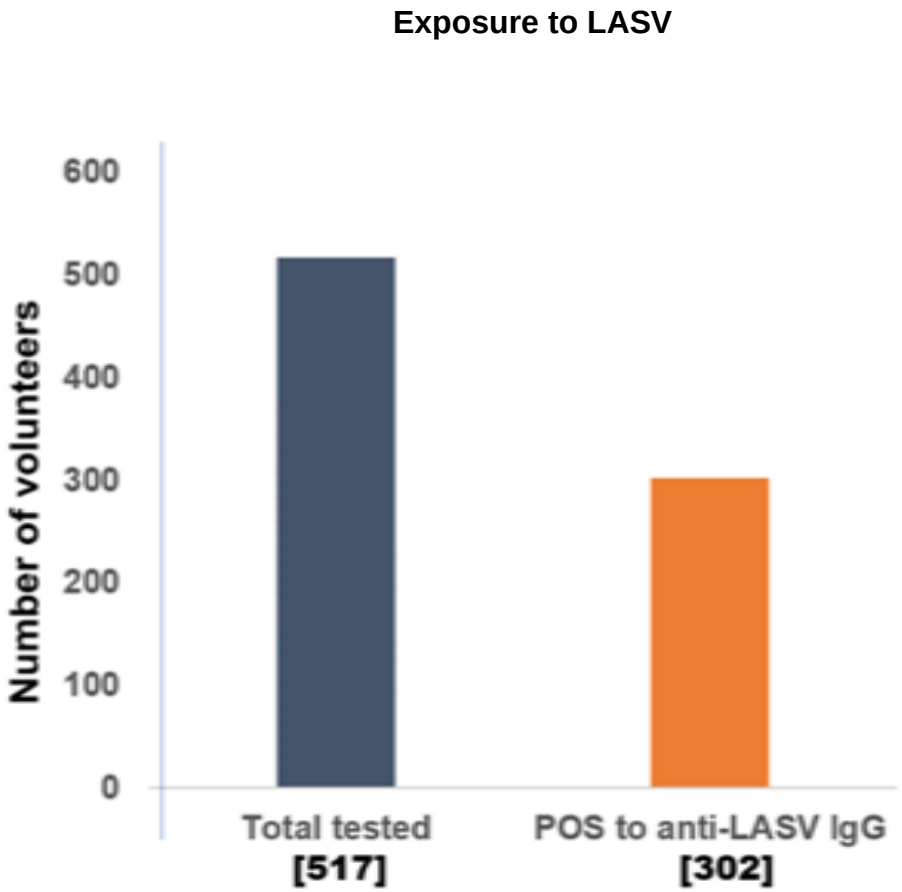


Figure 34: Lassa virus IgG detection in Macenta prefecture

Serum samples were processed and analysed as described in the Blackbox® assay protocol. We found that of the 517 samples tested 302 showed an antibody response to LASV.

When we mapped the seropositive result, it could be seen that cases were fairly diffused across our sample collection sites (**Figure 35**). However, in few sites we reported no anti-LASV antibody detection. Those unreported areas seen presented a few numbers of volunteers' participation with a total of 1 to 3 samples collected. We then assume that these seronegative results might have been caused by a lower participation rate in those places.

LASV seropositive results map

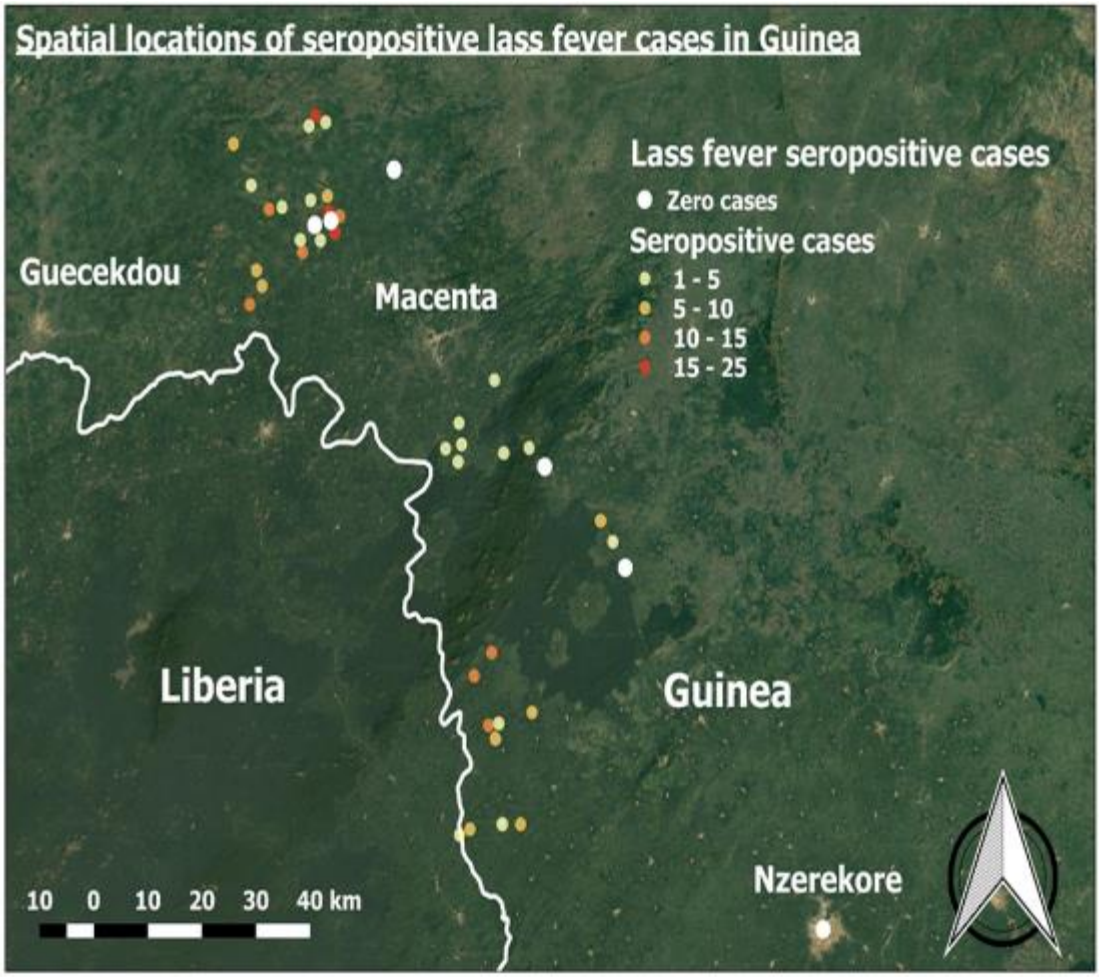


Figure 35: Localisation of LASV seropositive cases in Macenta

Spatial locations of LASV antibody seropositivity in Macenta prefecture. Positivity rate reported are simultaneously linked to the number of samples collected in each place. Places with no reported cases (zero case) may probably be related to the number of samples collected.

5.1.2 Lassa virus prevalence regarding age, sex and occupation groups

Males are likely considered to be more exposed to zoonotic pathogens due to their hunting activities. However, as LASV reservoirs are broadly spread in the region and closely share home habitats with people, human contamination often occurs when in contact with infected rodents' fluids or faeces. Consequently, there is no restriction of exposure to LASV infection in community (**Table 8**).

Table 8: Occupational data for study cohort participants.

High participation rate was respectively found in hunters group and household family members. Other activities are less practised in villages. Notably, no more than 2 healthcare workers are found in one village.

Occupation	Male	Female
Hunters	242	0
Household member	29	211
Healthcare worker	23	11
Workman	26	0
Traders	1	15

Our data findings resulted in hunters (59.91%, 145 of 242), households family members (58.29%, 116 of 199), healthcare workers (58.82%, 20 of 34), workmen (34.61%, 9 of 26) and traders (75.00%, 12 of 16). We found no major difference regarding sex group distribution and our data show 58.84% (166 of 292) in men and 60.44% (136 of 225) in female. We also did not see any significant correlation between age and LASV exposure (**Figure 36**).

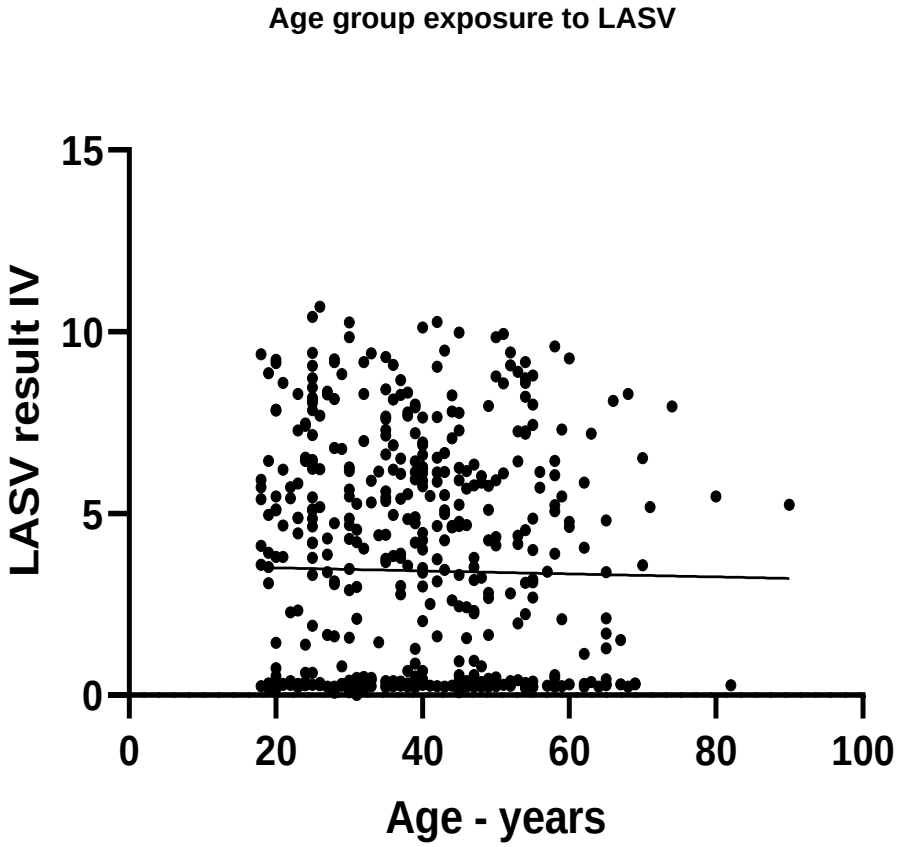


Figure 36: Age vs Anti-LASV IgG detection

Graph showing participant age vs ELISA in Index value (IV). The data show no significant correlation suggested by linear regression regarding age group.

5.2 Chapter discussion and conclusion

In this chapter, LASV-specific IgG antibodies were detected via ELISA test. The assay was performed using the BLACKBOX LASV IgG kit from BNITM as per the manufacturer's instructions.

Though bushmeat hunters and household family members represent our study's main target, people with other occupations such as healthcare workers, workmen and bushmeat traders were involved in the study. LASV is mainly spread through infected rodent fluids and materials (faeces). Despite its high prevalence in Macenta prefecture as previously reported in studies, Lassa haemorrhagic fever (LHF) infection disease has never been reported in the zone. Furthermore, regarding LASV diagnostic unavailability, hospitals in forested region of Guinea as Gueckedou, Macenta and Nzerekore might have encountered mild or asymptomatic unreported LASV cases[137]. The disease is often attributed to other tropical diseases such as malaria, yellow fever, influenza or typhoid fever. Therefore, developing LASV diagnostic capability for early detection in the zone is needed to avoid disease spreading throughout the community. Recently, in September 2018, one LASV infection case was suspected in Mamou and was laboratory confirmed in the capital city, Conakry[134]. Investigations later indicated that the subject originated from Kissidougou prefecture a town located at 180 km from Macenta.

Our study established 58.41% of LASV IgG detection in the population. It was conducted in a probable endemic area regarding the presence of LASV reservoir (*M. natalensis*). Study villages were randomly selected and the prevalence of LASV we found is likely to be representative of the local population in the zone. Koivogui *et al* reported 40% seropositivity to LASV in Faranah, the upper region of Guinea[205]. These findings were similar to those of Knobloch *et al* in Liberia (1982)[206] and McCornick *et al* in Sierra Leone (1987)[207]. The fact that LHF outbreaks do not occur in the zone may possibly be due to a milder LASV strain being in circulation in the zone or population genetics; however further works would be needed to confirm this. Alternatively, LASV pathogenicity regarding virus strain variation has been studied by Bowen *et al* in 2015[208] and M.D. Bowen, P.E. Rollin *et al* in 2000[138]. In addition, an experimental study using live LASV on animals to evaluate strain virulence was performed in Liberia by Jahrling *et al* 1985[209]. These studies have brought great insight about LASV pathogenicity in humans. Moreover, a broad range of non-pathogenic Arenavirus circulating may cross react in ELISA and it is not clearly defined why some Arenaviruses are pathogenic whereas others are not[210,211].

Our findings provide significant insight into LASV circulation in Macenta prefecture with no report of LHF infection outbreak. Further investigations on both human genetic makeup and LASV strain virulence should be conducted in forested Guinea to establish the potential reason for high LASV sero-positivity with low incidence of LHF absence in the zone.

With regards to LASV diagnostic challenges due to multiple LASV strains occurring in different regions, there has not be a validated assay to date[170]. It follows that one future strategy could be developing and validating LASV diagnostic assay performance according to geographic regions. Nevertheless, the BLACKBOX LASV IgG ELISA kit was developed using human serum and worked well in our assays. But unfortunately, the assay performance characteristics using blood, plasma, hemolyzed and icteric specimens have not been determined yet. As a result, using the BLACKBOX LASV IgG ELISA kit with icteric or hemolyzed serum in our study samples might have caused undetected errors.

Chapter 6: FINAL DISCUSSION AND CONCLUSIONS

6.1 EBOV circulation in the forested region of Macenta

From the results we presented in chapter three we would conclude that a number of EBOV infections, whether sub symptomatic, mild or unrecognized, may have occurred in remote rural villages deep in the forest and near large rivers within the forested region of Guinea. Within the forested region of Macenta there is poor healthcare and little record keeping with regards to infectious disease, therefore, we believe that this is the first such seroprevalence study on filoviruses to take place in the forested region of Guinea in Macenta prefecture.

Throughout this study, several challenges such as poor terrain and local sensitisation difficulty aroused. In addition, a lack of educated people in villages, absence of population awareness about haemorrhagic fever diseases, stigma associated to EVD disease, poor healthcare facilities in villages and difficult movement in the zone constitute major challenges to overcome.

We selected affected (previous report of EVD) and non-affected (no previous report of EVD) villages to evaluate exposure to EBOV via bushmeat hunting, manipulation and consumption. Community healthcare agent involvement and prefectoral health sanitary records contribute to identify whether villages were affected or non-affected with the past EVD outbreak of 2013-2016 in this zone. Unfortunately, these records are archived in DPSs and probably in the national health system but not published. Notably, DPSs monthly report health situation of their record to the ministry of health office in Conakry. We proposed a questionnaire to volunteers which provided important information about each participant regarding their age, locations, historical health issues, occupations and personal involvement and wildlife contact. To our knowledge, no such questionnaire had been used in previous studies. However, as this was the first seroprevalence study in the zone and with regards to the study target population exposure to EBOV infection, these questionnaires played a key role in selecting eligible volunteers. A total of 517 volunteers were questioned and blood donors would sign a consent form prior to sample collection. We performed ELISA using EBOV tetramer antigen from Oxford University. Furthermore, western blot and flow cytometric assays were performed to support our ELISA findings and to look for an antibody response to additional EBOV proteins. As part of our ELISA studies we used two blocking buffers to minimise background, ChonBlock buffer was found to give the lowest level of background noises. We used very high cut-off to define our sample positivity (5 times the standard deviation from the negative control mean). This conservative cut off gave us more confidence in our results.

Furthermore, our positive samples were performed on Western blotting and multiplex beads arrays assay. In total 67 samples were assayed on both tests. Western blot analysis additionally assayed for NP and VP40 proteins. A large number of samples showed detection to NP proteins (33 of 67 samples tested) and VP40 protein was detected in 18 samples. The abundance of NP protein detection may be related to its high level in viral particles and it has robust antigenicity[191]. However, EBOV GP is responsible for critical pathogenic differences among viral species[212]. Consequently, EBOV GP should be targeted as potential endpoint for EBOV infection establishment. In addition, we tested our sample on multiplex beads array and encountered significant IgG isotype response. The dominant IgG isotype was found to be IgG1 while IgG2-4 response were mixed.

Our findings suggest that 12.96% of participants showed a response to EBOV GP by ELISA using ChonBlock blocking buffer. These findings include both EVD affected and unaffected villages. On the other hand, we performed Western blot analysis to reinforce our positive samples from ELISA. We found only 2.12% of seropositivity with regards to anti-EBOV antibody response to EBOV GP. In both assays, we used a full length of EBOV antigen but found significant difference in antibody detection. Similar studies performed in Sierra Leone indicated significant differences in EBOV antibody detection using different methods. For example, Hearn *et al* performed IgG antibodies testing to specific viral pathogens in Kenema general hospital and found 5.2% seropositivity to EBOV and 50.2% to LASV [213]. Simultaneously, IgG antibodies were detected for other pathogens such as MARV-VP40 (10.2%), RVFV-NC (1.8%), CCHFV-N (2.0%), flaviviruses (52.9%) and alphaviruses (55.8%). Samples were collected from admitted people in Kenema government Hospital (KGH) in Sierra Leone for acute haemorrhagic fever illness and a multiplexed bead-based system (the MAGPIX platform) was performed for antibodies detection. Additionally, Halfmann *et al* detected EBOV GP antibodies in 34 volunteers over 267 giving 12.7% among EVD close contacts in Makeni, Sierra Leone[194]. These findings suggested that these people might have experienced mild or asymptomatic EVD infection. Unlike these studies performed in Sierra Leone, we collected our samples amongst highly exposed people to zoonotic pathogens, bushmeat hunters and household family members with no report of any illnesses. With regards to this significance differences of antibody detection between assays, developing a reliable and standardized accepted assay for EBOV antibody detection should be considered for future studies.

Our study was specifically tailored to occupational groups involving hunters and their household family members, workmen, bushmeat traders and healthcare. Respectively, our findings indicate bush meat hunters 2.48% (6 of 242), household family members 2.50% (5 of 199) and healthcare workers 5.88% (2 of 34) have been exposed to EBOV. In contrast, there was no antibody detection in workmen and traders' groups. However, it is important to note that these differences in antibody detection within occupational groups may be associated with the number of samples collected. It is unfortunate that we could not establish whether this prevalence is related to a specific animal hunted or associated with population occupations in the zone and this may be an avenue of future research.

Considering previous exposure to the virus in population, we found no major differences between affected and unaffected villages. Positive cases were found right across the study zone, suggesting there was no localised hot spot. These findings may be related to the ecological conditions in the zone which might result in the easy circulation of the virus reservoir species or intermediate host at low level. Although, it might be associated with movement of exposed people from affected places during the past 2013-2016 EVD outbreak in the zone. Those people might have experienced a subclinical EBOV infection. Notably, the area found in our study with the highest concentration of seropositive people may be related to the high number of samples collected compare to other locations. Especially, the chosen area is a non-affected village located deep within the forest. Also, it is the native village of the principal investigator of this study which could have played a key role in greater participation. It is important to note that volunteers were randomly selected and could include at least one couple from each family within the village. Future studies should consider including all villages in Macenta prefecture without restriction as this may result in more anti-EBOV GP detection in the zone. Nevertheless, in the study zone, the shortest distance between two villages is about 3 to 5 km. This explains the high connectivity between villages which enhances disease spread in community through human to human contact in the event of first exposure (index case).

Although, EBOV is widely known as zoonotic infection; however it remains unclear whether viral infection occurs directly from the virus reservoir or through an intermediate host which might be considered as virus amplifier[214,215]. Nonetheless, to date bats are thought to be an EBOV reservoir. During participant interview we found no particular activity related to bat manipulation in the zone. Commonly, bats have been subject of hunting and consumption in the entire study zone over past years. Our search established high antibody detection in hunters and household family members. Those people seem to be in daily contact with a variety of wildlife consequently

have higher exposure rate to potentially infected animals. Rimoin *et al* performed a statistical analysis to investigate population exposure to wildlife via daily occupations[214]. The study was done in general population (n=4574) and include 14 villages in Sankuru district in the DRC. The data reported population exposure to wild pathogens through eating, cooking, butchering and hunting. So, the association between wildlife contact and people's occupations with EBOV antibody detection via laboratory analysis should be thoroughly investigated for possible reservoir or intermediate host existence.

Our study is likely the first to demonstrate the possible circulation of EBOV prior to the 2013-2016 EVD outbreak in Macenta zone of Guinea. Similar serologic survey of rural population was performed in Gabon and involved 4,349 volunteers from 220 villages[191]. The study was conducted to understand EBOV circulation and transmission to human and includes both epidemic and non-epidemic regions. Collected samples were assayed using an ELISA method with *Zaire ebolavirus* antigen and established 15.3% seropositivity to EBOV IgG antibody. In addition, to better understand EBOV IgG reactivity, the study applied Western blot techniques with 138 samples that previously show antibody response on ELISA. The assay revealed 21% of antibody response to at least one EBOV proteins including GP, NP, VP40, VP30 and VP24. In contrast to the Gabon study, which was performed with regards to ecological factors, we conducted our study regarding previous exposure to EVD and population risk factors with regards to habitat and occupation. Unfortunately, our selected villages share similar ecological environment; therefore, we could not differentiate the variation of EBOV prevalence in the zone.

Our seroprevalence study conducted in Macenta prefecture, in forested Guinea to determine the possible circulation of EBOV prior to the 2013-2016 West African EVD outbreak provided novel data. Our data likely represent the level of prevalence of EBOV in both affected and unaffected areas. Regarding the high case fatality proportion associated to EVD, our seroprevalence results might be associated either to the past EBOV infection or a pre-existing unrecognized infection in the zone. A potential limitation to the study is that we failed to get information on previously unknown haemorrhagic deaths in those villages and this kind of information could have helped support the view that EBOV is in circulation at low levels. Considering the possible pre-exposure to EBOV, these unreported cases might have been linked to (i) a minor exposure to the virus which might have been cleared-up by host immune-response (ii) an exposure to known or unknown species or related filovirus of less pathogenicity circulating in the zone, such as RESTV which is known to be asymptomatic in humans and NHPs (iii) finally, it might be associated to a cross-reactivity to virus-like particles

which generally produce several nonspecific bindings of the antibody and consequently leading to false positive reaction[216].

To conclude, since early EVD symptoms are non-specific and are similar to that of many tropical illnesses such as malaria, influenza or typhoid fever, EVD may often go undiagnosed and consequently not reported. Providing available diagnostic facilities such as molecular diagnostics in communities of the forested region of Guinea could greatly help to prevent EVD outbreak spill over event by early detection of occurring infection. Although, regarding the persistence of stigma in the population associated with EVD, adequate outreach is crucial in preventing population mistrust for future epidemiological studies in outbreak zones.

6.2 Lassa virus IgG antibody detection

West Africa is a home-based to numerous pathogens but well-trained staff and proper infrastructures for disease surveillance are severely limited. Thus, understanding disease prevalence in a region is necessary in order to make appropriate preparations. Such preparedness could include an effective diagnostic system, proper care and treatment of infected people. These would help to reduce disease transmission and spill over in communities.

In the forested region of Guinea, the population often suffer and die from several viral diseases whether known or unknown. Therefore, knowing about febrile illnesses and diagnosing the causative agent early will contribute to controlling diseases and prevent an outbreak occurrence in communities. In order to determine the prevalence of LASV circulation in Macenta prefecture, we applied a serological assay (ELISA) on a panel of 517 serum samples collected mainly among hunters and household family members in remotes rural areas. Little is known with regards to LASV prevalence in this area and we hoped to add to this limited body of information. Selected study areas were composed of villages with previous report of EVD infection and villages with no previous report of EVD infection. However, to date there have been no previous report of LASV infection in the entire study zone and surrounding environment.

We analysed our samples using recombinant Lassa virus nucleoprotein (r-NP), which is an antigen from the BLACKBOX kit, to detect specific anti-LASV IgG antibody. This kit is known to be efficient and valuable for qualitative detection of LASV IgG antibody in human and non-human primate. It is being manufactured by BNITM and provides serological evidence of an acute or past LASV infection.

Using this kit our data indicates 58.41% seropositivity to LASV which gives strong evidence of population exposure to the virus in Macenta prefecture. Regarding the literature, we found that LASV antibody prevalence varies significantly between studies and assays performed. In July 1993, a broad search on LASV prevalence in Guinea which included the main geographic regions of Guinea established high prevalence rate of 25-55% in the forested and savannah regions of Guinea[217]. The study performed ELISAs using LASV nucleocapsid protein as the antigen of choice. It was found that there was a high prevalence of anti LASV -NP antibodies amongst samples from the Forested and Savannah regions and this might be associated with the presence and consumption of rodents in those communities. These rodents are largely distributed in the zone and include the *M. natalensis* known as a potential reservoir of LASV. In contrast, the lower prevalence 7%, was found in the middle and lower regions of Guinea[217]. These regions encounter less contact with rodents, perhaps, because they are forbidden for consumption amongst certain ethnic groups within the region. In Muslim religions, some animals including pork, frog and rodents are regarded as unclean, as a result, these animals are excluded from consumption and manipulation. On the other hand, LASV contamination is also known to occur through contact with contaminated rodents fluids and feces. Therefore, people from other regions might be exposed to LASV infection from rodents that live nearby. However, migrated people from the Forested region of Guinea remain the main consumers of rodents in other parts of Guinea. Further investigations on LASV seroprevalence in other parts of Guinea should consider the origin of people detected with LASV antibody. This will bring more understanding about LASV infection occurrence in rodents' consumers, manipulators and close contact.

Another study carried out in the forested region of Guinea including Gueckedou, Lola and Yomou prefectures (n=977) indicated 12.9% and 10% of seropositivity to anti-LASV IgG respectively in rural and urban areas[136]. The study used indirect immunofluorescence (IFA) slides from Bernhard Nocht Institute of Hamburg and acetone-fixed Vero cells infected with LASV antigen of Josiah strain. The main purpose of the study was to estimate the prevalence of LASV antibody in human population and establish risk factors for LASV infection. In contrast to our study, samples were collected from general population within selected villages regarding people exposure to wildlife (e.g. meat consumption) and contact with someone with haemorrhagic symptoms. Despite these findings and that of ours, to date Guinea has never officially recorded a LASV outbreak. These serological detected cases might have been mild or unrecognized. Alternatively, they might have been attributed to common tropical febrile diseases such as malaria or typhoid fever because of lack of proper diagnostic. In the records, one sporadic LASV infection disease case was notified in Mamou in

January 29, 2019[134]. Active contacts tracing and follow up likely indicate no further transmission to close relatives and healthcare workers.

As we only detected IgG antibodies, we suggest that future work should look at a number of different targets to confirm prior infections as these results may have occurred from cross reaction to other viruses specifically non-pathogenic arenaviruses. It is unfortunate that we could not define the period of exposure as samples were collected from healthy individuals with no report of fever symptoms. Nevertheless, this high prevalence rate highlights the severe extent of risk to public health within the Macenta zone. It is important to note that our study volunteers entirely claimed having reported febrile symptoms several times which might be related either to LASV infection or other illnesses. It follows that educating illiterate population about reporting febrile illnesses could play a key role in early detection of haemorrhagic infection diseases in communities. Although there have been no reported LASV disease outbreaks at present, measures should be taken to prevent an unpredicted LASV outbreak in the forested region of Guinea. This would include the availability of proper haemorrhagic fever diagnostic capabilities in different prefectures of the Forested Guinea as that of the European mobile laboratories newly established in Gueckedou prefecture (Forested Guinea). Consequently, further studies should strictly investigate LASV strain pathogenicity, population behaviour and immune response to the virus in Guinea. This will provide more understanding on the absence of LASV disease outbreaks in the country regardless its high seroprevalence reported in literature. In addition, investigating on the virus reservoir, a multimammate mouse, which is broadly spread throughout the country will provide clear insight into potential Guinea-LASV strain mutations. Finally, based on our findings and that of previous studies, we suggest that possibly there is an unrecognized or mild LASV infection circulating in the zone. This gives evidence of LASV strain circulation probably with less pathogenicity or virulence in Guinea.

In general, cross-sectional studies encounter various limitations due to their nature. As communities are constantly exposed to infections during their lifetime, our study cohort involved general population of rural areas including exclusively bushmeat hunters and their household family members. However, with regards to the study selection criteria and for ethics reasons; chronic diseases, pregnant women and infants under 16 of age would not take part of the survey. Blood samples were then collected from 517 volunteers from 44 villages around Macenta prefecture.

To conclude, our data provides a strong evidence of LASV circulation or related arenavirus that induces cross reactive NP antibody response in forested region of Guinea. The high prevalence rate of 58.41% indicates population exposure to the

virus. This could then alert national health authorities (e.g. The Ministry of health) and international partners on the necessity of taking further measures to prevent LASV infection disease outbreak occurrence in Guinea. Regarding its high prevalence which makes it a major concern, more studies to understand LASV and its silent circulation in Guinea are needed. Investigations should include LASV-Guinea strain pathogenicity, Guinea population immune response nature to the virus and the virus strain mutation in intermediate host.

6.3 Final conclusions and future work.

Macenta prefecture, the site of our cross-sectional seroprevalence study is in the Forested region of the Republic of Guinea (Surface area: 7.046 km^2. Population: 278,456 people. Latitude: 8°32'59.99''. Longitude: -9°27'59.99''W). It encounters high biologic diversity of fauna and flora which are almost identical through the zone.

There are different factors that can promote the transmission of zoonotic viruses to humans, ranging from geographic distribution of the animal reservoirs or intermediate hosts to the mode of exposure and the susceptibility of hosts. In this study, we tried to determine the seroprevalence of highly exposed people (e.g. bushmeat hunters and their household family members) to EBOV (filovirus) and LASV (arenavirus). Though the LASV reservoir is known to be the *M. natalensis*, the dynamic mode of transmission to humans and the pathogenicity of different viral strain is poorly understood in most affected West African countries including Guinea, with no accurate record of Lassa fever outbreaks. In contrast, the EBOV reservoir remains unknown despite few discoveries of the viral RNA in some fruit bats[12,55,218,62]. Therefore, the zoonotic transmission of EBOV continues to be a great challenge for scientific world. Thus, the zoonotic transmission is only effective via contact (direct or indirect) between human and wildlife which are actually frequent in communities. However, the increase in human activities (e.g. hunting, butchering, skinning), the demographic increase in population and serious climate change (e.g. deforestation) constitute factors that impact zoonoses as they promote direct contact between human and animals.

Our data is the first to investigate the possibility of EBOV circulation in the Macenta prefecture prior to the past 2013-2016 EVD outbreak in the country. It also brings more evidence about LASV circulation in the Forested region of Guinea as reported in previous studies[132,136,217,137]. The study population comprises the rural and urban areas of Macenta prefecture. It is mainly composed of farmers which are involved in wildlife animals manipulation through hunting, butchering and consumption. It follows

that our findings contribute to understand human behaviour in wild pathogens transmission and disease spill over in communities. It is easily perceived that some of the non-significant sociodemographic prevalence differences observed in our study participants groups might be real, but the potential limit of sample size collected per group might had dramatically reduced the power needed for detection. Therefore, oral swab sampling for antibody detection could be a first line of sero-prevalence studies. This is due to the fact that more people can be sampled giving enough possibility to investigate a large number of samples. Joseph WS Timothy et al. collected oral swab from 27 households out of 30 in Meliandou village the index site of the 2013-2016 outbreak[219].

Our data shows evidence of EBOV antibody detection; however, only a small proportion of EBOV antibody prevalence was found in the zone. Moreover, as the study was performed during a non-active EVD outbreak period, we established the prevalence of EBOV IgG antibody of past human exposure to EBOV whether from the 2013-2016 EVD outbreak in West Africa or related to a pre-existing unreported exposure. Further investigations to understand EBOV dynamic transmission to human and virus reservoir discovery should be conducted in the forested region of Guinea.

Despite the absence of reported LASV infections outbreak in forested Guinea, our data and that of others previously published[132,205] indicate the possible circulation of LASV in Macenta zone over a long period of time. Comparing our findings to that of Lamine et al in 2008; it is consistent that LASV have been actively circulating over the entire 9-year time frame (2008 to 2017). This suggests that many LASV infections are mild or unreported because of the lack of diagnostic availability in the zone. In addition, it indicates that the virus is endemic and has been maintained in the zone over years. The presence of LASV IgG antibody indicate the possible existence of unrecognised LASV infection or subclinical exposure in the population. Similar approach to detect haemorrhagic fever antibodies was performed by Jean-Paul Gonzalez in 6 central African countries[220]. In this study, samples were collected from both human and animals (wild and domestic) and tested for six (6) antigens amongst which LASV and EBOV were included. The study conducted antibodies detection via indirect immunofluorescence assay using Vero E6 cells. Report indicated 0.2% and 4.5% antibody detection respectively for LASV and EBOV. In contrast, we used EBOV antigen and recombinant LASV nucleoprotein to detect respectively anti-EBOV GP (12.96%) and anti-LASV IgG (58.41%) antibodies via ELISA test. Further, we found 2.51% of seropositivity to EBOV via Western blot analysis.

With regards to public health concerns, Macenta prefecture as well as other prefectures of the Forested region of Guinea need proper investment to improve haemorrhagic fever infection disease surveillance. So, developing EBOV and LASV diagnostic facilities will provide efficient methods of surveillance to evaluate pathogen presence, spread and risk of contamination in communities. Although, limiting people's contact with virus reservoirs (wildlife) will contribute to the eradication or reduction of zoonotic infections. In remote places or rural areas where population rely on bushmeat for food and trade business, there is high frequency of exposure to multiple viral species. To prevent future zoonotic disease outbreaks such as EBOV and LASV, effective public health interventions and campaigns that minimizes the risk of infected animal contact need to be diffused and directed to both age and sex groups. Although, further investigations are needed to better understand human complex relationships and exposure to wildlife animals.

This study, supported by the United State Food and Drug Administration program and the University of Kent, uses sero-prevalence to look at the footprint of human infection by EBOV and LASV viruses in the Forested region of Guinea in Macenta prefecture. The study looked upon the population of rural areas which appears to be highly exposed to multiple viral infections through constant contact with wildlife either by hunting, trapping or butchering. We performed antibody detection via ELISA, Western blot test and Multiplex beads array assay (Flow cytometry). A number of seropositive individuals were detected for LASV whereas only few EBOV antibody positive persons were observed. In accordance with these findings, (i) we suggest that EBOV has been circulating in the forested region of Guinea prior to 2013-16 outbreak; (ii) that LASV is being circulating in Macenta prefecture as previously described in studies with no record of outbreak.

Finally, this study has provided further insight regarding mild or unrecognized viral infections in communities. It has helped to identify practises that promote the transmission of zoonotic pathogens such as hunting activities.

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Appendix 1: Consent form
(European Mobile Laboratory consortium, EMLab)

Seroprevalence of emerging disease in Forested Guinea, Macenta zone

INVESTIGATORS AND INSTITUTIONS

Professor Miles W. CARROLL, Public Health England, Porton Down-UK.
Professor Mandy Kader Konde, FOSAD – CEFORPAG, Conakry, Guinea.
Professor Stephan Gunther, Bernhard Notch Institute of tropical medicine, Hamburg- Germany.
Mr Joseph Akoi BORE, Ministry of health Guinea.

What is the purpose of this sero-prevalence research in Guinea?
Emerging diseases are often viral hemorrhagic fevers infections that usually occur as epidemics. The past epidemic (2014-2016) of Ebola virus disease mainly affected three countries, Guinea, Liberia and Sierra Leone, with more than 28,600 reported cases and 11,300 deaths with social and economic impacts. The disease spread beyond these areas with few cases reported in other countries of West Africa, Europe and America.
The aim of this research is to identify, understand and prevent the emergence or re-emergence of these hemorrhagic fever diseases as Ebola and Lassa virus diseases. The results of this research may be applicable in the future, in case of re-emergency of the virus and will be extremely beneficial.
The only way to document this study is to analyze the blood to see if you have had any contact with viruses. We will thus take (10 to 12 ml) on people living in areas (villages) where the Ebola disease has had effects and also in the zones (villages) where no case of Ebola was declared. These unaffected areas will be considered as a control. By doing this, we will have a better understanding of the sero prevalence induced by Ebola and Lassa infection. The samples will be processed at the EMLab Laboratory in Gueckedou and sent to research institutions such as Public Health England (PHE-UK) and Bernhard Notch Institute (BNI-Hamburg) for deep analysis.

What is the European Mobile Laboratory (EMLab) mission?

EMLab is a member of the consortium "H2020 Evident" which also includes Doctor without border (MSF). EMLab in partnership with the World Health Organization (WHO) actively participated in the GOARN mission of the WHO in Guinea in order to control the Ebola virus epidemic. Since March 2014, EMLab was among the first field workers deployed in Guinea as requested by Guinea Government. At the end of the epidemic, EMLab performed more than half of all Ebola virus disease diagnosis in Guinea. The implementation of this virologic platform diagnosis has greatly helped to eradicate the impact of disease on local populations.

What is the aim of these researches?

Unlike a usual therapeutic approach, the goal of this research is to identify the best ways to prevent an emergence or reemergence of hemorrhagic fever diseases like Ebola, and then determine the immune response duration in peoples cured of Ebola. The results of this research will be applied in the future in case of re-emergence of the virus and will be beneficial for everyone. Ebola and Lassa diseases are viral hemorrhagic fevers that occur in the form of epidemics. The current outbreak (2014) Ebola mainly affected three countries, Guinea, Liberia and Sierra Leone, with more than 28,600 reported deaths. The disease has spread beyond these areas with reported cases in other west African countries, Europe and America.

We want to have access to low amount of blood samples (12ml) of people living in areas (villages) where the Ebola disease has had effects and also in areas (villages) where no Ebola case was declared. These non-affected areas will be considered as negative. By doing this, we will have a better understanding of the sero prevalence induced by infection with Ebola and Lassa eventually. The samples will be processed first at the Laboratory in Gueckedou and sent to research institutions such as Public Health England (PHE-UK) and Bernhard Notch Institute (BNI-Hamburg) approved by the European Union consortium for further analysis.

What did that mean to me?

If you accept to participate in this study:

Health monitoring: a doctor will regularly check your general health during study time and offer medical examinations for you if necessary. Each person will be individually and secretly informed about his results after analyzes.

What are the risks for me and my health?

Our priority is your wellbeing. Sampling shall be conducted by an experienced biologist with sterile equipment while following pre-established safety procedures by the care center.

What are my benefits of participating in this study?

Your participation will help to develop effective control measures against Filoviruses such as Ebola and Lassa virus diseases that will benefit your family, friends and future generations. If you are diagnosed with any disease, we will direct you to the appropriate treatment center recommended by your government.

You will receive support as a sign of our gratitude for your time and all expenses related to your participation in this study (travel and food).

If you refuse to participate to this study?

Your participation is on a voluntary basis and contributes to your well-being. You are free to decide whether to take part or not. That will not affect your health or personal or professional situation in the future.

What will happen with your blood samples?

Blood samples will carry a code (identification number of the study) and will therefore remain anonymous (no names or other personal information). The sample-patient relationship will only be done by a few people directly involved in the study which will respect your anonymity and your privacy. Samples will first be analyzed by the laboratory in Gueckedou, Guinea, and then they will be transported to Europe for further analysis.

Who will have access to the information related to this research?

All research data is stored on secure computers in rooms dedicated for this purpose and protected with passwords. Only a few people directly involved in

this research will be allowed to have access to information of the participants and in order to verify and ensure that the study is proceeding according to defined international standards and that the confidentiality and anonymity of participants is preserved. We will only share information and data summarized in code, and in constant collaboration with WHO. The identity of the participants will never be revealed.

Who allowed this research to take place?

These studies were approved by a committee of local experts in Guinea. This committee has ensured that these studies will be conducted according to very well-defined standards and safety, welfare and rights of participants are always respected. The committee reviewed the research carefully, confirmed that they were important for the Guinean population and they followed national and international guidelines.

Other questions?

You can ask any other question to our team involved in this study, and at any time. You can also contact the people responsible for the implementation of these studies.

I, ____Volunteers___, certify having a detailed explanation of the subject of this study. I understood perfectly what I've read, everything was explained to me and I had a complete response and understood all my questions answers. I understand that I may change my mind at any time of the study.

- ☐ Thanks to tick. **I agree to participate in this study.**
- ☐ Thanks to tick. **I agree to give 10 to 20 ml of my blood.**
- ☐ Thanks to tick. **I agree that my samples be stored.**
- ☐ Thanks to tick. **I agree that my samples be exported to Europe.**

Participant signature:

Date:

Time:

Appendix 2: Sample collection questioning

Questionnaire

First and last name:	
Gendre / Sexe 1- Male : …… 2- Female: …… 3- Poids : …… 4- Age : …….	
Marital statues 1- Are you married? (if yes) 2- how many children: ……	
Volunteers localisation 1- Préfecture : …… 2- Sous-Préfecture : …… 3- Village : ……..	
Affected village….. Unaffected village…… (if yes) 1- How many people survived: …… 2- How many people died: ……	
Main Activities 1- …… 2- ……	
Hunter (if yes, how do you hunt them?) 1- I use gun: …… 2- I use trap: …… 3- Other: ……	
Type of animal hunted	

Did anyone in your family practice any kind of hunting activity? (if yes) 1- What is your relationship to that person? ……	
Brush meat consumption (if yes, how did you get them?) 1- From my relative: ……. Specify: …… 2- I buy it: …… Specify: ……	
Clinical history - Have you ever been diagnosed with any chronicle disease or sickness? If yes, what was it about? …… - What is your actual health status?	
Last questions: 1- As you were told, did you agree to be part of the research by giving your blood? 2- Did you willingly sign the consent form? 3- Do you have any question or concern? (if yes, see questions/concern below)	
Blood quantity obtained	
Investigator:	

Appendix 3: Ethics approval

REPUBLIQUE DE GUINEE

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TRAVAIL – JUSTICE – SOLIDARITE

COMITE NATIONAL D'ETHIQUE POUR LA RECHERCHE EN SANTE

(CNERS)

Conakry, le 16 Mars 2017

N° : 012/CNERS/17
Objet : Examen Protocole

LA PRESIDENTE

A Dr Joseph Akoi BORE
Responsable au Laboratoire Mobile
Guéckédou, Guinée.

Monsieur,

Le Comité National d'Ethique pour la Recherche en Santé (CNERS), en sa session du 16 Mars 2017, a procédé à l'examen de la version corrigée de votre protocole de recherche intitulé: **«Incidence et prévalence des filovirus en Guinée Forestière, Macenta et Guéckédou».**

Le Comité note avec satisfaction la prise en compte de ses observations.

Il autorise la mise en œuvre de votre étude dans le respect des principes éthiques y énoncés et vous invite à lui transmettre un rapport annuel de l'étude. Cette approbation est valable pour une période d'un (1) an à compter de sa date de signature.

Le CNERS tient à être informé de toute modification du présent protocole au cours de sa mise en œuvre.

Veuillez agréer l'expression de ma considération distinguée.

La Présidente

COMITE NATIONAL D'ETHIQUE POUR LA RECHERCHE EN SANTE
La PRESIDENTE
CNERS - GUINEE

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