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Impact of KIR and HLA genotypes on the outcome and susceptibility to Ebola virus disease

Auteur : Lhermitte, Valentine
Promoteur(s) : Willems, Luc; 5481
Faculté : Gembloux Agro-Bio Tech (GxABT)
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VALENTINE LHERMITTE

TRAVAIL DE FIN D'ÉTUDES PRÉSENTÉ EN VUE DE L'OBTENTION DU DIPLÔME DE

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Abstract

Ebola virus (EBOV) induces a zoonotic disease with a high fatality rate, which affects human and non-human primates. First appeared in 1976, the Ebola Virus Disease (EVD) continues to take a lot of people life, mainly in Africa where the health infrastructures are poor, weak and not adapted. The 2014-2016 EBOV outbreak in West Africa was the largest and the most complex outbreak since its apparition. A better understanding of ebolavirus, the development of innovative treatments and the discovery of an efficient EBOV vaccine could help to prevent future EBOV outbreaks and the spread of this life-threatening infection.

EBOV carries a negative-sense RNA genome and has glycoprotein spikes, which are very important for the virus entry into host cells. The envelope, the matrix and the nucleocapsid are also components of this virion, and can be recognized by the innate and adaptive immune cells.

Natural Killer (NK) cells are among the important components of the innate immune system and provide the first line of defense against infected and transformed cells. They own activating and inhibitory receptors on their membrane in order to induce an appropriate innate immune response. Killer Immunoglobulin-like Receptors (KIRs) are one of the main receptors of NK cells. Human Leucocyte Antigen (HLA) class I molecules are located on nucleic cell membrane and are the KIR proteins ligands. Some combinations of KIR and HLA molecules could influence the susceptibility and outcome of several viral infections.

The objective of this master's thesis is to assess the association of KIR and HLA genotypes with EVD outcome by comparing the genotypes of different groups of patients: contacts, survivors and fatalities of EVD.

Results show that some KIR and HLA combinations correlate with the outcome of EVD. First, these KIR combinations "2DL1-4, 2DS4-001/3, 3DL1-3, 2DP1 and 3DP1-004", "2DL1-4, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004" and the last one "2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004" and these HLA combinations "HLA-C1" and "HLA-C1/2 and HLA-B-Bw4Ile" might provide a permissive immune microenvironment promoting a positive outcome of EVD. Secondly, survivors tend to own less activating KIR, which can confer a protection against EVD. Finally, KIR2DS4-003 and KIR2DS2 might increase the susceptibility to develop a fatal EVD.

These results highlight the fact that KIR and HLA genes may play a key role in the susceptibility and outcome of some viral infections and especially EVD.

Résumé

Le virus Ebola provoque une maladie zoonotique, avec un très haut taux de mortalité qui affecte les primates humains et non humains. Apparue pour la première fois en 1976, la maladie à virus Ebola continue à faire de nombreuses victimes, principalement en Afrique où les infrastructures sanitaires sont mauvaises, faibles et inadaptées. L'épidémie du virus Ebola en Afrique de l'ouest de 2014-2016 a été la plus grande et la plus complexe depuis l'apparition de la maladie. Une meilleure compréhension du virus, le développement de traitements innovants et la découverte d'un vaccin efficace pourraient aider à prévenir une nouvelle épidémie du virus Ebola et la propagation de cette infection potentiellement mortelle.

Composé d'un génome ARN antisens, le virus Ebola exprime des glycoprotéines qui s'avèrent être très importantes lors de l'infection. L'enveloppe, la matrice et la nucléocapside sont également des composants de ce virion, et peuvent être reconnus par les cellules des systèmes immunitaires inné et adaptatif.

Les cellules tueuses naturelles, appelées également cellules NK (*Natural Killer*), sont des cellules du système immunitaire inné. Elles sont les premières cellules impliquées dans la défense contre les cellules infectées et transformées. Elles possèdent des récepteurs activateurs et inhibiteurs sur leur membrane afin de pouvoir induire une réponse immunitaire innée appropriée. Les *Killer Immunoglobulin-like Receptor* (KIRs) font parties de ces récepteurs. Les antigènes de leucocyte humain (*Human Leucocyte Antigen*, HLA) appartenant au complexe d'histocomptabilité de classe I sont situés sur la membrane des cellules nucléées et sont les ligands des récepteurs KIR. Certaines combinaisons de molécules KIR et HLA pourraient influencer la susceptibilité et l'issue de certaines infections virales.

L'objectif de ce travail de fin d'études est d'évaluer l'association des génotypes KIR et HLA avec le développement de la maladie à virus Ebola en comparant les génotypes de différents groupes de patients : contacts, survivants et décès de la maladie du virus Ebola.

Les résultats montrent que certaines combinaisons de KIR et HLA corrèlent avec l'issue de la maladie à virus Ebola. Premièrement, les combinaisons KIR "2DL1-4, 2DS4-001/3, 3DL1-3, 2DP1 et 3DP1-004", "2DL1-4, 2DS4-001, 3DL1-3, 2DP1 et 3DP1-004" et finalement "2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 et 3DP1-004" ainsi que les combinaisons HLA "HLA-C1" et "HLA-C1/2 et HLA-B-Bw4Ile" pourraient fournir un microenvironnement immunitaire permissif favorisant une issue positive face à la maladie à virus Ebola. Deuxièmement, les survivants ont tendance à posséder moins de KIR

activateurs, ce qui peut conférer une protection face à la maladie à virus Ebola. Finalement, KIR2DS4-003 et KIR2DS2 pourraient augmenter les risques de mortalité due au virus.

Les résultats soulignent le rôle clé joué par les gènes KIR et HLA dans la susceptibilité et l'issue de certaines infections virales, et en particulier la maladie à virus Ebola.

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List of abbreviations

Abbreviation	Signification
%	Percentage
°C	Celsius degree
µl	Microliter
3D	Tridimensional
∞	Infinity
AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen-presenting cells
APS	Ammonium persulfate
BEBOV	Bundibugyo ebolavirus
bp	base pairs
C_3H_3N	Acrylonitrile
C_3H_5NO	Acrylamide
CatB	Cathepsin B
CatL	Cathepsin L
CCL	Chemokine Ligand
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 4
CD94	Cluster of differentiation 94
CH_3COO^-	Acetate
CS	Contact sample
CTL	Cytotoxic T cells
DAMPs	Damage-associated molecular pattern
DAP12	DNAX activation protein of 12kDa
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DRC	Democratic Republic of the Congo
EBOV	Ebola Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ELISPOT	Enzyme-linked Immunospot
EtBr	Ethidium bromide
EVD	Ebola Virus Disease
g	Gramm
GP	Glycoprotein
h	Hour
H_2O	Water
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HF	Fatal sample
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
Ig	Immunoglobulin
IL	Interleukine
Ile	Isoleucine
INF	Interferon
ITAM	Immunoreceptor tyrosine-based activating motif
ITGB1	Integrin beta-1
ITIMs	Immunoreceptor tyrosine-based inhibitory motifs
kb	Kilobase
kbp	Kilobase pair

kDA	Kilodalton
KIR	Killer Immunoglobulin-like Receptor
L	Viral polymerase
LB	Lymphocyte B
LRC	Leukocyte Receptor Complex
LT	Lymphocyte T
M	Molar
mA	Milliampere
MDA	Multiple Displacement Amplification
MHC	Major Histocompatibility Complex
ml	Milliliter
mRNA	Messenger Ribonucleic Acid
mV	Millivolt
m/V	Mass/Volume
Na	Sodium
NaAc	Sodium acetate
NC	Negative control
NK	Natural Killer
NKG2	Natural killer Group Protein 2
nm	Nanometer
NP	Nucleoprotein
PAGE	Polyacrylamide Gel Electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMOs	Phosphorodiamidate morpholino oligomers
PO_3	Phosphate
PPRs	Pattern recognition receptors
p-value	Probability value
REBOV	Reston ebolavirus
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rVSV-ZEBOV	Recombinant Vesicular Stomatitis Virus Zaire Ebolavirus
s	Second
SEBOV	Sudan ebolavirus
sGP	Small Glycoprotein
siRNAs	Small interfering RNAs
SS	Survivor sample
TAE	Tris-Acetate-EDTA
Taq	Thermus aquaticus
TBE	Tris-Borate-EDTA
TEBOV	Taï Forest ebolavirus
TEMED	Tetramethylethylenediamine
Thr	Threonine
Tm	Melting temperature
TNF	Tumor necrosis factor
Tris	Trishydroxymethylaminomethan
UV	Ultraviolet
VP	Viral Protein
WHO	World Health Organization
x g	x centrifuge force
ZEBOV	Zaire ebolavirus

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Introduction

1. Ebola Virus Disease

Ebola virus disease (EVD) is a fatal zoonotic disease that affects human and non-human primates. First appeared in 1976, several research topics have been addressed but many aspects of its history remain in obscurity (Weyer et al., 2015). The first outbreaks of EVD were recorded simultaneously in Southern Sudan (Nzara) and Northern region of Democratic Republic of the Congo (DRC) (Yambuku). From 1976 to 2018, 37 outbreaks of EVD occurred in Africa with a mortality rate ranged between 25 to 90% (*Table 1*). These periodic outbreaks affected different countries including Sudan, DRC, Ivory Coast, Gabon and Uganda (Weyer et al., 2015; Brettin et al., 2017).

Despite its high mortality rate, the disease was still considered as a low public health problem compared to malaria, tuberculosis or human immunodeficiency virus (Kaner et al., 2016). Indeed, the West African outbreak started in March 2014 caused panic and fear that the virus could spread to other continents including Europe and America. This outbreak was the largest and fatal outbreak recorded to date since the discovery of Ebola virus (EBOV) in Yambuku (DRC) in 1976. The West African EBOV outbreak emerged in Guinea and spread in Liberia and Sierra Leone (Weyer et al., 2015). On 8th August 2014, the World Health Organization (WHO) declared that this outbreak is an international public health emergency of international concern because more than 900 people died. According to the WHO report of March 30, 2016, the total number of confirmed, probable and suspected EVD cases during this outbreak is 28, 646 with 11, 323 reported deaths. Different factors such as poor health infrastructures, limited number of health care workers, high level of population movement across porous borders, cultural beliefs, and behavioral practices contributed to the spread of EBOV in West Africa. Usually, health care workers are most often the first victims at the beginning of epidemic. Beyond its direct impact on individuals, families and communities, these outbreaks highly affected the health and economic systems in these countries (Brettin et al., 2017).

Table 1: Chronology of EBOV outbreaks from 1976 to June 2018 (World Health Organization, 2018).

Year	Country	Ebolavirus species	Cases	Deaths	Case fatality
2018 (May-June)	Democratic Republic of Congo	Zaire	55*	28	51%
2017	Democratic Republic of Congo	Zaire	9	3	33%
2015	Italy	Zaire	1	0	
2014	DRC	Zaire	66	49	74%
2014	Spain	Zaire	1	0	
2014	UK	Zaire	1	0	
2014	USA	Zaire	4	1	25%

2014	Senegal	Zaire	1	0	
2014	Mali	Zaire	8	6	75%
2014	Nigeria	Zaire	20	8	40%
2014-2016	Sierra Leone	Zaire	14124*	3956*	28%
2014-2016	Liberia	Zaire	10675*	4809*	45%
2014-2016	Guinea	Zaire	3811*	2543*	67%
2012	Democratic Republic of Congo	Bundibugyo	57	29	51%
2012	Uganda	Sudan	7	4	57%
2012	Uganda	Sudan	24	17	71%
2011	Uganda	Sudan	1	1	
2008	Democratic Republic of Congo	Zaire	32	14	44%
2007	Uganda	Bundibugyo	149	37	25%
2007	Democratic Republic of Congo	Zaire	264	187	71%
2005	Congo	Zaire	12	10	83%
2004	Sudan	Sudan	17	7	41%
2003 (Nov-Dec)	Congo	Zaire	35	29	83%
2003 (Jan-Apr)	Congo	Zaire	143	128	90%
2001-2002	Congo	Zaire	59	44	75%
2001-2002	Gabon	Zaire	65	53	82%
2000	Uganda	Sudan	425	224	53%
1996	South Africa (ex-Gabon)	Zaire	1	1	
1996 (Jul-Dec)	Gabon	Zaire	60	45	75%
1996 (Jan-Apr)	Gabon	Zaire	31	21	68%
1994/1995	Democratic Republic of Congo	Zaire	315	254	81%
1994	Côte d'Ivoire	Taï Forest	1	0	
1994	Gabon	Zaire	52	31	60%
1979	Sudan	Sudan	34	22	65%
1977	Democratic Republic of Congo	Zaire	1	1	
1976	Sudan	Sudan	284	151	53%
1976	Democratic Republic of Congo	Zaire	318	280	88%

*Include suspect, probable and confirmed EVD cases

There were also some exported cases of EVD in different countries such as United Kingdom, Nigeria, Russia, Senegal, Spain, United States of America, Germany and Mali due to health care workers or laboratory experiments (Weyer et al., 2015).

On May 8, 2018, the Ministry of Public health of DRC officially declared a new outbreak of EVD in Bikoro health zone, located in Equateur province in DRC. This outbreak is the ninth EBOV outbreak in DRC since the discovery of EBOV. A total of 54 cases including 38 confirmed, 16 probable cases, and 33 deaths have been reported on June 26, 2018 (World Health Organization, 2018) and the DRCs' Ministry of Public health declared the end of this EBOV outbreak on July 24, 2018. The announcement has been done 42 days (two 21-day incubation cycles of the virus) after the last confirmed case of EVD.

2. Ebolavirus

2.1 Generalities

Ebolavirus belongs to the genus Ebolavirus in family *Filoviridae*. Five distinct species have been ascribed to the Ebolavirus genus: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Taï Forest ebolavirus* (TEBOV), *Reston ebolavirus* (REBOV) and *Bundibugyo ebolavirus* (BEBOV) (Weyer et al., 2015). Differences between these species are geographical expanse, virulence and pathogenicity in human and non-human primates. Ebola virus, which belongs to the *Zaire ebolavirus* species (ZEBOV) has the highest fatality rates (Weyer et al., 2015).

EBOV attacks monocytes/macrophages and dendritic immune cells, which are known as the early replication sites during EBOV infection. The virus can also infect other cells such as fibroblasts, hepatocytes, adrenal and epithelial cells that play a key role by disseminating the virus out of the spleen and lymph nodes to other tissues (Falasca et al., 2015).

2.2 Structure and viral proteins of EBOV

Filoviruses are enveloped, filamentous and non-segmented viruses that contain a negative sense single-stranded RNA. The virus shape is variable, with long tubes, turns and branches. Its diameter is 80 nm, the length ranges from 600 to 1400 nm, and the peak infectivity is associated with 805 nm particles. Three compartments characterize these viruses: the nucleocapsid, the matrix space and the envelope (Sullivan et al., 2003; Owolabi et al., 2016).

The ribonucleic acid (RNA) genome is only 1% of the mass of EBOV. The genome is around 19 kb long, composed of seven genes coding for eight proteins including structural proteins: the envelope glycoprotein (GP), the nucleoprotein (NP); and the matrix proteins VP 24 and VP 40. The non-structural proteins are VP 30 and VP 35, which are respectively the transcription activator and the polymerase cofactor. The genome includes also the viral polymerase (L) (Sullivan et al.,2003; Falasca et al., 2015; Kaner et al, 2016) (*Figure 1*).

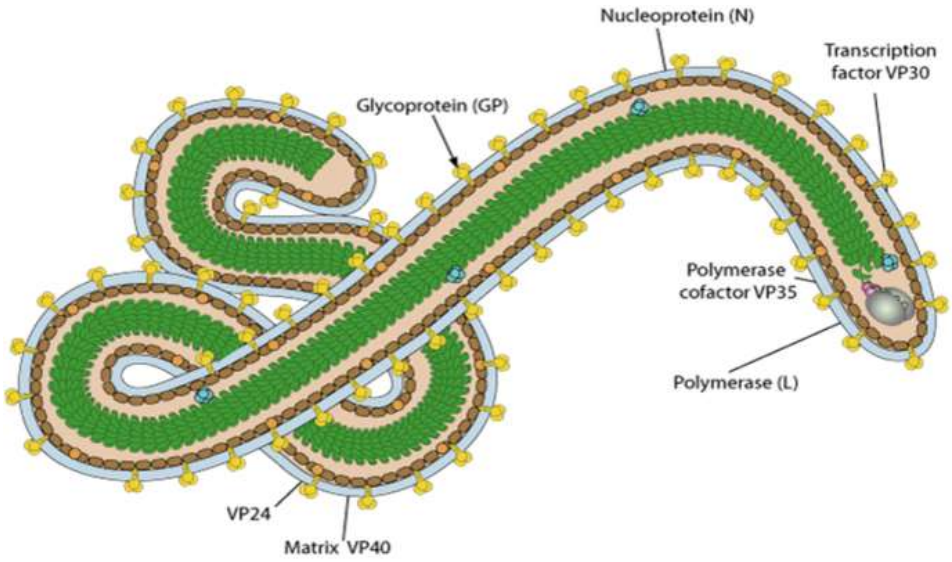


Figure 1: Model of the ebolavirus particle (Owolabi et al., 2016).

The glycoprotein (GP) is type I transmembrane protein complex composed of two proteins (GP1 and GP2) linked by a disulfide bond. However, this bond is cleaved by cathepsin B and L into the endosome (Falasca et al., 2015). It is reported that the GP gene is very important for virus infection and pathogenesis, and its expression is well regulated during virus replication. Indeed, the expression level of both proteins, GP1 and GP2, regulates production and release of the virus (Sullivan et al., 2003).

An mRNA editing mechanism of GP gene gives rise to a soluble 60-70 kDa protein (sGP), and another protein of 150 to 170 kDa protein (GP) that forms spikes on the viral membrane. This GP is required for viral cellular entry and cell fusion protein; it is also relevant to the viral pathogenesis. The sGP is only secreted from the infected host cell. GP appears to form a trimeric complex and bind preferentially to endothelial cells. sGP gives rise to a dimeric protein that interacts with neutrophils and can alter the immune response by inhibiting the activation of neutrophils (Sullivan et al., 2003; Owolabi et al., 2016).

2.3 Genome organization

The negative-sense RNA genome is 19 kb long and it is encapsidated by nucleoprotein and other viral proteins to form a helical nucleocapsid. This nucleocapsid is important for the virus assembly because it plays a role of scaffold. It also acts as a template for genome transcription and replication (Wan et al., 2017).

There are seven open reading frames coding for eight proteins and each gene encodes a single polypeptide, except the glycoprotein gene, which encode for GP and sGP after the mRNA editing as shown below (Owolabi et al., 2016) (*Figure 2*).

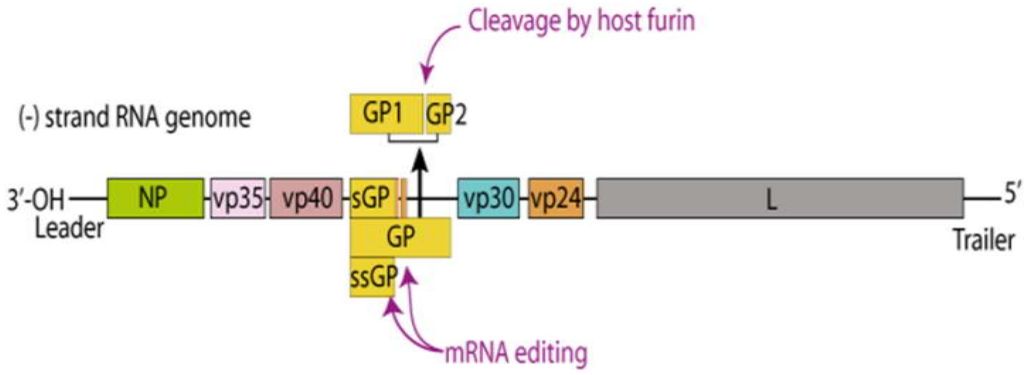


Figure 2 : The structure of the ebolavirus genome (Owolabi et al., 2016).

2.4 Replication cycle of EBOV

The virus can enter the human body via mucosal surfaces, abrasions and injuries in the skin or by direct parental transmission. Afterwards, it can infect all human cells using different attachment mechanisms such as lipid raft, receptor mediated endocytosis and micropinocytosis. Cell surface receptors β1-integrins encoded by the integrin beta-1 (ITGB1) gene are also a group of proteins involved in the virus entry (Falasca et al., 2015).

EBOV uses its viral spikes GP to bind to the cell surface receptors on the cell surface. The virus does not fuse immediately with the host cell membrane. It uses the conventional endolysosomal pathway. The membrane fusion is mediated by GP2, which is a part of GP after its proteolytic cleavage. Endosomal proteases cathepsin B (CatB) and cathepsin L (CatL) are responsible for this cleavage. Thanks to this membrane fusion, the viral nucleocapsid can be released into the cytoplasm of the infected cell. The negative-sense viral RNA of EBOV will be first transcribed into a positive-sense RNA that acts as viral messenger RNA (mRNA) which is involved for protein synthesis. This viral mRNA will also be replicated in the infected host cell (Falasca et al., 2015) (*Figure 3*).

Eight proteins are synthetized in the infected host cell by the viral genome. These proteins will be the bricks to form new viruses, released in the intercellular environment. These new viruses are able to infect other host cells.

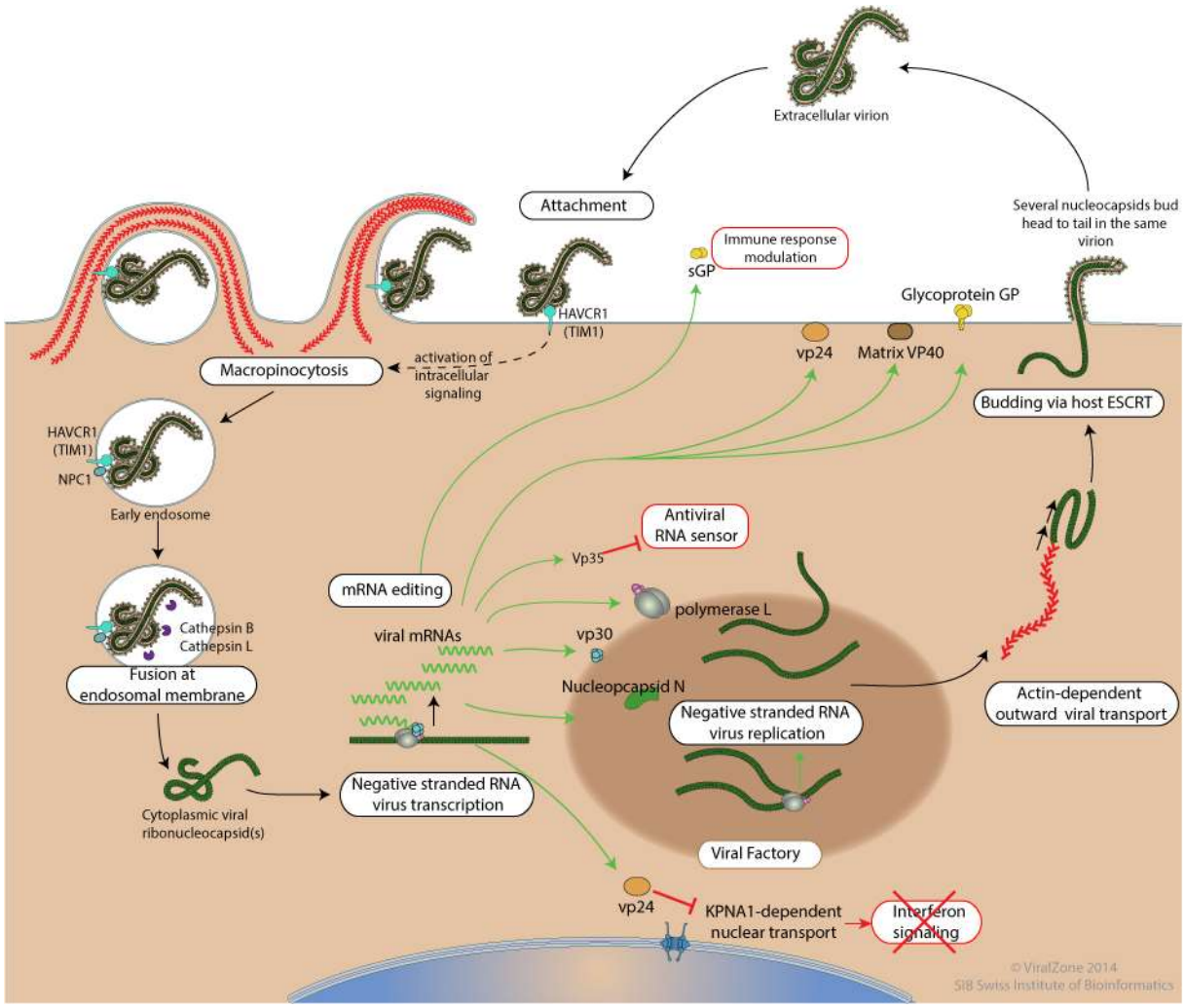


Figure 3 : Ebolavirus replication cycle (ViralZone, www. viralzone.expasy.org, Swiss Institute of Bioinformatics, consulted on 14/06/2018)

2.5 Ecology of EBOV

EBOV outbreaks were often associated with reports of deaths of animals. EBOV was introduced into human population through animals such as chimpanzees, gorillas, bats, monkeys through direct contacts with blood, secretions, organs or body fluids from this infected dead animals. To prevent future outbreaks, it is important to have a good surveillance system for wildlife death, which tends to precede human infections (Kaner et al., 2016; Weyer et al., 2015; Delgado et al., 2018).

Research on the evidence of EBOV infection in different animals (bats, monkeys, gorillas) captured in African countries failed to provide the correct answer about the exact natural reservoir. However, fruit bats remain to be the potential natural reservoir of EBOV because some antibodies (Immunoglobulin G) specific for EBOV were detected in their sera (Kaner et al., 2016).

3. Transmission of EVD

Human-to-human contact is the major mode of transmission of EVD through blood or secretions from infected patients. Other risk factors associated with human-to-human transmission are infection from contaminated materials, particularly sharps, preparation of victims during the burial ceremonies, and occasionally sexual contact. The most infectious materials are blood, feces and vomit from ill patients with EVD (Kaner et al., 2016; Brettin et al., 2017; Owolabi et al., 2016).

4. Clinical symptoms of EVD

The clinical manifestations of EVD can be confused with other infectious diseases such as malaria, typhoid fever, meningitis, Marburg and Lassa fever (Owolabi et al., 2016). The incubation period for EVD is fourteen to twenty-one days (Sullivan et al., 2003).

The onset of EVD is non-specific with symptoms like fever, headache, flue, and extreme malaise; the patient is able to transmit the disease at this point (Brettin et al., 2017). Patients can also develop weakness, muscle pain and abdominal pain. Gastrointestinal symptoms are developed in most patients between the second and the fourth day of illness, with abdominal pain, and cramping followed by vomiting and diarrhea. Bleeding, coagulation disorders, hypotension, lymphopenia, thrombocytopenia, hypovolemic shock, and a cytokine storm response are caused by a multi-organ failure (Sullivan et al., 2003). Survivors of EVD can suffer of fatigue, bulimia, hearing loss, headache, amenorrhea and tinnitus (Owolabi et al., 2016).

5. Laboratory diagnosis

The laboratory diagnosis of EVD is based on virus isolation, antigen detection and serology, and molecular assays. Biological safety level 4 containment facilities are recommended for any procedure such as isolation where virus is amplified. Indeed, EBOV has a high epidemic behavior and it is necessary to avoid contamination of researchers (Falasca et al., 2015). EBOV is isolated from serum, blood or tissue specimens then stored at -70°C, in Vero E6 cells (Owolabi et al., 2016; World Health Organization, 2018). For diagnosis purpose, most of these assays are actually replaced by RT-PCR, which is used on blood and any other body fluids and tissues. It is rapid, highly sensitive and specific, and can be performed safely in the field (Leroy EM et al., 2000).

6. Prevention and control of EVD

There are currently no antiviral drugs or approved vaccines available against EVD. A broad spectrum of antiviral agents is ineffective, such as interferon and ribavirin. However, the standard treatment is focusing on clinical symptoms; rehydration and symptomatic treatments to reduce vomiting, fever, diarrhea and pain are used for early supportive care and improve survival (Owolabi et al., 2016; Kaner et al., 2016; World Health Organization, 2018).

There is no treatment yet, but a range of blood, immunological and drug therapies are tested and under development. Vaccine development is also an interesting option for scientist and researchers (Kaner et al., 2016).

There is currently a trial vaccine that passed safety and immunogenicity tests “recombinant Vesicular stomatitis virus Zaire ebolavirus vaccine” (rVSV-ZEBOV) (Bettrin et al., 2017; Bornholdt et al., 2018). The efficacy rate was estimated between 74.7% to 100% in a ring vaccination conducted in Guinea (Henao-Restrepo et al., 2017). The effect of rVSV-ZEBOV for the long-time protection is still unknown. However, it is reported that humans who participated in the clinical trial have maintained high level of specific EBOV antibodies for 1-2 years after administration of this vaccine (Bornholdt et al., 2018). rVSV-ZEBOV generates antibodies which target the trimeric ebolavirus surface glycoprotein that initiates cellular attachment and facilitates viral entry. Future EBOV vaccine designs would target not only Zaire ebolavirus but also the entire group of EBOV.

There is still a possibility to have another EBOV outbreak because the natural reservoir of the virus is yet unknown, the dynamic transmission between healthy individuals and the reservoirs is still debated and transmission strategies to other species remain questions to be answered. Moreover, specific treatment and preventive vaccines need to be scrutinized for increasing the rate of survivors and preventing the spread of the virus. The improvement of healthcare infrastructures, and networks of healthcare professionals are of the utmost importance (Kaner et al., 2016).

7. Host innate and adaptive immune responses against EVD

7.1 Innate and adaptive immunity

The immune system has two arms, which function cooperatively to provide a response against antigen attacks: the innate and the adaptive immune system (*Figure 4*).

The innate immunity is involved in the immediate response when a virus enters into the body. The innate immunity has four types of defensive barriers including the anatomic barrier (skin, mucous), the chemical barriers (acid secretions), the cellular barriers (epithelial cells, macrophages, dendritic cells, mastocytes) and finally the humoral barriers (complement factors) (Janeway et al., 1997). The immune response is initiated by the recognition of conserved motifs, such as pathogen-associated molecular patterns (PAMPs) on pathogens, or damage-associated molecular pattern (DAMPs) on stressed or dead cells. These motifs are detected by a panel of membrane or cytosolic receptors, the pattern recognition receptors (PPRs), which are expressed on the surface of immune cells (Janeway et al., 1997).

The adaptive immunity takes more time for its activation, with a mean of five to six days. Moreover, this immunity is more specific, with specific and unique receptor for antigens. One of its characteristics is the ability to build an immunologic memory, which permits a rapid and more efficient response during a future contact with the same antigen. It is initiate by the antigen-presenting cells (APCs) and led by lymphocytes. Lymphocytes are lymphoid cells originated from the bone marrow. There are two types of lymphocytes: T lymphocyte (LT) and B lymphocyte (LB). T lymphocytes are responsible for cellular response while B lymphocytes are implicated in the humoral response. LT can be helper, T CD4+ helper, or cytotoxic, T CD8+. All lymphocytes need to be activated by the APCs to be functional. LT CD8+ play a role in the immune regulation and activate the LB, LT CD8+ and macrophage. The role of LT CD8+ is to eliminate pathogens. In addition, they are also able to induce a programmed cell death of infected or tumoral cells (Janeway et al., 1997).

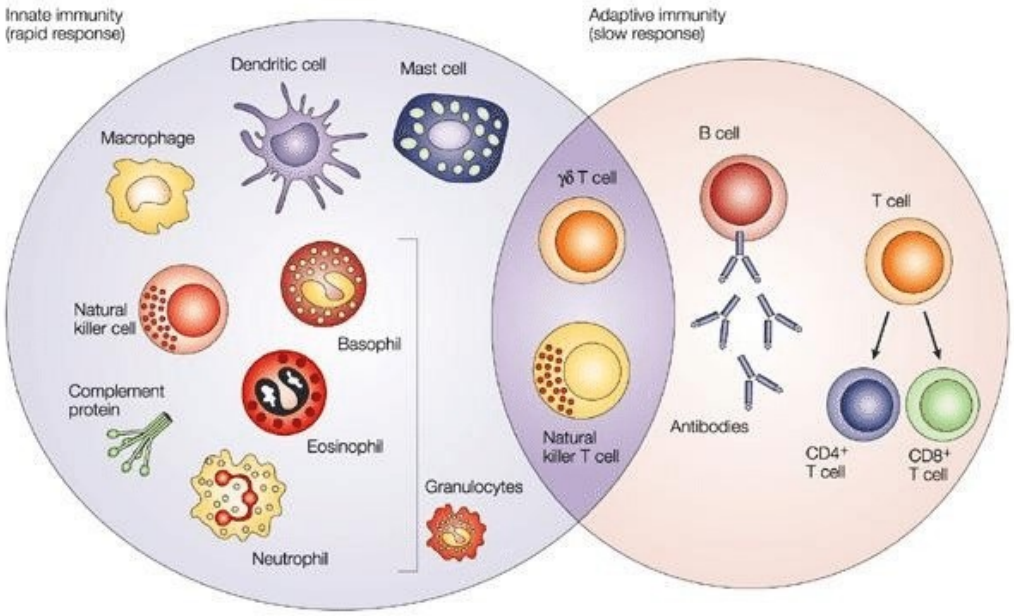


Figure 4: Innate and adaptive immunity. The innate and adaptive immunity are represented by their specific cells. The innate system is represented by different cells which are able to provide a rapid response against pathogens. The adaptive immune system is represented by specific cells (T and B cells) (Dranoff et al., 2004).

7.2 Natural Killer cells

Natural killer (NK) cells are among the first implicated in the innate immune response. They attack infected or transformed malignant cells via their cytotoxicity effects (Rajalingam et al., 2011). NK cells cause the apoptosis of infected or transformed cells through cytolytic granules, such as perforin proteins and granzymes proteases. Perforin forms pores in the cell membrane of the target cell, which permit the granzymes to come into the cell and induce apoptosis (Kumar et al., 2018).

Cytokines such as interferons, interleukins, chemokines, are cellular signaling soluble substances. These 10 to 40 kDa proteins can act from a distance to regulate the activity and the function of other cells. The target cell can recognize the signal via receptors on their surface for these cytokines (Zwirner et al., 2010) (*Figure 5*).

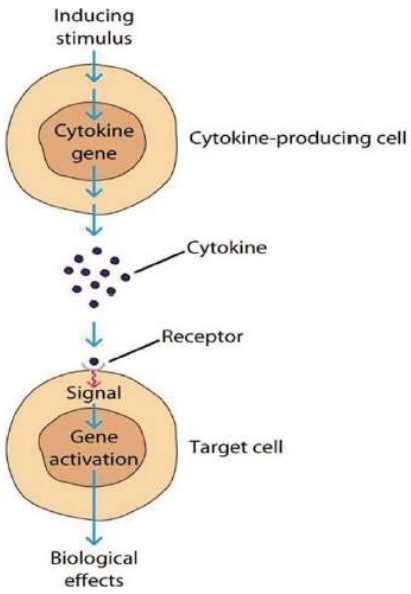


Figure 5: Cytokine functions. Cytokines are produced following a stimulus. They bind to receptors on the target cells that trigger the signal transduction pathway that ultimately influences the target cell and induces effect (Waykole et al., 2009).

Certain interleukins (IL) such as IL-12, IL-15, IL-18 and IL-2 and chemokine ligand 5 (CCL5) are involved in the NK activation. NK cells are also able to secrete many cytokines to control viral infections. Interferon gamma (INF-γ) is used to activate the macrophages, and tumor necrosis factor alpha (TNFα) acts to promote tumor cell killing by NK cells (Kumar et al., 2018; Zwirner et al., 2010).

Their activity is regulated by the interaction between activating or inhibitory membrane receptors and major histocompatibility complex (MHC) class I antigens expressed by the host cells (Rajalingam et al., 2011).

NK cells and CD8+ cytotoxic T cells (CTL) are originated from a common lymphoid progenitor and are both necessary for defense against virus and tumor-transformed cells. They both induce apoptosis of infected cells and share several features especially their morphology and cell surface phenotype. NK cells are also educated during their development, they are able to undergo clonal expansion, and can generate long-lived memory cells, such as T and B cells of adaptive immunity (Rajalingam et al., 2011). However, compared to CTLs, NK cells use a range of different inhibitory or activating receptors on their surface, depending on the ligands presented by the target cells. Sometimes, both receptors with inhibitory or activating functions are needed to recognize their ligand on target cell. The balance between these signals determines the action of NK cell. Concerning CTLs, activation is only when the T cell receptor recognize a specific antigen peptide laden by human leucocyte antigen (HLA) class I molecule on the infected cell (Rajalingam et al., 2011) (*Figure 6*).

The cells surface receptors of NK cells belong to C-type lectins-like group (CD94/NKG2) on the chromosome 12q1.3-13.4 and the immunoglobulin-like superfamily on the chromosome 19q13.4. Killer cell immunoglobulin-like receptors (KIRs) belong to the latest family (Middleton et al., 2010). KIRs and human leucocyte antigen (HLA) class I molecule are highly diverse and variable. The susceptibility to pathogens or disease varies due to this large diversity of both proteins and their interactions (Kulkarni et al., 2008).

NK cells do not express the entire set of NK receptors of an individual; it means these NK receptors are expressed in a random combination (Kulkarni et al., 2008; Middleton et al., 2010). However, they express at least one inhibitory receptor for self-HLA class I molecules to provide tolerance to healthy cell. Indeed, NK cells circulate in the peripheral blood and are able to deliver effector function whenever and anywhere. In absence of an inhibitory receptor, healthy cells could also be destroyed by NK cells (Rajalingam et al., 2011).

NK cells have the ability to kill tumor cells and infected cells, which express insufficient histocompatibility complex (MHC) class I molecules. Some tumor cells, virus or a stress state can induce a down regulation of HLA class I molecule on the surface of a human cell, to escape from CTL responses. However, this conducts to their destruction by NK cell via the “missing-self” mechanism (Middleton et al., 2010; Rajalingam et al., 2011) (*Figure 6*).

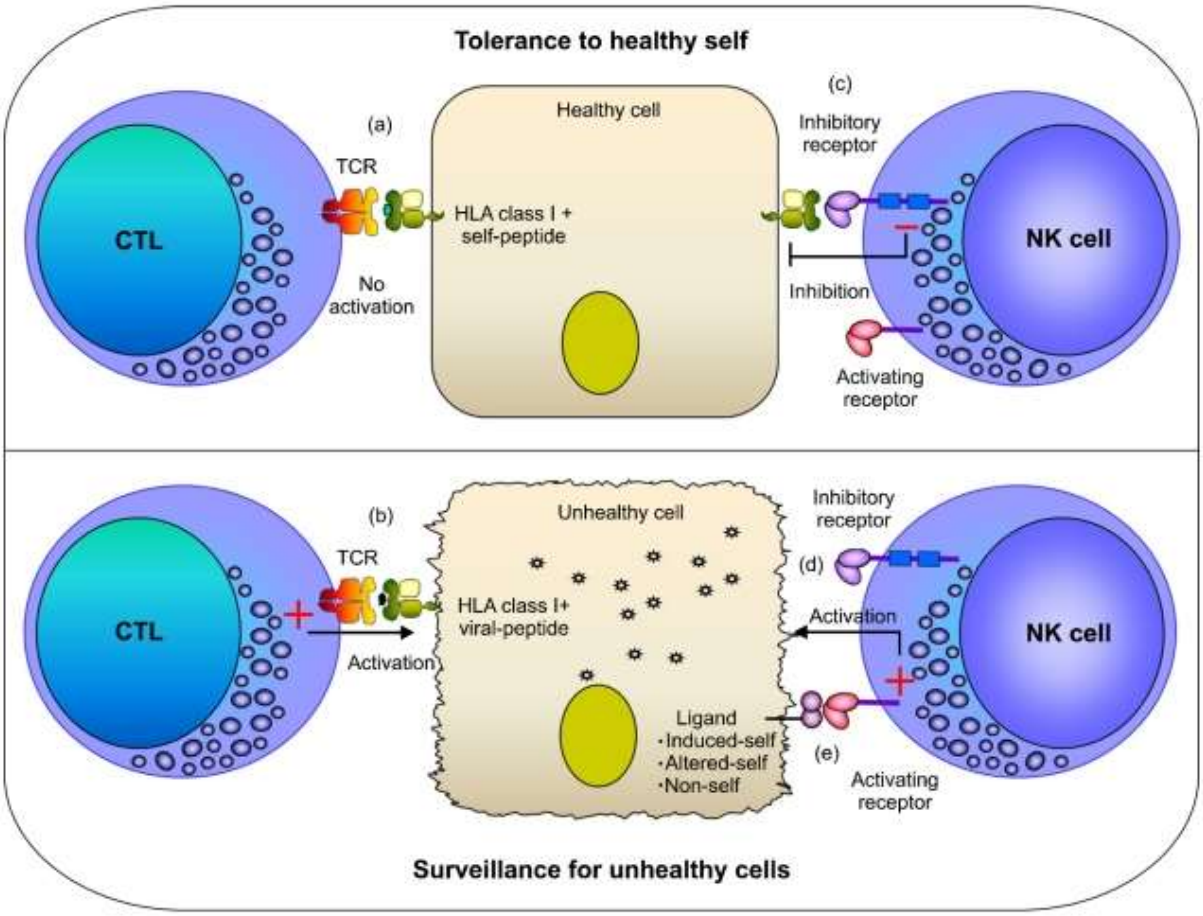


Figure 6 : Natural killer cell and cytotoxic T cell responses. CTLs have activation T cell receptors (TCR) and NK cells express activating and inhibitory receptors. Lack of HLA expression due to viral infection or tumor transformation can also be recognize by NK cells, compared to CTLs (Rajlingam et al., 2011).

7.3 Killer cell immunoglobulin-like receptor genes

Killer cell immunoglobulin-like receptors (KIRs) are members of the Immunoglobulin superfamily type I receptors, which are expressed on the surface of NK cells and a subset of T-cells (Carringhton et al., 2003). KIR genes are located on chromosome 19q13.4 within the leukocyte receptor complex (LRC). This family of highly polymorphic genes exhibits diversity at both allelic and haplotypic levels. To date, a total of sixteen KIR genes have been identified, of which eight are inhibitors (2DL1-5, 3DL1-3), six are activators (2DS1-5, 3DS1) and two are pseudogenes (2DP1 and 3DP1). The number and type of KIRs varies considerably between individuals, which contain seven to twelve KIRs for each haplotype. The diversity contributes to an individual-specific KIR gene content and makes it important for genotyping study. Four KIR genes including KIR2DL4, KIR3DL2, KIR3DL3, and KIR3DP1 are known to be always present in any DNA profile of a human being (Middleton et al., 2010). Within each gene, alleles can be present with different frequencies.

The A and B haplotypes are the most common. The B haplotype is more variable and defined by the presence of KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR2DL2, and KIR3DS1 while the A haplotype is less variable and contains nine genes: KIR2DS4, KIR3DL3, KIR2DL3, KIR2DP1, KIR2DL1, KIR3DP1, KIR2DL4, KIR3DL1, and KIR3DL2. The B haplotype encodes more activating KIRs than the A haplotype, which have just one activating KIR receptor (KIR2DS4). The frequency of KIR haplotypes varies between populations (Carringhton et al., 2003; Kulkarni et al., 2008; Middleton et al., 2010; Hong et al., 2011; Jamil et al., 2011; Rajalingam et al., 2011) (*Figure 7*).

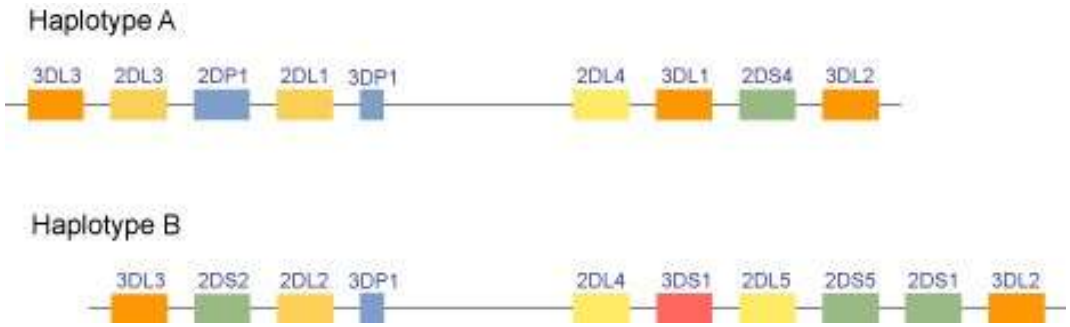


Figure 7 : Gene order of the two haplotypes, A and B. Here is the repartition of the genes along the chromosome on the two distinct haplotypes (Carringhton et al., 2003).

The evolution of KIR genes is not only based on the genetic level but also on the functional level. Indeed, there are different alleles having different protein expression levels, and these proteins have different affinity with their HLA ligand (Middleton et al., 2010).

The resulting proteins of KIR genes are members of the immunoglobulin (Ig) superfamily of receptors. Their name is related to their structure. The first number describes the number of Ig-like extracellular domains, which can be two or three domains (2D or 3D) whilst the letter means the length of the cytoplasmic tail (L for long and S for short). The P letter means pseudogene, which is defined as a gene having a sequence similar to KIR genes but not encoding proteins (Carringhton et al., 2003). The last number indicates the gene encoding a protein with this structure (Middleton et al., 2010; Jamil et al., 2011). Sometimes, additional numbers are present if there is a genetic variation in the exon sequences (for example KIR2DS4-001 and KIR2DL4-003) (Middleton et al., 2010).

KIR receptors confer some specificity to NK cells since they can inhibit or activate the function of NK cells. The long cytoplasmic tail contains the immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which trigger the inhibitory signal pathway after the ligand-receptor binding. In contrast, activating receptors own a transmembrane lysine residue that is required for pairing with immunoreceptor tyrosine-based activating motifs (ITAMs), which is associated with a tyrosine kinase binding protein

called DAP12 (a transmembrane protein of 12kDa). DAP12 is encoded at the centrometric end of the leucocyte receptor complex (LRC) (Carringhton et al., 2003; Jamil et al., 2011) (*Figure 8*).

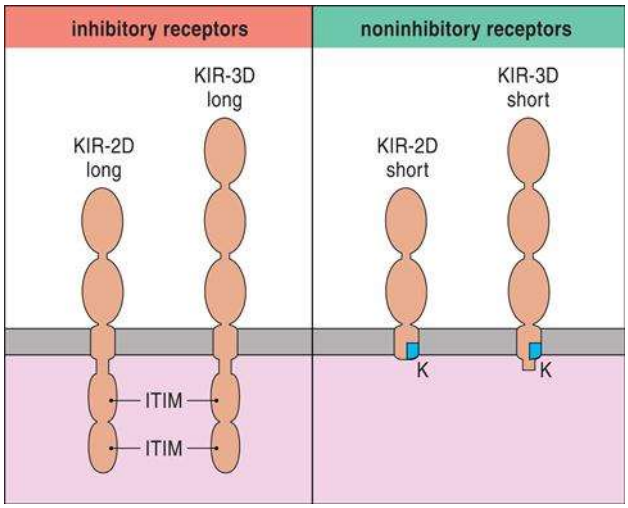


Figure 8 : Schematic representation of killer immunoglobulin-like receptors (KIRs). Two or three extracellular domains are characteristic for both inhibitory and activating KIRs. However, the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) are only present only on the inhibitory KIR, whilst the activating KIRs have a transmembrane lysine residue (Male et al., 2006).

As mentioned previously, sixteen KIR genes have been identified, of which eight are inhibitors (2DL1-5, 3DL1-3), six are activators (2DS1-5, 3DS1) and two are pseudogenes (2DP1 and 3DP1) (*Table 2*). Pseudogenes are defined as defunct copies of genes that have lost their function at mRNA or protein level. The loss of function is usually caused by a failure of transcription or translation, or production of a defective protein (Carringhton et al., 2003). 2DL4 is the only KIR which exhibit the inhibitory motif (ITIM) and can also interact with the activating motif (ITAM). Thus, this KIR2DL4 is an activating and inhibitory cell membrane receptor (Kulkarni et al., 2008).

Table 2 : Killer immunoglobulin-like receptors (Carringhton et al., 2003).

Activating receptors	Inhibitory receptors	Pseudogenes
3DS1	3DL1	2DP1
2DS1	3DL2	3DP1
2DS2	3DL3	
2DS3	2DL1	
2DS4	2DL2	
2DS5	2DL3	
2DL4	2DL5	
	2DL4	

Furthermore, KIR genes exhibit some polymorphisms which can influence the expression, the ligand binding and their functional capacity. A total of 614 KIR nucleotide sequences encoding 321 distinct KIR proteins are known to date (“European Molecular Biology Laboratory”, https://www.embl.org/ or

"GenBank nucleotide sequence databases", https://www.ncbi.nlm.nih.gov/genbank) (Rajalingam et al., 2011).

Another diversity of KIR genes is based on their expression on NK cells. Each NK cell expresses some of the KIR genes while only one KIR (2DL4) is present on all NK cells. Their KIR gene expression depends on DNA methylation process. Indeed, when the promoter region is unmethylated, KIR gene will be expressed. In contrast, DNA methylation leads to the non-expression of KIR genes (Middleton et al., 2010).

7.4 Human leucocyte antigen

The human leucocyte antigen (HLA) class I molecules are known as KIR protein ligands. They are encoded by genes located on chromosome 6, within the major histocompatibility complex (MHC) (Rajalingam et al., 2011). This genetic information is expressed via surface proteins on all nucleic cells. HLA is essentially present on antigen-presenting cells (APCs) such as B lymphocytes, macrophages and dendritic cells. They are heterodimers and consist of two polypeptides: alpha and beta-microglobulin chains. Theses chains are not covalently linked (Hickey et al., 2016) (*Figure 9*).

HLA class I molecules play a key role by presenting the antigenic peptide fragments to immune effector cells, such as cytotoxic T lymphocytes (CTL) or NK cells (Hickey et al., 2016).

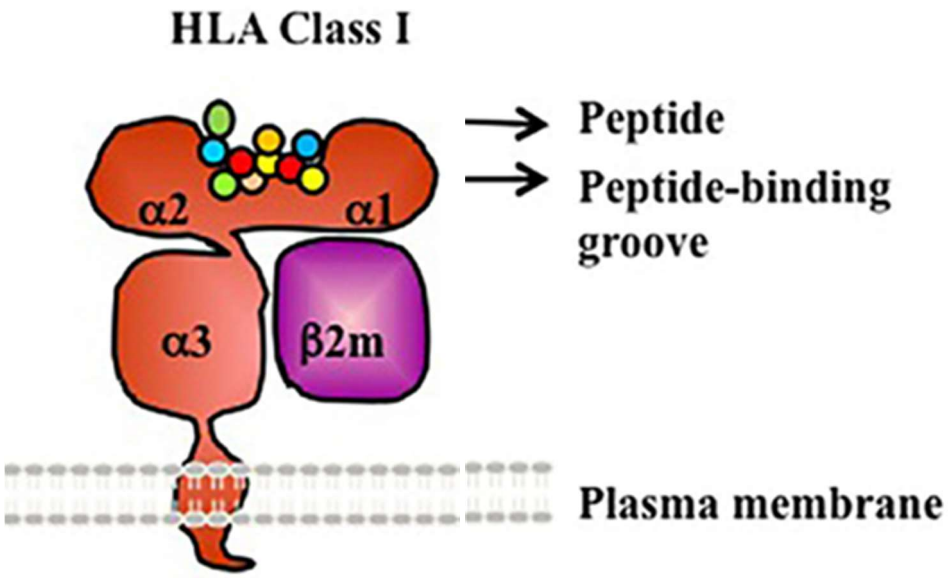


Figure 9 : HLA class I schematic representation. HLA is made of a heavy alpha chain and beta-microglobulin. This structure present antigenic of self-peptide to immune effector cells (Hickey et al., 2016).

HLA class I ligands are extremely polymorphic. Three HLA genes are located on the MHC gene region: HLA-C, HLA-B and HLA-A. On the α1 chain of these three classic molecules, one specific amino acid is

involved in their interaction with KIRs. HLA-C is the dominant and provides ligand for many different KIR receptors. They all have valine at position 76 in common and there is a polymorphism at amino acid position 80. Indeed, there are two groups of HLA-C alleles, which differ by the amino acid at position 80 of the molecule. HLA-C group 1 has asparagine at position 80. This is the ligand for KIR2DL2 and KIR2DL3. HLA-C group 2 provides the ligand for KIR2DL1 with a lysine at position 80. KIR2DS1 has similar Ig-like domains to KIR2DL1 and can also bind to HLA-C2 with lower affinity. KIR2DS4 can bind to HLA-C allotypes and HLA-A11. It is reported that KIR2DL2 and KIR2DL3 can bind to HLA-C group 2 with different binding affinity (Kulkarni et al., 2008; Middleton et al., 2010; Jamil et al., 2011; Rajalingam et al., 2011).

KIR3DL1 binds to HLA-B molecules that carry a Bw4 epitope (HLA-Bw4) with a lower affinity binding. Inhibition is stronger when KIR3DL1 binds to HLA-Bw4 with isoleucine at position 80 (HLA-Bw4-Ile) compared to the binding with HLA-Bw4-Thr (Threonine at position 80). KIR3DL2 and KIR3DS1 are highly specific for HLA-A3/A11 and HLA-Bw4-Ile respectively. All individuals are carrying HLA-C that seems to be more important than HLA-A and HLA-B ligand (Kulkarni et al., 2008; Middleton et al., 2010; Jamil et al., 2011; Rajalingam et al., 2011).

HLA-G and HLA-E are the non-classic HLA molecules. Indeed, HLA-E is expressed during a stress state and it is recognized by NKG2A, another NK cell receptor, belonging to C-type lectin family (Kumar et al., 2018). HLA-G plays a crucial role during pregnancy and it interacts with KIR2DL4 (Kulkarni et al., 2008) (*Table 3*).

Table 3 : KIRs molecules and their HLA ligands (Jamil et al., 2011).

KIRs	Ligand
2DL1	HLA-C2
2DS1	HLA-C2
2DL2	HLA-C1
2DL3	HLA-C1
2DS2	HLA-C1
2DL4	HLA-G
2DL5	Unknown
2DS3	Unknown
2DS4	HLA-A11, HLA-C
2DS5	Unknown
3DL1	HLA-Bw4
3DS1	HLA-Bw4-Ile
3DL2	HLA-A3, A3,11
3DL3	Unknown

It is known that KIR-HLA interaction favors NK and T cells activation, and protects human healthy cells against NK cells. NK cells express on their surface at least one inhibitory receptor for self-HLA class I molecule (Carringhton et al., 2003).

HLA class I molecules provide also a ligand for other immune cells such as CTLs from adaptive immune system. T cell receptors (TCR) interact with HLA through antigenic peptide, which is destroyed by CTL thanks to their cytolytic activities (Rajalingam et al., 2011).

As stated earlier, HLA class I expression can be down-regulated via the pathogen or the virus. This leads to the non-death of cell by CTL, even if this cell is infected. However, NK cells induce its death thanks to the "missing-self" hypothesis (Middleton et al., 2010; Rajalingam et al., 2011).

7.5 KIR and HLA combinations

Previous studies have demonstrated that some KIR-HLA combinations are associated with infectious or auto-immune disease outcome. Some combinations can also influence the pregnancy or transplantation complications. It is reported that the presence of a specific KIR or HLA ligand, can influence the disease susceptibility or outcome, but also the therapy response (Middleton et al., 2010; Hong et al., 2011).

State of the Art & Aim of the project

KIR and HLA gene families are highly variable because they are unlinked and located on different chromosomes, 6 and 19 respectively. Each individual has its own KIR/HLA genes, yielding a high diversity in the number and type of KIR-HLA combinations. All these combinations can modulate the outcome of diseases (Rajagopalan et al., 2005).

Several studies have been conducted on KIR genotypes and their interaction with HLA class I molecules to investigate the correlation between KIR-HLA combinations and the development or outcome of various infectious diseases. Some examples are mentioned below but there are many other diseases for which the KIR-HLA combinations are still under scrutiny.

In 2002, Martin et al. exposed that the interaction between KIR3DS1 and HLA-Bw4-Ile can delay the progression of AIDS in HIV-infected individuals (Martin et al., 2002).

Khakoo et al. showed in 2004 that the interaction between HLA-C1 and KIR2DL3 is associated with the resolution of chronic hepatitis C virus. It was hypothesized that KIR2DL3 binds to HLA-C with lower affinity than other KIR receptors, which decreases the inhibition activity of NK cells against infected cells (Khakoo et al., 2004). Moreover, Winter et al. showed that the binding of KIR2DL1 to HLA-C2 confers a stronger inhibitory response to NK cells than the binding between KIR2DL3 and HLA-C2 (Winter et al., 1998).

Like HCV, the hepatitis B virus (HBV) also causes hepatitis. Despite the fact that it can cause the disease, the virus is phylogenetically unrelated to HCV. Lu et al. and Gao et al. reported that the KIR2DL3-HLA-C1 interaction was protective while the KIR2DL1 and HLA-C2 combination was associated with a susceptibility to develop the disease. Moreover, they also highlighted that the frequency of B haplotype was higher in infected HBV patients than non-infected patients (Lu et al., 2002; Gao et al., 2010).

Some predispositions to autoimmune diseases have been highlighted with KIR/HLA combinations. Nelson et al. reported that psoriatic arthritis, a long-term inflammatory arthritis due to the autoimmune disease “psoriasis”, could be promoted by the combination of KIR2DS1 and/or KIR2DS2 coupled with HLA-C group homozygosity (Nelson et al., 2004). Van der Slick et al. demonstrated that the interaction between KIR2DS2 and HLA-C1 in the absence of HLA-C2 and HLA-Bw4 increases the susceptibility to type I diabetes (Van der Slick et al., 2003).

All these researches are important for preventing and/or drug development against infectious, malignant and autoimmune diseases. By finding the KIR-HLA combinations that trigger NK cells or T cells activation, we can improve the resistance to certain viruses. Moreover, understanding these combinations is up most important for treatment and vaccine strategies. However, some parameters must be considered: for example, it is important not to overlook the fact that some of these combinations can develop autoimmune diseases (Kulkarni et al., 2008).

Since its first apparition in 1976, ebolavirus has been studied by many researchers to better understand its pathogenesis, ecology and to develop an effective vaccine against this virus. However, many aspects remain unclear.

Vaccine development started in the late 1970s, and the first results were published by Lupton et al. in 1980 (Lupton et al., 1980). Before 2014, clinical trials on EBOV vaccines were not so important because the disease was underestimated but the 2014-2016 West African EBOV outbreak caused panic and people noticed that EBOV can spread everywhere, even in Europe and America. The current vaccine is effective and protects against Zaire ebolavirus strain and the same principles will be applied to develop another vaccine against EVD caused by the remaining ebolavirus strains (Kaner et al., 2016).

Antiviral drugs have also been developed. By targeting viral transcripts, blocking translation or neutralizing the virus to inhibit viral replication, researchers want to fight ebolavirus. Indeed, Kulgelman et al. have worked with small interfering RNAs (siRNAs), phosphorodiamidate morpholino oligomers (PMOs) and antibodies (Kulgelman et al., 2015). Butler reported in *Nature News and Comment* in 2014 that passive immunotherapy treatments such as blood transfusion from survivors provide antibodies against ebolavirus (Butler et al., 2014). Büttner et al. studied in 2014 the extracorporeal elimination of the virus and its viral glycoproteins by lectin affinity plasmapheresis: it consists on a mechanical filtering of patient blood with the concept of affinity chromatography (Büttner et al., 2014).

In 2010, Wauquier et al. found that KIR2DS1 and KIR2DS3 are associated with a fatal outcome of Ebola virus disease (Wauquier et al., 2010).

All these studies are not exhaustive, but show that the KIR-HLA combination is very important because it can influence the outcome of Ebola virus disease.

The main goal of this project is to investigate the presence and/or absence of particular KIR and HLA genes in order to define the KIR-HLA genotyping of EVD contact, survivor and fatal patients, and to assess the outcome of EVD.

We are assessing the susceptibility to EVD by comparing the KIR/HLA interactions between different groups of EVD patients. “Contacts” are defined as people who had a proven close contact with an ebolavirus-infected patient but did not develop the disease (asymptomatic). They reacted strongly in Elispot and ELISA against ebolavirus. “Survivors” are defined as persons that survived an ebolavirus disease, and “fatals” are people that have been infected with EBOV and did not survive the disease.

Material & methods

1. Study samples

This study was conducted at the REGA Institute for Medical research, KU Leuven University (Belgium).

The blood samples originated from three groups of EVD patients: “contact group” (CS), “survivor group” (SS) and “fatal group” (HF). All samples were collected in Guéckédou (Guinea).

The blood samples were collected between December 2015 and September 2017 in Guéckédou (Guinea), among contact (n=56), survivor (n=102) and fatal (n=121) cases of EVD.

The peripheral blood mononuclear cells (PBMCs) consist of B and T lymphocytes, NK cells and monocytes, known as cells with a round nucleus. They were isolated in Guinea using a Ficoll density gradient centrifugation of whole blood samples (Figure 10). The layer containing the lymphocytes was carefully removed from the others. To recover lymphocytes, this layer was washed with a salt solution (PBS with 5% fetal calf serum) to remove platelets. Afterwards, PBMCs were transferred into microfuge tubes containing *DNAgard® blood solution* (*Sigma-Aldrich*, Belgium) with the ratio blood/DNAgard provided by the manufacturers.

Finally, the samples were shipped to Belgium in order to perform the following analyses.

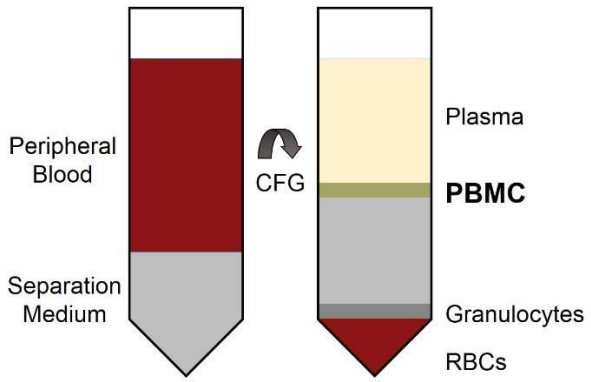


Figure 10 : Obtained layers after blood separation with a medium and a centrifugation. PBMCs are separated by centrifugation on a gradient of *Ficoll*, a hydrophilic polysaccharide. The blood is separated into a top layer of plasma, then PBMCs, followed by polymorphonuclear cells such as neutrophils, and finally red blood cells (RBCs), known also as erythrocytes (Kleiveland, 2015) (Human peripheral blood mononuclear cells, Cell application, www.cellapplications.com, consulted on 15/06/2018)

2. DNA extraction and purification

2.1. Principles

Genomic DNA is extracted from PBMCs and the "Spin column protocol for DNA purification" is performed with *QIAamp® DNA Mini Kit*. This kit is used to purify nucleic acid from tissues, swabs, whole blood, serum, body fluids, and washed cells from urine relying on the fact that nucleic acids binds to the solid phase of silica column under certain conditions. PCR inhibitors, such as divalent cations and proteins are removed by two washing steps. The purified DNA is eluted in a buffer provided with the kit.

2.2. Protocol

The first step consists in pipetting 20 µl of *QIAGEN Protease* into the bottom of a 1.5 ml microcentrifuge tube. Afterwards, 200 µL of sample is added followed by 200 µL of *buffer AL*. The mixture is pulse vortexing for 15 seconds.

After incubating it at 56°C for 10 minutes, the tube is briefly centrifuged to remove drops from the inside of the lid. Then 200 µl of ethanol (100%) is added and the solution is mixed by pulse vortexing for 15 seconds, and briefly centrifuged.

The mixture is then transfer carefully into a *QIAamp® mini spin column* with a 2 ml collection tube and centrifuge at 4650 x g for 1 minute. The resulting filtrate is discarded and the *QIAamp® mini spin column* is placed in a clean 2 ml collection tube.

Then 500 µl of the *buffer AW1* (wash buffer 1) is added, and the mixture is centrifuged at 4650 x g for 1 minute. As previously, the filtrate is discarded and the mini spin column is placed in a clean collection tube.

500µl of the *buffer AW2* (wash buffer 2) is then added and centrifuged at 14240 x g for 3 minutes. The spin column is placed in a new 2 ml collection tube and the filtrate is discarded. The same centrifugation at 14240 x g is applied to this tube.

The elution step is done with 200 µl of elution *buffer AE* (10mM Tris-Cl, 0.5mM EDTA, pH 9) added to the *QIAamp® mini spin column,* which is placed in a new 1.5 ml microcentrifuge tube. This is incubated at room temperature for 1 minute. Then the mixture is centrifuged at 4650 x g for 1 minute. The *QIAamp® mini spin column* can be discarded and the 1.5 ml microcentrifuge tube containing the pure DNA can be stored at -80°C (*Figure 11*).

Nanophotometer Implen N60/N50 is used for measuring the DNA concentration and its purity. This is usually realized to verify the correctness and cleanness of the extraction.

Figure 11 : Simplified DNA extraction protocol diagram. First the sample is lysed to release genomic DNA. This DNA is then bound on the column. The contaminants and impurities are then removed in the flow through. The purified DNA is then eluted (Blood genomic DNA isolation Mini Kit, Biosynthesis, www.biosyn.com, consulted on 15/06/2018).

3. Whole genome amplification

3.1. Principles

As we have several PCRs to perform for each DNA sample, the whole genome amplification is conducted using *QIAGEN Repli-g® Mini kit* according to the manufacturer's instructions.

REPLI-g® uses *Multiple Displacement Amplification (MDA*), which consists in an isothermal genome amplification. This involves the binding of random hexamers to denatured DNA. It is followed by strand displacement synthesis at a constant temperature. A high-fidelity enzyme Phi 29 polymerase (enzyme from bacteriophage Phi29 of *Bacillus subtilis*) is used to amplify the genomic DNA. To obtain high yields of amplification of DNA, additional priming events occur on each displaced strand which serves as a template (*Figure 12*).

This amplification method does not require different temperatures and DNA fragments greater than 2 kb are obtained at the end of the reaction.

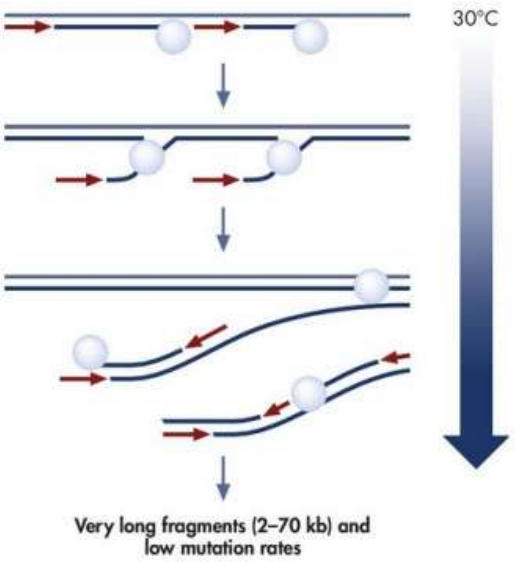


Figure 12 : Mechanism of the *REPLI-G® amplification*. Primers are represented by arrows. Phi 29 polymerase moves along the DNA template strand and extends these primers at 30°C. After, the complementary strand becomes a template strand from replication (Repli-G Principle and Procedures, Qiagen, www.qiagen.com, consulted on 15/06/2018).

3.2. Protocol

The first step consists in preparing sufficient *Buffer D1* (denaturation buffer: 9 µl of reconstituted *buffer DLB* and 32 µl of nuclease-free water) and *Buffer N1* (neutralization buffer: 12 µl of stop solution and 68 µl of nuclease-free water) for the number of amplification reactions.

5 µl of template DNA and 5 µl of *Buffer D1* are added in a 0.2 ml PCR tube. This tube is then vortexed and briefly centrifuged before being incubated at room temperature for 3 minutes. 10 µl of *Buffer N1* is added. Then 30 µl of *Master Mix* (29 µl of *REPLI-g mini reaction buffer* and 1 µl of *REPLI-g® mini DNA polymerase*) is added to the 20 µl of denaturated DNA.

All samples are incubated at 30°C for 10 to 16h.

To inactivate *REPLI-g® mini DNA polymerase*, the tube is heated during 3 minutes at 65°C. A clean up by ethanol precipitation method is useful to obtain the clean and pure amplified DNA (*Figure 13*). Finally, the DNA concentration is measured by Nanodrop.

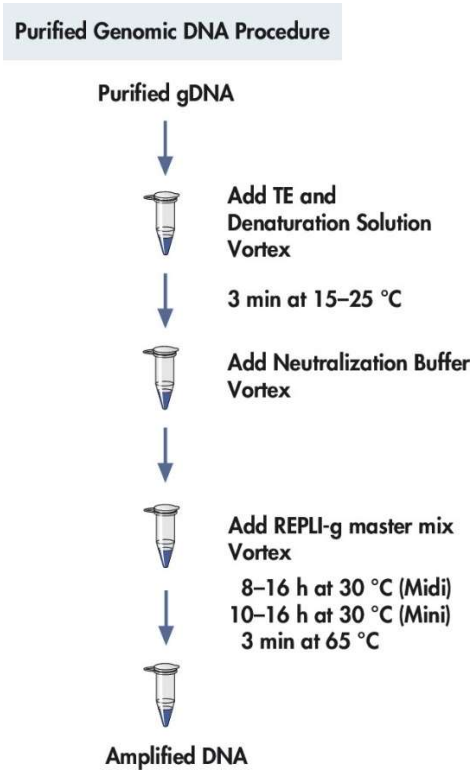


Figure 13 : Amplification of genomic DNA. First, the sample undergoes alkaline denaturation, then it is neutralized before being incubated with the *REPLI-G® Master Mix* to be amplified (Repli-G Principle and Procedures, Qiagen, www.qiagen.com, consulted on 15/06/2018).

4. Cleanup of the amplified genomic DNA by ethanol precipitation

4.1. Principles

Ethanol precipitation is a method used to purify DNA, RNA and polysaccharides. The protocol is based on the addition of salt (a commonly used is sodium acetate) and absolute ethanol to the aqueous solution, which contains the amplified DNA from *REPLI-g®* reaction. This addition induces the nucleic acids to precipitate and a centrifugation step at maximum speed allows the separation of nucleic acids with the rest of the solution (Fregel et al., 2010) (*Figure 14*).

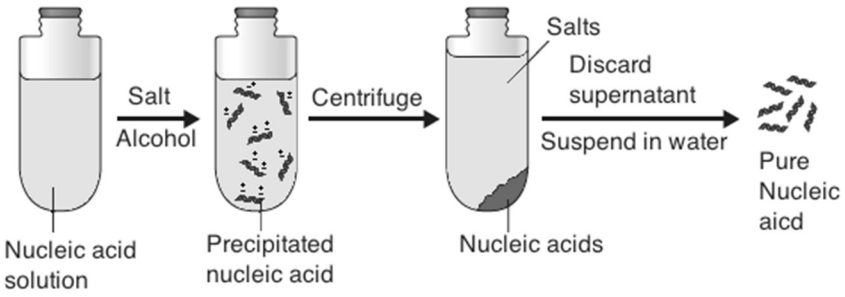


Figure 14 : Ethanol DNA precipitation steps (Zumbo, 2013).

How does it work? First the nucleic acids are soluble in water because water and DNA are polar molecules (*Figure 15*). They can interact electrostatically with each other, which leads to the dissolution of DNA into water. Indeed, nucleic acids are hydrophilic due to the negatively charged phosphate (PO_3^-) groups (*Figure 16*).

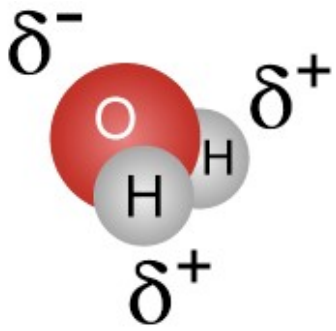


Figure 15 : Representation of H_2O molecule with the electrostatic charges. Water is negatively charged near the oxygen atom due to the unshared pairs of electrons. It is positively charged near the hydrogen atoms (Oswald N., 2017, Ethanol precipitation of DNA and RNA: How it works, BitesizeBio, www.bitesizebio.com, consulted on 12/06/2018).

By adding salt in the solution (sodium acetate, NaAc), the negatively charged phosphate (PO_3^-) groups are neutralized. Indeed, sodium acetate breaks up into Na^+ and $[CH_3COO]^-$ in solution. The positively charged ions neutralize the negative charge of the nucleic acid to make it less soluble in water, less hydrophilic.

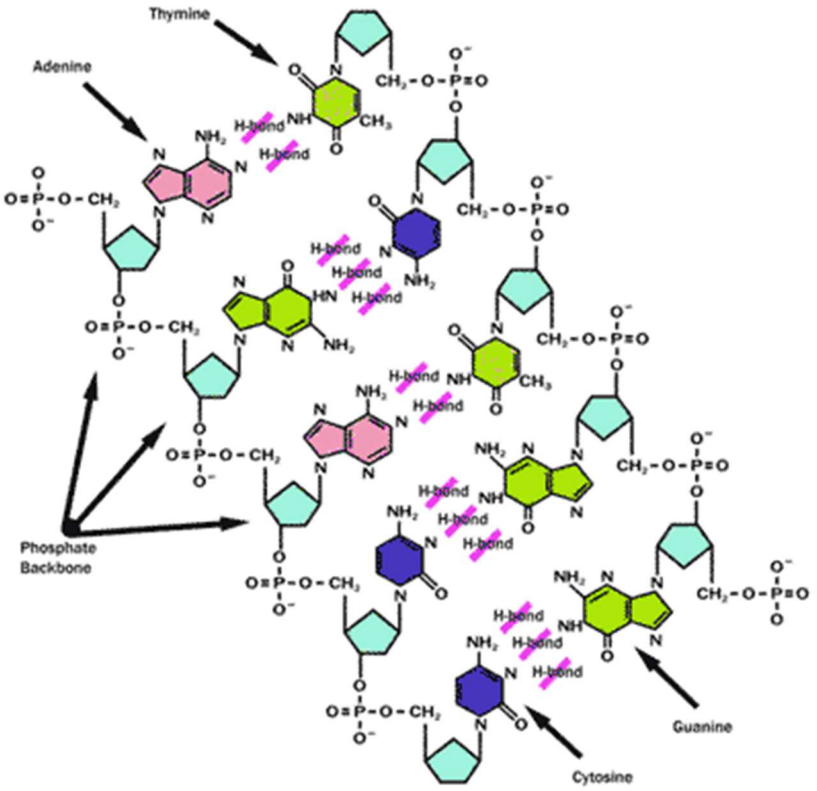


Figure 16 : Representation of DNA. DNA is negatively charged due to the phosphate group (PO_3^-) (Gel electrophoresis-Negative to positive, 2011, www.tymkrs.tumblr.com, consulted on 15/06/3018).

The electrostatic attraction between the Na^+ and PO_3^- are dictated by Coulomb's Law. The dielectric constant of water is high, so it is difficult for Na^+ and PO_3^- to come together. This is why there is the addition of ethanol, which has a dielectric constant much lower that water. It is much easier for Na^+ and PO_3^- to interact, which allows the drop out of solution.

This precipitation is often realized at low temperatures to avoid DNA degradation. The time and the temperature depend on the length and concentration of DNA in the tube. For this project, an optimization was performed, and the high yield of pure DNA was obtained at a temperature -80°C during 2 hours.

Finally, it is important to wash the pelleted DNA with 70% ethanol to remove any residual salt.

4.2. Protocol

The first step of *REPLI-g® products purification* consists in adding 5 µl of NaAc 3M and 100 µL of 100% ethanol in each 0.2 ml PCR tubes containing the amplified DNA from de REPLI-g® reaction. Then the tubes are inverted well to mix and stored at -80°C for at least 2 hours. The samples are then centrifuged at 14240 x g for 3000 minutes at 4°C.

The supernatant is removed and the pellet is washed with 200 µl of 70% ethanol and then centrifuged again at 14240 x g for 30 minutes.

For each samples, the liquid phase is removed and the samples are air-dried at 50°C. Later they are suspended again with 50 µl *QIAamp® elution buffer AE*.

The final dilution is 1:10 for all the samples in order to perform PCR analysis with a DNA concentration range between 20 and 60 ng/µl.

5. PCR Amplification

5.1. Principles

Polymerase Chain Reaction (PCR) is a common technique used to amplify DNA fragments between 0.1 and 10 kbp. With a single copy of a DNA segment, this technique can generate thousands to millions of copies of a particular DNA sequence. This method is based on thermal cycling, which involves exposing the reactants to cycles of repeated heating and cooling (Bartlett et al., 2003).

PCR consists of 20 to 40 repeated cycles with different temperatures. The success of PCR is mainly based on the melting temperature (*Tm*), the type of DNA polymerase, the two specific primers, the concentration of deoxynucleoside triphosphates (dNTPs), the number of cycles, and the quality of DNA samples.

Beforehand, a *Master Mix* is prepared with the reaction mixture. It contains a DNA polymerase, specific primers, dNTPs, and a buffer solution to provide a suitable chemical environment. The DNA sample will finally be added to the above *Master Mix*.

The PCR program consists of three different steps including a denaturation, an annealing and an extension (*Figure 17*). A pre-denaturation step is performed, which consists on heating the reaction chamber to a temperature of 94-96°C during 1-10 minutes to activate the thermostable polymerase.

Denaturation step is carried out at 94-95°C (20 to 30 sec) for separating the two strands of DNA template from one another. The hydrogen bonds are broken between complementary bases to permit the separation of the double-stranded DNA template.

The next step is the annealing which is carried out between 53-58°C for 20-40 seconds. The oligonucleotide primers are able to hybridize to each single-stranded DNA template. Two primers are necessary, a forward and a reverse for the single-stranded DNA containing the target region. This step is critical because the temperature must be optimal to have an efficient and specific annealing. If it is too high the hybridization cannot take place, and if it is too low, it is not specific enough. As primers are very specific, they hybridize the complement single-stranded DNA. Generally, the optimal temperature is about 3-5°C below the *Tm* of the primers used.

After hybridization, the temperature is increased to 72°C, which is the optimal temperature for DNA polymerase (*Taq polymerase*). During this elongation/extension step, a new DNA strand is synthetized by the DNA polymerase. This strand is complementary to the DNA template and is made by addition of free dNTPs from the reaction mixture in the 5’ to 3’ direction. The new DNA strands that are generated become DNA templates for the next cycles of denaturation, hybridization and elongation, leading to exponential amplification of the target sequence. The duration of the elongation step depends on the type of DNA polymerase used and the length of the targeted region.

A final elongation step is carried out at 70-74°C for 5-15 minutes to ensure that all single-stranded DNAs are fully elongated.

The last step after all the PCR reaction cycles consists of cooling the chamber to 4-15°C to stop any reaction. This step can be used to store the PCR products for a short time.

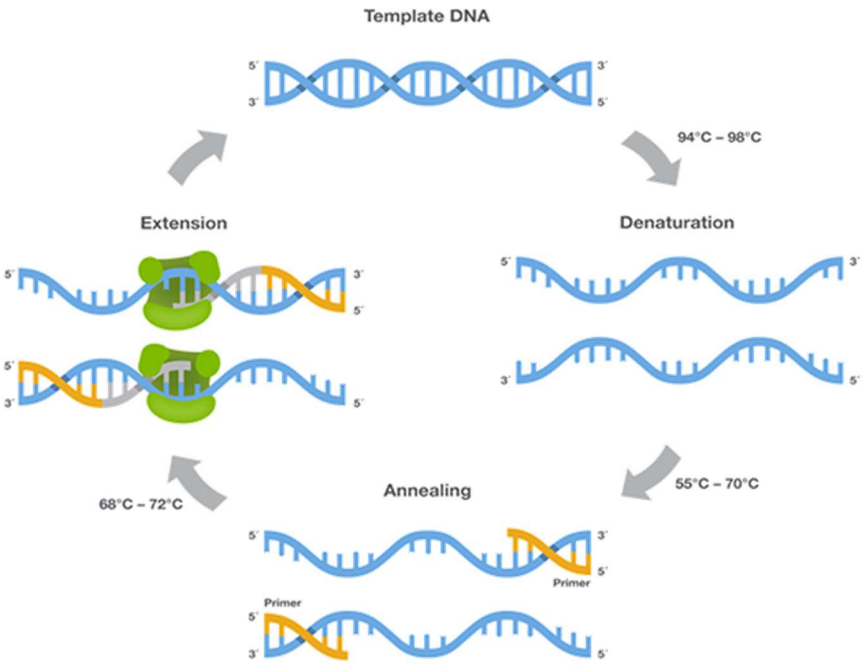


Figure 17 : PCR amplification cycle (PCR cycling parameters- six key considerations for success, ThermoFisher scientific, www.thermofisher.com, consulted on 15/06/2018).

Despite its high sensitivity and specificity, any PCR reagent can be contaminated, which leads to an amplification of wrong DNA products. It is important to work with good laboratory practices and an optimal temperature for each step to obtain a good yield of PCR products.

5.2. Protocol

The amplification of 18 KIR genes and 5 HLA genes is performed with the *Biometra TRIO Thermocycler (Westburg*). Based on the paper of Nazari et al., all primers used and amplicon lengths are reported in the tables below (*Table 4, Table 5*).

Table 4 : Respective length and nucleotide sequence for each pair of selected KIR genes primers (Nazari et al., 2015).

Gene	FP- Forward (5'-3')	RP-Reverse (5'-3')	Size (bp)
2DL1	TTGGTCAGATGTCATGTTTGAA	TCCCTGCCAGGTCTTGCG	143
2DL2	AAACCTTCTCTCTCAGCCCA	GCCCTGCAGAGAACCTACA	142
2DL3	ACAAGACCCTCAGGAGGTGA	GCAGGAGACAACTTTGGATCA	160
2DL4	TCAGGACAAGCCCTTCTGC	GACAGGGACCCCATCTTTC	130
2DL5	GCGCTGTGGTGCCTCG	GACCACTCAATGGGGGAGC	214
2DS1	GTAGGCTCCCTGCAGGGA	ACAAGCAGTGGGTCACTTGAC	148
2DS2	CTGCACAGAGAGGGGAAGTA	CAGAGGGTCACTGGGAGC	177
2DS3	ACCTTGTCCTGCAGCTCCT	AGCATCTGTAGGTTCCTCCT	160

2DS4-001	CAGCTCCCGGAGCTCCTA	TGACGGAAACAAGCAGTGGA	224
2DS4-003	CTTGTCCTGCAGCTCCATC	TGACGGAAACAAGCAGTGGA	202
2DS5	TGATGGGGTCTCCAAGGG	TCCAGAGGGTCACTGGGC	125
3DL1	TGAGCACTTCTTTCTGCACAA	TAGGTCCCTGCAAGGGCAA	129
3DL2	AAACCCTTCCTGTCTGCCC	TGGAAGATGGGAACGTGGC	134
3DL3	GCAATGTTGGTCAGATGTCAG	AGCCGACAACTCATAGGGTA	199
3DS1	TCCATCGGTTCCATGATGCG	GACCACGATGTCCAGGGGA	111
2DP1	ACATGTGATTCTTCGGTGTCAT	GTGAACCCCGACATCTGTAC	167
3DP1-001	GGTGTGGTAGGAGCCTTAG	GAAAACGGTGTTTCGGAATAC	280
3DP1-004	CGTCACCCTCCCATGATGTA	GAAAACGGTGTTTCGGAATAC	385

Table 5 : Respective length and nucleotide sequence for each pair of selected HLA genes primers (Nazari et al., 2015).

Gene	FP- Forward (5'-3')	RP-Reverse (5'-3')	Size (bp)
HLA-C1	GAGGTGCCCGCCCGGCGA	CGCGCAGGTTCCGCAGGC	332
HLA-C2	GAGGTGCCCGCCCGGCGA	CGCGCAGTTTCCGCAGGT	332
HLA-B-Bw4-Thr	GGAGCGAGGGGACCGCAG	GTAGTAGCGGAGCGCGGTG	344
HLA-Bw4-Ile	GAGCGAGGGGACCGCAG	GTAGTAGCGGAGCGCGATC	343
HLA-A1	TGGCGCCCCGAACCCTCG	GCTCTGGTTGTAGTAGCGGA	456

In order to perform PCR amplification with the best yield and in the most efficient way as possible, the PCR conditions are based on the papers of Ashouri et al. and Hong et al. with some modifications. Indeed, the PCR conditions are optimized for each KIR genes to obtain the best DNA amplicons (Ashouri et al., 2014, Hong et al., 2011).

We used a *QIAGEN One-Step RT-PCR kit* for typing both KIR and HLA genes. The *QIAGEN OneStep RT-PCR Enzyme mix* of this kit used for all the reactions permits us to ensure higher sensitivity, longer PCR products and higher yields compared to conventional Taq DNA polymerase.

For each sample of HF, SS and CS, 23 PCR amplifications are performed, 18 KIR genes and 5 HLA genes. Each one corresponds to one of the different KIR or HLA genes under investigation.

The Master Mix is prepared with the *QIAGEN One Step RT-PCR kit* with the same composition for each PCR reaction (*Table 6*).

Table 6 : Reagents and relative quantities used for a single sample PCR amplification reaction.

Reagents	X1 sample
H_2O	10µL
QIAGEN OneStep RT-PCR Buffer 5x	5µl
dNTPs mix 10mM	1µl
Primer Forward 10µM	2µl
Primer reverse 10µM	2µl
QIAGEN OneStep RT-PCR Enzyme mix (5-10U/µl)	1µl
Total	21µl

For every PCR reaction, 4µl (15 to 25 ng) of DNA sample is added to a 0.2 ml PCR tube containing 21 µL Master Mix.

The used cycling parameters are reported in the table below (*Table 7*). These parameters are used for 3DS1, 2DS1, 2DS2, 2DS5, 2DL4, 3DL1, 3DL2, 3DL3, 2DL1, 2DP1, 2DS4-0003 and 3DP1-0004.

Table 7 : PCR amplification parameters for the PCR with two different annealing temperature.

Step	Temperature	Time
Initialization	95°C	5min
Cycle 10x		
Denaturation	95°C	30 s
Annealing	58°C	45s
Elongation	72°C	59s
Cycle 30x		
Denaturation	95°C	30 s
Annealing	55°C	45s
Elongation	72°C	59s
Final elongation	72°C	10min
Final hold	4°C	∞

Two different annealing temperatures are used in order to have the expected DNA amplicons. In fact, thanks to a higher annealing temperature during the first 10 cycles, we obtain a better specificity for primer hybridization to the target region. By keeping the annealing temperature higher for all the

cycles, it is not possible to obtain the expected bands. That is why the annealing temperature is decreased after the first 10 cycles in order to have the specific DNA fragment.

For some other KIRs, the annealing temperature and the number of cycles are not the same as mentioned above. It was observed that the specific amplicons are obtained with one annealing temperature. The following parameters are used for 2DS3 and 3DP1-001.

Table 8: PCR conditions (one annealing temperature) of KIR2DS3 and KIR3DP1-001. The surrounded temperature was changed following the different KIR genes.

Step	Temperature	Time
Initialization	95°C	5min
Cycle 40x		
Denaturation	95°C	30 s
Annealing	54°C	45s
Elongation	72°C	1 min
Final elongation	72°C	10min
Final hold	4°C	∞

Table 8 is the same for the following KIR genes, except the annealing temperature (the surrounded temperature). Concerning 2DL2 and 2DL5, the optimum annealing temperature is 57°C; 55°C is the best for 2DL3; and finally, 58°C is chosen for 2DS4-001.

Concerning the 5 HLA genes, the PCR conditions are given in the following table (*Table 9*).

Table 9 : PCR amplification parameters for the HLA genes.

Step	Temperature	Time
Initialization	95°C	5min
Cycle 10x		
Denaturation	95°C	30 s
Annealing	57°C	45s
Elongation	72°C	59s
Cycle 30x		
Denaturation	95°C	30 s
Annealing	61°C	45s
Elongation	72°C	59s
Final elongation	72°C	10min
Final hold	4°C	∞

RNA free water as a negative control is included in each PCR run to ensure the absence of carryover contamination.

Note that a DNA positive control from Human Embryonic Kidney cell lines was used during PCR optimization of KIR genes. The efficiency of our PCR was assessed thanks to an internal control from the Human Growth hormone gene.

6. Gel electrophoresis of amplicons

6.1. Polyacrylamide Gel Electrophoresis (PAGE)

6.1.1. Principles

After PCR amplification, the PCR products are run on 4% polyacrylamide gel electrophoresis in order to determine the presence or absence of KIR genes in our samples. The results are reported into an *Excel* file.

PAGE is a widely used technique to separate biological macromolecules such as proteins and nucleic acids. This method is based on the electrophoretic mobility, which depends on the length, the conformation and the charge of a molecule (Stellwagen et al., 2009).

The formation of acrylamide molecules (C_3H_5NO) is done by hydration of acrylonitrile (C_3H_3N) to produce acrylamide thanks to nitrile hydratase. By mixing distilled water with acrylamide monomers in a powder state, the monomers polymerize and form polyacrylamide. This mechanism is helpful to form polyacrylamide gels with a different pore size in order to separate different weights of macromolecules. In fact, when the concentration of acrylamide increases, the pore size decreases. Small pore sizes are required for small molecules, while bigger pores are useful for large molecules (Stellwagen et al., 2009).

PAGE has many characteristics that make it attractive: it is synthetic, thermo-stable, transparent, strong, chemically inert, cheaper than agarose and can have a large ranch of pore sizes.

As DNA molecules are negatively charged due to the phosphate group (PO_3^-), the applied electric field causes the migration of DNA from the negative to the positive pole. Depending on the size, each DNA molecule will move differently through the gel matrix. Smaller molecules move farther down the gel (*Figure 18*).

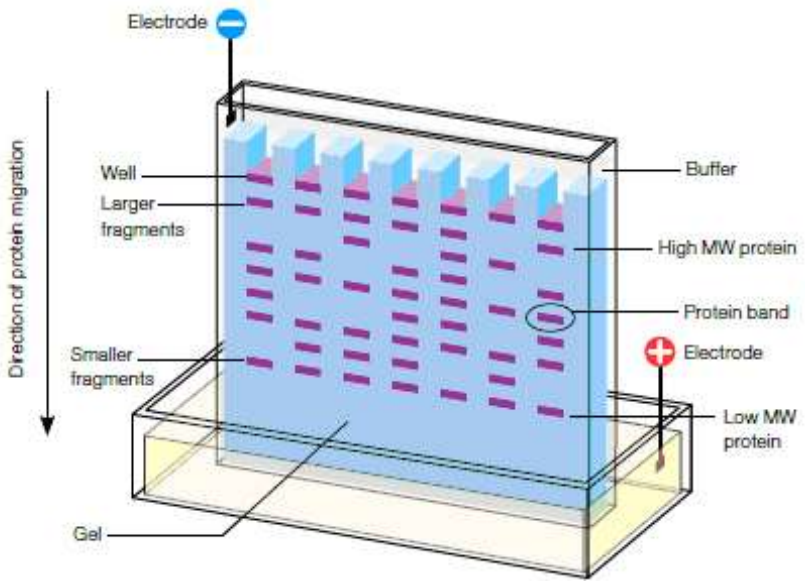


Figure 18 : Polyacrylamide gel electrophoresis. In most PAGE application, there are two buffer chambers and the vertical gel is between. The wells of the PAGE are made by inserting a comb into the PAGE. The electrical field forces the DNA to migrate thought the gel. The small DNA molecules migrate faster because they can easily pass through the pores of the gel than the big one (Protein Electrophoreses Methods, Bio-Rad, www.bio-rad.com, consulted on the 18/05/2018).

TBE buffer (Tris-Borate-EDTA) is the most commonly used buffer for the electrophoresis. Tris base and borate acid keep DNA deprotonated and soluble in water because of its basic properties, and the chelator EDTA plays a role against enzymes that degrade the DNA.

DNA markers or ladders are defined as DNA fragments of known length and are run in the same PAGE in order to hold the calibration. These markers are loaded in a separate lane and provide the approximate molecular length of unknown samples (*Figure 19, Figure 20*).

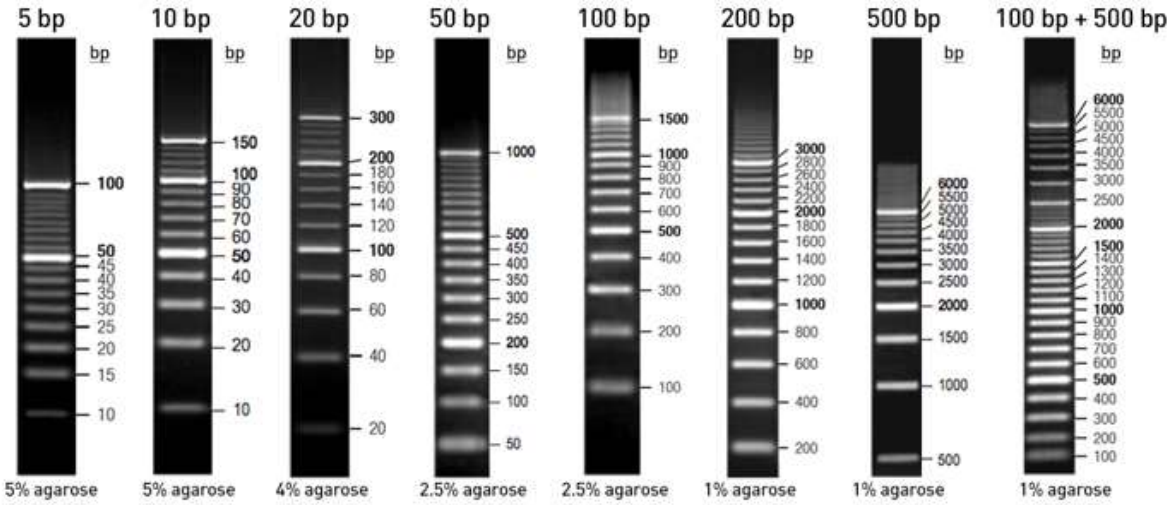


Figure 19 : Different types of DNA markers. The markers have higher density of bands at specific DNA size ranges to be suitable for an accurate sizing of DNA (O'RangeRuler Ladders, ThermoFisher scientific, www.thermofisher.com, consulted on 14/05/2018)

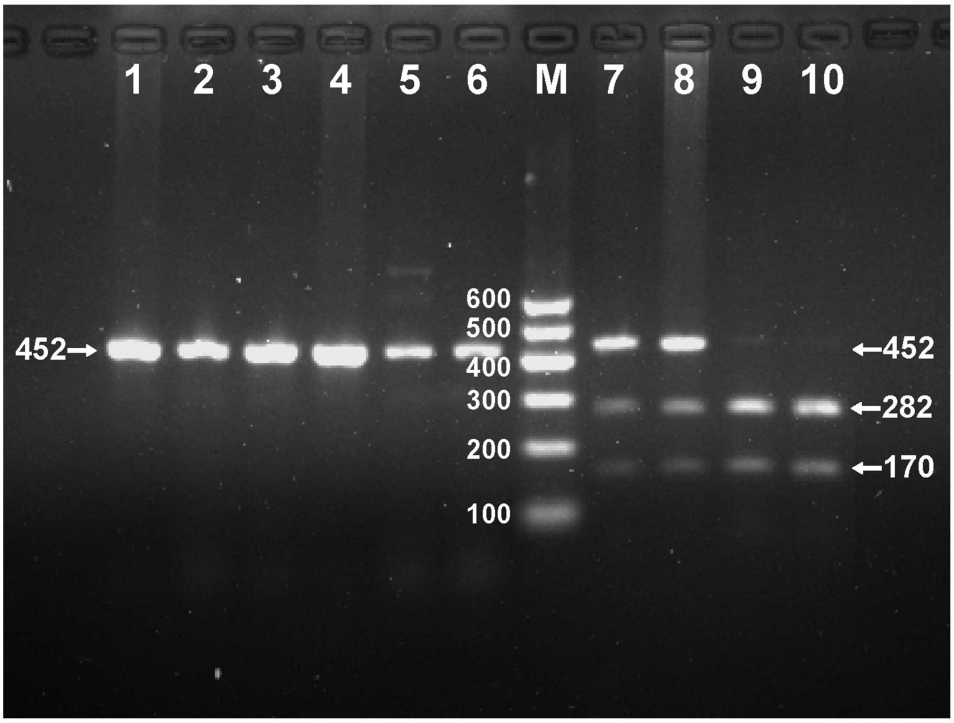


Figure 20 : Making size estimation of DNA samples thanks to DNA marker. M means "marker", and the numbers correspond to different DNA samples. To find the correct weight of a DNA fragment, a comparison between the marker and the sample bands is made (Wang et al., 2014).

A loading dye is used to ensure that the sample easily sinks into the well. It contains different dyes such as bromophenol blue (BPB, 3',3'',5',5'', tetrabromophenolsulfonphthalein), xylene cyanol FF, or orange G to track the DNA migration during electrophoresis. It also contains glycerol that increases the density of samples and EDTA for binding the divalent metal ions that may interfere with electrophoresis. EDTA also halts the DNA degradation by metal-dependent nuclease.

Ethidium bromide (EtBr) is an intercalant additive, used to detect DNA under ultraviolet (UV) light for decades. EtBr intercalates into the major groove of DNA (*Figure 21*). The light is released when the electrons of the aromatic ring of EtBr return to the ground state, after being excited by UV light. The intensity of bands shows the concentration of DNA (Sigmon et al., 1996). This dye is inexpensive, sufficiently sensitive and very stable. However, it is mutagen, and can pose a major health hazard to the user due to its toxicity.

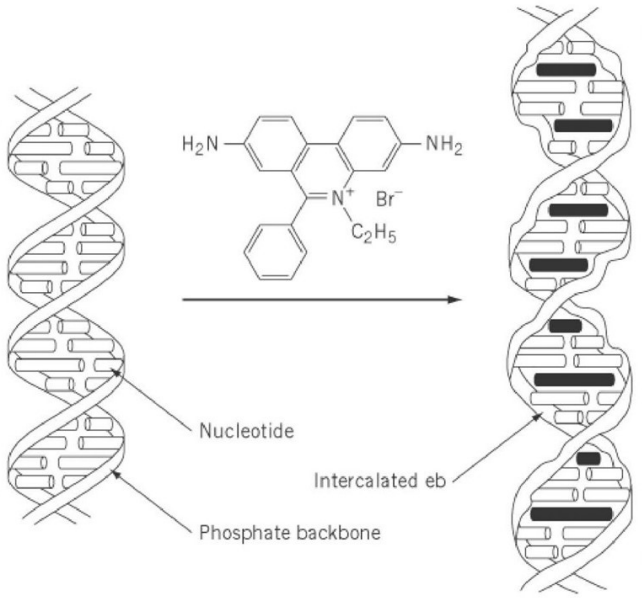


Figure 21 : The intercalation of EtBr into the DNA double helix. To the right, there are the normal DNA strands and to the left EtBr intercalates the DNA helix (Ethidium bromide, What-When-How- Depth tutorials and information, www.what-when-how.com, consulted on 12/06/2018).

EtBr has been replaced by Midori Green, which is a safe, non-carcinogenic and less mutagenic dye than EtBr. Midori Green contains double distilled water, fluorescent stain and hydrochloric acid (final concentration 0.3M). It possesses novel chemical features to minimize the interaction between the dye and the nucleic acid in living cells.

6.1.2. Protocol

Each well is loaded with 8 µl of amplicon, 1.5µl of 10x *Loading Dye* (*ThermoFisher DNA Gel Loading Dye- bromophenol blue and xylene cyanol FF)* and 0.5 µl of *Midori Green Direct DNA stain (Nippon Genetics®*). A 50 base pair ladder (*DNA Molecular Weight Marker XIII*) is used as DNA marker. The PAGE is run at 200 mV, 200 mA for 36 minutes and the PCR products are visualized under UV light using the gel imager camera.

The used buffer is *UltraPure™ TBE Buffer 10x (ThermoFisher*) which contains 1M Tris, 0.9M Boric acid and 0.01M EDTA. It is diluted 10x prior to its use.

For the gel preparation, safety measures must be followed because of the toxicity to the human nervous system of acrylamide. The preparation is realized under the hood. For a 150 ml preparation of acrylamide gel, 22.5 ml of 40% Acrylamide/Bis-acrylamide (AA/BA), 15 ml of 10x TBE and 6 ml of 1.6% ammonium persulfate (APS) is placed in a 250 ml cylindrical tube. Then, the 250 ml cylindrical tube is filled with distilled water up to 150 ml. 150 µl of Tetramethylethylenediamine (*TEMED- Sigma*

Aldrich) is finally added to catalyze the acrylamide polymerization. After having briefly inverted the cylindrical tube, the mix is poured in the mold. The gel was conserved at 4°C.

6.2. Agarose gel electrophoresis

6.2.1. Principles

Agarose gel electrophoresis has the same functions and mechanisms like PAGE. Its 3D matrix is made with agarose, one of the main components of agar. Helical agarose molecules in supercoiled bundles held together with hydrogen bonds form this 3D structure, with channels and pores, which allow DNA molecules to pass through. The pores of agarose are bigger than PAGE, making it suitable for large molecules (Lee et al., 2012). It is usually used for DNA fragments range between 50 and 20,000 bp. However, the resolving power is lower than PAGE.

The tray of agarose electrophoresis allows to run many samples at the same time, that is why we use it for some KIR and HLA genes. It is not used for all KIR genes because of its resolution and the DNA marker is usually not as well separated as on PAGE.

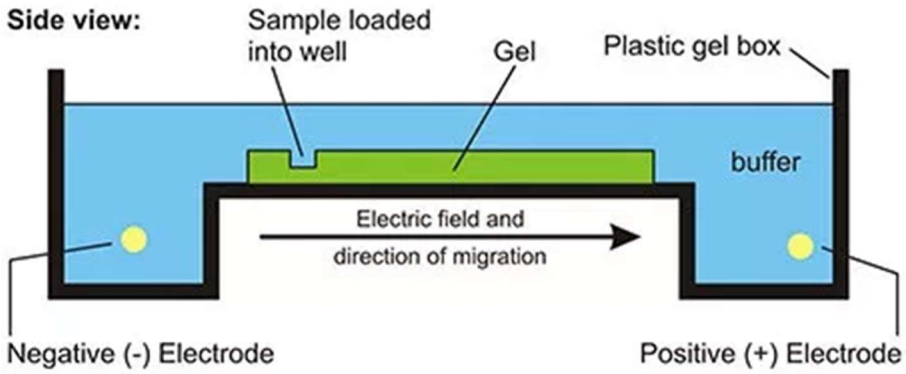


Figure 22 : Representation of the agarose gel electrophoresis. The gel is horizontal and completely covered by a 1x TBE buffer. Samples are loaded into wells preformed with a comb. The horizontal migration is due to an electrical field (Agarose gel Electrophoresis, Orbit BioTech Training, 2018, www.orbitbiotech.com, consulted on 25/05/2018).

A TBE buffer is also used for making the gel and migration of the PCR products. A TAE buffer (Tris-acetate acid- EDTA) can also be used and linear DNA can run faster in TAE than in TBE buffer. However, TBE buffer is better and finally used after optimization.

6.2.2. Protocol

First of all, we prepare the 1.5% m/V agarose gel. This concentration is based on the size of DNA amplicons.

To make 1.5% m/V of agarose gel, we add 1.5 g of agarose powder (*SeaKem®GTG® Agarose, Lonza*) into 100 ml of 1x TBE buffer (*UltraPure™ TBE Buffer 10x ThermoFisher*). To solubilize agarose powder into the buffer, the mix is heated in a microwave for a while. When the solution is totally clear and slightly warm, 3 to 5µl of *Midori Green Advance DNA stain (Nippon Genetics®)* is added. Afterwards, we pour the solution into the mold and a comb of 24 wells was placed. Within 15 to 30 minutes, the agarose gel is solidified and can be used (*Figure 23*).

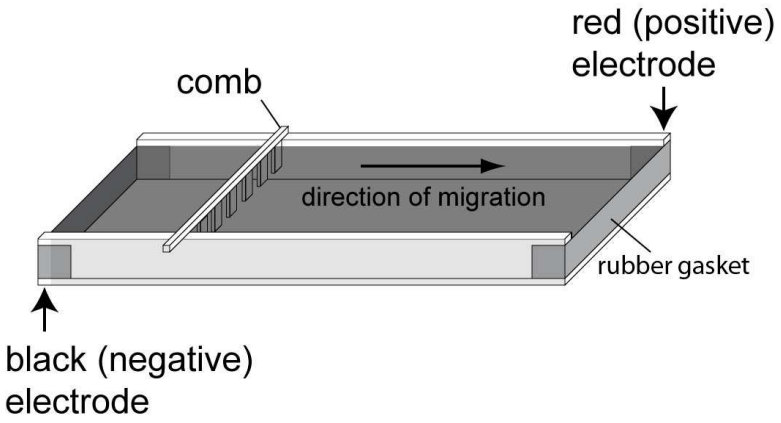


Figure 23 : Representation of an agarose gel electrophoresis. The comb is placed when the gel is still liquid near the negative electrode to form the well in the gel (B.Beason, 1999, Protocol for Agarose Gel Electrophoresis, Rice University, www.owlnet.rice.edu/~bios311/bios311/bios413/agarose.html, consulted on 18/06/2018).

Each well is loaded with 8 µl of amplicon and 2µl of 10x *Loading Dye* (*ThermoFisher DNA Gel Loading Dye- bromophenol blue and xylene cyanol FF)*. A 50 base pair ladder (*DNA Molecular Weight Marker XIII*) is used as DNA marker. The agarose gel is run at 120 mV, 400 mA for 45 minutes and the PCR products are visualized under UV light using the gel imager camera.

7. Visualization of PCR products

After the electrophoresis migration (agarose and PAGE), an UV light detection is performed in order to visualize the DNA fragments that are completely colorless. As mentioned before, the UV detection of the amplicons is possible thanks to Midori Green.

The UV detection *Westburg machine* is used for all of our migrations. Results of KIR and HLA genes are analyzed and reported as present (1) or absent (0) in an *Excel* file.

8. Data management

KIR and HLA genes are define as “present” or “absent” and results are reported on an *Excel* file. Due to sample size, the complexity and number of genes to analyze for each DNA sample, the statistical analysis is conducted by a biostatistician. The two-tailed Fisher Exact Test is performed in order to compare the three groups of EVD patients. This test is chosen to highlight possible links between the presence of KIR of HLA genes and the three groups of EVD patients. The null hypothesis is the equal proportions of KIR and HLA genes between the three groups, and the deviation from this null hypothesis is considered statistically significant when the p-value is < 0.05.

As preliminary results, we determine the genotype of 18 KIRs and 5 HLA class I molecules (HLA-A, HLA-B and HLA-C) in 52 fatal, 63 survivor and 56 contact samples of EVD.

Results

1. PCR amplification and gel electrophoresis

The picture of PAGE on *Figure 24* shows some fatal samples (HF 57 to 69) about KIR2DS1 with the DNA marker XIII (50 bp) as reference and a negative control (NC). The size of KIR2DS1 is 148 bp. The samples 58, 59, 63, 65, 66, 68 and 69 are negative because of the absence of the white band. These patients do not have KIR2DS1 in their genome. The white bands at the correct molecular weight for the other samples (57, 60, 61, 6, 64 ad 67) report the presence of KIR2DS1 in their genome. We can conclude on the quality of these results thanks to the negative control. It concludes that the Master Mix was not contaminated, and any unexpected DNA was amplified.

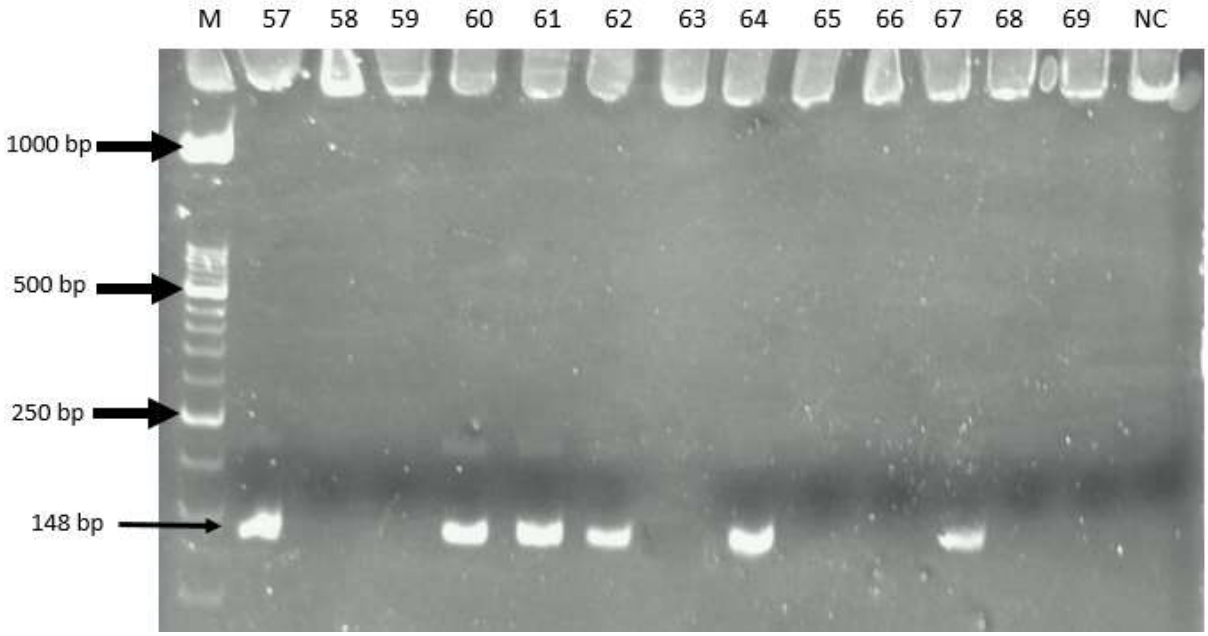


Figure 24 : PAGE electrophoresis of PCR showing the 50 bp DNA ladder (M), DNA samples (lanes 57-69) and the negative control (NC). The white bands are the light emits by the Midori after its excitation by the UV light. It means that the amplicons of 148 bp (KIR2DS1) are present in some of those samples.

Figure 25 shows the picture of agarose gel of some fatal samples (HF 66 to 80) about KIR3DL1 (129 bp), the negative control (NC) and the fatal samples (HF 42 to 48) about KIR3DL2 (134 bp), with DNA marker XIII (50 bp). The conclusions are drawn with the same way as above PAGE gel.

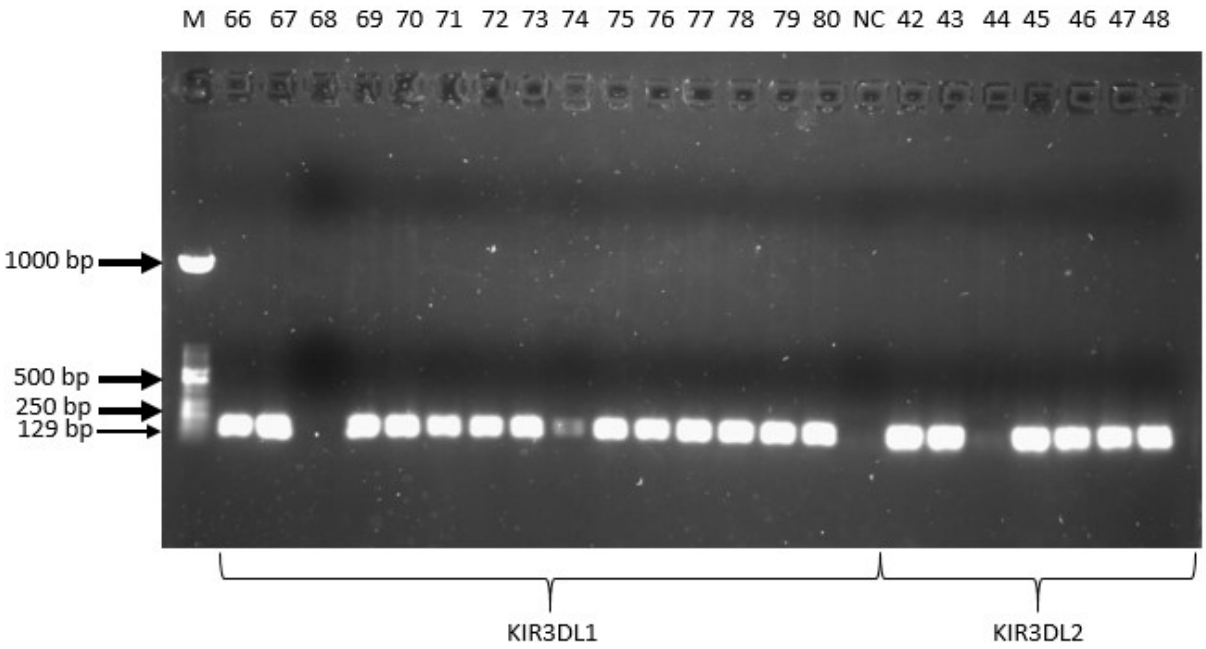


Figure 25 : Agarose gel electrophoresis of PCR showing the 50 bp DNA ladder (M), DNA samples (lanes 66 to 80 and 42 to 48) and the negative control (NC). The white bands are the light emits by the Midori after its excitation by the UV light. It means that the amplicons of 129 bp (KIR3DL1) and 134 bp (KIR3DL2) are present in some of those samples

From time to time, the results of some KIR are not easy to interpret because of the presence of a weak band or the presence of several nonspecific bands. For example, on *Figure 25*, the sample HF 74 shows a weak band. Every time, a band was not clear or weak, the corresponding sample was run again on the gel. If more than one band were present, either the sample was run again, or the sample was amplified one more time for having a better and clearer result.

Moreover, if the results were not clear after these repetitions, the PCR conditions were reviewed. The annealing temperature was increased or decreased in order to obtain the expected and specific band.

The *Figure 26* shows a picture of PAGE with several nonspecific bands, due to a nonspecific hybridization during PCR. Indeed, we obtained several nonspecific bands instead of one. An optimization of PCR amplification was required to increase the annealing temperature for getting one and specific DNA fragment.

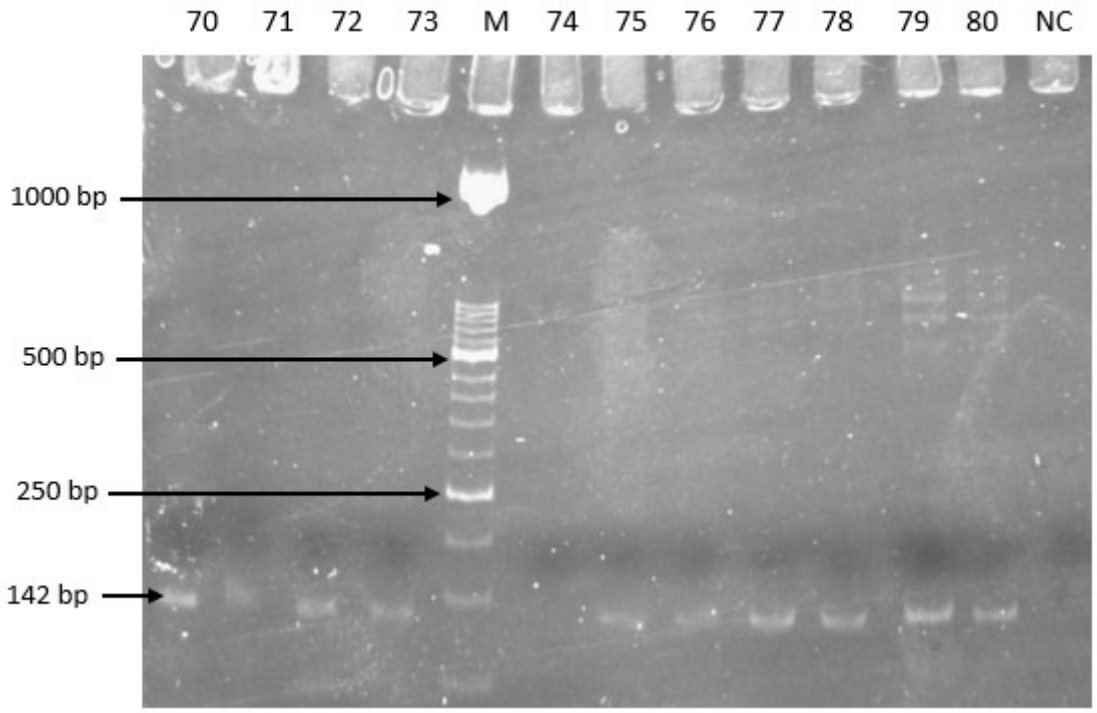


Figure 26 : PAGE electrophoresis of PCR showing the 50 bp DNA ladder (M), fatal DNA samples (lanes 70-80) and the negative control (NC). The white bands are the light emits by the Midori after its excitation by the UV light. It means that the amplicons of 142 bp (KIR2DL2) are present in some of those samples but nonspecific bands are also present in this gel.

Regarding HLA genes, they were all run in 1.5% agarose gel and the results were interpreted the same way as KIR genes (*Figure 27*). The DNA marker XIV (100 bp) was used in order to have a better separation and more reliable reference.

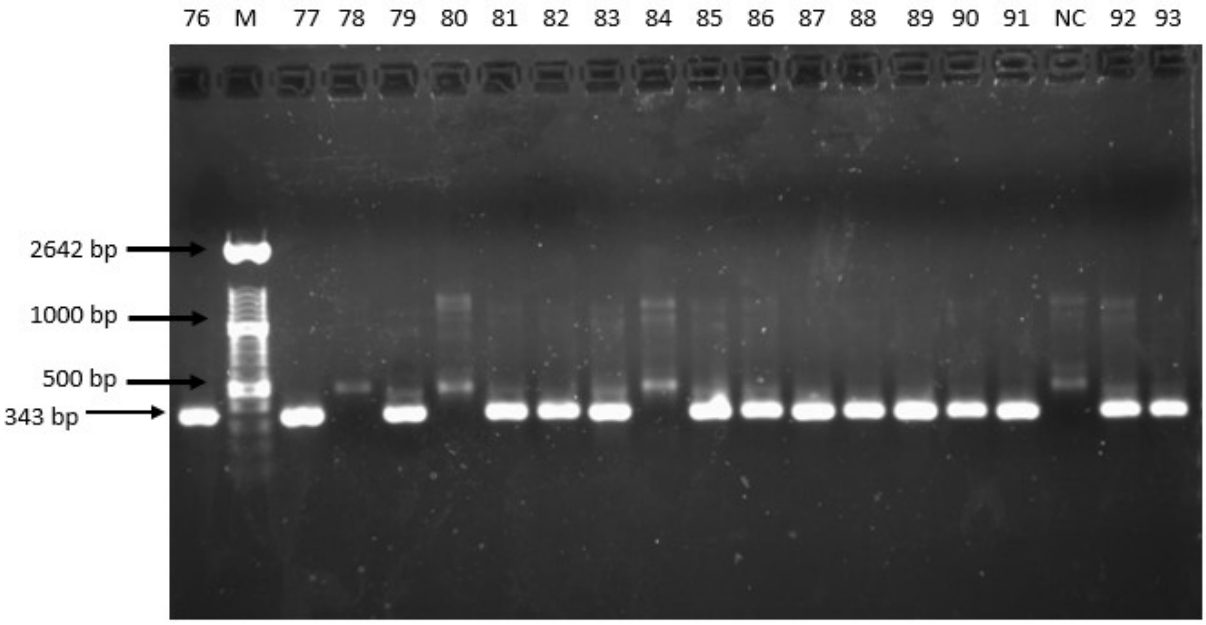


Figure 27: Agarose gel electrophoresis of PCR showing the 100 bp DNA ladder (M), survivor DNA samples (lanes 76-93) and the negative control (NC). The DNA bands are the light emits by the Midori after its excitation by the UV light. It means that the amplicons of 343 bp (HLA-B-Bw4-Ile) are present in some of those samples.

2. Statistical analysis

The results are based on the genotype of 18 KIRs and 5 HLA class I molecules (HLA-A, HLA-B and HLA-C) in 52 fatal samples, 63 survivor samples and 56 contact samples of EVD.

2.1 Frequency of KIR and HLA Genes

All different KIR and HLA genes are represented in each group, but in different proportions. The aim of this analysis is to determine the frequency of KIR and HLA genes in our three EVD groups. In fact, we want to spotlight if some genes could influence the susceptibility and/or outcome of EVD with the help of the Fisher Exact Test. The p-value <0.05 reports the significant deviation with the null hypothesis.

As you can notice on *Table 10*, with a presence of 100% for survivors against 84.6% and 82.1% for the fatalities and contacts respectively, KIR2DL2 is significantly more present in the survivor patients than in the fatal and contact patients (p-value= 0.0057).

KIR2DS4-001 is also significantly more present in survivor patients than in fatal and contact patients (p-value= 0.019), with a presence of 100% in survivors compared to 92.3% and 87.5% for the other groups.

In contrast, KIR2DL5 is significantly less present in survivor patients than in fatal and contact patients (p-value= 0.0076), with only a presence of 41.3% for the survivors against 69.2% and 60.7% for the fatalities and the contacts respectively.

Regarding KIR2DS2, the presence in fatalities is 51.9% against 47.6% and 42.9% for survivors and contacts respectively. KIR2DS2 is significantly more present in fatal patients (p-value= 0.007).

Concerning HLA genes, any significant difference is noticed between the three groups of EVD patients.

Table 10 : KIR and HLA gene frequency in each EVD patient group. The p-values considered statistical significant (< 0.05) are indicated by red color.

	Fatalities (n= 52)		Survivors (n=63)		Contacts (n=56)		
Inhibitory KIR genes	n	%	n	%	n	%	**p-value**
2DL1	52	100,0	63	100,0	56	100,0	NA
2DL2	44	84,6	63	100,0	46	82,1	0,0057
2DL3	48	92,3	62	98,4	50	89,3	0,12
2DL4	52	100,0	62	98,4	56	100,0	0,42
2DL5	36	69,2	26	41,3	34	60,7	0,0076
3DL1	51	98,1	63	100,0	56	100,0	0,32
3DL2	52	100,0	63	100,0	55	98,2	0,36
3DL3	52	100,0	63	100,0	56	100,0	NA
Activating KIR genes							
2DS1	35	67,3	31	49,2	35	62,5	0,12
2DS2	27	51,9	30	47,6	24	42,9	0,007
2DS3	18	34,6	17	27,0	19	33,9	0,61
2DS4-001	48	92,3	63	100,0	49	87,5	0,019
2DS4-003	30	57,7	26	41,3	30	53,6	0,18
2DS5	19	36,5	16	25,4	18	32,1	0,43
3DS1	4	7,7	1	1,6	6	10,7	0,12
Pseudogenes							
2DP1	50	96,2	63	100,0	56	100,0	0,099
3DP1-001	3	5,8	2	3,2	5	8,9	0,41
3DP1-004	51	98,1	63	100,0	55	98,2	0,55
HLA genes							
C1	36	69,2	50	79,4	39	69,6	0,37
C2	41	78,8	45	71,4	42	75,0	0,66
B-Bw4Ile	7	13,5	8	12,7	5	8,9	0,73
B-Bw4Thr	14	26,9	23	36,5	25	44,6	0,16
A1	15	28,8	15	23,8	18	32,1	0,59

2.2 KIR genotypes

In this study, 54 different KIR genotypes are highlighted from 52 samples of fatalities group, 63 samples of survivors group and 56 samples of contacts group (*Table 11*).

For making this *Table 11*, all genotypes were scrutiny observed in order to classify all these combinations of genes. Seven KIR genes (KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1 and KIR3DP1-004) are not mentioned in this table because they are almost present in our three groups.

This table shows the diversity of KIR genotypes among different EVD patients. The proportion of genotypes A and B is also showed and data show that the AB heterozygous and BB homozygous genotypes are most frequently associated with EVD patients. Some genotypes are present only in one group (for example the genotype number 54 which is only present in the contact patients) others are present in two groups (for example the genotype number 50 which is present in the fatal and contact patients) and others are present in each group in the same proportion (for example the genotype number 7) or in different proportion (for example the genotype number 13).

However there is an interesting trend about the AA genotype, which is not present in DNA samples from survivors.

Some genotypes are more represented in EVD patients than other genotypes. For example, the genotype number 5 (2DL1-4, 2DS4-001/3, 3DL1-3, 2DP1 and 3DP1-004) and number 12 (2DL1-4, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004) are represented respectively 23 and 20 times for a total of 171 patients. The percentage for genotypes number 5 and 12 are respectively 13.4% and 11.7%. Moreover, they are represented generously more among survivor patients.

The genotype number 13 (2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004) seems also very interesting. In fact, it is mainly represented in the survivor's group at 11.1% while this proportion is 5.8% and 1.9% for fatality and contact patients respectively.

Moreover, the survivor patients have a lower variability in KIR genotypes than the two others. In fact, the variability of survivor's genotypes is only 30% against 54% for the fatalities and 66% for the contacts.

Table 11 : KIR genotype of the three groups of EVD patients. 54 different KIR genotypes are highlighted which differed by the presence (shaded box) and absence (white box) of ten KIRs and one pseudogene.

		KIR genes											Fatalities (n=52)		Survivors (n=63)		Contacts (n=56)	
	Genotype	2DL2	2DL3	2DL5	2DS1	2DS2	2DS3	2DS4-001	2DS4-003	2DS5	3DS1	3DP1-001	n	%	n	%	n	%
1	AA		■					■	■				4	7,7	0	0,0	3	5,4
2			■					■					1	1,9	0	0,0	3	5,4
3			■						■				0	0,0	0	0,0	1	1,8
4	AB/BB	■	■	■	■	■	■		■				2	3,8	0	0,0	1	1,8
5		■	■					■	■				5	9,6	13	20,6	5	8,9
6		■	■	■				■	■				1	1,9	0	0,0	0	0,0
7		■	■	■	■	■		■		■		■	1	1,9	1	1,6	1	1,8
8		■	■	■	■				■	■			1	1,9	0	0,0	0	0,0
9		■	■	■	■	■	■	■		■	■		2	3,8	1	1,6	0	0,0
10		■	■	■	■			■	■	■			1	1,9	1	1,6	0	0,0
11		■	■		■			■					1	1,9	1	1,6	1	1,8
12		■	■					■					3	5,8	13	20,6	4	7,1
13		■	■	■	■	■	■	■					3	5,8	7	11,1	1	1,8
14		■	■	■	■	■		■		■	■		1	1,9	0	0,0	0	0,0
15		■	■	■		■		■	■	■			1	1,9	0	0,0	0	0,0
16		■	■	■	■	■		■	■	■		■	1	1,9	1	1,6	0	0,0
17		■	■	■	■	■	■	■	■				6	11,5	4	6,3	4	7,1
18			■	■	■			■					1	1,9	0	0,0	0	0,0
19		■	■	■	■			■		■			2	3,8	3	4,8	0	0,0
20			■	■	■			■		■			1	1,9	0	0,0	0	0,0
21		■	■	■	■	■		■	■	■			2	3,8	1	1,6	2	3,6
22		■	■	■	■	■		■		■			3	5,8	1	1,6	3	5,4
23			■		■			■					1	1,9	0	0,0	1	1,8
24		■		■	■	■	■	■	■				2	3,8	0	0,0	1	1,8
25		■	■	■				■	■	■			1	1,9	0	0,0	0	0,0
26		■		■	■	■		■	■	■			1	1,9	1	1,6	0	0,0
27		■	■	■	■		■	■	■		■		1	1,9	0	0,0	0	0,0
28		■		■	■	■			■	■			1	1,9	0	0,0	0	0,0
29		■	■			■		■				■	1	1,9	0	0,0	0	0,0
30		■	■			■		■	■				0	0,0	3	4,8	1	1,8
31		■	■	■	■	■	■	■		■			0	0,0	2	3,2	1	1,8
32		■	■	■	■	■	■	■		■			0	0,0	2	3,2	1	1,8
33		■	■		■	■		■		■			0	0,0	2	3,2	0	0,0
34		■	■		■			■					0	0,0	2	3,2	1	1,8
35		■	■			■		■					0	0,0	3	4,8	0	0,0
36		■	■	■	■			■		■			0	0,0	0	0,0	1	1,8
37		■	■	■	■			■		■	■		0	0,0	0	0,0	1	1,8
38		■	■	■	■		■	■			■	■	0	0,0	0	0,0	1	1,8
39		■	■	■	■			■		■		■	0	0,0	0	0,0	1	1,8
40		■	■	■	■		■	■		■	■		0	0,0	0	0,0	1	1,8
41		■		■	■	■	■	■	■	■			0	0,0	0	0,0	1	1,8
42		■	■			■			■			■	0	0,0	0	0,0	2	3,6
43		■		■	■		■	■		■			0	0,0	0	0,0	1	1,8
44		■	■		■			■	■				0	0,0	0	0,0	1	1,8
45		■		■	■			■		■			0	0,0	0	0,0	1	1,8
46		■		■	■	■	■	■					0	0,0	0	0,0	1	1,8
47		■	■	■	■			■	■		■		0	0,0	0	0,0	1	1,8
48			■	■			■	■	■				0	0,0	0	0,0	1	1,8
49		■	■	■	■	■		■	■				0	0,0	0	0,0	1	1,8
50		■	■	■	■		■	■					1	1,9	0	0,0	3	5,4
51		■		■		■			■	■			0	0,0	0	0,0	1	1,8
52			■	■	■			■	■	■	■		0	0,0	0	0,0	1	1,8
53		■	■	■	■	■			■				0	0,0	0	0,0	1	1,8
54		■	■	■	■	■	■		■	■	■		0	0,0	0	0,0	1	1,8
										Number of genotypes			28		19		37	

2.3 HLA genotypes

A total of 23 different HLA genotypes are found in our study. The genotype number 9 (HLA-C1/2, HLA-B-Bw4Thr and HLA-A1) is the most present, in all groups, and present 40 times. However, the proportion of this genotype is the same in the three groups. We can speculate that this genotype number 9 does not play a key role on susceptibility or outcome of Ebola virus disease.

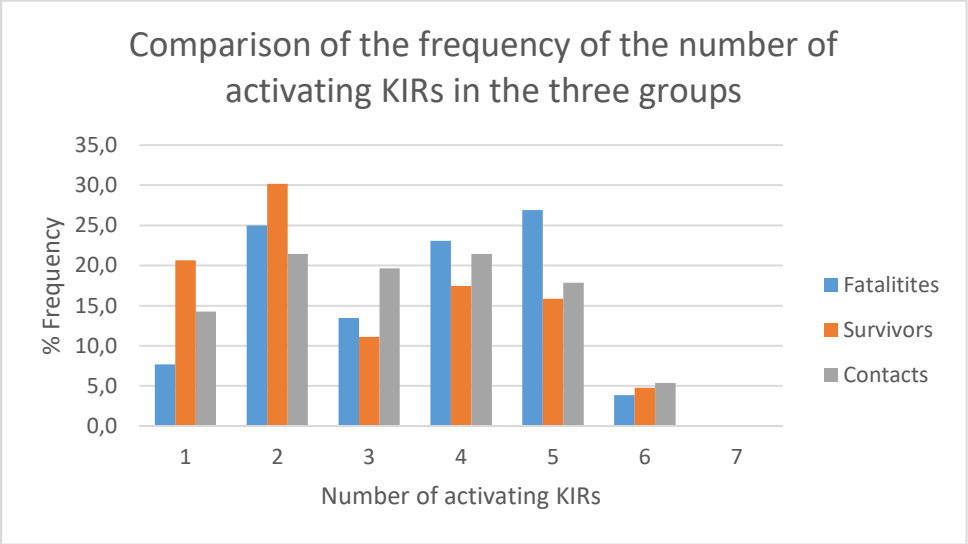
There are two genotypes which are more present in survivor patients: the number 1 (HLA-C1) with 11.1% for the survivors against 5.8% and 1.8% for the fatalities and contacts respectively, and the number 10 (HLA-C1/2 and HLA-B-Bw4Ile) with 7.9% for the survivor patients against 1.9% and 1.8% for the fatalities and contacts respectively.

Table 12 : HLA genotype of the three groups of EVD patients. 23 different HLA genotypes are highlighted which differed by the presence (shaded box) and absence (white box) of five HLA genes.

	HLA genes					Fatalities (n=52)		Survivors (n=63)		Contacts (n=56)	
	C1	C2	B-Bw4Ile	B-Bw4Thr	A1	n	%	n	%	n	%
1	■					3	5,8	7	11,1	1	1,8
2	■	■		■		2	3,8	4	6,3	7	12,5
3	■			■		3	5,8	7	11,1	5	8,9
4	■				■	3	5,8	2	3,2	4	7,1
5	■	■			■	5	9,6	4	6,3	2	3,6
6		■		■		2	3,8	4	6,3	4	7,1
7		■				7	13,5	6	9,5	5	8,9
8	■	■				14	26,9	14	22,2	12	21,4
9	■	■		■	■	1	1,9	5	7,9	3	5,4
10	■	■	■			1	1,9	5	7,9	1	1,8
11		■		■	■	1	1,9	0	0,0	3	5,4
12	■		■			1	1,9	1	1,6	1	1,8
13						1	1,9	0	0,0	0	0,0
14		■	■	■	■	1	1,9	1	1,6	0	0,0
15		■			■	3	5,8	1	1,6	4	7,1
16	■	■	■	■	■	1	1,9	1	1,6	0	0,0
17	■	■	■	■		2	3,8	0	0,0	0	0,0
18		■	■	■		1	1,9	0	0,0	1	1,8
19	■	■	■		■	0	0,0	1	1,6	0	0,0
20				■		0	0,0	1	1,6	0	0,0
21	■		■	■		0	0,0	0	0,0	1	1,8
22	■		■		■	0	0,0	0	0,0	1	1,8
23	■			■	■	0	0,0	0	0,0	1	1,8
Number of genotypes						18		16		17	

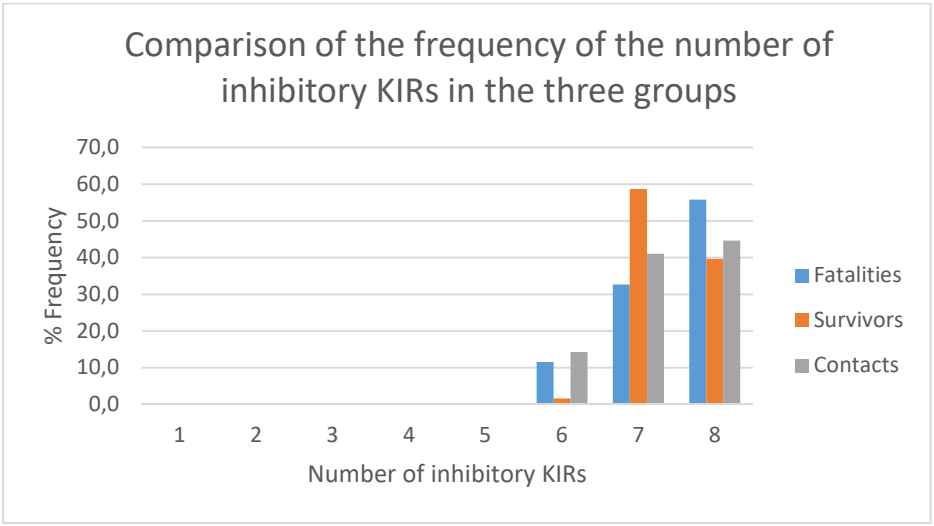
2.4 *Proportion of activating and inhibitory KIRs*

The frequency of activating KIR genes varies between the three groups (*Graph 1*). However, the survivor patients tend to carry less activating KIRs than the fatal and contact patients. This trend can play a role in the development and outcome of EVD.



Graph 1 : Comparison of the frequency of activating KIRs in the three groups of EVD patients.

The frequency of inhibitory KIRs varies also in the three groups (*Graph 2*). However, each group has at least 6 inhibitory KIR receptors. Moreover, carrying more inhibitory KIRs can play an important role in the resistance against the EVD. Indeed, fatalities group own more genotype with 8 inhibitory KIRs than the two other groups.



Graph 2 : Comparison of the frequency of inhibitory KIRs in the three groups of patients.

3. Statistical analysis of all results

These results are based on the genotype of 18 KIRs and 5 HLA in all DNA samples, including 56 contact, 102 survivor and 121 fatal samples.

Thanks to two-tailed Fisher Exact Test, some significant differences are highlighted between the frequency of the different genes in all samples from contact, survivor and fatality patients.

First of all, 2DL2 is significantly more frequent in fatal and in contact groups compared to survivor group (p-values = 0.0001 and 0.0006).

Moreover, 2DL5, 2DS4-001, 2DS4-003 and HLA-B-Bw4 all have significant differences between fatal and survivor groups. 2DL5 is significantly more frequent in fatal group than in survivor group (p-value= 0.0217). 2DS4-003 is significantly less frequent in survivor than in fatal samples (p-value= 0.0436). Concerning 2DS4-001, it is less frequent in fatal than in survivor samples, but this difference is not significant (p-value= 0.0868).

Finally, HLA-B-Bw4-Ile is significantly less frequent in fatal than in survivor samples (p-value= 0.0192).

Discussion & Perspectives

As mentioned in previous studies, the presence or absence of KIR and HLA genes and their interactions are interesting because of the correlation with the development and outcome of some viral infections (Rajagopalan et al., 2005; Khakoo et al., 2004; Martin et al., 2002).

The aim of this study is to determine the KIR and HLA genotypes of EVD patients from three different groups and to assess the association of KIR/HLA combinations with the development and outcome of EVD. The KIR and HLA genotypes of contacts, survivors and fatalities were scrutiny observed and compared in order to highlight some KIR and HLA combinations that might play a role in the susceptibility of EVD.

Using PCR amplification, we obtained the KIR and HLA genotypes of all DNA samples from EVD contact, survivor and fatal patients. Thanks to this, we have spotted some tendency that might be relevant for further investigations.

1. KIR gene observations

First of all, by ranking the samples into different KIR genotypes, we find that the variability of KIR genotypes is lower in the survivor group than in the other groups.

Moreover, some KIR genotypes are more represented in the survivor group compared to fatalities and contacts. These genotypes are composed by these KIR genes: “2DL1-4, 2DS4-001/3, 3DL1-3, 2DP1 and 3DP1-004”, “2DL1-4, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004” and the last one is “2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004”. Knowing that the variability of KIR genotypes in survivor patients is low, and these three genotypes are more present in this survivor group, we hypothesize that these three above genotypes might confer a protection against EVD by providing an immune microenvironment fostering a positive outcome of EVD.

We also observe that KIR2DL2 and KIR2DS4-001 are significantly more present in survivor patients, while KIR2DL5 and KIR2DS2 are significantly more present in fatal patients. This meets the observation made on the most observed genotypes in the survivor group except for one genotype (2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004), which own the KIR2DL5 and KIR2DS2.

KIR2DS2 has been previously associated with the susceptibility to type I diabetes when it interacts with HLA-C1 (Van der Slik et al., 2003) and KIR2DL5 has been associated with the detrimental impact on

head and neck squamous cell carcinoma in Iranians (Barani et al., 2018). We hypothesize that KIR2DS2 and KIR2DL5 might also increase the mortality risk of EVD.

The trends concerning KIR2DL5 and KIR2DS2 exposed with the entire set of results are similar to the partial set of results. However, KIR2DL2 is significantly more represented in survivor group when the results are partial rather than all results are taken into account. Moreover, KIR2DS4-001 is significantly more frequent in the survivor group, while it is not significantly different in the entire set of results. In contrast, KIR2DS4-003 does not show a significant difference between fatalities and survivors in the preliminary results, while it is significantly less frequent in the survivors than in fatalities in the entire set of results. All these differences are to notice because they show the importance of the size of sampling to conclude on the quality of the results.

The interaction between KIR2DL2 and HLA-C1 confers a risk of hepatitis C virus-related hepatocellular carcinoma in younger patients (Saito et al., 2018). With the data of our study showing that KIR2DL2 is significantly more frequent in fatalities and contacts, we hypothesize that KIR2DL2 may be a marker for increased the risk of mortality by EVD.

KIR2DS4 has been associated with a protective impact on head and neck squamous cell carcinoma in Iranians (Barani et al., 2018). It has been also showed that KIR2DS4 may be involved in the development of hepatitis C by inducing damage to the hepatic parenchyma (Podhorzer et al., 2018). However, with the data of our study showing that KIR2DS4-003 is significantly less frequent in the survivors than in fatalities, it is possible that KIR2DS4-003 might increase the fatal EVD outcome.

Concerning the KIR genotypes, we observe that the AB heterozygous and BB homozygous genotypes are the most represented genotypes in our study samples. Moreover, the AA homozygous genotypes are only present in fatalities and contacts, while survivor patients do not own this genotype. Previous study shows the association of AA genotype with a protective impact on head and neck squamous cell carcinoma in Iranians (Barani et al., 2018). It is up most important to investigate this tendency because owning AA homozygous KIR genotype can be associated with the mortality risk of EVD. Indeed, this AA genotype can also provide a certain permissive immune microenvironment fostering a fatal outcome of EVD. However, the study of Wauquier et al., in 2010, showed the opposite trends and demonstrated that the AA homozygous genotypes are the most represented genotypes (Wauquier et al., 2010). Moreover, Wauquier et al. illustrated that AA homozygous KIR genotypes can be present in survivor patients, while we notice its absence in our survivor samples. It has been also demonstrated that KIR2DS1 and KIR2DS3 can have a potential effect in fatal outcome of EVD while this significant difference is not observed in our study (Wauquier et al., 2010). The sample size and origin of samples might be the reason of these differences between our study and Wauquier's study. Indeed, KIR genes

are considerably variable, and KIR haplotypes varies between individuals but also between populations.

Regarding the activating and inhibitory KIR receptors, survivors tend to own less activating KIR genes than the other groups, while fatalities tend to own more inhibitory KIR receptors. These trends were also observed by Wauquier's study and strengthen the observation that KIR genes could play a role in the susceptibility and outcome of EVD (Wauquier et al., 2010). However, the presence of more than seven inhibitory KIR genes has been previously associated with a protection against reactive arthritis (Sun et al., 2018). The presence of less activating KIRs in survivor patients may be linked to the three highlighted KIR genotypes and confer a protection against EVD.

The preliminary results of the same project conducted few months ago in Prof. Maes' lab at the REGA Institute for Medical research, KU Leuven University (data not published, "The association of KIR genes with the outcome of Ebola virus disease in Guinea") showed exactly the same trends. This study confirms and expands preliminary results from the lab.

2. HLA gene observations

Less observations are made with the HLA genotypes. Some HLA genotypes seem to be more represented in the survivors groups. These genotypes are composed by these HLA genes: "HLA-C1" and "HLA-C1/2 and HLA-B-Bw4Ile". Moreover, one HLA genotype (HLA-C1/2, HLA-B-Bw4Thr and HLA-A1) is highly represented in our EVD patients.

With all results, we have observed that HLA-B-Bw4Ile is significantly more present in the survivor group than in the two others. Moreover, Martin et al. showed that this HLA gene can delay the progression of AIDS if it interacts with KIR3DS1 (Martin et al., 2002). We hypothesize that this HLA gene can confer a protection against EVD.

Similarly to KIRs, HLA genes also show sequence diversity. This influences the expression and the interaction with KIR receptors. Moreover, patients can carry the HLA-B-Bw4Ile gene but do not express it, explaining the fatality of certain patients who own this HLA-B-Bw4Ile gene.

3. KIR-HLA interactions

HLA-C is the most represented HLA gene in individuals and is the KIR ligand for KIR2DS4-001, KIR2DS2 and KIR2DL2, which are more present in fatal patients.

It is also showed that HLA-B-Bw4Ile correlates with the development of ebolavirus. However, its KIR ligand is KIR3DS1, which is almost not be present in the EVD patient's genotypes from Guinea.

4. Perspectives

Since the variability of KIR and HLA genes is considerable, and genotypes varies amongst but also between the populations, we propose to analyze additional individuals in the same region but also in other affected countries.

To enhance and get a better understanding of KIR and HLA combinations that confer a protection against EVD or influence the EVD outcome, the DNA-sequencing of relevant KIR and HLA genes needs to be done in order to find the genetic variations or polymorphisms that could predict the susceptibility to EVD.

A further study on other genes that are involved in the innate immune response for EVD (Toll-like receptor, cytokines, TNF-alpha, NKG2) can also be very relevant. This new investigation can bring new observations of genetic susceptibility to EVD.

Conclusion

Ebolavirus infection is often characterized by a rapid depletion in NK cells and lymphocytes caused by an excessive activation of the immune response (Wauquier et al., 2010). This master's thesis shows a correlation between some KIR and HLA combinations and the susceptibility and outcome of EVD. The presence of activating KIR genes, in interaction with their appropriate ligands, promotes the activation of NK cells. Indeed, the activating KIRs act by destroying infected cells, and participate to the over-activation of the immune response during EBOV infection.

The three KIR genotypes, "2DL1-4, 2DS4-001/3, 3DL1-3, 2DP1 and 3DP1-004", "2DL1-4, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004" and the last one "2DL1-5, 2DS1-3, 2DS4-001, 3DL1-3, 2DP1 and 3DP1-004", which are more represented in survivor patients, support our hypothesis. Indeed, by presenting only few activating KIRs, they prevent the over-activation of the immune response and confer a protection against EVD.

Moreover, survivors tend to own less activating KIR receptors. This observation is also boosted by this above hypothesis and the presence of less activating KIRs correlate with a protective effect against EVD.

KIR2DS4-003 and KIR2DS2 are activating KIR receptors and might increase the fatal EVD outcome. This observation supports also our hypothesis because these KIRs can participate to the over-activation of the immune response by fostering the activation of NK cells.

Furthermore, the results suggest that these two HLA combinations "HLA-C1" and "HLA-C1/2 and HLA-B-Bw4Ile" are more represented in survivor patients and could provide with their appropriate ligands an immune microenvironment fostering a positive outcome of EVD.

In summary, there is evidence that KIR and HLA combinations may influence the susceptibility and outcome of EVD.

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