FROM EBOLA VIRUS DISEASE TO BACTERIAL TOXICITY

An in silico and in vivo study

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1. INTRODUCTION

The next research project is an approach to Reverse Vaccinology and how it can be used to improve the current vaccines. I will focus this technology on the possible creation of a new vaccine against the Zaire Ebolavirus, which is the most lethal disease among the other species for the genus Ebolavirus. Zaire Ebolavirus, also known as ZEBOV, is responsible for the last Ebola outbreak in West Africa, dated 2014-2016.

The main questions which will be answered on this project are the following ones:

- What is Ebola?
- Which are its signs and symptoms?
- How has it evolved through the years?
- How does our body answer to an Ebola infection?
- Why is so important to develop a vaccine against it?
- What is Reverse Vaccinology?
- How does it improve the current vaccines?

I thought that it was important to perform a practical part which involved experiments and laboratory techniques. The main problem was the lack of a Biosafety level 4 laboratory next to Barcelona. In fact, there is only one Level 4 laboratory in Spain, but it is located near Madrid. Highly dangerous illness -such as the Ebola virus disease, the smallpox virus, the Hantavirus and the Lassa haemorrhagic fever- can only be studied in this kind of laboratories.

Finally, the back-up plan consisted in working with some crustaceans called *Daphnia magna* and study how some bacteria interacted and infected these specimens. This study of the phagocytosis in *Daphnia magna* could help me to understand how our body protects itself from an Ebola infection.

The different objectives that this project follows are the next ones:

1. The creation of figures to explain a ZEBOV infection
2. To collect statistical data on ZEBOV infections and to present data in tables and use geolocation tools using heat maps and choropleth maps.
3. To explain the defense mechanisms of our body.
4. To analyse the social knowledge about ZEBOV with a survey.
5. To explain the use of Reverse Vaccinology in a ZEBOV infection.
6. To create a scientific article and a scientific poster.
7. To use *in silico* procedures to study the ZEBOV proteins.
8. To create a website with interactive image maps.

2. MATERIALS AND METHODS

- **RStudio:** R professional software is used for statistical computing and developing graphics and plots. Different Heatmaps in this paper (Figures 3 and 4) were developed using the "plotly" library for R. In the annexed scientific article, RStudio is more widely used.

- **GIMP 2.8:** This open-source software was used to develop figures which helped to visualize the EBOV procedures (for example, Figure 1). It is also capable to develop basic image maps.

- **PAINT 3D:** It is one of the easiest 3D applications to use and it is completely free.

- **Sublime text:** This text editor for code was used to finishing the image maps which were first created with GIMP, adding new features as highlighting areas when the mouse goes above them. The image maps could be saved as HTML files and then uploaded to my web page at neocities.org.

- **CSS:** Cascading Style Sheets. It was used to change the style of the website which was developed in this project.

- **Bootstrap:** Bootstrap is a web framework for designing websites and web applications. It was used to design the website.

- **Mapper.js:** This plugin can be used through Sublime text and it is used to highlight areas when the mouse goes above them.

- **Neocities.org:** It is a web hosting service where different websites can be created. HTML files can be uploaded to it.

- **Google Fusion Tables:** This web application is used to visualize sets of data geolocating these sets. Heatmaps are one of its functions.

- **Google Forms:** It was used to create and analyse the survey that it is shown in this paper.

- **Different software packages by the Swiss Institute of Bioinformatics:** This includes SwissDock, SwissParam, SwissSidechain, SwissBioisostere, SwissTargetPrediction, SwissADME and SwissSimilarity. The most remarkable ones are SwissDock, used to study the interactions between two molecules and the energy their links have; SwissSimilarity, used to find similar molecules to the one that it's being studied; SwissADME, used to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules; and SwissTargetPrediction, used to predict the targets of a small molecule. All these software packages only work with small molecules.

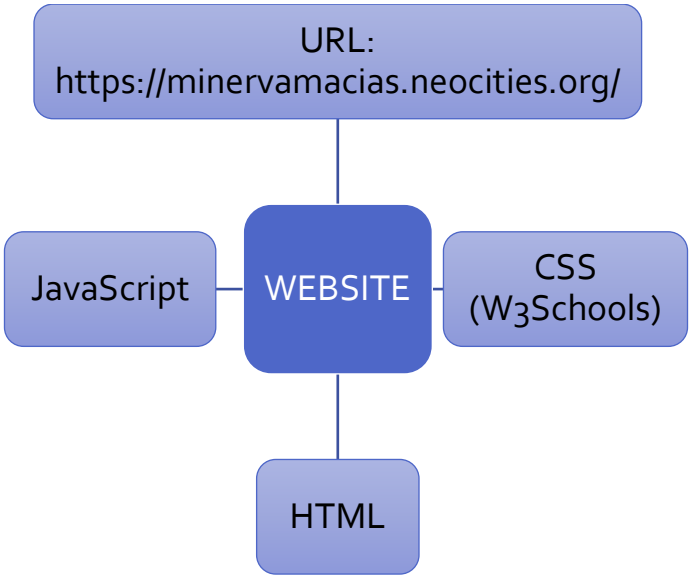
- **Vaxign and Vaxitop:** This software was the first web-based vaccine design system that predicted vaccine targets based on genome sequences. When it was first released, the Vaxign database contained a prediction of vaccine targets for >70 genomes. In January 2018, this number has increased to >350 genomes

- **NERVE:** NERVE is the acronym of New Enhanced Reverse Vaccinology Environment and it consists on a software which identifies the best vaccine candidates from the whole proteome of a pathogen. The software is remarkably complete because it shows the vaccine candidates on an HTML table coming with relevant information and links to the candidate data. The software chooses the candidates taking into account their safety and whether they are easy to experiment with or not.

- **RANKPEP:** It ranks all the peptides the user uploads using the PSSM coefficients. The PSSM means position-specific scoring matrix and it is used to distinguish binding sites from non-functional sites which have similar sequences. This way, the scientists are able to predict the peptide bonding. It is similar to Vaxign but not as accurate.

- **Immune Epitope Database and Analysis Resource (IEDB):** It compiles information about immune epitope information to facilitate the developing of tools, diagnostic techniques, vaccines and therapeutics.

- **Scheme of the methods used in the creation of the website:**



- **The nature of our project:**

  Our experiments were based on *in silico* procedures.

3. RESULTS

3.1. THE EBOLA VIRUS DISEASE (EVD)

The EVD is a virus which first infected wild animals as great primates and cephalophus [1] among many other animal species. It was first studied in 1976, when it appeared in South Sudan and in the Democratic Republic of Congo at the same time [2].

3.1.1 SYMPTOMS

According to the World Health Organization (WHO), the main symptoms and the first signs of the illness are periods of fever, feeling weak and without any energy, headaches, muscle pain and sore throat (Figure 1). It takes two to twenty-one days for these symptoms to appear. This is because the incubation period may vary among patients [3].

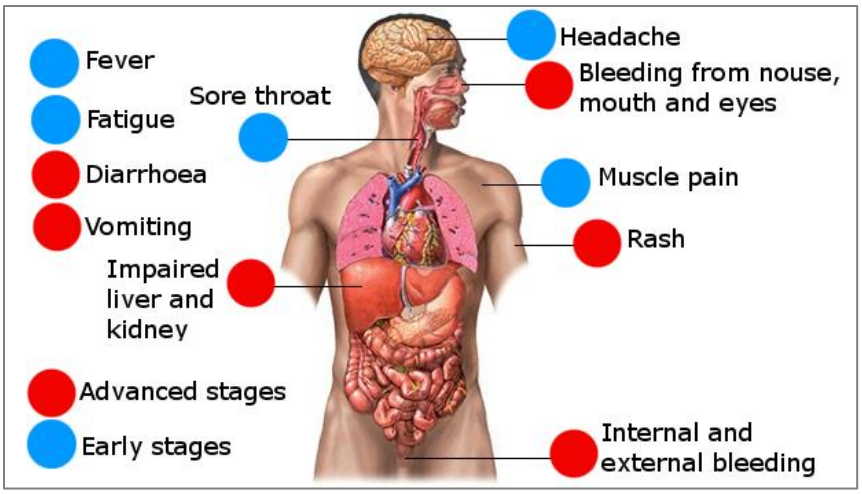


FIGURE 1. EBOLA SYMPTOMS THROUGH ITS STAGES. BLUE DOTS REPRESENT THE SYMPTOMS WHICH TAKE PLACE ON THE EARLY STAGES WHILE THE RED DOTS REPRESENT THE ADVANCED STAGES SYMPTOMS. THE INFORMATION WAS EXTRACTED FROM THE WORLD HEALTH ORGANIZATION'S WEB PAGE. THE SCHEME WAS DONE USING THE GIMP 2 SOFTWARE.

After that first symptoms, diarrhoea, rashes, impaired liver and kidney and vomiting are the next symptoms which usually appear on the infected people. In some cases, in the final stages, it could also appear internal and external bleeding.

3.1.2 CHRONOLOGY OF EBOLA VIRUS DISEASE OUTBREAKS (1976-2014)

YEAR	COUNTRY	EBOLAVIRUS SPECIES	CASES	DEATHS	CASE FATALITY
1976	Democratic Republic of Congo	Zaire	318	280	88%
1976	Sudan	Sudan	284	151	53%
1977	Democratic Republic of Congo	Zaire	1	1	100%
1979	Sudan	Sudan	34	22	65%
1994	Gabon	Zaire	52	31	60%
1994	Côte d'Ivoire	Taï Forest	1	0	0%

1995	Democratic Republic of Congo	Zaire	315	254	81%
1996 (Jan-Apr)	Gabon	Zaire	31	21	68%
1996 (Jul-Dec)	Gabon	Zaire	60	45	75%
1996	South Africa (ex-Gabon)	Zaire	1	1	100%
2000	Uganda	Sudan	425	224	53%
2001-2002	Gabon	Zaire	65	53	82%
2001-2002	Democratic Republic of Congo	Zaire	59	44	75%
2003 (Jan-Apr)	Democratic Republic of Congo	Zaire	143	128	90%
2003 (Nov-Dec)	Democratic Republic of Congo	Zaire	35	29	83%
2004	Sudan	Sudan	17	7	41%
2005	Democratic Republic of Congo	Zaire	12	10	83%
2007	Uganda	Zaire	264	187	71%
2007	Democratic Republic of Congo	Bundibugyo	149	37	25%
2008	Democratic Republic of Congo	Zaire	32	14	44%
2011	Uganda	Sudan	1	1	100%
2012	Uganda	Sudan	24	17	71%
2012	Uganda	Sudan	7	4	57%
2012	Democratic Republic of Congo	Bundibugyo	57	29	51%
2014-2016	Guinea	Zaire	3811*	2543*	67%
2014-2016	Liberia	Zaire	10675*	4809*	45%
2014-2016	Sierra Leone	Zaire	14124*	3956*	28%
2014	Nigeria	Zaire	20	8	40%
2014	Mali	Zaire	8	6	75%
2014	Senegal	Zaire	1	0	0%
2014	USA	Zaire	4	1	25%
2014	UK	Zaire	1	0	0%
2014	Spain	Zaire	1	0	0%
2014	Democratic Republic of Congo	Zaire	66	49	74%
2015	Italy	Zaire	1	0	0%

*Includes Suspect, Probable and Confirmed EVD cases.

TABLE 1. TABLE SHOWING ALL THE OFFICIAL EVD'S OUTNREAKS THROUGH THE YEARS. TABLE EXTRACTED FROM THE WORLD HEALTH ORGANIZATION.

To view all this information represented on an interactive map, go to: **https://minervamacias.neocities.org/InteractiveOutbreaks/**

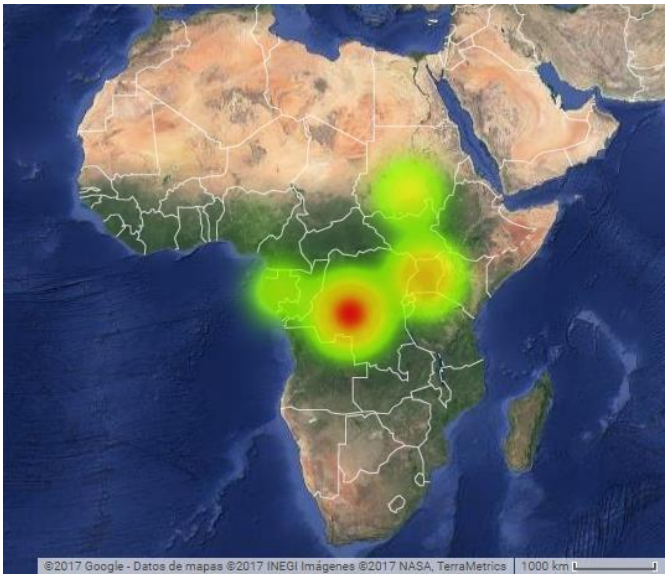


FIGURE 2. HEATMAP SHOWING THE PRINCIPAL EBOLA OUTBREAKS ON AFRICA. MAP CREATED USING GOOGLE FUSION TABLES AND GOOGLE MAPS. GUINEA, LIBERIA AND SIERRA LEONE OUTBREAKS ARE EXCLUDED BECAUSE OF THE LACK OF RIGOUR ITS DATA HAS.

The most important outbreaks of Ebola have taken place in the Democratic Republic of Congo, Uganda, Sudan and Gabon (Figure 2, Table 1). The lack of rigour on the Guinea, Liberia and Sierra Leone's outbreaks make impossible to measure how important and fatal had these infections been.

All the African countries which have suffered from Ebola are near the main Megabat colonies, also known as fruit bats. At first, they were thought to be the main source of infection. These first hypothesis have been successfully proven, now including not only bats, but also other animals as duikers, gorillas and chimpanzees [4, 5] (Table 2). Eating these dead bodies, a practise which is very extended among African indigenous peoples, could be one of the main ways of getting infected.

OUTBREAK LOCATION, YEAR	COUNTRY	EBOLAVIRUS SPECIES	NUMBER OF CASES	NUMBER OF DEATHS	ANIMAL SOURCE
Cote d'Ivoire, 1994	Cote d'Ivoire	EBOV-IC	1	0	Chimpanzee
Mekouka, 1994	Gabon	EBOV-Z	52	31	Chimpanzee, gorilla
Mayibout, 1996	Gabon	EBOV-Z	33	23	Chimpanzee
Booué, 1996	Gabon	EBOV-Z	60	45	Chimpanzee
Mekambo, 2001-2002	Gabon	EBOV-Z	65	53	Chimpanzee, gorilla, duiker
Mbomo Kelle, 2001-2002	Republic of Congo	EBOV-Z	59	44	Chimpanzee, gorilla, monkey
Kelle, 2003	Republic of Congo	EBOV-Z	143	128	Gorilla, duikers
Mbdanza Mbomo, 2003	Republic of Congo	EBOV-Z	35	29	Monkey, duikers

TABLE 2. TABLE SHOWING THE EBOLA OUTBREAKS IN CENTRAL AFRICA FROM 1976 TO 2007. THIS TABLE ONLY SHOWS THE 8 OUTBREAKS WHERE THE ANIMAL SOURCE COULD BE IDENTIFIED OUT OF 17 TOTAL OUTBREAKS. THE OUTNREAKS WERE CLEARLY LINKRF EITH DEAD BODIES OF CHIMPANZEES, GORILLAS, DUIKERS AND MONKEYS WHICH COULD BE FOUND ON THE FORESTS SURROUNDING THE OUTBREAKS LOCATIONS. EBOV-Z MEANS *ZAIRE EBOLAVIRUS*, EBOV-IC MEANS *TAÏ FOREST EBOLAVIRUS*. TABLE EXTRACTED FROM "HUMAN EBOLA OUTBREAKS RESULTING FROM DIRECT EXPOSURE TO FRUIT BATS IN LUEBO, DEMOCRATIC REPUBLIC OF CONGO, 2007" BY LEROY ET AL., PUBLICHED BY VECTOR-BORNE AND ZOONOTIC DISEASES ON VOLUME 9, NUMBER 6, 2009, PAGES 723-728.

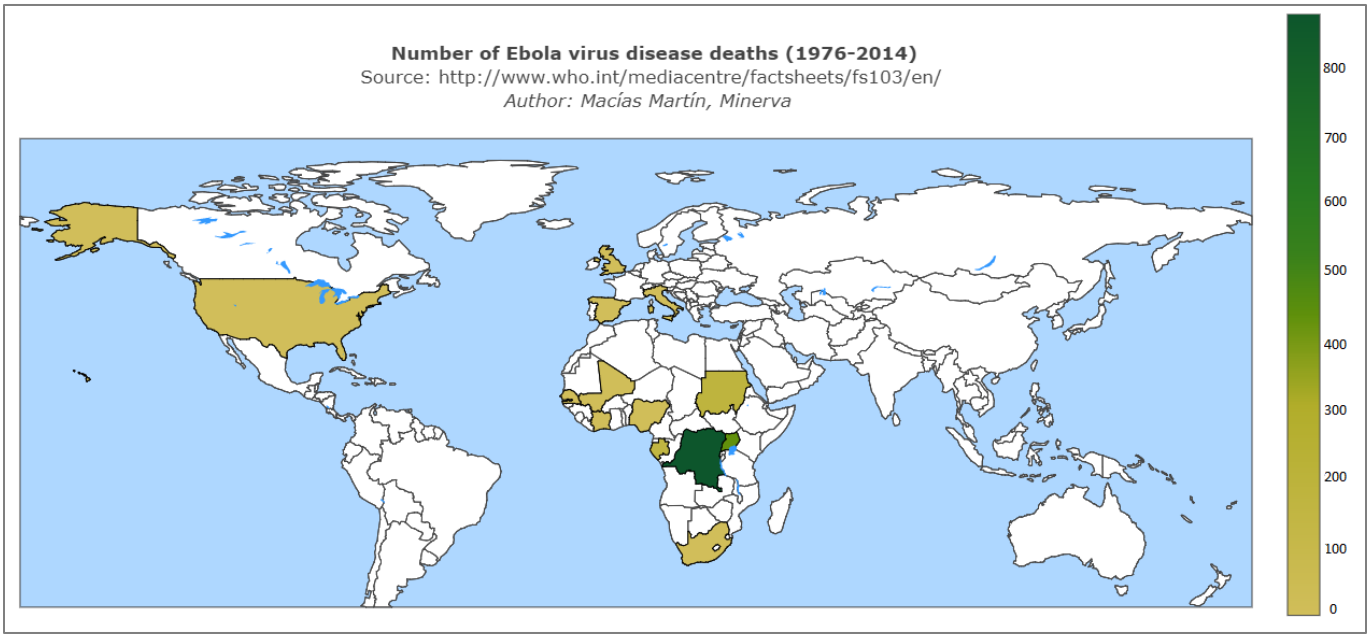


FIGURE 3. CHOROPLETH MAP SHOWING THE DEATHS CAUSED BY EVD IN DIFFERENT COUNTRIES FROM 1976 TO 2014.

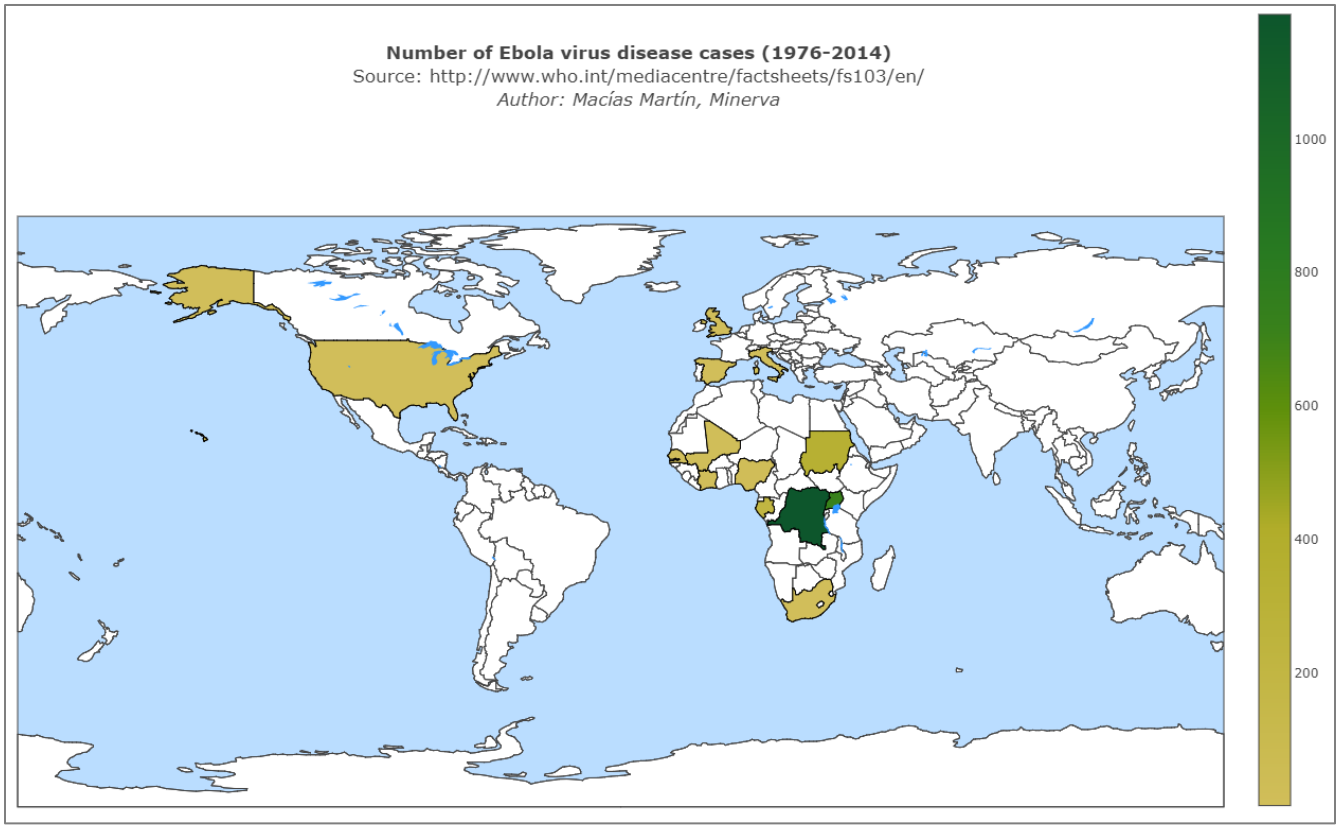


FIGURE 4. CHOROPLETH MAP SHOWING THE CASES OF EVD IN DIFFERENT COUNTRIES FROM 1976 TO 2014.

These choropleth maps show the impact that the Ebola virus disease have had around the world from its first study to the last outbreaks (2014). *Figure 3* shows the number of deaths that EVD has caused on each country where there has been at least one outbreaks, while *Figure 4* shows all the cases that had

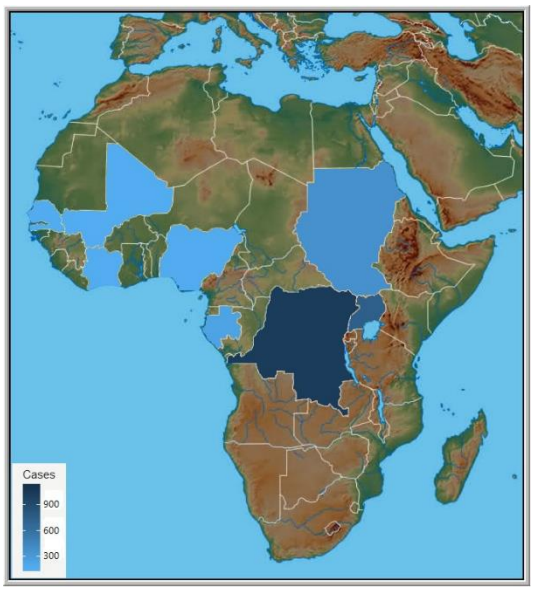


FIGURE 5. CHOROPLETH MAP SHOWING THE COUNTRIES WHICH HAVE SUFFERED FROM EBOLA VIRUS. DATA EXTRACTED FROM THE WORLD HEALTH ORGANIZATION. MAP CREATED USING R SOFTWARE, GIMP AND SUBLIME TEXT. INTERACTIVE MAP UPLOADED TO NEOCITIES.ORG (HTTPS://MINERVAMACIAS.NEOCITIES.ORG/INTERACTIVEAFRICA/)

been registered. Guinea, Liberia and Sierra Leone outbreaks are excluded because of the lack of rigour its data has. Both maps (Figure 3 and Figure 4) were made with the "plotly" package from RStudio.

There are three distinct species of bats, which have demonstrated suffering an EBOV infection without developing the disease. These are the *Hypsignathus monstrosus*, the *Epomops franqueti* and the *Myonycteris torquata.* These bats could act as a reservoir of the virus since they can carry the disease from the forest to the towns without dying themselves.

There is a strong correlation between the maps showing where these bats live (Figures 6, 7 and 8) and the choropleth map showing the locations of the main Ebola outbreaks in Africa (Figure 5). All the countries where Ebola have been detected are near rainforests, mangrove forests, swamps or dry savanna; the places where these bats live [6].

All the maps showing the range of the different bats have been extracted from The IUCN Red List of Threatened Species.

FIGURE 6. *EPOMOPS FRANQUETI* RANGE. THESE BATS CAN BE FOUND ACROSS A WIDE VARIETY OF LANDSCAPES, INCLUDING WET, DRY, AND MANGROVE FORESTS, SWAMPS, AND DRY SAVANNA.

FIGURE 7. *HYPSIGNATHUS MONSTROSUS* RANGE. ALSO KNOWN AS THE HAMMER-HEADED BAT, IT HAS A LONG BUT VERY NARROW DISTRIBUTION ACROSS THE TROPICAL BELT OF AFRICAN RAIN FOREST.

FIGURE 8. *MYONYCTERIS TORQUATA* RANGE. THESE BATS PREFER WET LOWLAND FORESTS AND WET SAVANNA

FIGURE 9. EPOMOPS FRANQUETI. IMAGE FROM INATURALIST.ORG. (RIGHT IMAGE)

FIGURE 10. HYPSIGNATHUS MONSTROSUS. IMAGE UPLOADED TO FLICKR BY BART WURSTEN. (LEFT IMAGE)

FIGURE 11. MYONYCTERIS TORQUATA. PHOTO MADE BY PIOTR NASKRECKI, ENTOMOLOGIST, PHOTOGRAPHER AND AUTHOR, BASED AT THE MUSEUM OF COMPARATIVE ZOOLOGY, HARVARD UNIVERSITY (CAMBRIDGE, MA, USA.) [7].

3.1.3 DEFENSE MECHANISMS

First of all, it is important to understand how does the EVD infect our body. People can become infected by butchering, cooking and eating infected animals or through contact with bodily fluids of infected humans being the most usual the next ones: blood, stool, urine, saliva and semen. These fluids can enter the body through wounds or mucous membranes. Soiled clothes, bed linen, gloves, protective equipment and medical waste which has been in contact with these fluids can also transmit the illness [8].

Once inside the body, this virus penetrates the healthy cells of the individual and precludes them from performing an active defense. The different defense mechanisms that our body can use to protect itself are divided into three groups: physical and chemical barriers, innate responses and acquired responses (Figure 12). It is important to underscore the importance of the immune system on the defense against the illness.

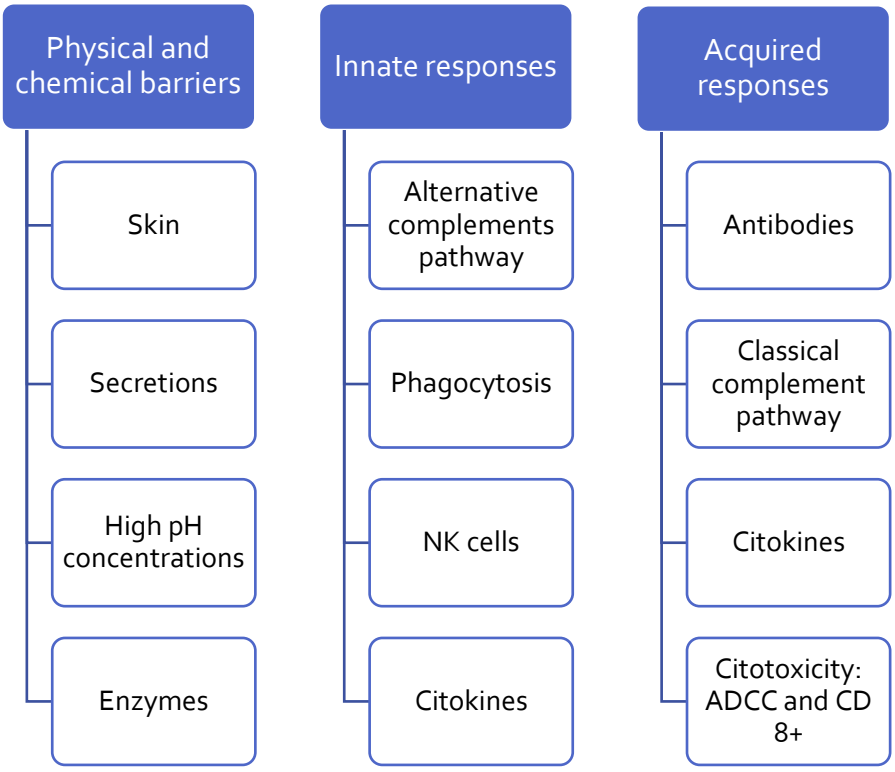


FIGURE 12. SCHEME SHOWING THE DIFFERENT DEFENSE MECHANISM THAT OUR BODY USES TO PREVENT AN EBOLA INFECTION.

The skin would be the first wall which tried not to let the virus enter the body. Little wounds and the mucous membranes are the only parts of our body which the virus can use to penetrate it. Secretions, higher pH concentrations and enzymes would try to denature the 7 proteins which form the virus.

The immune system consists in the innate immune system and the adaptive immune system, also known as acquired immunity. The first line of defense is the innate responses.

This system of protection needs to identify foreign particles which don't belong to our body. The molecules which are part of or made by the body are not the target of the immune system. On the other hand; bacteria, viruses, parasites, pollen, dust and toxic chemicals are examples of non-self-molecules that can be found inside our body and could be highly harmful. These harmful proteins create special proteins called antigens which inform the body that they could cause damage. These antigens, then will cause an immune response. The initiation of the response is helped by the cytokines [9]. The cytokines are small glycoproteins that measure between 8 and 70 kilodaltons [10]. These molecules communicate with other cells that an immune response is going to be performed, among other functions (Figure 13). If these cytokines are released by already infected cells in order to warn healthy cells of the threat are called chemokines.

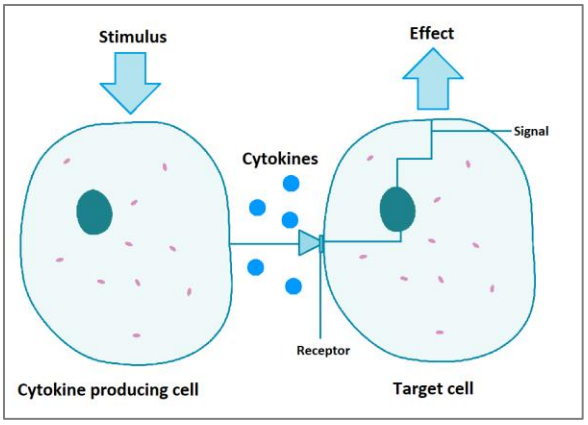


FIGURE 13. SYSTEM OF COMMUNICATION USED BY THE CYTOKINES. AN STIMULUS ENTERS A CYTOKINE PRODUCING CELL. THIS CELL CREATES VARIOUS CYTOKINES WHICH WILL TRAVEL BY THE BODY FLUIDS UNTIL THEY ARRIVE TO THE RECEPTOR OF A TARGET CELL. ONCE THEY HAVE ARRIVED, A SIGNAL WILL BE TRANSMITTED CAUSING AN EFFECT AS A RESPONSE. SCHEME DESIGNED USING GIMP 2.8 SOFTWARE.

The innate immune system englobes the physical and chemical barriers mentioned before and the innate responses. This system is always nonspecific and is activated by the presence of antigens.

The first innate response noted in the above scheme (Figure 7) is the alternative complement pathways. The Alternative pathway (AP), as well as the Classical complement pathway, is one of the three pathways of the complement system [11]. It plays an important role in the innate immune system. It is a complex system formed by different proteins that, when are activated, start following different steps (opsonisation, chemotaxis, cell lysis and agglutination) to facilitate the search and the removal of antigens, making it easier for other parts of the immune system to do their functions [9].

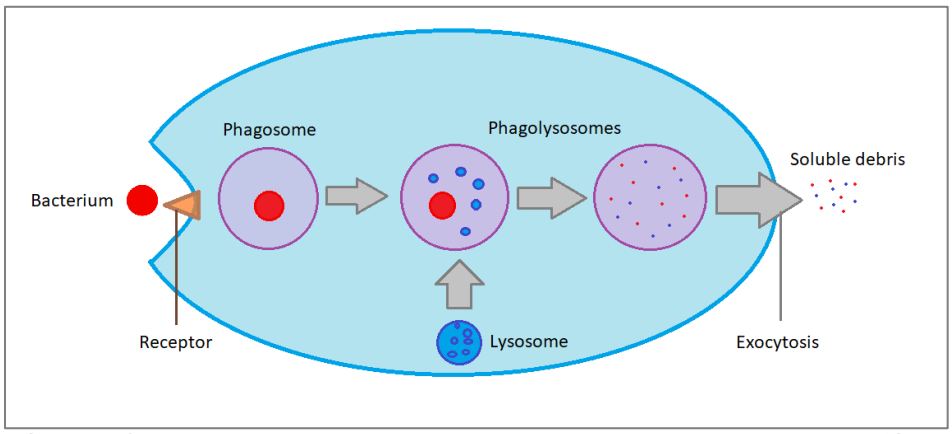


FIGURE 14. SIMPLIFIED DIAGRAM OF THE PHAGOCYTOSIS AND DESTRUCTION OF A BACTERIAL CELL (GIMP 2.8).

Phagocytosis is a process where a cell called phagocyte engulfs a potentially dangerous molecule, in this case the Ebola, and destroys it (Figure 14). To do this, the phagocyte deforms its surface, creating an invagination where the virus is placed. Later,

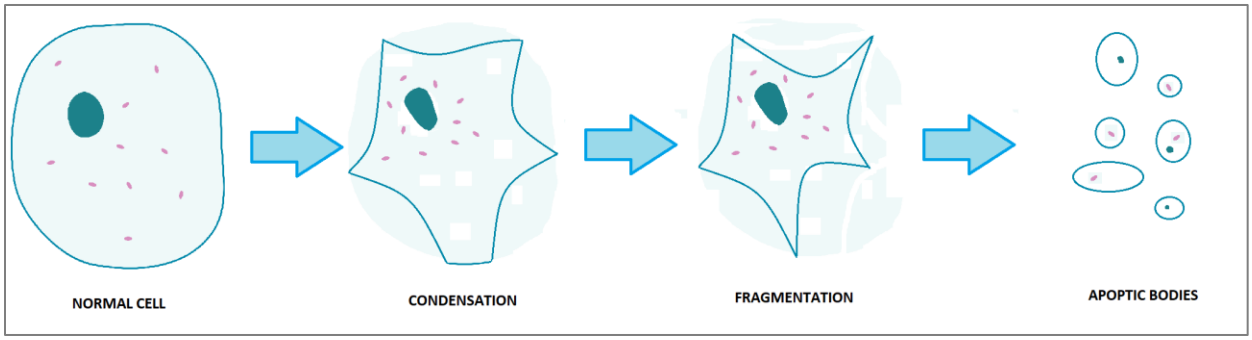


FIGURE 15. THE APOPTOSIS CYCLE. THIS CYCLE IS CARRIED OUT BY THE NK CELLS. IMAGE FROM STEM CELL THAILAND WEB PAGE.

the invagination is closed and the virus rests in a phagosome inside the cell, where it will be destroyed thanks to the lysosomes and its enzyme ([9]. The phagolysosome would be extracted from the cell by a process called exocytosis. This process was discovered by Methcnikoff as it is explained in the last chapter of this project.

The NK cells, or natural killer cells, are not able to attack the threats directly. Their function is to destroy the infected cells, known as host cells, in order not to let the illness spread itself. The destruction is usually caused by lysis or by apoptosis (Figure 15).

On the other hand, the adaptive immune system is activated when it detects pathogens and it can learn about these pathogens and enhance its response to future exposures [12]. There are two types of cells, which form the adaptive immune system: B cells and T cells. Both of them are made in the bone marrow and need to mature to be ready to do their functions.

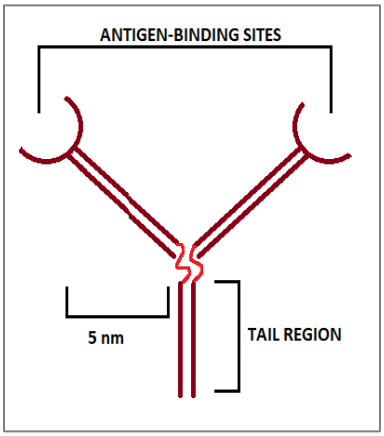


FIGURE 16. STRUCTURE OF A SIMPLE ANTIGEN MOLECULE. IT IS IMPORTANT TO HIGHLIGHT THE FACT THAT BOTH ANTIGEN-BINDING SITES ARE IDENTICAL, BOTH MEASURING 5NM ($1X10^{-7}$CM).

- **B cells:** they are the only cells which synthetize antibodies. Antibodies are molecules which defend the health of the individual by inactivating viruses or microbial toxins. This inactivation is possible because the antibodies are able to bind to these pathogens. There are billion forms which an antibody can adopt, each one with a different amino acid sequence and a different antigen-binding site. However, all the antibodies made by the same B cell have the same antigen-binding site (Figure 16). It means that all the antibodies made by this cell will interact with the pathogens in the same region of their surface through noncovalent bonding [13].
- **T cells:** once formed, the T cells move themselves to the thymus. While they are developing themselves there, they start to express two kinds of receptors. The first ones are called T cell receptors, also known as TCRs, and they are in every T cell. Then, the T cell expresses one of these two receptors: CD4 or CD8. It is not possible for a T cell to express both of them [12]. In contrast to B cells, they are not capable of linking themselves directly to an antigen. They can only recognize antigens which are linked to a receptor called "Major Histocompatibility Complex" (MHC), either class 1 or class 2. The CD4 or CD8 help the cell to recognize these complexes and

to bind themselves to them. After the TCRs rearrange in their own right, the T cells will have to pass two selection processes in order to stay alive. These processes are performed to protect the individuals healthy cells against their own immune response.

After these processes, the T cells are divided into three different groups (Figure 17):

- **Helper T cells:** they express CD_4 and help to activate B cells among other immune cells.
- **Cytotoxic T cells:** express CD8 and remove pathogens and infected host cells.
- **T regulatory cells:** express CD_4 and CD25 and help distinguish self and non-self-molecules. Thanks to them, the risk of autoimmune diseases drastically decreases.

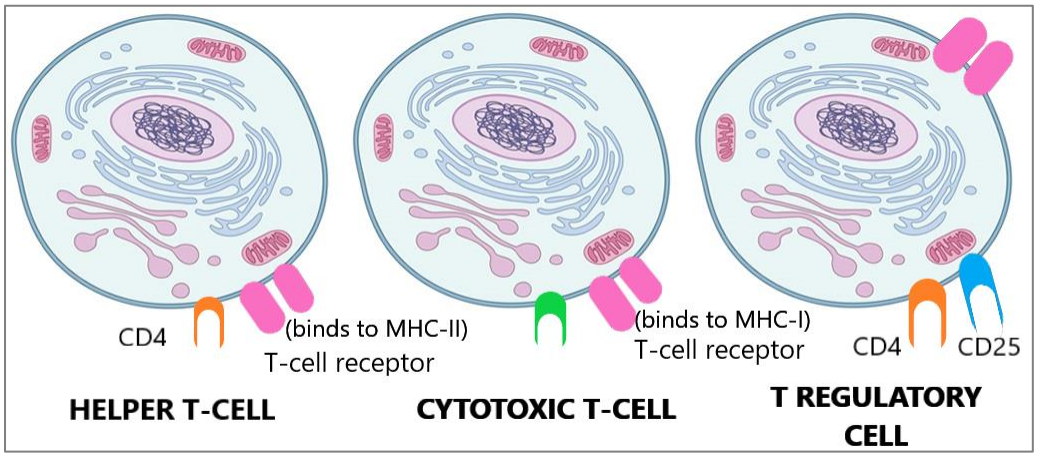


FIGURE 17. DISTINCT KINDS OF T CELLS AFTER THEY HAVE PASSED THE TWO SELECTION PROCESSES (PAINT 3D).

The cytotoxic T cells, also known as CD8+ cells, are critically important for the defense against pathogens like the EVD. When they detect the EVD antigen and become activated, they can adopt different mechanisms to kill the infected host cells. One of them is the secretion of cytokines. It has been explained before what these molecules are. Besides transmitting the message, informing that an immune response is going to be performed, these molecules have anti-viral effects [14].

Another major function of CD8+ cells is the release of cytotoxic granules which contain perforin and granzymes proteins. Perforin is able to form a pore in the membrane of the target infected cell. The granzymes then enter the cell by this pore and stop the production of viral proteins causing ultimately the apoptosis of the cell (Figure 11) [14].

The acronym ADCC stands for Antibody-dependent Cellular Cytotoxicity. It is the killing of an antibody-covered cell by the release of cytotoxic granules or the expression of cell death-inducing molecules [15]. This way, the infected host cells could be destroyed. The ADCC includes NK cells (explained before), macrophages (large white blood cells that play an essential role in our immunological system) [16], dendritic cells (cells which from a cellular network which shapes adaptive immune responses) [17] and CD8+ T Cells, among others.

3.1.4 TREATMENT AND VACCINES

Although EBOV has been studied extensively, today there is neither a licensed vaccine nor treatment available. The most important vaccine known for preventing Ebola virus is called rVSV-ZEBOV, but it is not commercialized yet and it has been only tested on the Zaire Ebolavirus disease. It consists on a vesicular stomatitis virus (VSV) (Figure 19) which has been genetically modified to express an immune response if the body detects Ebola antibodies. This response is provoked thanks to a surface glycoprotein of Zaire Ebolavirus which has been added to the VSV (Figure 18).

FIGURE 19. VESICULAR STOMATITIS VIRUS. IMAGE BY DR. FRED. A. MURPHY, COURTESY OF PUBLIC HEALTH IMAGE LIBRARY (PHIL).

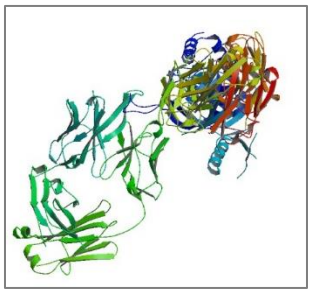


FIGURE 18. CRYSTAL STRUCTURE OF PROTECTIVE HUMAN ANTIBODIES 100 AND 114 IN COMPLEX WITH EBOLA VIRUS FUSION GLYCOPROTEIN (GP) (5FHC). IMAGE FROM THE PROTEIN DATA BANK.

It is the most important vaccine now because it has been tested in contacts and contacts of contacts of infected people in Guinea, according to an article published in the British magazine *The Lancet* on February 4th, 2017 [18]. It is the first vaccine to prevent from the EVD. The trial was made under a ring vaccination design, the same method that was used to eradicate smallpox [19]. Ring vaccination tries to control an outbreak of an infectious disease, like the EVD, by vaccinating the people around each infected individual (a ring of people) [20]. The clinical trial was registered as PACTR201503001057193 on the Pan African Clinical Trials Registry [21].

The test was a cluster-randomised trial. It was also open-labelled, which means that both the researchers and participants knew which treatment was being administered.

In the first part of the trial, 98 clusters were randomised and divided in two groups. The first one was assigned to immediate vaccination, while the second group were assigned to delayed vaccination. After excluding non-eligible contacts (individuals under 18 years, individuals who didn't provide basic information for cluster definition, pregnant or breastfeeding and severely ill individuals), people who didn't give their consent and absent people, **2119 individuals** were immediately vaccinated. No cases of Ebola virus occurred 10 days or more after the vaccination among the individuals who were vaccinated. In contrast, 23 cases of Ebola virus disease occurred among all the individuals in the delayed clusters. **The vaccine efficacy was 100%.**

More people were vaccinated on the following months. **5837 individuals** in total received the vaccine, including 194 children. These individuals were followed up for 84 days [18]. No cases of Ebola occurred among these patients.

On the negative side, more than a half of vaccinated people **(3149 out of these 5837)** reported at least one adverse event in the 14 days after vaccination, being the most usual headaches, fatigue and muscle pain [22]. However, all of them recovered with no future physical consequences.

Apart from the high protection that the vaccine gave to those vaccinated, it indirectly protected as well non-vaccinated individuals who were part of these rings (Figure 20). This kind of protection is called herd immunity. It is defined as the process which gives protection and immunity to individuals which have not been related to the trials because a sizable portion of a population have been vaccinated. This is due to the fact that the virus has few susceptible people to infect and it makes it difficult for it to spread [22]. This type of immunity is decisive for very young children, people with immune system problems and those who are severely ill since they cannot be vaccinated, and they would be highly exposed to the EVD. Nonetheless, the authors explained that the trials were not intended to measure these effects, so another trial will be needed to verify the impact of herd immunity [19].

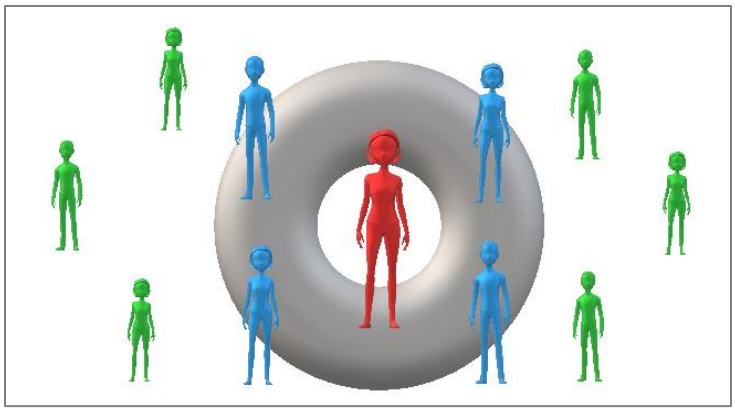


FIGURE 20. RING VACCINATION SCHEME. THE INFECTED AND HIS CONTACTS (RED AND BLUE BODIES) IMMEDIATELY RECEIVE THE VACCINE. THE CONTACTS OF THESE CONTACTS (GREEN BODIES) RECEIVE IT LATER.

3.1.5 SOCIAL CONSEQUENCES

FIGURE 21. SAA SABANS. IMAGE BY NIGERIAN TRIBUNE.

Ebola has built itself an image of a fatal and terribly contagious disease, and it is indeed. People can't help themselves from being terrified when a new outbreak happens. The worst part of all this chaos and dread is suffered by the patients who manage to evade the fatal end. They are often isolated from the society, even when they are fully recovered and suffer humiliations and hate due to people's fear of getting infected. Extracted from *www.vice.com*, there is an extract of an interesting interview with Saa Sabans, a man who has survived the illness (Figure 21).

VICE: Then, ¿what happened when you started to feel better?

SAA: When they (the doctors) let me go home, they gave me clothes and *Médecins Sans Frontières* (also known as Doctors Without Borders) took me home. When I left the car, they took my hand to show people that I couldn't infect them to avoid being discriminated. Some of them were afraid of me, so the fact that the doctors took my hand was a recovery signal. They also gave me a certificate which demonstrated that I was fully recovered and that no one should be afraid of me. After that, my friends cheered me up and took my hand as well. I thank God for that. Now, some people call me "The survivor", "The anti-Ebola man" or "The one who came back".

VICE: ¿Could you talk a bit more about the discrimination that is suffered by the survivors of the Ebola virus?

SAA: My family was discriminated while I was in the hospital. The discrimination is caused by the fear of getting infected. When I noticed that, I started to feel more secure of myself again. I want to show you a specific example: now, I'm working hand in hand with people from Germany, France, the United States, England, etc, in order to educate them about the Ebola. If anyone of them knew that I had been infected before, they wouldn't mind continuing working with me. That's why is very important for me going from a village to another with the Red Cross fighting against this kind of discrimination. I am the example. I tell people: "Do you think that people from all around the world would work with me if I was contagious?". A lot of them answer: "We understand it, but it's still frightening. The discrimination against the people who suffered the illness and recovered is decreasing.

To read the original interview go to: https://www.vice.com/es_mx/article/nnpw8q/entrevista-con-un-hombre-que-sobrevivio-al-ebola

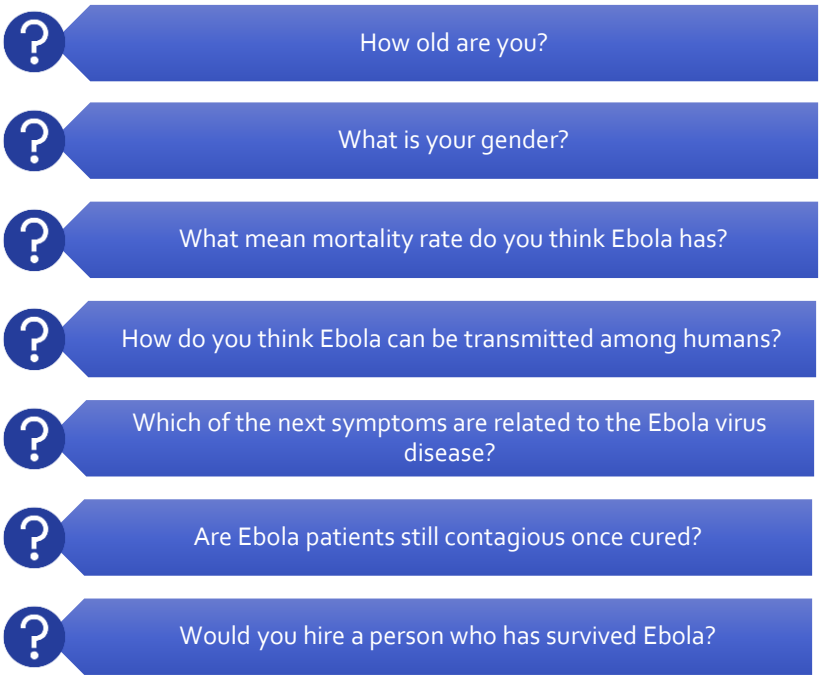
To read a translated version go to:

https://cmcminerva.wordpress.com/2017/07/30/translated-interview-with-saa-sabans-the-man-who-survived-the-ebola-virus-disease/

To understand better how this discrimination works and to understand how much do the people now about this illness, I made a survey with the next questions:

https://goo.gl/forms/naKGxAwrVM806Jds1

The survey tried to show how well or how bad are people informed about the Ebola virus disease. There were 7 questions in the survey:



The survey was posted online on the 9th August 2017, and it was closed on 22nd September 2017. Between these dates, 1038 people answered the questions of the survey. The population surveyed was formed by 238 men (23%) and 800 women (77%) (Figure 22).

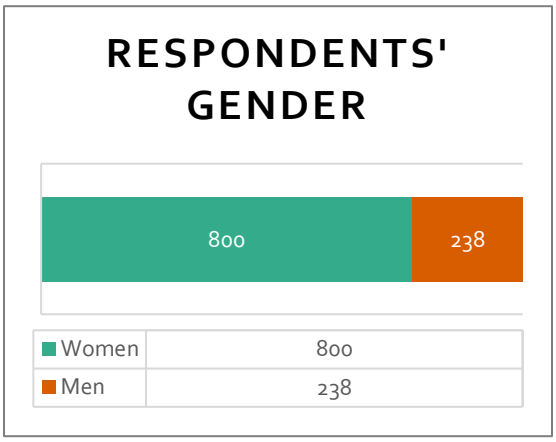


FIGURE 22. RESPONDENT'S GENDER SHOWED IN A PERCENTAGE STACKED BAR CHART.

The age range which had more participants were the ones who had less than 18 years old, possibly because of their most common use of the social networks, with a 63% of the total population, while only the 1% of the population was more than 60 years old (Figure 23).

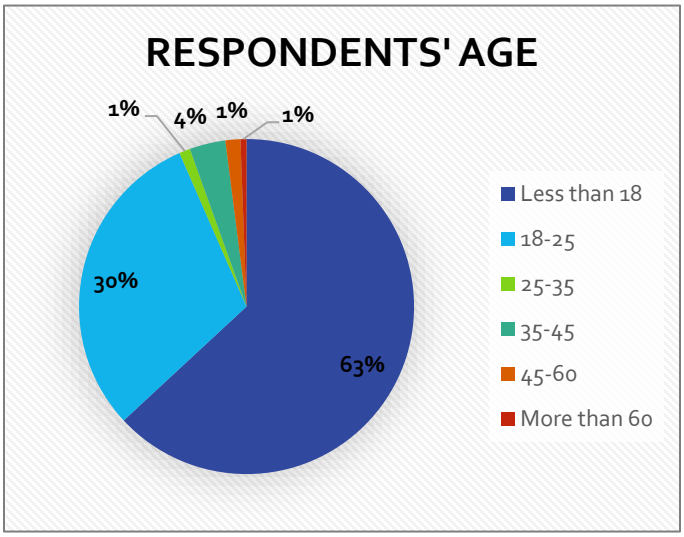


FIGURE 23. RESPONDENT'S AGE SHOWED IN A PIE CHART.

The average mortality rate reported by the WHO is around 50% [3], although some outbreaks went from the 25% to the 90%. Despite a not so higher mortality rate for most of the cases, the respondents showed a strong tendency to exaggerate these numbers. The 55% of the surveyed chose between the 100%, 90% and 75% options (Figure 24). This could be because some sensationalist news, which were on the front page of big newspapers announcing the EVD as a "calamity" [24] and even relating the disease with some zombie paranoia [25]. These massive misunderstanding -only the 22% (231 out of 1038) of the respondents answered right- shows the high misinformation about the Ebola virus disease that rules the western society.

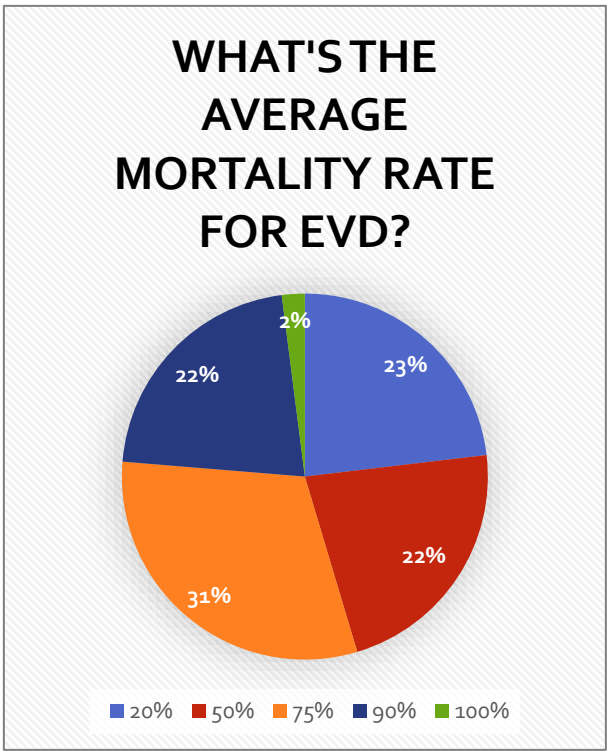


FIGURE 24. RESPONDENTS ANSWERS FOR THE MEAN MORTALITY RATE FOR EVD QUESTION. PERCENTAGES OUT OF 1038 RESPONDENTS.

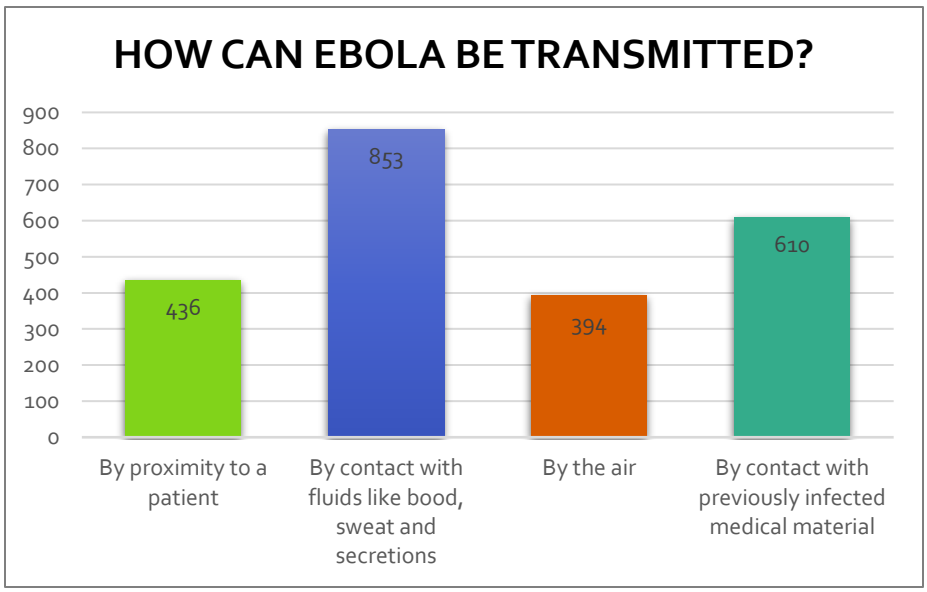


FIGURE 25. RESPONDENTS ANSWERS TO THE WAYS OF TRANSMISSION QUESTION.

The fourth question of the survey tested the general knowledge of the respondents about the potential ways of transmissions of the EVD. More than one answer could be chosen for this question. Although the right answers, which were "By contact with fluids like blood, sweat and secretions" and "By contact with previously infected material", were chiefly selected with 853 and 610 votes respectively, the two wrong answers had also a great support (Figure 25). These answers, which were "By proximity to a patient" and "By the air", had 436 and 394 votes out of 1038. The 42% and the 38% were misinformed about the topic. The misconception could be since some nurses and doctors get ill once they have treated some EVD patients. In spite of this, the infections are produced when infection control precautions are not strictly practiced, not because the proximity to the patient or because and not because of a hypothetical infected air.

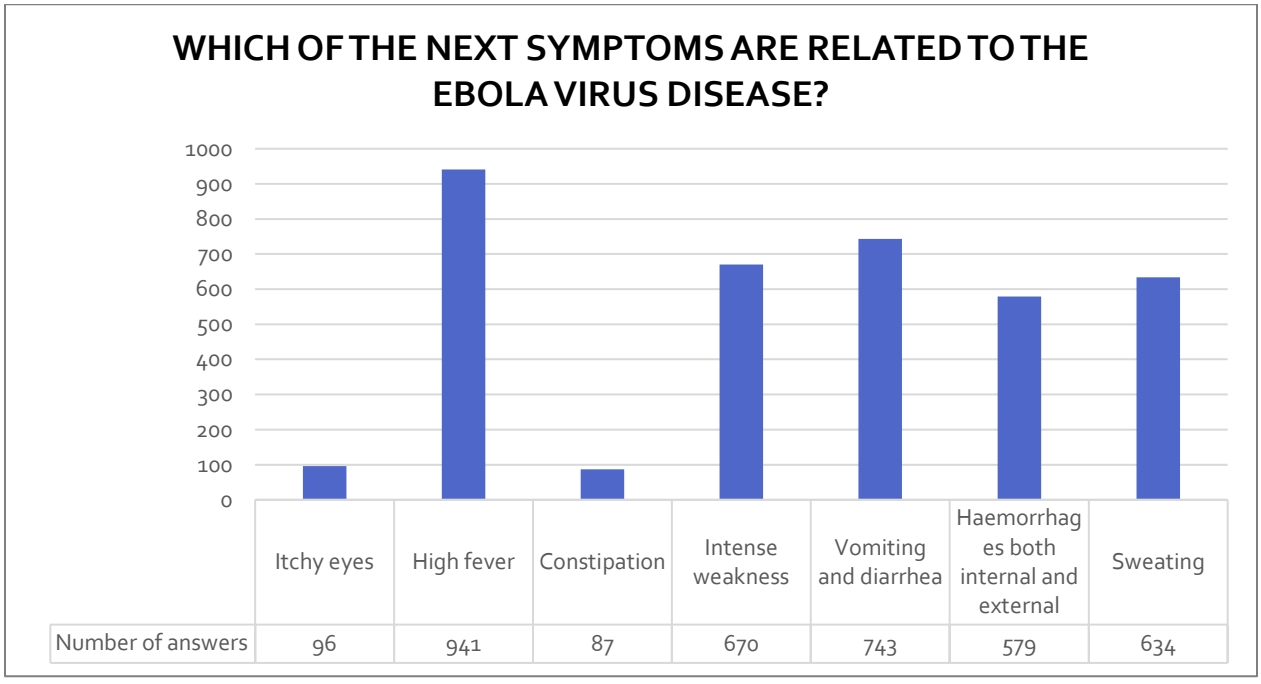


FIGURE 26. BAR CHART WITH THE RESULTS OF THE QUESTION FROM THE SURVEY: "WHICH OF THE NEXT SYMPTOMS ARE RELATED TO THE EBOLA VIRUS DISEASE". "HIGH FEVER" WAS THE MOST VOTED ANSWER (941 VOTES, 90.66%), BEING ONE OF THE RIGHT ANSWERS.

As it can be seen in the first chapter of the project, the main symptoms of the EVD are periods of fever, feeling weak and without any energy, headaches, muscle pain and sore throat. Diarrhoea, rushes, impaired liver and kidney, vomiting and internal and external bleeding are also usually present in the infected patients (Figure 1). The right answers ("High fever", "Intense weakness", "Vomiting and diarrhoea" and "Haemorrhages both internal and external") received most of the votes (Figure 26). On another note, "Sweating" received a lot of votes despite being a fake answer. The confusion could be due to the fact that humans can become ill from being exposed to the fluids of infected individuals, being one of them sweat, but, as a matter of fact, sweating is not a symptom of the Ebola virus disease.

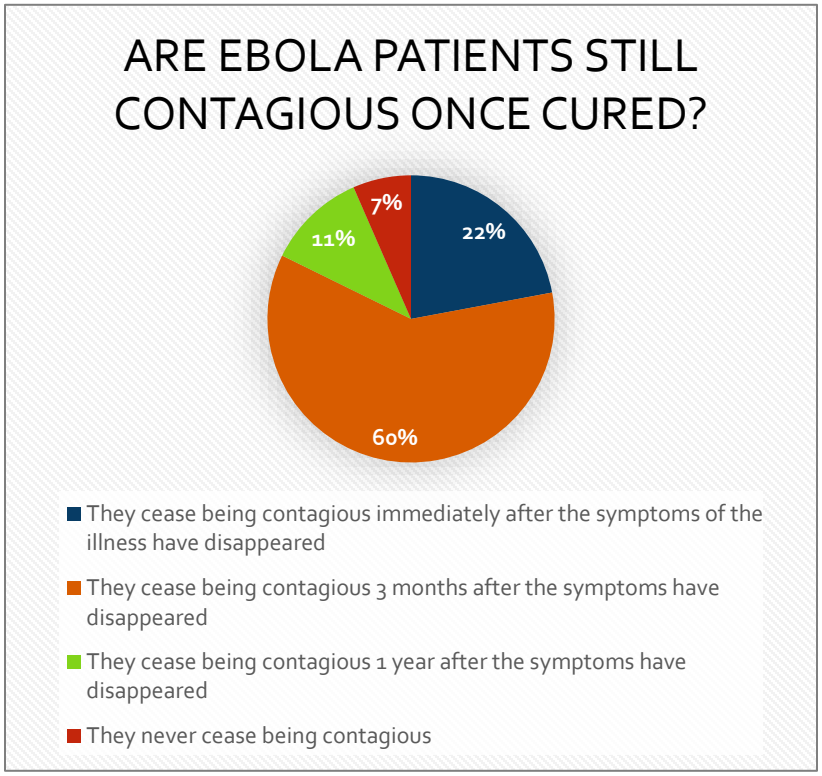


FIGURE 27. PIE CHART SHOWING THE ANSWERS FOR THE "ARE EBOLA PATIENTS STILL CONTAGIOUS ONCE CURED?" QUESTION.

The next question asked the respondents when they thought the recovered patients ceased being contagious (Figure 27). The different options were: "Immediately after the symptoms of the illness have disappeared", "3 months after the symptoms have disappeared", "1 year after the symptoms have disappeared" and "They never cease being contagious". There's not an exact answer for this question because the virus is able to stay different amounts of time depending on the fluid it is living. The virus can remain alive in the semen of cured patients up to 90 days (3 months) one the symptoms have faded. It is believed that in fluids like blood or saliva this period would be shorter. Taking that into account, the most accurate answer would be "They cease being contagious three months after the symptoms have

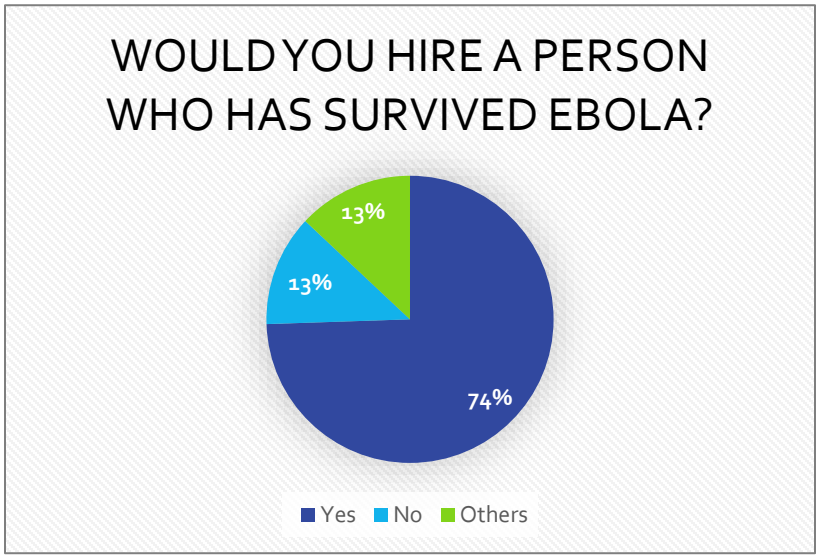


FIGURE 28. PIE CHART SHOWING THE ANSWERS FOR THE "WOULD YOU HIRE A PERSON WHO HAS SURVIVED EBOLA?" QUESTION.

disappeared", which in its turn have received the greatest support from the people who took the survey.

The last question was the most personal one (Figure 28). Almost none of us has asked himself whether he would hire a survivor of such a dangerous virus as Ebola is. 74% of the population answered that they would do it, meanwhile another 13% of the population asked they would not. The range of opinions is wide and most of the people couldn't find his own opinion on the matter reflected on a "yes" or "no" answer. This is reflected on another 13% who wrote different opinions. The views which stand out the most were: "I don't know", "It depends on how long the patient has been cured" and "I would need to be properly informed about the illness".

Considering that the 11% of the respondents thought that the survivors needed more than a year to be fully recovered and to stop being a threat and that the 7% thought they never stopped being contagious, I expected more people to answer they wouldn't hire an Ebola survivor. In addition, 55% of the surveyed granted Ebola more than a 75% mortality rate and there were 436 respondents who considered that just by proximity to a patient they could get infected. This calamitous tendency is not connected with the last question answers. This could possibly be because it is a hypothetical situation and the surveyed are not thoroughly evaluating it. If the same population found themselves in a real situation where they must decide whether to hire an Ebola survivor or not, the graph would be drastically different.

3.2 REVERSE VACCINOLOGY

Vaccines have been existing for more than two centuries. They were introduced into Western medicine in 1796 by Edward Jenner although this practice has been practiced in Asia since ancient times [26]. It was Jenner who introduced the terminology "Vaccine". This word comes from the Latin word *vacca* which means "cow". This is because he used infected materials from cows to immunize against smallpox [26]. On the other hand, Louis Pasteur – a French microbiologist and chemist – was the man who started its development proposing to "isolate, inactivate and inject the microorganism" to the people who were getting vaccinated (Figure 29). This procedure became the basic rule of Vaccinology and many diseases were controlled this way, such as diphtheria (Glenny and Hopkins, 1923) and tetanus (Ramon, 1924).

FIGURE 29. LOUIS PASTEUR RESTORED PORTRAIT. PHOTOGRAPH BY THE FRENCH PHOTOGRAPHER GASPARD-FÉLIX TOURNACHON, BETTER KNOWN AS NADAR.

Vaccines provide acquired immunity to different diseases. This process is called *Immunization* and, according to the World Health Organization, it is "one of the most powerful and cost-effective of all health interventions" [27]. Other organizations, such as the Copenhagen Consensus, have also extolled the virtues of this kind of immunization, saying that "vaccination may be the most effective public health intervention of all time" [28].

Before the vaccination method was openly used, infectious diseases such as diphtheria smallpox and pertussis were those responsible for the 20% of the infant mortality in the U.S [29]. We could think that nowadays, with all the technology which are in the hands of the doctors and researchers, the deaths from infectious diseases are a small percentage of the total deaths. However, the truth is that studies show that infectious diseases caused 18.5% of all human deaths [30] (Table 3). Why is this rate still so high in the 21st century?

Disease	Number of deaths in children under the age of five, 2002
Pneumococcal disease	716.000
Rotavirus infection	402.000
Hib Infection	386.000

TABLE 3. THE NUMBER OF DEATHS IN CHILDREN UNDER THE AGE OF FIVE CAUSED IN 2002 BY THREE DIFFERENT INFECTIOUS DISEASES (PNEUMOCOCCAL DISEASE, ROTAVIRUS INFECTION AND HIB INFECTION). TABLE EXTRACTED FROM GUIDE TO GIVING BY THE COPENHAGEN

Although the vaccines have proven to be one of the most effective methods against numerous diseases, they are far from perfect. Neglecting all the ethical issues surrounding vaccines, the standard method used to develop vaccines does not meet the needs of the global population. The most relevant issue is the time that a group of researchers needs to develop an effective vaccine. This process can take as long as ten or fifteen years [31]. The outbreaks of deadly diseases claim urgent measures and the infected ones cannot wait for so much time. Other issues would be the failure of the system when the

pathogens can't be cultivated *in vitro* or when the antigens are variable in its sequence [32]. The only vaccine which could be develop without cultivating its pathogen was the hepatitis B vaccine. For this vaccine the pathogen was recovered from the plasma of infected people [33].

Nowadays, most of the vaccines that could be developed using these technologies had been developed already. This entails that innovative technologies were required to immunize people against the remaining pathogens. The primary technique that has been developed until today is called "Reverse Vaccinology" and its main inventor is the Italian scientist and researcher Rino Rappuoli [32].

3.2.1 DEFINITION

Rappuoli presents a new process to create vaccines starting the research using the genetic information and *in silico* procedures rather than the pathogen itself. This way, it is not necessary to cultivate the pathogens. That would open the possibility to create new vaccines which couldn't be created before because it was not possible to grow the pathogens that caused the illness. It was first used against Serogroup B *meningococcus* [34].

3.2.2 METHODS

As it has been said before, Reverse Vaccinology starts working with the pathogen's genome, which provides all the genes in the microorganism. Thanks to the computer analysis, this genomic sequence is analysed and the antigens which are more likely to be vaccine candidates are identified (Figure 30). The approach does not differentiate very or less abundant antigens or if they are expressed or not *in vitro*. It could also happen that the analysis highlighted some antigens which are not immunogenic during the infection, that is to say the antigen wouldn't be able to produce an immune response during the infection [32].

This way, a wider variety of proteins can be discovered. However, as it works with the

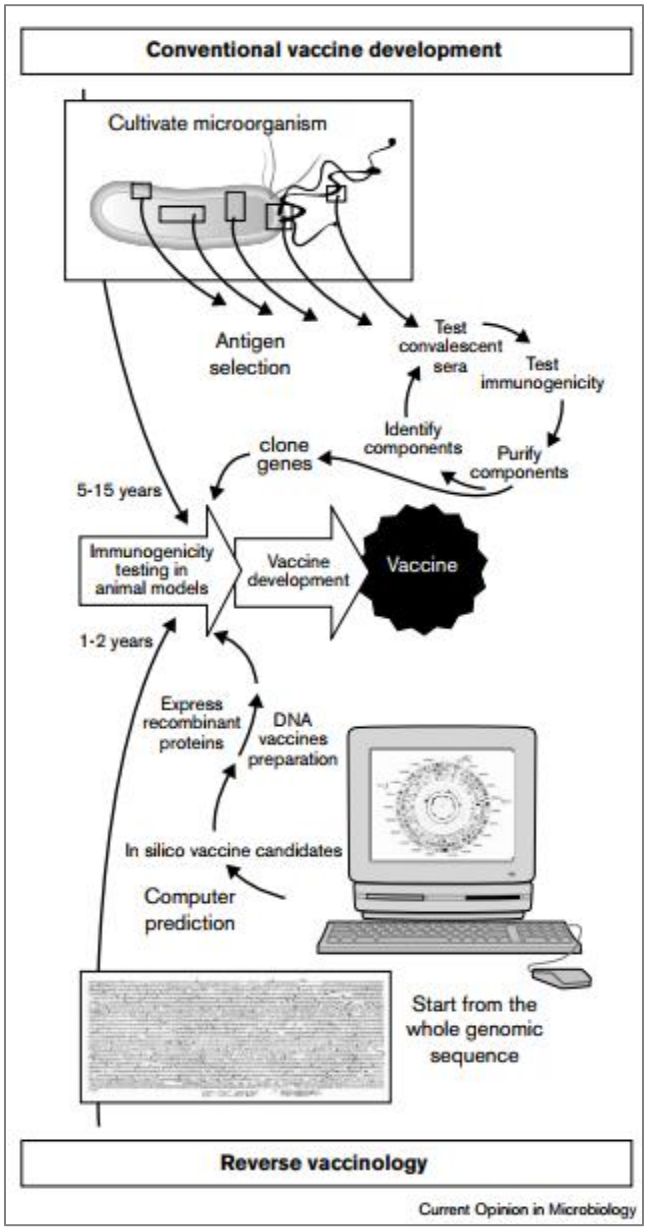


FIGURE 30. SCHEMATIC REPRESENTATION OF THE ESSENTIAL STEPS OF VACCINE DEVELOPMENT BY THE CONVENTIONAL APPROACH AND BY REVERSE VACCINOLOGY [32].

genome, only proteins are considered. Polysaccharides, lipopolysaccharides nor glycolipids-based vaccines can't be developed using this method.

To carry out Reverse Vaccinology performances is absolutely necessary to dispose of systems that are able to detect protective immunity. This kind of systems would allow the scientists to test all the genes of a pathogen. Nevertheless, the current knowledge of the scientific community on vaccine immunology, which is very narrow, limits the evolution of Reverse Vaccinology.

The available software nowadays is very limited, but it has been constantly increasing since Reverse Vaccinology was first carried out. The main software packages are noted and explained on the "Materials and methods" chapter.

- Different software packages by the Swiss Institute of Bioinformatics.
- Vaxign [35].
- NERVE [36].
- RANKPEP [37, 38].

To test some of this software, we decided to test the different Ebola proteins (Table 4) with these programs.

EBOLA PROTEIN	PDB ID	SHORT DESCIRPTION
Glycoprotein	2EBO	It is responsible for binding to target cells and subsequent fusion of the viral and host-cell membranes.
VP40 (matrix)	4LDD	It shapes the virus and drives the process of budding.
VP40 N-terminal	1H2C	It binds to RNA and regulates viral transcription
VRR-NUC domain	4QB0	It is a nucleocapsid protein that protects the genome at the centre of the virus.
VP24	3VNE	It is a nucleocapsid protein that protects the genome at the centre of the virus.
VP35 Interferon Inhibitory Domain	3FKE	It is a nucleocapsid protein that protects the genome at the centre of the virus.
C-terminal domain of Ebola virus VP30	2I8B	It is a nucleocapsid protein that protects the genome at the centre of the virus.

TABLE 4. THE SEVEN DIFFERENT PROTEINS WHICH BUILD THE EBOLA VIRUS WITH THEIR PROTEIN DATABANK ID AND A SHORT DESCRIPTION OF THEIR FUNCTION EXTRACTED FROM THE PDB WEBSITE [39].

For a better visualization of the EBOV structure, Table 4 was converted to an image map which is available in:

https://minervamacias.neocities.org/InteractiveEBOV/

First of all, the seven protein sequences were compiled. Their sequences were in a FASTA sequence file. The FASTA format represents peptide sequence using a single-letter code that was created by the IUPAC (Table 5). When this was done, the docking could start.

We started with the RANKPEP software which ranks the different peptides depending if they are likely to be binding sites or not. Among the seven proteins cited in table 4, the one which resulted to be more active was the VP35 (3FKE on PDB) (Table 6). A certain peptide of it showed the highest score among all the peptides that shapes the virus. The peptide was the following one: AKDLRNIMY. Although being the most likely peptide to act as a binding site, it only scored 26.077 points, being the optimal score given by the program 50.449 (51,69% probability) [41, 42, 43].

As the RANKPEP outcome noted the VP35 protein as the most likely to be a binding site, I proceeded to analyze this protein with Vaxitop, one of the main tools of Vaxign. It predicts epitopes in both MHC class I and II. I adjusted the P value cutoff to 0.0005 and the MHC host species to human. Any allele of both MHC could be predicted. As it can be seen in the next page table (Table 7), 4 different epitopes were shown. With a P value as little as the one used, they are highly likely to work with such protein.

Amino Acid Code	Meaning
A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
Q	Glutamine
E	Glutamic acid
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

TABLE 5. 1-LETTER AMINO ACID CODE EXTRACTED FROM THE INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY (IUPAC) [40].

RANK	POSITION	N	SEQUENCE	C	MW (Da)	SCORE	% OPT.
1	10	DIS	AKDLRNIMY	DHL	1105.32	26.077	51.69%
2	44	DSN	SLDIIHAEF	QAS	1026.17	10.56	20.93%
3	78	IFQ	DAAPPVIHI	RSR	914.08	4.898	9.71%
4	119	VFQ	LQDGKTLGL	KI	926.07	1.395	2.77%
5	71	QIT	KRVPIFQDA	APP	1055.25	-0.621	-1.23%
6	49	DIT	HAEFQASLA	EGD	955.05	-1.445	-2.86%
7	39	CKL	GKDSNSLDI	IHA	929.98	-1.507	-2.99%

TABLE 6. OUTCOME OF THE RANKPEP ASSESSMENT. IT CAN BE SEEN THAT THE AKDLRNIMY PEPTIDE HAS THE HIGHEST SCORE AMONG THE OTHER PEPTIDES WHICH SHAPE THE VP35 PROTEIN.

EPITOPE	EPITOPE LENGTH	MHC CLASS	MHC ALLELE	P VALUE	LOCATION	
					FROM	TO
IPRACQKSL	9	I	HLA-B*07:02	0.0000382	92	100
IMYDHLPGF	9	I	HLA-A*26:02	0.000117	16	24
IMYDHLPGF	9	I	HLA-B*15:01	0.000497	16	24
PPSPKIDRG	9	II	HLA-DQA1*03:02/DQB1*04:01	0.000413	104	112

TABLE 7. OUTCOME OF VAXITOP FOR THE VP35 PROTEIN FROM EVD. THE EPITOPES SHOWING THE HIGHEST PROBABILITY OF WORKING AS EPITOPES ARE SHOWED FIRST ON THE TABLE. THE IPRACQKLS PROTEIN OF THE MHC CLASS I SHOWED THE HIGHEST PROBABILITY AMONG ALL THE PROTEINS BUILDING UP THESE COMPLEXES.

To contrast this information, I tested the Ebola protein again, but this time using the IEDB analysis resource tools. The MHC source species were again human and using the information which was provided by the Vaxitop outcome, I selected to only take into account the HLA-B*07: 02 allele from the MHC class I. Once again, the most probable epitope was IPRACQKSL (Table 8).

START	END	LENGTH	PEPTIDE	PROTEASOME SCORE	TAP SCORE	MHC SCORE	PROCESSING SCORE	TOTAL
92	100	9	IPRACQKSL	1.63	0.37	-0.59	2.00	1.41
101	109	9	RPVPPSPKI	1.09	0.23	-1.87	1.32	-0.55
5	13	9	KPDISAKDL	1.47	0.37	-2.77	1.84	-0.93
61	69	9	SPQCALIQI	1.08	0.18	-2.22	1.26	-0.96
106	114	9	SPKIDRGWV	0.82	0.09	-1.98	0.91	-1.07
20	28	9	HLPGFGTAF	1.35	1.07	-3.86	2.42	-1.44
9	17	9	SAKDLRNIM	1.37	0.14	-2.97	1.51	-1.46
68	76	9	QITKRVPIF	1.47	1.11	-4.23	2.58	-1.65
16	24	9	IMYDHLPGF	1.52	1.15	-4.32	2.66	-1.66

TABLE 8. OUTCOME OF IEDB MHC-I PROCESSING PREDICTIONS FOR THE VP35 PROTEIN FROM EVD. THE EPITOPES SHOWING THE HIGHEST PROBABILITY OF WORKING AS EPITOPES ARE SHOWED FIRST ON THE TABLE. THE IPRACQKSL PROTEIN SHOWED THE HIGHEST PROBABILITY AMONG ALL THE PROTEINS BUILDING UP THIS PROTEIN OF ZEBOV. IN RED, EPITOPES WHICH ALSO APPEARED ON THE VAXITOP OUTCOME.

In fact, if these epitopes are searched in the "Immune Epitope Database", two of them appear to be referenced epitopes with at least one assay done concerning them. The ones who are in the database are IPRACQKSL (epitope ID: 227193) and PPSPKIDRG (epitope ID: 227473), both in larger forms.

The epitope IPRACQKSL has been studied as a part of the GDIPRACQKSLRPVP epitope. Two qualitative binding B Cell assays have tested it. These assays were carried out by Pierre Becquart in 2014. The assays were based on the ELISA method, but none of them showed positive results [(44)].

The epitope PPSPKIDRG was studied in the same year by the same scientists. It was also studied as a part of a larger epitope (PVPPSPKIDRGWVCV). Two assays based on the ELISA method neither showed any positive result.

3.2.3 REVERSE VACCINOLOGY VS CONVENTIONAL VACCINE DEVELOPMENT

The favourable and unfavourable aspects of each way of vaccine development are compiled in the following table (Table 9). It collects all the information showed in this chapter of the study.

CONVENTIONAL VACCINE DEVELOPMENT	REVERSE VACCINOLOGY
It only works with pathogens that can be cultivated *in vivo.*	It does not need to cultivate the pathogens since it works with its genomic sequence.
It needs to identify the components building the pathogen one at a time and then finding the ones which are suitable for vaccine development. It does not work if the antigens can be purified in enough quantity.	It does not need to identify the pathogens' components nor purify them as it works with the whole genomic sequence.
The most abundant proteins are often not suitable vaccine candidates and the less abundant ones, which could be the suitable ones, cannot be identified.	It does not need to identify any protein as it works with the whole genomic sequence [45]. Therefore, antigens not expressed *in vitro* can be identified.
It takes from five to fifteen years to develop a vaccine.	It only takes two years at most.
It can find multiple biomolecular targets as polysaccharides and proteins, among others.	It can only target proteins.
Lipopolysaccharide-based vaccines are possible.	
Glycolipids and other CD1-restricted antigens can be used.	
It only considers structural proteins.	All proteins are considered.
The process of designing a new vaccine requires a lot of money.	The process becomes cheaper.

TABLE 9. TABLE SHOWING AND COMPARING THE CHARACTERISTICS OF BOTH CONVENTIONAL VACCINE DEVELOPMENT AND REVERSE VACCINOLOGY.

3.3 STUDY OF PHAGOCYTOSIS

The experimental part of my Research project is based on an essential experiment carried out by Metchnikoff (1845-1916). Thanks to the experiments and conclusions obtained by this Russian scientist the phagocytosis was discovered.

Phagocytosis is a type of endocytosis by which special cells called phagocytes engulf particles or other cells as food or distinct kinds of bacteria. In some animals such as sponges or amoebas, it is a way of nutrition, but in higher forms of life, such as mammals, phagocytosis works as a defensive reaction against infections and invasions of antigens. The cells which oversee doing this process are called phagocytes. Metchnikoff was who discovered how phagocytes worked.

In 1882, Metchnikoff was observing starfish larvae when he observed some cells which were moving inside the larvae. He thought then: "*These wandering cells in the body of the larva of a starfish, these cells eat food… But they must eat up microbes too! Of course, the wandering cells are what protects the starfish from microbes! Our wandering cells, the white cells of our blood – they must be what protect us from invading germs.*"[46].

He then threw thorns from garden plant into the pot where he had the larvae. The next morning, the cells he had noticed were aggregated around the antibodies trying to drive them out. He was observing an immune response of this protecting cells. Metchnikoff coined the term phagocyte, from the Greek words "phages" (to eat) and "cite" (cell).

This was not the only experiment he did. Later, he mixed *Daphnia magna* with spores of infectious fungus. The reaction was similar to the one observed in the starfish experiment: some cells engulfed the infectious spores. This reaction was then compared with the white blood cells aggregating an infected wound.

I tried to replicate this last experiment using *Daphnia magna* (Figure 32) and some bacteria as *Escherichia coli* and *Serratia*

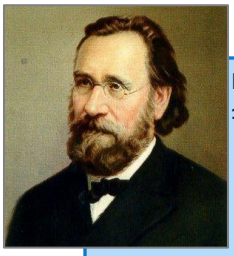


FIGURE 31. ILIÁ MÉCHIKOV (1845 - 1916), RUSSIAN MICROBIOLOGIST.

Iliá Méchnikov

Iliá Ilich Méchnikov (Figure 31), often written as Elie Metchnikoff, was a Russian microbiologist who was born in Járkov, the Russian Empire, on 1845. He won the Nobel in Physiology or Medicine on the 1908 in recognition of his work on the immunity field. He also studied Zoology and Gerontology.

He was involved with a lot of institutions through his life, such as the Kharkiv University, the University of Giessen, the Munich Academy and the Pasteur Institute where he was named vice director [47], among others.

He is known for coining the terms "gerontology" and "thanatology" and for being one of the primary researchers of life-extending methods, showing how the immune system (phagocytes) and the microbiome affects in degenerative aging processes.

He died in 1916 in Paris after a difficult life and two suicide attempts which both failed. The cause of his death was a heart failure at the age of 71.

marcescens, among others. To show the procedure and conclusions extracted from my experiment, I wrote a scientific article which would be attached to this document.

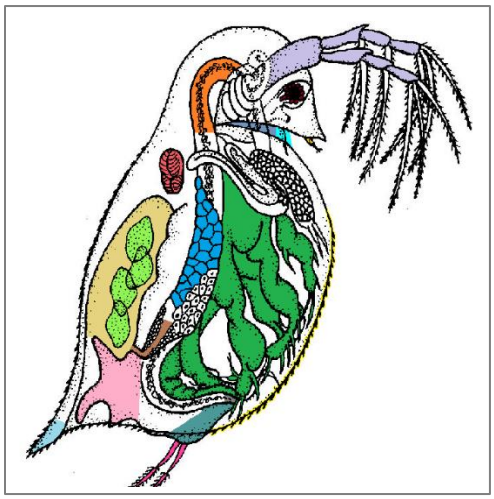


FIGURE 33. IMAGE USED AS A BASE TO THE IMAGE MAP. IT SHOWS THE BASIC STRUCTURES ON THE BODY OF DAPHNIA MAGNA.

FIGURE 32. PAIR OF DAPHNIA MAGNA WHERE THE EMBRYOS ARE EASILY DETECTABLE ON THE BIGGER ONE. PHOTO BY HARALD OLSEN FROM THE NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY.

To start the investigation, I first did a brief study *Daphnia magna's* biology. Following the information from an article by Ebert. D published in 2005 [48] and a webpage ran by Sai S., Bharat V., Vikash K., and Sai M, I did an image map of the basic biology of this animal. The image map can be seen following the link below:

https://minervamacias.neocities.org/InteractiveDaphnia

To create this image map I used the image editor GIMP (version 2.8.22) and Sublime text, a text editor for HTML code. To highlight the different body parts of the animal when the mouse goes over them, I used Mapper. js, a script of the german programming group Netzgesta. On Figure 34, it is shown the HTML aspect of the image map which had to be written from the start. The same process was used to create the Ebola cronology image map showed before and all the other image maps.

```
<HTML>

<script type="text/javascript" src="wz_jsgraphics.js"></script>

<script type="text/javascript" src="mapper.js"></script>

<img src="Daphnia.png" class="mapper" width="900" height="703" border="0"
usemap="#map" />

<map name="map">

<area shape="poly"
coords="283,209,274,207,267,212,261,227,264,248,265,266,277,274,293,273,295,267,29
7,249,296,227,294,215,293,214" class="noborder icolorff0000" title="Heart.

Daphnia&apos;s beats 200 times per minute at 20ºC, slowing down at lower
temperaturess. They have an open blood circulation system with the heart located
dorsally." nohref="nohref" />

<area shape="poly"
coords="225,317,210,333,210,355,220,378,216,386,204,378,192,406,193,435,189,453,18
0,465,190,483,196,500,204,499,218,476,212,444,227,421,225,409,248,399,253,375,243,
359,251,348,251,327,246,303" class="noborder icolor009933" title="Parthogenic
Embryo.

Females produce a clutch of parthenogenetic eggs every molt (3/4 days) by asexual
reproduction (apomixis). In a typical growth season, the eggs are diploid (2n).
The new Daphnia are released by the mother through ventral flexion of the post-
abdomen." nohref="nohref" />

[…]

</map>

</HTML>
```

FIGURE 34. HTML ASPECT OF THE DAPHNIA MAGNA'S IMAGE MAP. (SUBLIME TEXT, GIMP).

As it can be seen, an HTML file always starts with **<HTML>** and ends with **</HTML>** (Figure 34). It indicated where the code starts and where it ends. In the next two lines, two different scripts are added. Thanks to these scripts, when the mouse goes over a determinate area, it gets highlighted.

The next line describes the general characteristics of the image map. First of all, we write what image do we want to be used for the map. It is indicated with "**img src=**". If the image is saved in the same folder as the HTML file, we only have to write its name and its filename extension. In this case it is "Daphnia.png". "**class=**" tells the program that we are designing a map and "**height=**" and "**width=**" describe the dimensions of the map. We decided that our map would have no border. **<map name="map">** indicates where does the map begin, similar to what **</HTML>** does.

The next lines all start with "**area shape= "poly""**, which means that all our areas are polygons. Next to it we find "**coords=**" followed by the multiple coords that define our area. "**class="noborder icolorffoooo"**" indicates the program that when our mouse goes over that area, it must be highlighted in the FFoooo color. This color code is called HEX color and it defines all the colors with only six signs, either numbers or letters. All the text inside "**title="** is the text that it is going to be seen once we go over the

area with our mouse. **"nohref="** would lead us to a link if we wrote a direction, but as it can be seen in this image map we didn't write anything.

Some symbols are not included in the HTML language and they must be written on a special form. For example, to write an apostrophe, **"'"** needs to be written as it can be seen on line 10 (Figure 34).

Once I had a brief idea of *Daphnia magna* biology (Figure 33), I started to design the experiment. The main question which was trying to be resolved was: "How do some bacteria affect *Daphnia magna*? How do they kill *Daphnia*?". We set an independent variable (presence or not of bacteria) and a dependent variable (number of living *Daphnia* after 24 hours in the same water tank). To avoid counting deaths non-related to bacteria, a control tank in the same conditions as the infected tank was observed all the time. If some *Daphnia* in the control tank died, the deaths in the bacteria tank couldn't be solely due to bacteria, thus the experiment was considered void. Only if there were no deaths in the control tank, the experiment could be considered meaningful.

Each bacterial infection was replicated three times in order to avoid errors and be as accurate as possible. Replicating the experiment several times were the only way to eliminate randomness or unknown factors.

At the end of the 24-hour period, the living *Daphnia* were counted and returned to the *Daphnia* tanks. The data were registered on a table (Table 10). The conclusions of these sets of data can be seen in the scientific article.

Bacterium	**Nº of *Daphnia* at the starting point**	**Living *Daphnia* after 24 hours. (1st experiment)**	**Living *Daphnia* after 24 hours. (2nd experiment)**	**Living *Daphnia* after 24 hours. (3rd experiment)**	**Average percentage of mortality (%)**
Bacillus cereus	20	17	15	13	25%
Bacillus megaterium	20	8	11	9	53%
Escherichia coli	20	8	9	8	58%
Micrococcus luteus	20	0	3	5	87%
Pseudomonas fluorescens	20	10	9	11	50%
Staphylococcus epidermidis	20	8	10	7	58%
Serratia marcescens	20	0	0	0	100%

TABLE 10. DATA OBTAINED FROM THE MULTIPLE EXPERIMENTS CARRIED OUT FROM AUGUST TO OCTOBER 2017. THE TABLE SHOWS THE BACTERIUM WHICH INFECTED *DAPHNIA* ON EACH EXPERIMENT, THE LIVING SPECIMENS AFTER 24 HOURS ON EVERY INFECTION AND THE AVERAGE PERCENTAGE OF FATALITY THAT EACH BACTERIUM CAUSED. AS CAN BE SEEN ON THE TABLE, THE BACTERIUM WHICH CAUSED THE HIGHEST MORTALITY WAS *SERRATIA MARCESCENS*, EVEN THOUGH OTHER BACTERIA ALSHO SHOWED A HIGH DEATH RATE.

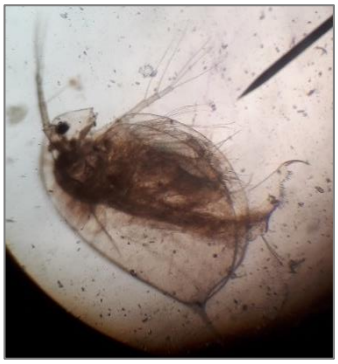


FIGURE 35. DAPHNIA MAGNA SPECIMEN OBSERVED THROUGH A ZEISS MICROSCOPE.

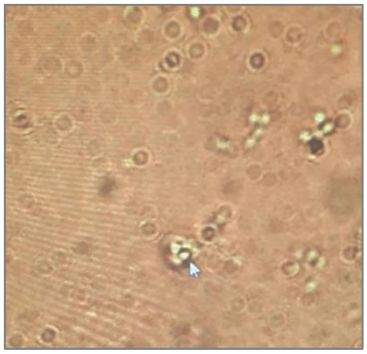


FIGURE 36 BACILLUS MEGATERIUM (X630). PHOTOGRAPH TAKEN THROUGH A MOTICOM CAMERA CONNECTED TO A ZEISS MICROSCOPE IN UNIVERSITAT DE BARCELONA. THE BACTERIUM IS POSSIBLY IN A DEFENSE STRUCTURE.

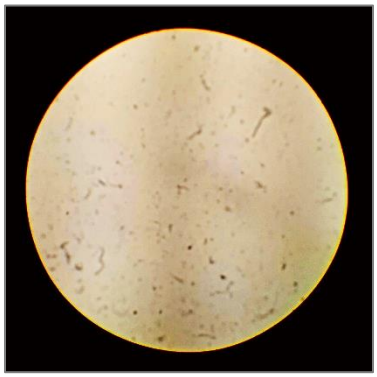


FIGURE 37. ESCHERICHIA COLI (X 1000). PHOTOGRAPH DONE THROUGH A ZEISS MICROSCOPE IN UNIVERSITAT DE BARCELONA.

4. DISCUSSION

The main question now is the next one: why is rVSV-ZEBOV not being used to prevent more Zaire ebolavirus outbreaks? The astonishing protection rate that the vaccine provided to contacts and contacts of these contacts which were related with infected patients should be enough to consider this medicine as a key discovery in the fight against EVD. However, it is not being like that and rVSV-ZEBOV is unavailable for commercial use.

The vaccine was first created in 2003 and until 2009 it had been tested in three animal trials. The human trials started back in 2014. The phase I was first done with a high dose which caused arthritis and rashes. The clinical trial was halted, and it started again with a lowest dose [49]. The phase II treated health workers who had been in contact with Ebola patients while phase III was a ring vaccination (Figure 20). It was phase III which reached the 100% efficacy at the time of protecting people susceptible of contracting the disease.

But this rate was strongly discussed once the vaccine got to the committee of approval. The committee stated the lack of a placebo arm and a delayed treatment group which would work as a control group, but which was after showing elevated levels of protection (supposed herd immunity). These facts left the trial with rather weak reliability. Under an intention-to-treat analysis [50]. The authors of the 2016 report said that it was impossible to affirm the 100% efficacy.

That would be the reason that explains why the vaccine is not commercialized yet and why it has been only used in emergency outbreaks: its efficacy has been highly doubted by the scientific community.

Otherwise, our results of the *in silico* study of the disease did not clarify if there's a protein in the virus which could work as a vaccine. The RANKPEP (Table 6) results did not totally match with the results of Vaxitop (Table 7) and IEDB (Table 8). However, the three of them situated VP35 as the most likely protein to become a vaccine to prevent the Ebola virus disease.

The three epitopes which were most repeated were IPRACQKSL, SPKIDRGWV and IMYDHLPGF. Two of them, IPRACQKSL and SPKIDRGWV, have been already tested. This fact supports our investigation, although there haven't been positive results yet. These studies looked for efficient epitopes in 4 proteins (glycoprotein, nucleoproteins, VP40 and VP35). Human sera from different survivors of EVD and from non-infected people were tested. Sera from survivors collected 7 days after the end of symptoms and from survivors collected in 2008 reacted with 14 VP35 peptides.

On the other hand, AKDLRNIMY was the most likely protein to become a vaccine by RANKPEP. Pierre Becquart and his team also made two assays concerning that epitope but, again, there were no positive results. However, Pierre talks about an epitope which is positioned from amino acid 4 to 13 that it has been one of the 14 active VP35 peptides. AKDLRNIMY is positioned from amino acid 10 to 19 so it will be partially active taking into account Becquart's studies [51].

The lack of any protein which could be targeted by now makes us think about the impossibility of developing a vaccine based on an Ebola protein. Maybe the substance that a lot of scientists are looking for is not a protein but another substance. Polysaccharides, lipopolysaccharides, glycolipids or CD1-restricted antigens could be that substance. Going back to Table 6, it can be observed that this kind

of targets cannot be identified by Reverse Vaccinology methods. That means that a lot of money and a lot of time must be invested to develop a new vaccine against EBOV.

The results of our survey showed interesting results. Most of the people who were surveyed knew nearly anything about such an important and lethal disease. Putting aside this fact, it seems that men were more concerned that women about the illness. The percentages of participation were the following ones: 77% women and **23**% men. The men who were surveyed tended to be more fatalistic because in both answers concerning the mortality rate of the illness, the proportion of the answers varied. 57 men answered that it had a 90% mortality rate (**25.3**% of the total responses to this question) and 6 of them answered that it had a 100% (**28.57**% of the total answers for this question). Analysing the same data but now taking into account the age, the older groups (from 45 to 60 and older than 60) showed disperse results: 38% voted to less than 20% mortality rate, 19% to 50%, 23.8% to 75%, 14.44% to 90% and 4.76% to 100%

5. CONCLUSSIONS

1. Original images using GIMP software (Figures 1, 5, 13, 14, 15, 16 and 33) and using PAINT 3D (Figures 17 and 20) have been created to introduce this research project on Ebola virus (EBOV), immune system, Reverse Vaccinology, *Daphnia magna* toxicity test and bacteria. Also microphotographs taken with microscopes from CESIRE and School of Pharmacy of both bacteria and *Daphnia* are also available (Figures 35-37)

2. Choropleth maps R programming language and library *Plotly* and a heat map using Google Fusion Tables have been created to study EBOV disease distribution. The heat map shows a concentration of EBOV disease in the area of Central Africa, more specifically in the Democratic Republic of Congo with over 1187 cases (Table 1, Figure 2). The choropleth map shows the same information including some outbreaks which are located in Europe and America (Figures 3-5).

3. A website created with HTML, CSS and JavaScript programming is available for this research project at **https://minervamacias.neocities.org/presentation.HTML**. Website include interactive image maps created with JavaScript library mapper.js and Google Fusion Tables on *Daphnia* (**https://minervamacias.neocities.org/InteractiveDaphnia/**), the Africa map (**https://minervamacias.neocities.org/InteractiveAfrica/**), a worldwide outbreaks map (**https://minervamacias.neocities.org/InteractiveOutbreaks/**) and the EBOV virus (**https://minervamacias.neocities.org/InteractiveEBOV/**) in order to understand *Daphnia's* and EBOV physiology and what areas do the virus affect the most.

4. Results of a survey (N=1038) showed 38% think EBOV disease is transmitted by air, when it is not like this. 55% of the respondents thought that EBOV caused a higher mortality than it actually has (nearly 50%). Despite these negative views that the disease suffers, the 74% of the people said they would hire a recovered patient.

5. In silico studies on 7 EBOV proteins with RANKPEP have shown the most suitable protein to create a vaccine was the VP35 (Table 6). Vaxign along with IEDB shows the best peptide belonging to this protein which is most suitable to create a vaccine is IPRACQKSL (Tables 7 and 8).

6. A scientific article (annex 1) and a scientific poster have been created with our main research results (annex 2) about bacterial toxicity related with Metchnikoff's phagocytosis.

7. *Daphnia* ecotoxicology tests (ISO 6341) have shown a toxic effect produced by bacteria after a 24h infection with mortality rates of 100% on *Serratia marcescens*, being followed by *Micrococcus luteus* (87%), *Escherichia coli* (58%), *Staphylococcus epidermis* (58%), *Bacillus megaterium* (53%), *Pseudomonas fluorescens* (50%) and *Bacillus cereus* (25%).

8. *Serratia marcescens*, in 8 hours, produced a reduction in the heart rate on an infected *Daphnia* of 5.25% (from 199 beats/minute to 180 beats/minute) ($p<0.05$).

9. *In silico* studies of docking energies of *Serratia marcescens* chitinases A, B, C and chitobiase using Swiss Bioinformatics Institute software showed the best binding energy is chitobiase with chitoriose (15.39 kcal/mol) and it statistically lower than chitinase B (18.5 kcal/mol) probably because chitobiose interacts with dimers of chitin while chitinases interact with whole and larger chains of chitin. Probably Chitinases from *Serratia* could destroy Daphnia chitin carapace.

6. GLOSSARY

All the glossary entries follow the same structure:

Word *(POS):* Brief definition (page or pages where it appears).

ADCC *(noun):* Antibody-dependent cell-mediated cytotoxicity. A type of immune reaction in which a target cell or microbe is coated with antibodies and killed by certain types of white blood cells. The white blood cells bind to the antibodies and release substances that kill the target cells or microbes. Also called antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (16).

Antibody *(noun):* A blood protein produced in response to and counteracting a specific antigen. Antibodies combine chemically with substances which the body recognizes as alien, such as bacteria, viruses, and foreign substances in the blood (15, 16).

Antigen *(noun):* A toxin or other foreign substance which induces an immune response in the body, especially the production of antibodies (14, 15, 16, 26, 30, 31, 36).

Apoptosis *(noun):* The death of cells which occurs as a normal and controlled part of an organism's growth or development (15, 16).

B-cell *(noun):* B-lymphocyte. A lymphocyte not processed by the thymus gland, and responsible for producing antibodies (15, 16, 29).

Binding site (*noun*): A location on a macromolecule or cellular structure at which chemical interaction with a specific active substance takes place (6, 25, 27, 28, 29, 38).

Butcher, to *(verb):* Slaughter or cut up (an animal) for food (13).

Cell agglutination *(noun):* Clump together (14).

Cell lysis *(noun):* Disintegration or decomposition of cells (14, 15).

Cephalophus *(noun):* Mammal genus which contains two species of duiker, a type of small antelope (8).

Chemokines *(noun):* Any of a class of cytokines with functions that include attracting white blood cells to sites of infection (14).

Chemotaxis *(noun):* Movement of a motile cell or organism, or part of one, in a direction corresponding to a gradient of increasing or decreasing concentration of a particular substance (14).

Complement system *(noun):* A group of proteins present in blood plasma and tissue fluid which combine with an antigen–antibody complex to bring about the lysis of foreign cells (14).

Cytokine *(noun):* Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells (14, 16).

Dendritic cell *(noun):* Cell that haves a short-branched extension, along which impulses received from other cells at synapses are transmitted to the cell body (16).

ELISA *(noun)*: Enzyme-linked immunosorbent assay, an immunological assay technique making use of an enzyme bonded to a particular antibody or antigen (29).

Endocytosis *(noun):* The taking in of matter by a living cell by invagination of its membrane to form a vacuole (31).

Exocytosis *(noun):* A process by which the contents of a cell vacuole are released to the exterior through fusion of the vacuole membrane with the cell membrane (15).

Genome *(noun):* The haploid set of chromosomes in a gamete or microorganism, or in each cell of a multicellular organism (6, 26, 27).

Genus *(noun):* A principal taxonomic category that ranks above species and below family and is denoted by a capitalized Latin name (4).

Herd immunity *(noun):* The resistance to the spread of a contagious disease within a population that results if a sufficiently high proportion of individuals are immune to the disease, especially through vaccination (18).

Hypothesis *(noun):* A supposition or proposed explanation made on the basis of limited evidence as a starting point for further investigation (10).

Immune *(adjective):* Resistant to a particular infection or toxin owing to the presence of specific antibodies or sensitized white blood cells (6, 13, 14, 15, 16, 17, 18, 26, 29, 31, 37).

Immune system *(noun):* The organs and processes of the body that provide resistance to infection and toxins. Organs include the thymus, bone marrow, and lymph nodes (13, 14, 15, 18, 37).

In silico (adjective): technique of performing on computer or via computer simulation (4, 7, 26, 37, 38).

***In vitro (adjective)*:** Latin for *within the glass,* technique of performing a given procedure in a controlled environment outside of a living organism (7, 26, 30).

In vivo (adjective): Latin for *within the living*, experimentation using a whole, living organism as opposed to a partial or dead organism (30).

Kidney *(noun):* Each of a pair of organs in the abdominal cavity of mammals, birds, and reptiles, that excrete urine (8, 23).

Liver *(noun):* A large lobed glandular organ in the abdomen of vertebrates, involved in many metabolic processes (8, 23).

Macrophage *(noun):* A large phagocytic cell found in stationary form in the tissues or as a mobile white blood cell, especially at sites of infection (16).

Medical waste *(noun):* Subset of wastes generated at health care facilities, such as hospitals, physicians' offices, dental practices, blood banks, and veterinary hospitals/clinics, as well as medical research facilities and laboratories which may be contaminated by blood, body fluids or other potentially infectious materials (13).

MHC (*noun*): Major histocompatibility complex. A genetic system that allows large proteins in immune system cells to identify compatible or foreign proteins. It allows the matching of potential organ or bone marrow donors with recipients (15, 28, 29).

Microbiome *(noun):* The microorganisms in a particular environment (including the body or a part of the body) (31).

Mucous membrane *(noun):* An epithelial tissue which secretes mucus, and lines many body cavities and tubular organs including the gut and respiratory passages (13).

NK cell *(noun):* A lymphocyte able to bind to certain tumour cells and virus-infected cells without the stimulation of antigens and kill them by the insertion of granules containing perforin (15).

Opsonise *(verb):* Make (a foreign cell) more susceptible to phagocytosis (14).

Outbreak *(noun):* A sudden occurrence of something unwelcome, such as war or disease (4, 8, 9, 10, 11, 12, 17, 19, 21, 25, 37).

Pathogen *(noun):* A bacterium, virus, or other microorganism that can cause disease (6, 15, 16, 26, 27, 30).

Phagocytosis *(noun):* The ingestion of bacteria or other material by phagocytes and amoeboid protozoans (4, 14, 31, 37).

Phagolysosome *(noun):* A structure formed in the cytoplasm of a cell by the fusion of a phagosome and a lysosome (15).

Phagosome *(noun):* A vacuole in the cytoplasm of a cell, containing a phagocytosed particle enclosed within a part of the cell membrane (15).

Proteome *(noun):* The entire complement of proteins that is or can be expressed by a cell, tissue, or organism (6).

P value *(noun)*: Probability for a given statistical model that, when the null hypothesis is true, the statistical summary would be the same as/greater magnitude than the actual observed results (28,29).

Reverse vaccinology *(noun):* Improvement on vaccinology that employs bioinformatics, pioneered by Rino Rappuoli and first used against Serogroup B meningococcus (4, 6, 25, 26, 27, 30, 36, 37).

Ring vaccination *(noun):* Vaccination of people or animals in contact with or proximity to infected individuals, as a strategy for preventing the spread of an outbreak of disease (17, 18).

Smallpox *(noun):* An acute contagious viral disease, with fever and pustules that usually leave permanent scars. It was effectively eradicated through vaccination by 1979. Also called variola (4, 17, 25).

Soiled *(adjective):* Dirty; stained (13).

T-cell *(noun):* T-lymphocyte. A lymphocyte of a type produced or processed by the thymus gland and actively participating in the immune response (15, 16).

Trial *(noun):* A test of the performance, qualities, or suitability of someone or something (17, 18).

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