UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXI CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

IDENTIFICATION AND PRODUCTION OF RECOMBINANT ANTIBODIES TARGETING WEST NILE VIRUS THROUGH NAÏVE AND IMMUNIZED PHAGE-DISPLAY LIBRARIES

Settore scientifico-disciplinare: **BIO/13**

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Abstract

West Nile virus (WNV) is a member of the Flavivirus genus, which includes several human pathogens transmitted by arthropod vectors. The maintenance in nature of WNV is possible through a bird-mosquito life cycle, in which mosquitoes act as transmission vectors and birds as virus amplification hosts. Humans are not able to contribute to WNV transmission cycle, therefore are defined accidental or dead end hosts.

The 20% of WNV infected patients develop a mild disease with influenza like symptoms known as West Nile fever, while in the 1% of cases the viral infection results in a severe neuroinvasive disease, which can lead to death.

The most widely used approaches for WNV diagnosis relay on the detection of specific antibodies against viral antigens and are generally based on ELISA format assays. The weak points of the immunodiagnostic tests are the false positive results deriving by cross-reactive antibodies. The rise of such antibodies is caused by the structural similarity of flaviviruses. The diagnosis becomes particularly complicate in geographical areas interested by the co-circulation of more than one flavivirus or vaccination programs leading to the immune system pre-exposure to flaviviral antigens, as occurs in some European areas interested by the co-circulation of WNV and Tick-Borne Encephalitis virus (TBEV). In addition, the vaccination against TBEV is extensively applied in north Eastern Europe countries.

In order to improve the detection of WNV infection, the needed reagents for immunodiagnostic applications have been produced. Such reagents include viral antigens and highly specific antibodies against the antigens. As most of commercial kit for WNV diagnosis provide the use of the inactivated virus, requiring specialized equipment and extensive production procedures, a fast and easy protocol has been developed for viral antigens expression and purification. The quality of the produced antigens, in terms of purity, integrity and antigenicity, has been confirmed by SDS PAGE, Western Blot and ELISA assays with murine immunized sera. The isolation of antibodies specifically targeting the WNV antigens has been based on two strategies: the selection of a naïve phage library and the construction and screening of immunized phage libraries from murine samples. In addition to libraries construction, the murine samples have been used for immune repertoires analysis by Next Generation Sequencing. Both the approaches have led to the identification of highly sensitive and specific antibodies. Two of them have been characterized, defining their binding and affinity properties for the target through epitope mapping, ELISA and Western Blot assays.

All the collected data suggest the high potentiality of the produced reagents for WNV immunodiagnostic applications.

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1. INTRODUCTION

1.1. WEST NILE VIRUS AND THE FLAVIVIRUS GENUS

West Nile virus (WNV) is a member of the Flavivirus genus, one of the three genera composing the *Flaviviridae* family. The Flavivirus genus includes more than 70 arthropod-borne viruses among which WNV represents one of the most important human pathogenic flaviviruses, together with Zika virus (ZIKV), Dengue virus (DENV), Tick-borne encephalitic virus (TBEV), Yellow fever virus (YFV) and Japanese encephalitis virus (JEV) [1, 2].

Depending on the transmission vector, the flaviviruses are classified into mosquito-borne or tick-borne viruses. WNV belongs to the mosquito-borne group, whose main transmission vectors are mosquitoes of the *Culex* and *Aedes* species. The viruses transmitted by *Culex* mosquitos, like WNV, are generally associated with neurotropic diseases in humans, whereas the *Aedes* species are mainly responsible of viscerotropic and hemorrhagic viruses transmission [3].

The discovery of the main flavivirus members and the related human diseases date back to several years ago, but the few number of cases and the generally attenuated illness symptoms have limited the attention on this group of viruses. The increase in international travels and climatic changes have contributed to the adaptation of flaviviruses arthropod vectors into new geographic areas and the consequent expansion of the affected countries by flaviviral infections [4].

WNV represents the best example of the rapid and efficient adaptation of a flavivirus member in a new environment. After its first isolation in 1937 in Uganda, WNV was disseminated in West African nations and was responsible of mild, self-limiting febrile illness. In 1958 WNV arrived in Europe, maintaining a sporadic and limited activity. It was in the summer of 1999 that the virus dramatically changed its epidemiologic history, appearing for the first time in the New York City and spreading across the American continent [5, 6]. Since 1999, WNV changed also the severity and intensity of the caused outbreaks, becoming the most geographically widespread flavivirus leading to neuroinvasive diseases [7].

1.1.1. Geographical distribution

The dependence of specific vectors for their transmission and maintenance in nature, determines the geographical distribution of flaviviruses. Among the mosquito-borne group, ZIKV circulates in the Americas, Southeast Asia, and the Pacific Islands [8], whereas DENV is endemic in Africa, America and Southeast Asia [9], overlapping with the geographical areas of YFV in Africa and south-America [2].

WNV, as mentioned before, is the widest distributed flavivirus found in almost all the continents [10, 11] (figure 1).

Figure 1. Global geographical distribution of West Nile virus.
Global geographical distribution of West Nile virus indicated in yellow. Adapted from *Viruses* 2013, 5(9), 2079-2105.

On the other hand, TBEV, the main member of the tick-borne group, is prevalent in temperate zones of Europe and Asia, overlapping with some WNV geographic areas in the European continent [12], in which both the viruses are responsible for several human infections (figure 2).

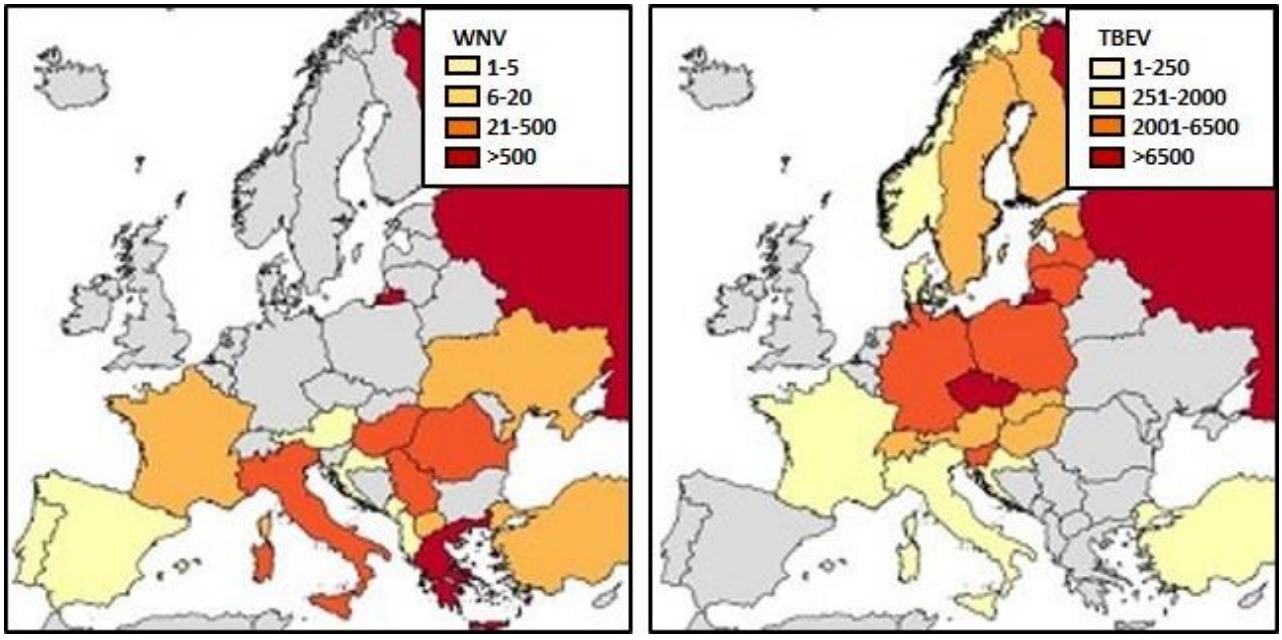

Figure 2. Geographical distribution and case report of West Nile and Tick-borne Encephalitis viruses in Europe.

Geographical distribution of West nile virus (left panel) and Tick-borne Encephalitis virus (right panel) in Europe. The colour legend of the number of reported cases is indicated at the top right of each panel. Adapted from *Int. J. Environ. Res. Public Health* 2013, 10, 6049-6083.

The co-circulation of flaviviruses in the same geographic area is one of the main problems in both surveillance programs and infections diagnosis. The existing antigenic correlation between the flavivirus members, in fact, leads to cross-reactive immune responses and false positive results. This problem becomes huge in areas interested by vaccination programs, as occurs for TBEV [4, 13].

1.1.2. Molecular structure and biogenesis of flaviviruses

Flaviviruses are small enveloped viruses with a diameter of 50 nm. The genome is a positive-stranded RNA of 11,000 nucleotides containing a unique open reading frame (ORF) and two non-coding regions at the 5' and 3' ends. The non-coding regions conserved secondary RNA structures involved in the genome replication and protein translation enhancement. The viral ORF codes for a single polyprotein, later processed into three structural and seven non-structural proteins by viral and host proteases [14] (figure 3).

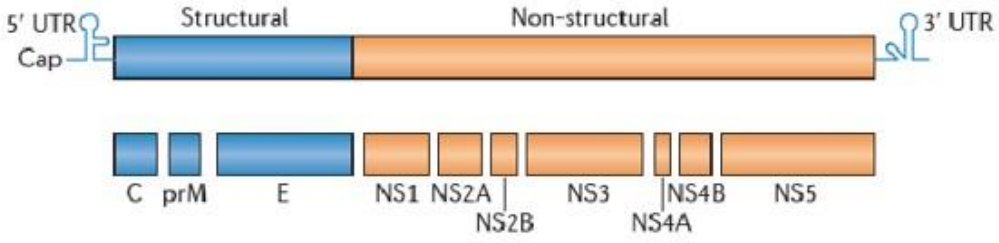

Figure 3. Flavivirus genome organization.

Structure of the flavivirus genome. Two non-coding regions forming secondary structures are reported at 5' (Cap) and 3' ends. The coding regions for structural and non-structural proteins are indicated in blue and orange, respectively. The name of each protein is also indicated. *Int. J. Environ. Res. Public Health* 2013, 10, 6049-6083.

The three structural proteins are indicated as E (Envelope), prM/M (precursor Membrane/Membrane) and C (Capsid). The E and prM/M proteins constitute the envelope covering the viral membrane, while multiple copies of the C protein associate to form the electron-dense nucleocapsid enclosing the RNA genome [14].

During the infection of a host cell, the E protein mediates the attachment of the virus on the cell membrane through specific cell receptors. After the internalization by clathrin-mediated endocytosis, the acidic pH of the endosome determines conformational changes of the viral envelope leading to the fusion of the viral and endosome membranes with the consequent release of the RNA genome

into the cytoplasm [15, 16]. The virus starts its replication cycle and assembles as non-infectious immature virions into membranes derived from endoplasmic reticulum, where the capsid protein can associate with the viral genome. Once completed the assembling of the immature particles, virus maturation proceeds into trans Golgi membranes [17]. During the transport of the virions through the exocytosis pathway, the pH changes lead to E protein rearrangement on the viral surface and the cleavage of the prM into pr peptide and M protein by the host cellular protease furin. Such event determines the maturation and release of infectious viruses in the extracellular milieu, where the pr peptide dissociates from the viral surface [18] (figure 4).

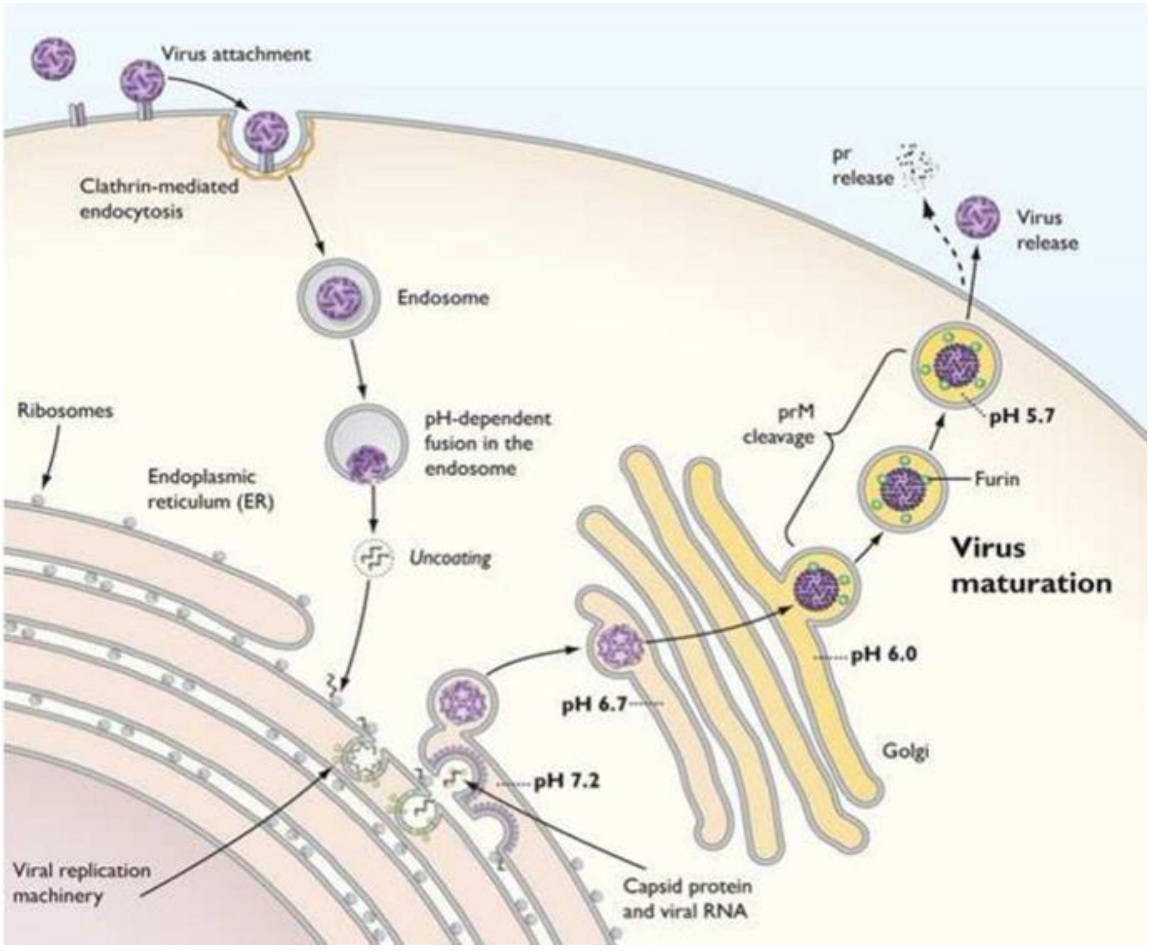

Figure 4. Flavivirus host cell infection and maturation.

Flaviviruses enter cells via a clathrin-dependent endocytic pathway and fuse with endosomal membranes. After viral-endosome membranes fusion, the RNA genome is released into cytoplasm. Immature virions assemble on ER-derived membranes and complete the maturation process during the transport in the trans Golgi network. *Curr Opin Virol*. 2012, 2(2): 168–175.

The viral envelope in the mature and immature forms presents different conformations. In the immature phase, 60 spikes of trimers of prM-E heterodimers characterize the viral surface. After the

prM cleavage in the Trans-Golgi Network (TGN), the virus rearranges the envelope proteins to finally be secreted in its mature status characterized by a smooth envelope surface with a herringbone-like simmetry of 90 antiparallel E dimers organized into 30 rafts of three dimers each [19, 20] (figure 5).

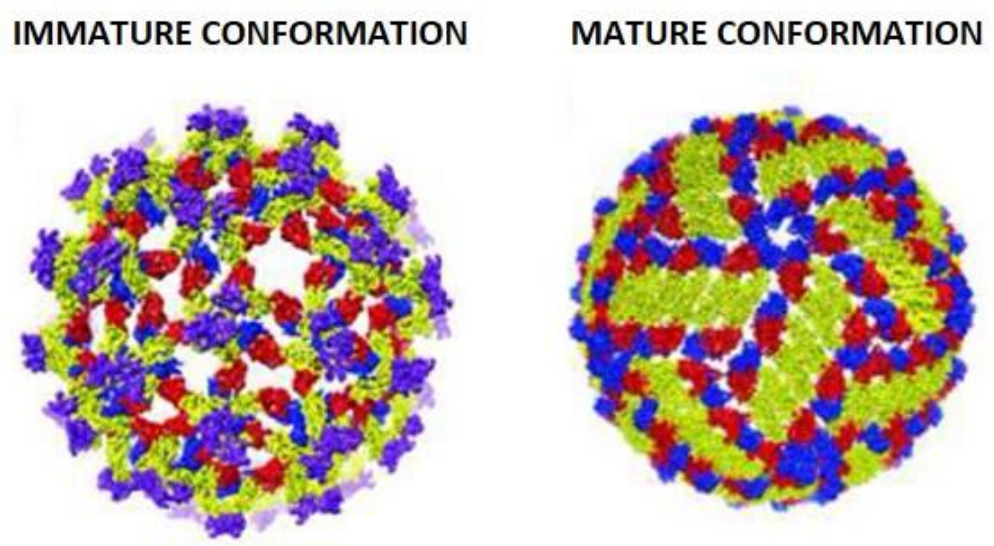

Figure 5. Structure of the flavivirus envelope in the immature and mature conformations.
Organization of prM and E proteins on the viral envelope in immature and mature conformations. prM is indicated in purple. The three domains of E monomer are indicated in blue, red and yellow. Left: arrangement of prM and E proteins on the fully immature virion. Each immature virion is composed of sixty prM-E heterotrimeric spikes. Right: arrangement of E proteins on the mature virion. Each particle is composed of 30 rafts of three antiparallel dimers in a herringbone pattern. *Curr Opin Virol*. 2012 April ; 2(2): 168–175.

1.1.3. The envelope protein

Molecular structure

The E protein monomer contains 500 amino residues and presents a final molecular weight of 53-60 kDa depending on the glycosylation status. The protein is organized into three domains indicated as DI, DII and DIII, connected by flexible hinges. The DIII is followed by a stem region and two transmembrane domains for the anchoring of the protein to the viral membrane (figure 6) [21, 22].
The DI domain contain 120 residues and is flanked by the other two domains. It participates in the protein conformational changes and carries a glycosylation site at residue Asn 154, consistent with most flaviviruses, included WNV [23]. The DIII domain, also known as immunoglobulin-like domain, is located at the C-terminus; it contains 100 amino acids and epitopes involved in the host cell receptor binding to promote the viral entry [24]. The DII is the dimerization domain and carries a highly conserved peptide indicated as fusion loop (FL) [25]. In the E dimeric form, the DIII and DI

domains of each monomer provide a hydrophobic pocket protecting the FL of the close monomer (figure 6).

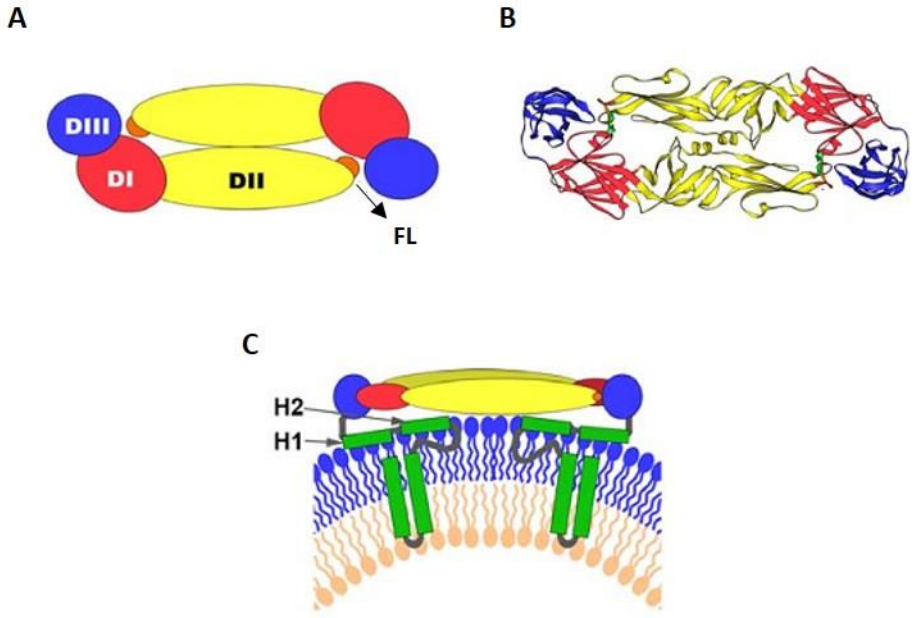

Figure 6. Different representations of the E dimer.

A. Top view of the E protein dimer organization as present on the surface of mature virions. **B.** Crystal structure (top view) of the E dimer. **C.** Side view of the E dimer on the surface of mature virions. The α-helices (H1, H2) of the stem anchor and the two transmembrane domains are indicated in green. Adapted from *PLoS Pathog* 2007, (2): e20.

Starting from the host cell invasion, the E protein undergoes several conformational changes. As mentioned in 1.1.2, the virus endocytosis exposes the E homodimer to the acidic pH of the endosome, triggering its dissociation. The resulting rotation of the E domains leads to the exposure of the FL and its insertion into the host membrane promoting the fusion with the viral membrane [16]. The reorganization of the viral surface occurs also during the transit of the virus through the acidic compartments of the TGN. The low pH exposes the cleavage site on the prM protein recognized by the cellular protease furin. After the cleavage, the pr peptide remains attached to the viral surface, until the secretion of the virus into the neutral extracellular pH allows the release of the peptide and the maturation of the virus in its final conformation characterized by a smooth surface of E dimers [26] (figure 7).

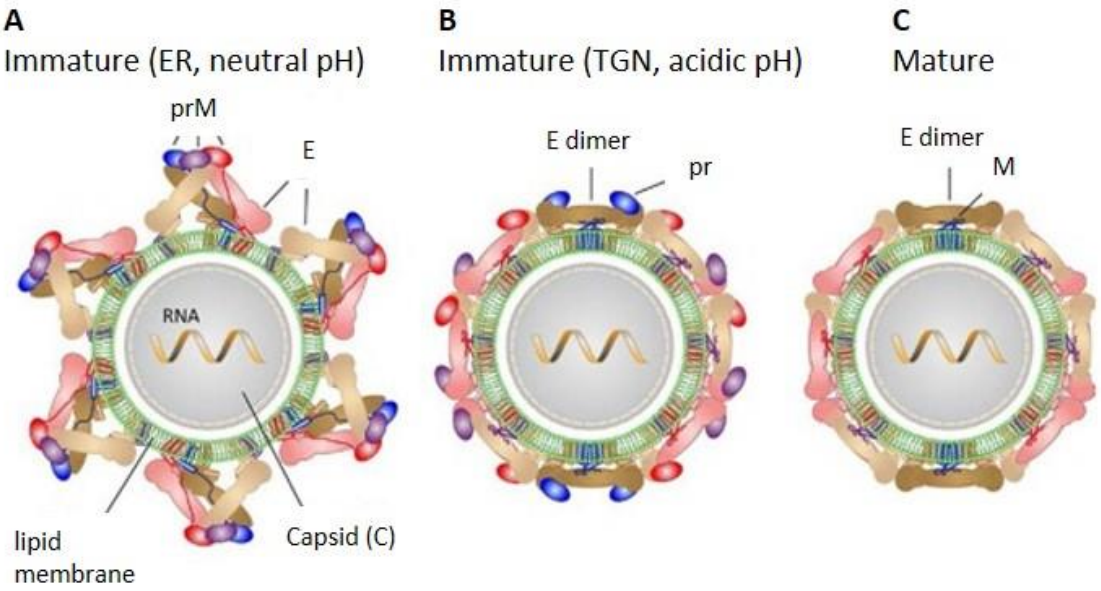

Figure 7. Schematics of envelope conformational changes.

A. Immature virion after budding in the Endoplasmic Reticulum (ER) (neutral pH). **B.** Immature virion after exposure to low pH in the Trans-Golgi Network (TGN) and rearrangement of the envelope proteins. **C.** Mature virion after pr peptide cleavage and secretion from infected cells. *Curr Opin Virol*. 2017, 24: 132–139.

Role of the E protein in host cell infection

The E protein, predominantly exposed on the viral surface, is responsible of the attachment and receptor-binding processes occurring during the host cell entry, as well as the membranes fusion and virions assembly mechanisms described before.

The binding to specific receptors, is preceded by contacts between the E protein and attachment factors that concentrate the virus particles on the host cell membrane. The mainly known attachment factors are Glycosaminoglycans (GAGs) such as the heparan-sulfate proteoglycan. The interactions involve the negative charges of the sulfate groups on the polysaccharide and clusters of positively charged residues on the surface of the E glycoprotein [27, 28].

Less is known about the cellular receptors mediating the virus endocytosis. A role was demonstrated for the $\alpha_v ß_3$ integrin during the infection of mammalian cells by WNV [29], but the use of different lineages of the virus in more recent studies has shown that the integrin is not required in all the infection contexts [30]. The CLR (C-type lectin receptors), the TIM (T-cell immunoglobulin and mucin domain) and TAM (TYRO3, AXL, MER) are the protein families best characterized for their implication in flavivirus binding [31].

Several members of the CLRs family are expressed on myeloid cells, including monocytes, macrophages and dendritic cells (DCs) and recognize carbohydrate profiles on pathogens to promote

their clearance to the immune defences. The N-linked glycan on the E protein of WNV is able to interact with the two C-type lectins DC-SIGN and L-SIGN, promoting the virus internalization [32]. In the case of the TIM and TAM receptor families, the E protein is involved in indirect manner. As the physiological functions of these receptors is to recognize lipids negatively charged in apoptotic cells and to trigger their endocytic engulfment by phagocytic cells, the interactions during virus entry occur with the negatively charged lipids, such as phosphatidylserine (PS), in the viral membrane. The process is known as 'apoptotic mimicry' [33].

E protein antigenicity

The E protein has a fundamental role in host cell invasion and therefore is the main target of the antibodies elicited after flavivirus infection. The neutralization mechanisms performed by anti-E antibodies to contain the infection occur by preventing the virus attachment to the host cell and/or the membranes fusion and several studies have been conducted to identify the E epitopes involved in the neutralization processes.

Antibodies able to inhibit the viral infection have been mapped on all the three domains of the E protein, but those directed against the DIII show the highest neutralizing potency [34, 35]. The DIII domain protrudes the farthest from the surface of the virus and studies have demonstrated that mutations in the DIII can affect the virus tropism or virulence, highlighting its primary involvement in virus attachment to the target cell [36, 37]. These DIII properties suggest a mechanism of neutralization based on the blockade of the domain to prevent virus binding to the host cell. In addition, the DIII is the E-domain presenting the highest sequence variability among flaviviruses, a feature that correlates with the high specificity observed for anti-DIII antibodies [35].

Although the DIII domain contains epitopes eliciting the most specific and neutralizing antibodies, the immune response after flavivirus infection develops antibodies primarily against the DI and DII domains. These antibodies have lower neutralizing activity and higher cross-reactivity if compared to anti-DIII antibodies [35, 38–40]. One of the targeted epitope within the DI/DII region is the FL. As mentioned before, the FL sequence is highly conserved among flaviviruses and this feature explains the low specificity characterizing most of the anti-FL antibodies [41, 42]. However, a cross-reactive antibody targeting the FL and showing strong neutralizing activity has been described [43].

The analysis of the immune response elicited after flavivirus infection has led to the identification of a more complex class of epitopes on the viral envelope. These sites are quaternary structures exposed at the E-dimer interface, targeted by highly neutralizing antibodies. In this case, the mechanism of neutralization is based on the inhibition of the envelope structural rearrangement required for viral infection. The first human antibody mapping on the E-dimer has been described for WNV [44].

Recently, human antibodies binding quaternary structures have been found also for DENV and ZIKV [42, 45, 46]. The surprising discovery emerged from the characterization of anti-DENV antibodies targeting E-dimer sites, is their ability to be highly neutralizing against both DENV and ZIKV infections [46].

The strong neutralizing properties described for some anti-E antibodies have led to their evaluation as therapeutic drugs for flavivirus infections. The most promising antibodies under clinical trial and their respective recognized epitopes are summarized in the following table.

Table 1. Neutralizing antibodies under clinical trial targeting the E protein of flavivirus members and proposed as therapeutic drugs.

Antibody name	E epitope	Virus	Reference
E16	DIII	WNV	Nybakken G.E. et al., 2005
CR4374	DIII	WNV	Throsby M. et al., 2006
DENV1-E105, E106	DIII	DENV1	Shrestha B. et al., 2010
Ab513	DIII	DENV1-4	Robinson L.N. et al., 2015
ZKA64	DIII	ZIKV	Stettler K. et al., 2016
ZV54	DIII	ZIKV	Zhao H.Y. Et al., 2016
ZV67	DIII	ZIKV	Zhao H.Y. Et al., 2016
Z23	DIII	ZIKV	Wang Q.H. et al., 2016
2A10G6	FL	WNV, DENV1-4, ZIKV, YFV, TBEV	Deng Y.Q. et al., 2011 Dai L.P. et al., 2016
HM14c10	E-dimer interface	DENV1	Teoh E.P. et al., 2012
2D22	E-dimer interface	DENV2	De Alwis R. et al., 2012
ZIKV-117	E-dimer interface	ZIKV	Hasan S.S. et al., 2017
EDE1-B10	E-dimer interface	DENV/ZIKV	Fernandez E. et al., 2017

1.1.4. Life cycle of WNV and immune response after infection

The maintenance of WNV in nature is possible through a life cycle involving vectors and host reservoirs. As mentioned in the section 1.1, WNV belongs to the mosquito-borne Flavivirus group and its main vectors are mosquitoes of the *Culex* species. The predominant host reservoir are wild birds of the *Corvidae* family, which generally become infected without signs of disease and act as virus carriers. Some bird species are more susceptible to the virus infection showing symptoms of illness, which can lead to the animal death [47].

The acquisition of the virus by mosquito occurs during blood meal from an infected bird. The virus begins the replication process in the midgut epithelia and travels through the mosquito haemolymph

to the salivary glands, where accumulates. During the mosquito feeding on a mammalian host, the high level of viremia in the saliva results in virus transmission. Humans are defined "dead-end" host, as the low level of viremia after infection is not sufficient for the virus transmission to another mosquito vector. For this reason, humans are also defined "accidental" host of WNV, as they do not participate in the virus maintenance in nature [48].

In addition to the classical WNV life cycle, other possible routs of viral transmission have been studied. The contact transmission between birds has been examined in laboratory with several species, but only for few of them (mainly in *Corvidae* and *Laridae* families) a direct infection has been observed [49, 50]. Concerning the direct human-human infection, cases of transmission by blood transfusion have been documented, particularly after the WNV outbreaks occurred after 1999 in the United States [51], leading the American blood collection agencies to screen all donated blood for WNV since then. However, based on the CDC (Centres for Diseases Control and prevention) guidelines on WNV, the risk of human-human infection through blood or other routs such as pregnancy or breast-feeding, is very low due to the limited period of viremia and viral load.

The acquisition of WNV by mosquitoes results in a persistent infection allowing virus accumulation and transmission to mammalian hosts, although evidences suggest that also the mosquito activates an immune response against the virus. These evidences derived mainly by studies on *Drosophila melanogaster*, but the analysis of mosquito immune system reveals corresponding proteins and mechanisms. The signalling pathways proposed as anti-WNV infection in mosquitoes are the Toll, IMD (Immune Deficiency) and JAK-STAT (Janus Kinase-Signal Transducer and Activator of transcription). WNV can be sensed by Toll receptors, activating a serine protease cascade leading to the activation of the MyD88-dependent signalling cascade. The IMD pathway is activated by the virus via PGRP (peptidoglycan recognition protein) and both the Toll and IMD pathways culminate in the expression of antimicrobial peptides mediated by NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells). Also the JAK-STAT pathway activation leads to the expression of antiviral genes mediated, in this case, by the nuclear translocation of STAT [52]. In addition to the signalling pathways, another anti-WNV mechanism of mosquitoes is the RNA interference (RNAi). Replicative dsRNA intermediates activate the RNAi pathway starting with their cleavage into 20-25 bp long RNA termed viRNA (virus induced siRNA) by the RNase III Dicer-2. One strand of the viRNA is degraded, while the other is integrated into the RISC complex (RNA Induced Silencing Complex) and targeted to complementary sequences on the viral single stranded RNA for the degradation. The activation of the RNAi mechanism by WNV has been demonstrated in *Drosophila* [52] and mosquitoes of the *Culex pipiens* species [53]. However, WNV encodes for an RNAi

suppressor in the form of a highly structured subgenomic RNA, that acts as a decoy substrate for Dicer and thereby prevents induction of the RNAi response [54].

Human immune responses to WNV infection

In humans, the majority of WNV infections are asymptomatic (80%), while the 20% of infected patients develop signs of disease that include fever, headache, and tiredness. Among the symptomatic patients, less than the 1% develop a severe disease form known as neuroinvasive, characterized by high fever, meningitis, encephalitis and/or acute flaccid paralysis (*World Health Organization* data, October 2017).

The pathogenesis of WNV in humans has been studied in animal models, defining three phases: the initial infection and spread, the peripheral viral amplification in visceral organs and the neuroinvasion. The first phase begins after the injection of the virus into the skin by an infected mosquito. During the feeding, the mosquito vector releases also saliva factors that alter the local immune responses, reducing inflammation and facilitating the viral spread [55]. The primary host cells for WNV replication are keratinocytes and Langerhans cells, which enter afferent lymphatics and migrate to draining lymph nodes [56, 57]. Here, WNV amplification leads to viremia and spread to secondary lymphoid and visceral organs, including spleen and kidney as primary sites. After amplification in peripheral organs, the virus can enter the circulation and cross the blood brain barrier (BBB), reaching the central nervous system (CNS) [58]. The outcome of WNV infection depends on the balance between the rate of viral dissemination and the innate and adaptive immune responses activated to contain the infection. The type I interferons (IFN-α and IFN-β), secreted by most cell types following viral infection, play a key role as innate immune regulator, inducing neighbouring cells to express antiviral genes. Cells recognize the viral RNA through several sensors, including the TLR3 (Toll-like receptor 3), the RIG-1 (retinoic acid-inducible gene I protein) and the MDA5 (melanoma differentiation antigen 5). Binding of viral genome to these pathogen recognition receptors results in downstream activation of transcription factors such as the IRF3 (IFN regulatory factor 3) and IRF7 (IFN regulatory factor 7) and expression of IFN-stimulated genes. However, WNV uses a passive evasion strategy to mask its genome accumulating the replication complexes in ER membrane vesicles [59]. Other innate defences are mediated by immune cells, among which macrophages and dendritic cells (DCs) acting as link between innate and adaptive responses. After infection, activated macrophages and DCs release pro-inflammatory cytokines and chemokines such as type I INF, TNF (tumour necrosis factor), IL-1 and IL-8 (interleukins1 and 8) that regulate innate cell-mediated responses involving natural killer (NK) cells, neutrophils and γδ T cells. The exact functional role of neutrophils and NK cells in WNV infection is unclear due to contrasting results

observed in animal models, whereas γδ T cells promote protective immunity secreting IFNγ and perforin, implicated in the clearance of WNV-infected cells. The absence of γδ T cells increases the susceptibility to the infection, resulting in a rapid spread of WNV to peripheral organs and the CNS [60, 61].

Another immune response elicited by WNV infection is the complement system, a large family of serum and surface proteins recognizing pathogen structures, altered self-ligands and immune complexes. Its activation occurs through three pathways inducing antiviral mechanisms of the immune system such as the pathogen opsonisation, recruitment of phagocytes, priming of B cells and increase of T cells-mediated killing of infected cells. The viral NS1 protein, secreted by infected cells, is able to attenuate the complement activation binding different factors such as the regulator factor H to facilitate the inactivation of its target C3b [62]. This leads to C3b degradation and a decrease in the membrane attack complex formation on infected cells, required to promote their disruption. Other factors recognized by NS1 to prevent complement activation are the C1s and C4 [63].

As demonstrated in several studies, the adaptive response consisting of antibodies production by B cells and activities carried out by immune CD4+ and CD8+ T cells is essential to mediate protection against WNV infection. Mice lacking B cells uniformly die after WNV infection [64], while naïve mice are protected by passive transfer of immune sera [65]. CD8+ T cells are responsible of the direct killing of infected cells and this function is sustained by CD4+ T cells that, in addition, prime the antibodies response against WNV. Mice deficient for CD8+ or CD4 + T cells show a higher viral load in the CNS [66]. As mentioned in 1.1.3, the majority of the antibodies elicited after infection are directed against the envelope protein and their protective efficacy depends on the ability of blocking viral replication. The classic primary immune response to an antigen results in the production of IgM antibodies followed by a rise of IgG, which can persist for years. In humans WNV infection, the IgM levels, detectable in serum or cerebrospinal fluid (CSF) 3 to 8 days after illness onset, persist up to 90 days. IgG antibodies are generally detected shortly after IgM rise and persist for many years following infection. The absence of IgG and the presence of IgM is invariably an accurate marker of a very recent WNV infection, while the presence of both IgM and IgG antibodies per se provides limited clinical information on the time since infection, as IgM persistence for period longer than 90 days have been documented (CDC data, February 2018).

1.1.5. Diagnosis of WNV infection

The diagnostic methods for WNV infections are divided essentially into two categories: the direct and indirect tests. The first are based on the detection of the viral genome, while the second rely on the detection of antibodies against the virus. The main feature distinguishing the two groups of tests

is the time-window in which they can be applied. The WNV RNA is detectable in blood or CSF by nucleic acid amplification from 2-3 days after infection, when the viremia has reached its pick, and generally decreases few days later, at the time of symptoms onset (figure 8). On the other hand, as mentioned before, IgM are detectable approximatively 3 days after symptoms onset and persist for more than one month, while IgG titre increases two days later and persist for years. For these reasons, the most widely used approaches for WNV diagnosis are the detection of antibodies against the virus in serum or CSF [67].

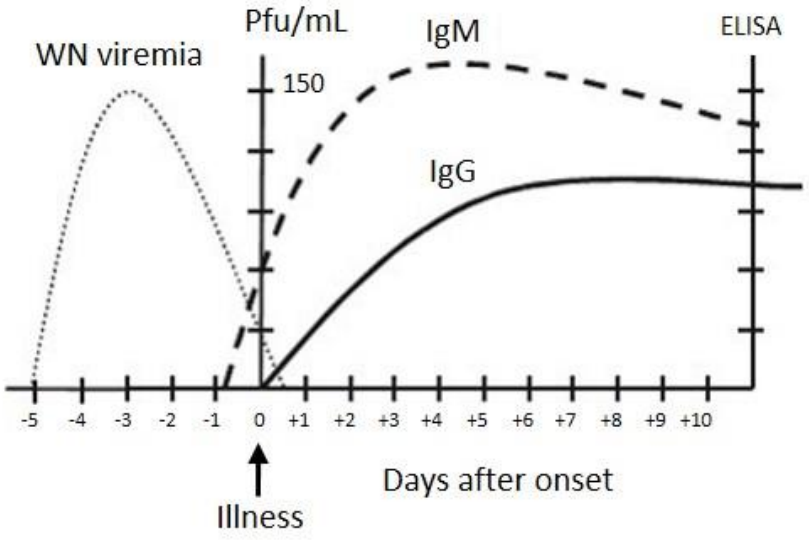

Figure 8. WNV viremia and IgM/IgG response after infection.

Timelines of WNV viremia indicated as pfu (plaque forming unit)/mL and immune response (IgM/IgG) after infection. IgM and IgG are detectable by ELISA assay 3 and 5 days after symptoms onset, respectively. *Mayo Clin Proc.* 2003, 78(9):1137-1144.

Despite viral RNA detection is generally limited to few days, it remains a fast and specific method for WNV diagnosis, especially in immunocompromised patients unable or poorly efficient to develop antibodies against the virus. The Food and Drug Administration has approved two tests for nucleic acid amplification of WNV genome: the Cobas TaqScreen West Nile Virus test (manufactured by Roche Diagnostic) [68] and the Procleix West Nile virus assay (manufactured by Gene-Probe and distributed by Novartis Diagnostic) [69]. The first is a real-time method, based on reverse transcription polymerase chain reaction (RT-PCR), while the second is a transcription mediated amplification (TMA), an isothermal amplification system based on the use of two enzymes (RNA polymerase and reverse transcriptase) able to produce billions copies of the target sequence within 15-30 minutes [69]. The target sequence of the tests is not available to users. Both the tests are fully

automated on high throughput instruments and are able to detect less than 100 genome copies/ml. Due to their high sensitivity, the two molecular tests are used in asymptomatic persons (whose hypothetical viral load could be very low) and to screen blood donations for biological safety testing [70]. As in symptomatic patients the viral load is expected to be higher than in asymptomatic subjects and thus requires less sensitive tests, other molecular diagnostic methods have been developed [71–73].

Among the indirect methods for WNV diagnosis, the detection of specific antibodies based on enzyme-linked immunosorbent assays (ELISAs) is widely used due to their relative applicability in routine laboratory. However, the broad antigenic cross-reactivity between flaviviruses can lead to false positive results and represents the main weakness of the serologic immunoassays.

The ELISA formats mostly used to detect antibodies raised after WNV human infection are the IgM Capture ELISA (MAC-ELISA) and the indirect IgG ELISA [67] (figure 9).

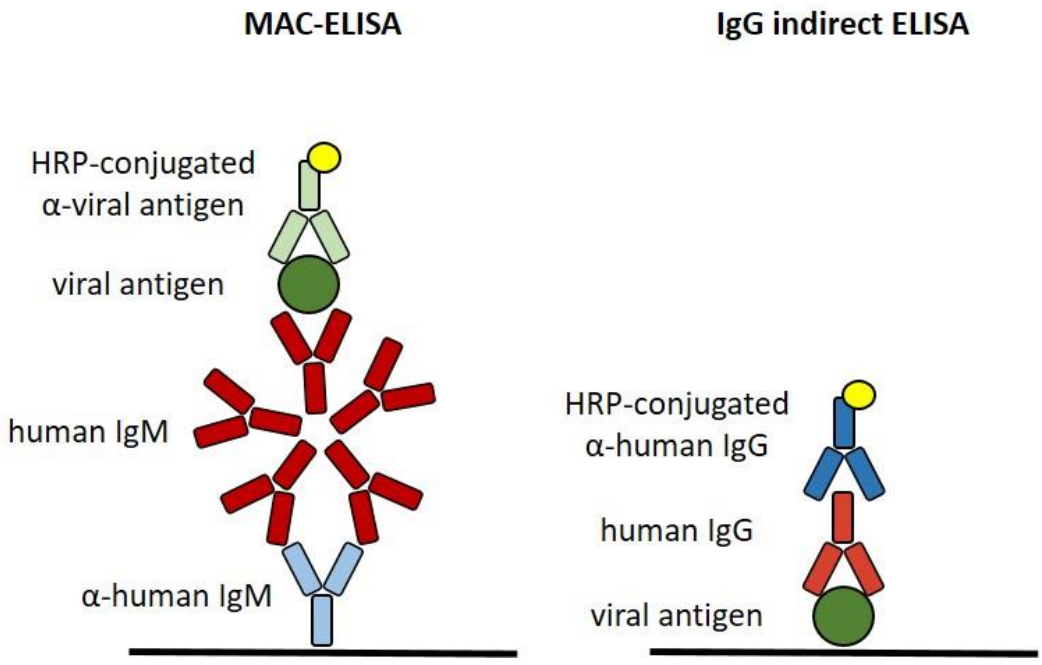

Figure 9. MAC-ELISA and indirect IgG ELISA assays formats for flaviviruses infection diagnosis.
Schematic representation of IgM Antibody Capture ELISA (MAC-ELISA) and indirect IgG ELISA used for flaviviruses infection diagnosis.

The MAC-ELISA uses anti-IgM antibodies to immobilize the serum/CSF-derived IgM on ELISA plate; a WNV antigen (generally the whole inactivated WNV or the viral recombinant envelope protein) is then incubated and, if anti-WNV IgM are present, the antigen is linked. A secondary

antibody against WNV detects the eventually present immune-complexes [74]. This test is useful for diagnosis of acute or recent infection, as IgM production is the first response to the infection and persist in serum and CSF for more than a month. For CSF samples, in particular, the detection of IgM is a clear confirm of an acute phase of viral neuroinvasion because this antibody isotype is unable to cross the blood brain barrier and thus is locally produced by infiltrated lymphocytes in the central nervous system. In addition to the possible specificity problems mentioned before [75], another limit of the MAC-ELISA is the decreased sensitivity occurring when IgM are produced in response to other parallel infections [76].

In the IgG ELISA format, the WNV antigen is directly coated on the plate (generally as immune-complex with an anti-WNV monoclonal antibody). The biological sample is added on the plate and, if anti-WNV IgG are present, the antibodies linked to the viral antigen are detected by the anti-IgG secondary antibody. Despite the high sensitivity of the test, also in this case the major weakness point is the restricted clinical specificity due to extensive cross-reactions observed in presence of IgG elicited by vaccination/past infections with other flaviviruses [77].

One of the main factors affecting the sensitivity and specificity properties of the tests is the viral antigen used. As mentioned in 1.1.3, the DIII domain of the envelope protein contains virus-specific epitopes and different studies have been conducted to evaluate its ability to distinguish different flaviviruses infections and its potential employment in diagnostic tests [78–80].

In order to improve the reliability of the tests, besides the identification of more appropriate reagents, efforts have been made to develop diagnostic test formats for rapid and multiplex analysis. These features are of particular importance in the flaviviruses field, considering their antigenic correlation, co-circulation in some cases and rapid adaptation to new geographic areas, which require continuous epidemiological serosurvay. Fluorescent microspheres immunoassays potentially reflect these properties. Such tests consist of the direct or antibody-mediated covalent attachment of multiple viral antigens to fluorescent and differentiable microspheres; the analysis is performed in microfilter plates and the binding of sample-derived antibodies is detected through a flow cytometer [76]. However, the requirement of specialized laboratories and the expensive costs have limited the application of this technology.

1.2. RECOMBINANT ANTIBODIES PRODUCTION TECHNOLOGIES

1.2.1. Antibodies structure and function

The immunoglobulins (antibodies) are antimicrobial proteins secreted by B lymphocytes which serve as critical participants in the adaptive immune response. The main function of antibodies is to bind foreign molecules in the serum or other body fluids to promote their removal [81]. From a structural

point of view, antibodies are heterodimeric proteins consisting of two heavy (H) and two light (L) chains. Each chain contains a variable (V) domain involved in the antigen binding and constant (C) domains that specify effector functions such as activation of complement or binding to Fc receptors. Each V domain can be split into three regions of sequence variability known as complementarity determining regions, or CDRs, and four regions of relatively constant sequence termed the framework regions, or FRs. The three CDRs of the H chain are paired with the three CDRs of the L chain to form the antigen-binding site (figure 10). Both the L chains of the immunoglobulins contain only one C domain, while the H chains contain either three or four such domains, depending on the specific immunoglobulin isotype. In particular, five main classes of H chain C domains define the immunoglobulins isotypes indicated as IgM, IgG, IgA, IgD and IgE. [82]. A given V region can become associated with any C region through the mechanism of isotype switching occurring during B-cell activation. As IgM can be expressed without isotype switching, the first antibodies to be produced in a humoral immune response are always IgM. These early immunoglobulins form pentamers, whose 10 antigen-binding sites can bind simultaneously to the target and compensate the relatively low affinity of IgM monomers. Concerning the other immunoglobulins classes, while the IgA is the principal isotype in secretions, the most important being those of the mucus epithelium of the intestinal and respiratory tracts, the IgG represents the main immunoglobulin class produced during an immune response [83].

As mentioned before, the antibodies effector activity depends on the Fc domain. In particular, after antigen binding, the immune complex is able to activate the complement system through the Fc interaction with the C1q molecule. Such interaction leads to a cascade of events known as “classical pathway” of complement activation. A more complex mechanism involving the Fc domain is based on its binding to Fc receptors (FcRs), which connects the antibody mediated immune response to cellular effector functions. FcRs are widely expressed by several effector leukocyte types and their binding to immunoglobulins Fc leads to the induction of phagocytosis by phagocytic leukocytes and antibody-dependent cellular cytotoxicity by NK cells [84].

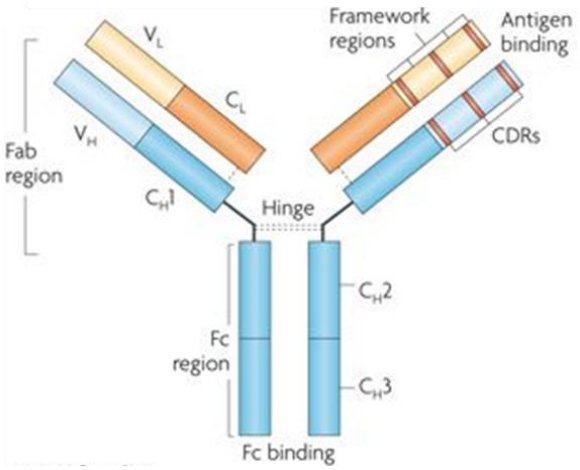

Figure 10. Structure of immunoglobulin.
Adapted from *Nature Reviews Drug Discovery* 2010, 9: 325–338.

1.2.2. Production of monoclonal antibodies

The generation of antibodies represents a field of huge interest for its application in basic research, diagnostics and above all therapy.

The demonstration of immunoglobulins power dates back to a century ago from the studies of Emil von Behring and Shibasaburo Kitasato with polyclonal antibodies contained in the serum of immunized animals and their efficacy to prevent diphtheria [85].

It was in 1975 that the development of hybridoma technology set a milestone in the generation of monoclonal antibodies. The method is based on the fusion of myeloma and B-cells of immunized animals, which leads to the continuous production of monoclonal antibodies by the derived hybrid cell lines [86]. Despite the advantage of producing homogeneous monoclonal antibodies, the hybridoma technology presents some limits. The antibodies produced by hybridoma are mostly of murine species, which can be used for research and diagnostic applications, but as therapeutics have demonstrated relevant side effects. The repeated administration of murine antibodies in humans can stimulate the production of anti-murine antibodies [87]. Such immunogenic reaction was observed with Orthoclone OKT3, the first murine monoclonal antibody approved by the FDA as therapeutic, able to prevent organ rejection after transplantation by blocking the activation of T-cells [88]. In addition, the murine antibodies poorly interact with the human immune effector system [89]. To overcome this problems, different strategies have been developed, such as the combination of non-human variable chain with human constant domains leading to chimeric antibodies [90], the replacing of the complementarity-determining regions (CDRs) of human antibodies with those from mice [91] or the use of transgenic mice whose original antibody gene repertoire is replaced with human

immunoglobulins loci [88, 89]. An example of chimeric antibody is Rituximab, another FDA approved murine monoclonal immunoglobulin derived by hybridoma technology. It is used for several diseases such as non-Hodgkin's lymphoma, chronic lymphocytic leukemia or rheumatoid arthritis and has been partially humanized by the substitution of the murine constant region with the human counterpart [94]. However, the maintenance of murine domains can still lead to human anti-murine antibodies production [95].

In addition to problems related to immunogenicity, the production of antibodies by hybridoma technology is difficult to apply for immunization antigens that are toxic or unstable in the animal systems [96].

Other methods developed to generate monoclonal antibodies are based on *in vitro* display technologies, including the phage, yeast, ribosome and bacterial display systems. Such methods are based on the construction of DNA libraries coding for binding proteins and their expression and selection against the target molecule. Since all the passages are performed *in vitro*, they are independent from the immune response or the nature of the antigens, which are the main limits of *in vivo* technologies as mentioned before.

1.2.3. The phage display technology

The phage display is a technology introduced by George P. Smith in 1985 and recently awarded with the chemistry Nobel prize (October 2018), based on the use of the filamentous bacteriophage M13 to express peptides in fusion with the surface phage proteins [97]. As the coding sequence of each phage-displayed peptide is included in the phage genome, the selection of a binder leads to the concomitant isolation of its DNA sequence.

The discovery that antibodies can be expressed in smaller formats on the phage surface preserving their binding capability allowed the great development of this technology. Today, the most widely used antibody format is the single-chain fragments variable (scFv) comprising the variable domains of light and heavy chains (VL and VH) taken together by a linker sequence [94, 95].

The phage structural protein generally used for the antibody display is the pIII, located on one tip of the phage surface in 3-5 copies (figure 11, A). The phage system mostly adopted relies on the phagemid vector, which uncouples the expression of the fusion proteins scFv-pIII from the expression of the other phage proteins. In addition to the scFv-pIII expression cassette, the vector contains a bacterial origin replication site, the replication signal for the assembly into phage particles and an antibiotic resistance sequence. The co-presence of a helper phage provides the additional phage proteins required for the packaging [100].

For the selection process, known as panning, the target can be immobilized on different solid surfaces: column matrixes, magnetic beads, polystyrene tubes or microtiter wells. In addition, biotinylated antigens allows the selection in solution by streptavidin beads. The collection of phages-displayed antibodies is added on the antigen and while the non-binding phages are removed by washes, the antigen-linked antibodies can be eluted by pH shift or *E.Coli* cells. The collected phages can be amplified and used to repeat the selection cycle, increasing the specificity of the identified clones for the antigen. Generally, after 2-3 panning rounds, the final screening of single clones on the antigen is performed by ELISA assay (figure 11, B) [101].

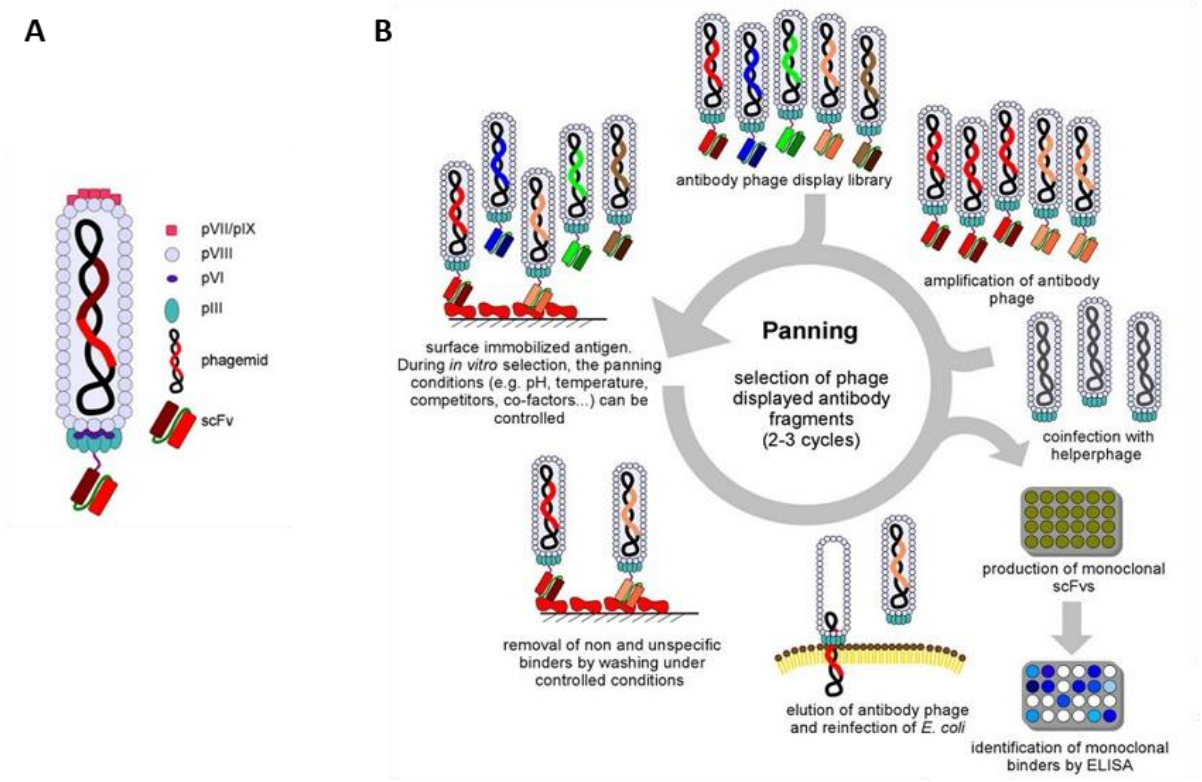

Figure 11. scFv phage display particle and panning cycle of antibodies phage display library.
A. scFv phage display particle. **B.** Illustration of panning cycle of phage display library. Adapted from *Proteomics Clin Appl.* 2016, 10(9-10):922-948.

An important consideration on the *in vitro* phage display technology is the possibility to control the conditions for the antibodies selection. The adding of competitors to the library before the panning together with the number and kind of washes allows subtracting undesired antibodies and directs the available phages to specific epitope or protein conformations. In addition, once selected, the clones sequences can be further engineered to improve the affinity for the target and sub-cloned into suitable vectors for their expression as full antibody or other antibody structures (figure 12) in accordance with the expected applications [97, 98].

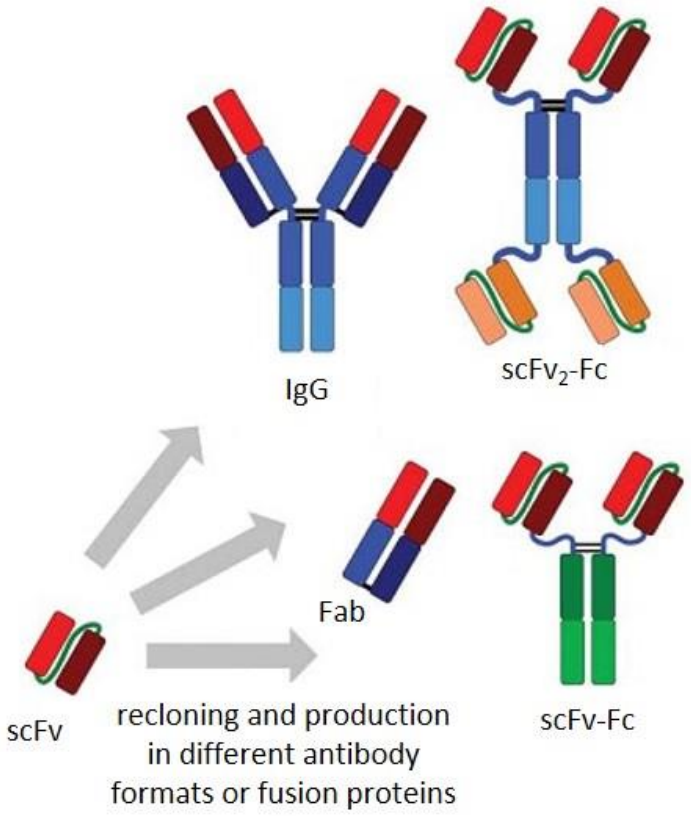

Figure 12. Antibodies formats for scFv production.
Adapted from *mAbs.* 2016, 8:7, 1177-1194.

1.2.4. Naïve and immunized antibodies libraries

The gene fragments for antibodies libraries construction can derive from either B-cells of non-immunized donors or immunized/infected donors. In the first case, the library uses the natural rearranged V genes repertoire, which have been *in vivo* selected for functional B cells receptors, or synthetic sequences. Different approaches have been applied to obtain fully synthetic libraries, such as the use of fixed VL and VH frameworks genes, whose CDRs regions are replaced by randomized or rationally designed CDRs cassettes [99, 100]. In addition, the combination of natural and synthetic antibodies sequences results in the so-called semi-synthetic libraries. The construction of such libraries can use natural unrearranged V genes of pre-B cells or only one antibody framework, whose CDRs are endowed with randomized sequences [101, 102]. A naïve libraries is expected to represent the entire immune repertoire and to be selected ideally against any target, therefore the number of independent clones is crucial for the success of the selection [107].

The immune libraries can be constructed from immunized animals against a specific antigen or infected donors and, with respect to naïve libraries, represent a repertoire of V-genes already affinity matured, improving the possibility to select specific clones for the target. The choice of the B-cell source is generally based on the final purpose, although in the case of human immune libraries from infected patients, the availability of genetic material has clear limitations. However, human immune libraries have been constructed against a wide range of targets, such as the type 1 human immunodeficiency virus (HIV) [108], the hepatitis B virus [109], tumour markers [110], the flu virus H5N1 [111].

The use of immunized animals represents a more usable approach and different species have been successfully employed, ranging from mouse [112], rabbit [113], camel [114], chicken [115], shark [116] and many others. Some animals present unique features, like camels and sharks, which possess homodimeric antibodies of only heavy chains presenting a higher thermo-stability with respect to human antibodies or single-chain fragments [110, 113].

As mentioned in 1.2, non-human derived antibodies have some limitations as therapeutics due to potential immunogenic reactions. However, manipulations aimed to minimize the non-human antibody sequences can be applied, as pairing the selected scFv with a human constant region, resulting in a chimeric structure, or "humanize" the whole antibody, maintaining only the animal CDRs regions [118].

Animal-derived antibodies find less limitation in the diagnostic field, due to the lack of an *in vivo* interaction between the recombinant antibody and the immune system, avoiding the possibility of immunogenic reactions [119].

1.2.5. Applications of the antibody phage display technology

Several antibodies under clinical studies or approved by the FDA derive by phage-display technology and the wide range of their targets, including bacteria, viruses, eukaryotic pathogens, toxins, cytokines and receptors, demonstrates the great applicability of such method for diagnostic and therapeutic purposes [101].

Adalimumab (Humira) is the first human monoclonal antibody approved by FDA for therapy and derives by phage display. It prevents the activation of inflammatory processes binding to the tumour necrosis factor (TNF) and is used for rheumatoid arthritis treatment. The identification of Humira is an example of the optimization processes feasible through the phage display technology. It derives from an already existing murine anti-TNF antibody, modified to improve its immunogenic and bio distribution properties. The VH and VL of the murine antibody have been used to construct two independent scFv libraries, which one combining the murine fixed domain with human variable

domains library. After selection, the hybrid clones from the two libraries have been combined to construct a third library carrying only human domains, whose selection has led to Humira [120].

Different strategies to guide the antibodies identification process by phage display have been adopted also for viruses, which can be used as selection targets, as mentioned before. Dengue virus is one of the most important Flavivirus member with respect to public health impact and efforts have been made to isolate antibodies for diagnostic and therapeutic aims. It circulates in four serotypes presenting the 70% of sequence homology [121] and while highly specific antibodies are required to detect each serotype, the neutralization of all the viral forms for therapeutic purposes is possible through cross-reactive antibodies [122]. The strategy adopted by Lebani K. and co-workers to isolate serotype-specific antibodies for the Dengue NS1 protein (a marker of the viral infection) was based on the subtracting of cross-reactive phages. This was achieved pre-incubating a naïve library on the NS1 proteins of the undesired three serotypes, before the final selection on the NS1 of the target serotype with the specific not-subtracted phages [123]. On the contrary, in order to obtain antibodies recognizing the NS5 protein of all Dengue serotypes (implicated in viral replication), the Vasudevan S. G. group screened a naïve library alternating the serotype of the viral target at each panning cycle and re-selecting the bound phages, which resulted in the isolation of cross-reactive antibodies [124].

Different was the approach used by Throsby M. and co-workers to isolate neutralizing antibodies against WNV. They started with the construction of an immune library deriving by three convalescent infected donors. Then, in order to reproduce all the possible epitopes of the viral surface, they prepared the target in three different versions: as whole-inactivated virus, as E recombinant protein and as recombinant virus-like particles. Finally, two selection cycles were performed on the three antigen versions in the nine possible combinations [35].

The examples described above demonstrate the versatility of the phage display technology for antibodies discovery. Differently from hybridoma-derived antibodies, the *in vitro* library screening allows to guide the process of antibodies selection and, thanks to the concomitant isolation of the antibody gene sequence, to modify the antibody format by molecular biology techniques [102].

1.3. HIGH-THROUGHPUT SEQUENCING FOR ANTIBODIES REPERTOIRE ANALYSIS

The advent of the Next Generation Sequencing (NGS) technologies has revolutionized the understanding of the antibodies repertoire, revealing new tools for antibodies discovery and immune profiling. Through high-throughput sequencing methods, in fact, it is possible to analyse the entire immunoglobulins genes diversity of B cell populations and its changes after particular events like vaccination or infection. The first NGS repertoire sequencing of B cell populations was done on

zebrafish in 2009 [125] and since then efforts have been made to develop experimental procedures and bioinformatics tools for the analysis of massive sequencing data.

The complexity of the antibodies repertoire is found in the two variable regions of the heavy and light chains, deriving from multiple maturation processes and implicated in the antigen recognition. During B-cells development, the variable (V), joining (J) and diversity (D, only for the heavy chain) gene segments undergo recombination and somatic hypermutation events, generating the antigen-binding domains of heavy and light chains [122, 123]. Among the three CDRs included in each variable domain, the heavy chain CDR3 mainly determines antibodies diversity [128], leading most studies on immunoglobulins repertoire to focus on this region.

Three main platforms are employed for antibodies repertoire sequencing: Illumina, Roche/454 and Pacific Bioscience. Roche/454 is the first NGS platform launched on the market and relies on capture beads and emulsion PCRs. The DNA fragments are captured on beads through adaptors and amplified in individual wells of picotiter plate (emulsion PCR). The incorporation of each nucleotide determines the release of a pyrophosphate, which light signal allows the nucleotide deciphering (pyrosequencing technology) [128]. The advantage of the technique are the relatively long reads that range from 600 to 700 bp, hindered by the tendency to introduce errors known as INDELs (bases insertion/deletion). A high base error rate characterized also the Pacific Bioscience platform, which in contrast presents the unique capability of sequencing very long reads (more than 10 Kb) [129].

The Illumina technology is considered more suitable for antibodies repertoire sequencing with respect to the platforms described above due to its relatively low error rate (dominated by substitution errors) and costs. It is based on the immobilization of single DNA molecules on a surface (flow cell) through adaptors and their *in situ* amplification. The amplification generates up to 1000 identical copies of each template molecule called “cluster”. The clusters are then sequenced by synthesis technology, which uses fluorescently-labeled nucleotides. During each sequencing cycle, the four labeled deoxynucleosides triphosphate (dNTPs) are added and serves as reversible terminators for the polymerization reaction. After the incorporation of the first dNTP, the fluorescent dye is imaged and the emitted fluorescence from each cluster, corresponding to a specific base, is registered. The dye is then enzymatically cleaved to allow the incorporation of the next dNTP. The cycle is repeated “n” times to generate a read length of “n” bases, derived by the fluorescence signals analysis (figure 13). The sequencing is generally performed from both the ends of each DNA molecule (“pair-end sequencing”) and the sequences obtained, that do not have overlap, can be associated thanks to their same physical location on the surface. Actually the sequence coverage has reached the 2 x 300 bp and tens of millions of clusters can be analysed in parallel [129].

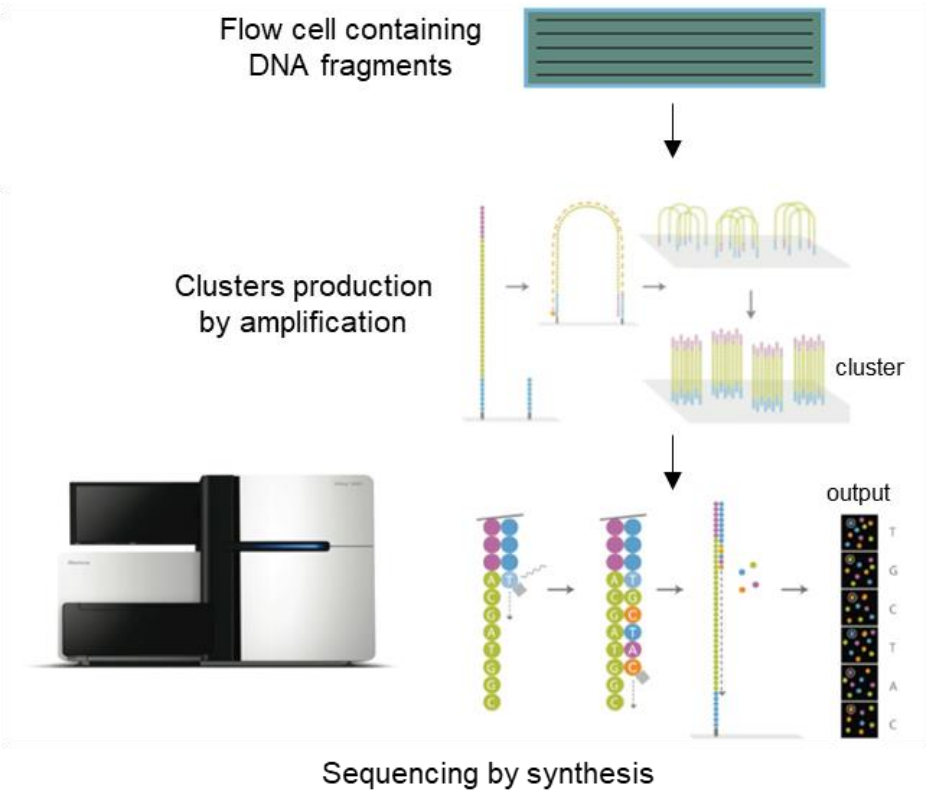

Figure 13. Illustration of Illumina technology.

Illumina technology: immobilized DNA fragments on flow cell are amplified by *in situ* PCR to produce multiple copies of the same sequence (cluster). The clusters are sequenced by synthesis through fluorescent-labeled nucleotides. The light emission at each nucleotide insertion deciphers a specific base.

The source of B cells for antibodies repertoire sequencing in humans is limited to peripheral blood, containing only the 2% of the total B cells population. In other compartments like lymph nodes, spleen and bone marrow the B cells percentage increases to 28%, 23% and 17%, respectively. Such compartments are accessible using animal models [130].

1.3.1. Application of NGS technology for specific antibodies identification

The main goal in antibodies repertoire analysis by NGS is the discovery of candidates against specific target without the need of display libraries construction and selection or hybridoma screening. The use of antibodies expression libraries like phage-display, although widely and successfully employed, requires multiple steps including immunoglobulins genes amplification, cloning and transformation in bacteria, repeated panning cycles on the target. Each step of the library construction is crucial to reach a wide clones diversity and representation, essential requisites to isolate different antigen-specific antibodies.

The sequencing of whole immunoglobulins repertoires by NGS provides two important information: the antibodies sequences and their relative abundance in the analysed B cells population [130]. Such information are particularly useful analysing repertoires of immunized or infected subjects, in which the most represented sequences presumably identify antigen-specific antibodies. However, also for antibodies discovery by NGS technologies, several factors have to be considered. One of them concerns the purity and abundance of the B cell population, especially for humans, whose B cells source is generally limited to peripheral blood, as discussed before. To improve the repertoire analysis, in addition to use B cells enriched compartments like spleen, lymph nodes and bone marrow from animal models, it is possible, through cellular surface markers, to sort the B cells subsets from heterogeneous samples [131]. Other technical parameters affecting the immune repertoire analysis concern the errors introduced during sample preparation and the final sequencing. The VL and VH genes amplification, in fact, is generally performed with primers mix, which can introduce sequence errors and artefacts due to differential amplification of some fragments. In addition, all the NGS platforms are characterized by an error rate, as already mentioned [132].

At the end of the high-throughput sequencing, the data have to be filtered using bioinformatics tools aimed to exclude non-productive sequences and pair the VL and VH coming from the same antibody. One of the first works demonstrating the applicability of NGS technology for antibodies discovery was published by Reddy S. T. et al. in 2010. The researchers analysed the V genes repertoire derived from antibodies secreting bone marrow plasma cells (BMPCs) of immunized mice, previously sorted for the analysis. As general observations on the repertoires profiles, they found that while more than the 90 % of the CDRH3 sequences were unique for each mouse (also comparing mice immunized with the same antigen), the CDRL3 sequences presented a lower degree of diversity and, in some cases, were in common between mice of different groups of immunization. In additions, mice immunized with a certain antigen presented preferential V-gene families usage. The pairing of VL and VH sequences for antigen-specific antibodies discovery was based on their frequency similarity derived by NGS data. The resulting scFv sequences were synthetized, expressed in *E. Coli* and confirmed for their antigen specificity in the 78% of cases (on a total of 27 scFv tested) [133].

An interesting work was published two years later by Saggy I. and colleagues, which compared the antibodies identified by NGS analysis and those isolated by phage display library selection, using the same immunized mice. In this case, the B cells for repertoire sequencing and library construction derived by spleens without pre-sorting. They found that among the clones selected by library panning, the sequence of the most reactive and specific for the antigen was absent in the NGS data, while the others were poorly represented, in terms of number of reads. On the other hand, the three scFv coming from the pairing of the most frequent VL and VH sequences were expressed and purified in fusion

with maltose-binding protein, confirming their specificity for the target. Interestingly, the same scFv cloned into a phagemid vector, resulted poorly displayed on the phage surface [133].

The works described above are only examples of the NGS technology applications for antibodies discovery. Many other publications have demonstrated the usefulness of high-throughput immune repertoire sequencing, both in humans [130, 131] and animal models [132–134].

The second work described (Saggy I. et al.), in particular, highlights the complementary of the NGS and phage display technologies, which one characterized by different bias introducible during the experimental design but both successfully applied for different antibodies discovery.

2. MATERIALS AND METHODS

Abbreviations

AP: Alkaline phosphatase

APS: Ammonium persulfate

CHO: Chinase hamster ovary

DTT: Dithiothreitol

EDTA: Ethylene diamine tetra acetic acid

ELISA: Enzyme-linked immunosorbent assay

GST: Glutathione S-transferases

HRP: Horseradish Peroxidase

IPTG: Isopropil-β-D-1-tiogalattopiranoside

SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

scFv: single chain fragment variable

TMB: 3,3′,5,5′-Tetramethylbenzidine

Materials

1. Bacterial strain used is *Escherichia coli* DH5αF' [F'/endA1 hsd17(rK_mKþ) supE44 thi-1 recA1gyrA (Nalr) relA1 _(lacZYA-argF) U169 deoR (F80dlacD-(lacZ)M15)].
2. GenElute™ Plasmid Miniprep Kit (Sigma) for plasmid DNA preparation, following the instructions of the manufacturer.
3. Stock solutions of antibiotics (Sigma) are prepared by dissolving kanamycin at 50 mg/mL in water and ampicillin at 100 mg/mL in water. Kanamycin and ampicillin stocks are filtered with 0.22 µm filter device and stored at –20°C.
4. 2xTY liquid broth: 16 g bacto-tryptone, 10 g bacto-yeast and 5 g NaCl to 1L of ddH_2O. Final pH 7.0. Agar plates are prepared by adding 1.5% bacto-agar to 2xTY broth.
5. Restriction endonucleases, T4 DNA ligase and buffers are purchased from New England Biolabs and used according to the manufacturer suggestions and standard molecular biology procedures.
6. GenElute™ Gel Extraction Kit and GenElute PCR Clean-Up Kit (Sigma) for DNA purification from agarose gel and restriction reactions, respectively, following the instructions of the manufacturer.

7. The DNA Clean & Concentrator™-5 kit (Zymo Research) for purification and concentration of the ligation reaction, following the instructions of the manufacturer.
8. TG1 Electrocompetent Cells (Lucigen) and 1 mm gap cuvette (BTX) for ligase transformation, following the instructions of the manufacturer.
9. Helper phage M13KO7 (GE Healthcare).
10. Solution for precipitation of phages: 20% (w/v) polyethylene glycol (PEG) 6000 in 2.5 M NaCl. The solution is filtered through a 0.22 µm filter before use.
11. PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1L H_2O, final pH 7.4.
12. 2xTYAG: 2xTY liquid broth supplemented with 100 µg/mL ampicillin and 1% of glucose.
13. 2xTYAK: 2xTY liquid broth supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin.
14. PBS/Tween: PBS supplemented with 0.1% (v/v) of Tween-20.
15. 2% MPBS: 2 g non-fat milk powder /100mL PBS.
16. 4% MPBS: 4 g non-fat milk powder /100 mL PBS.
17. HRP-conjugated antibodies: purchased from Jackson ImmunoResearch.
18. AP-conjugated antibodies, α-GST and α-FLAG antibodies: purchased from Sigma.

Methods

The common procedures of SDS PAGE, Western Blot and ELISA are described at the end of this section.

2.1. Antigens production and purification

Cloning of the sE and DIII coding sequences of WNV and TBEV

The codon-optimized sequences of the ectodomain of the Envelope protein (sE) of TBEV (Neudoerfl Western Serotype, NCBI reference genome NC_001672.1) and WNV (strain NY99, NCBI reference genome: NC_009942.1) were synthesized and obtained in the pMAT vector (Invitrogen), each one flanked by the restriction sites BssHII and NheI. The DIII domain sequences were obtained by PCR using the pMAT-sE plasmid as template and a primer pairs mapping on the DIII region adding the BssHII and NheI restriction sites:

TBEV_DIII_Forward: 5’ - ATGCGGCGCGCATGCCGGCCTGACCTACACCATGTGC - 3’;

TBEV_DIII_Reverse: 5’ - ATGCGGGTACCGCTAGCCTTCTGGAACCACTGGTG - 3’;

WNV_DIII_Forward 5’ - ATGCGGCGCGCATGCCCTGAAGGGCACCACCTACGG - 3’;

WNV_DIII_Reverse: 5’ - ATGC GGTACCGCTAGC GCTCTTGTGCCAGTGGTG 3’.

The sequences were digested with BssHII and NheI enzymes. The prokaryotic expression vector pGEX 4T-1 (GE Healthcare), previously modified to introduce the BssHII and NheI restriction sites, was digested with the same enzymes. The ligation reaction of each antigen sequence with the expression vector was transformed in chemically competent bacteria.

Expression of the recombinant proteins

The colonies carrying the recombinant genes were confirmed by PCR using forward and reverse primers mapping on pGex vector:

pGex_Forward: 5' - GGGCTGGCAAGCCACGTTTGGTG - 3';

pGex_Reverse: 5' - CCGGGAGCTGCATGTGTCAGAGGTTTTCACC - 3'.

The positive colonies were inoculated in 2xTY medium supplemented with 100 µg/ml of Ampicillin and 0.1% of glucose. The growing was conducted on shaker platform at 30 °C and bacteria induced for protein expression at OD_{600nm} = 0.6 with 0.2 mM IPTG. Three hours after induction, bacteria were centrifuged 20 minutes at 3000 x g at 10 °C and supernatants discarded. Pellets were weighted, resuspended with 10 ml/g of lysis buffer (20mM Tris pH 8, 500mM NaCl, 0.1% Triton X100) and frozen at -80 °C.

Purification of the recombinant proteins

After thawing, 400 µg of lysozyme per ml of lysate were added and incubated on ice for 30 minutes, shacking. 100 µg/ml of DNase and 1mM EDTA were added to lysates and incubated on ice for 45 minutes, shacking. Lysates were sonicated three times for 30 seconds and centrifuged 20 minutes at 18000 x g at 10 °C. Supernatant were discarded and pellets frozen at -80 °C. After thawing, pellets were resuspended with 1/10 of the starting culture volume of wash triton buffer (50mM Tris pH 8, 100mM NaCl, 1mM EDTA, 1mM DTT, 0.5% Triton X100), sonicated three times for 30 seconds and centrifuged at 11000 x g 15 minutes at 10 °C. The wash was repeated a second time and pellets were resuspended with 1/50 of the starting culture volume of wash buffer without triton. Four volumes of UREA 6M were added to one volume of sample in wash buffer to finally dialyzed in PBS O/N at 10 °C. The purified proteins were checked by SDS PAGE and western blot.

2.2. Antibodies phage display libraries construction and screening

2.2.1. Construction of the antibodies libraries from immunized mice

Notes:

- All the procedures *in vivo* were performed at ICGEB (International Centre for Genetic Engineering and Biotechnology, Trieste)
- The sequencing by Illumina technology was performed at CBM (Consorzio per il centro di Biomedicina Molecolare, Trieste)

Mice immunization

Four 5-6 weeks old Balb/c mice were DNA immunized against WNV. The immunization DNA were prepared cloning the sE_WNV sequence into pcDNA3.1 vector (Invitrogen), previously modified to remove the neomycin resistance gene. 1 μm of gold nanoparticles coated with 1 μg of DNA were biolistic delivered by Genegun technology (Bio-Rad, Hercules, CA, USA) into each mouse. Two weeks after the first immunization, the procedure was repeated for three times at one-week intervals. Blood samples were collected one day prior each boost of DNA immunization and one week after the final boost by sub-mandibular puncture. The last bleeding was checked by ELISA.

RNA extraction from splenocytes and cDNA retrotranscription

After mice sacrifice, cells lysates from murine spleens were collected in trizol. 1.5 mL of cells in trizol were mixed with 500 μl of chloroform and left at room temperature (RT) for 2 minutes. After centrifugation at 11000 x g, 4°C for 15 minutes, the upper aqueous layers were transferred into a new 1.5 ml tube. The nucleic acids were precipitated adding 750 μl of isopropanol and leaving at RT for 10 minutes. After centrifugation at 11000 x g, 4°C for 10 minutes, supernatants were discarded and pellets washed with 1.5 ml of 75% cold ethanol. After centrifugation at 7500 x g, 4°C for 5 minutes, ethanol was discarded and each RNA pellet resuspended with 20 μl of sterile RNase-free water (Thermo Fisher Scientific). After quantification by Nanodrop, 1μg of each RNA sample was used for cDNA preparation (Maxima First Strand cDNA synthesis kit, Thermo Fisher Scientific).

Amplification of the variable domains genes

The variable domains genes of heavy (VH) and light (VL) chains were separately amplified performing two PCR reactions:

1. PCR with forward and reverse primers mixes (table 1) designed for murine total V genes amplification and carrying fixed sequences at the 5' ends;
2. PCR re-amplifying the VL and VH products obtained from the first reactions with forward and reverse primers (table 1) overlapping with the fixed sequences. The primers carry also a restriction site at the 5' end (BssHII in the forward, NheI in the reverse) and overlapping regions at the 3' ends.

Each PCR reaction 1 was performed in a volume of 50 µL for VL and 5 x 50 µL for VH, using: 1 µL of cDNA as template and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to manufacturer's instructions. Cycling parameters: 98°C for 10 sec (denaturation), 65°C for 30 sec (annealing) and 72°C for 30 sec (extension) for 31 cycles. 50 µL of each chain were loaded on a 1.5% agarose gel and purified. The remaining VH PCR 1 reactions (4 x 50 µL) were mixed and sequenced by Illumina technology. The purified VL and VH products were quantified and 10 ng used as template for the respective PCR 2, maintaining the same reaction conditions of PCR 1. The VL and VH products were purified from gel as described above.

Assembly of the single-chain variable fragments (scFv)

The assembly of VL and VH genes to generate the scFv library occurs through the 3' overlapping regions added in the PCR 2, which generate the linker coding sequence. The assembly PCR reaction was performed with 25 ng of each variable genes library in 50 µL setting 8 cycles without primers followed by 25 cycles in the presence of external primers (table 1). Cycling parameters are 98°C for 10 sec (denaturation), 60°C for 30 sec (annealing) and 72°C for 30 sec (extension). The resulting scFv product was purified as described above.

Table 1: List of primers used for VL/VH genes amplification

Primer mixes for PCR 1			
VH PRIMERS_MIX_FORWARD		**VH PRIMERS_MIX_REVERSE**	
HB1	TTATCCTCGAGCGGTACCGAKGTRMAGCTTCAGGAGTC	HF1	GATTGGTTTGCCGCTAGCTGAGGAGACGGTGACCGTGGT
HB2	TTATCCTCGAGCGGTACCGAGGTBCAGCTBCAGCAGTC	HF2	GATTGGTTTGCCGCTAGCTGAGGAGACTGTGAGAGTGGT
HB3	TTATCCTCGAGCGGTACCCAGGTGCAGCTGAAGSASTC	HF3	GATTGGTTTGCCGCTAGCTGCAGAGACAGTGACCAGAGT
HB4	TTATCCTCGAGCGGTACCGAGGTCCARCTGCAACARTC	HF4	GATTGGTTTGCCGCTAGCTGAGGAGACGGTGACTGAGGT
HB5	TTATCCTCGAGCGGTACCCAGGTYCAGCTBCAGCARTC		
HB6	TTATCCTCGAGCGGTACCCAGGTYCARCTGCAGCAGTC		
HB7	TTATCCTCGAGCGGTACCCAGGTCCACGTGAAGCAG		
HB8	TTATCCTCGAGCGGTACCGAGGTGAASSTGGTGGAAT		
HB9	TTATCCTCGAGCGGTACCGAVGTGAWGYTGGTGGAGTC		
HB10	TTATCCTCGAGCGGTACCGAGGTGCAGSKGGTGGAGTC		
HB11	TTATCCTCGAGCGGTACCGAKGTGCAMCTGGTGGAGTC		
HB12	TTATCCTCGAGCGGTACCGAGGTGAAGCTGATGGARTC		
HB13	TTATCCTCGAGCGGTACCGAGGTGCARCTTGTTGAGTC		
HB14	TTATCCTCGAGCGGTACCGARGTRAAGCTTCTCGAGTC		
HB15	TTATCCTCGAGCGGTACCGAAGTGAARSTTGAGGAGTC		
HB16	TTATCCTCGAGCGGTACCCAGGTTACTCTRAAAGWGTSTG		
HB17	TTATCCTCGAGCGGTACCCAGGTCCAACTVCAGCARCC		
HB18	TTATCCTCGAGCGGTACCGATGTGAACTTGGAAGTGTC		
HB19	TTATCCTCGAGCGGTACCGAGGTGAAGGTCATCGAGTC		
VL PRIMERS_MIX_FORWARD		**VL PRIMERS_MIX_REVERSE**	

LB1	AGCAAGCGGCGCGCATGCCGAYATCCAGCTGACTCAGCC	LF1/2	GAAGTTATGGTCGACCCTCCGGATTTKATTTCCAGCTTGG
LB2	AGCAAGCGGCGCGCATGCCGAYATTGTTCTCWCCCAGTC	LF4	GAAGTTATGGTCGACCCTCCGGATTTTATTTCCAACTTTG
LB3	AGCAAGCGGCGCGCATGCCGAYATTKTGMTVACTCAGTC	LF5	GAAGTTATGGTCGACCCTCCGGATTTCAGCTCCAGCTTGG
LB4	AGCAAGCGGCGCGCATGCCGAYATTGTGYTRACACAGTC	LFLAM	GAAGTTATGGTCGACCCTCCGGATAGGACAGTCAGTTTGG
LB5	AGCAAGCGGCGCGCATGCCGAYATTGTRATGACMCAGTC		
LB6	AGCAAGCGGCGCGCATGCCGAYATTMAGATRAMCCAGTC		
LB7	AGCAAGCGGCGCGCATGCCGAYATTCAGATGAYDCAGTC		
LB8	AGCAAGCGGCGCGCATGCCGAYATYCAGATGACACAGA		
LB9	AGCAAGCGGCGCGCATGCCGAYATTGTTCTCAWCCAGTC		
LB10	AGCAAGCGGCGCGCATGCCGAYATTGWGCTSACCCAATC		
LB11	AGCAAGCGGCGCGCATGCCGAYATTSTRATGACCCARTC		
LB12	AGCAAGCGGCGCGCATGCCGAYRTTKTGATGACCCARAC		
LB13	AGCAAGCGGCGCGCATGCCGAYATTGTGATGACBCAGKC		
LB14	AGCAAGCGGCGCGCATGCCGAYATTGTGATAACYCAGGA		
LB15	AGCAAGCGGCGCGCATGCCGAYATTGTGATGACCCAGWT		
LB16	AGCAAGCGGCGCGCATGCCGAYATTGTGATGACACAACC		
LBLAM AGCAAGCGGCGCGCATGCCGACAGGCTGTTGTGACTCAGGAATC			
Primers for PCR 2			
VH PTL_FORWARD		**VH PT1_REVERSE**	
GGAGGGTCGACCATAACTTCGTATAATGTATACTATACGAAGTTATC CTCGAGCGGTA		CCAGGCCCAGCAGTGGGTTTGGGATTGGTTTGCCGCTA	
VL PT1_FORWARD		**VL PTL_REVERSE**	
CGCTGGATTGTTATTACTCGCAGCAAGCGGCGCGCATGCC		ACCGCTCGAGGATAACTTCGTATAGTATACATTATACGAAGTTATGG TCGACCCTCC	
Primers for scFv assembly			
VL PT2_FORWARD		**VH PT2_REVERSE**	
TACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTC		TGGTGATGGTGAGTACTATCCAGGCCCAGCAGTGGGTTTG	

Ligation and electroporation of the scFv library

Both phagemid cloning vector pDAN5 [139] and purified scFv fragments library of were sequentially digested with BssHII and NheI enzymes for for 2 h at 50°C and for 4 h at 37°C, respectively. The ligation reaction was prepared in 70 µl volume with 1 µg of double-digested and purified vector and 0.5 µg of double digested and purified scFv (phagemid: insert molar ratio of 1:3), incubating at 16°C overnight. The reaction was purified and concentrated in 7 µl using a commercial kit (Zymo research). The ligation mix was electroporated into TG1 Electrocompetent Cells (Lucigen) following the instructions of the manufacturer. Transformations were pooled, plated on three 2xTY agar plates with ampicillin (30 cm diameter each) and grown O/N at 28°C. Two dilutions were also plated to estimate library diversity. The next day colonies were scraped up in 2xTY 20% glycerol and frozen in 1 mL aliquots and working aliquots of 100 µl.

2.2.2. Screening of the naïve and immunized antibodies libraries

Rescuing phagemid particles from library

One aliquot of the library was diluted in 11 mL (1 mL for OD_{600nm} calculation) of 2xTYAG medium and grown with shaking (200 rpm) at 37°C. After reaching OD_{600nm} 0.5, bacteria, concentrated as $5x10^8$/mL, were infected with a 20 fold excess of helper phage and left at 37°C for 45 min, standing. Bacteria were centrifuged for 15 min at 2800 x g, supernatant discarded and cells pellet dissolved in 40 mL of 2xTYAK medium. Bacteria were grown with shaking (200 rpm) at 28°C overnight. The next day, bacteria were centrifuged for 20 min at 6300 x g, 10 °C. The supernatant containing the phage particles were mixed with 1/5 volume of PEG/NaCl solution and left on ice for 45 min. The concentration of phages particles in the supernatant of culture medium is usually 10^{11-12} per mL. The precipitated phages were centrifuged for 20 min at 4500 x g, 10°C and, after supernatant discard, dissolved in 1 mL of sterile PBS.

Panning of the library on the WNV target

Note: the phages particles of the naïve library [139] were stored in ready-to-use aliquots for selection at -80°C.

The day before selection, 10 µg of the sE_W antigen in 1 mL of PBS was coated in immunotube (Nunc) and incubated overnight at 4°C. The day of selection, antigen solution was discarded and unspecific sites blocked adding 4 mL of 2% MPBS to the immunotube for 1h. At the same time, 10^{11-12} of phages particles were saturated in 1 mL of 2% MPBS containing 100 µg of sE_T (used to subtract unspecific phages). After blocking, the immunotube was washed once with PBS and phages in milk added to the tube. For the first selection cycle, phages were incubated on the antigen for 30 min on rotation and 1.5 hrs standing, followed by 10 washes of the immunotube with PBS/Tween and 10 with PBS to remove unbound phages. The antigen-linked phages were eluted by adding 1 mL of DH5αF' cells at $OD_{600\ nm}$ 0.5 to the immunotube and incubating at 37°C for 45 min, standing. At the same time, 0.5 mL of cells were infected with the starting phages particles. After incubation at 37°C, the cells infected with the starting phages were plated in serial dilutions on 2xTY agar plate with ampicillin to estimate the number of infecting particles (input). The cells infected with the selected phages (output of the selection) were plated on a 30 cm 2xTY agar plate with ampicillin. A small volume of the output was used to plate serial dilutions and estimate the number of selected phages. Plates were incubated O/N at 28°C and the next day the output colonies were scraped up in 2xTY 20% glycerol and frozen in 1 mL aliquot and working aliquots of 50 µl. For the second selection

cycle, the procedure described above (starting from the rescuing of phagemid particles) was repeated. The second panning differed from the first for an additional washing step adding 4 mL of PBS/Tween to the tube and leaving on rotation for 10 min, before the final 10 washes with PBS. In addition, the output dilutions were used for the final screening on the antigen by phage-ELISA.

Screening of the selected phages on the antigen by phage-ELISA

96 random colonies coming from the second cycle of selection were grown in microtiter plate in 130 µl/well of 2xTYAG medium O/N at 37°C, shaking (200 rpm). The next day, a copy plate was prepared from the first by transferring a small volume (5-10 µl) of culture from each well into the corresponding wells of a second microtiter plate containing 2xTYAG medium. The copy plate was grown at 37°C for 3 hrs, shaking (200 rpm). After growing, 50 µl of 2xTYAG medium containing the helper phage (helper phage to bacteria ratio 20:1) was added to each well and incubated 45 min at 37°C, standing. The bacteria were spun down by centrifugation for 30 min at 1000 x g, the supernatant discarded and each pellet resuspended with 120 µl of 2xTYAK medium. The copy plate was grown O/N at 28°C, shaking. A 96 plate (Nunc, MaxiSorp) was coated with 100 µl/well of the sE_WNV antigen in PBS (final concentration 5 µg/mL) and incubated O/N at 4°C. The next day, the coating solution was discarded and unspecific sites blocked with 120 µl/well of 2% MPBS for 1h. At the same time, the copy plate was centrifuged for 30 min at 1000 x g. After blocking, 50 µl of the copy plate supernatants (containing the phages) were transferred from each well on the corresponding wells of the antigen plate and mixed with 50 µl of 4% MPBS. The phages were incubated for 1.5 hrs and the unbound particles removed by three washes with PBS/Tween and three washes with PBS. HRP-conjugated anti-M13 mAb (GE Healthcare) was used as indicated by manufacturer's instructions for antigen-linked phages detection. After washing (three times with PBS/Tween and three times with PBS), 70 µl of TMB were added to each well. The reaction was developed for 5-20 min and blocked with 30 µl/well of 2N H2SO4. Absorbance was measured at 450 nm.

Analysis of the positive clones by PCR and fingerprinting

The clones resulted positive from phage-ELISA screening were firstly analysed by PCR to confirm the presence of the scFv. The amplification of each clone was performed with VLPT2 and VHPT2 primers in 20 µl of reaction volume, adding 1 µl of bacteria from master plate as template (the denaturation step allows the release of DNA from bacteria) and PCR BIO HiFi polymerase (PCR BIOSYSTEM) according to manufacturer's instructions. The PCR products were loaded on a 1.5% agarose gel and those presenting the correct length were digested with frequently cutting restriction enzyme (BstNI or HaeIII, according to manufacturer's instructions). The obtained products were

loaded on a 3% agarose gel to compare the digestion patterns of the clones. In the case of identical patterns, coming from redundant phages with the same scFv sequence, only one representative clone was further analysed.

Confirmation of the positive clones by phage-ELISA

The isolated clones were inoculated in 2 mL of 2xTYAG medium in single 13 mL tubes and grown at 37°C, shaking until $OD_{600\ nm}$ = 0.5 was reached. Bacteria were infected with helper phage (ratio to bacteria 20:1) and incubated for 45 min at 37°C, standing. Bacteria were centrifuged for 15 min at 2800 x g, resuspended in 1 mL of 2xTYAK medium and grown O/N at 28°C, shaking. The 96-plate coating was prepared as described before. The next day, bacteria were centrifuged for 20 min at 4500 x g, 10°C. The supernatant of each clone was tested in duplicate on three proteins: sE_W (specific antigen), sE_T and GST alone. The incubations of blocking, phages and α-M13 mAb were performed as described before. The clones resulted cross-reactive on the tested proteins were excluded from further analysis.

Sequencing of the positive clones

The scFv of the clones resulted positive and specific for the sE_W antigen were amplified by PCR with VLPT2 and VHPT2 primers in 50 µl of reaction volume, using BIOSYSTEM DNA polymerase (according to manufacturer's instruction). The PCR products, after check on agarose gel, were purified in solution and sequenced by Sanger technology.

2.3. Production of the positive clones as scFv-Fc

Sub-cloning of the scFv in eukaryotic expression vector

The pcDNA 3.1 vector (Invitrogen) was previously modified to introduce the rabbit CH2-CH3 domains sequences (Fc) downstream the NheI restriction site and replace the Neomycin with Hygromycin resistance gene [140]. The modified vector was called pMB Rabbit. The plasmid preparations of each selected scFv and pMB rabbit were sequentially digested with BssHII and NheI. The ligation reaction was prepared in 10-20 µl of reaction volume with 100 ng of double-digested and purified vector and 50 ng of double digested and purified scFv (vector: insert molar ratio of 1:3), incubating O/N at 16°C. Each ligation reaction was transformed into 50 µl of chemically competent bacteria. After O/N growing on agar plate with ampicillin, the obtained colonies were checked by PCR, using scFv external primers mapping on pMB rabbit vector.

Transfection of CHO cells with scFv-Fc plasmids

Chinese Hamster Ovary (CHO) cells were seeded in 24-well plate in CHO-S-SFM II medium (GIBCO, Invitrogen) at the final concentration of 2 x 10^5 cells/mL and a final volume of 1 mL/well, for each transfection. After 1 day growing at 37°C, 5% CO_2, 1 µg of plasmid DNA in 100 µl of CHO-S-SFM II was mixed with 100 µl of CHO-S-SFM II containing 1 µl of FreeStyleTM Max Reagent (Invitrogen) and left at RT for 15'. The transfection mix was added to cells and after 48-72 hrs the cells medium was checked by ELISA for scFv-Fc production. The cells resulted positive for scFv-Fc production were expanded in T75 tissue culture flasks in Pro CHO (Lonza) medium supplemented with 10 U/mL penicillin (Sigma), 1 µg/mL streptomycin (Sigma), 2mM glutamine (Sigma) and Hygromicin B (200ug/ml) (Sigma).

Purification of the scFv-Fc molecules

Each scFv-Fc molecule was purified from 60-90 mL of cells culture medium. The collected medium was filtered through a 0.22 µm filter device and mixed with 1 M Tris/HCl pH 8.0 (1/10 of the final volume). The medium pH should range from 7.5 to 8.5 after Tris/HCl adding. 50 µl-100 µl of protein A-conjugated resin (POROS Mab Capture A Select, Applied Biosystems) were washed twice by transferring into a tube containing 8 mL of 0.1 M of Tris/HCl pH 8.0 and centrifuging for 5 min at 800 x g, 10 °C. The resin was incubated with the medium on rotation O/N, at 10°C. The next day, the medium was transferred into a plastic column (Biorad) allowing the package of the resin. After flowing of the medium, 10 mL of 0.1 M Tris/HCl pH 8.0 were added to wash the resin. Finally, the resin-linked scFv-Fc molecule was eluted by flowing 1 mL of 0.1 M Glycine pH 3.0 through the resin and collecting 3-5 fractions of 200 µl each. The eluted scFv-Fc molecules were checked by SDS PAGE, western blot and ELISA assay to determine.

Phage ELISA competition assay

The competition assay determines if the selected clones against the same antigen recognize the same protein epitope. It was performed as the phage ELISA assay (described before) with an additional step: after blocking of the 96-plate coated with the sE_W antigen, wells were incubated with 100 µl of purified scFv-Fc (competitor) at a final concentration of 2µg/mL in 2% MPBS for 1h at RT. In parallel, wells were incubated without competitor (positive controls). 10 µl from each well were replaced with the same volume of phages supernatant and incubated for 90 min at RT. The subsequent incubation with HRP-conjugated α-M13 mAb and reaction developing were performed as described before.

COMMON PROCEDURES

SDS PAGE and Western Blot

Acrylamide gels were prepared at 4% stacking and 12% resolving acrylamide concentration by Mini-PROTEAN, Tetra Vertical Electrophoresis Cell (Bio-rad) with a thickness of 0.75mm. In particular, the stacking was prepared with 30% acrylamide/bis-acrylamide solution (Sigma), 130 mM Tris/HCl pH 6.8, 0.1% SDS, 0.1% APS and 0.01% TEMED (N,N,N',N'tetrametiletilendiamina) (Sigma). The resolving was prepared with 30% acrylamide/bis-acrylamide solution (acrylamide 29.2 g/ bis-acrylamide 0.8 g in 100mL of H2O), 400 mM Tris/HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.01% TEMED 0,01%. The protein samples were mixed with denaturing buffer (tris-HCl 61.5mM pH 6.8, SDS 2.5%, Glycerol 10%, bromophenol blue 0,0025%, β-mercaptoethanol, ratio sample to buffer 6:1), boiled for 5 min and loaded on gel. The run was conducted in running buffer (50 mM Tris, 384 mM glycine, 0.1% SDS) at 15 mA until proteins reached the running gel, then the amperage was increased to 20 mA. The proteins separation on gel was monitored through pre-stained molecular standard (Smobio).

For western blot analysis, the proteins were transferred to nitrocellulose membrane (GE Healthcare, Amersham UK) using a Mini Trans-Blot module (Bio-rad). The transfer was performed at 250 V for 45 min in transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol, pH 8.3). The membrane was blocked in 2% MPBS for 1h at RT, washed in PBS and incubated with primary antibody diluted in 2% MPBS (according to manufacturer's instruction, for commercial Abs) for 1h at RT. After three washes in PBS/Tween and three washes in PBS, the membrane was incubated with alkaline phosphatase-conjugated antibody diluted in 2% MPBS (according to manufacturer's instruction) for 1h at RT. After washes (as described above), the membrane was developed with 0,3 mg/ml of BCIP (5-Bromo-4-Chloro-3-Indolylphosphate, Sigma) and 0,6mg/ml of NBT (Nitro Blue Tetrazolium, Sigma) in 10 mL of alkaline phosphatase buffer (100 mM Tris, 0.1 M NaCl, 5 mM $MgCl_2$).

ELISA assay

96-wells plate (Nunc MaxiSorp) were coated with 100 µl/well of antigen in PBS at a final concentration of 5 µg/mL and incubated O/N at 4°C. In the case of coating with cells medium, a dilution of 1:10 in PBS was used. The next day, antigen solution was discarded and plate incubated with 120 µl/well of 2% MPBS for 1h at RT. Wells were washed with PBS and incubated with 100 µl/well of primary antibody diluted in 2% MPBS (according to manufacturer's instruction for commercial Abs) for 1h at RT. In the case of cells medium, a dilution of 1:10 in 2% MPBS was used. Wells were washed for three times with PBS/Tween and three times with PBS and incubated with

100 µl/well of HRP-conjugated antibody diluted in 2% MPBS (according to manufacturer's instruction) for 1h at RT. After washes (as described above), the reaction was developed adding 70 µl/well of TMB and blocked with 30 µl/well of 2N H2SO4. Absorbance was measured at 450 nm.

3. AIM OF THE THESIS AND EXPERIMENTAL DESIGN

The flavivirus genus, belonging to the Flaviviridae family, includes arthropod-borne viruses responsible of a wide range of clinical symptoms in humans, including encephalitic and haemorrhagic manifestations. The increase in international travels and the climatic changes of the last decades have promoted the spreading of flaviviruses into new geographic areas and their adaptation to different vectors. The widest applied methods for the diagnosis of human flavivirus infections are based on the detection of anti-viral antibodies in serum or cerebrospinal fluid. The "gold standard" test to screen infected patients is the PRNT (Plaque Reduction Neutralization Test), which measures the capability of serum antibodies to neutralize or reduce the viral infection rate on cells cultures. However, the assay presents several limitations mainly due to the need of highly specialized operators and appropriate equipment to manipulate live viruses. In addition, one week is required for test results. The available commercial kits developed for the most pathogenic flaviviruses are generally in ELISA format, due to its fast and easy application. The weak points of the immunodiagnostic tests are the false positive results derived by cross-reactive antibodies. The rise of such antibodies is caused by the structural similarity of flaviviruses. The diagnosis becomes particularly complicate in geographical areas interested by the co-circulation of more than one flavivirus or vaccination programs leading to the immune system pre-exposure to flaviviral antigens. This is the case of West Nile Virus (WNV), one of the most widespread and important member of the flavivirus genus, in terms of human health impact, which co-circulates with Tick-Borne Encephalitis virus (TBEV) in some European areas. In addition, the vaccination against TBEV is extensively applied in north Eastern Europe countries.

In order to improve the detection of WNV infection, we aimed to produce the needed reagents for immunodiagnostic applications. Such reagents include viral antigens and highly specific antibodies against the antigens.

The choice of the WNV target and its expression system has considered different parameters: antigenic property, time and cost of production, as most of the flaviviruses diagnostic kit rely on the whole inactivated virus requiring extensive production procedures and specialized equipment. On the other hand, the isolation of antibodies against the WNV antigen has been based on the screening of naïve and immunized antibodies libraries.

The two planned strategies and the related main points to reach the proposed purposes are summarized below:

1. **Isolation of α-WNV antibodies by naïve library screening**
    - Production of the viral antigens
    - Screening of the naïve library on the viral antigen
    - Production and characterization of the identified antibodies

2. **Isolation of α-WNV antibodies by immunized libraries screening**
    - Immunization of mice α-WNV through Gene gun technology
    - Analysis of the murine antibodies repertoires by Next Generation Sequencing
    - Construction of the murine immunized libraries
    - Screening of the murine immunized libraries on the viral antigen

4. RESULTS AND DISCUSSION – 1

ISOLATION OF ANTIBODIES α-WNV BY NAÏVE LIBRARY SCREENING

The first planned strategy to produce the needed reagents for WNV diagnostic purposes was the screening of a human naïve antibodies library on WNV target. Therefore, the first step was the identification and production of the viral antigen, followed by the library screening and the characterization of the identified clones.

4.1. VIRAL ANTIGENS PRODUCTION

The target identified as potential reagent for WNV diagnostic applications is the ectodomain (sE) of the envelope protein, which assembles the surface of the virus. The sE represents the main antigen of flaviviruses, as most of the immune response after infection is directed against the viral envelope. The sE is structurally organized into three domains indicated as DI, DII and DIII. The DIII, in particular, presents the highest degree of sequence diversity among flaviviruses and previous works demonstrated its capability to better discriminate between different viral infections [137–139]. Therefore, also the single DIII domain was produced. The same antigens (sE and DIII) were produced for TBEV, in order to check the specificity of the antibodies response.

4.1.1. Production of the constructs for viral antigens expression

Most commercial kit for WNV infection diagnosis use the inactivated virus as antigen [144], which requires extensive production procedures and specialized equipment. On the contrary, prokaryotic systems for proteins expression present several advantages, such as reduced production time and costs, together with common needed reagents and equipment. Therefore, WNV and TBEV antigens were expressed and purified from bacterial cells.

The proteins expression constructs were obtained by cloning the sequence of each antigen into a previously modified pGEX vector allowing the fusion of GST protein at the N-terminus and FLAG tag at the C-terminus (figure 14).

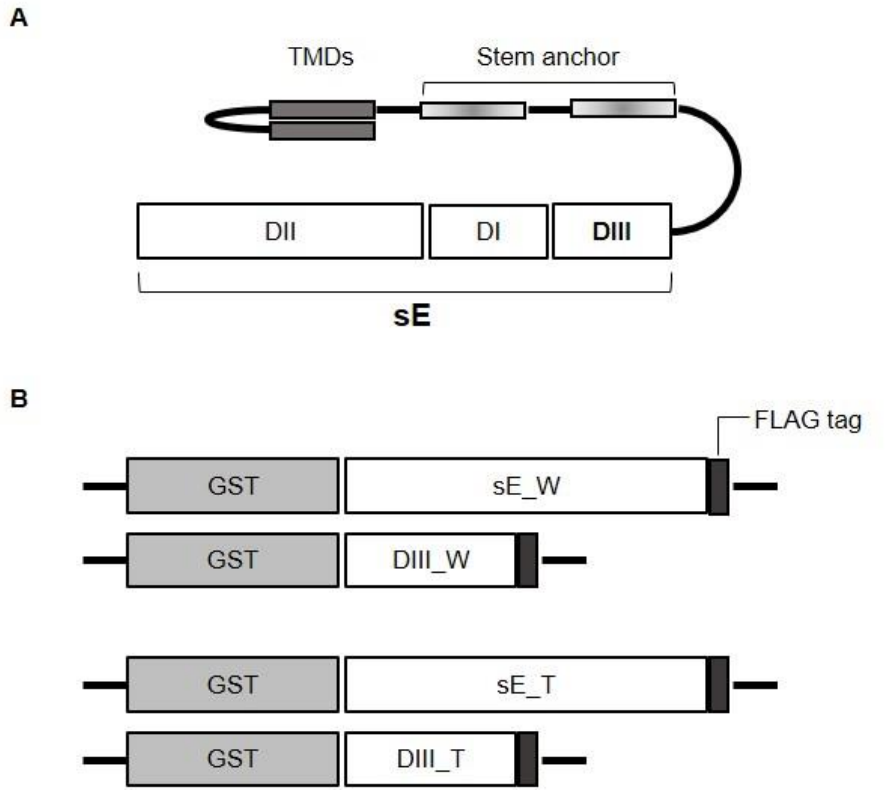

Figure 14. **WNV and TBEV envelope protein structure and produced constructs for viral antigens expression.** **A.** Schematic structure of the envelope protein of flaviviruses including two transmembrane domains (TMDs) linked by a stem anchor region to the DI, DII and DIII domains (sE). **B.** Schematic representation of the constructs produced for WNV and TBEV antigens expression reporting the GST protein at the N-terminus and the FLAG tag at the C-terminus.

After cloning of the sE_W and sE_T synthetic sequences, the correct length of the viral genes (1224 bp for sE_W and 1080 bp for sE_T) was checked by PCR reaction on the transformed colonies. The reaction was performed using external primers mapping on pGEX vector, which amplify an additional region of 150 bp (figure 15). The DIII sequence of each virus was obtained by PCR reaction on the respective sE synthetic gene with specific primers and cloned in pGEX vector. After cloning, the expected sequence length (300 bp for DIII_W and 285 bp for DIII_T) was verified by PCR with pGEX external primers (figure 15).

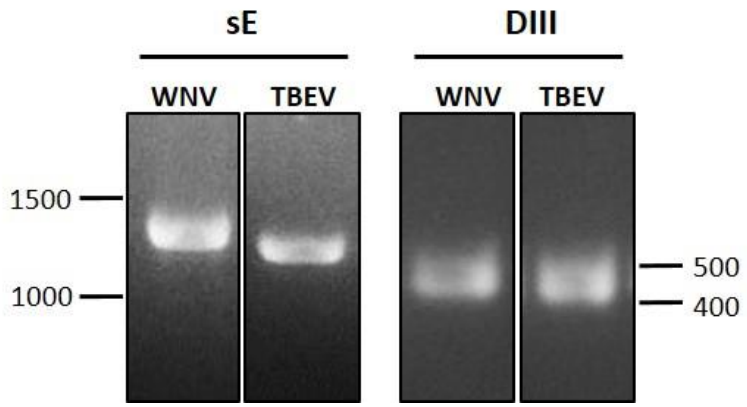

Figure 15. **PCR products of WNV and TBEV antigens sequences after cloning in pGEX vector.**
PCR products of WNV and TBEV antigens sequences amplified after cloning in pGEX vector and analyzed on 1.5% agarose gel.

4.1.2. Expression of the viral antigens

In order to establish the best conditions for proteins expression, different parameters were analyzed in terms of culture temperature, IPTG concentration for expression induction and time of induction. The protocol optimization revealed that the two most relevant parameters affecting proteins stability were the bacteria growing temperature and the time of induction, finally set at 28°C and 3 hours, respectively. After induction of proteins expression, bacteria were lysed and centrifuged, obtaining two protein fractions: the soluble (supernatant) and insoluble (pellet). Both the fractions were analyzed for all the antigens by Western Blot to determine which fraction presented the higher protein expression rate. As shown in figure 16 (A), the sE antigen of TBEV was detected in both the analyzed fractions, although most of protein yield was found in the insoluble fraction. The same protein analyzed for WNV was detected only in the insoluble fraction, suggesting that the antigen was poorly expressed as soluble product. Similar results was obtained for the DIII (figure 16, B). Also in this case, in fact, the antigen of the two viruses was mainly detected as insoluble protein. Therefore, all the viral antigens were purified from insoluble aggregates, Inclusion Bodies (IB).

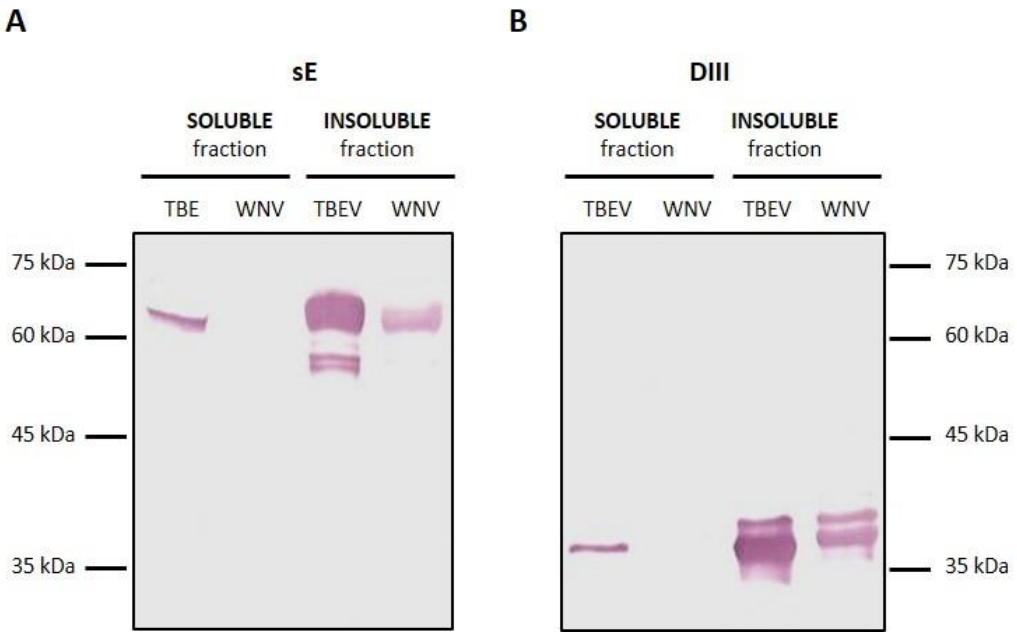

Figure 16. Western Blot on TBEV and WNV recombinant antigens from different protein fractions.
Western Blot on the recombinant antigens sE (**A**) and DIII (**B**) of TBEV and WNV expressed in *E.Coli* and obtained in soluble and insoluble fractions after cells lysis. Detection with anti-FLAG tag antibody.

4.1.3. Purification of the viral antigens from IBs

As mentioned before, one of the aims in WNV antigens production for diagnostic applications was the developing of a fast and easy purification protocol feasible with common reagents and equipment. After lysis of the induced bacteria, the passages followed for protein purification from IBs (indicated in figure 17) were the washes of the insoluble protein fraction, the solubilization of protein aggregates with denaturing buffer and the buffer exchange by dialysis.

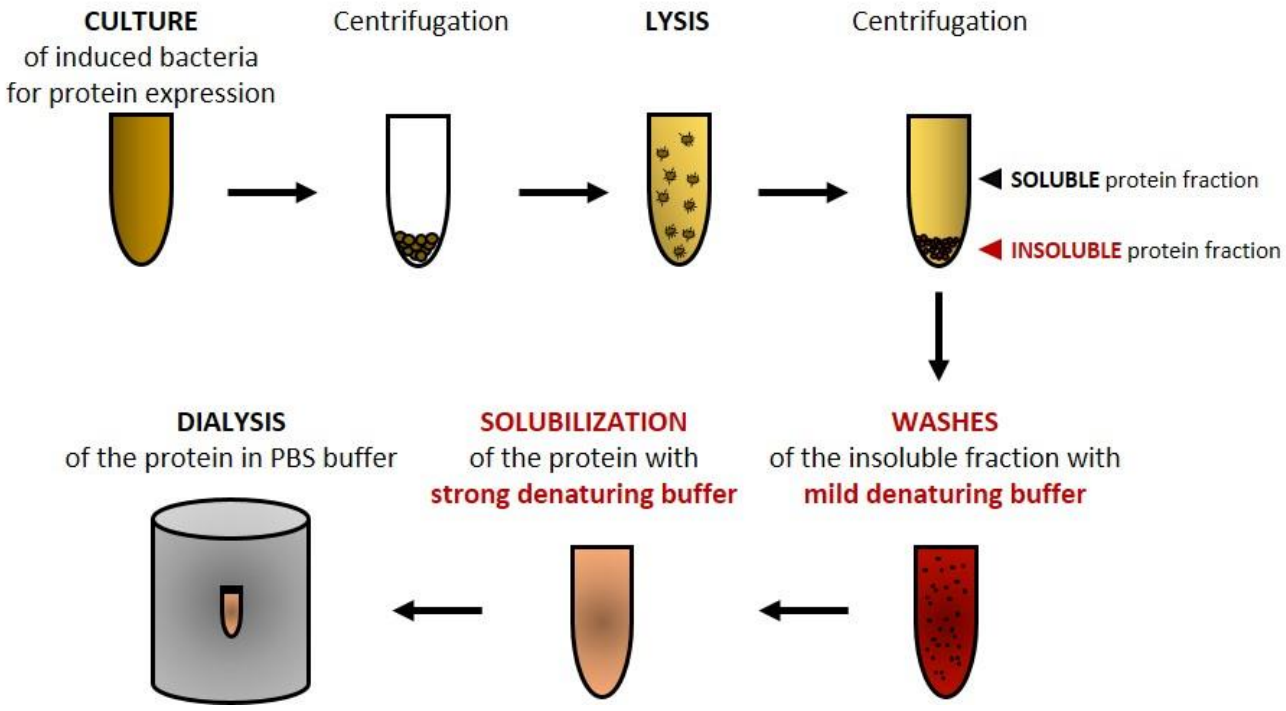

Figure 17. Workflow for WNV and TBEV antigens purification from Inclusion Bodies.
The culture of bacteria collected after induction of protein expression is centrifuged and lysed both enzymatically and mechanically. Protein aggregates (insoluble fraction) are centrifuged and washed with mild denaturing buffer. The protein aggregates are finally solubilized with strong denaturing buffer and dialyzed in PBS.

The wash buffer, containing denaturing reagents and detergent, allows the gradually crumbling of protein aggregates and the removal of bacterial contaminants but at the same time, determines a loss of protein yield proportional to the number of washes. The best balance between protein amount and degree of antigen purity was found after three washes, the last one performed without detergent, while the complete solubilitazion of protein aggregates was performed with PBS/urea solution 6 M. Due to the high concentration of urea in protein samples which could led to antigens precipitation, the dialysis was performed in PBS/urea buffer through gradual decrease of urea concentration or directly in PBS buffer. Although a small volume of protein precipitation products was observed in both cases, the two methods led to equal yield of dialyzed protein, therefore the faster process was adopted.
Differently from previous published works [141–143], which provide for an additional chromatographic step after protein solubilization, the developed protocol is based exclusively on sequential washes of the insoluble protein fraction followed by aggregates solubilization and direct dialysis.
The antigens before and after dialysis were firstly analyzed by SDS PAGE (figure 18).

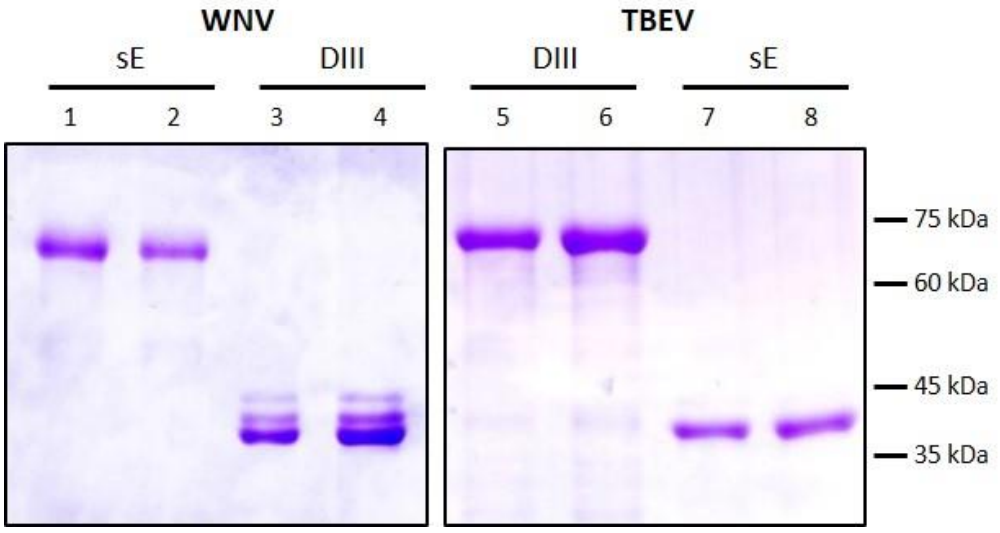

Figure 18. SDS PAGE and Coomassie Blue stain of WNV and TBEV purified sE and DIII proteins.
Purified sE and DIII proteins of WNV and TBEV analysed on 12% acrylamide gel and Coomassie Blue stained. Lanes 1, 3, 5 and 7: protein samples pre-dialysis; Lanes 2, 4, 6 and 8: protein samples post-dialysis.

The obtained bands showed the expected molecular weight of 70 kDa for sE and 36 kDa for DIII, including the GST-fused protein. Comparing each antigen before and after dialysis, the final products maintained approximately the same concentration, suggesting limited proteins degradation during buffer exchange. In addition, the protein samples appeared cleaned from secondary products, with the exception of DIII_W, which presented a main band at 36 kDa and two additional products of higher molecular weight. The protein yield estimated on the final products was 50 mg/L for sE and 100 mg/L for DIII.
The purified antigens were further analyzed by Western Blot (figure 19) using α-FLAG tag mAb, mapping at the C-terminus of the recombinant proteins.

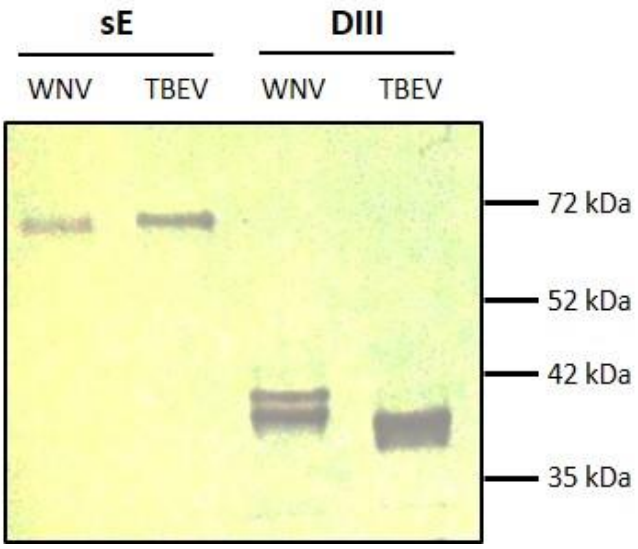

Figure 19. Western Blot on purified sE and DIII proteins of WNV and TBEV.
Western Blot on the recombinant sE and DIII purified proteins of WNV and TBEV. Detection with anti-FLAG tag antibody.

The bands obtained in Western Blot reflected those observed in SDS PAGE and confirmed the full-length of the final products, as the binding site for detection was located at the protein C-terminus. The DIII_W antigen was the only one protein presenting two positive bands for α-FLAG mAb, which could indicate a possible partial degradation occurred during antigen expression.
The antigenicity of all the produced antigens was further evaluated with murine immunized sera (section 5.1).

4.2. ISOLATION OF ANTIBODIES α-WNV BY NAÏVE LIBRARY SELECTION

The sE antigen of WNV was used to screen a human naïve antibodies library [139] by phage display technology. The scFv of the library are cloned into a phagemid vector and their expression is possible through the co-infection of bacteria with a helper phage, providing the needed proteins for phages particles assembly (figure 20, A). The selection cycle (illustrated in figure 20, B) is based on the incubation of the library phages with the antigen, followed by washes to remove unbound particles and the elution of the antigen-linked phages. The eluted phages can be re-amplified and the selection cycle repeated to improve the specificity of the isolated antibodies. At the end of the selection process, random clones are expressed as single phages and checked by phage-ELISA on the antigen.

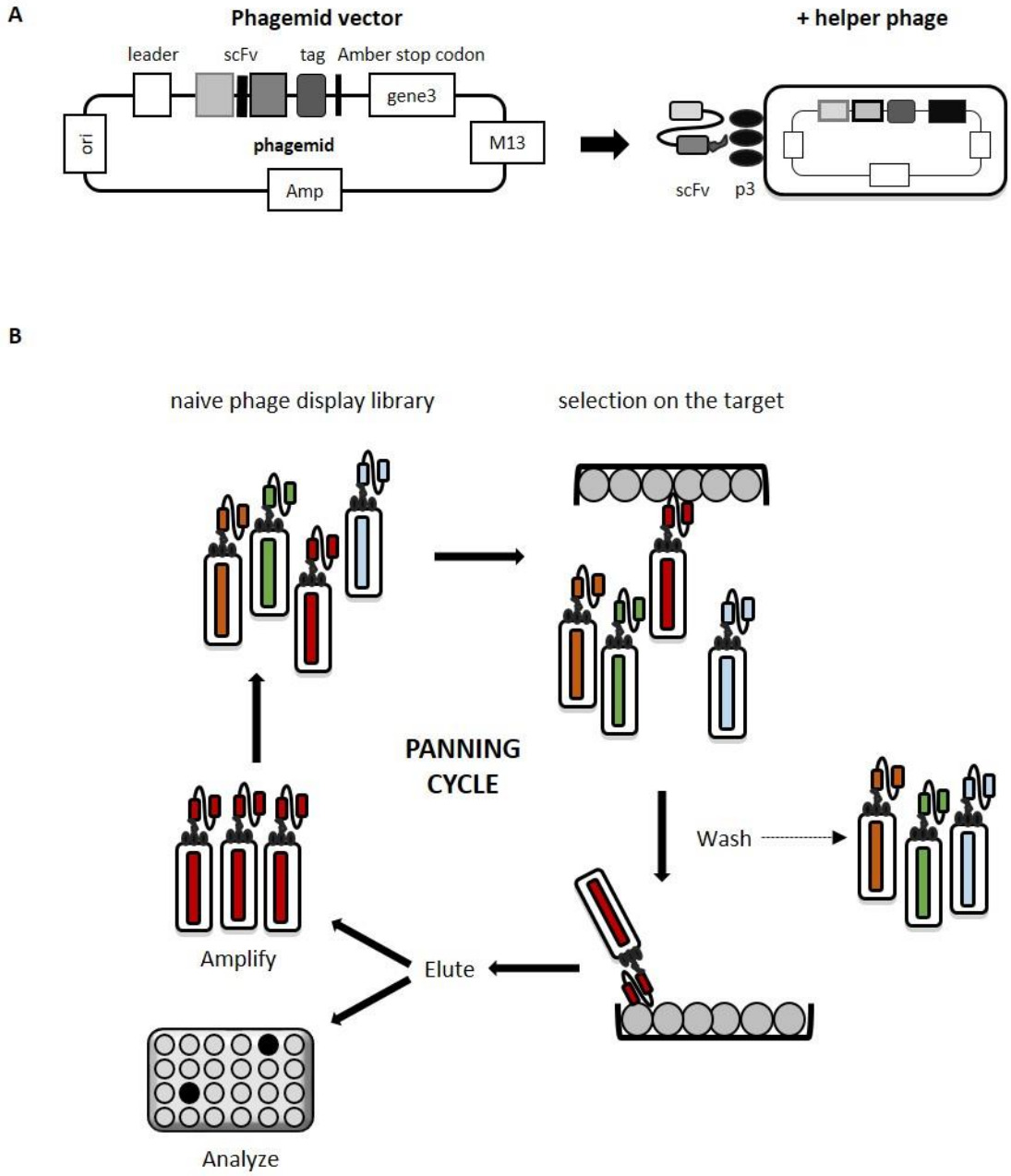

Figure 20. **The phage display technology.**

A. Structure of phagemid vector (left) and phage displaying the scFv by helper phage addition (right). **B.** Panning cycle of phage display library.

4.2.1. Screening of the naïve library on the sE_W antigen

The naïve library was selected on the sE of WNV by two panning cycles. As described in the *Introduction* section, multiple parameters ranging from the number of selection cycles, time and condition of phages incubation on the target, number and condition of washes, affect the selection process. Such parameters are set in accordance with the final aim of the selection. In this case, the

main purpose was the selection of α-sE_W clones with high affinity and specificity properties. To this aim, the library phages were pre-incubated with the sE_T protein before each selection cycle on the target. In particular, a competitor concentration 10-fold higher was used with respect to WNV coated antigen. This step allowed the subtraction of most cross-reactive antibodies. In addition, extensive washes were performed during the second panning cycle to remove poorly reactive or unspecific clones. After two selection cycles, 96 random clones were checked on the sE_W antigen by phage-ELISA. The obtained output (figure 21) showed that almost the 25% of the tested clones resulted highly positive for the target.

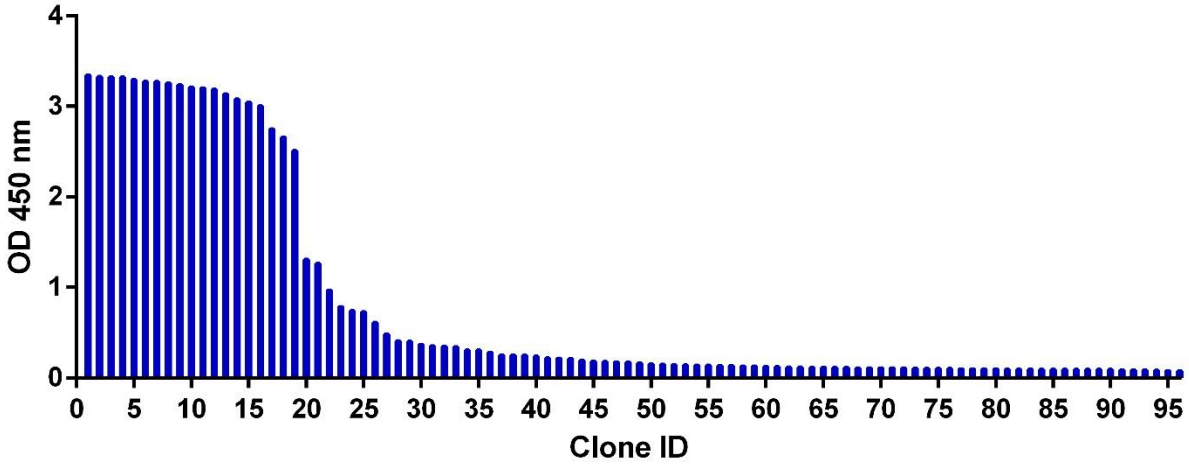

Figure 21. **Output of phage-ELISA assay of 96 random clones on sE_W antigen.**
Output of 96 random clones tested by phage-ELISA assay on sE_W antigen after two cycles of selection.

4.2.2. Analysis of the identified clones

One of the main advantages of phage display technology is the concomitant isolation of target-specific clones and their coding DNA sequence. Therefore, the α-WNV isolated phages were first analyzed by PCR reaction amplifying the scFv fragment. Among the 25 analyzed clones, 21 were confirmed for the full-length of the scFv, while 4 clones presented PCR products of lower length (400 bp) probably due to the loss of a single variable domain.

In order to determine the specificity of the selected antibodies, the 21 positive clones were amplified and tested by phage-ELISA on sE_W, sE_T and GST proteins. As shown in figure 22, with the exception of five clones (F4, E6, H8, D3, C5) cross-reactive on both WNV and TBEV antigens and the G10 clone which was not confirmed as positive for the target, all the selected antibodies specifically recognized the sE_W antigen.

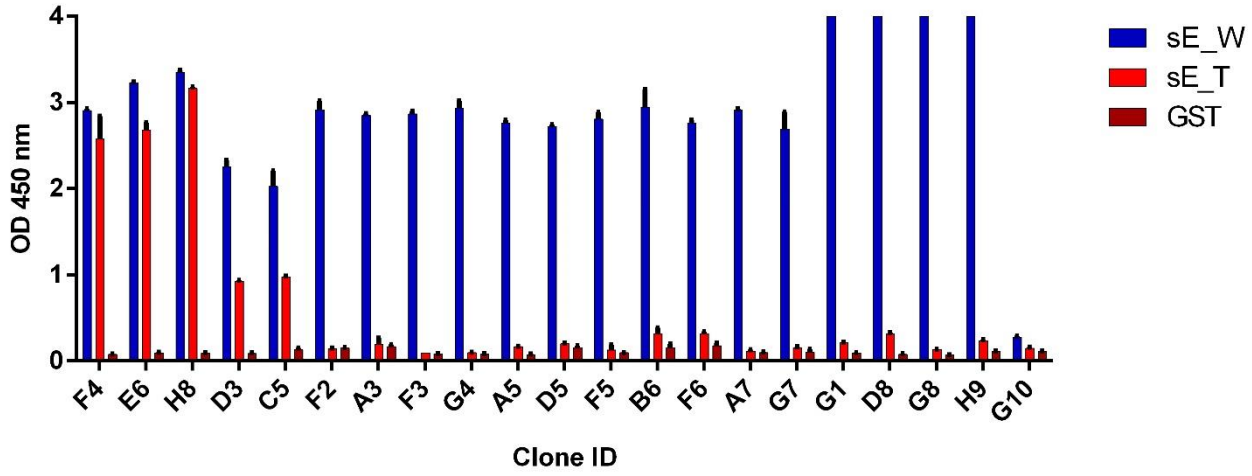

Figure 22. **Analysis of the clones specificity for sE_W target.**

Analysis of the clones specificity for sE_W by phage-ELISA assay. Each clone is tested on the target, sE_T and GST. The OD and standard deviation values are calculated on the average of duplicates.

The scFv of the clones specific for sE_W target were sequenced and analyzed by IMGT/V-Quest database, providing genomic information on the analyzed sequences. The V gene family and the CDR3 amino acid sequences of VL and VH are reported in table 2.

Table 2. V gene family and CDR3 amino acid sequences of the scFv selected on sE_W antigen.

CLONE ID	VL gene family	VL_CDR3	VH gene family	VH_CDR3
A3, D5, B6	IGKV1-39*01	CQQSYSTPVTF	IGHV1-69*04	CARDLWGQQLPYDAFDIW
G1, H9	IGKV1-39*01	CQQSYSTPRTF	IGHV1-18*01	CARTYNWNDLWFDPW
G4, F5, F6, A7	IGLV3-21*03	CQVWDTSSANVVF	IGHV6-1*01	CARGSRNYVGGMDVW
G7, D8, G8, A5	IGLV1-44*01	CAAWDDSLDAWVF	IGHV3-64D*06	CAKLRERRYSSSSVGWGYFDLW
F2	IGLV3-21*02	CQVWDSSSDLVF		Out of frame
F3	IGKV1-39*01	CQQSYSTPMTF		Out of frame

The first evidence after sequencing analysis was that among the 15 positive clones, the number of unique scFv sequences were four (highlighted in blue in the table). Therefore, multiple clones presented the same VL and VH chains. The subsequent sequences alignment for V gene families identification provided two main information: while the VH sequences of the four selected scFv derived from different V gene families, the VL sequences of two scFv (the first represented by A3, D5, B6 clones and the second represented by G1, H9 clones) belonged to the same gene family (IGKV1-39*01). The CDR3 of such VL chains differed for only one amino acid residue.

The sequences analysis evidenced also that two clones (F2 and F3) reported a frameshift in the VH chain, which could be due to an intrinsic sequence error produced during library construction or a technical error during the sequencing. However, the two clones were excluded from further analysis. The redundancy of some clones observed after sequencing is an expected consequence of phage panning which confirmed the occurred enrichment of target-specific clones. Multiple cycles of selection, in fact, increase the affinity of the selected scFv for the target and, at the same time, decrease the range of sequence diversity.

4.3. PRODUCTION AND CHARACTERIZATION OF THE SELECTED α-WNV CLONES

Antibodies in phage format are not compatible with functional assays. Even though produced phages can be stored at -80° C, their structure results unstable for long period and can be affected by contaminant proteases. Therefore, the scFv of three clones selected for their specificity for sE_W antigen and presenting unique sequence were produced in fusion with an IgG immunoglobulin constant region (Fc), consisting in the CH_2-CH_3 domains. The adding of the Fc region allows the stabilization of the scFv fragment, which can be expressed as scFv-Fc molecule in eukaryotic cells (figure 23).

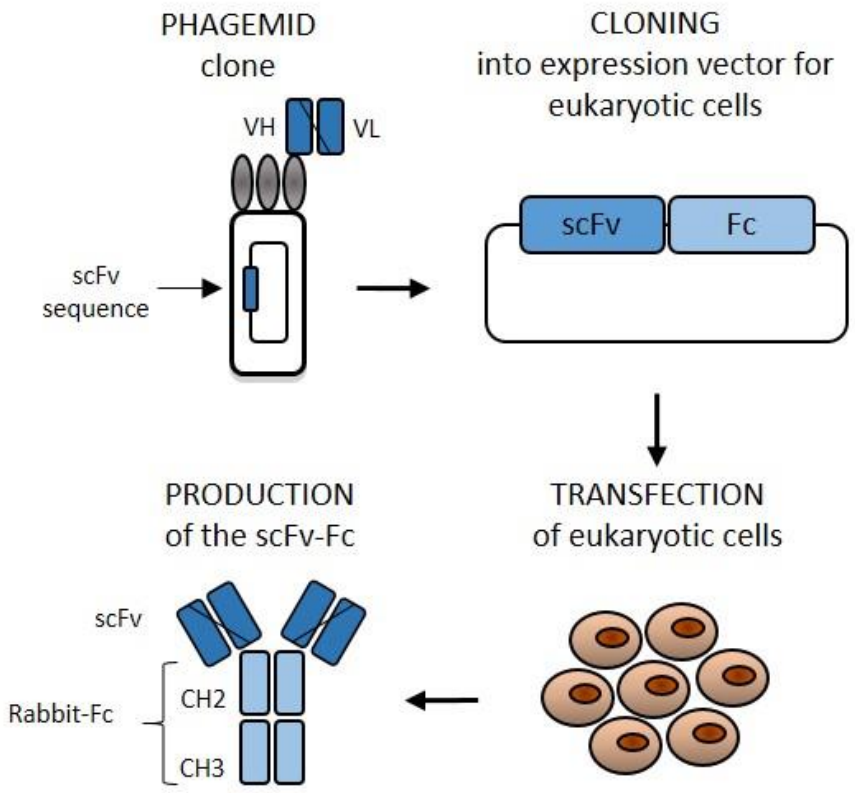

Figure 23. **Workflow for production of phagemid clones as scFv-Fc molecules.**
The scFv sequence of the selected phagemid clone is sub-cloned into an expression vector for eukaryotic cells adding the coding sequence of rabbit constant region (Fc). The plasmid carrying the scFv-Fc sequence is transfected into eukaryotic cells for scFv-Fc molecule production.

Once obtained as scFv-Fc molecules, the selected antibodies were characterized by different tests. The first analysis determined if the scFv recognized different epitopes on the sE_W antigen. The epitope mapping is of particular importance in the case of antibodies employment in sandwich ELISA test, which requires at least two antibodies targeting different epitopes of the target. Subsequently, the antibodies were tested on a larger panel of antigens, including not only the sE protein but also the DIII domain of the two viruses and the sE_W antigen produced through a different expression system. Such tests allowed confirming the specificity of the selected antibodies for the target and, at the same time, localizing the recognized epitope. The capability of the scFv to bind the denatured form of the antigen was also evaluated. Finally, the binding affinity properties of the antibodies were determined on the recognized target.

4.3.1. Expression of scFv-Fc molecules in eukaryotic cells

Each scFv sequence was sub-cloned from phagemid vector to expression vector for eukaryotic cells. The pMB expression vector used [140] allows the production of the scFv fragment in fusion with the IgG Fc region of rabbit species. In addition, the presence of a signal sequence upstream the cloning sites, leads to the scFv-Fc molecule secretion into the cells medium.
After cloning, the plasmid preparation of each antibody was transfected in CHO (Chinese Hamster Ovary) cells seeded in 24-well plate. In order to verify the production of the scFv-Fc molecules, two ELISA assays were performed on the culture medium, 72h after cells transfection (Figure 24).

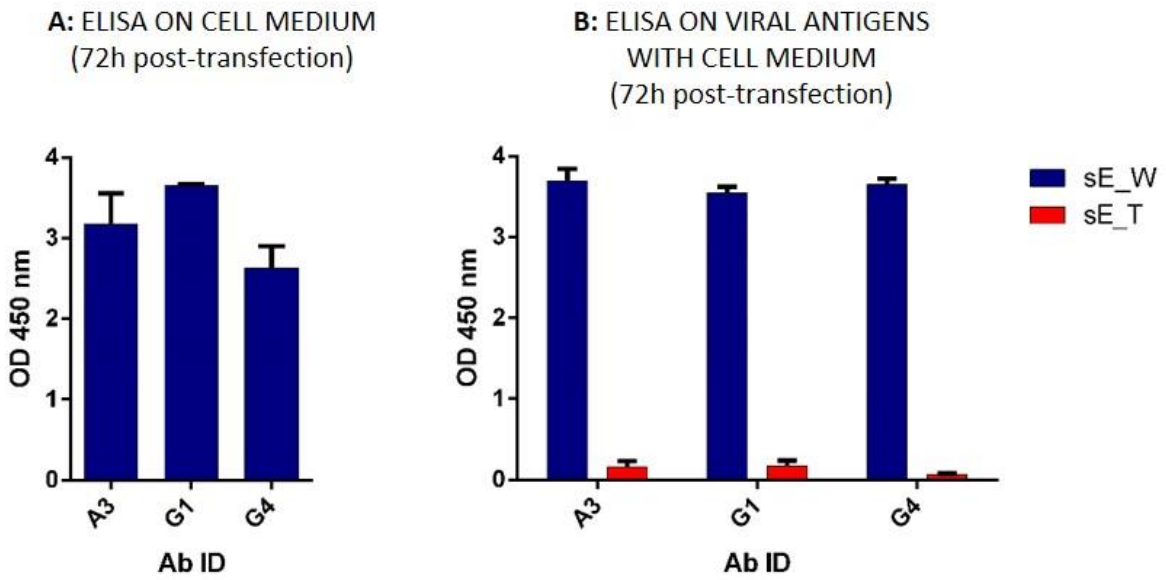

Figure 24. **ELISA assays on medium of cells transfected for scFv-Fc production.**
A: detection of the scFv-Fc molecules in the medium of transfected cells directly coated on plate and incubated with HRP-conjugated Ab anti-Rabbit Fc. **B:** analysis of functionality and specificity of the scFv-Fc molecules tested by incubation of medium on the target (sE_W) and sE_T antigen. The OD and standard deviation values are calculated on the average of duplicates.

In the first test, the medium of transfected cells was diluted (1:10 in PBS) and directly coated on plate. The presence of the scFv-Fc molecules were detected by HRP-conjugated Ab α-Rabbit Fc. As shown in figure 24, panel A, all the tested samples resulted positive, confirming the expression of the transfected constructs in cell medium.
In the second ELISA assay, the functionality and specificity of each scFv-Fc molecule were tested. Both sE_W and sE_T antigens were coated on plate and the medium of each sample was diluted (1:10 in PBS) and incubated on the two proteins. The antigen-linked scFv-Fc molecules were detected with Ab α-Rabbit Fc. The results (figure 24, B) confirmed that the scFv-Fc molecules expressed in the medium were functional, as they recognized the target sE_W. In addition, the specificity observed in the phage formats, was confirmed for the scFv-Fc molecules, which did not react on the sE_T antigen.

4.3.2. Purification of the scFv-Fc molecules

The cells expressing the scFv-Fc molecules were expanded in T75 culture flasks and medium checked for antibodies expression and functionality before storing. The collected medium (80-90 mL) was used for scFv-Fc purification by Protein A resin, which presents high affinity for the Fc of different species, included rabbit. Both resin and medium were conditioned in 0.1 M of Tris buffer and incubated O/N on rotation. The medium mixed with the resin was loaded on a plastic column allowing

resin packaging. The elution of the protein A-linked antibodies was performed by adding Glycine buffer at acidic pH on the resin and collecting three or four fractions, which final pH was corrected to 7.4 with Tris buffer. The eluted fractions were analyzed by SDS PAGE, loading 1% of each fraction volume on gel (figure 25).

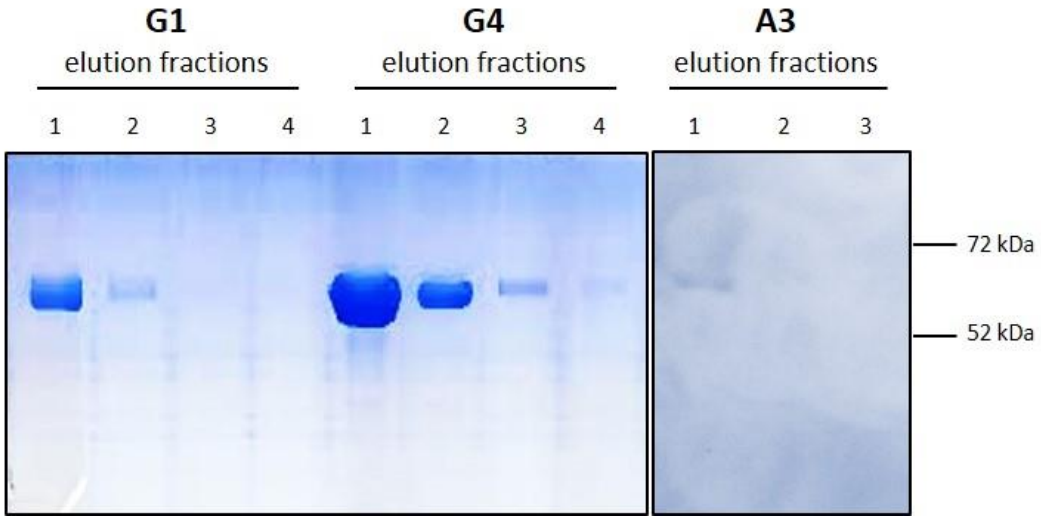

Figure 25. **SDS PAGE of purified scFv-Fc molecules.**
Analysis of the fractions obtained after scFv-Fc molecules purification by SDS PAGE. Each protein fraction is loaded on gel in denaturing buffer and bands detected by coomassie blue stain.

As shown in SDS PAGE, the purified scFv-Fc molecules presented the expected molecular weight of almost 60 kDa, which includes the two variable domains and the constant region. Most of the scFv-Fc molecules were eluted in the first fraction, while a progressive decrease of protein amount was observed in the subsequent fractions, suggesting the complete elution of all protein A-linked antibodies. Although all the three antibodies were purified, a variable protein yield was obtained from cells medium, which corresponded to 0.1 ug/mL for A3, 0.4 ug/mL for G1 and 1 ug/mL for G4. Such variability could be due to different percentages of transfection efficiency or different expression rate of the scFv-Fc by transfected cells.

4.3.3. Epitope mapping analysis

In order to determine if the selected scFv recognize different epitopes on the sE_W antigen, a phage-ELISA competition assay was performed. This test provides the incubation of the antigen with one of the produced scFv-Fc molecule, defined competitor. After incubation, the other scFv in phage format are added on the antigen: if the phage scFv is directed against the same antigen epitope recognized by the competitor, it is not able to bind the antigen, due to the presence of the competitor

itself. On the contrary, if the recognized epitope is different from competitor, the phage scFv is able to bind the antigen. After phages incubation, the unbound clones are removed by washes and detection performed by HRP-conjugated mAb α-M13. In parallel, the phages are incubated on the antigen without competitor (positive control) and with a scFv-Fc molecule which does not recognize the antigen, to confirm that the binding between phages and target is not affected by the presence of the competitor. A schematic illustration of the competition test is shown in figure 26.

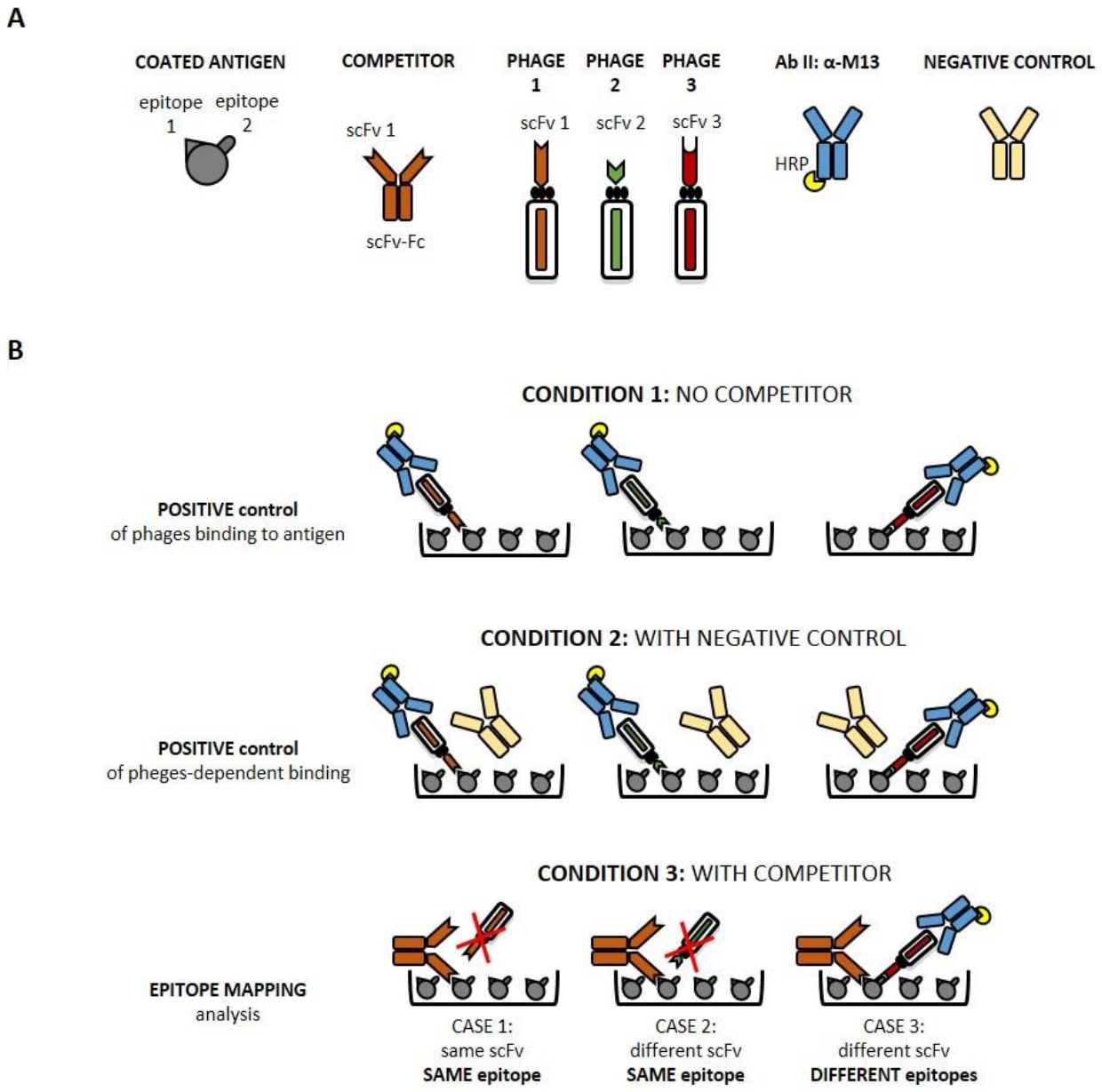

Figure 26. Schema of phage-ELISA competition assay.

A. Components of phage-ELISA competition assay. **B.** Illustration of the three test conditions evaluating the epitope binding of clones without competitor (condition 1), with negative control (condition 2) and with competitor (condition 3).

The phage-ELISA competition assay with the selected scFv was performed using the G1 scFv-Fc molecule as competitor. The obtained results are shown in figure 27.

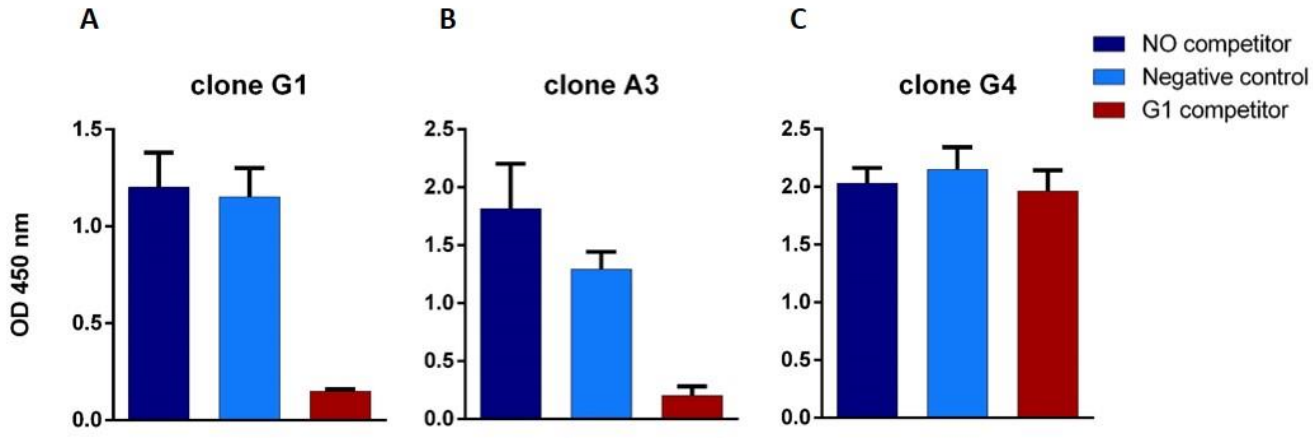

Figure 27. **Phage-ELISA competition assay on α-sE_WNV scFv.**

Phage-ELISA competition test with the three selected α-sE_W clones (G1, A3 and G4) and G1 scFv-Fc as competitor. Blue bars indicate the phages-antigen binding in the absence of competitor; light blue bars indicate the phages-antigen binding in the presence of a scFv-Fc molecule unable to link the antigen; red bars indicate the phages-antigen binding obtained in the presence of G1 scFv-Fc competitor. **A.** Competition test with G1 phage clone. **B.** Competition test with A3 phage clone. **C.** Competition test with G4 phage clone. Each OD and standard deviation values are calculated on the average of triplicates.

As first observation, the values obtained without competitor (blue bars) and in the presence of a scFv-Fc molecule not recognizing the target (light blue bars) were comparable for each tested clones. This demonstrated the reliability of the assay, as the scFv phages α-sE_W were able to bind the target also in the presence of the unspecific scFv-Fc molecule. The results of G1 clone (panel A) represented an additional control of the test. As the G1 phage format has the same scFv of the competitor, the absence or reduction of antigen binding was expected (red bar). Such binding decrease was observed also for the A3 clone (panel B), suggesting that its scFv was directed against the same G1 epitope. However, another possible explanation which can not be excluded is the recognition of a different binding site near the G1 epitope, whose binding is prevented by the steric hindrance of the G1-scFv. On the contrary, the G4 clone (panel C) did not present any change in binding signal on the target, leading to the conclusion that while A3 and G1 probably recognized the same sE_W epitope, G4 scFv was directed against a different binding site.

4.3.4. Analysis of the scFv binding properties for the target

The results of the competition assay evidenced one antigen epitope recognized by G4 and a second epitope targeted by both A3 and G1. Considered the lower amount of purified A3 scFv-Fc obtained with respect to G1 (figure 25), only G4 and G1 antibodies were further analyzed.

The G1 and G4 purified scFv-Fc molecules were tested by ELISA assay on all the produced antigens: the sE and DIII domain of WNV and the same proteins of TBEV. As mentioned before, the DIII is a small domain of the sE protein, therefore this test allowed not only to confirm the specificity of the selected antibodies for the target but also to localize the epitopes recognized by G1 and G4. The two scFv-Fc molecules were tested in different dilutions on the four antigens and the results are shown in figure 28.

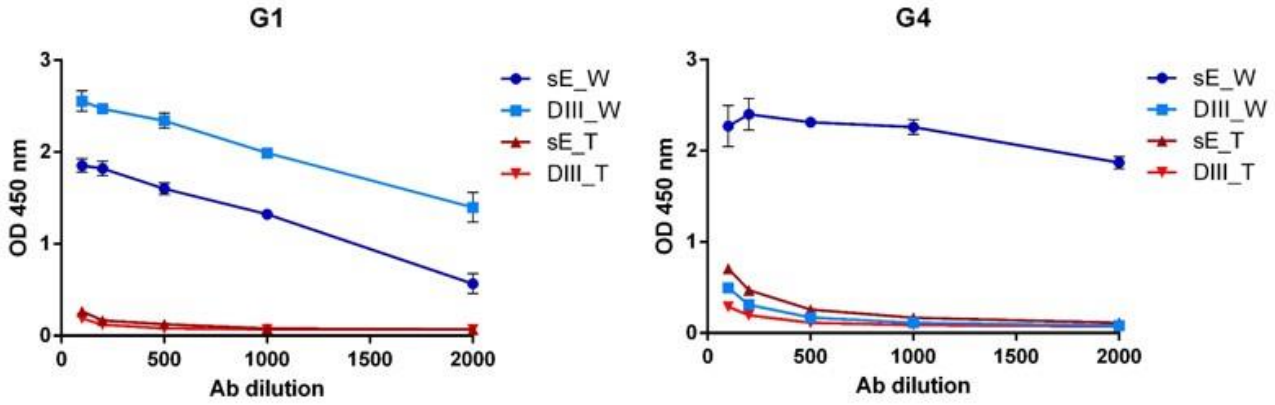

Figure 28. ELISA assay on WNV and TBEV antigens with G1 and G4 antibodies.

G1 and G4 antibodies tested in different dilutions (X-axis) by ELISA assay on four antigens: sE_W, DIII_W, sE_T and DIII_T. The OD and standard deviation values are calculated on the average of duplicates.

All the dilutions of both G1 and G4 antibodies resulted negative on TBEV antigens, while the sE_W was recognized at each dilution point, confirming the specificity of the purified antibodies observed also in the unpurified form (medium of transfected cells) and phage format. A progressive decrease of the binding signal was observed for G1 in accordance with the increase of antibody dilution, while G4 showed high reactivity also at lower concentration. In addition, the G1 antibody resulted positive on both the sE and DIII antigens of WNV, indicating that its binding site is located on the DIII_W domain. On the other hand, the G4 antibody was not able to recognize the single DIII_W antigen, suggesting that the recognized epitope maps between the DI and DII envelope domains.

As described in the previous results section, the G1 and G4 antibodies were selected on the sE_W antigen purified from bacteria as GST-fused protein. In order to confirm that the binding capability

is independent from the antigen expression system, the scFv-Fc molecules were tested on the sE_W antigen produced in eukaryotic cells and purified by His tag (figure 29). The sE_His protein includes the same viral envelope domains (DI, DII and DIII) of sE_GST. The absence of the fusion protein determines a lower molecular weight ranging from 45 to 50 kDa depending on its glycosylation status.

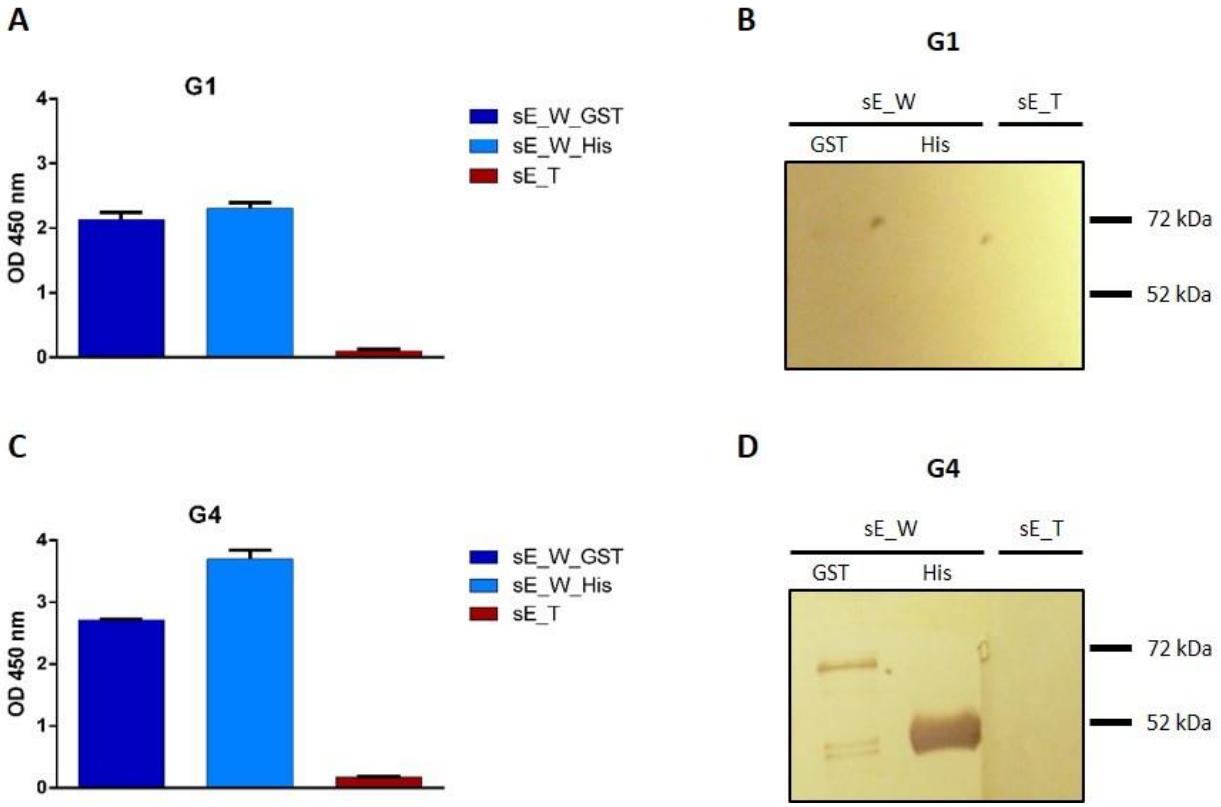

Figure 29. **Analysis of G1 and G4 antibodies binding properties on the sE_W target produced in different expression systems.**

ELISA and Western Blot assays on sE_W antigen produced in bacteria (sE_W_GST) and eukaryotic cells (sE_W_His). sE_T is used as negative control. **A.** ELISA assay with G1 scFv-Fc. **B.** Western Blot with G1-scFv-Fc. **C.** ELISA assay with G4 scFv-Fc. **D.** Western Blot with G4 scFv-Fc. The OD and standard deviation values of ELISA assays are calculated on the average of duplicates.

The two versions of sE_W antigen were checked by ELISA and Western Blot assays. As shown in the figure, the ELISA results of G1 scFv-Fc (panel A) demonstrated that capability of the antibody to bind the sE_W protein produced in both the expression systems. In particular, a comparable binding signal was observed on the two WNV antigens, without cross-reactivity on the sE of TBEV used as negative control. The same antigens were checked by Western Blot using G1 as primary antibody (panel B). In this case, the G1 scFv-Fc was not able to recognize the sE_W antigens, suggesting the loss of the target epitope conformation in denaturing conditions.

Similar ELISA test results was obtained for G4 scFv-Fc (panel C), which resulted positive on both sE_W_GST and sE_W_His proteins, confirming its specificity for the target and demonstrating the independence from the expression system used for antigen production. The signal on sE_T was negative, as expected. Moreover, the two versions of WNV antigen were recognized by G4 also in Western Blot assay. As shown in the figure (panel D), the antibody detected the two target proteins at the expected molecular weight of 70 kDa (sE_W_GST) and 50 kDa (sE_W_His).

The results obtained from binding assays with the selected antibodies (summarized in figure 30) allowed determining the localization and predicting the conformational status of the two different recognized epitopes. The capability of G4 to target the antigen in denaturing condition, in fact, suggested a linear conformation of the binding site. On the other hand, the antigen recognition by G1 occurred only in native condition, indicating its dependence from a particular epitope conformation.

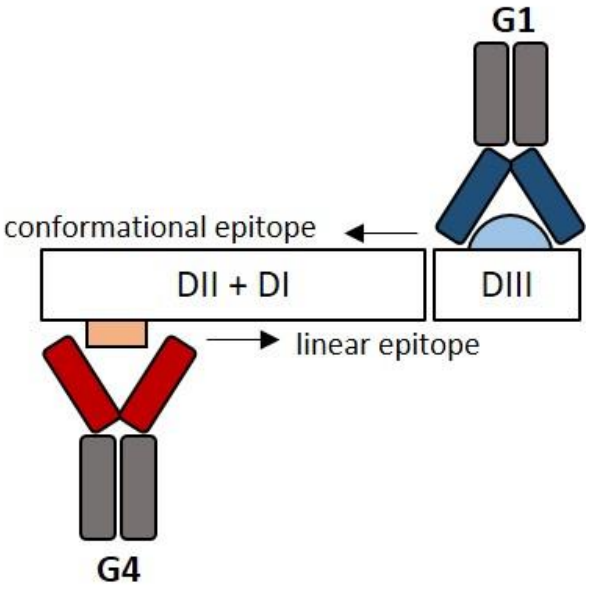

Figure 30. Localization and conformation of the epitopes recognized by G1 and G4 antibodies.
Schematic illustration of the localization and conformation of the two epitopes recognized by G1 and G4 antibodies on sE_W antigen.

4.3.5. Analysis of the scFv binding affinity for the target

In order to determine the affinity of the two antibodies for the target, the G1 and G4 scFv-Fc molecules were tested in ten different concentrations on WNV antigens by ELISA assay (figure 31).

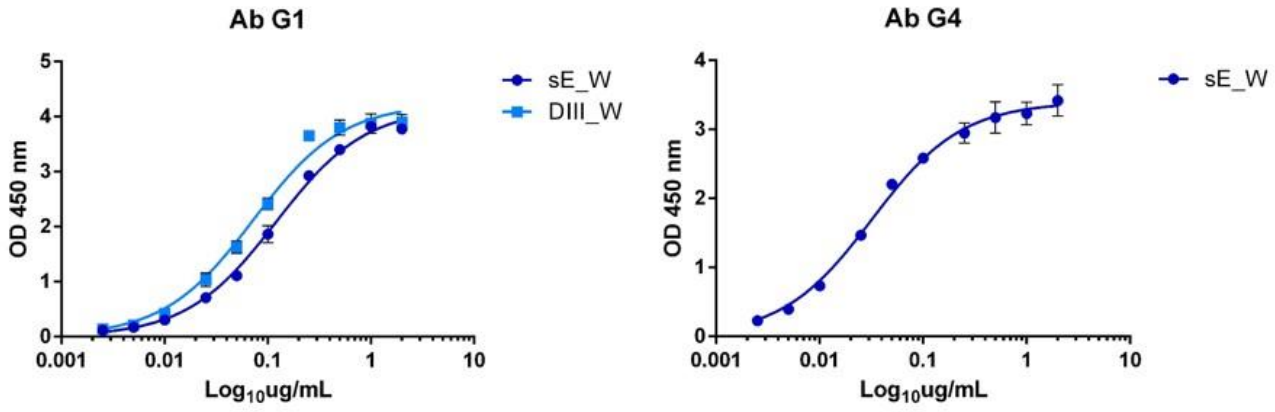

Figure 31. **Affinity of G1 and G4 antibodies for sE_W target.**

G1 and G4 antibodies tested by ELISA assay in different concentrations (X-axis) on the specific targets: sE_W and DIII_W for G1, sE_W for G4. The OD and standard deviation values are calculated on the average of duplicates.

The curves obtained with G1 antibody presented a similar trend on the two WNV antigens, although a higher affinity value was observed for the DIII domain. The 50% of binding saturation, in fact, was reached at a concentration of 100 ng/mL on sE_W and 70 ng/mL on the single DIII domain. Comparing the curves of the two antibodies on sE_W antigen, the G4 presented a higher affinity for the target, as it reached the 50% of binding saturation at a concentration of 30 ng/mL. The K_d affinity values calculated on each curve are reported in table 3.

Table 3. K_d values of G1 and G4 antibodies for the target.

	Ab G1		Ab G4
	sE_W	DIII_W	sE_W
K_d (nM)	1.7	1.05	0.45

4.4 PLAQUE REDUCTION NEUTRALIZATION TEST WITH G1 AND G4 scFv-Fc MOLECULES

Although antibodies for diagnostic test aim to recognize the target without additional functions, G1 and G4 scFv-Fc molecules were further characterized by Plaque Reduction Neutralization Test (PRNT) in order to determine if their binding on the antigen could affect the infection capability of WNV.

The PRNT is an *in vitro* assay, which measures the presence of neutralizing antibodies in serum samples of flavivirus infected patients and represents the most specific serologic test for flaviviruses diagnosis. However, the need of highly trained staff and specialized equipment for live virus manipulation, the expensive costs and the long time required (generally one week) limit the assay application.

The classical PRNT protocol is based on the preparation of serum serial dilutions, each one mixed with a fixed number of live viral particles. The mixes containing antibody-virus complexes are then added on a monolayer of cells susceptible for viral infection. The effect of antibodies on the virus infection rate is calculated by comparison with control cells infected with the virus alone. In particular, the parameter used for results evaluation is the number of plaques derived from infected cells. An overview of the PRNT test is shown in figure 32.

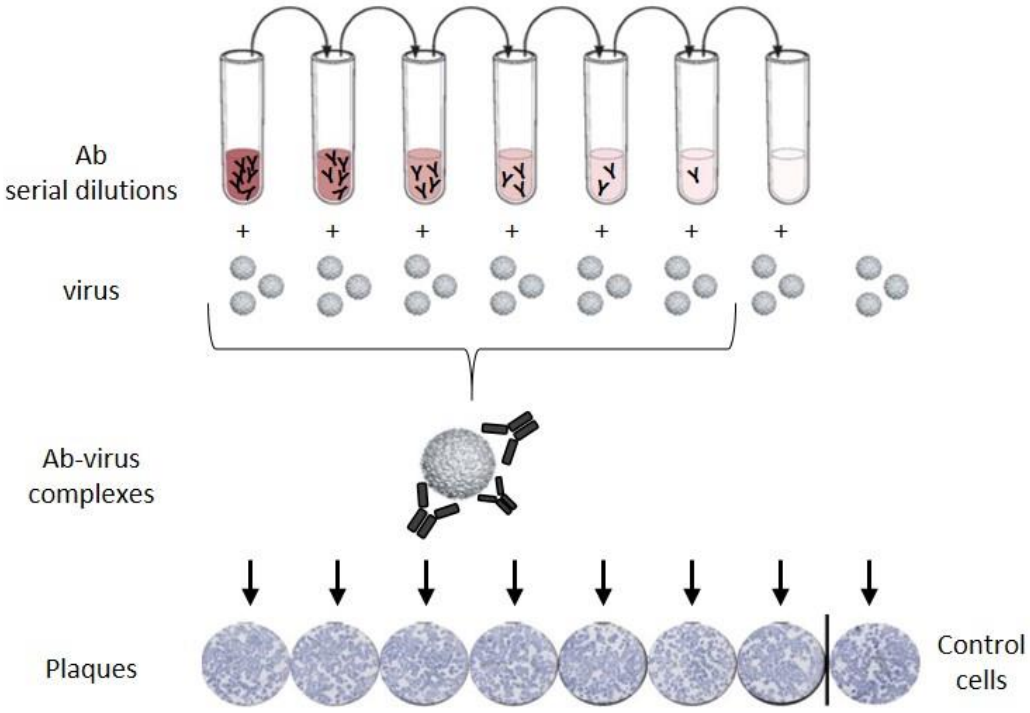

Figure 32. Schematic representation of Plaque Reduction Neutralization Test.

Serial dilutions of the antibody are prepared and mixed with fixed concentration of virus. The antibody-virus complexes are added on monolayer of cells. The infection rate is calculated comparing the number of plaques between treated and control cells.

The PRNT was performed on Vero cells using the two α-WNV antibodies G1 and G4. In addition, the 4G2 antibody, known for its cross-reactivity on different flaviviruses and unable to affect WNV infection [148], was used as control of the test. The obtained results are shown in figure 33.

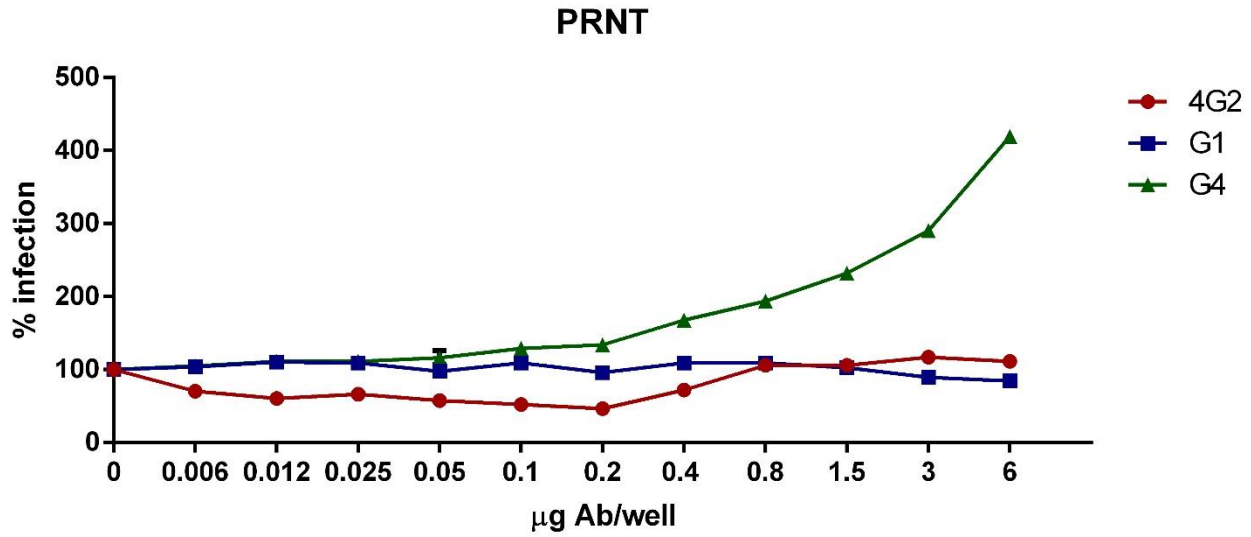

Figure 33. Plaque Reduction Neutralization Test with G1 and G4 antibodies.

Plaque Reduction Neutralization Test with G1 and G4 antibodies. 4G2 antibody is used as negative control. The amount of antibody used for each treatment is indicated on X-axis. The percentage of cells infection is indicated on Y-axis. The percentage and standard deviation values are calculated on the average of duplicates.

The reported graph show the percentage of cells infection obtained with the virus alone (100%) or after treatment with the antibody-WNV complexes at different dilutions.

As expected, the cells treatment with 4G2-WNV complexes did not affect virus activity, as all the dilutions used led to infection rates comparable to untreated cells. The comparable infection rates obtained for 4G2 antibody confirmed the reliability of the test.

A similar trend was observed for G1 antibody, suggesting that its binding on the viral surface does not influence the infection capability of WNV. On the contrary, a proportional increase in cells infection was obtained with growing amount of G4 antibody. Comparing with control cells, in fact, a four-fold higher percentage of infection was observed.

The antibody-dependent enhancement of infection (ADE) is a phenomenon extensively studied in flaviviruses infections. It refers to not neutralizing antibodies linked to the viral envelope, which mediate the endocytosis of the virus through the attachment of the antibody-virus complexes on FcγR-bearing cells, increasing the viral infection rate [149]. As the PRNT with G1 and G4 antibodies was performed on FcγR-negative cells, the enhancement of WNV infection mediated by G4 depends on a different mechanism. The independence of antibody-induced infection from FcγR was observed in previous works on West Nile [150] and Dengue [151] viruses, but only recently the involved

molecular events have been described for TBEV [152]. The proposed mechanism is based on the exposure of the fusion loop of the viral envelope caused by antibody binding, which leads to viral and cellular membranes fusion and thus to infection enhancement. As the G4 antigen epitope maps on DII/DI domains region and the fusion loop is located on the DII, the described mechanism could explain the G4-induced enhancement of WNV infection.

5. RESULTS AND DISCUSSION – 2

ISOLATION OF ANTIBODIES α-WNV BY IMMUNIZED LIBRARY CONSTRUCTION AND SCREENING

The second planned strategy to produce the needed reagents for WNV diagnostic purposes was the construction and screening of murine immunized antibodies libraries on the viral target. The first step was the mice immunization against WNV. In order to have a deeper knowledge of the immune response triggered against the virus, the antibodies repertoires resulted positive for immunization were analyzed by Next Generation Sequencing (NGS) through Illumina technology. The same murine samples were used for antibodies libraries construction and the subsequent screening on WNV antigen.

5.1. MICE IMMUNIZATION α-sE_W ANTIGEN

The immunization against WNV was performed on four mice through the Gene gun technology. This method is based on the delivery of the DNA antigen sequence into animal, which provides itself for the antigen production. In particular, the DNA expression construct containing the antigen sequence is coated on gold particles used as carriers. The DNA-coated particles are shot on animal epidermis by the gene gun, leading to antigen expression and, therefore, immune response stimulation. With respect to classical immunization procedures, the replacement of antigen administration as purified protein with the *in vivo* production limits the variability problems related to antigen quality and immunogenicity and represents the main advantage of Gene gun technology.

A schematic representation of the construct used and the timeline followed for mice immunization is shown in figure 34.

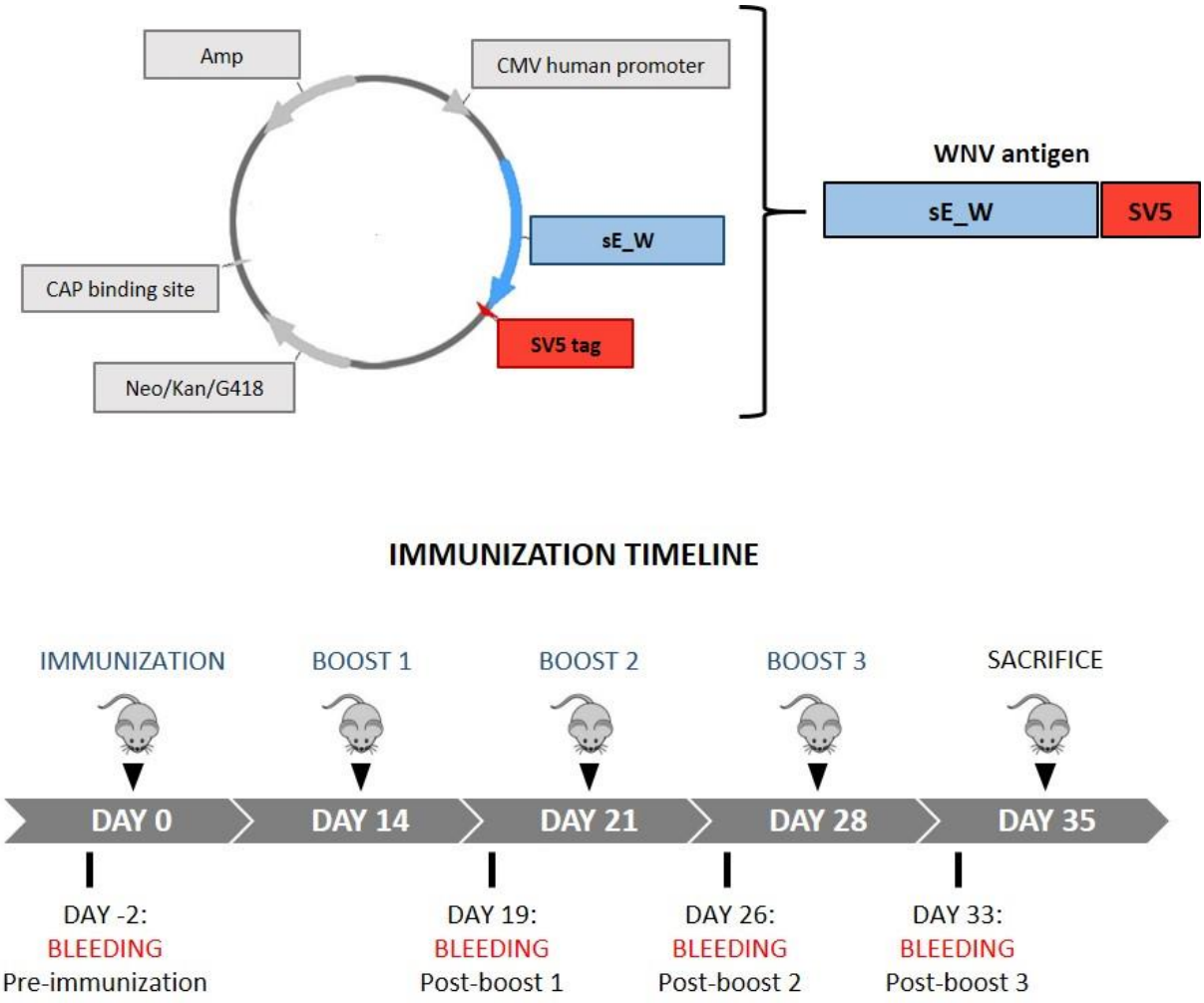

Figure 34. Mice immunization against WNV by Gene gun technology.

Top: schematic representation of the construct used for Gene gun immunization and the related antigen product. Down: immunization timeline.

As indicated in the figure, the DNA coding sequence of sE_W antigen was cloned into an expression vector for mammalian cells reporting a C-terminus tag (SV5). The obtained construct was used for mice immunization. In particular, 14 days after the first immunization, three boost were administered at one-week intervals. Blood samples were collected before immunization and 5 days after each boost. In order to verify the positivity and specificity of the immune response against the WNV antigen, all the collected serum samples of mouse 1 were checked by ELISA assay on the produced viral antigens and BSA, as negative control (figure 35).

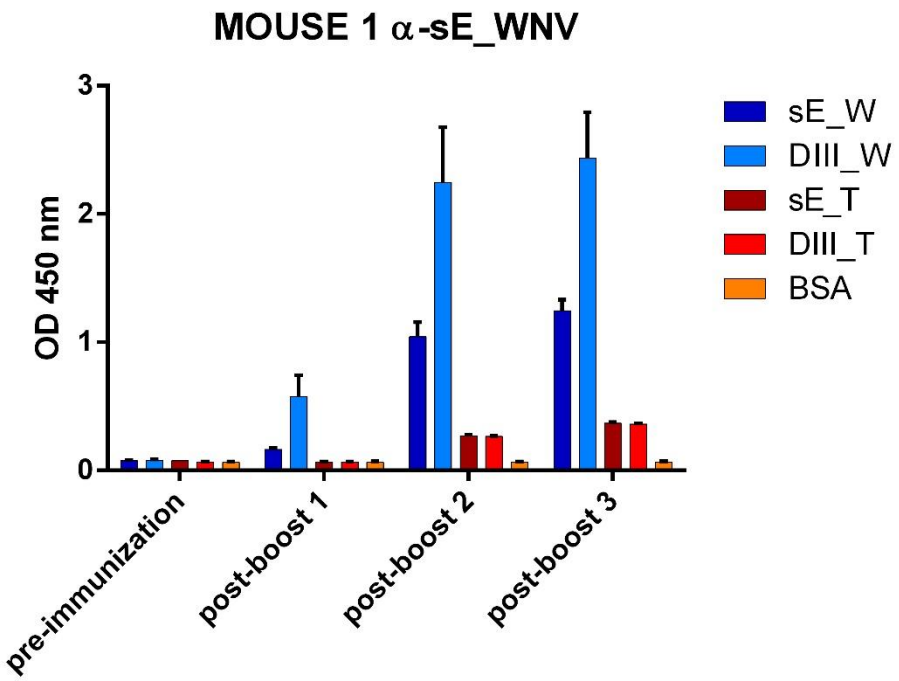

Figure 35. Time course of α-sE_W serum samples from mouse 1 tested on different viral antigens.
Time course of α-sE_W serum samples collected before and after three boost of immunization on mouse 1. The collected samples are tested on WNV and TBEV antigens. BSA is used as negative control. The OD and standard deviation values are calculated on the average of duplicates.

As shown in the figure, while the pre-immunization serum resulted negative on all the coated proteins, the signal on WNV antigens was positive for all the post-immunization sera. As expected, a proportional increase of the antibodies response was observed in accordance with the progress of the immunization procedure represented by the three serum samples collected after each boost. In addition, the sera resulted negative or poorly reactive on TBEV antigens, demonstrating the response specificity triggered by α-WNV immunization.

Once obtained the preliminary data on the immunization efficacy, the final sera of all immunized mice were tested on WNV antigens and BSA (negative control). As additional controls of the response specificity, the sera of a α-sE_T immunized mouse and a not immunized mouse were checked on both TBEV and WNV antigens (figure 36).

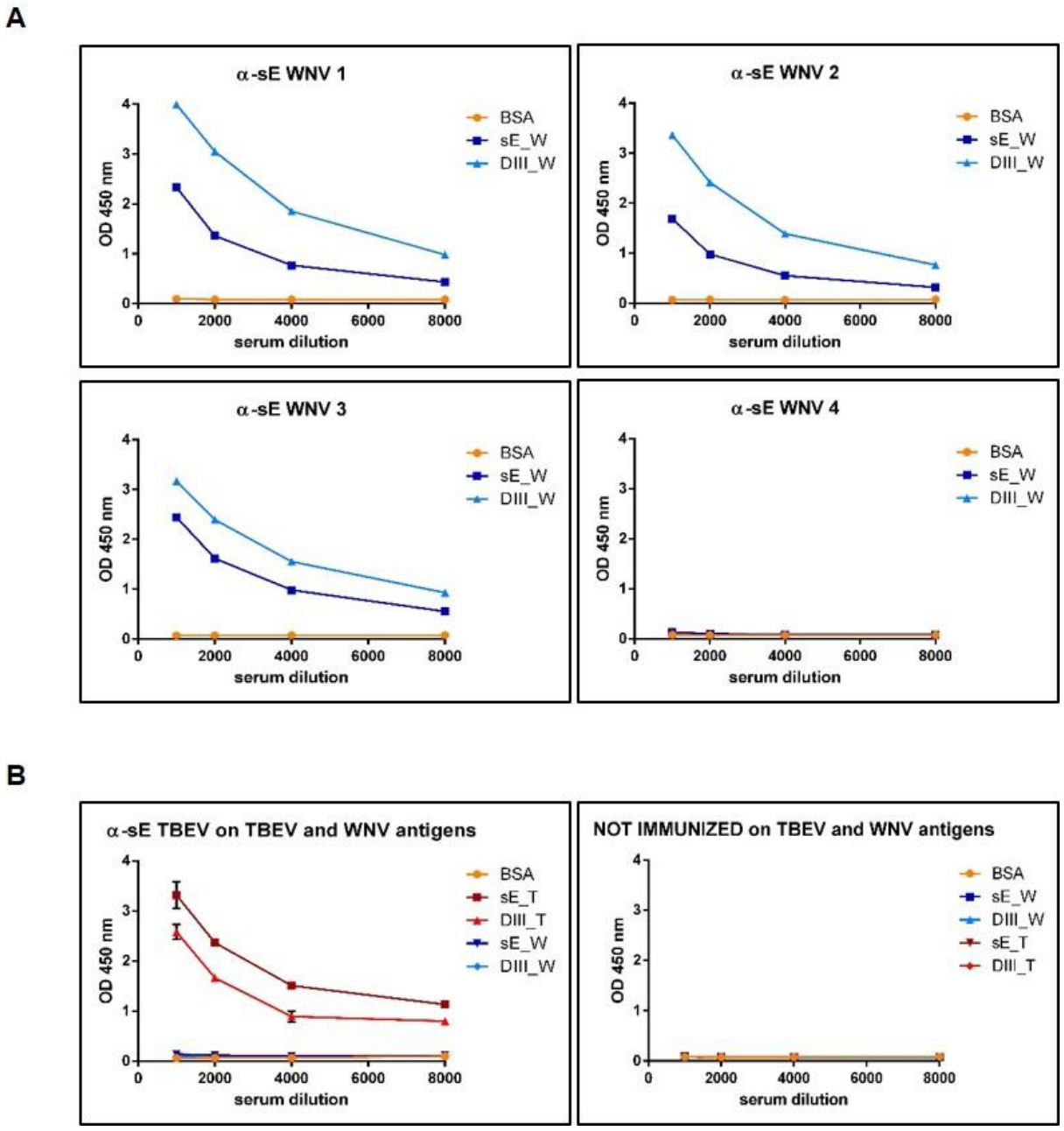

Figure 36. Serologic analysis of α-sE_W immunized mice.

ELISA assays with murine sera collected after α-sE_W immunization. **A.** α-sE_W sera of four immunized mice tested in different dilutions on BSA (negative control) and WNV antigens (sE and DIII). **B.** Sera of α-sE_T immunized mouse (left) and not immunized mouse (right) tested in different dilutions on BSA (negative control), TBEV and WNV antigens (sE and DIII). The OD and standard deviation values are calculated on the average of duplicates.

The four α-sE_W sera were tested in serial dilutions on the proteins (figure 36, A). Among them, three resulted highly positive on WNV antigens, with a progressive signal decrease in accordance with serum concentration decrease. One sample did not show any response on the antigens, including the lower serum dilution point. Considered the efficiency of the DNA immunization method observed for three mice, the negative sample suggests a possible technical error occurred during the

immunization procedure. The signal on BSA was negative for all α-sE_W sera, as expected. On the other hand, the α-sE_T serum specifically recognized the TBEV antigens, without cross-reaction on WNV antigens and BSA (figure 36, B). The response specificity was further confirmed by not immunized serum (figure 36, B) resulted negative on all the coated proteins.

5.2. ANALYSIS OF THE ANTIBODIES REPERTOIRES OF α-WNV IMMUNIZED MICE

The antibodies repertoires of mice resulted positive for immunization were analyzed by NGS through Illumina technology. In particular, as antibodies diversity is mainly determined by CDR3 of heavy chain [128], the sequencing was focused on this region.

After mice sacrifice, spleens were extracted for lymphocytes isolation and the RNA obtained from lymphocytes was used for cDNA retrotranscription. Using primers mixes specifically designed for the amplification of murine variable antibodies regions (details in section 2.2.1) the VH genes were obtained by PCR using the cDNA as template. The VH repertoire of a not immunized mouse was also amplified and used as background during data analysis. The same VH gene repertoires analyzed by NGS were used for phage libraries construction (discussed in the next section). The workflow describing the use of murine samples for both repertoires analysis and libraries construction is reported in figure 37.

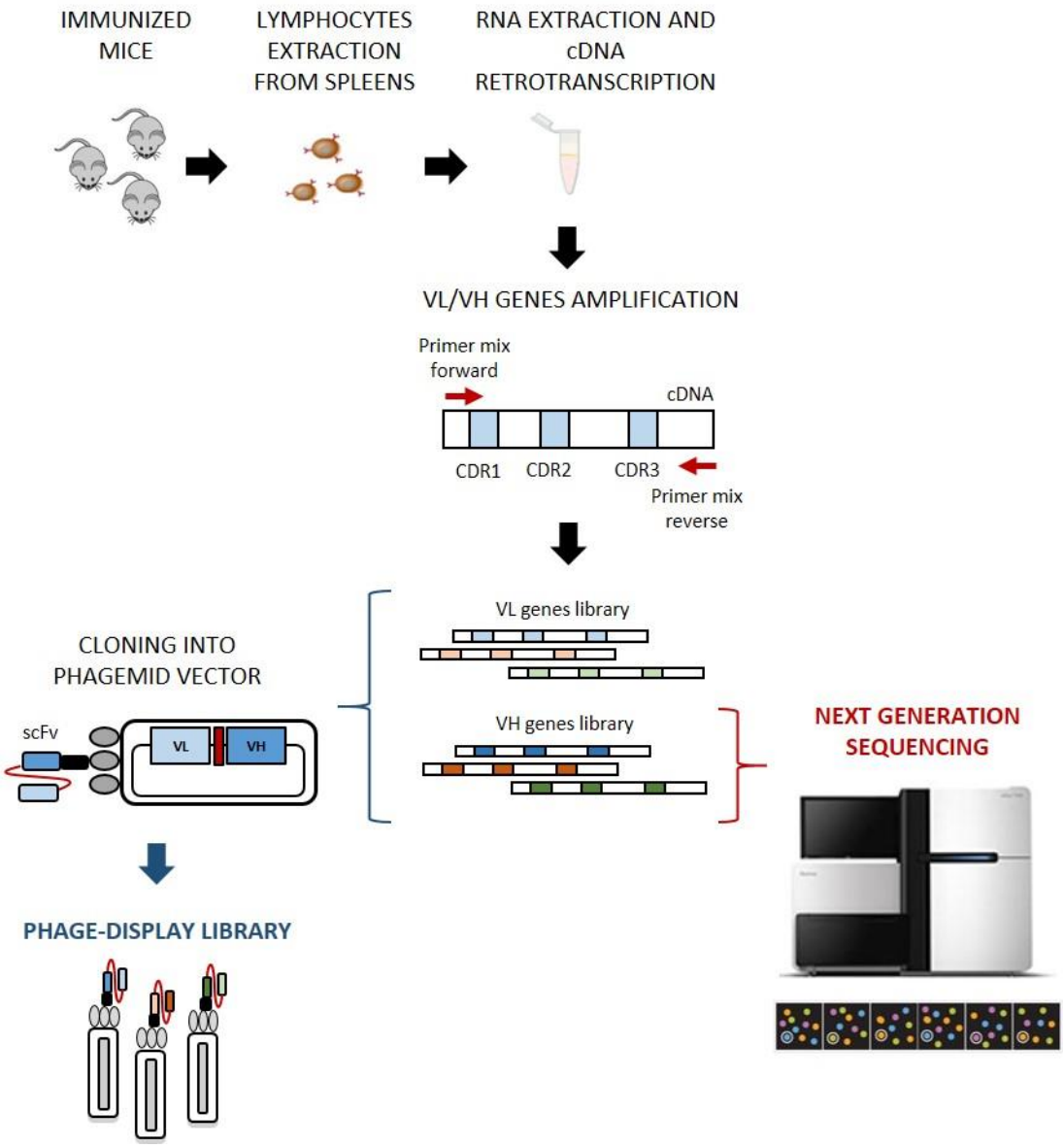

Figure 37. Workflow for immunized phage library construction and VH repertoire sequencing.
The spleens of α-WNV immunized mice are used for lymphocytes isolation. The RNA of lymphocytes is extracted and retrotranscribed into cDNA. The VL and VH genes are amplified from cDNA through mixes of forward and reverse primers. The VH genes library is sequenced by Illumina technology. The same VH repertoire is assembled with VL genes into scFv fragments by PCR, cloned into phagemid vector and transformed into electrocompetent cells to obtain the phage library.

The analysis of the murine VH repertoires performed through Illumina technology provided the DNA sequences of the CDR3s regions and the related frequency of each sequence expressed as number of reads. The number of reads obtained for the three murine samples are reported in the following table.

Table 4. Number of reads obtained by Next Generation Sequencing of three VH murine repertoires

VH repertoire ID	Mouse 1	Mouse 2	Mouse 3
Number of reads	12×10^6	25×10^6	27×10^6

As shown in table 4, the total DNA sequences for each repertoire was comparable between the three samples, despite the lower number of reads of sample 1. However, the range of sequences obtained was in accordance with the expected output of Illumina technology. As the murine samples derived from an immunization process, previously confirmed by serologic analysis, it was expected an enrichment of a subset of CDR3s within the repertoire, likely corresponding to antibodies raised against the target. Therefore, the frequency of the CDR3s of each repertoire was analyzed by AbMining ToolBox software [153]. The results are reported in figure 38.

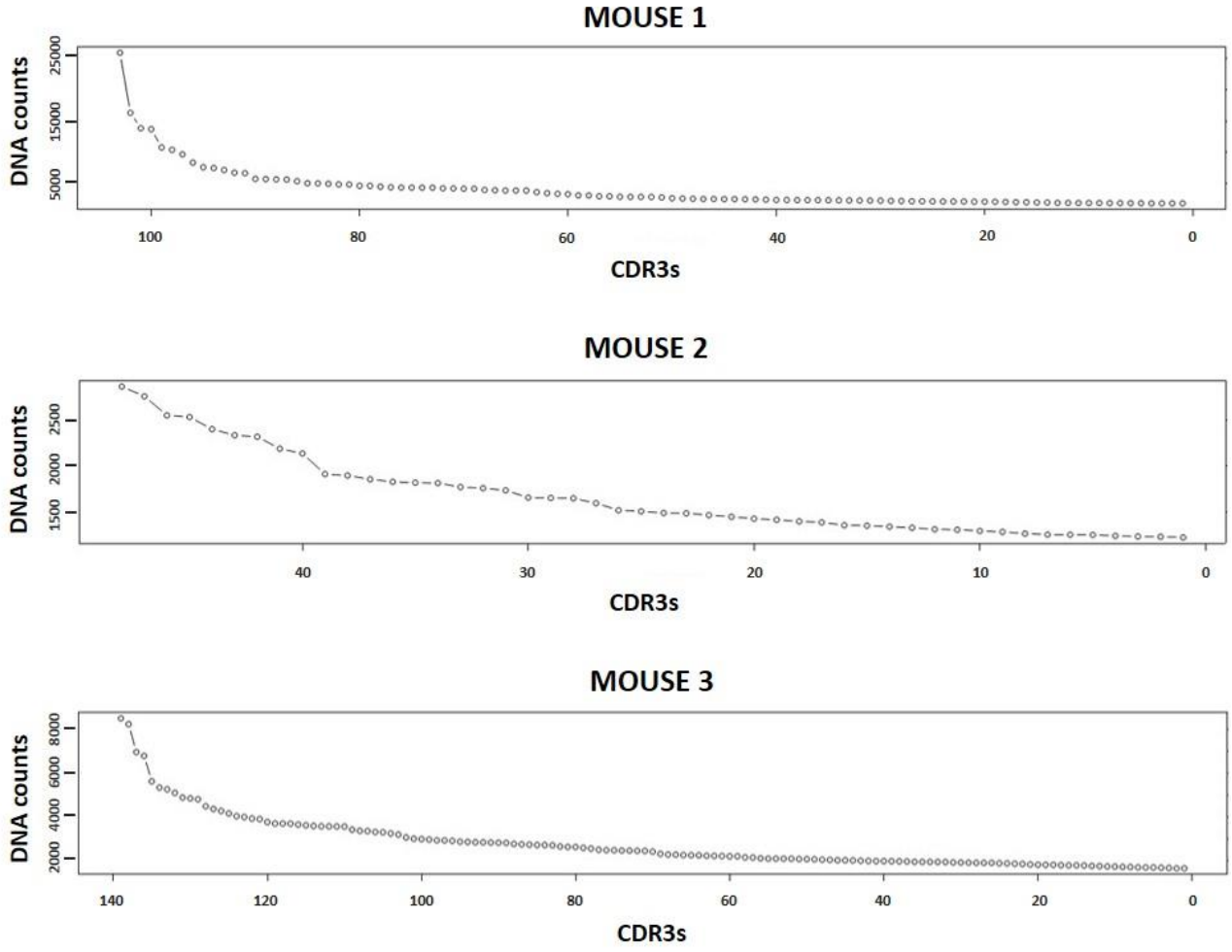

Figure 38. Frequency of CDR3s sequences in VH murine repertoires.
Frequency of the CDR3s sequences identified by NGS on the three murine immunized repertoires.

The CDR3s frequency range obtained for each repertoire was highly variable among the three samples. While in samples 2 and 3 the most represented CDR3s showed frequency values of 3000 and 8000 reads, respectively, in sample 1 the most frequent CDR3 reached more than 25000 reads. Comparing the most represented CDR3s of the analyzed repertoires, no common sequences were found, suggesting that mice immunized against the same antigen develop a private response. Previous studies on murine repertoires analysis reported contrasting data [129, 150], which support both the possibilities of private and public VH CDR3s between mice immunized against the same antigen.
The three most frequent CDR3s of each repertoire were further analyzed in order to determine if different DNA sequences coded for the same CDR3. As shown in figure 39, each analyzed CDR3 from the three repertoires was coded by a variable number of DNA sequences. In particular, for each CDR3 of samples 1 and 3 almost 30 different DNA sequences were found, while the CDR3s of sample 2 presented a lower number of different DNA sequences. However, the sequence variants resulted poorly represented, while the dominance of a single DNA coding sequence was observed for all the CDR3s.

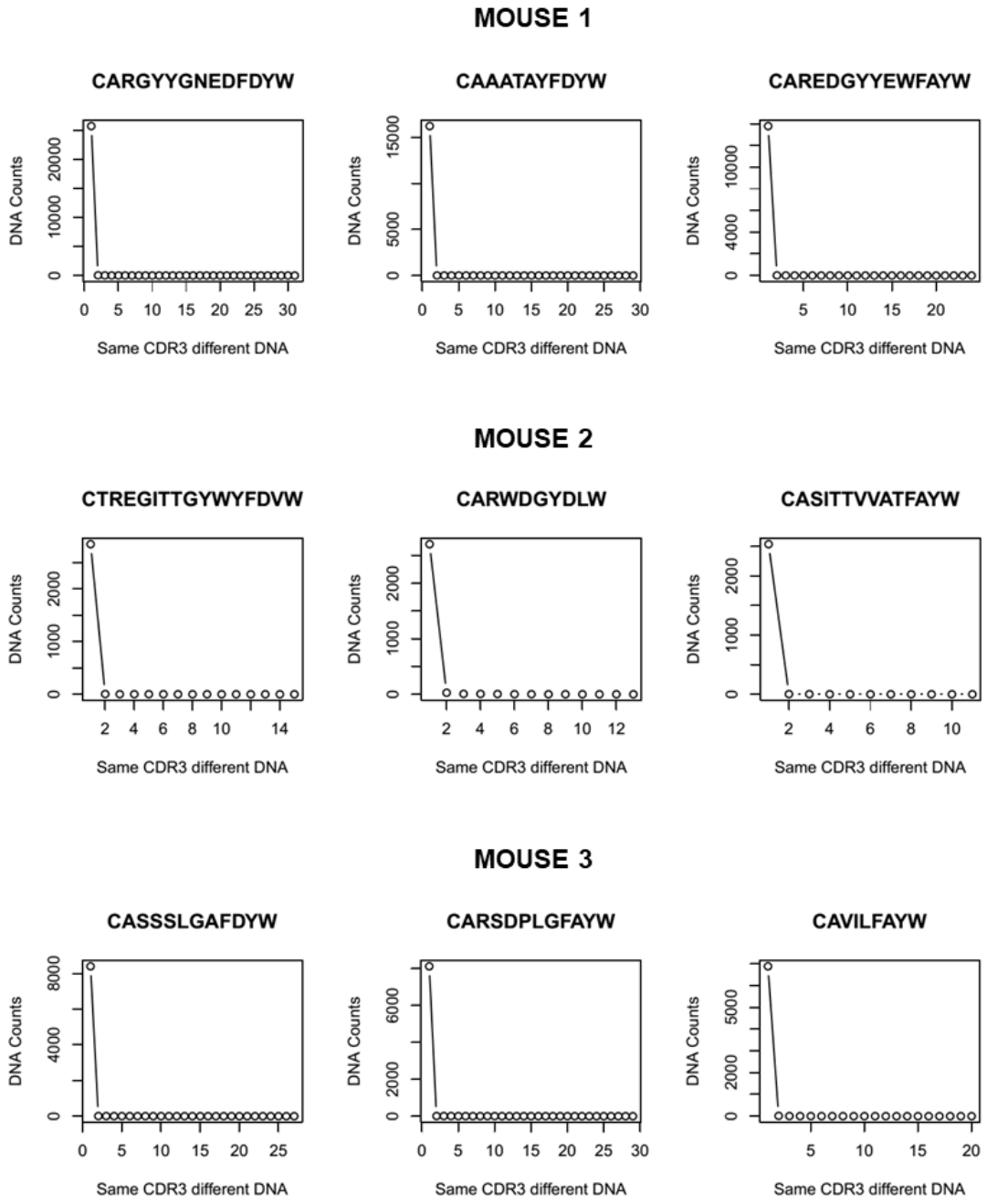

Figure 39. Number and frequency of DNA sequences coding for the same CDR3.

Number and frequency of the DNA sequences coding for the three most represented CDR3s in each murine repertoire.

5.3. IMMUNIZED LIBRARIES CONSTRUCTION AND SCREENING

The same mice analyzed by NGS, were used for the construction of antibodies libraries. As for VH genes, the VL genes amplification was performed by PCR using the cDNA of lymphocytes as template and mouse-specific forward and reverse primers mixes. The variable genes were assembled as scFv fragments through PCR reaction, cloned into phagemid vector and finally transformed into electrocompetent cells, obtaining a titer of 10^7 for each library.

Before proceeding with the selection process on the WNV target, the libraries were subjected to quality control test to confirm the presence and variability of the scFv fragments within the phagemid vector. To this aim, PCR reactions amplifying the scFv fragment and finger printing analysis were performed on random clones for each library. The obtained results evidenced that the 90 % of the tested clones reported full length scFv with different digestion patterns, suggesting a good representation of the immunized antibodies repertoire and therefore acceptable conditions for clones screening on the target. The PCR products and the related digestion pattern of four representative random clones for each library are shown in figure 40.

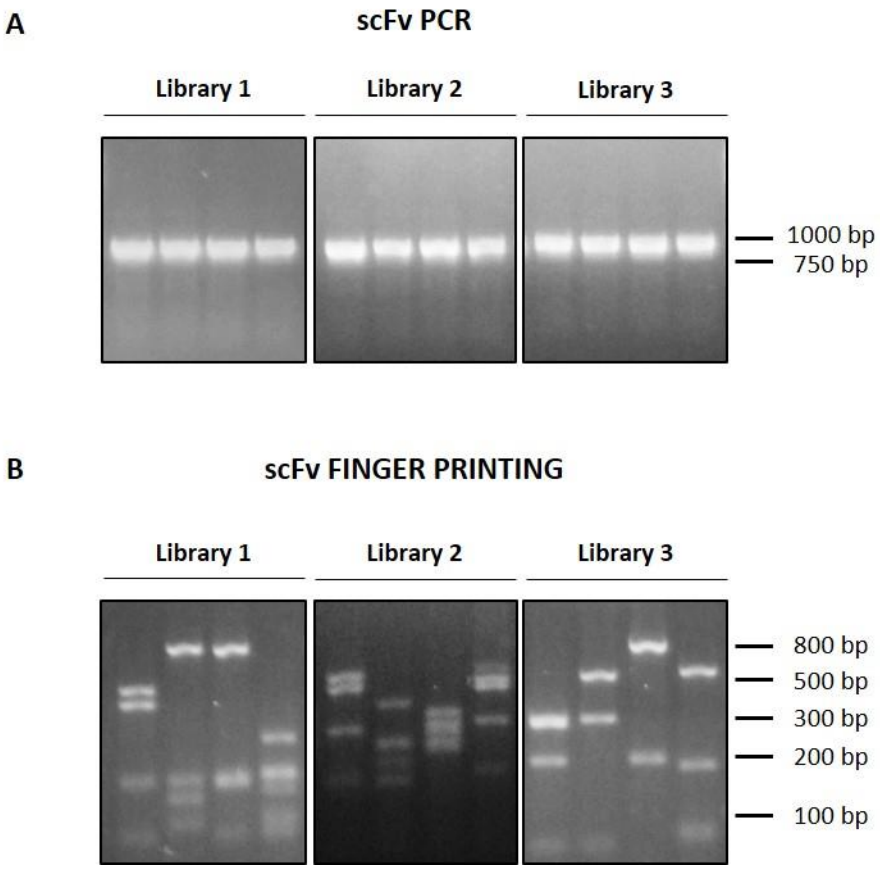

Figure 40. **PCR product and finger printing fragments on random clones from murine immunized libraries.** **A.** PCR products of scFv amplification of four random clones for each murine immunized libraries separated on 1.5% agarose gel. **B.** DNA fragments from enzymatic digestion of the scFv PCR products separated on 3% agarose gel.

5.3.1. Screening of the immunized murine library on sE_W antigen.

The selection on sE_W antigen was performed using one of the three library. The scFv expressing-phages were selected on the target through two panning cycles, maintaining the same selection

parameters used for naïve library screening. After the second cycle of selection, 96 random clones were tested on sE_W antigen by phage-ELISA. The output of the screening is reported in figure 41.

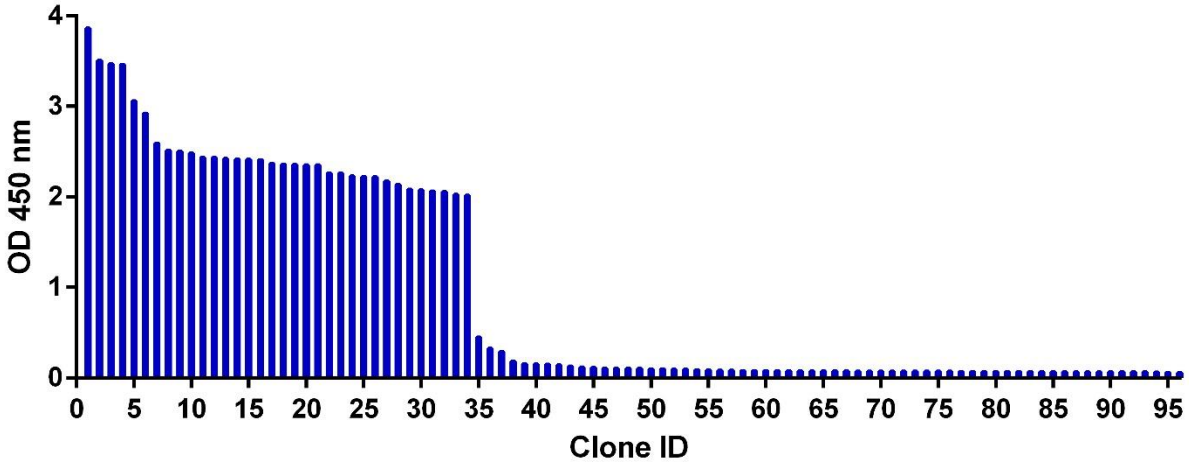

Figure 41. **Output of phage-ELISA assay of 96 random clones on sE_W antigen.**
Output of 96 random clones tested by phage-ELISA on sE_W antigen after two cycles of selection.

As shown in the figure, almost the 50% of the tested clones resulted positive for the target.
The obtained clones were checked by PCR and finger printing analysis (figure 42), which evidenced three different digestion patterns corresponding to three different scFv.

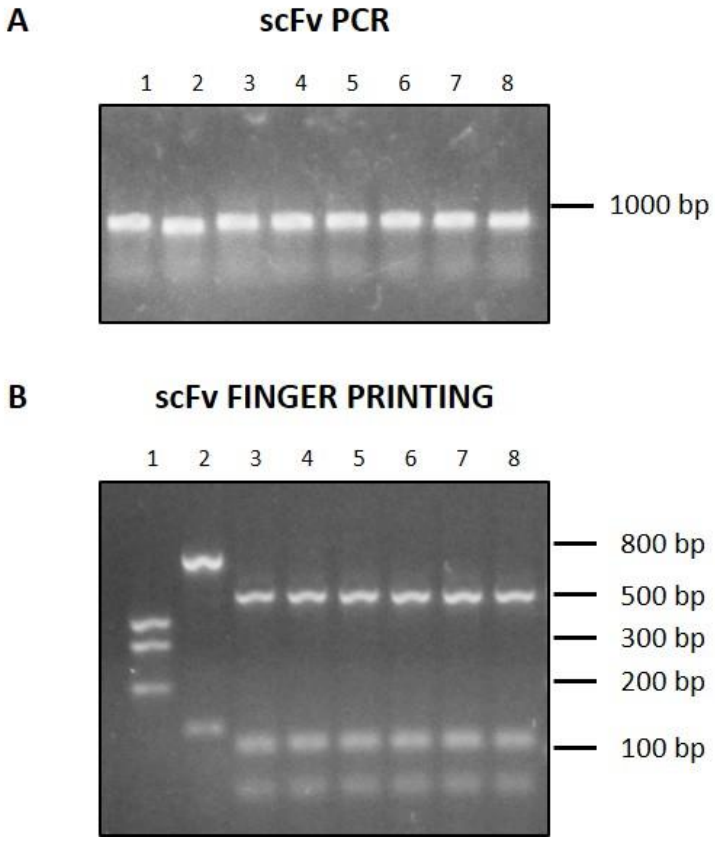

Figure 42. **PCR products and finger printing fragments on eight positive clones from murine immunized library selection on sE_W antigen.**

A. PCR products of scFv amplification of eight positive clones from murine immunized library selection on sE_W antigen separated on 1.5% agarose gel. **B.** DNA fragments from enzymatic digestion of the scFv PCR products separated on 3% agarose gel.

The figure reported the PCR of eight positive clones and their relative digestion patterns. Several PCR and finger printing analysis were performed on the other positive clones identified by phage-ELISA on sE_W, but the obtained patterns reflected the three shown in the figure. Therefore, the high number of positive clones obtained from the screening corresponded to three redundant antibodies.
As expected, comparing with naïve library screening performed with the same selection condition, the immunized library gave a higher rate of redundancy. The panning cycles on the target, in fact, enriches a subset of specific scFv which are already affinity matured on the antigen in the case of immunized library.

5.3.2. Analysis of the identified clone

The most represented and reactive phage clone on the target, identified as A4, was further tested by phage-ELISA on WNV antigens (sE and DIII). The sE_T was used as control of the clone specificity

for WNV antigen, while GST as negative control. In addition, the G1 clone selected from naïve library and targeting the DIII_W domain was used as positive control. The obtained results are shown in figure 43.

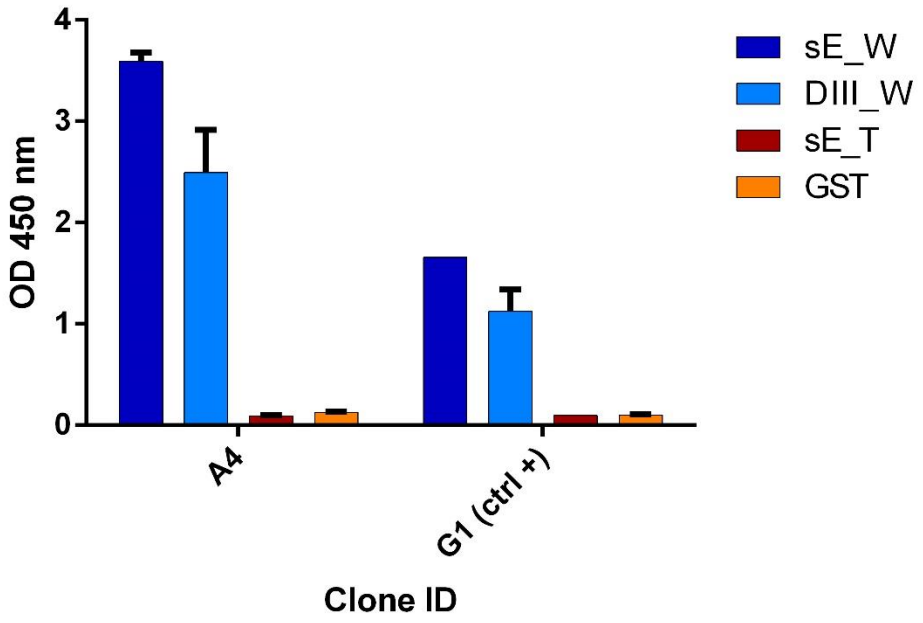

Figure 43. **Specificity analysis of the selected clone for sE_W target.**
Phage-ELISA assay of the selected clone on the target (sE_W), DIII_W, sE_T and GST proteins. Each OD and standard deviation values are calculated on the average of duplicates.

As shown in the figure, the A4 clone resulted positive for both WNV antigens, suggesting that the binding site maps on DIII domain. In addition, the negative signal on sE_T and GST proteins confirmed the specificity for the target. The selected clone was further analyzed by sequencing of the scFv and compared with the NGS data of the corresponding murine repertoire. Surprisingly, although the VH_CDR3s sequence selected by phage display was found in the corresponding murine repertoire, it resulted poorly represented, with a frequency of 50 reads. An explanation of the obtained data can rely on a possible bias introduced during libraries construction. The scFv, in fact, derived from multiple PCR reactions amplifying the variable genes and their final assembly into unique fragments. Such passages, performed with primers mixes, can led to preferential amplifications of VL/VH genes subsets which do not reflect the sequences frequency identified by NGS. In addition, during the library panning on the target, an intrinsic selection of scFv occurs on the base of their compatibility as phage-display particles, toxicity and solubility properties in bacteria [151–153].

However, the preliminary data presented above need to be completed with a deeper characterization of the selected clone and the screening of all the available murine libraries, in order to have a complete overview on the second approach used for α-WNV antibodies isolation and the potential application of the obtained antibodies as diagnostic reagents for WNV infection.

6. CONCLUSIONS

The presented work aimed to produce the needed reagents to improve the diagnosis of WNV infection.

The most common assays to detect the infection rely on the measure of the immune response against the virus and are mainly in ELISA format. The weak point of the available test is represented by false positive results due to the presence of cross-reactive antibodies. Such unspecific immune responses derive from the structural similarity between WNV and other members of the same genus. In addition, the co-circulation of more than one flavivirus in the same geographic area and, thus, the pre-exposure of the immune system to flaviviruses through infection or vaccination events, affect diagnosis specificity. Such condition concerns WNV, which overlaps with some European areas interested by TBEV circulation and vaccination programs against the virus.

The needed reagents for a diagnostic test based on detection of α-WNV antibodies are the viral antigen together with sensitive and specific antibodies against the antigen. As most of commercial kit for WNV detection provide the use of the inactivated virus, requiring specialized equipment and extensive production procedures, we developed a fast and easy protocol for viral antigens purification from bacteria. The quality of the produced antigens, in terms of purity, integrity and antigenicity, was positively checked by SDS PAGE, Western Blot and ELISA assays with murine immunized sera.

On the other hand, the isolation of antibodies specifically targeting the WNV antigens was based on two strategies: the selection of a naïve phage library and the construction and screening of immunized phage libraries from murine samples.

The first strategy led to the identification of three antibodies, which specifically recognize the WNV target. The selected antibodies were produced as scFv-Fc molecules and characterized by epitope mapping test. The obtained results demonstrate that two antibodies are directed against the same antigen epitope, while the third antibody recognizes a different binding site. Once identified the localization of the recognized epitopes on the target, the binding capability of the antibodies for the antigen produced as recombinant protein in bacteria was demonstrated also on the glycosylated form of WNV antigen. In addition, Western Blot assays on both the two versions of WNV antigen demonstrated that one of the selected antibodies is able to link the target in denaturing condition. The affinity of the produced scFv-Fc for the viral antigen was finally defined through ELISA assays. As additional characterization assay, the two antibodies were tested on live WNV through Plaque Reduction Neutralization Test, demonstrating that one scFv-Fc molecule is able to affect viral infection.

All the collected data on the antibodies isolated with the first strategy suggest that they are highly specific for the target and independent from the expression system used for antigen production. The different recognized epitopes suggest also that the antibodies can be employed in sandwich ELISA assay format. Indeed, the binding property of one selected antibody to link the denatured form of the target increases its potential applications in WNV detection test, including assays providing the use of the antigen in non-native conformation. Such tests include immunohistochemistry assays on infected tissues mainly performed on animal models for WNV pathogenesis or during epidemiologic studies.

The second strategy based on the construction and screening of immunized murine libraries on WNV antigen, although partially completed, led to preliminary results. After mice immunization, the elicited response against the target was confirmed by immunoserologic assays. A deeper knowledge of the murine antibodies repertoires was obtained by Next Generation Sequencing. The collected data evidenced the enrichment of a subset of VH_CDR3s for each mouse, which likely correspond to antibodies raised after immunization. In addition, a private immune response was observed for each murine sample, suggesting that the screening of the related libraries potentially generate different antibodies. A first confirm of such consideration was obtained after phage libraries construction, analyzing random clones by finger printing. The different digestion patterns observed for the tested clones, in fact, represent an indication of libraries diversity. Finally, the screening of one library on WNV antigen led to the isolation of a highly reactive clone, whose sequence was found in the immune repertoire of the corresponding murine sample. However, the preliminary results obtained from the second strategy need to be confirmed through the screening of all the libraries and subsequent antibodies characterization.

The presented data highlight the validity of both the planned procedures to identify and produce the needed reagents for WNV diagnostic purposes. As future perspectives, the purified viral antigens together with the produced antibodies will be tested on human samples and compared with commercial available kit in order to define their potential employment for diagnostic applications.

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